Estrogen Receptor Signaling in the Endometrium
pathway analysis of agonists and antagonists

Oestrogeen receptor signaaltransductie in het endometrium
werking van agonisten en antagonisten

Proefschrift

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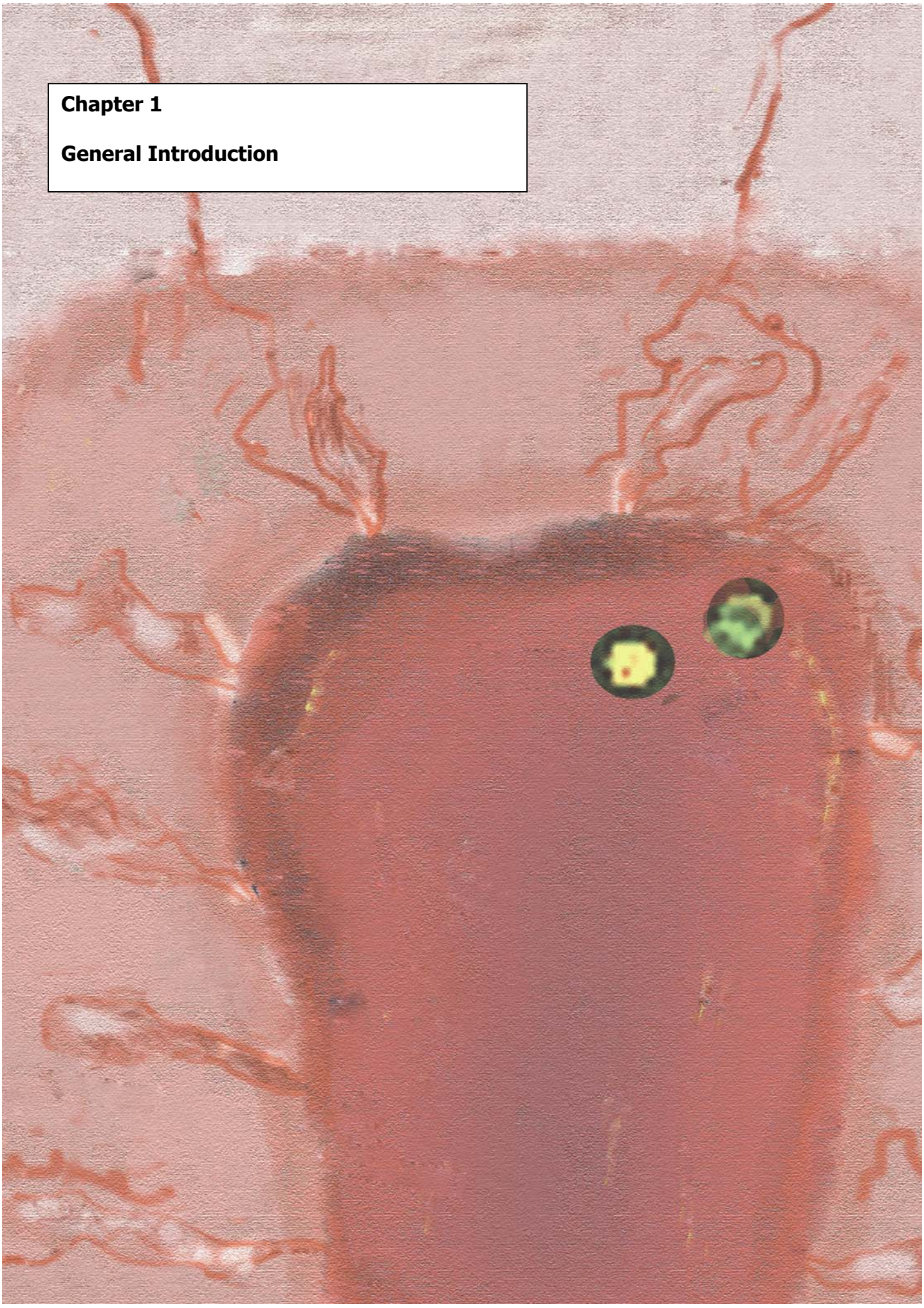
Chapter 1

General Introduction



1.1 The endometrium and endometrial cancer

Throughout female life physiological levels of the ovarian hormones estrogen and progesterone are important regulators of reproduction, bone density, coronary vascular reactivity and general well-being. Aberrant exposure to estrogens or estrogen-like hormones, however, results in an increased risk for the development of breast and endometrial cancer.

In order to understand the course of events leading towards the development of a cancer, the elucidation of the sequence of the human genome and the discovery of many mutated genes in the general population was very important. However, the next step, defining the precise molecular mechanisms underlying each particular form of cancer, seems even more complicated.

In this chapter, the role of several genetic and epigenetic factors in endometrial carcinogenesis, will be discussed. Because the ovarian hormones estrogen and progesterone play a prominent role in regulation of endometrial homeostasis, the role these hormones play in the development of endometrial cancer is highlighted most.

1.1.1 Normal endometrium

Sexual development of the human embryo starts with an indifferent stage, in which bipotential gonads and anlagen for both the male and the female genital reproductive tract, the Wolffian and Müllerian ducts respectively, are present (Swain and Lovell-Badge, 1999). In females, the Wolffian ducts regress in the absence of testosterone, while the Müllerian ducts develop into the Fallopian tubes, the uterus and the upper part of the vagina. In the ninth week of development, the uterus is formed, by fusion of the caudal tips of the Müllerian duct into a single-lumen tube, the uterovaginal canal (Fig. 1.1) (Larsen, 1993). This canal fuses with the sinovaginal bulb, resulting in formation of the uterus, cervix and vagina. At birth, the uterus of a female baby is temporarily enlarged by maternal estrogens that have crossed the placenta during pregnancy. This may result in vaginal bleeding. However, several weeks after birth, the uterus shrinks and remains dormant until puberty (Larsen, 1993).

The endometrium is the inner layer of the uterus, covering the uterine cavity (Fig.1.1). The endometrium consists of two distinct layers, the basal layer and the functional layer. The basal layer directly contacts the myometrium (muscle layer) and undergoes only minor changes during the menstrual cycle. The functional layer surrounds the lumen of the uterine cavity and changes extensively during the menstrual cycle. The functional layer of the endometrium consists of two different cell types, the glandular cells, which form the endometrial glands, and the surrounding stromal cells (Ludwig and Spornitz, 1991).

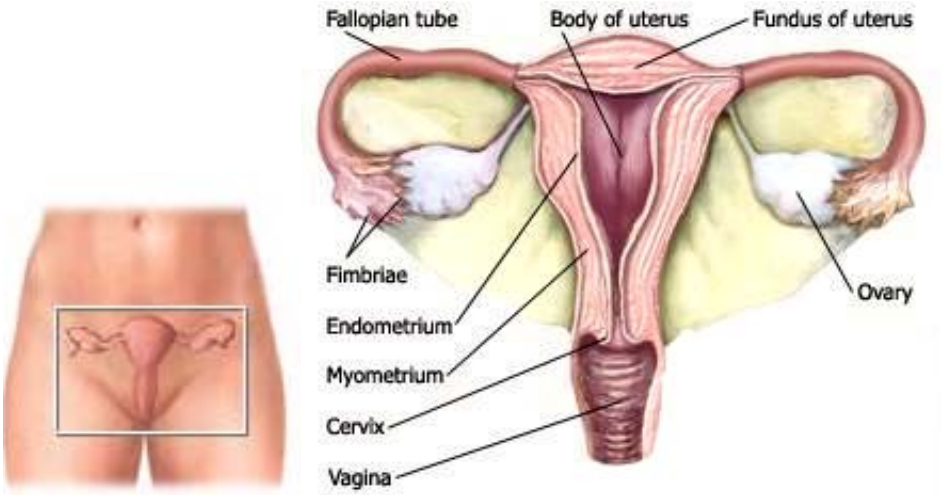


Figure 1.1 Schematic representation of the female genital tract.

Starting at menarche (the first menstruation), the uterus undergoes monthly cyclic changes caused by differential production and secretion of the ovarian hormones, estrogen and progesterone (Fig. 1.2). The menstrual cycle can be divided into three phases, the proliferative or follicular phase, the secretory or luteal phase and the menstrual phase. During the proliferative phase, increasing quantities of estrogens, secreted by the growing follicles in the ovary, induce proliferation, gland formation and vascular growth of the functional layer of the endometrium. Around day 14, a surge in luteinizing hormone (LH), induces ovulation and a reduction in circulating estrogens. This is the start of the secretory phase, which is characterized by high levels of progesterone, produced by the corpus luteum. Progesterone now stimulates differentiation of the glandular cells of the endometrium. If no pregnancy occurs, the corpus luteum regresses, which leads to a drop in circulating levels of estrogen and progesterone. In response to the decreased hormonal levels, the spiral arteries in the endometrium constrict, resulting in ischaemia of the tissue, which eventually leads to sloughing of the functional layer of the endometrium (menses).

Approximately at the age of 50, the menstrual cycle becomes irregular and ovulation eventually completely stops (menopause). Because of this, there is a steep decline in production of estrogen and progesterone by the ovaries, with the result that the endometrium no longer undergoes cyclic changes and goes into regression. In some women, however, for various reasons, the level of estrogen remains relatively high and as a result the endometrium starts to proliferate again. In these postmenopausal women estrogen-induced proliferation of the endometrium is no longer balanced by progesterone and this may lead to endometrial hyperplasia and eventually even endometrial cancer. Postmenopausal is defined as no menstrual bleedings for at least one year.

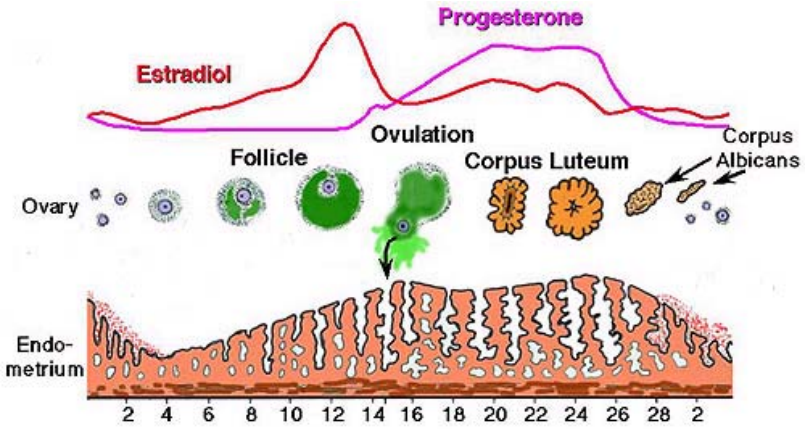


Figure 1.2 The menstrual cycle. Changes in the endometrium in relation to changes in the ovarian cycle.

1.1.2 Endometrial hyperplasia

Endometrial hyperplasia is defined as an overgrowth of both endometrial glands as well as endometrial stroma, and is characterized by a proliferative glandular pattern with or without different degrees of atypia (Mutter, 2000). Relatively increased estrogen levels, as found in polycystic ovary syndrome (PCOS), estrogen-secreting ovarian tumors and obesity (Montgomery et al., 2004), seem to induce hyperplasia. Atypical hyperplasia can be treated with either progestins (to antagonize the estrogenic effects) or surgery (hysterectomy) (Jadoul and Donnez, 2003). Without treatment, 25% of patients with atypical hyperplasia will develop endometrial cancer (Kurman et al., 1985).

1.1.3 Endometrial cancer

Endometrial cancer is one of the most common gynecological malignancies in the Western world. In the Netherlands, the incidence (2001) has been set on 1541 new cases, compared to 1119 for ovarian cancer and 591 for carcinomas of the cervix uterus (www.ikcnet.nl). Endometrial cancer

develops mainly in postmenopausal women, with the highest incidence in the age category of 60-80 years.

Risk factors for the development of endometrial cancer are, as for endometrial hyperplasia, mostly related to increased estrogen levels, either endogenous (such as late onset of menopause or estrogen-producing tumors) or exogenous (mainly estrogen-only hormone replacement therapy). Other risk factors include: obesity, polycystic ovary syndrome, nulliparity and diabetes mellitus. These factors are, however, also associated with increased estrogen levels (Akhmedkhanov et al., 2001; Hale et al., 2002; Schottenfeld, 1995). Furthermore, women who use tamoxifen, a selective estrogen receptor modulator, have a 2-7 fold increased risk for the development of endometrial cancer (Cohen, 2004). The effects of tamoxifen on the endometrium will be further discussed in section 1.2 (Tamoxifen and the endometrium).

There is evidence for a genetic predisposition for the development of endometrial cancer. One example is the Lynch syndrome II, which is characterized by a high incidence of hereditary non-polyposis colorectal cancers (HNPCC). In this syndrome a higher risk for the development of extracolonic tumors, of which carcinomas of the endometrium are the most common, are observed compared to the general population (Aarnio et al., 1995). Mutations in the breast cancer susceptibility gene BRCA1 have also been associated with an increased risk for the development of endometrial cancer; in a multinational cohort study involving 11,847 individuals, a two-fold increased risk was observed in women carrying a mutation in the BRCA1 gene (Thompson et al., 2002).

1.1.4 Histology and Typing

Based on histopathology, defined by the World Health Organization and the International Federation of Gynecology and Obstetrics (FIGO), endometrial cancers are classified into several subgroups (http://www.figo.org).

Histopathologic classification

Endometroid carcinoma
- Adenocarcinoma
- Adenocanthoma
- Adenosquamous carcinoma

Non-endometroid 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
G2	Moderately differentiated
G3	Poorly or undifferentiated

Surgical staging (Stage)

1A	Tumor is limited to the endometrium
1B	Invasion to less than half of the myometrium
1C	Invasion equal to or more than half of the endometrium
2A	Endocervical glandular involvement only
2B	Cervical stromal invasion
3A	Invasion of serosa of the corpus uteri and/or adnexa and/or positive cytology
3B	Vaginal metastasis
3C	Metastases to pelvic and/or para-aortic lymph nodes
4A	Tumor invasion of bladder and/or bowel mucosa
4B	Distant metastasis, including intra-abdominal metastases and/or inguinal lymph nodes

Table 1.1 FIGO-guidelines for classification of endometrial tumors. A complete overview for staging of gynecological cancers can be found at (http://www.figo.org).

The majority of tumors arise from glandular cells and are known as adenocarcinomas. The adenocarcinomas are further subdivided into two groups, endometroid tumors and non-endometroid tumors. The endometroid type is the most common type, accounting for approximately 90% of all tumors (Platz and Benda, 1995), and risk factors are all associated with unopposed estrogenic action. Non-endometroid tumors include papillary serous carcinoma (3-10%), mucinous adenocarcinoma, clear cell carcinoma and mixed Müllerian tumors (Bristow, 1999) (Table 1.1). Also stromal cells can become malignant, the endometrial stromal sarcomas.

Besides histopathologic grouping, tumors are also classified based on histological grade and surgical staging using the FIGO classification (http://www.figo.org) (Table 1.1).

1.1.5 Pathogenesis

Nowadays, the molecular mechanisms underlying endometrial carcinogenesis are extensively studied. Since research described in this thesis is performed mainly on endometroid endometrial cancers, the emphasis of the following sections will be on those tumors.

1. ER signaling and PR signaling

Endometroid type tumors are essentially estrogen-related tumors, since risk factors for the development of endometroid-cancer are all related to high estrogen levels. In most endometroid-tumors both estrogen receptors (ERα and ERβ) and progesterone receptors (PRA and PRB) are expressed (Oehler et al., 2003).

In the normal endometrium, ERα is much higher expressed compared to ERβ (approximately 100:1). During the menstrual cycle the expression of both ERα and ERβ are decreased in the late secretory phase compared to the proliferative phase (Mylonas et al., 2004; Taylor and Al-Azzawi, 2000). Expression of ERα and ERβ decreases with age in the endometrium of postmenopausal women (Koshiyama et al., 1996), but expression in endometrial polyps and in well-differentiated endometrial cancers remains high (Pickartz et al., 1990; Sant'Ana de Almeida et al., 2004; Sivridis and Giatromanolaki, 2004).

In endometrial cancer, a selective loss of ERα is observed going from low-grade tumors (G1 and G2) to high-grade tumors (G3 and G4) (Fujimoto et al., 2002; Utsunomiya et al., 2000). Apparently, because of a selective loss of ERα, other factors, like growth factors, have become more important for tumor growth. Since high-grade endometrial tumors still express ERβ, also signaling via ERβ may be an important factor.

Expression patterns of PRA and PRB vary during the menstrual cycle. Highest levels of expression are found during the second half of the proliferative phase, although variation in expression is much higher for PRB (20-fold) as for PRA (5-fold) (Arnett-Mansfield et al., 2001; Mote et al., 1999).

For endometrial cancer, it is generally agreed on that expression of the PRA and PRB is usually lost during progression of the tumor towards a more poorly differentiated phenotype. However, it is still under debate if the altered expression ratio between PRA and PRB will affect the behavior of the tumor. Hanekamp et al. have shown that endometrial cancer cell lines containing only PRB are more invasive *in vitro* and *in vivo* compared PRA-only or PRA/PRB cell lines). Furthermore, they have shown that differences in invasive capacity between these cell lines are due to regulation of different sets of genes (Hanekamp et al., 2005; Hanekamp et al., 2002; Hanekamp et al., 2004). One of these differentially expressed genes is E-cadherin (Hanekamp et al., 2005) and it was observed that inhibition of expression of E-cadherin either by antibody-neutralization or by PRA expression, results in reduction of the invasive capacity of the manipulated endometrial cancer cells.

The molecular mode of action of ER and PR signaling will be further discussed in section 1.3 of this chapter.

2. Common genetic alterations.

For the initiation and further development of endometroid carcinoma and its precursor endometrial hyperplasia, besides changed ER and PR signaling, other genetic alterations have been indicated to be (potentially) involved: microsatellite instability (MI) occurs in 25% to 30% of cases, PTEN mutations in 37% to 61%, RAS mutations in 10% to 30%, and ß-catenin (CTNNB1) mutations with nuclear accumulation in 25% to 38% of endometroid cancers (Bussaglia et al., 2000; Lax and Kurman, 1997; Matias-Guiu et al., 2001; Sherman, 2000) (Fig. 1.3).

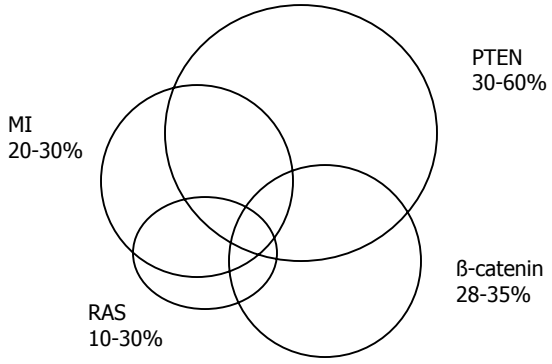


Figure 1.3 Main molecular alterations found in endometroid carcinomas. Frequency range of microsatellite instability (MI), and mutations in PTEN, RAS and ß-catenin genes. (Figure adapted from Matias-Guiu et al. 2001)

A. Microsatellite instability.

Microsatellite DNA sequences are short-tandem repeats (CA_n is the most common repeat in humans) that are distributed throughout the genome. Due to their repeating nature, these microsatellites are particularly prone to errors during replication, leading to instability of parts of the genome (microsatellite instability) (Aaltonen et al., 1993; Ionov et al., 1993). The genes shown to be responsible for Microsatellite Instability (MI) encode proteins involved in DNA mismatch repair (MLH1, MLH2, PMS1 and PMS2) (Salvesen et al., 2000). Mutations of these genes alter the ability of the cells to repair errors during replication. In case of loss of function of DNA repair genes, genes with microsatellite DNA sequences are easily mutated (Catasus et al., 1999).

In tumors of patients with the hereditary nonpolyposis colon cancer syndrome (HNPCC), MI was initially observed (Ichikawa et al., 1999). Patients from HNPCC families have an inherited germ line mutation in either MLH1 or MLH2 (Aaltonen et al., 1993; Ionov et al., 1993).

Endometrial cancer is the second most common cancer in patients with HNPCC, and the occurrence of MI in endometrial cancers of HNPCC patients is 75% compared to 25% to 30% in sporadic endometrial cancers (Aarnio et al., 1995). However, endometrial cancers rarely show mutations in the MLH1 or MLH2 DNA-repair genes (Katabuchi et al., 1995; Kowalski et al., 1997; Simpkins et al., 1999). Therefore it is assumed that in these tumors functional loss of the MLH1 and MLH2 genes is accomplished through methylation of the promoter of these genes, which may result in MI (Salvesen et al., 2000).

B. PTEN mutations

Loss of PTEN function (either partial or complete) seems to be an early event in carcinogenesis, since it is detected in both endometrial hyperplasias as well as endometrial cancers (Kinzler and Vogelstein, 1997; Mutter et al., 2000). The tumor suppressor gene PTEN (phosphatase and tensin homologue deleted from chromosome 10) is located on chromosome 10q23.3. PTEN encodes a member of the tyrosine phosphatase family and inhibits the PI3K/AKT signaling pathway. Because of this, activated PTEN is involved in inducing apoptosis. Loss of PTEN function

results in less apoptosis, and therefore PTEN may stimulate tumour progression (Downward, 2004).

In endometrial cancers loss of heterozygosity (LOH) at the locus of the PTEN gene, is found in 40% of tumors (Jones et al., 1994; Peiffer et al., 1995). Besides loss of PTEN expression due to LOH, also somatic PTEN mutations are found in endometrial tumors; the occurrence is 37% to 60%, and almost exclusively in endometroid tumors (Kong et al., 1997; Tashiro et al., 1997a). Loss of expression of PTEN in the absence of LOH on locus 10q23.3 may be caused through aberrant promoter methylation (Matias-Guiu et al., 2001; Salvesen et al., 2001).

C. RAS mutations

Mutations of the k-ras gene have been identified in 10% to 30% of endometrial cancers, mainly in tumors of endometroid type (Matias-Guiu et al., 2001). Since RAS mutations are, like PTEN mutations, found in endometrial hyperplasias and endometrial cancers, these mutations might also be an early step during carcinogenesis (Lagarda et al., 2001; Sun et al., 2002). The proto-oncogene RAS is involved in cell proliferation; RAS activates the MAP kinase signaling pathway and subsequently several growth factor systems are activated (Hill et al., 1993).

D. ß-catenin mutations

Mutations in the ß-catenin gene have been reported in 28%-35% of endometroid carcinomas (Matias-Guiu et al., 2001). ß-catenin complexes together with E-cadherin and is in this form associated with the cell membrane, playing a major role in cell-cell adhesion. Another function of ß-catenin is that it is a key component in the canonical Wnt signaling pathway. The general Wnt signal transduction cascade is as follows: secreted Wnt protein binds to its receptor (frizzled) in the plasma membrane. Through a cascade of events, including activation of dishevelled (DSH) and inactivation of GSK3β, the β-catenin degradation complex (consisting of APC, Axin, GSK3β and β-catenin) falls apart, enabling β-catenin to translocate from the cytoplasm to the nucleus. There it teams up with the transcription factors TCF/LEF, resulting in transcriptional activation of Wnt target genes (Bienz, 2002).

As depicted in figure 1.3, in many cases of endometroid tumors MI, PTEN and RAS mutations coexist. Mutations in the ß-catenin gene, however, are less generally associated with the other alterations.

E. Genetic alterations in non-endometroid tumors

For the initiation and development of non-endometroid endometrial cancer, mutations in the TP53 gene and LOH on several chromosomes, seems more important (Sherman et al., 1995; Tashiro et al., 1997b; Tritz et al., 1997). Mutations in the TP53 gene are described in up to 90% of non-endometroid tumors. The tumor suppressor gene TP53 functions as a G1-S cell cycle checkpoint: it induces a G1 arrest thereby creating extra time for DNA repair mechanisms. If DNA-repair fails, TP53 can initiate cell death via apoptosis (Lane, 1992). If TP53 is inactivated through mutations or deletions, DNA repair will not proceed as planned, and mutations may be introduced that can lead to a more aggressive cancer type, while the second line in defense, apoptosis, is not induced.

It has also been suggested that development of non-endometroid tumors is caused by dedifferentiation of a preexisting endometroid tumor, since tumors are described which contain both endometroid as well as non-endometroid features.

Although the classification of endometrial cancers into endometroid tumors and non-endometroid tumors is relevant to most cases, it is nonetheless artificial and exceptions do occur. Actually, some endometrial cancers are in the gray zone in which overlap is observed in clinical, morphological, immunohistochemical and molecular features between endometroid and non-endometroid tumors. Recently, more advanced molecular research tools were used for the classification of endometrial tumors. It turned out to be quite uncomplicated to classify endometrial tumors based on gene expression profiles. Interestingly, these classifications

resemble the classification according to FIGO-stage (Mutter et al., 2001; Smid-Koopman et al., 2004).

1.1.6 Diagnosis and Screening

The classical symptom for women with endometrial cancer is abnormal vaginal bleeding. In the Netherlands, all women with unexplained vaginal bleeding are evaluated in a standardized way. As a first step a Papanicolou (PAP) smear, to exclude cervical pathology, and a transvaginal ultrasound, to evaluate endometrial thickness, are performed. If the endometrial thickness (equivalent of both layers of the endometrium) is more then 4mm, an endometrial biopsy with or without hysteroscopy is performed (http://www.nvog.nl).

1.1.7 Therapy

Endometrial tumors are normally diagnosed at an early stage, since the most common symptom vaginal bleeding, is usually alarming enough to seek medical attention. Therefore, at diagnosis approximately 75% of tumors are confined to the uterus (Stewart and Kleihaus, 2003). The choice of treatment is based on FIGO staging and the general health condition of the patient.

Stage 1 and 2: In general, hysterectomy with bilateral salpingo-oophorectomy (BSO) is curative for these stages of disease. The value of adjuvant radiotherapy in patients with stage 1 disease remains unclear. In the PORTEC (Post Operative Radiation Therapy in Endometrial Carcinoma) study, patients with stage 1 endometrial cancer were randomized to either receive adjuvant radiotherapy or no further treatment after surgery. Adjuvant radiotherapy did reduce the loco-regional recurrence rate, but it had no effect on overall survival (Creutzberg et al., 2000).
Stage 3 and 4: Treatment of these patients is a combination of surgery with postoperative radiotherapy. If the tumor has metastasized outside the pelvis, in addition, chemotherapy or progesterone-treatment can be used, however, response rates are low and the overall prognosis is poor (see also section 1.3.4). Chemotherapy in the form of adriamycin, cisplatin, paclitaxel, cyclophosphamide and/or ifosfamide is effective in 14%-30% of tumors, with a median progression-free period of approximately four months (Elit and Hirte, 2002). Other forms of endocrine therapy currently investigated for treatment of advanced and recurrent endometrial cancer are selective estrogen receptor modulators (SERMs), gonadotrophin-releasing hormone (GnRH) analogues and aromatase inhibitors (Rose et al., 2000; Sugiyama et al., 2003). However, again with limited results so far.

Overall, because of the early detection and effective treatment of endometrial cancer, in The Netherlands, five-year survival rate is as high as 86% (Stewart and Kleihaus, 2003).

1.2 Tamoxifen and the endometrium

Tamoxifen-use is asoociated with an increased incidence of endometrial pathologies, including endometrial cancers. However, the benefits of tamoixfen far outweigh the risk of use and is therefore first choice adjuvant treatment for postmenopausal breast cancer patients. The 1998 EBCTCG (Early Breast Cancer Trialists' Collaborative Group) overview, showed the following benefits of tamoxifen-use in women who had an estrogen receptor positive breast cancer; tamoxifen-use for five years reduced the risk of relapse of disease after 10 years by 37% in women age 50 to 59, and by 54% in women age 60 to 69. Furthermore, for women under 50 years, the reduction in risk of recurrence is 45% .

1.2.1 Histology and typing

The mechanism of action of tamoxifen in breast cancer patients is that tamoxifen inhibits the growth of cancer cells by competitive antagonism of estrogen at ERα or ERβ. In the endometrium, tamoxifen has an effect that varies with the ambient concentration of estrogen; in premenopausal women (high estrogen levels), tamoxifen displays an estrogen-antagonistic effect, while in postmenopausal women (low estrogen levels), tamoxifen displays an estrogen-

agonistic mode of action (Bergman et al., 2000; Chang et al., 1998; Kedar et al., 1994; Mourits et al., 2001). Because of this, in postmenopausal tamoxifen-treated breast cancer patients, the incidence of endometrial pathologies, including hyperplasia, polyps, carcinomas and sarcomas, is as high as 36% (Cohen, 2004). The most common endometrial pathologies are hyperplasias (incidence 2.15 to 30.3%) and polyps (incidence 5.38% to 36%) (Table 1.2).

For the development of endometrial cancer in postmenopausal breast cancer patients using tamoxifen, the relative risk is estimated at 2-7 fold compared to non-users (Bergman et al., 2000; van Leeuwen et al., 1994). Most of the endometrial cancers were adenocarcinomas, with a lower frequency of endometroid carcinomas and a higher frequency of clear cell carcinomas and serous carcinomas compared to non-treated patients (Mignotte et al., 1998; Silva et al., 1994).

Authors	Tamoxifen-treated patients	Non-treated patients	Endometrial hyperplasia			Endometrial polyps			Sampling Method
			+Tam	-Tam	p-value	+Tam	-Tam	p-value	
1	51	52	4	2	n.s.	36	10	0.004	pros. case control
2	93	20	2.2	0	n.i.	5.4	0	n.i.	pros. case control
3	175	27	12	11	n.s.	8	7.4	n.s	pros. case control
4	33	23	30.3	4.3	n.i.	21.2	0	0.025	pros. case control
5	124	104	13.7	0	<0.0001	13.7	0	<0.0001	pros. case control
6	58	68	6.9	7.4	n.s.	17.2	16.2	n.s.	case control

Table 1.2 Incidence of endometrial hyperplasias and endometrial polyps in postmenopausal tamoxifen-treated breast cancer patients compared to postmenopausal nontreated breast cancer patients. 1= (Lahti et al., 1993), 2= (Cohen et al., 1994), 3= (Cohen et al., 1997), 4= (Cheng et al., 1997), 5= (Maugeri et al., 2001), 6= (McGonigle et al., 1996). Numbers are percentages of numbers to total group. n.i. =non indicated and n.s.=non significant. (Figure adapted from (Cohen, 2004) .

1.2.2 Correlation between patient characteristics and histology

Although tamoxifen-use increases the incidence of endometrial pathologies, most women using tamoxifen will not develop any. Several factors were tested to evaluate their positive predictive value for the occurrence of endometrial pathologies in tamoxifen-users.

The occurrence of endometrial pathologies is positively associated with vaginal bleeding and spotting (Cohen et al., 1999; Deligdisch et al., 2000). Endometrial thickness of more then 5mm, also increases the chance to find pathologies (Marchesoni et al., 2001). Combining these two factors, will even better predict occurrence of endometrial pathologies (Marchesoni et al., 2001).

The factors dose and duration of tamoxifen-use are also positively correlated with the incidence of endometrial abnormalities. In patients who received tamoxifen for five years, the frequency of endometrial pathology increased throughout the observation period. Furthermore, the occurrence of endometrial cancer was significantly higher in patients who received tamoxifen for 5 years then in the 2-year group (Fornander et al., 1989). From dose-response curves it has been clear, that in women receiving 20mg of tamoxifen daily, endometrial pathologies develop after longer periods of treatment, than in women treated with 40mg daily (Ismail, 1994).

Currently, these clinical features are used as guidelines for performing hysteroscopy and biopsies in women using tamoxifen. However, in most women with vaginal bleeding and endometrial thickness of more then 5mm, no endometrial pathology is found in biopsies. Furthermore, in non symptomatic tamoxifen-treated patients endometrial cancers do occur (Cohen, 2004).

In conclusion, although several factors are positively associated with an increased risk for the appearance of endometrial pathologies in tamoxifen-users, it is impossible to predict which tamoxifen-receiving patient will eventually develop endometrial pathologies. The molecular mode of action of tamoxifen will be further discussed in the next section.

1.2.3 Pathogenesis

The frequency of endometrial pathologies is significantly higher among breast cancer patients who use tamoxifen compared to breast cancer patients who did not receive tamoxifen. Furthermore, the increased incidence of endometrial cancers in tamoxifen-users is complicated by the occurrence of more tumors with a less favorable stage; most cancers are of the endometroid type, but a higher frequency of clear cell carcinomas and serous carcinomas is observed in tamoxifen-users. Many studies have tried to unravel the molecular mechanism of tamoxifen in the endometrium.

1. ER signaling and PR signaling.
In benign endometrium of breast cancer patients who use tamoxifen, the expression of both ERα and ERβ and PRA and PRB, evaluated with immunohistochemistry, is higher compared to non-tamoxifen users (Elkas et al., 2000; Mourits et al., 2002b). This may implicate that the uterotrophic effects of tamoxifen are possibly due to potentiation of signaling via ERα and ERβ. As both PRs are known estrogen-responsive genes, up-regulation of the PRs in the tamoxifen-group strengthens this hypothesis.
In endometrial carcinomas of tamoxifen-users a selective loss of ERα compared to ERβ, was observed (Wilder et al., 2004). This suggests that in these tumors, ERα-signaling becomes less important. However, it may also indicate that signaling via ERß becomes more important. PR expression is reported to be higher in endometrial cancers of tamoxifen-users compared to non-users (Mourits et al., 2002b)**.**

2. Common genetic alterations.
Pathologic classification of endometrial tumors, based on FIGO classification, is different for tamoxifen-users compared to non-users (see section 1.2.1). If tumors of tamoxifen-users and sporadic tumors are matched for stage and grade, however, the incidence of microsatellite instability and mutations in the RAS, PTEN and ß-catenin genes, is similar in both groups (Prasad et al., 2005).

3. Other.
Several other genes were studied to investigate the effects of tamoxifen in the endometrium. In benign endometrium (atrophic, hyperplastic, proliferative or polyps) expression of TGFß1, p27, Cathepsin D and CA125 is different in tamoxifen-users compared to non-users (Carmichael et al., 2000; Mylonas et al., 2003a; Mylonas et al., 2003b; Siufi et al., 2003). Furthermore, the proliferation/apoptosis index, as measured by expression of Ki67, FAS, FASL and BCL2 is higher in benign endometria of tamoxifen-users compared endometria of non-users (Mourits et al., 2002a; Mourits et al., 2002b). In endometrial cancer samples, however, no differences were found in gene-expression profiles between similarly staged tamoxifen-associated tumors compared to spontaneous tumors (Ferguson et al., 2004).

In conclusion: in the benign endometrium, tamoxifen induces the expression of specific genes and pathways, resulting in an increased incidence of endometrial pathologies. This increased proliferation may even lead to endometrial cancer. Those cancers are, however, indistinguishable from sporadic tumors when matched for stage and grade.
In chapter 6 tamoxifen-specific effects in benign endometrium will be further discussed.

1.3 Estrogen and progesterone receptor

Estrogen and progesterone-induced growth and differentiation are essential for maintenance of the human endometrium. Most risk factors for the development of endometroid endometrial cancer are related to exposure to high levels of estrogen or estrogen-like compounds (such as tamoxifen). As in the normal endometrium, progesterone causes differentiation of endometrial cancer cells and thereby growth inhibition. The cellular actions of estrogens and progestagens

are mediated through binding to their specific receptors, the estrogen (ER) and progesterone (PR) receptors.

1.3.1 General structure

The estrogen receptor (ER) and progesterone receptor (PR) are members of the steroid hormone receptor family, and can act as hormone-dependent activators of transcription. Two ERs are described, ERα and ERβ (Green et al., 1986; Kuiper et al., 1996). ERα (60-66kD protein) and ERβ (51-61kDa protein) are translated from two different genes (located at chromosome 6 and chromosome 14, respectively). Furthermore, they display considerable homology at the protein level: 96% in the DNA-binding domain and approximately 60% in the ligand-binding domain (Fig.1.4).

The progesterone receptor exists as two isotypes, PRA and PRB, that are transcribed form two distinct promoters in the same gene, located on chromosome 11 (Kastner et al., 1990). The PRA (94kDa protein) is a truncated form of the PRB (114kDa protein), lacking the first 164 amino acids at the N-terminus.

In both ERs and PRs several functional domains can be distinguished, like the transcription regulating domain (TRD), DNA binding domain (DBD), hinge region (H), ligand binding domain (LBD), activation function domain (AF1-3) and an inhibitory domain (ID) (Fig.1.4).

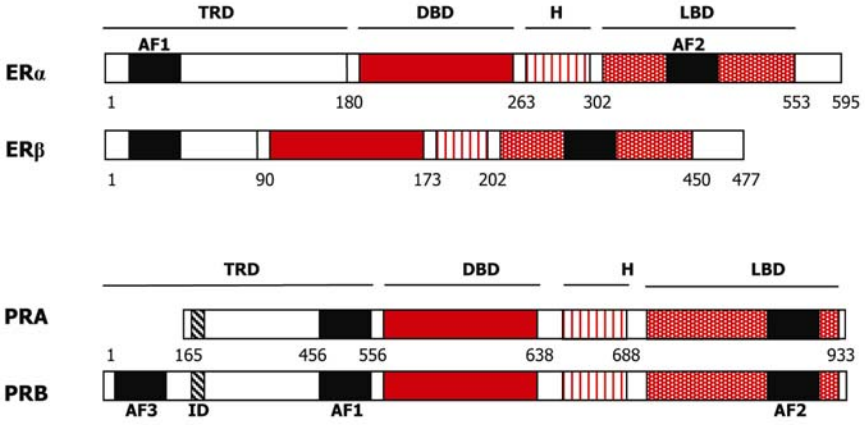


Figure 1.4 Structure of estrogen receptor (ERα and ERβ) and progesterone receptor (PRA and PRB). TRD: transcription regulating domain; DBD: DNA-binding domain; H: hinge region; LBD: ligand-binding domain; AF1, AF2, AF3; activation function 1, 2, and 3; ID: inhibitory domain. Numbers indicate amino acid location.

The TRD, located in the amino-terminal (NH_3) part of the receptor, seems to be involved in modulation of transcription. The DBD contains two zinc finger motifs, and binds to specific hormone-response elements (HRE) in the genome, and the LBD, located at the carboxyl-terminal (COOH) part of the receptor, is responsible for ligand binding. Between the DBD and LBD lies the hinge region, which contains a nuclear localization signal, and is involved in receptor dimerization. Several activation function (AF) domains are located within these receptors. These domains act as docking sites for interaction with several cofactors and transcription factors. Upon ligand binding in the LBD, the family of SRC cofactors (steroid receptor co-activators or p160), interact with AF-2 to stimulate transcription (McKenna and O'Malley, 2000). AF-1, however, is involved in ligand-independent activation of transcription. AF-1 can act either dependent or independent of AF-2; but after binding of ligand, these two AF domains act synergistically (Giangrande and McDonnell, 1999; Tzukerman et al., 1994).

Only the PRs, not the ERs, contain an inhibitory domain (ID), and PRB also contains an additional AF domain, AF-3. Because of the conformation of PRA, the ID partly prevents

transcription activation through AF-1 and AF-2. In the PRB, the additional AF-3 domain is not capable of activating transcription on its own, and therefore it is hypothesized that AF-3 acts indirectly through enhancing the activity of AF-1 and AF-2. Furthermore, it is thought that in the PRB this AF-3 prevents the inhibition by ID of transcription activation (Sartorius et al., 1994). Since in the PRA the ID is not inhibited (no AF-3 is present), this explains why the transcriptional activity of PRA is less then of PRB. Furthermore, it can also help to explain why PRA can even function as a dominant repressor of PRB (Giangrande et al., 1997; Vegeto et al., 1993). In agreement with this, if the ID in the PRA is deleted the receptor mutant is functionally indistinguishable from the PRB (Giangrande et al., 1997).

1.3.2 Mode of action

The general mode of action of the ERs and PRs is depicted in Figure 1.5. In the absence of ligand, the receptors are associated with heat shock proteins. After ligand binding, the conformation of the receptor changes and a dissociation of heat shock proteins occur. The receptor-hormone complex forms dimers, of which both homodimers (for example ERα-ERα) as well as heterodimers (for example ERα-ERβ) can be formed. In the absence of ligand both ERα and ERβ are located in the nucleus of the cell, while unliganded PR can be present in either the cytoplasm or the nucleus; GFP-bound PRB is localized mainly in the cytoplasm and GFP-bound PRA is localized more in the nucleus (Lim et al., 1999; McDonnell, 2004). After binding of ligand, however, the receptor is activated (mainly through phosphorylation) and both receptors (ER and PR) predominantly localize to the nucleus. In the nucleus the dimers bind to specific hormone response elements (ERE or PRE) on the DNA. After binding of the receptor-dimer to the DNA, several co-activators, co-repressors and/or transcription factors bind, after which target genes are transcribed.

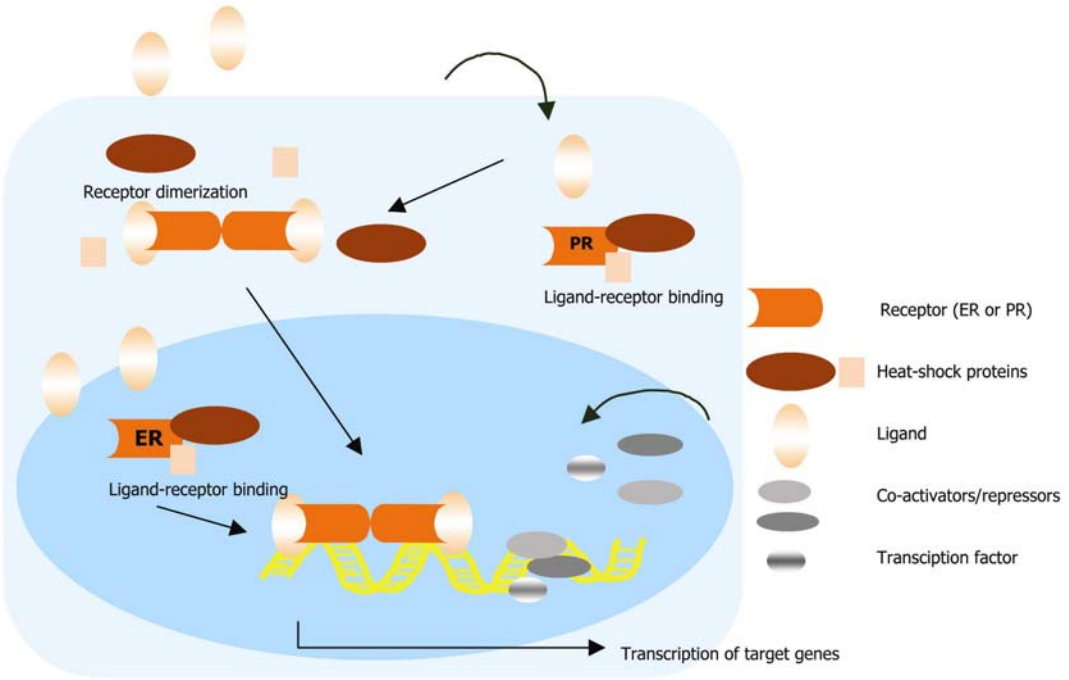


Figure 1.5 General mode of action of estrogen and progesterone receptors. In the absence of ligand the ERs and PRs are bound to heatshock proteins. While the ERs are located in the nucleus, the PR can be localized in either the nucleus or the cytoplasm. Upon ligand binding, the receptor dissociates from the heat shock proteins, dimerizes and is activated (mainly through phosphorylation). The receptor-dimer relocates to the nucleus, recruits several cofactors, and transcription of target genes follows.

Around one third of genes in humans that are regulated by ERs do not contain ERE-like sequences (O'Lone et al., 2004). Besides the classical transcription route, ERs can regulate transcription via modulation of other transcription factors through protein-protein interactions in the nucleus, like activator protein 1 (AP-1) transcription complex (Gottlicher et al., 1998).

Already for many years there are data indicating that the effects of estrogen and progesterone are not only a result of transcription activation through respectively ERα or ERβ or PRA or PRB, but also through cross-talk of the ERs or PRs with several intracellular signaling pathways, like the MAP-kinase/RAS/RAF/PI3-kinase and IGF1 receptor signal transduction pathways (Bramley, 2003; Zhang et al., 2004). In these, so called non-genomic effects, estrogen or progesterone binds to receptors that are located in or near the membrane. It has been postulated that in this situation it are the ERs and PRs themselves that translocate to the vicinity of the plasma membrane. However, although evidence indicated the presence of these membrane-linked receptors, these studies may suffer from technical limitations. In most studies transient transfection systems are used, in which, due to the high amounts of molecules in the cell, the receptors may artificially be localized at non-physiological sites in the cell (Simoncini and Genazzani, 2003).

1.3.3 Estrogen, SERMs and anti-estrogens

In a wide variety of tissues, like the male and female reproductive organs, and in the cardiovascular and neural tract, estrogens (mainly 17ß-estradiol) are major regulators of many important biological functions (Hess et al., 1997; Korach, 1994; Turner et al., 1994). The production of 17ß-estradiol takes place mainly in the ovaries, with concentrations varying throughout the menstrual cycle (see also section 1.1.1). At the end of fertile life (post menopause), no growing follicles are left in the ovary, and a decline in circulating estrogens is found. Reduced estrogen levels may cause hot flushes and negatively affect cognitive functioning and bone homeostasis and are difficult to correct for because substitution with exogenous estrogens will cause a significant increment in endometrial cancer incidence. Therefore, development of synthetic ER ligands displaying only the beneficial effects of estradiol (E_2), has intensified over the last decades. These compounds are called Selective Estrogen Receptor Modulators (SERMs), and tamoxifen and raloxifene are currently used most .

The activities of SERMs are tissue specific. Tamoxifen, as discussed in section 1.2 of this chapter, is used for treatment of breast cancer patients. In breast tissue, tamoxifen exerts estrogen-antagonistic properties, while in the uterus it displays partial estrogen-agonistic effects, resulting in an increased risk for development of endometrial cancer. Raloxifene, initially developed to prevent osteoporosis, is also used as adjuvant therapy for breast cancer patients. Currently, the efficiency of raloxifene over tamoxifen for treatment and prevention of breast cancer is investigated (STAR trial) (Jordan et al., 2001). Raloxifene, however, does not seem to increase the incidence of endometrial cancer (Delmas et al., 1997; Ettinger et al., 1999; Jordan et al., 2001). The pure estrogen-antagonist ICI182780 has been successfully used for treatment of women who developed tamoxifen-resistant breast cancer (Osborne, 1999). Interestingly, treatment of these patients with ICI182780, revealed no stimulation of the endometrium (Wardley, 2002).

	Uterus	**Bone**	**Breast**	**Cardiovascular**	**Brain**
E_2	Agonist	Agonist	Agonist	Agonist	Agonist
Tamoxifen	Partial Agonist	Partial Agonist	Antagonist	Agonist	Antagonist
Raloxifene	Antagonist	Agonist	Antagonist	Agonist	Antagonist
ICI182780	Antagonist	Antagonist	Antagonist	Antagonist	Antagonist

Table1.3 Agonistic/antagonistic effects of E_2 and synthetic ER ligands in target tissues

The molecular mechanism of action of SERMS has been studied extensively and over the years it has become clear that these compounds act differentially from estrogens at several levels from receptor activation to subsequent modulation of transcription. These experiments are performed with ERα.

1. Conformation of the receptor; Differences in conformation of ERα upon ligand binding, probably forms the basis for the selective action of SERMs. E_2 induces a conformational change of ERα characterized by refolding of the ligand-binding pocket (called the mouse trap model). Upon E_2 binding, helix 12 is positioned over the ligand binding pocket formed by helices 3, 5/6 and 11. Both tamoxifen and raloxifene prevent folding of helix 12 over the ligand-binding pocket (Brzozowski et al., 1997). This conformational change of the receptor after binding of tamoxifen causes a blockade of the activity in the AF-2 domain of ERα whereas AF-1 activity is only partially decreased (Shiau et al., 1998). Interestingly, however, in some tissues only AF-1 activation is necessary for an agonistic mode of action of SERMs (uterus), while in other tissues full agonistic activity is only achieved if both the AF-1 and AF-2 domain of ERα are activated (breast) (Berry et al., 1990; Kuiper et al., 1999). This may explain the tissue-selective mode of action of SERMs.

2. Interaction with cofactors: The effect of activation of AF-1 and AF-2 is cell-, and tissue-dependent, and therefore the relative distribution of cofactors (co-activators and co-repressors) in the different tissues may play a role. Co-activators such as SRC1, GRIP1, AIB1 (p160 family) CBP, p300 and pCAF interact primarily with agonist-activated ERα. In several studies it has been shown that overexpression of p160 co-activators in target cells converts tamoxifen from an ER-antagonist into an ER-agonist (Smith et al., 1997). Furthermore, Shang et al. (2002) have shown that the agonistic-effect of tamoxifen on the expression of the IGF1 gene and the MYC gene in endometrial cells may be due to high expression of SRC1. Whereas, in breast cancer cells, where tamoxifen displays an antagonistic-effect on the IGF1 gene and MYC gene, expression of SRC1 is low (Shang and Brown, 2002).

In contrast to co-activators, co-repressors like NCOR, DAX1, RIP140 and SMRT interact preferentially with an antagonist-activated ERα. In mice bearing a genetic disruption of NCOR, it was shown that tamoxifen displays full agonistic properties (Jepsen et al., 2000). However, it is not completely clear, whether co-repressors, like co-activators, are physiological determinators of agonist/antagonistic activation of ERα, or are only engaged when the receptor is bound to a synthetic antagonist (McDonnell, 2004).

3. Receptor phosphorylation: ERα is phosphorylated after binding of ligand, which will increase ligand binding capacity, and induce dimerization and binding of the receptor to the DNA. Some data indicate that this aspect of ERα-signaling also plays a role in determining the effects of SERMs, although results are conflicting. It was shown, that in the presence of E_2, tamoxifen inhibited ER phosphorylation, while in the absence of E_2, tamoxifen, but also the pure anti-estrogen ICI164384, increased phosphorylation of the ER (Ali et al., 1993; Auricchio et al., 1987). Furthermore, in endometrial cancer cells SRC kinase seems to potentiate tamoxifens agonistic activity through phosphorylation of serine 167 which stabilizes ERα (Shah and Rowan, 2004).

4. Receptor dimerization: Estrogen and tamoxifen promote dimerization of ERα, which increases binding to the DNA and subsequent activation of transcription. For the pure anti-estrogen ICI182780, however, several studies have shown that it prevents ERα-dimerization, while other studies show reduced dimerization upon ICI182780 binding (Chen et al., 1999; Wang et al., 1995).

5. Receptor-promoter interaction: Liganded-ERα can initiate transcription directly via binding to an ERE consensus site or indirectly via physically associating with AP-1 or Sp-1 complexes (also see section 1.3.2). Data on the mechanism of estrogen-agonist activity of tamoxifen in the endometrium are controversial. Several authors have shown that in endometrial cancer cells tamoxifen functions as an agonist on promoters containing an AP-1 consensus site, whereas it functioned as a pure antagonist when analyzed on ERE-containing promoters (Norris et al., 1997; Shang and Brown, 2002; Uht et al., 1997; Webb et al., 1995). However, others have claimed an agonistic mode of action of tamoxifen via ERE-containing promoters (Barsalou et al., 1998; Klinge

et al., 2002) (chapter 4). A problem in interpretation of these data is that all studies differ from each other with respect to the used cell lines, reporter constructs and whether the reporter constructs were stable or transient transfected into the cell systems.

6. ERα and ERβ: ERα is more effective in activation of an ERE reporter construct than ERβ, while ERβ is more effective at activating an AP-1 element when occupied with tamoxifen (An et al., 1999; Paech et al., 1997). Furthermore, in bone cells stable transfected to express either ERα or ERβ, it was found that the majority of genes regulated by ERα differs form those genes regulated by ERβ in response to E_2, tamoxifen and raloxifene. Therefore differences in expression levels of ERα and ERβ may also affect the cellular response to estrogens, SERMs and anti-estrogens (Kian Tee et al., 2004).

In conclusion, several factors are summarized that are proposed to play a role to determine the pharmacological activities of ER ligands. However, literature is sometimes conflicting and most data have been derived from artificial systems. In Chapter 4,5 and 6 new insights into the molecular mechanism underlying the mode of action of estrogen, SERMs (tamoxifen and raloxifene), and pure anti-estrogens (ICI182780) in the endometrium, are presented.

1.3.4 Progestins

Progesterone is produced in the ovaries, the adrenal glands, and from conversion of precursors in peripheral tissues. During the menstrual cycle, circulating levels of progesterone are high in the secretory phase because of production in granulosa cells of the corpus luteum. In case of pregnancy, the placenta takes over the production of progesterone from the corpus luteum, after 6-8 weeks (section 1.1.1). As with estrogen, also circulating levels of progesterone are low after menopause because no follicles are growing in the ovaries anymore.

Endometrial hyperplasia and endometrial cancer are associated with high levels of estrogens, in the absence of the growth-inhibitory and differentiative actions of progestagens (Saegusa and Okayasu, 1998). In order to treat patients with hyperplasia or cancer, the progestagen, Medroxy progesterone acetate (MPA) is used. In endometrial cancer, however, response rates for treatment with MPA are low (10-15%) probably due to a transition of hormone-controlled growth toward hormone-independent growth (Lentz 1994; Rose, 1996). The average response duration is only four months, and mean survival is 10 months following the institution of therapy. Primary treatment with MPA has been used in premenopausal women with endometrial cancer who were determined to preserve fertility. Interestingly, in this group the response rate for treatment with high levels of MPA appeared to be in the order of 60% (Kim et al., 1997).

1.3.5 Progestins in tamoxifen-users

Despite the fact that progestins have an anti-mitotic effect on the endometrium, clinical studies could not show a beneficial effect of progesterone for prevention and treatment of tamoxifen-associated endometrial pathologies (Powles et al., 1998). Local treatment with a levenorgestrel-releasing intrauterine device resulted in decidualisation of the endometrium in 85% of women, fewer new polyps were found. However, endometrial thickness and ultrasonographic appearance remained unchanged (Gardner et al., 2000). This observation is similar to that reported after exposure to systemic progestagens, and seems in contrast with the observation that treatment results in decidualisation of the endometrium (Powles et al., 1998). However, current observations suggests that the sonolucent areas, seen after treatment with tamoxifen, probably represent structural changes caused by cystic dilatation of glands within the endometrium and within areas of adenomyosis, and this could explain that a change from a tamoxifen-induced oestrogenic environment to a predominantly progestagenic environment does not change the appearance seen with ultrasound (McGonigle et al., 1998). More prospective trials have to be

done to test the efficacy of progesterone therapy on development of endometrial pathology in tamoxifen-users.

1.4 Growth factor signaling in endometrial cancer

Neoplastic transformation of the endometrium is associated with unbalanced estrogenic stimulation, which can be either endogenous (local conversion of estrogens in fat-tissue) or exogenous (hormone-replacement therapy or tamoxifen) (section 1.1.3 and section 1.2.1 to 1.2.3). Progression of normal cells to a malignancy requires the activation of oncogenes as well as the inactivation of tumor suppressor genes (section 1.1.5). Abnormal regulation of several genes, including growth factors and their receptors, is also frequently observed in several cancers, including endometrial cancer. It is not known, however, what the relation is between estrogenic stimulation and abnormal regulation of genes in endometrial cancer.

In the non-neoplastic endometrium, both stromal and epithelial cells, synthesize cytokines and growth factors, like insulin-like growth factor-1 (IGF1), epidermal growth factor (EGF), transforming growth factor-alfa (TGFα), tumor necrosis factor-alfa (TNFα), colony stimulating factor-1 (CSF1) and interleukins-1 and –6 (IL1 and IL6) (Giudice, 1994) (Gargiulo et al., 2004). These growth factors interact with ER and PR-signaling to actively regulate growth and differentiation of the endometrium and endometrial cancer.

In this section the interaction of IGF and EGF signaling pathways with ER and PR signaling in development and progression of endometrial cancer will be discussed.

1.4.1 IGF signaling

The IGFs are a family of hormones that share high homology with insulin (Rinderknecht and Humbel, 1976). IGFs (IGF1 and IGF2) are small polypeptides (70 amino acids for IGF1 and 67 for IGF2) with 62% homology in their amino acid sequence. The human gene for IGF1 is transcribed from chromosome 12 and the gene for IGF-2 is located on chromosome 11 (Druckmann and Rohr, 2002; Rosenfeld et al., 1990; Sara and Hall, 1990). In order to exert their action, IGFs bind to membrane-associated receptors; the IGF1 and IGF2 receptor. The IGF1 receptor binds both IGF1 and IGF2, although the affinity for IGF1 is higher. The IGF1 receptor is transcribed as a precursor, after which it is processed into an α-subunit and a β-subunit. The functional receptor contains two α-subunits and two β-subunits. Ligand binding induces a conformational change of the receptor, resulting in autophosphorylation of tyrosine residues in the β-subunits. This forms docking sites for adaptor proteins like insulin receptor substrate 1 (IRS1) and SHC. Subsequently, several downstream signaling pathways are activated, like PI3 kinase and the MAP kinase pathway (Surmacz et al., 1998) (Fig. 1.6).

The IGF2 receptor binds IGF2 and also serves as a receptor for mannose-6-phosphate-containing ligands. The IGF2 receptor does not have tyrosine kinase activity, and therefore the mechanism of activation of down-stream signal transduction is less clear. It is hypothesized that, because binding of IGF2 to the IGF2 receptor prevents binding of IGF2 to the IGF1 receptor, this is the biological function of the IGF2 receptor (Ellis et al., 1996; Sachdev and Yee, 2001).

The IGFs are normally bound to the IGF-binding proteins (IGFBPs). The IGFBP family consists of seven proteins, which have high affinity for the IGFs (the IGFBPs), and several lower affinity proteins, the IGFBP related proteins (Hwa et al., 1999). The IGFBPs regulate the bioavailability of IGFs by maintaining a circulating reservoir of IGFs, and by prolonging their half-life. Moreover, there is evidence that indicates effects of IGFBPs independent of IGF. The mechanisms of these IGF-independent effects are still under debate, although a putative receptor for IGFBP3 and IGFBP5 has been described (Hwa et al., 1999).

1.4.2 EGF signaling

The epidermal growth factor receptor (EGF receptor) belongs to the subfamily of protein tyrosine kinases, consisting of EGFR/ERBB, Her2/ERBB2, Her3/ERBB3 and Her4/ERBB4 (Burgess et al.,

2003). The EGF receptor can be activated by several ligands: epidermal growth factor (EGF), transforming growth factor alfa (TGFα), amphiregulin (AREG), heparin binding-EGF (HBEGF), cripto and betacellulin (Carpenter and Cohen, 1990; Pfeiffer et al., 1997). All these ligands share some structural homologies and, as they use the same receptor, their biological activities partly overlap. After ligand binding the EGF receptor homodimerizes or forms a dimere with other members of the subfamily. Dimerization of the EGF receptor activates the intrinsic kinase activity of the cytoplasmic tail of the receptor, which results in phosporylation of the other receptor. This way docking sites are created for adaptor molecules (Yarden, 2001). Subsequently, depending on the tissue type, several downstream signaling pathways can be activated, like the PI3 kinase and MAP kinase pathways (Fig. 1.6).

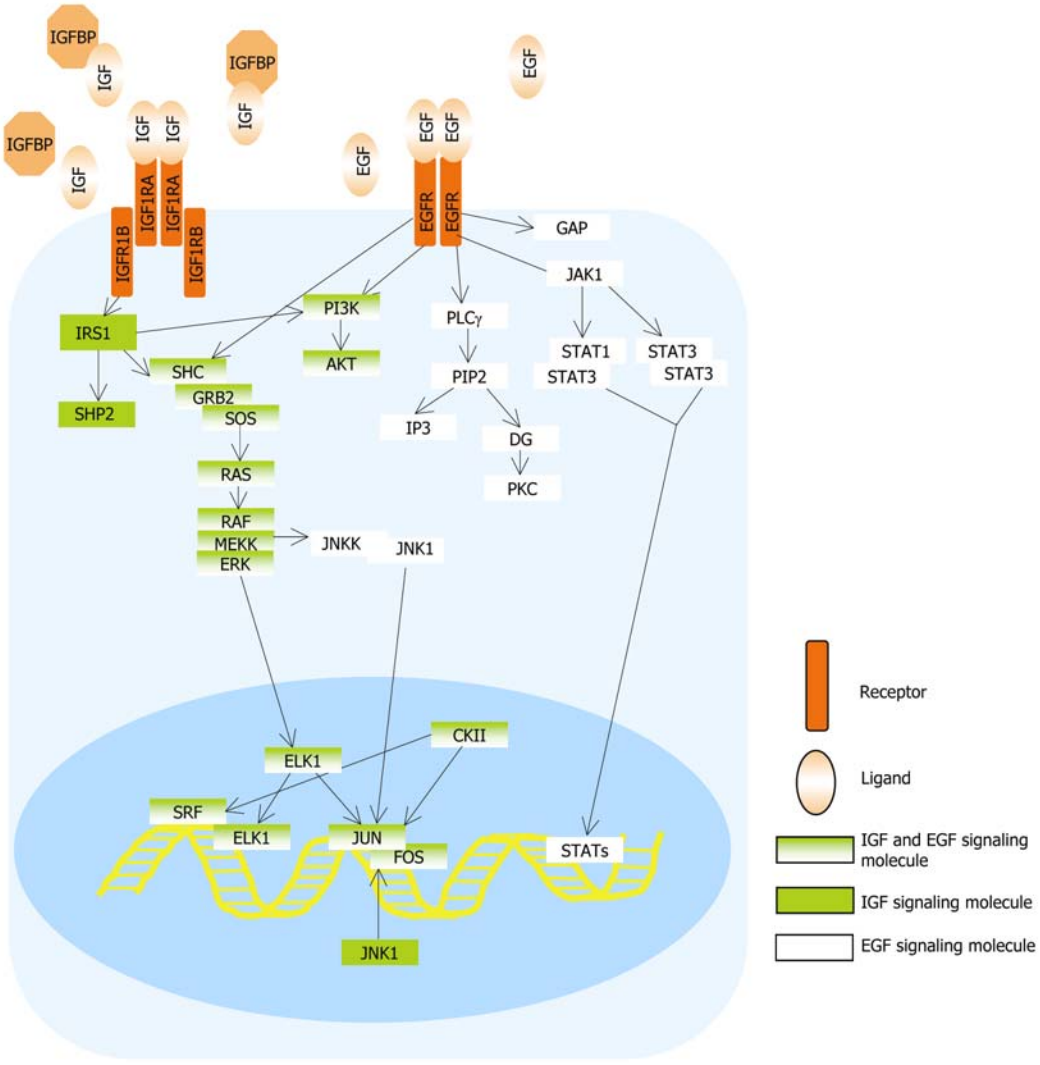


Figure 1.6 General mode of action of IGF and EGF receptor signaling. The IGF receptor and EGF receptor are activated upon binding of growth factor, IGF-ligands or EGF-ligands respectively. If the IGFs are not bound to the receptor, they are normally bound to IGFBPs. After ligand binding, depending on cellular context, diverse signal transduction pathways are activated. In this figure activation of some pathways is illustrated, like RAS that activates MAPK signaling pathway (RAF, MEKK, ERK), JAK kinases that activate STAT1 and STAT3, and phosphorylation of IRS1 and PI3 kinase. As is shown in the figure the IGF receptor and EGF receptor activate common but also different pathways.

1.4.3 IGF signaling in the endometrium and endometrial cancer

Several members of the IGF signaling pathway are expressed in the human endometrium and endometrial cancer. During the menstrual cycle, expression levels of IGF1 are high in the proliferative phase, IGF2 levels are high in the secretory phase, while the expression of the IGF1 receptor and the IGF2 receptor do not change. Furthermore, all six IGFBPs are expressed in the endometrium during the menstrual cycle, with IGFBP5 mainly expressed in the proliferative phase and the other IGFBPs presumably in the secretory phase (Giudice et al., 1991; Zhou et al., 1994).

In endometrial cancer, the levels of IGF1 and IGF2 are lower as during the menstrual cycle, while expression levels of the IGF1 receptor and IGF2 receptor seem to be increased (Maiorano et al., 1999; Roy et al., 1999). Besides measurement of members of the IGF signaling pathway in endometrial cancer tissues, several patient studies have tried to link serum levels of IGFs and IGFBPs to endometrial cancer risk. These data, however, are inconclusive (Augustin et al., 2004; Lacey et al., 2004; Lukanova et al., 2004; Oh et al., 2004; Petridou et al., 2003).

1.4.4 EGF signaling in the endometrium and endometrial cancer

The EGF receptor and its ligands are expressed in the normal endometrium, and for the EGF receptor, EGF and betacellulin, expression levels seem to be increased in the secretory phase compared to the proliferative phase (Moller et al., 2001; Srinivasan et al., 1999). Furthermore, expression of heparin binding-EGF is at its highest just before the window of implantation, suggesting that it plays a role in implantation of the human blastocyst (Chobotova et al., 2002).

In endometrial cancer the six ligands for the EGF receptor are also expressed. Expression of amphiregulin and TGFα is higher in endometrial cancers compared to normal tissues (Pfeiffer et al., 1997). Srinivisan et al. reported that expression of betacellulin is also elevated in endometrial cancer tissue (Srinivasan et al., 1999). As far as we know, there are no reports that link serum levels of EGF receptor ligands to endometrial cancer risk.

1.4.5 IGF and EGF signaling in the endometrium of tamoxifen-users

In postmenopausal women using tamoxifen, treatment for two months resulted in a decrease in serum IGF1 and an increase in IGFBP1 levels compared to matched controls (Bonanni et al., 2001). A certain caution is necessary regarding these data since patient numbers are low and it is reported only once. Furthermore, there is conflicting evidence whether the expression of IGF1 in normal endometrial tissues is different from that in tamoxifen-users and in non-users (Elkas et al., 1998; Laatikainen et al., 1995). However, it is clear that in endometrial cancers, expression levels of IGF1, IGF2 and their receptors, are similar between spontaneous arising tumors and tamoxifen-associated tumors (Roy et al., 2000).

1.4.6 Cross-talk between estrogen and growth factor signaling in endometrial cancer

Many examples of interactions between ER signaling and growth factor signaling have been reported. Three distinct mechanisms of interaction can be distinguished.

1. Activation of IGF1 and EGF receptor by ERα.

In COS7 and HEK293 cells, it has been shown that after addition of E_2 ERα rapidly induces phosphorylation of IGF receptors (Kahlert et al., 2000). More evidence for activation of the IGF receptor by ERα occupied with several ligands came from experiments performed in mouse models. In the uterus of wild type mice, stimulation with E_2, or tamoxifen, resulted in activation of the IGF1 receptor signaling pathway though phosphorylation of the receptor. Administration of ER ligands to ERα knockout mice did not activate the IGFI receptor, indicating that ERα is required for activation of uterine IGFI receptors (Klotz et al., 2000). Furthermore, in IGF1 knock out mice (IGF1KO), DNA synthesis occurred in the uterus after stimulation with E_2. However, the mitotic index in response to E_2, is much lower in IGF1KO mice compared to wild type mice, indicating that IGF1 is an important factor for the cells to progress properly through mitosis in response to E_2 (Adesanya et al., 1999).

ERα has also been shown to activate EGF receptor signaling in cultured endometrial cells by induction of phosphorylation of the EGF receptor (Ishihara et al., 1991). Whether in

endometrial cancer E_2 and tamoxifen also directly activate the IGF and EGF signaling pathways remains to be determined and was investigated in Chapters 3, 4 and 5 of this thesis.

2. Activation of the ER by IGF1 and EGF receptor

The IGF1 and EGF receptor have been implicated in regulation of the activity of the ERα in a number of observations. In a study of Kato et al. it was demonstrated that ERα is phosphorylated on Ser^{118} within 15 minutes after addition of IGF1 or EGF. Phosphorylation of this serine is necessary for full activity of the AF-1 domain of ERα (Kato et al., 1995). Furthermore, using transgenic mice carrying a luciferase gene driven by two estrogen response elements (ERE-luciferase mice), it was shown that treatment with IGF1 resulted in an increased luciferase signal. The luciferase signal was attenuated by addition of the pure anti-estrogen ICI182780, indicating that the ER was involved in activation of the ERE-reporter construct (Klotz et al., 2002). For EGF signaling it has been shown that EGF activated uterine DNA synthesis is also reduced by anti-estrogen treatment (Ignar-Trowbridge et al., 1992).

Whether activation of ERα by growth factor receptors also plays a role in endometrial cancer is not yet clear. Gehm et al. have shown that in Ishikawa cells, EGF activates an ERE-reporter construct, however, induction of this luciferase signal can not be inhibited by additional treatment of an anti-estrogen (Gehm et al., 2000). In Chapter 3 of this thesis activation of the ERα and ERβ signaling pathway by IGF1 in endometrial cancer cells is investigated.

3. Synergistic effects of hormones and growth factors on the expression and activation of several genes.

So far it has been shown that in several systems, including endometrial cancer cells, ER ligands as well as IGFs and EGF stimulate proliferation. Since ERα can activate the IGF1 and EGF receptor and vice versa, the IGF1 and EGF receptors activate the ERα, the question arises if ER ligands as well as growth factors activate the expression of similar genes. In breast cancer cells, it has been shown that both IGF1 and E_2 induce changes in the expression of cell cycle components, leading to activation of the cyclin E/cyclin-dependent kinase inhibitor, CDK-2 (Dupont et al., 2000; Lai et al., 2001). Furthermore, both IGF1 and E_2 significantly increase the expression of cyclin D1 (Hamelers and Steenbergh, 2003).

Again is not clear for endometrial cancer whether induction of proliferation by ER ligands and activation of the IGF1 or EGF pathway is accomplished by regulation of similar genes. In chapter 5 of this thesis gene-expression profiles were generated of E_2, Tamoxifen, IGF1 and the EGF receptor ligand amphiregulin to elucidate these mechanisms.

1.5 Micro-array

Micro-array analysis, a tool to analyze large-scale differences in mRNA expression between conditions or tissues, has recently been developed. RNA expression profiles of, for example treated cells versus untreated cells, or normal tissues versus different stages of cancer, are generated. A comparison between the expression levels of potentially all genes in the genome, for two or more conditions, can be made. The flowchart of the micro-array technique is depicted in Figure 1.7.

Generally, two types of micro-arrays exist (both were used in the current experiments):

1. Single probe hybridization arrays: This type of array is produced by Affymetrix and is manufactured under the name of "The Gene Chip". It contains hundreds of thousands of ordered, single-strand, synthetic oligonucleotides that are typically 25 bases in length and are synthesized on the glass surface of the chip. RNA samples are reverse transcribed into cDNA in which one nucleotide is tagged with a biotin label. Each micro-array measures the RNA abundance of thousands of different genes in one single RNA sample (no reference sample is used). The resulting data represent absolute levels of RNA (Fig.1.7 right panel).

2. Competitive dual-probe hybridization arrays: This type of array contains double-stranded oligonucleotides created by PCR, or synthesized, and spotted on the surface of glass sides (array). Two samples of RNA (treated versus non-treated cells for example) are reverse transcribed, labeled with two different dyes (for example treated cells with Cy5 and non-treated cells with Cy3), and simultaneously hybridized to one micro-array. The resulting data represent the relative concentration of a certain transcript in a treatment condition compared to a reference condition (treated versus non-treated cells) (Fig. 1.7 left panel).

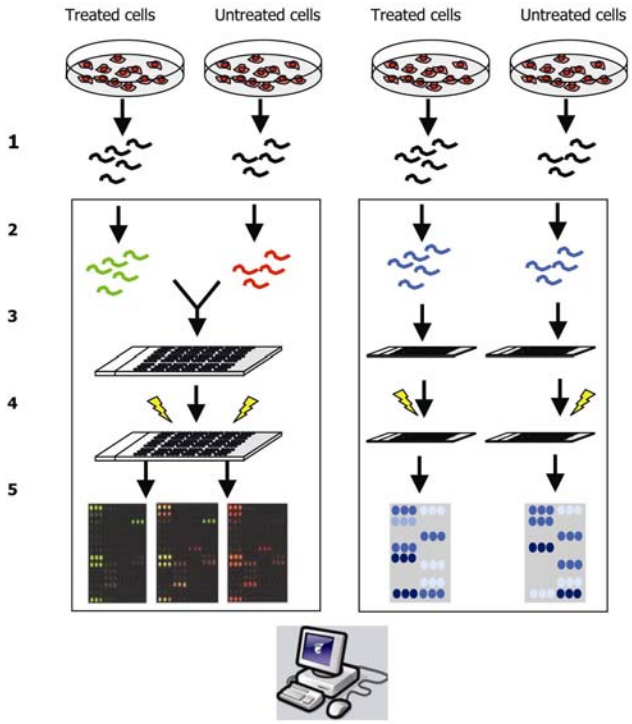


Figure 1.7. Flow chart of micro-array experiments. The left panel indicates the flow chart for competitive dual-probe hybridization and the right panel for single probe hybridization. 1.Two samples of RNA are isolated, from for example treated cells and untreated cells. 2. RNA is reverse transcribed into cDNA in which either different dyes, Cy3 and Cy5 (left panel) or similar dyes, biotin (right panel) are incorporated. 3. The samples are hybridized to one micro-array slide, or two separate micro-array slides. 4. After appropriate washing, the slides are scanned. 5. The generated data are either a combination of two channel-readings (left panel) or are two separate readings from one channel (right panel), and can be analyzed after normalization of the acquired signal intensities.

1.5.1. From cells to genes to biology

Several steps are essential to generate reliable results of micro-array experiments. After identification of differentially expressed genes, the next step is to study the biology of these genes. Since in a well-performed micro-array experiment an enormous amount of data is usually generated, computerized methods were developed that help to find relevant answers. For the micro-array data described in this thesis, the following steps were taken to evaluate results and to try to answer our research questions.

1. Processing of the raw expression data. This involves a systemic quality-check of each spot and the acquirement of background and foreground signals. The background signal is subtracted from the foreground signal, which results in the corrected value for each spot. Several methods are developed to determine the background signal; defining the local background near the spot

or determining the morphological opening, which determines the background at the actual spot location (Smyth and Speed, 2003). The main purpose is, however, to identify and remove unreliable spots, for example spots in which the background signal is higher then the foreground signal, or spots that have an aberrant shape, or distribution of pixel intensities.

2. Normalization of the data. Micro-array experiments should be normalized to adjust for systematic variation caused by other factors then differential expression. The two types of micro-arrays (single probe hybridization and competitive dual-probe hybridization) are normalized in a different way. For both types of arrays normalization can be performed using either data from user-selected genes (for example a group of housekeeping genes) or data from all genes on the array. Using all genes for normalization will introduce less bias (Kim et al., 2002).

Normalization of single probe hybridizations (Affymetrix array) is done by so called scaling which proposes that intensities should be scaled so that each array has the same average value.

Normalization of competitive dual-probe hybridization aims to remove systematic errors by balancing the fluorescence intensities of the two labeling dyes. This dye bias is caused by differences in labeling efficiency, heat and light sensitivity and from scanning procedures of the two dyes. In general, three normalization steps are performed: (i) within-slide normalization, (ii) paired-slide normalization for dye-swap experiments, and (iii) multiple slide normalization. For a comprehensive description of different normalization methods please see (Smyth and Speed, 2003) and (Yang et al., 2002).

3. Pattern determination to find interesting groups of genes for further analysis. Methods to identify genes that can answer your research question can be divide into two groups:
A. pattern discovery or unsupervised methods, to characterize internal structures or relationships in a data set without any previous knowledge.
B. class prediction or supervised approaches, to identify genes that fit a predetermined pattern.

Both methods provide visual graphics, which help to determine which genes to focus on in further research. The following programs were used: the Omniviz package (http://www.omniviz.com) and EPSCLUST (Expression Profile data CLUStering and analysis) at http://ep.ebi.ac.uk/EP/EPCLUST.

A. pattern discovery or unsupervised methods:
The unsupervised analysis method used in this thesis is cluster analysis. By performing cluster analyses, a collection of differential genes (for example from 20 patients of which ten have a type of cancer and the others have a pre-malignant histological abnormality) are grouped into clusters, such that those genes within each cluster are more closely related to one another than genes assigned to different clusters.

B. class prediction or supervised approaches:
In this method the biological samples are divided into groups with similar behavior of properties. In this way, specific genes are identified that correlate to the defined groups. The classification of cancer type samples into several groups based on histological type and grade is an example of this type of analysis.

4. Annotation, to determine the function of selected genes and their possible relation. If groups of interesting genes have been selected using clustering, the next step is to determine the biological function of these genes and their interrelationship. Again, several methods are developed to better understand the biological meaning of the generated data. We performed the following analysis:

A. comparison with literature: using all information from Pubmed, array data can be combined with data from literature. Data can, for example, be compared to a disease type, an important family of genes, a well-known biological function, and so on (www.pubmatrix.grc.nia.nih.gov).

B. biological classification: using the gene ontology (GO) database, genes can be classified into several groups based on the biological process, cellular component or molecular function in which they take part. The next level is then to assign the genes into different biological pathways based on GO classifications (www.geneontology.org).

C. biological network: the basis for the construction of a genetic network is the assumption that if two genes are co-mentioned in a MEDLINE record there is an underlying biological relationship. What this program does is to visualize these relationships in a network, which then represents biological knowledge in a formalized form with the focus on how proteins, cellular processes and small molecules interact, modify and regulate each other.

We used the Ingenuity database (www.analysis.ingenuity.com/pa/) and Pathway Assist (www.ariadnegenomics.com/products/pathway)

1.6. Aims and outline of this thesis

1.35% of women develop endometrial cancer during their life. Although in most cases the tumor is confined to the uterus, in approximately 25% of cases the tumor has already spread beyond the uterus upon surgery and eventually these patients will die of metastasis.

In the normal endometrium, growth and differentiation is controlled by the ovarian hormones estrogen and progesterone. After menopause, the absence of follicle recruitment in the ovary results in a decline in serum levels of estrogen and progesterone, and consequently results in an atrophic/inactive state of the endometrium. However, in some women increased levels of estrogen (endogenous or exogenous) are present, which will stimulate the endometrium. And these high estrogen-levels are associated with an increased risk of endometrial pathologies, including endometrial cancer. As in the normal endometrium, progesterone inhibits growth of endometrial cancer cells and is therefore used in the clinic as adjuvant therapy. Concerning the role of estrogen and progesterone signaling in normal and aberrant endometrial growth, we addressed the following question.

1. What are the molecular mechanisms underlying estrogen-induced growth stimulation and progesterone-induced growth inhibition of endometrial cancer cells?

In Chapter 2, gene expression profiles were produced of endometrial cancer cell lines cultured with estrogens and/or progestagens in order to reveal molecular mechanisms underlying endometrial growth regulation by these two hormones.

Activation of the ER signaling pathway as well as activation of EGF and IGF signaling pathways stimulates proliferation of the endometrium and endometrial cancer. Concerning this, we asked the following question:

2. Does activation of the ER signaling pathway result in activation of IGF or EGF signaling, and *vice versa*, does activation of the IGF and EGF signaling pathways result in activation of ER signaling?

In Chapter 3, the role of IGF signaling was determined during estrogen-induced growth stimulation and progesterone-induced growth inhibition. Furthermore, experiments were conducted to study cross-talk between ER signaling and IGF1 signaling during proliferation. In Chapter 5, the cross-talk between ER signaling, and the IGF1 and EGF signaling pathways was studied in several ways. First of all, direct activation of the IGF1 and EGF receptor by estrogen

and tamoxifen was studied. Secondly, the overlap in the regulation of genes, after long-term culture in the presence of estrogen, tamoxifen, IGF1 and amphiregulin was studied.

Besides estrogens, also the estrogen-agonistic effects of tamoxifen induce growth of the endometrium. This is in contrast to the activities of another SERM, raloxifene, and the anti-estrogen ICI182780, which do not induce growth of the endometrium. Because it has been documented that SERMs display different characteristics in different tissues, the question was raised what the molecular mechanism underlying the estrogen-agonism of tamoxifen in the endometrium could be? And more specific:

3. Which genes are regulated by estrogen, tamoxifen, raloxifene and the anti-estrogen ICI182780 in endometrial cancer cells, and do the four ER-ligands regulate similar genes, in the same cellular processes or pathways?

In Chapter 4, gene expression profiles were generated for estrogen, tamoxifen, raloxifene and ICI182780 stimulation of a well-differentiated estrogen-responsive endometrial carcinoma cell line. For each of these ligands, affected biological processes were studied, and the interrelationship between the regulated genes was assessed.

Women using tamoxifen experience a 2- to 7-fold higher incidence of endometrial cancer. Furthermore, tamoxifen-induced endometrial tumors in general are more aggressive and less differentiated than other endometrial tumors. Surprisingly, however, when tamoxifen induced endometrial tumors are compared to matched control endometrial tumors there are no differences detected in gene-expression profile. Therefore we studied the molecular mode of action of tamoxifen during the early benign stages that may result in tumor formation. The following question was raised:

4. Which genes are regulated in endometrial tissues of tamoxifen-users compared to non-users, and can we, based on the generated gene-expression profiles, elucidate which pathways are activated by tamoxifen during the early changes which may lead to endometrial cancer formation?

In Chapter 6, gene-expression profiles of endometrial tissues of tamoxifen-users and non-users were generated and analyzed. Furthermore, on the basis of a network analysis, the mode of action of tamoxifen in tumorigenesis was evaluated.

The results of these investigations, in the context of the above questions, are discussed in Chapter 7.

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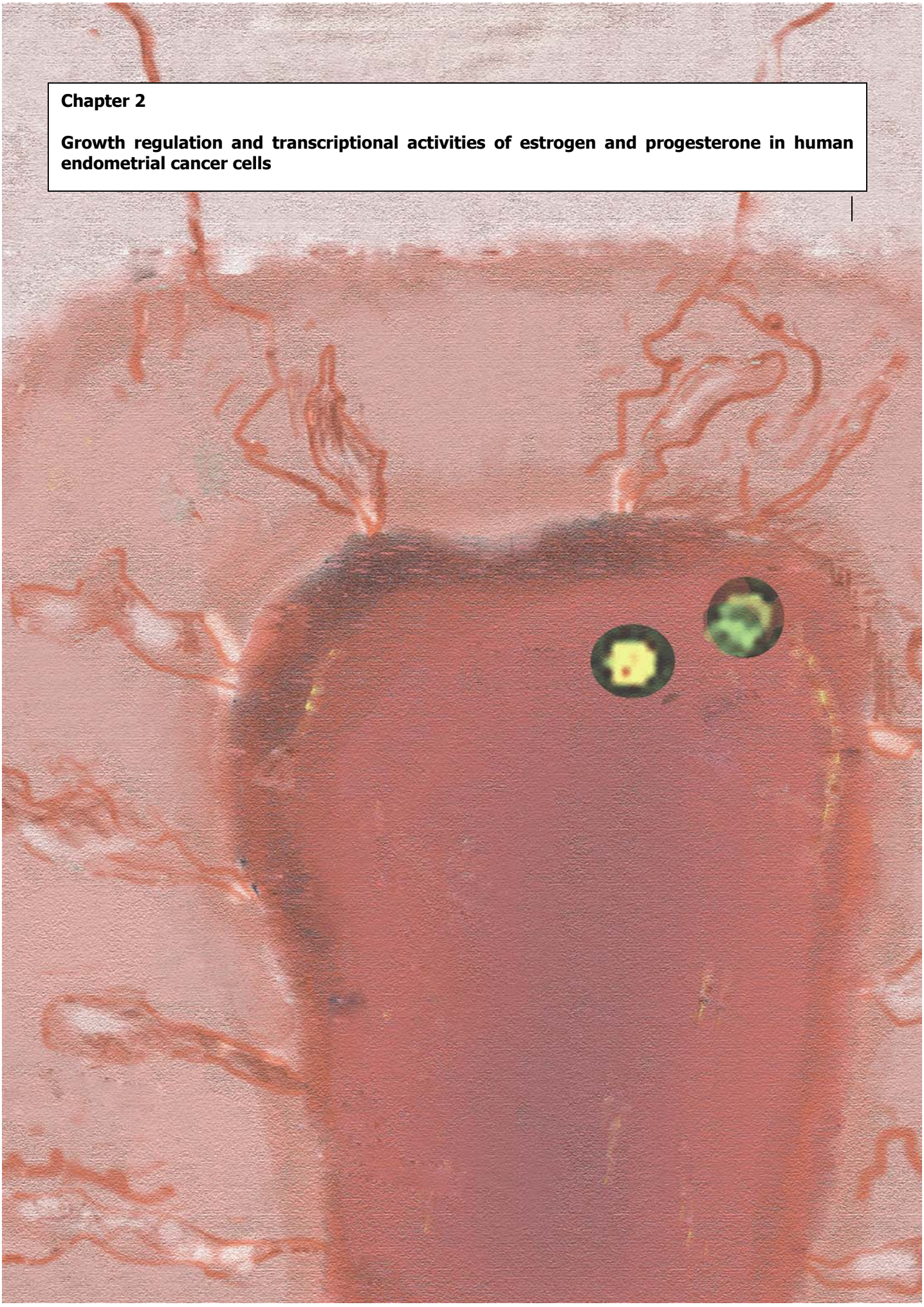
Chapter 2

Growth regulation and transcriptional activities of estrogen and progesterone in human endometrial cancer cells



Growth regulation and transcriptional activities of estrogen and progesterone in human endometrial cancer cells

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Abstract:

Estrogen stimulated growth of the malignant human endometrium can be balanced by the differentiating properties of progesterone. To study the molecular basis behind this, gene expression profiling was performed using cDNA microarray analysis.

In this study, the human endometrial cancer cell lines ECC-1 and PRAB-36 were used as models. The ECC-1 cell line, which expresses high levels of ERα and is stimulated in growth by estrogens, was used to study estrogen regulation of gene-expression. The Ishikawa sub cell line PRAB-36, expressing both PRA and PRB, and inhibited in growth by progestagens was used to study progesterone regulation of gene expression.

Using these two well-differentiated human endometrial cancer cell lines, 148 estrogen- and 148 progesterone-regulated genes were identified. After functional classification, the estrogen- and progesterone-regulated genes could be categorized in different biologically relevant groups. Within the group of "cell growth and/or maintenance" 81 genes were clustered, from which a number of genes could be involved in arranging the cross-talk which exists between estrogen and progesterone signaling.

On the basis of analysis of the current findings it is hypothesized that cross-talk between estrogen and progestagen signaling does not occur by counter regulation of single genes, but rather at the level of differential regulation of different genes within the same functional families.

Keywords: estrogen, progesterone, endometrium, micro-array, proliferation

2.1 Introduction

In the normal human endometrium, the growth stimulatory actions of estrogens are counteracted by the differentiative (growth inhibitory) properties of progestagens. For endometrial cancer, which is one of the most common gynecological malignancies in the Western world, it is well-established that risk factors are related to increased levels of exogenous and/or endogenous estrogen levels (Akhmedkhanov et al., 2001; Hale et al., 2002; Schottenfeld, 1995). As in the normal endometrium, progesterone is able to suppress growth of endometrial cancer cells (Persson et al., 1989). Therefore, in clinical practice, progesterone is used, in a palliative setting, as treatment of advanced and recurrent endometrial cancer (Rose, 1996).

The cellular actions of estrogens and progestagens are mediated through binding to their specific receptors, the estrogen (ER) and progesterone (PR) receptor. These receptors are members of the nuclear receptor superfamily and are, upon ligand binding, recruited to specific HRE (Hormone Response Elements) sequences on the DNA (Evans, 1988). From these sites they can stimulate or inhibit transcription of certain genes.

The human estrogen receptor (ER) exists as two isoforms, ERα (Green et al., 1986) and ERβ (Kuiper et al., 1996). ERα and ERβ are translated from two different genes (located at chromosome 6 and chromosome 14, respectively) and are both expressed in the uterus (Taylor et al., 2000). In the inner uterine layer, the endometrium, a much higher expression of ERα is observed compared to the level of ERβ. In endometrial cancer the situation is as follows: in well-differentiated endometrial cancer ERα is much higher expressed than ERβ (Fujimoto et al., 2002) but in poorly differentiated and/or metastatic disease, there seems to be a selective loss of ERα, which results in a relative up-regulation of ERβ (Matsuzaki et al., 1999; Matsuzaki et al., 2000; Utsunomiya et al., 2000). These observations indicate that estrogen signaling in the normal endometrium and in early stage endometrial cancer occurs through ERα, while in late stage disease ERβ could be more important.

The human progesterone receptor isoforms, PRA and PRB, are translated from the same gene located at chromosome 11 (Horwitz and Alexander, 1983). The PRA is a truncated form of the PRB, lacking the first 164 amino acids at the N-terminus (Kastner et al., 1990). In the glandular epithelium of the normal endometrium both isoforms are expressed during most stages of the menstrual cycle, but expression levels vary (Mote et al., 1999). In endometrial cancer, expression of the PR is inversely related to clinical grade and stage, with proportionally lower levels of PR in more advanced disease (Arnett-Mansfield et al., 2001).

The molecular mechanisms involved in estrogen-induced growth induction and progesterone-induced differentiation of the human endometrium and endometrial cancer are largely unknown. Gaining insight in these processes, will enable us to understand the steps leading to the induction and progression of endometrial tumors. This may eventually result in better diagnostic tools and treatment modalities.

To begin to understand estrogen and progesterone mediated signaling, expression profiling using cDNA micro-array technology was used. In the ER-expressing ECC-1 cell line, estrogen-induced growth stimulation and gene regulation was investigated, and in the PR-expressing Ishikawa sub-cell line PRAB-36, progesterone-induced growth inhibition and gene-regulation was studied. Based on analysis of the presented results it is hypothesized that estrogen-induced growth stimulation and progesterone-induced growth inhibition is achieved by differential regulation of different genes within the same functional families.

2.2 Materials and methods

Cell culture:
The ECC-1 cell line is derived from a well-differentiated human endometrial adenocarcinoma, transplanted into nude mice, and was a generous gift from Dr. B. van den Burg (Utrecht, The Netherlands) (Satyaswaroop et al., 1988). The PRAB-36 cells are Ishikawa cells which have been stably transfected to express high amounts of hPRA and hPRB (Blok et al., 2003). The original

Ishikawa cell line, Ishikawa clone 3H12, is derived from a well-differentiated human endometrial carcinoma and was obtained from Dr. M. Nishida, (Tsukuba, Japan) (Nishida et al., 1996). The PRAB-36 cells were maintained under selection pressure by neomycin (G418: 500µg/ml, Invitrogen Life Technologies, Breda, The Netherlands) and hygromycin (250µg/ml, Invitrogen Life Technologies). The T47D cell line is derived from a well-differentiated human breast cancer and was a generous gift from Dr. B. van den Burg. The cells were routinely maintained in DMEM/F12 + 10% Fetal Bovine Serum (FBS, Perbio Science, Helsingborg, Sweden), supplemented with penicillin/streptomycin in a 37ºC incubator at 5% CO_2.

Western immuno-blotting:
For these experiments, ECC-1 and PRAB-36 cell lines were cultured in phenol red free DMEM/F12 + 5% Dextran Coated Charcoal treated FBS (DCC-FBS). Cells were cultured for 72 hours in the presence or absence of 1nM estradiol (E_2) or 100nM of the progestagen medroxy progesterone acetate (MPA). The T47D cell line was cultured routinely. Cells were washed twice with Phosphate Buffered Saline (PBS), lyzed in RIPA buffer (40nM Tris-HCL (pH 7.4), 5mM EDTA (pH 8.0), 10% glycerol, 10mM sodiumphosphate, 10nM sodiummolybdate, 50mM sodiumfluoride, 0.5mM sodiumorthovanadate, 10mM DTT, 1% Triton, 0.08% SDS, 0.5% deoxycholate, and protease inhibitors: 6mM PMSF, 5mM bacitracin, 5mM leupeptin) and centrifuged for 15 minutes at 60.000xg at 4ºC. The proteins were separated on a SDS polyacrylamid gel and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH, USA). The membrane was washed with PBS-Tween (0.5%) and blocked for 1 hour with blocking solution, 5% skinned milk in PBS/Tween. The membrane was incubated with the following antibodies: ERα (sc-8002) mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:1000, PRA/B (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology) 1:2000, in blocking solution overnight at 4°C. The membrane was washed three times for 5 minutes with PBS-Tween. Antibody-peroxidase conjugate was diluted 1:2000 in blocking solution and incubated with the membrane for 1 hour. The membrane was washed 6 times for 10 minutes with PBS-Tween. The bands were detected using Dupont/NEN's Luminol chemiluminescence's procedure and visualized by exposing the blot to film (Kodak X-Omat, New Haven, Ct, USA) for at least 5 minutes.

Hormone binding assays:
All cell lines were cultured routinely in 12-well plates (Nunclon Surface, Nunc Brand Products, Denmark), to 70% confluence. For binding to the estrogen receptor, cells were incubated with 1nM ^{3}H-estradiol (NEN Life Science Products, Inc, Boston, MA, USA) in the presence or absence of 200nM non-labeled estradiol (Steraloids, Wilton, NH, USA). For binding to progesterone receptors, cells were incubated with 10nM ^{3}H-R1881 (NEN Life Science Products, Inc, Boston, MA, USA), in the presence or absence of 2µM non-labeled triamcinolone acetonide (Sigma, St.Louis, USA) or 2µM non-labeled R1881 (NEN Life Science Products). Cells were incubated for 2.5 hours in a 37°C incubator at 5% CO_2. Cells were washed five times with PBS at 0°C. The cells were lyzed in 500µ1 1M NaOH for 60 minutes at 37°C. 100µl of the lysate was diluted in 5ml Picofluor 15 (Packard Bioscience Company, Groningen, The Netherlands) and radioactivity was measured in a liquid scintillation counter (Packard Bioscience Company, type 2700TR). For all samples OD260 nm measurements were performed. Furthermore, for each cell line a standard curve was produced which indicates OD260 nm measurements per cell number, enabling us to estimate binding per 100,000 cells.

Growth studies:
Cells were passaged to 24-well plates, at 5000 cells per well, in phenol red free DMEM/F12 containing 5% DCC-FBS in the presence or absence of the indicated concentrations of estradiol (E_2), the partial anti-estrogen 4OH-tamoxifen (Sigma-Aldrich Chemie BV, The Netherlands), the progestagen Medroxyprogesterone Acetate (MPA) (Sigma-Aldrich), or the anti-progestagen Org-31489 (N.V. Organon, Oss, the Netherlands). After 10 days of culture, cells were washed twice in PBS and harvested in 150µl of 1M NaOH for 30 minutes at 60°C. After complete lysis of the cells,

850µl H_2O was added to each sample and OD260 nm measurements were performed in order to measure cell growth (Blok et al., 2003). The standard deviations were calculated from quadruplicate incubations within one representative experiment.

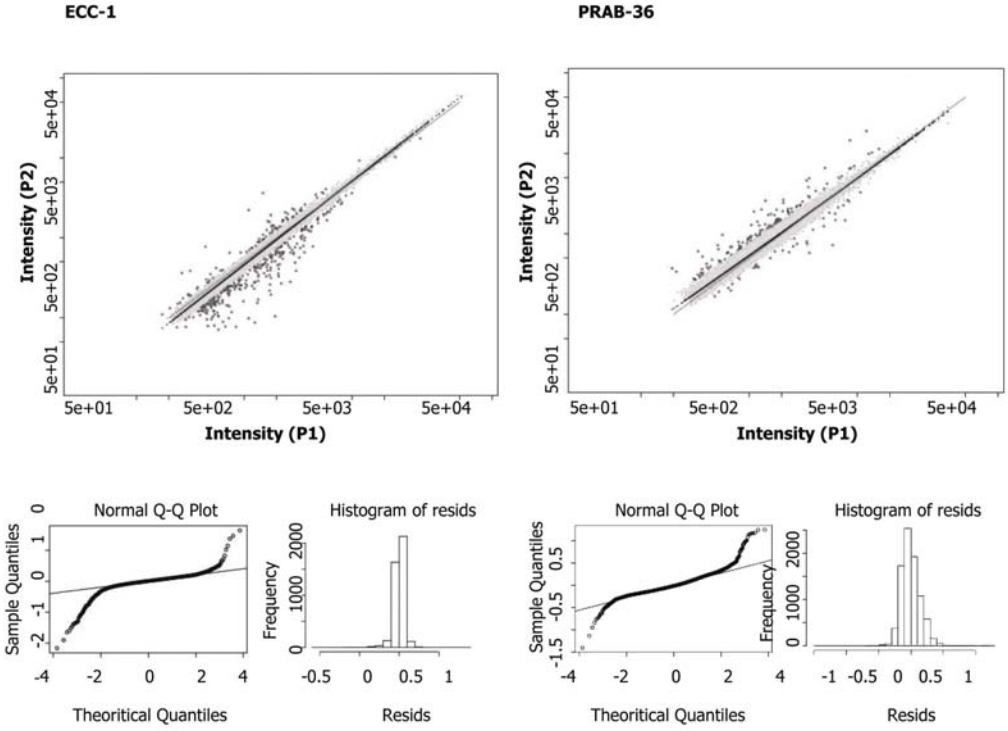

Figure 2.1
Differentially expressed genes in the experimental versus the control sample were selected by a statistical method derived from boxplots. Further, based on Q-Q plots and histograms genes are selected to be differentially expressed with a p value=0.995.

Micro-arrays:
The cell lines were cultured in phenol red free DMEM/F12 + 5% DCC-FBS. ECC-1 cells were cultured for 72 hours in the presence or absence of 1nM E_2. PRAB-36 cells were cultured for 48 hours in the presence or absence of 100nM MPA. Total RNA was isolated using the lithium chloride/urea method (Auffray and Rougeon, 1980). Micro-array hybridizations were performed with Human Unigene 1 (Incyte Genomics, Inc., St.Louis, MO, USA) containing 9.600 cDNA spots. Both labeling and hybridization were performed according to previously described protocols (Schena et al., 1995). Briefly: 200ng of polyA+ RNA from every experimental variant or reference sample were labeled using reverse transcriptase reaction in the presence of dNTPs and either a Cy3 or Cy5 random 9mer. Hybridization results on individual spots were selected for further analysis only when the intensity signal to background ratio was greater than 2.5 and when the area occupancy under the grid was greater than 40 percent. Following signal quantification, a signal correction algorithm was used to correct for systematic differences between the Cy3 and Cy5 labels. This algorithm applied a 2nd order polynomal regression model to the data by fitting a parabola through log-transformed Cy3 versus Cy5 intensities. The residuals of the regression model were taken as the new gene expression ratios. Genes that were significantly differentially expressed in the experimental versus the control sample were selected by a statistical method derived from boxplots, which are widely used to visualize the overall shape of a data set (Venables and Ripley, 1997) (Fig. 2.1). In our experiments, in which a single perturbation is compared to an isotypic reference, the expression of the majority of genes will not differ between the experimental sample and the control sample and their ratios will be in the center of the

distribution of expression ratios. Therefore, we computed the 1st (Q1) and 3rd (Q3) quartiles of the distribution of the residuals of the regression model and the inter-quartile range (IQR) of the distribution as a measure for the variation in the expression ratios of non-differentially expressed genes. Then, in analogy with box plots, an inner fence was set at Q1-(1.58 x IQR) and Q3+(1.58 x IQR). Using these criteria, genes within the inner fence have a probability of p=0.995 to be non-differentially expressed, whereas the outlier group will harbour the differentially expressed genes. Genes were selected for further study if they fell outside the inner fence (Fig. 2.1).

Northern blotting:
Cells were cultured in phenol red free DMEM/F12 + 5% DCC-FBS for 72 hours in the presence or absence of 1nM E_2, or for 48 hours with or without 100nM MPA. Total RNA was isolated using lithium chloride/urea (Auffray and Rougeon, 1980), separated using 1.5 % agarose gels and blotted to nitrocellulose membranes (Blok et al., 1995). The following ^{32}P-labeled probes were hybridized to the blot: AREG; IMAGE: 4277616, CCNG2; IMAGE: 249688 (kindly provided by Dr. G. Jenster), BTG1; IMAGE: 5211519, BTG2; IMAGE: 1249983, CCND1; IMAGE: 5240197 and SNK; IMAGE: 4750649, all acquired from RZPD, Germany. The hamster β-actin probe was used to verify equal loading of samples.

2.3 Results

Analysis of steroid receptors in ECC-1 and PRAB-36 cell lines
The actions of estrogens and progestagens are mediated through binding to their specific receptors, ER and PR. By performing Western blotting and ligand-binding assays, ER and PR expression levels were determined in our model cell lines. Because the ligand used for PR measurements (^{3}H-R1881) also effectively binds to androgen receptors, a 500-fold molar access triamcinolone acetonide was used. Triamcinolone acetonide binds with a high affinity to the progesterone receptor and not to the androgen receptor, which makes it possible to measure specific binding of ^{3}H-R1881 to the androgen receptor (Zava et al., 1979). No significant androgen receptor expression was observed. The T47D cell line was used as a positive control for ER and PR expression.

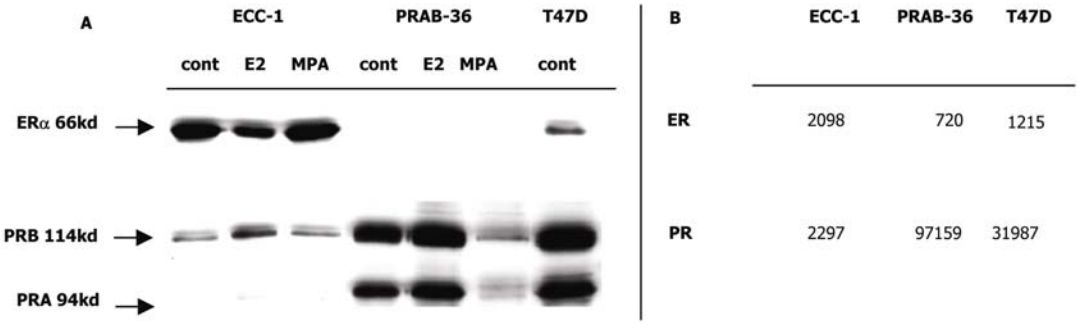

Figure 2.2 Expression of steroid receptors

A: Protein expression of estrogen and progesterone receptors (ER and PR) in ECC-1 and PRAB-36 cells. Cells were cultured in the presence or absence of either estradiol (E_2) or medroxy progesterone acetate (MPA) for 72 hours. As a control T47D cells were used.
B: Binding of ^{3}H-ligands to the ER and PR. For binding to the ER, cells were incubated with 1nM ^{3}H-E_2 in the presence or absence of 200nM estradiol. For binding to the PR, cells were incubated with 10nM ^{3}H-R1881 in the presence or absence of 2μM Triamcinolone Acetonide or 2μM R1881. Ligand binding is represented as disintegrations per minute (dpm) per 100.000 cells.

Using a specific antibody against ERα, it was observed that ECC-1 cells express high levels of ERα, T47D cells express low levels of ERα and PRAB-36 cells do not express detectable levels of ERα (Fig. 2.2a). Furthermore, culture in the presence of estrogen resulted in down-

regulation of ERα. When ERβ-expression was reviewed, we could only detect weak antibody binding to all cell lines (data not shown). Using radioactively labeled E_2, ligand binding was observed in ECC-1 and T47D cells, while PRAB-36 cells showed low ligand binding (Fig. 2.2b).

For detection of progesterone receptors, a polyclonal antibody was used which recognizes both PRA and PRB. PRAB-36 and T47D cells express the highest levels of PRA and PRB, and ECC-1 cells also express some PR (mainly PRB) (Fig. 2.2a). Specific ligand binding assays confirmed these Western blotting data (Fig. 2.2b). Furthermore, it was observed that estrogen treatment seems to induce some PRB expression in ECC-1 cells, while MPA is very efficient in reducing PRA and PRB expression in PRAB-36 cells (Fig. 2.2a).

Growth effects of steroids in the cell lines

To study growth modulation, ECC-1 and PRAB-36 cell lines were cultured for 10 days, in the presence or absence of the indicated hormones (Fig. 2.3). The ECC-1 cell line was stimulated in growth by estrogen in a dose dependent way (Fig. 2.3a). By using tamoxifen, which is a partial estrogen antagonist, the growth stimulating properties of E_2 could almost be abolished (Fig. 2.3b). Despite the fact that ECC-1 cells do express some PRB (Fig. 2a), no MPA-induced growth inhibition was observed (Fig. 2.3c). Also when ECC-1 cells were cultured with 1nM E_2 in combination with varying concentrations of MPA, the growth stimulatory properties of E_2 could not be reverted or otherwise influenced by MPA (Fig. 2.3c). Culturing the PRAB-36 cell line in the presence of MPA gives a dose dependent growth inhibiting effect (Fig. 2.3d). This growth inhibiting effect of MPA in the PRAB-36 cells could readily be reverted by using an anti-progestagen, Org-31489 (Fig. 2.3e). Culturing the PRAB-36 in the presence of E_2, or E_2 in combination with MPA, did not result in any E_2 effects on growth of the cells (Fig. 2.3f).

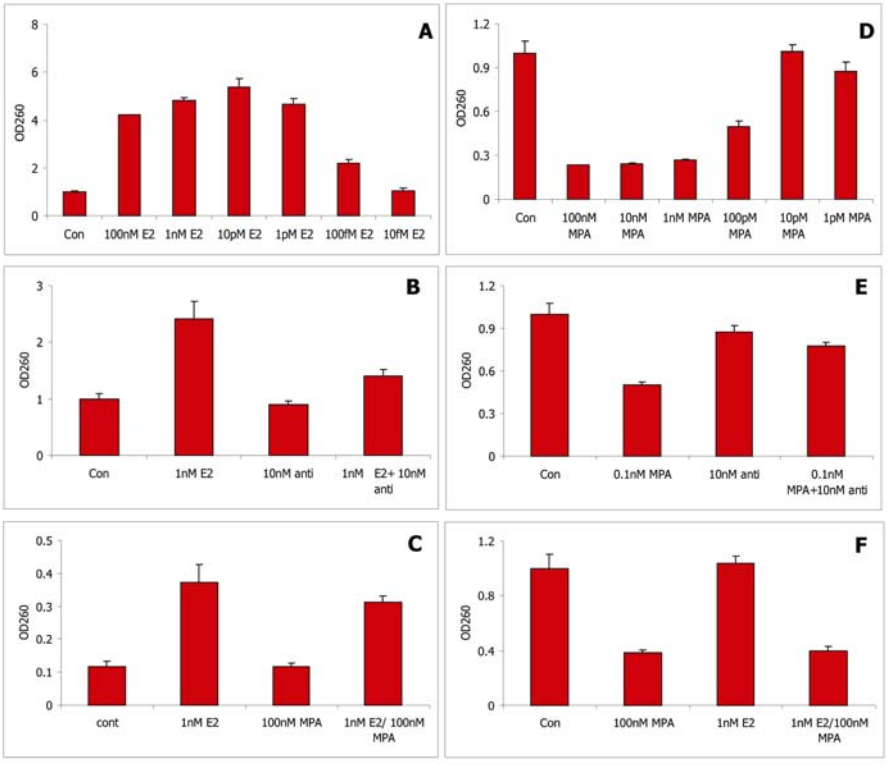

Figure 2. 3

ECC-1 cells (A, B, C) were cultured in the absence of hormone (Con), or in the presence of the indicated concentrations of estradiol (E_2) (A), E_2 and/or the anti-estrogen tamoxifen (anti) (B), E_2 and/or medroxy progesterone acetate (MPA) (C). PRAB-36 cells (D, E, F) were cultured in the absence of hormone (Con), or in the presence of the indicated concentrations of MPA (D), MPA and/or the anti-progestagen Org-31489 (anti) (E), MPA and/or E_2 (F). Cells were lysed in NaOH and OD260 values were measured.

Micro-array analysis of estrogenic and progestagenic effects

To study the molecular basis of the growth-promoting role of estrogen and the growth-inhibiting role of MPA in endometrial cancer, we performed two 9600 cDNA micro-arrays. One micro-array was performed using mRNA isolated from ECC-1 cells cultured for 72 hours in the absence or presence of estradiol. The other micro-array was performed using mRNA that was isolated from PRAB-36 cells, which were cultured for 48 hours in the absence or presence of MPA. These time-points were chosen because these were the first time-points at which growth induction or inhibition could be measured. Genes were analyzed as discussed in the material and methods section and Figure 4 shows box-plots, Q-Q plots and histograms to be able to verify quality of the current micro-array experiments. In total 288 genes were found to be regulated. In the ECC-1 cell line 148 estrogen-regulated genes were found. Of these genes 102 (69%) were down-regulated and 46 (31%) genes were up-regulated. In the PRAB-36 cell line 148 MPA-regulated genes were found, of which 34 (23%) were down-regulated and 114 (77%) were up-regulated.

Using the GO-ART program genes were categorized in groups according to their biological function (Smid and Dorssers, 2004). In this program, Gene Ontology (GO) annotations provide tree-structured networks of defined terms. A gene can have one or more biological functions in a cell and can therefore be classified into several branches of the network tree. Out of 288 estrogen- and progesterone-regulated genes, 243 genes could be classified using this program. The genes that could not be classified are expressed sequence tags (ESTs) or hypothetical proteins.

Functional group	**ECC-1**	**PRAB-36**
mechanosensory behavior	1	0
rhythmic behavior	1	0
cell communication	37	40
cell death	2	7
cell differentiation	0	2
cell growth and-or maintenance	40	41
cell motility	5	6
morphogenesis	14	6
pattern specification	0	1
reproduction	2	1
actin cytoskeleton reorganization	1	1
drug resistance	1	1
embryogenesis and morphogenesis	2	3
heavy metal sensitivity-resistance	1	0
histogenesis and organogenesis	0	1
oncogenesis	5	5
resistance to pathogenic bacteria	0	1
small molecule transport	7	4
bone remodeling	1	0
digestion	2	1
excretion	1	0
hemostasis	1	3
homeostasis	1	2
metabolism	61	53
pathogenesis	1	2
response to external stimulus	9	15
response to stress	3	10

Table 2.1.
Using the GO-ART program, genes regulated by estrogen (E_2) or medroxy progesterone acetate (MPA) were clustered according their biological function. The number of regulated genes per biological function is shown.

In Table 2.1 functional groups are listed with the number of genes categorized per group. Most genes are categorized in "cell communication", "cell growth and/or maintenance" and "metabolism". In order to find a molecular basis for growth modulation of endometrial cancer cells by estrogens and progestagens, we focused on the group of "cell growth and/or

maintenance" and subdivided this group further into 6 more specified categories (Table 2.2). All microarray data and full details including transcript identities can be found at (www2.eur.nl/fgg/rede/data.htm).

Cell growth and maintenance		**ECC-1**	**PRAB-36**
cell growth			
CTGF	Connective tissue growth factor precursor	n.s	1.6
IGFBP3	Insulin-like growth factor binding protein 3 precursor	n.s	-4.1
IGFBP4	Insulin-like growth factor binding protein 4 precursor	1.3	n.s
MIG2	mitogen inducible 2	-1.3	n.s
SLC3A2	solute carrier family 3, member 2	1.5	n.s
TGFB2	Transforming growth factor beta 2 precursor	-2.1	-1.5
cell homeostasis			
FTH1	Ferritin heavy chain	n.s	3.2
SRI	Sorcin	-2.1	1.5
cell organization and biogenesis			
CCR1	C-C chemokine receptor type 1	-1.3	n.s
CHD2	chromodomain helicase DNA binding protein 2	-1.3	n.s
HIST1H2AE	H2A histone family, member A	1.4	n.s
HMGA2	High mobility group protein HMGI-C	n.s	-1.7
HSPD1	60 kDa heat shock protein, mitochondrial precursor	1.3	n.s
MCP	Membrane cofactor protein precursor	-1.5	n.s
MID1	Midline 1 protein	n.s	-1.5
SMARCA1	SWI/SNF actin dependent regulator of chromatin, subfamily a, member 1	n.s	1.7
VIL2	Ezrin	n.s	1.6
cell proliferation			
AREG	Amphiregulin precursor	4	n.s
BTG1	B-cell translocation gene 1, anti-proliferative	-1.5	n.s
BTG2	BTG2 protein (NGF-inducible anti-proliferative protein PC3).	n.s	2.2
COL4A3	Collagen alpha 3(IV) chain precursor	n.s	3.2
FRAT2	GSK-3 binding protein FRAT2	1.4	n.s
FTH1	Ferritin heavy chain	n.s	3.2
HBP17	HBP17	n.s	-1.7
HK2	Hexokinase, type II	n.s	1.6
JAG1	jagged 1	n.s	1.6
NME1	Nucleoside diphosphate kinase A	1.6	n.s
NME2	Nucleoside diphosphate kinase B	1.6	n.s
PBEF	Pre-B cell enhancing factor precursor.	-1.4	n.s
PDZK1	PDZ domain containing 1	n.s	-1.5
PMP22	Peripheral myelin protein 22	-1.4	n.s
PPP1CB	Serine/threonine protein phosphatase PP1-beta catalytic subunit	n.s	2.7
TACSTD2	tumor-associated calcium signal transducer 2	-1.6	n.s
cell cycle			
CCND1	cyclin D1	n.s	-1.6
CCND2	G1/S-specific cyclin D2.	n.s	-1.6
CCNG2	cyclin G2	-1.5	n.s
CDK5R1	Cyclin-dependent kinase 5 activator 1 precursor	2.3	n.s
CDKN1A	Cyclin-dependent kinase inhibitor 1	n.s	1.5
CENPE	Centromeric protein E	-1.9	n.s
DIM1	Spliceosomal U5 snRNP-specific 15 kDa protein	1.5	n.s
G0S2	Putative lymphocyte G0/G1 switch protein 2.	n.s	-1.6
NBS1	Nijmegen breakage syndrome 1	n.s	1.8
RAD51	DNA repair protein RAD51 homolog 1	-1.7	n.s
REV3L	DNA polymerase zeta catalytic subunit	-1.4	n.s
SNK	Serine/threonine-protein kinase SNK	-1.5	-2.6
TGFB2	Transforming growth factor beta 2 precursor	-2.1	-1.5

other			
ABCC5	Multidrug resistance-associated protein 5	-1.3	n.s
ABCG2	ATP-binding cassette, sub-family G, member 2	-1.9	n.s
APOD	Apolipoprotein D precursor	-2.7	n.s
ARF3	ADP-ribosylation factor 3	-1.4	n.s
ARHC	Transforming protein RhoC	-1.4	n.s
ATP11B	Potential phospholipid-transporting ATPase IR	n.s	1.5
ATP1A1	Sodium/potassium-transporting ATPase alpha-1 chain precursor	n.s	2.1
ATP2B1	Plasma membrane calcium-transporting ATPase 1	-1.8	n.s
CLIC4	Chloride intracellular channel protein 4	n.s	1.6
FOXO1A	forkhead box O1A	n.s	1.9
FSTL3	Follistatin-related protein 3 precursor	n.s	1.6
GDI1	Rab GDP dissociation inhibitor alpha	n.s	1.5
GNAS	Guanine nucleotide-binding protein G(S), alpha subunit	-1.5	1.5
JUN	Transcription factor AP-1	n.s	-1.6
KCNJ8	potassium inwardly-rectifying channel, subfamily J, member 8	-1.7	n.s
KDELR3	ER lumen protein retaining receptor 3	n.s	1.5
KIAA0062	KIAA0062 protein	n.s	1.5
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	-1.5	n.s
MLLT2	myeloid/lymphoid or mixed-lineage leukemia; translocated to, 2	n.s	1.6
MYB	Myb proto-oncogene protein	2.8	n.s
OCLN	Occludin.	-1.4	n.s
PLAU	Urokinase-type plasminogen activator precursor	n.s	-1.9
RPL23	ribosomal protein L23	1.4	n.s
SEC22L1	Homo sapiens vesicle trafficking protein sec22b mRNA	n.s	1.7
SGK	Serine/threonine-protein kinase Sgk	n.s	2.5
SLC12A2	Solute carrier family 12 member 2	-1.7	n.s
SLC22A5	Organic cation/carnitine transporter 2	n.s	2.2
SLC6A3	Sodium-dependent dopamine transporter	n.s	1.6
SLC7A5	solute carrier family 7 , member 5	1.8	-1.7
SORT1	Sortilin precursor	n.s	1.7
SRP54	Signal recognition particle 54 kDa protein	n.s	2.3
TFF1	trefoil factor 1	5.2	n.s
VPS45A	Vacuolar protein sorting-associated protein 45	n.s	1.5

Table 2.2.
Genes regulated by estrogen (E_2) or medroxy progesterone acetate (MPA), clustered to the group of growth regulation and/or maintenance and further sudivided into the indicated 6 subgroups (cell growth, cell homeostasis, cell organization and biogenesis, cell proliferation, cell cycle and a rest group (other)). Column 3 and 4 gives fold induction (f.i) when present. n.s.= no significant change in expression level observed between control and treated cells.

Validation of gene-expression

The results from our micro-array studies have the potential to provide novel molecular mediators of hormonal regulation of endometrial cancer, biomarkers and therapeutic targets, Subsequently, validation of promising genes will be required. The estrogen and progesterone regulated genes identified by micro-array analysis have been validated in several ways. At first, all gene-expression data were compared to literature using PubGene and Pubmatrix databases (Becker et al., 2003; Jenssen et al., 2001). Pubgene is a gene-to-gene co-citation network for 13,712 known human genes and is generated by an automated analysis of titles and abstracts of more than 10 million MEDLINE records. The method is based on the pre-assumption that for most genes that are co-mentioned in a medline record there exists a biological relationship. PubMatrix is a database that rapidly and systematically compares any list of terms against any other list of terms in PubMed. It reports back the frequency of co-occurrence between the two lists.

Combining these two databases, 48 genes out of 288 regulated genes are linked to the endometrium and 41 genes to endometrial cancer. 43 of the estrogen-regulated genes are linked to ERα signaling and 33 of 148 progesterone-regulated genes are linked to PR signaling.

Secondly, we validated the expression of a select group of genes involved in "cell growth and/or maintenance" (Fig. 2.3). BTG1, BTG2, Cyclin D1, Cyclin D2, and SNK data are consistent with those data obtained through the micro-array analysis. For amphiregulin, regulation by estrogen in ECC-1 cells was confirmed but on northern blot some progesterone regulation was observed which had not been detected using the micro-array.

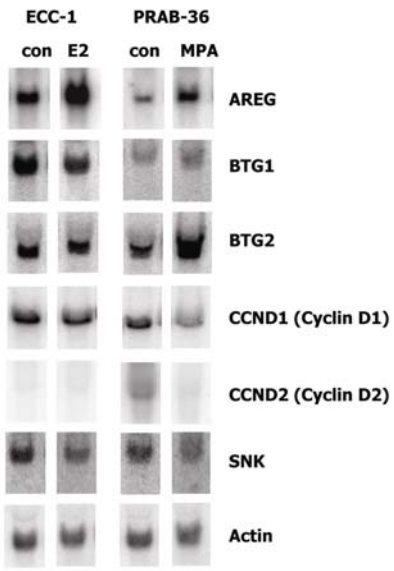

Figure 2.3.
ECC-1 cells were cultured for 72 hours in the presence or absence of 1nM estradiol (E_2). PRAB-36 cells were cultured for 48 hours in the presence or absence of 100nM medroxy progesterone acetate (MPA). RNA was isolated, electrophorezed and blotted. The ^{32}P-labeled probes, indicated in the figures and in the Materials and Methods section were hybridized with the blots.

2.4 Discussion

Unbalanced estrogenic stimulation is the main factor responsible for the development of endometrial cancer, and can, to some extent, be controlled through administration of progestagens (Lentz, 1994; Rose, 1996; Van Gorp and Neven, 2002). In the current study we investigated the molecular mechanism behind hormonal regulation of proliferation. Because there is no endometrial cancer cell line available that expresses both estrogen and progesterone receptors, we used two models. The ECC-1 cell line was used to study estrogen modulation of growth and gene-expression, and the Ishikawa cell line (clone PRAB-36) to study progesterone-induced growth inhibition and gene regulation.

The endometrial carcinoma cell line ECC-1 expresses high levels of ERα and very low levels of ERβ (Matsuzaki et al., 1999; Mote et al., 1999; Utsunomiya et al., 2000). In accordance to literature, expression of ERα was down-regulated by estrogen (Dardes et al., 2002). Furthermore, estrogen was capable of inducing growth. Despite the fact that the ECC-1 cells express low amounts of PRB, progestagenic effects on these cells could not be observed.

The Ishikawa cell line that was initially used (Ishikawa clone 3H12) (Nishida et al., 1996), did not express detectable amounts of PRA or PRB. It was therefore stably transfected with PRA and PRB, resulting in the PRAB-36 sub-cell line (Blok et al., 2003). In the current investigations it was shown that PRAB-36 cells express high levels of PRA and PRB, and no ER. As reported in literature, it was shown that expression of the two progesterone receptors was efficiently inhibited at the post-transcriptional level by MPA (which is the progestagen preferably used in the clinic) (Jacobsen et al., 2002). Furthermore, MPA-induced growth inhibition could readily be shown, while effects of estrogen administration were not observed in this cell line.

As in the normal endometrium and early endometrial cancer, the cell lines used in this study express high levels of ERα, PRA and PRB (Matsuzaki et al., 1999; Mote et al., 1999; Utsunomiya et al., 2000) and clear estrogen-induced growth and progesterone-inhibited growth was observed. Furthermore, when we performed micro-arrays on ECC-1 and PRAB-36 cells grown under control conditions, more than 95% of expressed genes were expressed by both cell lines at comparable levels (Hanifi-Moghaddam et al., 2005). Therefore, the currently used cell lines were considered suitable models to investigate the genetic background of growth modulation by estrogen and progesterone in endometrial cancer.

Exploring a 9600 cDNA micro-array, 140 genes were found to be exclusively estrogen-regulated, 140 genes were only MPA-regulated and 8 genes were found to be regulated by both estrogen and MPA. Analysis of these regulated genes revealed that 26% of regulated genes had already been recognized in literature to be regulated via estrogen or progesterone receptors, while the other group of genes was not known to be regulated by these hormone receptors. Furthermore, 16% of regulated genes are associated with the endometrium and 11% to endometrial cancer.

As indicated in Table 2.1, based on their biological function, regulated genes could be classified into different functional groups. Because, our research question was to find a molecular basis for steroid-induced growth modulation in endometrial cancer, we focused on genes that belong to the functional category of "cell growth and/or maintenance". The group of cell-growth and maintenance was further divided into 6 subgroups: cell growth, cell homeostasis, cell organization and biogenesis, cell proliferation, cell cycle and one rest-group (other) (Table 2.2).

Genes involved in estrogen-induced growth stimulation

In the ECC-1 cell line, amphiregulin (4.0-fold induction) was found to be up-regulated by estrogen. Amphiregulin is one of the ligands for the EGF receptor and can stimulate cell proliferation by activating the MAP-kinase pathway (Pfeiffer et al., 1997). Furthermore, Pfeiffer et al (Pfeiffer et al., 1997) reported that amphiregulin is relatively overexpressed in endometrial cancer as compared to the normal endometrium (Pfeiffer et al., 1997).

Cyclin G2 (CCNG2), B-cell translocation gene 1 (BTG1) and Spliceosomal U5 snRNP-specific 15 kDa protein (DIM1) were estrogen-regulated in the ECC-1 cell line. These genes are of interest, because cyclin G2 expression is up-regulated as cells undergo cell cycle arrest or apoptosis (Horne et al., 1997). Furthermore, DIM1 is known to be necessary in late G2 phase for successful entry into and progression through M phase in yeast (Berry and Gould, 1997) and BTG1 inhibits the transition from G1 to S phase (Kuo et al., 2003). Therefore, estrogen-induced down-regulation of cyclin G2 and BTG1 and up-regulation of DIM1 may positively effect cell cycle progression and thus endometrial cell growth.

Pre-B cell enhancing factor precursor (PBEF) and peripheral myelin protein 22 (PMP-22) were also found regulated by estrogen in de ECC-1 cell line. (Kitani et al., 2003) reported that in PC-12 and Swiss 3T3 cells, PBEF is expressed both in the nucleus and in the cytoplasm, but that a difference is seen in expression pattern between proliferating and non-proliferating cells, suggesting that PBEF is a cell cycle-associated protein. Furthermore, PMP22 has been identified as a growth arrest specific gene in NIH3T3 fibroblasts (Schneider et al., 1988). Therefore, these genes may also be important for regulating the cell cycle in the human endometrium.

Genes involved in progesterone-induced growth inhibition

Using the PRAB-36 cells, the gene for putative lymphocyte G0/G1 switch protein 2 (G0S2), that is involved in the G0/G1 switch of the cell cycle was found progesterone down-regulated (Russell and Forsdyke, 1991). Furthermore, cyclin-dependent kinase inhibitor p21 (CDKN1A) was found up-regulated by MPA. The genes for cyclin D1 (CCND1) and cyclin D2 (CCND2) were down-regulated by MPA. These findings are of interest because MPA-induced down-regulation of GOS2, may negatively affect progression of the cell cycle from GO to G1 phase, while the expression patterns of cyclin D1, cyclin D2 and CDKN1A, negatively affect the switch from G1 to S phase (Dai et al., 2002; Evron et al., 2001; Milde-Langosch et al., 2001). This may all result in inhibited endometrial cell growth

Other genes possibly involved in regulation of cell growth are BTG2 protein (BTG2) and jagged 1 (JAG1). Rouault et al. determined that BTG2 is preferentially expressed in quiescent cells and overexpression of this gene causes a decrease in the growth rate of NIH 3T3 cells (Rouault et al., 1996). Further, JAG1 is the ligand for the notch 1 receptor and binding of the ligand to the receptor is known to inhibit proliferation of $CD34^+$ macrophage progenitors (Masuya et al., 2002). As both genes were found to be up-regulated by MPA, this indicates that these genes could be important for MPA-induced growth inhibition of endometrial cells.

Cross-talk between estrogen and progesterone signaling in the endometrium

In endometrial cancer, the growth stimulatory properties of estrogen can be balanced by the growth inhibitory properties of progesterone. Therefore the question arises if in our experiments we can find indications for this cross-talk, in other words, are there genes which are up-regulated by estrogens on the one hand and down-regulated by progestagens on the other hand, or *vice versa.* In the current study 5 genes, ID1, EVA1, GNAS, SLC7A5 and SRI, showed an inverse regulation between ECC-1 and PRAB-36 cells. Based on literature, none of these 5 genes are candidates to explain the modulating effects of estrogens and progestagens on growth. Therefore, we explored the possibility of a different mechanism. 5 genes belonging to the cyclin superfamily, are regulated in our cell lines: cyclin G2 and CDK5R1 are estrogen-regulated and cyclin D2, cyclin D1 and CDKN1A are MPA-regulated. Furthermore, two members of the BTG family, which are genes involved in negative regulation of the cell cycle, are found regulated. BTG1 was found estrogen down-regulated and BTG2 MPA up-regulated. These results indicate that it is possible that the cross-talk between estrogen signaling and progestagen signaling does not occur at the single gene level, but rather at the level of different genes that fall in the same functional family.

We hypothesize that at least part of the hormonal regulation of endometrial cancer, has its molecular basis in the above described data. Recent applications of global gene profiling in endometrial cancer include the study of Mutter et al. (Mutter et al., 2001), in which gene-expression is reviewed in different stages of the menstrual cycle and endometrial cancer tissues. We anticipate that combining data obtained from both in vivo and in vitro experiments will expand our understanding of the nature of endometrial cancer.

In summary, using the micro-array approach we were able to identify 148 estrogen- and 148 progesterone-regulated genes in two well-differentiated endometrial cancer cell lines. Of these genes some are known to be ER, or PR signaling associated genes but for most genes it is a new finding. Furthermore, genes could be classified into functional groups based on GO annotiations. Based on this, growth modulation of estrogen and progesterone in endometrial cancer could be partly explained.

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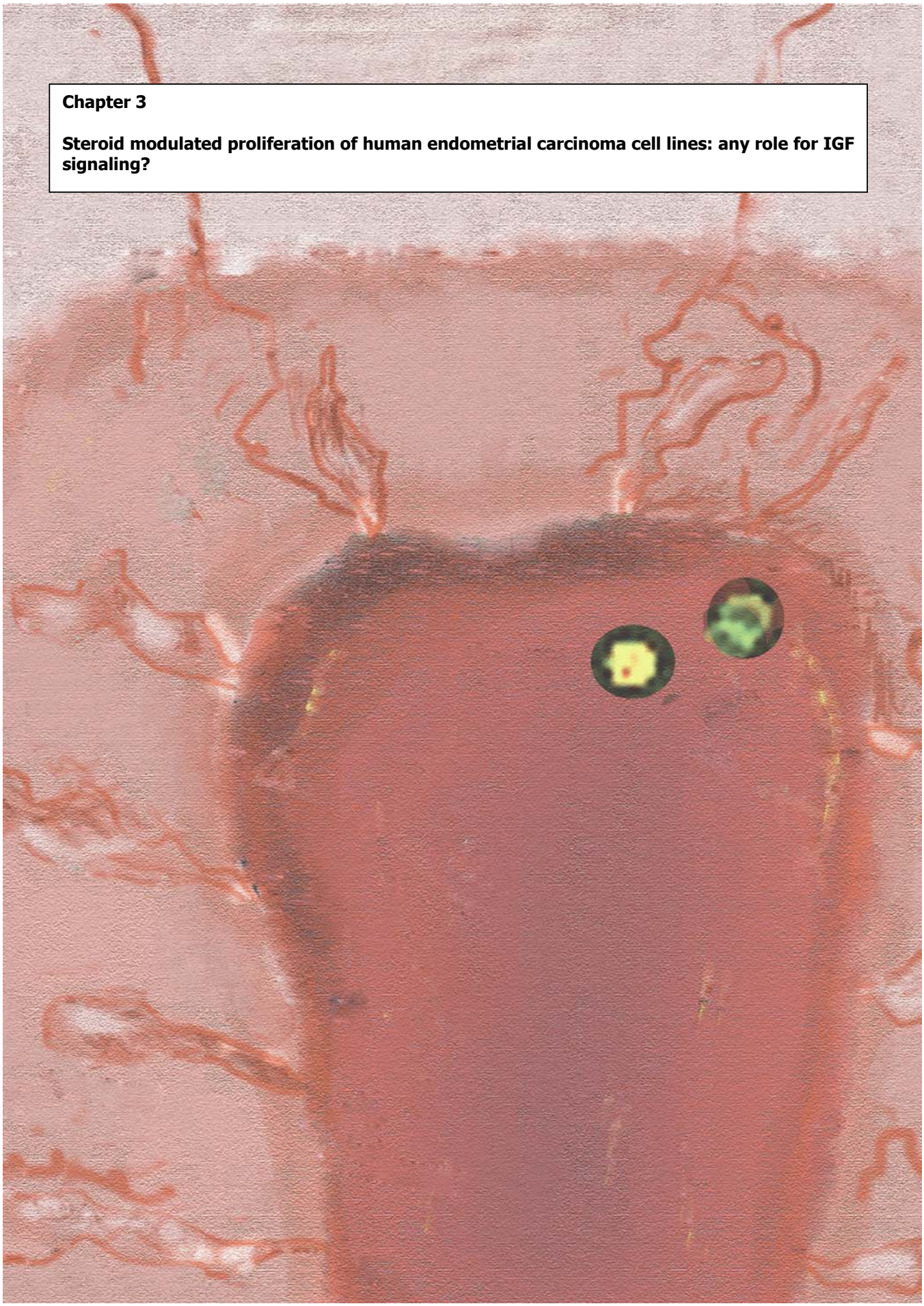
Chapter 3

Steroid modulated proliferation of human endometrial carcinoma cell lines: any role for IGF signaling?



Steroid modulated proliferation of human endometrial carcinoma cell lines: any role for IGF signaling?

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Abstract:
Objectives:
Estrogen-stimulated proliferation of the normal and malignant human endometrium is balanced by the differentiating properties of progesterone. This study evaluated the role of insulin like growth factor (IGF) signaling in steroid-induced modulation of endometrial cancer cell proliferation.
Methods:
We used the human endometrial, estrogen responsive ECC-1 and progesterone responsive PRAB-36 cell lines. Proliferation studies with IGFs in combination with either estrogen or progesterone were conducted. Furthermore, the mRNA and protein expression of insulin like growth factor binding proteins (IGFBPs) was evaluated.
Results:
Using the ECC-1 cell line, we observed that estrogen-induced proliferation is modulated via the IGF receptor signaling pathway, and that IGF1 induced stimulation of proliferation does not influence ER signaling. Furthermore, expression of the main modulators of IGF action, the IGFBPs, was found regulated by estrogen and progesterone in both cell lines. IGFBP4 was estrogen up-regulated in the ECC-1 cell line and IGFBP3 and IGFBP6 were progesterone down-regulated in the PRAB-36 cell line.
Conclusions:
Estrogen induced stimulation of proliferation of ECC-1 endometrial cancer cells is partly achieved via IGF signaling. Furthermore, the IGFBPs are regulated by estrogens as well as progestagens and could potentially play a role in the modulation of endometrial cancer cell proliferation.

Keywords: estrogen, progesterone, endometrium, proliferation regulation, IGF signaling, IGFBP

3.1 Introduction

In the human uterus, the endometrial layer undergoes cyclic changes that are controlled by the ovarian hormones, estrogen and progesterone. Estrogen stimulates proliferation and progesterone induces differentiation of the glandular cells. However, in the presence of elevated estrogen levels or in the absence of progestagens, a significant increase in the incidence of endometrial hyperplasia and endometrial cancer is observed (Van Gorp and Neven, 2002). Furthermore, progestins are used as an adjuvant therapy for endometrial cancer because of their proliferation inhibiting properties (Lentz, 1994; Rose, 1996).

For the endometrium, but also for many other organs, growth factors and growth factor receptors play a central role in mediating the effects of steroid hormones. Experiments described in literature suggest that for the endometrium one of these growth factor systems is the insulin like growth factor-system (Badinga et al., 1999; Druckmann and Rohr, 2002). IGFs (IGF1 and IGF2) are small (70 amino acids for IGF1 and 67 for IGF2) polypeptides with 62% homology in their amino acid sequence. (Druckmann and Rohr, 2002). The human gene for IGF1 is transcribed from chromosome 12 and the gene for IGF2 is located on chromosome 11 (Rosenfeld et al., 1990; Sara and Hall, 1990).

The mitogenic actions of the IGFs are mediated through binding to their receptors (mainly the type 1 receptor; IGF1 receptor), which upon binding forms a heterotetrameric complex composed of two α-subunits and two β-subunits. Binding of the ligand to the receptor results in an autophosphorylated β-subunit and phosphorylation of the major receptor substrate, IRS-1. This, subsequently, results in activation of downstream signaling pathways (Hwa et al., 1999; LeRoith et al., 1995). The role of the IGF 2 receptor (IGF-2 receptor) in mediating IGF action is less clear: it binds primarily IGF2, activates downstream signaling pathways, and also serves as a receptor for mannose-6-phosphate-containing ligands (Nissley and Lopaczynski, 1991).

In biological fluids IGFs are bound to insulin-like growth factor binding proteins (IGFBPs), of which 7 mammalian binding proteins have been characterized (IGFBP1 to 7). Besides prolonging the half-life of the IGFs, the binding proteins can also function as modulators of IGF availability and activity. This is caused by the much higher binding affinity of IGFs for IGFBPs than for the IGF receptors (Clemmons, 1997; Oh et al., 1996). Furthermore, there are *in vitro* and *in vivo* data supporting a role for IGF-independent actions of the binding proteins in regulating cell proliferation of normal and malignant cells (Kelley et al., 1996).

In the normal human endometrium, estrogen induces up-regulation of the expression of IGF1 and the IGF1 receptor, while expression of IGFBP1, known to be the most important IGFBP in the endometrium, is controlled by progesterone (Druckmann and Rohr, 2002; Zhou et al., 1994). Furthermore, mRNAs encoding IGFBP2 and IGFBP3 are differentially expressed in the proliferative and secretory phase of the menstrual cycle, suggesting that regulation of IGFBP expression by steroid hormones may exist (Giudice et al., 1991; Rutanen et al., 1994). In endometrial cancer, down-regulation of expression of IGF1 and up-regulation of expression of the IGF1 receptor has been reported (Roy et al., 1999; Rutanen et al., 1994). Furthermore, in vitro assays have shown a stimulating effect of IGF1 on proliferation of different cancer cell lines (RL95-2, KLE, HEC and Ishikawa), and estradiol seems to sensitize endometrial cancer cells to the effects of IGFs by elevating receptor levels and decreasing the (potentially inhibitory) expression of IGFBP3 (Kleinman et al., 1995; Pearl et al., 1993; Roy et al., 1999; Rutanen et al., 1994). These data indicate a role for IGF signaling in estrogenic and progestagenic regulation of the normal and malignant endometrium.

In order to understand the role of IGF signaling in steroid-induced modulation of proliferation of endometrial cancer, two well-differentiated endometrial cancer cell lines were used. ECC-1 cells were used to investigate IGF signaling in an estrogen-responsive cell line, while modified Ishikawa cells (PRAB-36) were used to investigate IGF signaling in a progesterone responsive endometrial cell line.

3.2 Materials and Methods

Cell culture:
The ECC-1 cell line is derived from a well-differentiated adenocarcinoma of human endometrium, transplanted into nude mice and was a generous gift from Dr. B. van den Burg (Utrecht, The Netherlands) (Satyaswaroop et al., 1988). The Ishikawa cell line is derived from a well-differentiated human endometrial carcinoma and was obtained from Dr. M. Nishida (Tsukuba, Japan) (Nishida et al., 1996). This cell line was stably transfected to express human progesterone receptor-A (hPRA) and hPRB, resulting in clone PRAB-36 (Blok et al., 2003). PRAB-36 cells were cultured under selection pressure by neomycin (G418: 500µg/ml, Invitrogen Life technologies, Breda, The Netherlands) and hygromycin (250µg/ml, Invitrogen Life Technologies). The cells were routinely maintained in DMEM/F12 + 10% fetal bovine serum (FBS, Perbio Science, Helsingborg, Sweden) supplemented with penicillin/streptomycin in a 37ºC incubator at 5% CO2.

Immunohistochemistry:
ECC-1 and PRAB-36 cells were cultured in phenol red free DMEM/F12 + 5% dextran-coated charcoal treated-FBS (DCC-FBS). Cells were passaged onto glass coverslips in a 24 well plate (Nalge Nunc International, Rochester, NY, USA) and cultured in the presence or absence of 1nM estradiol (E_2) for ECC-1 cells, and 100nM Medroxyprogesterone acetate (MPA) for PRAB-36 cells. After 72 hours, cells were washed twice with phosphate buffered saline (PBS) and fixed in methanol (100%) at 4˚C for 20 minutes. Cells were pre-incubated with 1% bovine serum albumin (BSA) in PBS and incubated with the following antibodies: IGF1 receptor α (sc-463), IGF1 receptor β (sc-713), IGF2 receptor (sc-14408), IGFBP3 (sc-6003), IGFBP4 (sc-6005) and IGFBP6 (sc-6007) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. Cells were washed 3 times for 5 minutes in PBS. After pre-incubation for 20 minutes in 10% serum (IGF1 receptor α, IGF1 receptor β: goat serum, IGF2 receptor and IGFBP3, 4, 6: rabbit serum), cells were incubated for 2 hours at room temperature with the secondary antibody diluted 1:200 in 1% serum (IGF1 receptor α: goat anti mouse-TRITC, IGF1 receptor β: goat anti rabbit-TRITC, IGF2 receptor and IGFBP3, 4, 6: rabbit anti goat-TRITC (Sigma, Saint Louis, Missouri, USA). After incubation with the secondary antibody, cells were washed 3 times with PBS and colored with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, USA). All stainings were compared to negative controls. For each cell line a representative negative control, in which no primary antibody was used, is shown in the figures (Fig. 3.1 and Fig. 3.5).

Proliferation studies:
In order to reduce steroid activity in the culture medium, 5 days before starting these experiments, cells were cultured in phenol red free DMEM/F12 containing 5% DCC-FBS. At day 1 cells were transferred to a 24 well plate (Nalge Nunc International, Rochester, NY, USA), at 5000 cells per well in 450µl medium. At day 2 and 4 the following compounds, diluted in 50µl medium were added: 10nM Insulin-like growth factor-1 (IGF1), 10nM Insulin-like growth factor-2 (IGF2) (Sigma-Aldrich, Saint Louis, Missouri, USA), 1nM E_2, 100nM MPA, 50ng IGF1 receptor antibody (Ab-1, Oncogene research products, San Diego, USA) and the anti-estrogen ICI182780 (0.1 µM, Tocris Cookson Limited, Bristol, United Kingdom). On day 6 medium was changed into serum free medium supplemented with ^{3}H-Thymidine (NEN Life Science Products, Inc, Boston, MA) at 12kBq (0,33µCi) per well. Again 50µl of the compound mix was added per well and cells were cultured overnight at 37ºC. At day 7, cells were washed twice for 5 minutes with PBS and harvested in 500µl of 1M NaOH for 60 minutes at 37°C. After complete lysis of the cells, 100µl of the lysate was diluted in 5ml picofluor 15 (Packard Bioscience Company, Groningen, The Netherlands) and radioactivity was measured in a liquid scintillation counter (Packard Bioscience Company, type 2700TR). Experiments were performed at least twice and standard deviations were calculated from quadruplicate incubations within one representative experiment. Oneway ANOVA tests were performed in order to measure statistical differences between groups.

Northern blotting:
Cells were cultured for the indicated times in the presence or absence of 1nM E_2 for the ECC-1 cell line and 100nM MPA for the PRAB-36 cells. Total RNA was isolated using the following method: Cells were lyzed with 3M Lithium chloride/ 6M Urea, purified by ultracentrifugation at 100.000g, separated using 1.5 % agarose gels and blotted to nitro-cellulose membranes (Auffray and Rougeon, 1980; Blok et al., 1995). The following ^{32}P- labeled probes were hybridized to the blot: human IGFBP1 (IMAGE: 2068836), human IGFBP6 (IMAGE: 753620) (required from RZPD, Germany), mouse IGFBP2 (emb X81580), mouse IGFBP3 (emb X81581), mouse IGFBP4 (emb X81582) and mouse IGFBP5 (emb X81583) (kindly provided by Dr. S.L.S. Drop, Department of Pediatrics, division of Endocrinology, Erasmus MC, Rotterdam, The Netherlands). A hamster β-actin probe was used to verify equal loading of samples. Blots were analyzed using IMAGE QUANT software program.

3.3 Results

The role of IGF in estrogen and progesterone modulation of endometrial cell proliferation
The actions of IGFs are mediated through binding to the IGF1 or IGF2 receptor. The IGF1 receptor is a precursor that is cleaved in an α-subunit and a β-subunit. By performing fluorescent immunohistochemistry, expression levels of IGF1 receptor-α, IGF1 receptor-β and IGF2 receptor were evaluated in the ECC-1 and the PRAB-36 cell lines. Moreover, to investigate the modulating effects of estrogens and progestagens on the expression of IGF receptors, cells were cultured for 72 hours in the absence or presence of these hormones. It was observed that both cell lines express detectable levels of IGF receptors, while the addition of estrogens or progestagens to the medium did not affect these expression levels (Fig.3.1). Western blotting confirmed these findings (not shown).

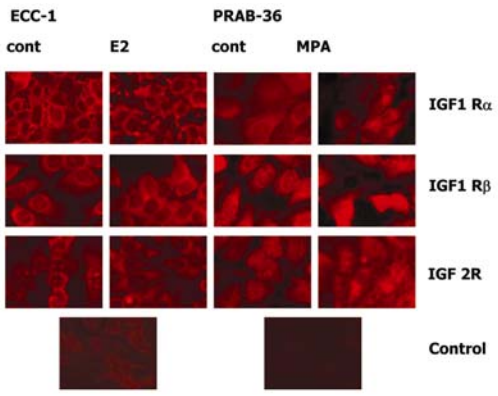

Figure 3.1 Protein expression of IGF1 receptor alfa (IGF1 Rα), IGF1 receptor beta (IGF1 Rβ) and IGF2 receptor (IGF2 R) in ECC-1 and PRAB-36 cells. ECC-1 cells were cultured with or without 1nM estradiol (E_2) and PRAB-36 cells with or without 100nM MPA (MPA) for 72 hours. Of each cell line a representative negative control, in which no primary antibody was used, is shown (control).

Since literature indicated a role for the IGFs in estrogen and progestagen modulated endometrial homeostasis, the proliferation-inducing effects of IGF1 and IGF2, in the presence or absence of estrogens or progestagens, were studied in ECC-1 and PRAB-36 cells. In ECC-1 cells an increased proliferation rate was seen for cells treated with IGF1, IGF2 and estradiol (Fig. 3.2). Furthermore, as shown in Figure 3.2 (A and B), incubation of the ECC-1 cells for 7 days in the presence of IGF1 or IGF2 in combination with E_2, did not result in an additive or synergistic effect. The PRAB-36 cell line, which expresses high levels of both PRA and PRB, is inhibited in proliferation, dose dependently, by MPA (Blok et al., 2003). Despite the fact that this cell line expresses all IGF receptors, no effects of either IGF1 or IGF2 were seen in the proliferation assays (Fig. 3.2C and 3.2D). Furthermore, even if cells were cultured in the presence of IGFs in medium containing 2% charcoal treated FBS, no proliferation-induction was observed (data not shown). When PRAB-36 cells were cultured in the presence of IGF1,

IGF2 and MPA simultaneously, only the MPA-induced inhibition of proliferation was observed (Fig. 3.2C and 3.2D).

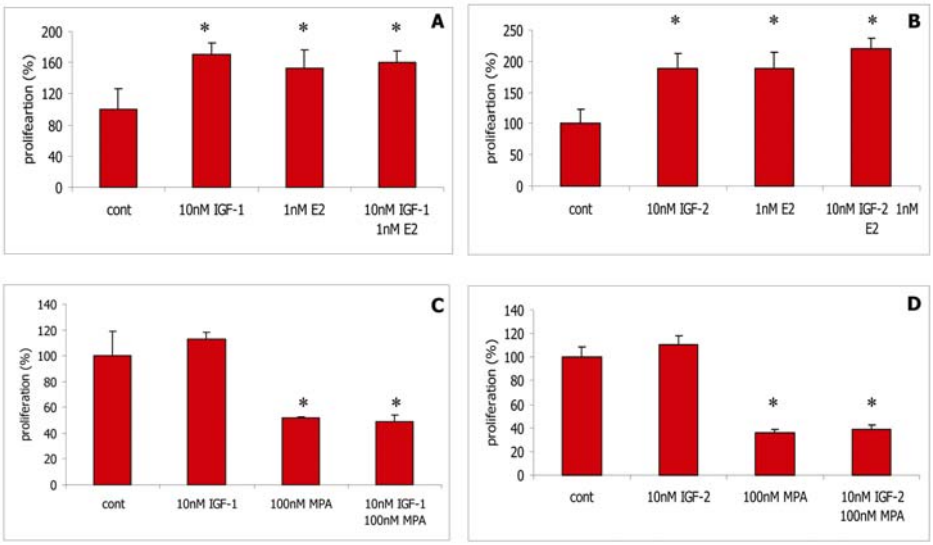

Figure 3.2 IGF1 and IGF2 enhance proliferation in ECC-1 cells. ECC-1 cells (panel A and B) were cultured in the absence of ligand (cont), or in the presence of the indicated concentrations of: insulin like growth factor 1 (IGF1); insulin like growth factor (IGF2); estradiol (E_2). PRAB-36 cells (panel C and D) were cultured in the absence of ligand (cont), or in the presence of the indicated concentrations of: insulin like growth factor 1 (IGF1); insulin like growth factor (IGF2); MPA (MPA). Proliferation is expressed as a percentage of control proliferation (proliferation %). Experiments were performed in quadruplicate and oneway anova analyses were performed. Differences between control and treatments were considered significant (*) at $p<0.01$.

Interactions between IGF and ER signaling pathways

Because we found that both IGFs and estrogen enhance proliferation of ECC-1 cells, we considered the idea that the IGF and estrogen signaling pathways are intertwined. Therefore experiments were conducted to measure whether estrogens are influencing IGF signaling and whether IGFs are influencing estrogen signaling. In order to do so, proliferation assays with IGFs in the presence of an anti-estrogen (complete ER antagonist ICI182780) and proliferation assays with E_2 in the presence of an IGF1 receptor blocking antibody were conducted (Rohlik et al., 1987). Since the antibody was unable to block IGF2 activity, the incubations were only performed with IGF1.

As indicated in figure 3.3, the anti-estrogen ICI182780 decreases the proliferation rate of ECC-1 cells and the antibody slightly increases the proliferation rate of ECC-1 cells (Fig. 3.3). Therefore, to be able to compare between the different experiments, increments in proliferation over the three control situations (cont, anti and ICI), were indicated in percentages at the top of the bars in the figure (Fig. 3.3). As expected, the IGF1 receptor antibody is inhibiting IGF1 induced proliferation.

Estrogen-induced proliferation was partly inhibited by the antibody (126% to 50%). From this observation it was concluded that estrogens partly act via an IGF1 receptor mediated pathway. When the cells were incubated with the anti-estrogen ICI182780 it was observed that estrogen-induced proliferation could indeed be blocked by the anti-estrogen (126% to 9%). IGF1 induced proliferation however, was not inhibited by the anti-estrogen. From this observation it was concluded that IGF1, in the current cellular context, does not induce proliferation through a ligand-independent activation of the estrogen receptor.

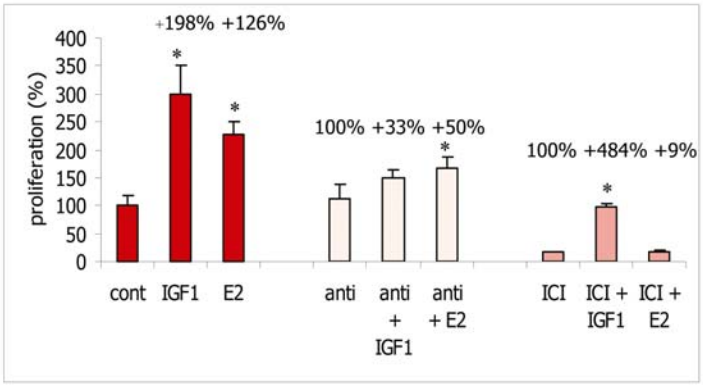

Figure 3.3 Estrogen mediated proliferation is inhibited by blocking IGF1 signaling and IGF1 mediated proliferation is independent of ER-signaling. ECC-1 cells were cultured in the absence of ligand (cont), in the presence of 10nM IGF1 (IGF1) or 1nM E_2 (E_2). In the second panel IGF signaling is blocked by adding 50ng antibody against the IGF1R (anti). In the third panel estrogen signaling is blocked by adding 1µM of the anti-estrogen ICI182780 (ICI). Proliferation is expressed as percentage of control proliferation (proliferation %). Experiments were performed in quadruplicate and one way anova analyses were performed. Differences between control and treatments were considered significant (*) at $p<0.01$.

Analysis of insulin like growth factor binding protein (IGFBP) 1-6

From the experiments described in Figure 3.3 it was concluded that estrogens partly act through an IGF1 receptor mediated pathway, while for progestagens a possible role for IGF signaling remains to be resolved. The biological actions of IGFs are mediated through binding to their specific insulin like growth factor binding proteins (IGFBPs). Furthermore, IGFBPs have also been reported to affect cellular proliferation independent of IGF.

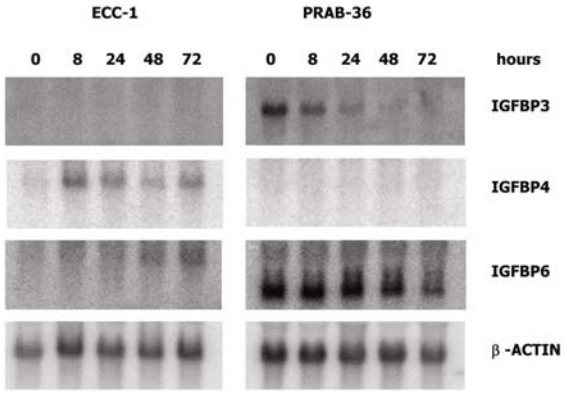

Figure 3.4 IGFBPs are regulated by estrogens and MPA. ECC-1 cells were cultured for the indicated times in the absence (0) or presence of 1nM estradiol (E_2). PRAB-36 cells were cultured for the indicated times in the absence (0) or presence of 100nM MPA (MPA). RNA was isolated, electrophorezed and blotted. The blots were hybridized with the ^{32}P-labeled probes as indicated in the Figure.

To investigate the possible modulating effect of estrogen and progesterone on IGF signaling via modulation of the expression of IGFBPs, we measured the mRNA and protein levels of IGFBP1 to 6 in ECC-1 and PRAB-36 cells, cultured in the presence or absence of estrogen or progesterone. The IGFBP2 and IGFBP5 genes were found to be expressed in both cell lines, but no regulation of expression by either estrogens or MPA could be observed, while for IGFBP1 no detectable expression levels were measured using Northern blot analysis (data not shown). The mRNA for IGFBP3 is highly expressed in the PRAB-36 cell line and the transcript is already effectively down-regulated after 8 hours of culture in the presence of MPA (Fig. 3.4). Furthermore, the IGFBP3 protein is localized predominantly in the cytoplasm and regulation of protein expression by MPA (Fig. 3.5) confirms the mRNA expression data (Fig. 3.4 and Fig. 3.5). In contrast to down-regulation of IGFBP3 in PRAB-36, IGFBP4 is clearly up-regulated by estrogen in the ECC-1 cell line (Fig. 3.4). Protein data (Fig. 3.5) again confirm

the estrogen-induced up-regulation of IGFBP4 expression at the mRNA level (Fig. 3.4 and Fig. 3.5). For IGFBP6 the situation is the following: in the PRAB-36 cell line an MPA-induced down-regulation of the mRNA and protein was observed (Fig. 3.4 and Fig. 3.5); in ECC-1 cells, no clear up- or down-regulation was observed at the mRNA level, but at the protein level IGFBP6 expression was elevated in the presence of estrogen.

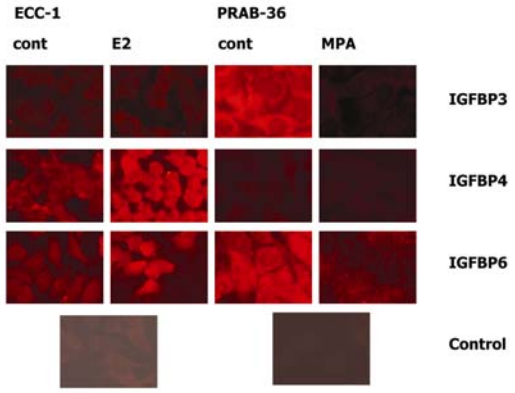

Figure 3.5 Protein expression of IGFBP3, IGFBP4 and IGFBP6. ECC-1 cells were cultured for 72 hours in the absence (cont) or presence of 1nM estradiol (E_2). PRAB-36 cells were cultured for 72 hours in the absence (cont) or presence of 100nM MPA (MPA). Of each cell line a representative negative control, in which no primary antibody was used, is shown (control).

3.4 Discussion

Several studies have tried to link changes in expression of IGFs, IGF receptors and IGFBPs to endometrial carcinogenesis. Ayabe et. al (1997) reported increased levels of IGF1 and decreased levels of IGFBP1 in patients suffering from endometrial cancer (Ayabe et al., 1997). Petridou et al. observed that the occurrence of endometrial cancer was associated with higher serum levels of IGF2 and lower serum levels of IGF1 (Petridou et al., 2003). In endometrial cancer tissues, overexpression of the IGF1 receptor and down-regulation of IGF1 and IGFBP1 have been reported (Maiorano et al., 1999; Roy et al., 1999). All these studies point to a significant role for IGF signaling in endometrial carcinogenesis.

Unbalanced estrogenic stimulation is the main factor responsible for the development of endometrial cancer, and can, to some extent, be controlled through administration of progestagens (Lentz, 1994; Rose, 1996; Van Gorp and Neven, 2002). In the current study we investigated the role of IGF signaling in estrogen-induced proliferation stimulation and in progesterone-induced proliferation inhibition of the endometrium. Ultimately we believe that more knowledge about the role of IGF signaling during steroid receptor signaling may improve understanding of endometrial cancer.

Role of IGF receptors and estrogen receptors in IGF and estrogen signaling

As summarized in Table 3.1, several components of the IGF signaling machine are regulated by estrogens and progestagens. One of the most interesting findings of the present investigations was that E_2 regulation of proliferation of ECC1 cells is partially mediated through the IGF pathway (IGF receptor antibodies inhibit E_2 induced proliferation), while IGF signaling is independent of the ER pathway (anti-estrogens do not affect IGF-induced proliferation stimulation).

Several reports in the literature have described connections between ER and IGF signaling pathways. Adesanya et al. (1999) have shown that in the uterine epithelium of IGF-1 knockout mice, the mitotic index after E_2 stimulation is lower than the index in wild type mice (Adesanya et al., 1999). Furthermore, IGF1 stimulation in estrogen receptor alfa knock out (ERKO) mice, does not result in DNA synthesis in the uterus (Klotz et al., 2002). While these two studies indicate that IGF signaling and estrogen-signaling are mutually dependent, our results indicate only that estrogen-signaling is partly accomplished by stimulating the IGF pathway and not that IGF signaling depends on activation of the ER. The basis for this difference is not easily explained, but there are more reports in literature that describe

findings similar to our results. In a study by Surmacz et al, it was observed that overexpression of IRS1, one of the principal substrates of the IGFIR, leads to estrogen-independent proliferation of MCF-7 breast cancer cells (Surmacz and Burgaud, 1995). Furthermore, Bartucci et al. (2001) reported that IGF1 promotes migration in ER-negative breast cancer cell lines (Bartucci et al., 2001).

	ECC-1	**PRAB-36**
E2	growth stimulation	no effect
MPA	no effect	growth inhibition
IGF receptors	yes, but no regulation	yes, but no regulation
IGF	growth stimulation	no effect
IGF + E2	growth stimulation	no effect
IGF+MPA	no effect	no effect of IGF,growth inhibition by MPA
E2 + anti-estrogen	inhibition of E2 effect	
IGF + IGFR antibody	inhibition of IGF effect	
E2 + IGFR antibody	partial inhibition of E2 effect	
IGF + anti-estrogen	no inhibition of IGF effect	
IGFBP1 expression	no	no
IGFBP2 expression	yes	yes
IGFBP3 expression	no	MPA-induced down-regulation
IGFBP4 expression	E2-induced up-regulation	yes
IGFBP5 expression	yes	yes
IGFBP6 expression	no effect at the mRNA level, protein level E2 up-regulated	MPA-induced down-regulation

Table 3.1 Overview of results presented in this study.

Role of differential regulation of IGFBP3, 4 and 6 in steroid receptor modulated endometrial proliferation

An alternative way through which estrogens and progestagens might regulate proliferation using the IGF signaling pathway, is by modulating the expression of different IGFBPs. In fact, in the present investigations we observed progesterone-induced down-regulation of IGFBP3 and 6, and estrogen-induced up-regulation of IGFBP4 (Table 3.1).

The function of the IGFBP3 gene has been studied extensively in other organs and tissues, and in general these findings suggest a proliferation-promoting role for IGFBP3. Neuenschwander et al. (1996) reported that in mice overexpressing IGFBP3, the process of involution in mammary glands after pregnancy is decreased through reduction of apoptosis (Neuenschwander et al., 1996). In another mouse model, overexpression of human IGFBP3 caused organomegaly in the heart, spleen and liver (Murphy et al., 1995).

Furthermore, in bovine fibroblasts and also in MCF-7 breast cancer cells, membrane bound IGFBP3 is found to act as an initiator of IGF function by activating phosphatidyl inositol 3 kinase (PI3K) (Chen et al., 1994; Conover et al., 2000). In the breast epithelial cell line MCF-10A, IGFBP3 is known to enhance the proliferation-stimulatory effects of EGF via increased EGF receptor phosphorylation and activation of p44/42 and p38 MAP kinase signaling pathways (Martin et al., 2003). Also IGF independent actions are described in which the transcriptional activity of IGFBP3 is mediated by cell membrane receptors such as the TGFβ receptor (Conover et al., 2000; Mohseni-Zadeh and Binoux, 1997).

For IGFBP4, promotion and inhibition of IGF dependent proliferation is described. The inhibiting actions appear to be a result of the sequestration of local IGFs, which prevents receptor interaction (Giudice et al., 2002). Proliferation-promoting effects of IGFBP4 are described in diabetic rat (Rodgers et al., 1995). Although the precise physiological role of IGFBP4 in cancer remains to be determined, it is tempting to speculate with regard to our currently used cell lines, that estrogen induced up-regulation of IGFBP4 increases the availability of IGF for the receptor, and that this results in activation of proliferation.

IGFBP6 is still a relatively unknown member of the IGF-family. IGFBP6 overexpression is described in many tumors including prostate, breast and colon carcinomas

(Drivdahl et al., 1995; Sheikh et al., 1993; Singh et al., 1994). It was also reported by Rutanen et al. that the expression of IGFBP6 is suppressed during the mid cycle of the human endometrium, suggesting regulation of expression by progesterone (Rutanen et al., 1994). Furthermore, an increase in expression of IGFBP6 in a more aggressive phenotype compared to a mild phenotype, was shown in meningeoma tissues (Nordqvist and Mathiesen, 2002).

In summary, the current investigations provide further evidence that IGF signaling pathway plays a significant role in mediating the effects of steroid hormones on the endometrium. Therefore, we hypothesize that differential regulation of different components of the IGF signaling pathway during development of endometrial cancer can possibly contribute to hormone-independent proliferation of more advanced endometrial tumors.

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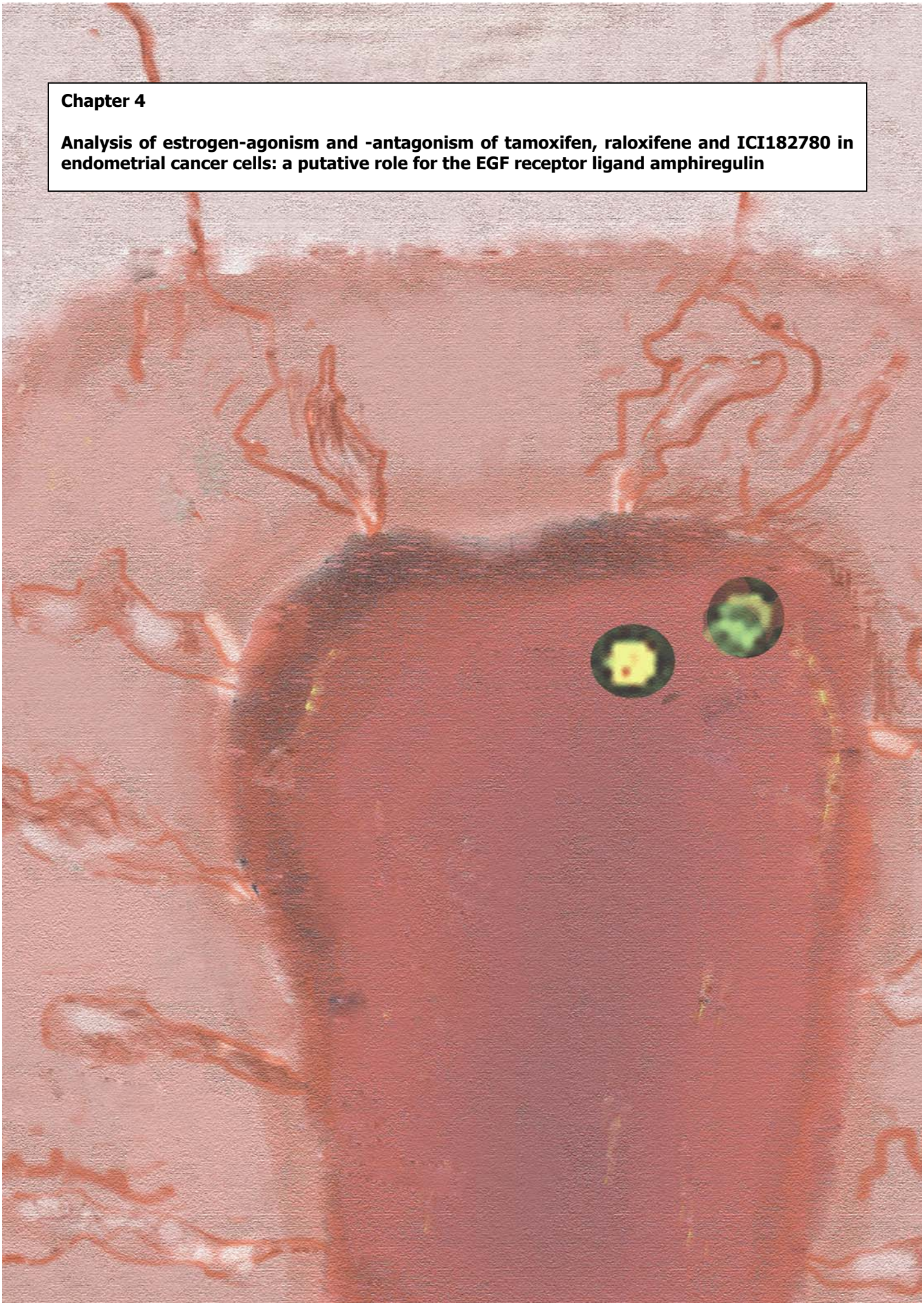
Chapter 4

Analysis of estrogen-agonism and -antagonism of tamoxifen, raloxifene and ICI182780 in endometrial cancer cells: a putative role for the EGF receptor ligand amphiregulin



Analysis of estrogen-agonism and -antagonism of tamoxifen, raloxifene and ICI182780 in endometrial cancer cells: a putative role for the EGF receptor ligand amphiregulin

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ABSTRACT

Objectives: In different tissues, estrogens, selective estrogen receptor modulators (SERMs) and anti-estrogens exert different biological activities. For the endometrium, estradiol and tamoxifen induce proliferation, and because of this, tamoxifen-treatment of breast cancer patients results in a 2-7 fold increased risk for development of endometrial cancer. Use of raloxifene, or the anti-estrogen ICI182780, does not result in such an increased risk. In this study the objective was to generate and analyze gene expression profiles that reflect the transcriptional response of the human endometrium to estradiol, SERMS like tamoxifen and raloxifene and anti-estrogens like ICI182780.
Methods: Transient transfections were performed to analyze the transcriptional response of ECC-1 cells to estradiol, tamoxifen, raloxifene and ICI182780. Subsequently, to reveal the molecular mechanism of action, gene-expression profiles were generated and some of the observed regulated genes were confirmed by Northern blotting. Biostatistical methods were employed to analyze the expression profile-results further, and amphiregulin effects on ECC-1 cell signaling were investigated using Northern and western blotting, and ^{3}H-thymidine incorporation.
Results: Analysis of the profiles revealed that estradiol, tamoxifen, raloxifene and ICI182780 influence the same biological processes, but do so via regulation of different sets of genes. Upon construction of a genetic network it was observed that the largest possible network centered on EGF receptor signaling. Furthermore, the EGF receptor ligand amphiregulin was differentially regulated by all four ligands. Next it was shown that amphiregulin indeed could stimulate EGF receptor signaling in ECC-1 cells. Based on these results, it was hypothesized that EGF receptor signaling could differentially be affected by estrogen, tamoxifen, raloxifene and ICI182780 because these four compounds differentially regulate the EGF receptor ligand amphiregulin.
Conclusions: Regulation of amphiregulin coincides with the described in vivo effect of the 4 ligands on the endometrium. Therefore it is possible that modulation of EGF receptor signaling is a significant player in estrogen-agonistic growth of the endometrium and needs to be investigated further.

Keywords: Estrogens, SERMs, anti-estrogens, endometrium, amphiregulin, EGF receptor

4.1 Introduction

17β-estradiol (E_2) is a major regulator of many normal biological functions in a variety of tissues, like breast, bone, uterus, brain and the vascular system. Aberrant exposure to estrogens and estrogen-like compounds, from prescribed drugs, environmental pollutants and dietary substances, however, is associated with an increased incidence of hormone-dependent breast, endometrial and testicular carcinogenesis (Akhmedkhanov et al., 2001; Heikaus et al., 2002).

The negative effects of estrogens have inspired the development of a new class of drugs, the selective estrogen receptor modulators (SERMs). These SERMs, such as tamoxifen and raloxifene, exhibit, depending on the tissue type, estrogenic or anti-estrogenic properties. Tamoxifen, which has an anti-estrogenic mode of action on breast cancer cells, is first choice adjuvant therapy for treatment of estrogen receptor positive (ER+) breast cancer. Furthermore, the results of several large trials have shown that tamoxifen also reduces the risk of cancer in the contralateral breast (Buzdar, 1998). Based on these findings, chemoprevention trials have been initiated in the United States and Europe to examine whether tamoxifen decreases the risk of developing breast cancer in healthy women at high risk of the disease (Powles, 1998; Veronesi et al., 1998). Besides an anti-estrogenic mode of action, tamoxifen also displays estrogenic activity. In bone, tamoxifen elicits beneficial estrogenic activity and thereby prevents osteoporosis (Love et al., 1992). However, in the uterus of postmenopausal women, tamoxifens estrogen-agonistic activity results in an increased risk for the development of endometrial hyperplasia and endometrial cancer (Bergman et al., 2000; Buzdar, 1998).

Like tamoxifen, raloxifene prevents osteoporosis in postmenopausal women and prevents breast cancer by acting as an estrogen-antagonist. However, raloxifene does not seem to increase the incidence of endometrial cancer (Delmas et al., 1997; Ettinger et al., 1999). Up till now, tamoxifen is still first choice adjuvant therapy for breast cancer patients, because the efficiency of raloxifene over tamoxifen for treatment and prevention of breast cancer is still under investigation (STAR trial) (Jordan et al., 2001).

Although tamoxifen is very effective for treatment of breast cancer, some patients develop tamoxifen-resistance (Tobias, 2004). The pure anti-estrogen ICI182780 (faslodex, fulvestrant) is the only currently available anti-estrogen to show clinically relevant activity in these tamoxifen-resistant patients (Osborne, 1999). Interestingly, treatment with ICI182780 of patients with tamoxifen-resistant breast cancer, revealed no stimulation of the endometrium (Wardley, 2002).

New paradigms have recently emerged regarding the molecular mode of action of estrogens, SERMs and anti-estrogens in exerting their tissue specificity. They all initiate their effects through binding to two estrogen receptors, ERα and ERβ (Green et al., 1986; Kuiper et al., 1996), after which recruitment of different co-activators and co-repressors results in differential transcriptional complex-formation (McDonnell and Norris, 2002). Although differences between estrogens, SERMs and anti-estrogens in recruitment of co-regulatory proteins to the ER-ligand complex and transcription activation of simple response elements in reporter plasmids are found, the distinct regulatory effects on gene-expression are largely unknown (McDonnell and Norris, 2002) (Shang and Brown, 2002; Shang et al., 2000). An exception to this is a study by Dardes et al. (2002) who showed differential effects of estrogen, SERMs and anti-estrogens on ERα, pS2 and VEGF expression in ECC-1 cells (Dardes et al., 2002). Therefore, the main objective of this study was to generate transcription profiles that reflect the response of the human endometrium to estradiol, SERMs like tamoxifen and raloxifene, and anti-estrogens like ICI182780.

Using the micro-array technology, we found that estradiol, tamoxifen, raloxifene and ICI182780, produce different expression profiles in the human endometrial cancer cell line ECC-1. Furthermore, it was observed that all ligands modulate the same biological processes, but do so mainly through modulation of a different set of genes. The EGF receptor ligand amphiregulin forms an exception to this rule and was investigated in more detail.

4.2 Materials and Methods

Materials:
Cell culture: Tissue culture flasks and plastic disposables were obtained from Nunc (Nunc A/S, Rosklide, Denmark). DMEM/F12 was purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Fetal calf serum (FCS) came from Greiner (Frickenhausen, Germany). The following compounds were used: estrogen, 4OH-tamoxifen (Sigma Chemicals co., St Louis, MO, USA), raloxifene (kindly provided by Dr. M. Hibner, Mayo Clinic Scottsdale, USA), ICI182780 (Tocris Cookson Inc., Ellisville, USA), human recombinant amphiregulin (R&D systems, Abingdon, UK) and AG1478 (Calbiochem, San Diego, CA, USA).
Western blotting: For Western blots the following antibodies were used: sc-8002 (ERα), sc-03 (EGFR), sc-7020 (p-Tyr) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and D7N (ERβ) (Zymed Laboratories Inc., San Francisco, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Sigma Chemicals Corporation (St Louis, MO, USA). ECL detection reagents were from Perkin Elmer (Wellesley, MA, USA).
Transfection: The pcDNA3.1 hygromycin and neomycin resistance marker containing vectors were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). FuGENE^{TM} 6 Transfection Reagent was obtained from Roche Diagnostics (Indianapolis, IN, USA), and the Dual-Glo^{TM} luciferase assay system and the Renilla Luciferase Reporter plasmid (pRL-SV40) were obtained from Promega (Madison, WI, USA).
Micro-array: Probes for arrays were designed by Compugen (Compugen Ltd., Jamesburg, NJ) and synthesized by Sigma–Genosys (The Woodlands, TX, USA), coated slides from UltraGAPS Coated Slides (Corning Inc., Acton, MA, USA) and a high-precision pin-spotting robot from Chipwriter Pro Virtek (San Carlos, CA, USA). Deoxy-dNTPs were obtained from Roche, $Pd(T)_{12-18}$ oligo primer, Cy3-dCTP and Cy5-dCTP from Amersham (Amersham Biosciences, Roosendaal, the Netherlands) and Superscript 2 Reverse Transcriptase enzyme kit from Gibco (Carlsbad, CA, USA). Riboshredder RNase blend was obtained from Epicentre (Biozym, Landgraaf, The Netherlands). SlideHyb was obtained from Ambion (Huntingdon Cambridgeshire, UK). RNA 6000 NanoAssay and a Bioanalyzer 2100 are from Agilent (Agilent Technologies Netherlands B.V. Amstelveen, The Netherlands). Tecan HS4800 hybridization station is from Tecan (Maennedorf, Switzerland). Micro-arrays were scanned on a ScanArray Express HT scanner (Perkin Elmer, Boston, USA).
Proliferation assays: ^{3}H-Thymidine (specific activity: 1.85TBq/mmol) was obtained from Amersham (Amersham Biosciences, Roosendaal, the Netherlands), UltimaGold from Perkin Elmer (Boston, USA). Liquid scintillation counter is from Packard, type A2500/01(Packard Bioscience Benelux NV, Asse, Belgium).

Cell culture:
The ECC-1 cell line is derived from a well-differentiated adenocarcinoma of a human endometrium transplanted into nude mice, and was a generous gift from Dr. B. van den Burg (Utrecht, the Netherlands) (Satyaswaroop et al., 1988). The cells were routinely maintained in DMEM/F12 + 5% FBS supplemented with penicillin/streptomycin in a 37ºC incubator at 5% CO_2. One week before the experiments started, cells were stripped of endogenous steroids and cultured onwards in phenol red free DMEM/F12 + 5% dextran coated charcoal treated (DCC) FBS.

Transient Transfection:
The hERα-cDNA cloned in the pSG5 expression vector was a generous gift from Dr. P. Chambon (Illkirch Cedex, France). A 1900 bp fragment containing the complete ERα-coding sequence, was isolated from pSG5-hERα by excision of the EcoR1-EcoR1 fragment and cloned in the pcDNA3.1+/hygromycine vector. The hERβ-cDNA cloned in the pSG5 expression vector was a generous gift from Dr. A.P.N. Themmen (Rotterdam, the Netherlands). A 1927 bp fragment was isolated from pSG5-hERβ by excision of the EcoR1-EcoR1 fragment, and, was then cloned in pcDNA3.1+/neomycine vector. The pS2-Luc reporter (a gift from Dr. V. Giguere, Montreal,

Canada) contains the estrogen-responsive pS2 gene promoter fused upstream of the luciferase reporter (Tremblay et al., 1997). C3-Luc contains the human complement 3 gene promoter and was a generous gift from Dr. D. P. McDonnell, Durham, USA (Fan et al., 1996).

For transient transfection assays, ECC-1 cells were seeded in a 48-well plate at 20.000 cells per well. The following day cells were transfected. Both reporter constructs (pS2-Luc and C3-Luc) only responded to hormonal stimulation when ER plasmids were co-expressed. Furthermore, using dilution curves first the optimal concentration of plasmids was established (data not shown). The transfection procedure was as follows: 0.75µl FuGENE, 1ng hERα-pcDNA3.1+, 3ng hERβ-pcDNA3.1+, 150ng reporter plasmid (pS2-Luc or C3-Luc) and pTZ19 was added to a total DNA concentration of 250 ng/well. As a control for transfection efficiency 0.1ng pRL-SV40 was cotransfected per well. The following ligands were added: 1nM E_2, 1µM 4OH-tamoxifen, 1µM raloxifene, 100nM ICI182780. After 24 hours of incubation cells were harvested in 50µl lysis buffer (25 mM Tris phosphate (pH 7.8), 15% glycerol, 1% Triton X-100, 8 mM MgCl, 1 mM dithiothreitol). After an incubation of 10 min, 25µl of the lysate was transferred to a 96-well plate and the luciferase assay was performed following instructions provided by the manufacturer. Luciferase activity was measured using a LUMAC Biocounter M2500. Experiments were performed at least twice, and standard deviations were calculated from the average of six incubations within one representative experiment.

Western Immunoblotting:
Cells were cultured for 48 hours in the presence of ethanol, 1nM E_2, 1µM 4OH-Tamoxifen, 1µM Raloxifene, 100nM ICI182780. To study phosphorylation of the EGFR, cells were cultured for 0, 1, 2.5, 5, 10, 15, 20, 30 and 45 minutes with amphiregulin. Western blotting was essentially performed as described by Blok (Blok et al., 2003).

Micro-arrays:
Cells were cultured for 48 hours in the presence or absence of the indicated hormones. The time frame (48h) and concentrations of hormones that were used were based on a series of pilot experiments. Furthermore, Dardes et al. (2002) used comparable hormone concentrations in some of their experiments (Dardes et al., 2002). Total RNA was isolated using the lithium chloride/urea method (Auffray and Rougeon, 1980). The oligoarrays were produced at the Erasmus Center for Biomics and contained 18,861 Compugen designed and Sigma–Genosys synthesized probes representing 17,260 unique genes. Oligo probes measure 60-mer in length and were designed according to sequence, melting temperature, and ATGC content. The oligo's were printed on UltraGAPS Coated Slides using a high-precision pin-spotting robot to make pin-spotted oligo arrays. Oligo's were crosslinked to the slides by UV treatment. Control spots include landmarks, spotting buffer, alien oligo's, poly $d(A)_{40-60}$, salmon sperm DNA, and human COT-1 DNA.

Before labelling, quality of RNA was ensured by analyzing 500ng of each sample using the RNA 6000 NanoAssay and a Bioanalyzer 2100. Samples with a 28S/18S ribosomal peak ratio of 1.6-2.0 were considered suitable for labelling. For samples meeting this standard, 60µg of total RNA from every experimental variant or reference sample was labelled using a reverse transcriptase reaction in the presence of dNTPs and either Cy3 or Cy5. Hybridization was performed in 60µl hybridization mix for 16h at 47°C in the Tecan HS4800 hybridization station. All experiments were performed in duplo using dyeswap. Micro-arrays were scanned on a ScanArray Express HT scanner at wavelengths 633nm for Cy5 Dye and 543nm for Cy3 dye, to obtain images of 10µm resolution.

Analyses of genes:
Raw expression data were analyzed using Imagene 5.6 software (http://www.biodiscovery.com/imagene.asp. To correct for intensity-dependent dye bias, self-normalization within dye-flip replicates was performed. As a second step, to remove the spot location-specific error, data were normalized using the LIMMA package from bioconductor

(http://www.bioconductor.org). After this within-slide normalization, per array and per individual gene, the log-scaled signal of the control RNA of a gene was compared to the average of all log-scaled signals for that gene in all arrays. This makes it possible to adjust for scale differences within different oligoarrays. We found that all log-scaled signals centered on zero, which indicates that there were no scale differences between the arrays. After normalization, up- or down-regulated genes were identified using the Rosetta Resolver System and the Microsoft® Excel program (http://www.rosettabio.com/) (http://www.microsoft.com). Genes with a p-value of ≤ 0.05 and a changed expression of at least 2-fold were defined as regulated.

Biological classification of genes:
To compare regulated genes to literature, PubMatrix, a system that systematically compares list of terms against other list of terms in PubMed, was used (http://pubmatrix.grc.nia.nih.gov/secure-bin/). For functional classification of genes we used FatiGo (http://fatigo.bioinfo.cnio.es/) and Pathway Assist 2.5 (Ariadne Genomics, Inc., USA). FatiGO is a web interface that carries out data-mining using Gene Ontology for micro-array data. The Pathway assist database contains biological knowledge represented in a formalized form focused on how proteins, cellular processes and small molecules interact, modify and regulate each other. Pathway Assist provides a method for searching objects individually by keyword, string or attributes. These include, for example, type (protein, enzym), effect (positive, negative, unknown), mechanism (transcription, phosphorylation), tissue type, biological process, belonging to cell structure, and others.

For building networks, Ingenuity Pathway analysis was used https://analysis.ingenuity.com/pa/. This database utilizes the Ingenuity Pathway Knowledge Base (IPKB) to computationally analyse datasets to identify networks or pathways.

Proliferation studies:
In order to reduce steroid activity in the culture medium, 7 days before starting these experiments, cells were cultured in phenol red free DMEM/F12 containing 5% DCC-FBS. At day 1, cells were transferred to a 24 well plate at 5000 cells per well in 450µl medium. At day 2 and 4 amphiregulin and EGF receptor-inhibitor AG1478, diluted in 50µl medium, were added. On day 7, medium was refreshed and supplemented with ^{3}H-Thymidine at 12kBq (0,33µCi) per well. Again 50µl of the compound mix was added per well and cells were cultured overnight at 37ºC. At day 8, cells were washed twice for 5 minutes with PBS and harvested in 500µl of 1M NaOH for 60 minutes at 37°C. After complete lysis of the cells, 100µl of the lysate was diluted in 5ml UltimaGold and radioactivity was measured in a liquid scintillation counter. Experiments were performed in quadruple, and were repeated twice. Oneway ANOVA tests were performed to assess p-values of differences between control and stimulated cells. A p-value < 0.05 was considered significant.

Northern blots:
Total RNA extraction (from an independent cell culture experiment) and Northern blotting was performed as described (Blok et al., 1995). Ethidium bromide staining was used to verify equal loading of total RNA.

4.3 Results

ECC-1 as a model for estrogen agonistic and antagonistic effects in the endometrium

The actions of estrogens, SERMS and anti-estrogens are mediated through binding to their receptor, the estrogen receptor (ER). Two ERs are known, ERα and ERβ. Expression of ERα and ERβ was analyzed in the ECC-1 cell line by performing Western Blotting. Both ERα and ERβ are expressed in ECC-1 cells. Furthermore, ERα is down-regulated in the presence of estrogen, while tamoxifen slightly reduces the expression of ERβ. Both ERα and ERβ are down-regulated in

the presence of ICI182780 (Fig. 4.1a). The results on the expression of ERα are in agreement with earlier findings by Dardes et al., (2002) (Dardes et al., 2002).

Transient transfections were performed to evaluate the effect of estradiol, the SERMS tamoxifen and raloxifene and the anti-estrogen ICI182780 on known estrogen-responsive promoters. Therefore ERα and ERβ in combination with either the pS2 promoter or the C3 promoter (placed in front of the luciferase reporter gene) were transfected into ECC-1 cells. It was shown that estrogen and tamoxifen activate the pS2 promoter as well as the C3 promoter. These results are in agreement with earlier findings using the same cell line but different reporter constructs (Dardes et al., 2002). Raloxifene and ICI182780 did not activate either of the promoters. Furthermore, as expected, when combinations are made with estrogen and either tamoxifen, raloxifene or ICI182780, it was clearly shown that the estrogenic activity could be inhibited (Fig. 4.1b).

These results demonstrate that using ECC-1 cells, estrogen-agonistic and antagonistic effects of tamoxifen can be measured at the transcriptional level. Therefore this cell line was used to study changes in gene-expression in response to estrogen, SERMs and anti-estrogen.

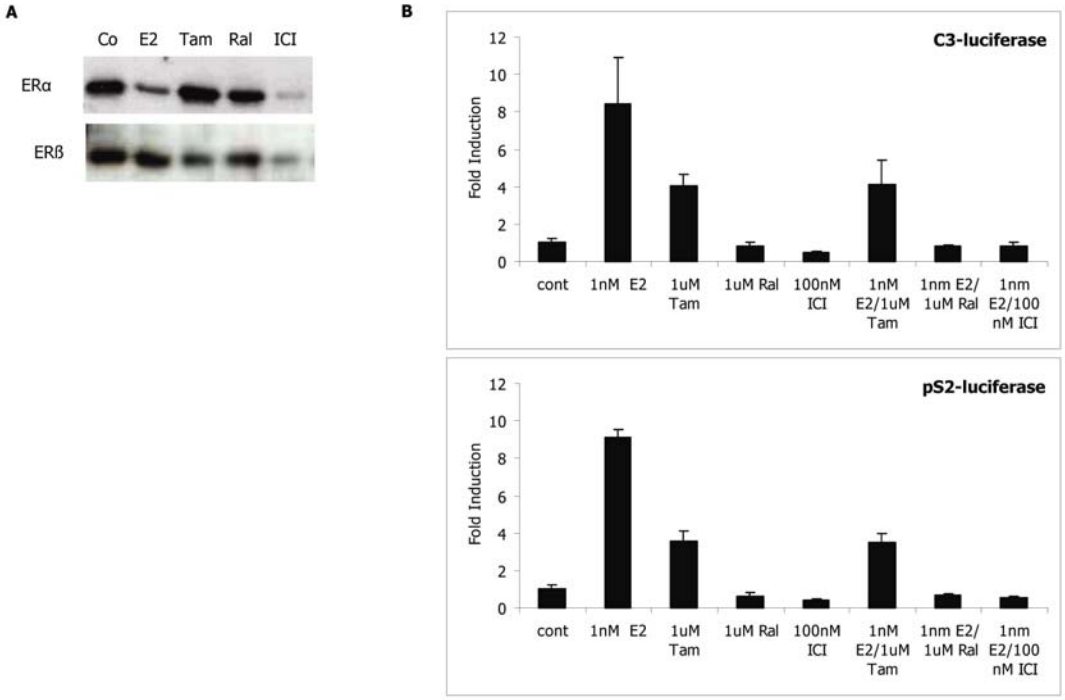

Figure 4.1. Estrogen-agonistic and –antagonistic signaling in ECC-1 cells.
A: The cells were cultured for 48h in the absence (Con) or presence of 1nm estradiol (E2), 1µm tamoxifen (Tam), 1µm raloxifene (Ral) or 100nM ICI182780 (ICI) before being harvested for western blotting and immunodetection of ERα and ERβ.
B: ECC-1 cells were transfected with a C3-Luciferase construct (upper panel) or a pS2-Luciferase construct (lower panel). Cells were incubated for 24h with 0.1% ethanol (con), 1nM estrogen (E2), 1µM tamoxifen (Tam), 1µM raloxifene (Ral) or 100nM ICI182780 (ICI) or combinations of these ligands.

Analyses of gene expression profiles

Identification of up- and down-regulated genes: In total 371 regulated genes were identified (Table 4.1). Of these genes, 188 genes were estrogen regulated (79 up and 109 down), 48 genes were tamoxifen regulated (28 up and 20 down), 96 genes were raloxifene regulated (44 up and 52 down) and 62 genes were found to be ICI182780 regulated (24 up and 38 down) (Table 4.1a). To identify which regulated genes were already known from literature and public databases to be regulated via the estrogen receptor, we composed a list of genes associated with this receptor. Using public databases (including our own database), a list containing 965 estrogen receptor linked genes, which were represented as oligo's on the array,

was composed. When the 371 regulated genes were compared to this list of 965 genes, 55 overlapping genes were found. As indicated in Table 4.1b, 35 (19%) of the estrogen-regulated genes, 7 (15%) of tamoxifen regulated genes, 14 (15%) raloxifene and 9 (15%) of the ICI182780 regulated genes were already known from literature to be regulated by the ER (Table 1b). The names and fold-induction of all genes regulated by estrogen, tamoxifen, raloxifene and ICI182780 are presented on our website (http://www2.eur.nl/fgg/rede/arraydata.htm).

A. regulated genes

total	up	down	
Estrogen	188	79 (42%)	109 (58%)
Tamoxifen	48	28 (58%)	20 (42%)
Raloxifene	96	44 (46%)	52 (54%)
ICI182780	62	24 (39%)	38 (61%)

B. ER-regulated genes from literature

total	up	down	
Estrogen	35 (19%)	14	21
Tamoxifen	7 (15%)	2	5
Raloxifene	4 (15%)	6	8
ICI182780	9 (15%)	2	7

Tabel 4.1. Overview of the number (total and in comparison to literature) of estrogen, tamoxifen, raloxifene and ICI182780 up- or down-regulated genes.
A: Total numbers of regulated genes.
B: Percentage of regulated genes that were also found in literature.

In order to verify that our micro-array data were indeed showing differentially regulated genes, expression of a number of regulated genes was measured using Northern blotting. The genes SNK, BPAG1, G1P3, MGP and RANBP3 all showed differential regulation corresponding to our original micro-array data (Fig. 4.2). For TFF3, regulation by raloxifene was confirmed. However, on Northern blot regulation of expression was also found for estrogen (up-regulation), and tamoxifen and ICI182780 (down-regulation) that had not been detected using the micro-array.

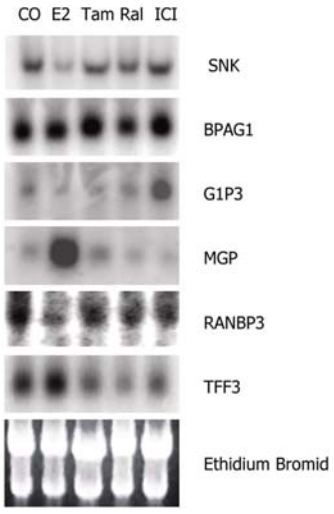

Figure 4.2. Validation of differential gene-expression by Northern blotting. Cells were cultured for 48 hours in the absence (Con) or presence of 1nM estradiol (E2), 1µM tamoxifen (Tam), 1µM raloxifene (Ral) or 100nM ICI182780 (ICI). RNA was isolated, electrophorezed and blotted. The ^{32}P-labeled probes, indicated on the right side of the figure and in the Materials and Methods section, were hybridized o/n with the blots. Hamster β-actin probe (Actin) and the ethidium bromide (EtBr) image was used to verify equal loading of the RNA samples on the different lanes of the gel.

Tamoxifen, raloxifene and ICI182780 block estrogen induced gene regulation: To examine what effect tamoxifen, raloxifene and ICI182780 have on E_2-regulated gene-expression in our model,

luciferase assays and micro-array experiments were conducted were ECC-1 cells were stimulated with E_2 in combination with either tamoxifen, raloxifene or ICI182780. As shown in Figure 4.1b, these ligands blocked estrogen activation of the pS2 and C3 promoter. Furthermore, from the micro-array experiments it was clear that when ECC-1 cells were stimulated with both estrogen and tamoxifen, modulation of 86% of estrogen-regulated genes was prevented. Using raloxifene in combination with estrogen 90% of E2 regulated genes was not modulated and for ICI182780 in combination with estrogen, transcription of 97% of estrogen-regulated genes was antagonized. Data from these micro-arrays are presented on our website (http://www2.eur.nl/fgg/rede/arraydata.htm).

Biological behaviour of regulated genes:

Estradiol, tamoxifen, raloxifene and ICI182780 regulate the same biological processes. The biological response of the endometrium to estrogens, SERMs or anti-estrogens is very diverse; therefore, the question was raised whether estradiol, tamoxifen, raloxifene and ICI182780 regulated different biological processes within cells. To answer this question, the differentially expressed genes were categorized, based on the cellular compartment in which they act (cellular component) and based on the biological function to which they are related (using the FatiGO database). Table 4.2 shows the annotated categories and the percentages of genes in each category. To find over- or under-representation of a category, unadjusted p-values were calculated of percentages of genes per indicated category (using Fisher exact tests). No significant differences were found between the four ligands when expressed genes were grouped based on the cellular component in which they act. Also when genes were grouped based on their biological function, besides one category (cell motility), no significant enrichment or depletion of a category was found. These results indicate that differences between the effects of estrogens, SERMs and anti-estrogens on the endometrium cannot be explained by the regulation of different biological processes.

		Estrogen	Tamoxifen	Raloxifene	ICI182780
Cellular component					
	intracellular	56.41%	66.67%	61.54%	41%
	membrane	46.15%	37.50%	41.03%	62.50%
	cell surface	1.28%	0%	0%	0%
	extracellular matrix	6.41%	0%	5.14%	0%
	extracellular space	7.69%	8.33%	0%	9.38%
Biological function					
	catabolism	10.89%	7.69%	8.89%	5.41%
	cell adhesion	1.98%	7.69%	2.22%	0%
	cell- cell signaling	7.92%	7.69%	13.33%	18.92%
	cell death	5.94%	7.69%	13.33%	2.70%
	cell growth and/or maintenance	39.60%	50%	35.56%	24.32%
	cell motility	1.98%	3.85%	13.33%	5.41%
	immune respons	4.95%	11.54%	8.89%	21.62%
	lipid metabolism	9.90%	3.85%	8.89%	5.41%
	nucleotide and nucleic acid metabolism	9.90%	19.23%	17.78%	13.51%
	organogenesis	12.87%	7.69%	15.56%	5.41%
	protein metabolism	21.78%	19.23%	15.56%	16.22%
	regulation of metabolism	10.89%	19.23%	15.56%	10.81%
	response to external stimulus	7.92%	11.54%	17.78%	2.70%
	signal transduction	32.67%	15.38%	20%	35.14%

Table 4.2. Biological classification of regulated genes.
Differentially expressed genes were categorized, based on the cellular compartment in which they act and based on the biological function to which they are related (using the FatiGO database). Percentages indicate the percentage of genes categorized in a particular cellular compartment or biological function.

Proliferation and apoptosis are differentially modulated by estradiol, tamoxifen, raloxifene and ICI182780. Since the four different ligands regulate similar biological processes, the question was then raised whether the biological responses of the ligands in the endometrium are generated by co- or contra-regulation of similar genes. To test this hypothesis, we studied the effect of estradiol, tamoxifen, raloxifene and ICI182780 on the biological processes proliferation and apoptosis/cell death. In Table 4.3 genes are summarized which are involved in proliferation (Table 3a) or apoptosis (Table 3b). Out off 32 genes that are involved in proliferation (Table 4.3a), only 6 genes are regulated by at least two ligands and out off 28 genes involved in apoptosis (Table 4.3b), only 4 genes are co- or contra-regulated. From these data we conclude that, although there exists some overlap between the treatment groups, most genes are regulated specifically by one of the ligands.

Proliferation

Gene	*Description*	*Accession*	*Fold-change*	*P-value*
Estrogen				
AREG	amphiregulin	NM_001657	5.2	0.001
DUSP4	dualspecificityphosphatase4	NM_001394	-2.4	0.029
ELF3	E74-like factor 3	NM_004433	-4.4	0.007
GAL	galanin-related peptide	NM_015973	2.9	0.015
GRB14	growth factor receptor-bound protein 14	NM_004490	-2.7	0.022
IL1R1	interleukin 1 receptor, typeI	NM_000877	-7.4	0.004
MME	membrane metallo-endopeptidase, transcript variant 2b	NM_007289	-4.4	0.012
MYBL1	a-myb	X66087	3.0	0.013
PGR	progesterone receptor	NM_000926	5.2	0.002
PRLR	prolactin receptor	AK023665	2.9	0.012
RUNX2	runt-related transcription factor 2	AL353944	2.4	0.048
SUFU	suppressor of fused	NM_016169	-3.8	0.014
TFP12	tissue factor pathway inhibitor 2	NM_006528	2.9	0.022
TIMP1	tissue inhibitor of metalloproteinase 1	NM_003254	-2.9	0.014
TRAF6	TNF receptor-associated factor 6	NM_004620	9.0	0.000
TFGB3	transforming growth factor, beta 3 .	NM_003239	-2.3	0.041
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	NM_002546	-3.6	0.006
Tamoxifen				
AREG	amphiregulin	NM_001657	2.4	0.032
CALR	calreticulin	NM_004343	-2.4	0.05
CCND1	cyclin D1	NM_001758	-2.3	0.05
FUS	fusion, derived from t (12;16) malignant liposarcoma	NM_004960	-3.1	0.016
Raloxifene				
CDT6	mRNA for angiopoietin-like factor	Y16132	-2.3	0.039
CLU	clusterin	NM_001831	2.2	0.049
ELF3	E74-like factor 3	NM_004433	2.4	0.034
GAL	galanin-related peptide	NM_015973	-2.7	0.023
GHR	growth hormone receptor	S97393	-3.7	0.005
IFNA2	interferon-alpha 2	M54886	-2.4	0.032
PPP1R13B	protein phosphatase 1, regulatory (inhibitor) subunit 13B	AB018314	-2.6	0.024
PYY	peptideYY	NM_004160	2.6	0.036
SEMA3B	Semaphorin A(V)	NM_004636	2.7	0.024
TBX2	T-box 2	NM_005994	2.2	0.046
TFF1	trefoilfactor 1	NM_003225	-5.3	0.001
TFF3	trefoilfactor 3	NM_003226	-3.3	0.008
TIMP1	tissue inhibitor of metalloproteinase 1	NM_003254	2.5	0.026
TNSF1 5	tumor necrosis factor superfamily,member 15	NM_005118	4.0	0.023
ICI182780				
AREG	amphiregulin	NM_001657	-2.2	0.044
MME	membrane metallo-endopeptidase, transcriptvariant 2b	NM_007289	4.2	0.003
PCNA	proliferating cell nuclear antigen	D17232	-3.2	0.009
TFF1	trefoilfactor 1	NM_003225	-4.2	0.003

Apoptosis				
Gene	*Description*	*Accession*	*Fold-change*	*P-value*
Estrogen				
ADBR1	beta-1 adrenergic receptor	AF272890	2.6	0.034
AREG	amphiregulin	NM_001657	5.2	0.001
DAPK1	death-associated protein kinase 1	NM_004938	-2.7	0.027
GAL	galanin-related peptide	NM_015973	2.9	0.015
IDS	iduronate 2-sulfatase, transcript variant 1	NM_000202	-2.3	0.037
MME	membrane metallo-endopeptidase, transcript variant 2b	NM_007289	-4.4	0.012
MT2A	metallothionein 2A	NM_005953	3.6	0.005
PRKRA	interferon-inducible double stranded RNA dependent activator	NM_003690	2.2	0.046
PSAP	co-beta glucosidase	J03077	-2.6	0.023
PTPN13	protein tyrosine phosphatase, non-receptor type 13	NM_006264	-3.5	0.013
SERPIN A1	alpha-1-antitrypsin	M26123	6.8	0.001
SGK	serum/glucocorticoid regulated kinase	NM_005627	2.6	0.032
TGFB3	transforming growth factor, beta 3	NM_003239	-2.3	0.040
TIMP1	tissue inhibitor of metalloproteinase 1	NM_003254	-2.9	0.014
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	NM_002546	-3.6	0.006
TP53INP1	tumor protein p53 inducible nuclear protein 1	AL133074	-3.3	0.008
Tamoxifen				
AREG	amphiregulin	NM_001657	2.4	0.032
CALR	calreticulin	NM_004343	-2.4	0.05
CBFA2T1	core-binding factor, runt domain, alpha subunit 2	AF131817	13.3	0.000
CCND1	cyclin D1	NM_001758	-2.3	0.05
Raloxifene				
BAK1	BCL2-antagonist/killer 1	NM_001188	-3.2	0.009
PDCD5	programmed cell death 5	NM_004708	2.7	0.020
PMS2	postmeiotic segregation increased 2	NM_000535	2.2	0.044
SERPIN B2	plasminogen activator inhibitor, type II	NM_002575	-22.5	0.000
T	T brachyury homolog	NM_003181	2.5	0.03
TFF3	trefoil factor 3	NM_003226	-3.2	0.007
TIMP1	tissue inhibitor of metalloproteinase 1	NM_003254	2.5	0.025
TNFRSF10D	tumor necrosis factor receptor superfamily, member 10d	NM_003840	-2.4	0.041
TNFSF1 5	tumor necrosis factor superfamily, member 15	NM_005118	4.0	0.023
GAL	galanin-related peptide	NM_015973	-2.7	0.023
ICI182780				
MME	membrane metallo-endopeptidase, transcript variant 2b	NM_007289	4.2	0.003

Table 4.3. Genes involved in proliferation and apoptosis.
A: Those genes regulated by estrogen, tamoxifen, raloxifene or ICI182780 that were observed (using Pathway Assist) to be involved in proliferation.
B: Those genes regulated by estrogen, tamoxifen, raloxifene or ICI182780 that were observed (using Pathway Assist) to be involved in apoptosis.

The EGF receptor ligand amphiregulin may play a central role:

Key role for EGF receptor signaling in estrogen, tamoxifen and ICI182780 networks. So far, it has been shown that estrogen, tamoxifen, raloxifene and ICI182780 modulate similar biological processes, but do so via regulation of different sets of genes. The next question, which was asked, was whether these different sets of genes belonged to the same signaling networks or to different networks. To study this question, genetic network models were constructed using the Ingenuity database. In such a genetic (biological) network, molecules are nodes, and different types of connections represent interactions between the different genes. Of estrogen, tamoxifen and ICI182780 regulated genes, the largest network that could be constructed, centred on the EGF- receptor (Fig. 4.3). Interestingly, the EGF receptor itself is not regulated by estrogen, tamoxifen or ICI182780 (its changed expression remains below our arbitrary 2-fold cut-off) but its ligand, amphiregulin, is. Raloxifene is not involved in this network because it does not

regulate the EGF receptor ligand amphiregulin. Down-stream from the activated EGF receptor, signal transduction markedly diverges for the three ligands (as shown by the estrogen-branch, the tamoxifen-branch and the ICI182780-branch of the network). For reasons of clearness, several non-regulated genes were also included in this network. It is possible, however, that some of these non-regulated genes are actually regulated by one of the ligands because they are not present on the currently used micro-array (19.000 oligo-array), or because they are regulated in a different timeframe than that was investigated in the current experiments (48 hours).

From the network analysis, two different conclusions can be drawn. First EGF receptor signalling is differentially affected by estrogen, tamoxifen and ICI182780 because the three compounds differentially regulate the ligand for the EGF receptor (amphiregulin). Second, down-stream from the activated EGF receptor, the network is also differentially regulated because estrogen, tamoxifen and ICI182780 regulate a different set of genes within the different branches of the network (Fig. 4.3).

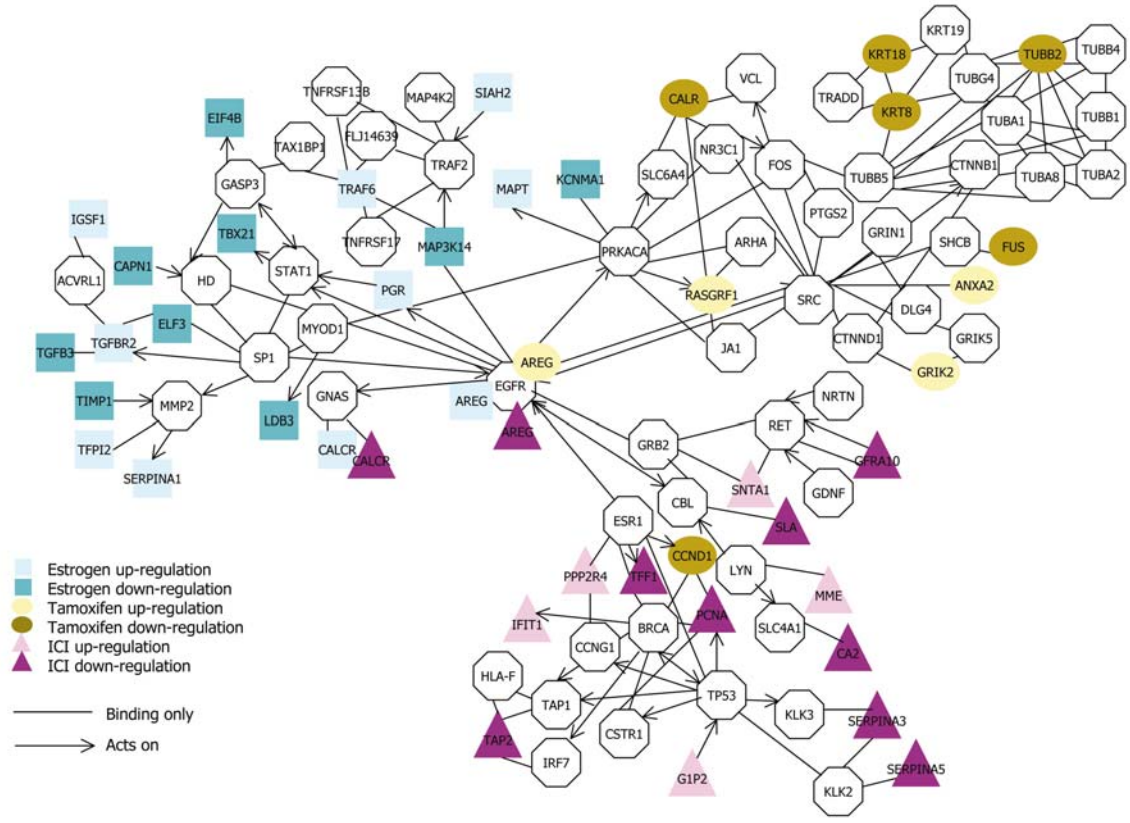

Figure 4.3. EGF receptor - Amphiregulin network, generated by using estrogen, tamoxifen and ICI182780 regulated genes and centred on the EGF receptor.
A genetic network was constructed using the Ingenuity database. The largest network that could be generated contained genes regulated by estrogen, tamoxifen and ICI182780, supplemented with non-regulated genes to make connections, and centred on the EGR receptor. The EGF receptor itself is not regulated by estrogen, tamoxifen or ICI182780 but its ligand (not above the 2-fold threshold), amphiregulin, is. Down-stream from the activated EGF receptor, signal transduction diverges for the three ligands (as shown by the blue estrogen-branch, the brown tamoxifen-branch and the purple ICI182780-branch of the network).

Amphiregulin is up-regulated by estrogen and tamoxifen and down-regulated by ICI182780.
Since EGF receptor-signaling was central in the constructed network, and since its ligand, amphiregulin was differentially regulated by estrogen, tamoxifen and ICI182780, this pathway was studied in more detail.

Our microarray data indicated that amphiregulin expression was enhanced by estradiol and tamoxifen, not effected by raloxifene and inhibited by ICI182780. From literature it is known that activation of the EGF receptor pathway by amphiregulin, may enhance proliferation. So, regulation of expression of amphiregulin in ECC-1 cells, potentially reflects the biological response of the human endometrium to administration of estrogens, SERMs and anti-estrogens. First, in order to confirm our microarray data, mRNA expression of amphiregulin was investigated by Northern blotting. The microarray observations could readily be confirmed this way (Fig. 4.4a).

Second, to investigate if amphiregulin could indeed activate the EGF-receptor, cells were cultured in the presence or absence of 20ng/ml amphiregulin. Evaluation of expression of the EGF-receptor upon stimulation by amphiregulin, revealed that the receptor is somewhat stabilized upon ligand binding (Fig. 4.4b. upper panel). Using an anti-phospho-tyrosine antibody, we observed an immediate increase of a phophorylated band running at the same height (of a similar molecular weight) in the gel as the EGF receptor, suggesting EGF-receptor autophosphorylation and potential subsequent activation of down-stream signaling (Fig. 4b. lower panel). Interestingly, administration of estrogen or tamoxifen to the cells did not induced EGF receptor phosphorylation. This may be explained because EGF receptor phosphorylation is usually only induced after a rapid administration of relatively large amount of ligand. It is very well likely that amphiregulin production under the influence of E2 or tamoxifen changes gradually and therefore does not result in a measurable change in phosphorylation of the EGF receptor.

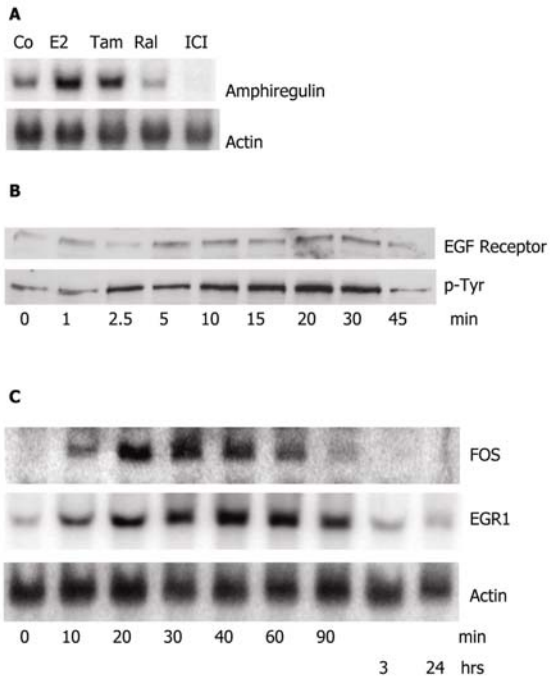

Figure 4.4. Amphiregulin is expressed by and can act on ECC1 cells.
A: Cells were cultured for 48 hours in the absence (Con) or presence of 1nM estradiol (E2), 1µM tamoxifen (Tam), 1µM raloxifene (Ral) or 100nM ICI182780 (ICI). RNA was isolated, electrophorezed and blotted. The Northern blot was hybridized to a labeled amphiregulin cDNA. Hybridization to Hamster β-actin cDNA was performed to validate equal loading of RNA on the different lanes of the gel.
B: Cells were incubated with 20ng amphiregulin for 0, 1, 2.5, 5, 10, 15, 20, 30 and 45 minutes. EGF receptor expression was determined (EGFR-ab), and an anti-phospho-tyrosine antibody (p-Tyr-ab) was used to detect autophosphorylation of the EGF receptor. Equal protein loading was verified using Ponceau S staining (not shown).
C: ECC-1 cells were incubated with 20ng/ml amphiregulin for 0, 10, 20, 30, 40, 60, 90 minutes or 3 and 24 hours before total RNA was isolated, electrophorezed and blotted. The ^{32}P-labeled EGR1 and c-FOS probes were hybridized to the blot. Hamster β-actin was used to verify equal loading of RNA samples to the lanes of the gel.

Third, since phosphorylation of the EGF-receptor seems to be induced by amphiregulin, we investigated whether this results in down-stream activation of the EGF receptor-signaling pathway. Therefore regulation of expression of two immediate early EGF-response genes, EGR-1 and c-FOS was studied. It was clearly shown that amphiregulin transiently stimulates the expression of EGR-1 and c-FOS (Fig. 4.4c).

Amphiregulin enhances proliferation of ECC-1 cells. Since amphiregulin can activate the EGF-receptor signaling pathway in ECC-1 cells, one would expect that amphiregulin affects the proliferation rate of these cells. Therefore proliferation assays were performed in which ECC-1 cells were cultured for 7 days in the absence or presence of amphiregulin. Culturing cells in the presence of amphiregulin resulted in enhancement of proliferation (Fig. 4.5a). Furthermore, since the specific inhibitor of the EGF-receptor, AG1478, abolishes induction of proliferation by amphiregulin, it was concluded that the EGF-receptor was indeed involved (Fig. 4.5b).

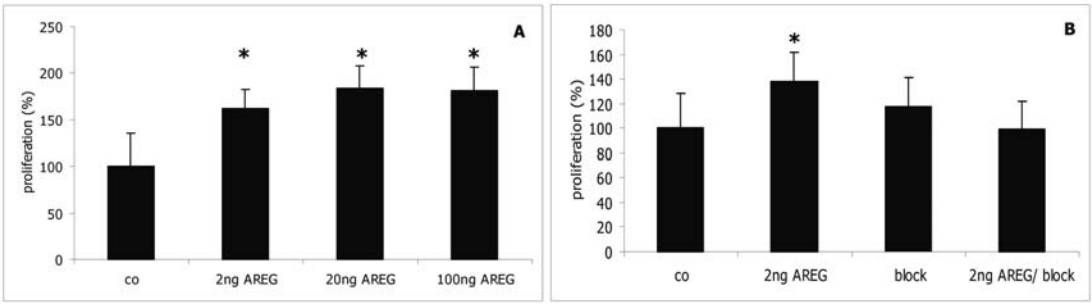

Figure 4.5. Amphiregulin enhances proliferation of ECC-1 cells. A: The cells were cultured for 7 days in the absence (Con) or presence of 2ng, 20ng or 100ng amphiregulin before assessment of growth by 3H-thymidine incorporation. B: The cells were cultured for 7 days in the absence (Con) or presence of 2ng amphiregulin (2ng), 600nM of the specific EGF receptor inhibitor AG1478, or a combination of the two (2ng/AG1478) before assessment of growth by 3H-thymidine incorporation. Differences between control and treatments were considered significant (*) when $p<0.05$.

4. 4 Discussion

In the current study, gene expression profiles were generated of a human endometrial cancer cell line responsive to estrogen, tamoxifen, raloxifene and ICI182780. The ECC-1 cell line, which was used, is derived from a well-differentiated endometrial cancer and expresses both estrogen receptors. In this cell line we could show that, promoters of well known estrogen-responsive genes (pS2 and C3) could be activated by estrogen and tamoxifen. This is in agreement with earlier work by Dardes et al., 2002 and indicates that in the ECC-1 cell line agonistic effects of tamoxifen could be measured at the gene regulatory level, which corresponds with the estrogen-agonistic activity of tamoxifen observed in endometrial tissues of postmenopausal women (Bergman et al., 2000; Dardes et al., 2002; Kedar et al., 1994). In contrast, estrogen mediated transcription, measured by luciferase assays and micro-array experiments, could be partly reversed by tamoxifen (86%) and raloxifene (90%) and completely reversed by ICI182780. This corresponds to the situation in endometrial tissues of premenopausal women, (who have high estrogen levels) where tamoxifen does act as an estrogen-antagonist (Chang et al., 1998; Mourits et al., 2001).

For these reasons, the ECC-1 cell line was considered a suitable model to investigate the transcriptional response to estrogen, tamoxifen, raloxifene and ICI182780 signaling in the human endometrium.

Differential regulation of proliferation and apoptosis

A remarkable finding of the current investigations was that the four ligands regulate similar biological processes in different ways. Upon studying the processes of proliferation and apoptosis in detail, the following was observed: estrogen, tamoxifen, raloxifene and ICI182780 regulate different sets of genes within these two biological processes. For example, only MME, ELF3, TIMP1, TFF1, GAL and AREG are co- or contra-regulated by more than one of the ligands. The fact that estrogen, tamoxifen, raloxifene and ICI182780 regulate different sets of genes is not unique for the endometrium, but was also observed in bone and breast cancer cells (Frasor et al., 2004; Kian Tee et al., 2004) and can be explained by differential cofactor binding after ER-activation by the different ligands (Brzozowski et al., 1997; Feng et al., 1998; Shiau et al., 1998). In breast cells, for example, where tamoxifen acts as an antagonist, the co-repressors NCoR and SMRT are recruited to the ER-tamoxifen complex, while in endometrial cells, were tamoxifen acts as an agonist, the co-activators SRC-1, AIBI and CBP are recruited to the ER-ligand complex (Shang and Brown, 2002; Shang et al., 2000). These observations further strengthen the concept that recruitment of different co-regulatory proteins to the ER-ligand complex is an important mechanism for the tissue-specific actions of SERMs.

Interestingly, the unique gene expression profile generated by tamoxifen, can also explain why tamoxifen-induced endometrial tumours have a different phenotype as compared to endometrial tumours induced by estrogens (Deligdisch et al., 2000).

Amphiregulin signaling potentially involved in estrogen-agonistic effects of tamoxifen

Network analysis revealed that although estrogen, tamoxifen, raloxifene and ICI182780 generate unique gene-expression profiles they modulate similar signaling networks. Upon reviewing the largest network, a central role for the EGF receptor and its ligand amphiregulin was observed, implicating that amphiregulin may be of more than normal importance for regulation of the endometrial response to estrogen, SERMS and anti-estrogens. In the current study, estrogen and tamoxifen increased amphiregulin expression; raloxifene had no effect on its expression while ICI182780 markedly reduced its expression. EGF receptor signaling is of interest for the current discussion because amphiregulin is a ligand for the EGF receptor and has been reported to be up-regulated in endometrial cancer tissues compared to normal endometrial tissues (Pfeiffer et al., 1997). However, there are six putative EGR receptor ligands and a change in the RNA level of one of six EGF-family ligands does not necessarily translate into a change in EGF receptor activation.

As stated above, culture of our endometrial cancer cell line in the presence of estradiol or tamoxifen resulted in increased expression of amphiregulin. Furthermore, when ECC-1 cells were cultured in the presence of amphiregulin this resulted in EGF receptor autophosphorylation, subsequent induction of expression of an early growth response gene, and eventually increased proliferation was measured.

Under physiological conditions, estrogens induce proliferation of the endometrium in the first half of the oestrous cycle and unopposed or sustained estrogen exposure often results in endometrial hyperplasia, which can derail in endometrial cancer. Interestingly, in pigs it was observed that expression of amphiregulin is increased in the first half of the oestrous cycle compared to the second half of the cycle (Kim et al., 2003) and as mentioned earlier, amphiregulin is increased in endometrial cancer (Pfeiffer et al., 1997). The observation that amphiregulin levels are enhanced by tamoxifen is, in light of these facts, an exciting new finding.

In contrast to the endometrium were tamoxifen displays estrogen-agonistic properties, in human breast tissue, tamoxifen is an estrogen-antagonist (Bergman et al., 2000). Interestingly, tamoxifen down-regulates the expression of amphiregulin in the breast (Frasor et al., 2004; Vendrell et al., 2004). Therefore, regulation of expression of amphiregulin by tamoxifen, may be an indicator of the tissue-specific activity of tamoxifen; estrogen-agonist in the endometrium or estrogen-antagonist in the breast.

Finally, it is possible that modulation of EGF receptor signaling, through regulation of amphiregulin expression, is important for estrogen-agonistic (tamoxifen-induced) induction of aberrant growth of the endometrium. Therefore, providing some of the more progressed (metastatic) endometrial cancer patients with specific EGF receptor modulators like gefitinib may be an interesting new treatment option, which is worthwhile pursuing on.

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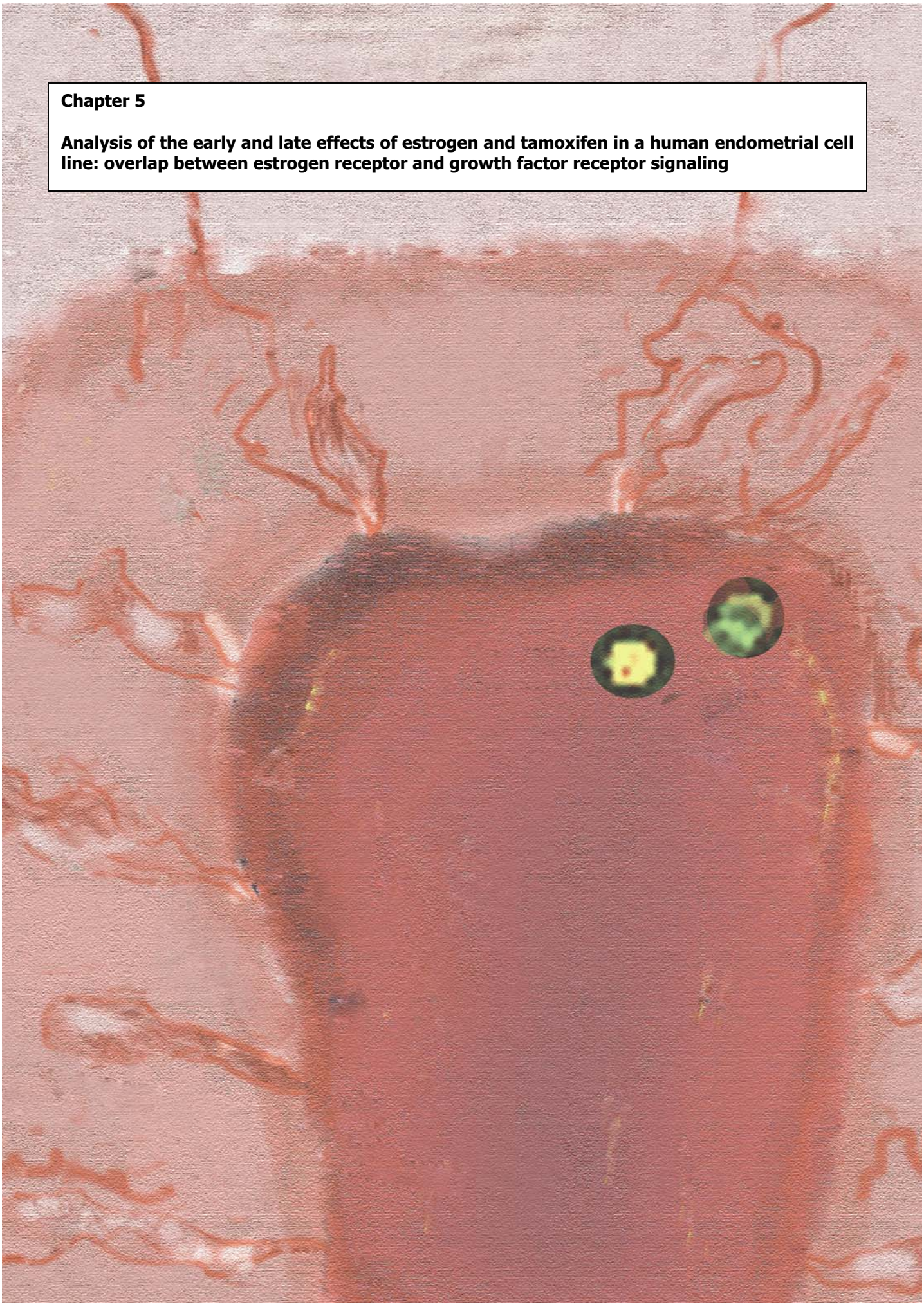
Chapter 5

Analysis of the early and late effects of estrogen and tamoxifen in a human endometrial cell line: overlap between estrogen receptor and growth factor receptor signaling



Analysis of the early and late effects of estrogen and tamoxifen in a human endometrial cell line: overlap between estrogen receptor and growth factor receptor signaling

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In Preparation

Abstract
Depending on the tissue, estrogen and tamoxifen exert different biological activities. For the endometrium, estradiol and tamoxifen induce proliferation. Tamoxifen-treatment of breast cancer patients results in a 2-7 fold increased risk for development of endometrial cancer. In the currently described experiments the role of activation of IGF1 receptor and EGF receptor signaling in mediating the effects of estrogen and tamoxifen in the endometrium was determined.

Micro-array analysis of ECC-1 cells treated for various lengths of time, indicated that rapid responses to treatment were very distinct from long-term responses to estrogen and tamoxifen treatment. Within the group of genes regulated upon treatment for 1hr, several IGF-1 receptor and EGF receptor target genes were found. Comparing long-term estrogen and tamoxifen regulated genes (24, 48 or 72 hr of treatment) with genes regulated by IGF-1 and the EGF receptor ligand amphiregulin revealed that also the late effects of estrogen and tamoxifen signaling were partly mediated via activation of IGF-1 and EGF receptor signaling pathways.

It was concluded that both early and late effects of estrogen and tamoxifen signaling are partly mediated via activation of the IGF1 receptor and the EGF receptor signaling pathways.

Keywords: Estrogen, tamoxifen, IGF1 receptor, EGF receptor, Micro-array

5.1 Introduction

Tamoxifen, which has an anti-estrogenic mode of action on breast cancer cells, is first choice adjuvant treatment for estrogen receptor positive (ER+) breast cancer in postmenopausal women. It has been shown that the survival rate of these tamoxifen-treated women goes up by as much as 50% (Early Breast Cancer Trialists' Collaborative Group, 1998). In the postmenopausal endometrium, however, tamoxifen appears to act as an estrogen-agonist, resulting in enhanced proliferation and an increased incidence of endometrial pathologies, including endometrial cancer (Bergman et al., 2000; Buzdar, 1998).

The classical mechanism of gene modulation by estrogens and tamoxifen is through direct transcriptional regulation via activation of the estrogen receptor, either ERα or ERβ. After binding of ligand, the ER dimerizes and binds to specific DNA sites, so called estrogen response elements (EREs), located in the promoter-regions of primary target genes (Nilsson et al., 2001). However, in humans around one third of genes regulated by ERs do not contain ERE-like sequences (O'Lone et al., 2004). Most of those secondary response genes are regulated by primary estrogen-regulated transcription factors. Alternative mechanisms of transcription regulation by the ER without DNA binding are through protein-protein interactions between the ER and other classes of transcription factors, like activator protein1 (AP-1) and Sp-1 (Gottlicher et al., 1998). Furthermore, besides these genomic effects of the ER, also non-genomic actions are described for this steroid receptor. Here, regulatory activities are the product of estrogen-activation of various protein-kinase cascades, like the MAP- and PI3-kinase signal transduction pathways (Bramley, 2003; Zhang et al., 2004).

For many organs, growth factor signaling plays a central role in mediating the effects of steroid hormones. In the endometrium, both insulin like growth factor receptor signaling (IGF1 receptor) and epidermal growth factor receptor signaling (EGF receptor) are important regulators of the effects of estrogens and tamoxifen (Giudice, 1994; Pfeiffer et al., 1997). Both the IGF1 receptor and the EGF receptor are membrane receptors with intrinsic tyrosine kinase activity. The IGF1 receptor consists of two α-subunits and two β-subunits, and ligand binding results in autophosphorylation of the β-subunits (Hwa et al., 1999; LeRoith et al., 1995). Binding of ligand to the EGF receptor results in dimerization of the receptor, which activates an intrinsic kinase activity in the cytoplasmatic tail of the receptor resulting in phosphorylation of the other receptor (Wells, 1999). For both the IGF1 and EGF receptors, phosphorylation induces the formation of docking sites for adaptor proteins and subsequently several downstream signaling pathways are activated, like PI3- and MAP-kinase pathways (Surmacz et al., 1998; Yarden, 2001).

Many examples of interactions between ER signaling and growth factor signaling have been reported. We, and others, have previously shown that both estradiol and insulin like growth factor 1 (IGF1) stimulate proliferation of endometrial cancer cells. The ER and IGF1 signaling pathways seem to be intertwined in mediating these effects, since blocking IGF signaling reduces the effect of estradiol (Gielen et al., 2005a). Visa versa, the effect of estradiol on the IGF1 signaling pathway seems more complicated. In Ishikawa cells, estradiol sensitizes the IGF1 effect by up-regulation of IGF receptors, and both estradiol and tamoxifen enhance IGF1 stimulated phosphorylation of the IGF1 receptor (Karas et al., 1995; Kleinman et al., 1995). However, IGF1 induced proliferation is not reduced by blocking ER signaling (Gielen et al., 2005a).

One of the EGF receptor ligands, amphiregulin (AREG), was found to be up-regulated by both estradiol and tamoxifen in the ECC-1 endometrial cancer cell line (Gielen et al., 2005b). Furthermore, it was also shown that in the ECC-1 cell line AREG induces phosphorylation of the EGF receptor resulting in regulation of EGF-responsive genes and subsequent proliferation of cells (Gielen et al., 2005b). These results indicate that possibly tamoxifens estrogen-agonistic effect is partly mediated via EGF receptor signaling. Interestingly, in the breast, where tamoxifen acts as an estrogen-antagonist, AREG is up-regulated by estrogen but down-regulated in the presence of tamoxifen (Gielen et al., 2005b; Vendrell et al., 2004).

If, for the human endometrium, estrogen and tamoxifen signaling depends to a certain extend on IGF1 and EGF receptor signaling, it becomes of interest to measure to what extend the

ER and growth factor signaling pathways are intertwined. The current study measures genomic and non-genomic effects of estrogen and tamoxifen signaling in a human endometrial cancer cell line and evaluates the role of growth factor signaling herein.

5.2 Materials and methods:

Cell culture:
The ECC-1 cell line is derived from a well-differentiated adenocarcinoma of the human endometrium transplanted into nude mice, and was a generous gift from Dr. B. van den Burg (Utrecht, the Netherlands) (Satyaswaroop et al., 1988). This cell line was stably transfected to express human progesterone receptor A (hPRA) and progesterone receptor B (hPRB), resulting in clone ECC-1/PRAB72 and was continuously cultured under selection pressure (250µg/ml hygromycin, Gibco Invitrogen Corporation, Carlsbad, CA, USA) (Hanifi-Moghaddam et al., 2005). Cells were routinely maintained in DMEM/F12 (Gibco) + 5% FBS (Greiner, Frickenhausen, Germany) supplemented with penicillin/streptomycin in a 37ºC incubator at 5% CO_2. One week before the experiments started, cells were transferred to phenol red free DMEM/F12 supplemented with 5% dextran coated charcoal treated FBS (DCC-FBS). The following hormones and hormone-like compounds were used: estradiol (E_2), 4OH-tamoxifen (Tam), Insulin like growth factor 1 (IGF1; Sigma-Aldrich Chemie BV, the Netherlands) and human recombinant amphiregulin (AREG; R&D systems, Abingdon, UK).

RNA Isolation, Amplification and Hybridization;
Total RNA of ECC1-PRAB72 cells was isolated using the LiCl/Ureum method (Auffray and Rougeon, 1980). Quality of RNA was ensured before labeling by analyzing 20ng of each sample using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent Technologies Netherlands B.V. Amstelveen, The Netherlands). Of each sample cRNA was synthesized and labeled according to the Affymetrix protocol, followed by hybridization to the U133 plus 2.0 GeneChip (Affymetrix, Santa Clara, CA, USA).

Data analysis
ECC1-PRAB72 cells were cultured for 1, 6, 12, 24, 48 and 72 hours in the presence of ethanol (control), 1nM estradiol (E_2) or 1µM 4OH-tamoxifen (Tam). For experiments with growth factors cells were cultured for 24 and 48 in the presence of ethanol (control), 10nM insulin like growth factor 1 (IGF1) or 20ng/ml amphiregulin (AREG). Raw expression values were analyzed using the GeneChip Operating Software (GCOS) provided with Affymetrix Genechip Service. Intensity values were scaled to an average of 100 per GeneChip according to the method of global scaling provided in the GCOS software, version 1.0. Using this method only reliable results are generated for samples with an average intensity value of 30 or more, and therefore all values between 0 and 30 were set to 30 (Valk et al., 2004). This procedure affected 46% of all intensity values, of which 98% were flagged as absent or marginal by he GCOS software, while 2% were flagged as present according to the GCOS software, indicating the reliability of this method.

The following steps were undertaken to normalize the data, using Microsoft® Excel software (http://www.microsoft.com). As a first step, per gene, the geometric mean of the hybridization intensities over all controls was calculated. Secondly, for all samples stimulated with estradiol, tamoxifen, IGF1 or AREG, the level of expression per gene was determined relative to the geometric mean of the controls for that gene. As a last step, the newly generated expression levels were log transformed (on a base 2 scale) to assign gene-expression levels with similar relative distances to the geometric mean. As a result of this, deviation from the geometric mean of the controls reflects differential gene-expression.

Clustering of estrogen and tamoxifen profiles
Using EPSCLUST (Expression Profile data CLUStering and analysis) at http://ep.ebi.ac.uk/EP/EPCLUST unsupervised cluster analysis was performed. Genes whose level of expression differed from expression level of the controls two-fold in at least one sample were used for analysis.

Biological classification of genes
For functional classification of genes we used Pathway Assist 2.5 (http://www.ariadnegenomics.com/products/pathway.html). The Pathway assist database contains biological knowledge represented in a formalized form focused on how proteins, cellular processes and small molecules interact, modify and regulate each other. Pathway Assist provides a method for searching objects individually by keyword, strings or attributes. These include, for example, type (protein,enzym), effect (positive, negative, unknown), mechanism (transcription, phosphorylation), tissue type, biological process, belonging to cell structure, and others.

For building networks, Ingenuity Pathway analysis was used https://analysis.ingenuity.com/pa/. This database utilizes the Ingenuity Pathway Knowledge Base (IPKB) to computationally analyze datasets to identify networks or pathways.

Quantitative PCR
Validation of micro-array expression data was accomplished by selection of 14 genes. First strand cDNA synthesis was performed using 2µg of total RNA and the Superscript 2 enzyme (Gibco, Carlsbad, CA, USA), according to a standardized protocol (protocol is available on request). Real-time PCR was performed using SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, USA) in the Opticon 2 apparatus (MJ Research, Bio-Rad laboratories Inc., Waltham, MA, USA). Of each sample 5ng cDNA was used for the PCR reaction. Per reaction, a melting curve analysis was performed following each experiment to ensure the presence of a single amplified product. All PCRs were performed in duplo. Starting quantity for each gene analyzed was determined using the Opticon monitor software. Using this quantity, the expression level of each gene was normalized to the expression level of the reference gene, β-actin.

5.3 Results

Estrogens and tamoxifen increase proliferation of the endometrium in postmenopausal women, which results in an increased risk for the development of endometrial cancer. Previously we have shown that in an endometrial carcinoma cell line, treated for 48 hours with estradiol or tamoxifen, different sets of genes were regulated by these ligands. Furthermore, estradiol seemed more potent than tamoxifen because estradiol regulated many more genes. However, since only one time-point was investigated, it is also possible that tamoxifen regulated more genes but at a later time-point. In order to investigate this, in the current investigations, the ECC-1/PRAB72 cell line was treated with estradiol or tamoxifen for 0, 1, 6, 12, 24, 48 or 72 hours. After treatment, total RNA was isolated and genome-wide gene-expression was measured using the Affymetrix U133 plus 2.0 GeneChip. The micro-array data obtained with RNA isolated after 12 hours of treatment with tamoxifen were of poor quality and therefore, these data were left out of the analysis.

Unsupervised clustering was performed for all genes which expression level differed at least 2-fold from the control (in one of the treatments and/or in one of the time points). In Figure 1 unsupervised cluster analysis resulted in a dendogram in which the treatment-groups were divided in three braches. From the two right-hand branches of the dendogram it was concluded that the gene-expression profiles generated after 24, 48 and 72 hours of treatment were different for estradiol compared to tamoxifen. Furthermore, again estradiol is more potent than tamoxifen in regulation of numbers of genes (for estradiol the average is 1284 regulated genes per time-point, while for tamoxifen the average is 490 genes per time-point) and tamoxifen-

induced gene modulation does not take effect slower or faster than estrogen-induced gene modulation.

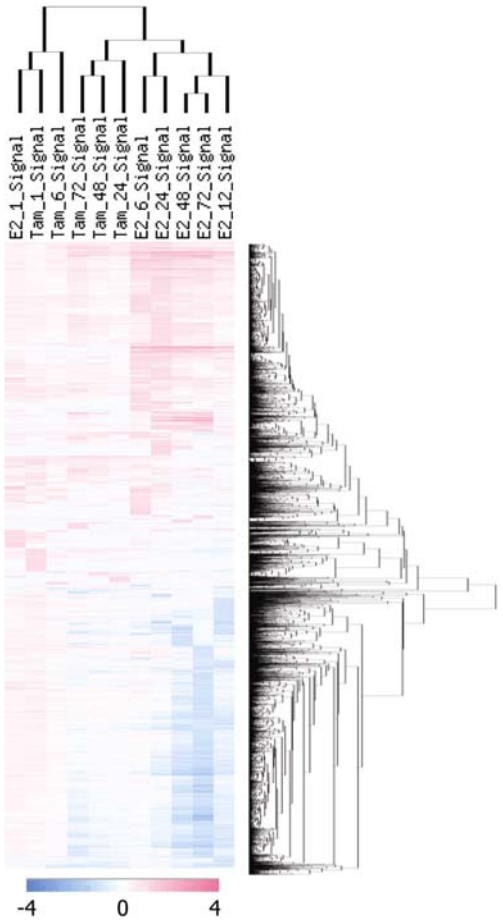

Figure 5.1. Gene-expression profiles are different between estrogen and tamoxifen treatment and between early and late treatment. Cells were cultured for 1, 6, 12, 24, 48 or 72 hours with or without estrogen (E2) or tamoxifen (Tam). Hierarchical clustering represent genes in which in at least one sample the ratio versus the average of 6 controls differs 2-fold. The dendogram at the top indicates the division of samples into several arbitrarily groups.

Interestingly, the left-hand branch in the dendogram only contains short-term treated groups: genes regulated by treatment for 1h with estradiol and 1h and 6h with tamoxifen cluster separately and away from the genes regulated at other time-points. Regulation of genes by steroid receptors after 1 hour of treatment is remarkable because gene regulation by steroid receptors is usually not that fast (Bjornstrom and Sjoberg, 2005). However, there are some reports in literature on non-genomic effects of steroid receptors on growth factor signaling and therefore these observations were investigated further (Hamelers and Steenbergh, 2003).

Early response to estrogen and tamoxifen: involvement of IGF receptor and EGF receptor signaling pathways.

Since treatment for 6hr with tamoxifen could probably also affect regulation of genes via transcriptional activation by the ER, we focused our investigations into the non-genomic effects of estradiol and tamoxifen on genes regulated after 1hr of treatment. Treatment of cells for 1 hour revealed regulation of 665 genes by estradiol and 794 by tamoxifen. Of these genes, 106 genes (approximately 15 %) overlap between the treatment groups. Functional classification of all identified genes using the pathway assist database (combination of a number of other databases like GO and KEGG), revealed that the regulated genes affect several cellular processes such as cell survival, focal contact, regulation of signal transduction and differentiation. Most genes, however, were involved in proliferation and apoptosis. Furthermore, comparing the regulated genes with literature we found that a high level of genes could be linked to the IGF1 and EGF receptor pathway. Of the estradiol-regulated genes, 50 genes were linked to the IGF1

receptor pathway and 69 genes to the EGF receptor pathway; of the tamoxifen-regulated genes, 35 genes were linked to IGF1 receptor signaling and 54 genes to EGF receptor signaling.

Early response to estrogen and tamoxifen: Activation of IGF receptor and EGF receptor signaling pathways.

Several mechanisms are hypothesized and reported to be responsible for the non-genomic interactions between ER signaling and growth factor signaling. As a first step, in order to measure direct effects of ER signaling on the IGF1 or EGF receptor, receptor phosphorylation was determined. Therefore, cells were cultured for 0, 5, 15, 30, 45, 60 minutes in the presence of E_2 or tamoxifen. Unfortunately, no increment in the phosphorylated forms of either receptors was observed, indicating that interactions between ER signaling and growth factor signaling is not realized via direct activation of growth factor receptors.

	E2_30 min	E2_1h	E2_6h	Tam_30 min	Tam_1h	Tam_6h
EGR3	14.7	3.1	5.7	6.9	12.4	7.5
NH4A1	19.7	12.3	0.8	8.6	10.3	2.0
FOS	2.9	2.1	1.9	1.8	1.5	3.6
MYC	8.5	8.8	5.8	3.5	1.8	2.6
EGR1	9.2	3.8	2.0	8.9	1.9	1.1
	IGF_30 min	IGF_1h	IGF_6h	AREG_30 min	AREG_1h	AREG_6h
EGR3	3.9	2.2	1.0	5.3	0.9	2.5
NH4A1	28.1	115.1	8.8	27.3	54.2	2.4
FOS	1.7	6.4	3.4	1.3	2.5	2.8
MYC	4.4	2.3	1.5	1.5	3.0	1.4
EGR1	18.8	22.3	3.5	29.6	10.8	0.6

Table 5.1. RT-PCR data on genes involved IGF receptor and EGF receptor signaling regulated by estrogen and tamoxifen. Cells were cultured for 0, 30 and 60 minutes in the presence of estrogen (E2), tamoxifen (Tam), insulin like growth factor 1 (IGF1), amphiregulin (AREG). Data represent the expression ratio of the indicated genes in stimulated cells versus non-stimulated cells.

Another mechanism of interaction between ER signaling and growth factor signaling that has been postulated is through interaction with MAPK and PI3K signaling pathways (Bjornstrom and Sjoberg, 2005). If growth factor signaling is activated by ER signaling through this mechanism, incubation of cells with ER ligands and growth factors should result in activation of similar down-stream acting genes in approximately the same timeframe. Therefore, cells were cultured for 0, 30 and 60 minutes in the presence of E_2, tamoxifen, IGF-1, or AREG (AREG is an EGF receptor ligand hypothesized to be involved in estrogen and tamoxifen regulation of the endometrium). The genes tested were selected on the basis of our micro-array data and linkage to EGF receptor and IGF receptor activation in literature. It was observed that expression of EGR3, FOS, IRS2, MYC and NR4A1 indeed showed a significant overlap between ER and growth factor signaling(Table 1). These experiments, however, need to be followed up upon.

Late response to estrogen and tamoxifen: involvement of IGF receptor and EGF receptor signaling pathways.

Since earlier work in human endometrial cancer cell lines indicated that growth factor signaling was significantly affected by estrogen receptor signaling, this was investigated in more detail (Gielen et al., 2005a; Gielen et al., 2005b). Additional micro-arrays were performed on cells treated for 24 or 48 hours in the absence or presence of IGF-1 or amphiregulin (AREG).

Unsupervised clustering of expression profiles of cells treated for 24, 48 or 72 hours with estrogen or tamoxifen and cells treated for 24 or 48 hours with IGF-1 or AREG revealed that growth factor regulated genes cluster separate and away from E2 or tamoxifen regulated genes (Fig.2). If then, the actual numbers of regulated genes are reviewed (genes were selected if regulation is at least 2-fold in one time-point) (Fig.3) it was observed that 3138 genes were estrogen-regulated, 1268 genes tamoxifen-regulated, and 347 genes were regulated by both

steroids. Furthermore, 1707 genes were IGF-1-regulated, 2451 genes AREG-regulated and 501 genes were regulated by both growth factors.

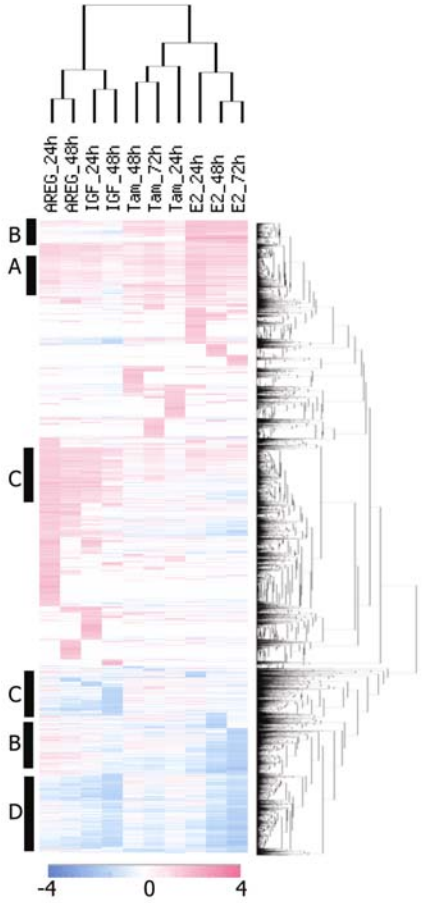

Figure 5.2. Estrogen, tamoxifen, IGF-1 and amphiregulin generate a specific gene-expression profile. Cells were cultured for 24, 48, 72 hours with or without estrogen (E_2), tamoxifen (Tam) and for 24 and 48 hours with or without insulin like growth factor 1 (IGF-1) of amphiregulin (AREG). Hierarchical clustering was performed for genes in which in at least one sample the ratio versus the average of 6 controls differed 2-fold. The dendogram at the top indicates the division of samples into several arbitrarily groups.

As a subsequent step, we aimed to identify regulated genes that potentially contribute to functional similarities and differences between ER signaling and growth factor signaling. In order to do so in figure 3 several informative clusters are indicated. In cluster A, genes are grouped that were regulated by all four ligands. Cluster B contains genes that were regulated by estrogen and tamoxifen, but were not regulated by IGF-1 or AREG and within cluster C, genes are grouped that were only regulated by IGF-1 and AREG, and not regulated by estrogen or tamoxifen. A very interesting group of genes is indicated in cluster D. Here genes are indicated which were regulated by estrogens and by IGF-1, but not regulated by tamoxifen or AREG. Furthermore, of the estrogen-regulated genes, 407 genes are also IGF-1 regulated, and 305 genes overlap with AREG regulated genes. For tamoxifen-regulated, 126 genes are also regulated by IGF-1 and 168 genes by AREG (Fig.4).

Results from the cluster analysis will be evaluated further in the discussion section. The complete list of genes will be made available upon publication.

Validation of micro-array data

In order to verify our micro-array data further, expression of a number of genes was measured using real time PCR. Expression of the following genes was tested: AREG, BCMP11, CTSD, EPAS1, IGFBP4, MGP and PDZK1 (Table 2).

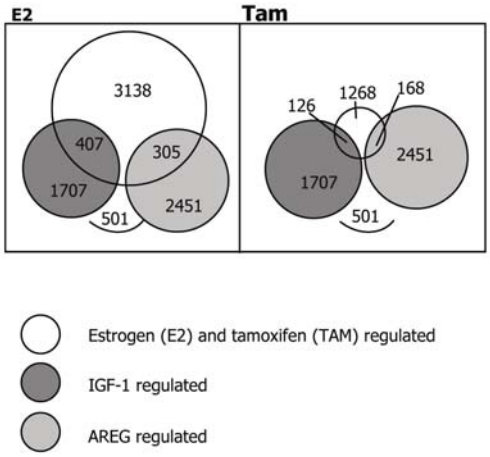

Figure 5.3. Overlap between genes regulated by estrogen, tamoxifen, IGF-1 and amphiregulin. Per treatment group a list was compiled of genes regulated at least 2-fold (log2, -1< or >1) in one time-point. The number of overlapping genes between treatment groups was determined.

5.4 Discussion

Gaining insight into the molecular mode of action of tamoxifen in the human endometrium is important, since exposure is associated with an increased risk for the development of endometrial cancer (Bergman et al., 2000; Buzdar, 1998). The current investigations started off as a detailed analysis of differences and similarities between estrogen and tamoxifen signaling. Doing this, it was discovered that both at early and at late time-points estrogen receptor signaling overlaps with growth factor signaling. Therefore it was decided to study the early and late effects of estrogen and tamoxifen in a human endometrial cell line, with specific emphasis on the overlap between estrogen receptor and growth factor receptor signaling.

One of the most striking findings was that estrogen receptor signaling at early time-points is quite different from signaling at later time points. A possible explanation for this observation is that rapid effects of steroids are due to the use of non-genomic routes, in which the transcription machinery is activated via direct interactions of the estrogen receptor with protein-kinase signaling pathways (Bramley, 2003; Zhang et al., 2004). Reviewing all genes regulated at 1 hour after estrogen or tamoxifen administration to the culture medium revealed regulation of many IGF-1 and EGF target genes. Upon measuring potential direct activation of the IGF-1 and EGF receptor by estrogens and tamoxifen, we could not show any increments in active forms of these receptors. Nevertheless, upon reviewing the expression of a number of early EGF and IGF-1 regulated genes using RT-PCR, regulation by estradiol or tamoxifen as well as regulation by AREG or IGF-1 could readily be confirmed. This indicates that rapid effects of estradiol or tamoxifen are not caused through direct activation of the IGF or EGF receptor, but might be due to interaction with MAPK or PI3K signaling pathways that subsequently activate the expression of early EGF and IGF-1 target genes.

A question that is fair to ask in this respect, is what the relevance may be of such a fast, non-genomic response to estrogen or tamoxifen stimulation. This question is not easily answered because during physiological processes, estrogen receptor signaling is a fairly sustained event that changes over a matter of days rather than over a matter of minutes. Therefore the overlap between estrogen receptor signaling and growth factor signaling was also analyzed at later time-points.

Cross-talk between ER signaling and growth factor signaling

Earlier work of our group and others indicated that growth factor signaling was significantly affected by estrogen receptor signaling. To test if IGF-1 and EGF receptor pathways are involved in mediating the cross-talk between growth factor signaling and ER signaling an the genomic level, gene-expression profiles were generated of cells treated for 24, 48 and 72 hrs in the presence or absence of estradiol, tamoxifen, IGF-1 or amphiregulin (AREG). These ligands were chosen on the basis of earlier experiments: IGF-1 enhances proliferation of ECC-1 cells, and

AREG was found to be up-regulated by estrogen and tamoxifen while culturing cells with AREG stimulated proliferation (Gielen et al., 2005a; Gielen et al., 2005b). Cluster analysis off all samples identified several interesting gene-clusters, including genes regulated by all ligands, growth factor specific genes and ER specific genes. Furthermore, subsequent analysis of overlapping genes indicated that 305 genes were regulated by estradiol and AREG, 168 genes by tamoxifen and AREG, 407 genes by estradiol and IGF-1 and 126 genes are regulated by tamoxifen and IGF-1. On the basis of this, we hypothesize that activation of IGF-1 and EGF receptors plays a role in mediating the effects of estrogen and tamoxifen in the endometrium. This will be subject of further experiments.

PCR

	E2_24hr	E2_48hr	E2_72hr	TAM_24hr	TAM_48hr	TAM_72hr	IGF_24h	IGF_48h	AREG_24h	AREG_48h
AREG	4.8	5.6	6.0	2.3	3.7	4.4	1.6	1.2	0.5	1.3
IGFBP4	1.9	0.7	1.6	1.3	1.2	0.9	-0.1	-0.3	-1.3	-0.7
BCMP11	4.6	4.5	4.1	4.6	4.8	5.4	-0.7	-1.1	-1.8	-1.0
CTSD	1.8	2.0	2.4	1.1	1.0	1.3	0.5	0.0	0.1	0.5
MGP	2.6	3.9	5.7	-0.2	-0.2	-0.1	-0.7	-1.3	-1.1	-1.0
EPAS	1.7	0.3	-1.2	2.0	0.9	0.6	0.4	-0.2	0.1	-0.9
PDZK1	2.9	1.4	2.2	2.3	2.2	2.5	-1.7	-2.1	-2.7	-1.9

Micro-array

	E2_24hr	E2_48hr	E2_72hr	TAM_24hr	TAM_48hr	TAM_72hr	IGF_24h	IGF_48h	AREG_24h	AREG_48h
AREG	2.2	3.1	3.7	0.0	2.2	3.2	0.8	0.3	1.0	0.7
IGFBP4	2.4	1.7	1.3	1.0	1.6	1.2	-0.4	-0.2	0.3	0.2
BCMP11	4.3	4.1	3.7	3.8	4.8	5.0	0.0	0.0	0.0	0.0
CTSD	2.6	2.1	1.8	1.0	1.1	1.8	1.2	1.0	1.8	1.4
MGP	1.8	3.2	4.1	0.2	-0.3	-0.2	0.0	0.0	1.1	0.0
EPAS1	-1.7	-2.2	-2.6	-0.7	-1.2	-1.2	0.8	0.0	1.7	1.0
PDZK1	1.7	2.1	2.0	1.5	2.1	1.9	-0.9	0.0	0.0	-1.0

Table 5.2. Validation of differential gene-expression by quantitative RT-PCR. Cells were cultured for 24, 48 or 72 hours in the presence or absence of estrogen (E_2) or tamoxifen (Tam), and for 24 or 48 hours in the presence or absence of amphiregulin (AREG) or insulin like growth factor 1 (IGF-1). Data represent the expression ratio of the indicated genes in
stimulated cells versus non-stimulated cells.

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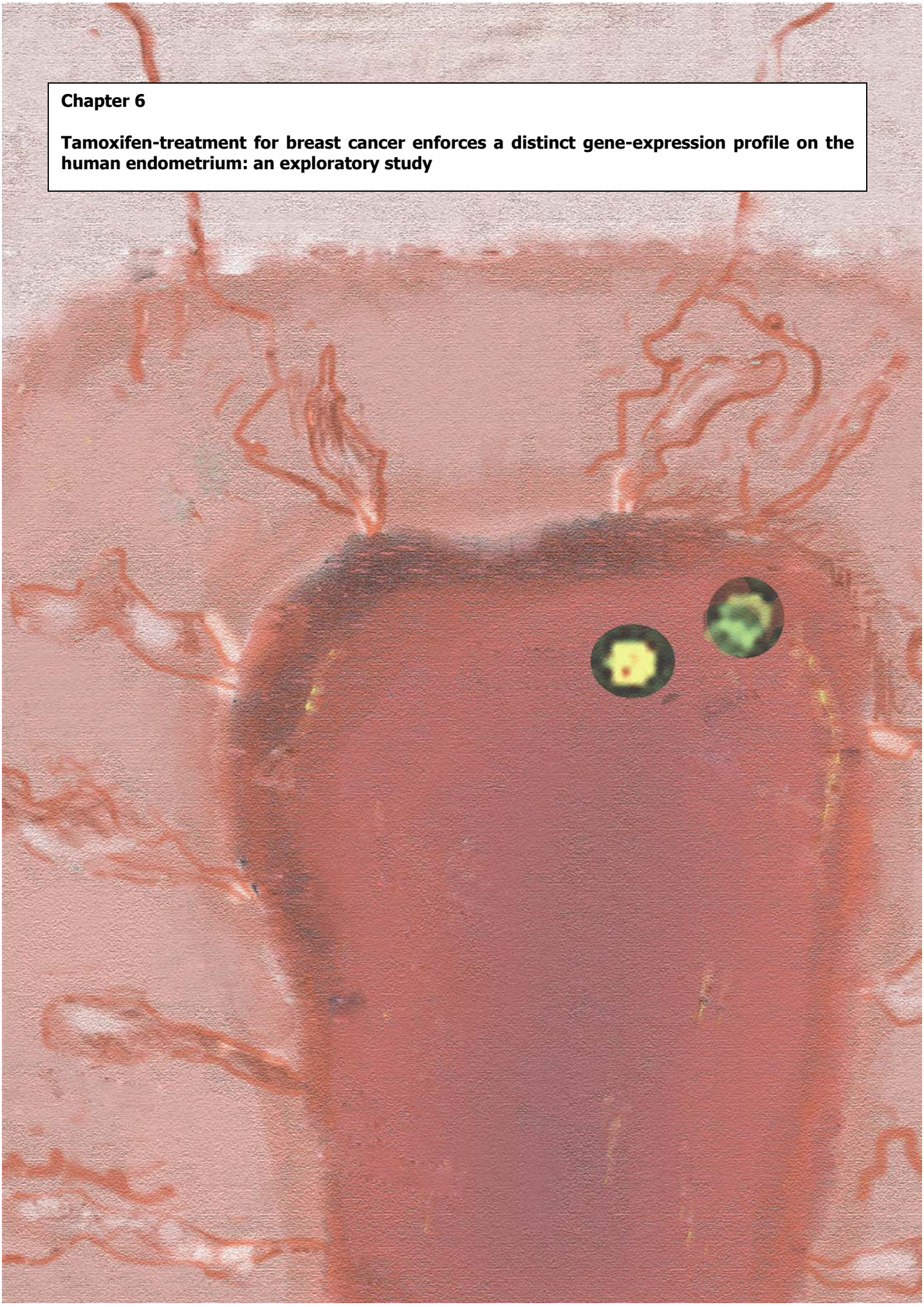
Chapter 6

Tamoxifen-treatment for breast cancer enforces a distinct gene-expression profile on the human endometrium: an exploratory study



Tamoxifen-treatment for breast cancer enforces a distinct gene-expression profile on the human endometrium: an exploratory study

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Endocrine-Related Cancer, accepted

Abstract:
Tamoxifen treatment for breast cancer increases proliferation of the endometrium, resulting in an enhanced prevalence of endometrial pathologies, including endometrial cancer. An exploratory study was performed in order to begin to understand the molecular mechanism of tamoxifen action. Gene-expression profiles were generated of endometrial samples of tamoxifen-users and compared to matched controls. The pathological classification of samples from both groups included atrophic/inactive endometrium and endometrial polyps.

Unsupervised clustering revealed that samples of tamoxifen-users were, irrespective of pathological classification, fairly similar and consequently form a subgroup distinct from the matched controls. Using SAM analysis (a statistical method to select genes differentially expressed between groups), 256 differentially expressed genes were selected between the tamoxifen- and the control-group. Upon comparing these genes with estrogen-regulated genes, identified under similar circumstances, 95% of the differentially expressed genes turned out to be tamoxifen specific.

Finally, construction of a gene expression network of the differentially expressed genes, revealed that 69 genes centred around 5 well-known genes; TP53, RELA, MYC, EGF receptor and ß-catenin. This could indicate that these well-known genes, and the pathways in which they function, are important for tamoxifen-controlled proliferation of the endometrium.

Keywords: human endometrium, tamoxifen, gene-expression network

6.1 Introduction

Tamoxifen is first choice adjuvant treatment, for primary estrogen receptor positive (ER+) breast cancer in postmenopausal women. It has been shown that survival rates in tamoxifen-treated women are improved as much as 50% (Early Breast Cancer Trialists' Collaborative Group, 1998). Furthermore, tamoxifen-use has also been shown to reduce the incidence of breast cancer in healthy women at high-risk for this disease (Mokbel, 2003; Powles, 1998; Veronesi et al., 1998). The mechanism of action of tamoxifen in breast cancer patients is that it inhibits cancer cell growth by competitive antagonism with estrogens for its receptor (Ring and Dowsett, 2004).

One of the most significant side effects of treatment with tamoxifen appears to be its proliferative effect on the endometrium (estrogen-agonistic effect) (Bergman et al., 2000; Buzdar, 1998). Several studies have evaluated the incidence of endometrial pathologies in tamoxifen-users, and although occurrence rates differ per study, a higher incidence in the tamoxifen group is generally agreed upon. Endometrial pathologies associated with tamoxifen-use include hyperplasia, polyps, carcinomas and sarcomas (Cohen, 2004).

The mechanism of action of tamoxifen is very complex. It is generally agreed that conformational change of the receptor after ligand binding differs between estrogen and tamoxifen, resulting in binding of other co-factors to the ligand-receptor complex (Brzozowski et al., 1997). The tissue-dependent mode of action of tamoxifen can then be explained by the relative abundance or paucity of co-factors in different tissues (McDonnell, 2004). For example, in breast cells the co-repressors NCoR and SMRT are recruited to the ER-tamoxifen complex, while in endometrial cells the co-activators SRC1, AIBI and CBP are recruited to the ER-tamoxifen complex (Shang and Brown, 2002; Shang et al., 2000).

The formation of the ER-tamoxifen complex results in downstream activation of genes and pathways. Several genes have been studied to investigate the effects of tamoxifen on the human endometrium. It has been suggested that the expression of TGFß1, p27, Cathepsin D and CA125 is different in benign endometrial samples of tamoxifen-users when compared to non-users (Carmichael et al., 2000; Mylonas et al., 2003a; Mylonas et al., 2003b; Siufi et al., 2003). In contrast, the expression of inhibin/activin alpha and beta in polyps was found to be similar in both groups (Mylonas et al., 2004). Furthermore, the apoptosis/proliferation index, determined by measuring the proliferation marker Ki67 and the apoptosis markers Fas, FasL and BCL2, is higher in benign endometria of tamoxifen-users compared to endometria of non-users (Mourits et al., 2002a; Mourits et al., 2002b).

Interestingly, differences in expression levels are not observed when endometrial carcinomas from tamoxifen-users and matched controls are compared. Micro-array analysis has so far not shown any difference in gene expression profiles between tamoxifen-associated tumours and matched controls (matched for stage, age and histology) (Ferguson et al., 2004). It is true, however, that endometrial tumours seem to develop sooner and are more aggressive in tamoxifen treated patients (Cohen, 2004).

In the current investigations, it was speculated that the effects of tamoxifen on gene-expression are specifically detected in benign endometria of tamoxifen-users. In order to explore this hypothesis further, a genome-wide micro-array was used to generate gene-expression profiles of samples containing 100% endometrium of women exposed to tamoxifen (7 patient samples) in comparison to matched controls (6 patient samples). Interestingly, even in this limited patient group, it was observed that the gene-expression profile of endometria obtained from women using tamoxifen was very distinct from endometria of matched controls. Furthermore, upon comparing the differentially expressed genes with estrogen regulated genes identified under similar circumstances, most of the differentially expressed genes turned out to be tamoxifen specific. Finally, to gain insights into the cellular effects of tamoxifen, we generated a gene expression network, which seems to point to relatively profound effects of tamoxifen on cell cycle regulation and cell survival.

6.2 Materials and Methods

Tumour samples and clinicopathologic characteristics:
The human subjects review board of the Erasmus MC, University Medical Center Rotterdam, The Netherlands, approved this study. Between 2002 and 2004 endometrial curettages and endometrial tissues from abdominal uterus extirpations were obtained from patients attending the gynaecologic oncology unit from the Erasmus MC University Medical Center (Department of Obstetrics & Gynaecology) for vaginal bleeding. All patients were postmenopausal, defined as no menstrual period in the preceding 12 months. There were two patient groups; one group using tamoxifen as adjuvant therapy for breast cancer (tamoxifen-users); and the other group (control) that had not used tamoxifen or any other sex-hormone related therapy. Because in The Netherlands, patients with node-positive breast tumours and a positive oestrogen and/or progesterone receptor status are all treated with tamoxifen, it was impossible to fully match the tamoxifen-users group with a control group consisting also of node positive and receptor positive breast cancer patients. Therefore, our control group did not contain any breast cancer patients.

Before using any of the surgical specimens, the histological classification of all curettage and hysterectomy specimens was revised using standardized guidelines by a pathologist experienced in gynaecopathology (Klaassens et al., 2005). The endometrium was assessed as inactive/atrophic or as containing any proliferative activity, hyperplasia or a polyp. Where the specimen was a curettage, the presence of a polyp was determined by assessment of the form of the fragment, presence of large blood vessels, nature of the stroma and comparison with adjacent endometrium. The appearance of the glands within the polyp was recorded.

In total, primary tissues from 17 patients who had used tamoxifen and 8 control patients, were collected; all specimens were snap-frozen and stored at -80°C. Sandwich sections were made of the samples to establish the percentage of endometrium (Smid-Koopman et al., 2004). Of the 17 endometrial tissues from tamoxifen-users, 9 samples were excluded for further evaluation; in 8 of these samples the amount of endometrial tissue was to low (percentage of endometrium less than 100%), and 1 sample was histological classified as endometrial cancer. From the remaining 8 samples RNA was isolated and the quality of RNA was verified. In one of these 8 samples the RNA turned out to be degraded. The remaining 7 samples were used for a micro-array experiment. These 7 samples were matched, based on histological classification, to tissue samples of patients attending the hospital for vaginal bleeding but without prior tamoxifen exposure (and without breast cancer). Initially 8 control samples were collected, from one sample the amount of endometrial tissue was to low and one sample was excluded because of malignant pathology. Patients that had used hormone replacement therapy were excluded from the control group. Patient characteristics (Table 1) of the tamoxifen-group and the control-group were compared using the one-way ANOVA test (SPSS version 11).

group	patient	age (years)	pathology	tamoxifen (months)
G1	**43**	47	inactive	27
G1	**45**	38	inactive	36
G2	**105**	57	polyp, cystic atrophy	24
G2	**112**	57	polyp, cystic atrophy	48
G2	**116**	55	polyp, simple hyperplasia without atypia	60
G2	**33**	87	polyp, simple hyperplasia without atypia	24
G2	**74**	63	polyp, largely inactive with focal simple hyperplasia without atypia	30
G3	**120**	49	disorded proliferation	-
G3	**122**	60	cystic atrophy	-
G3	**29**	63	cystic atrophy	-
G4	**123**	52	polyp, cystic atrophy	-
G4	**125**	61	polyp, cystic atrophy	-
G4	**44**	67	polyp, cystic atrophy	-

Table 1. Clinicopathologic characteristics of tissue samples.

RNA Isolation, Amplification and Hybridization;
Total RNA was isolated by sonification of the sample in TRizol buffer (Invitrogen Life Technologies, Carlsbad, CA, USA) for 1 minute at 4°C and then purified using RNeasy columns (QIAGEN Benelux BV, Venlo, The Netherlands). Quality of RNA was ensured before labelling by analysing 20ng of each sample using the RNA 6000 NanoAssay and the Bioanalyzer 2100 (Agilent Technologies Netherlands B.V. Amstelveen, The Netherlands). From each sample cRNA was synthesized and labelled according to the Affymetrix protocol, following hybridisation to the U133 plus 2.0 GeneChip (Affymetrix, Santa Clara, CA, USA).

Data normalization and analysis
Raw expression values were analysed using the GeneChip Operating Software (GCOS) version 1.0, provided with Affymetrix Genechip Service. Intensity values were scaled to an average of 100 per GeneChip according to the method of global scaling provided in the GCOS software. Using this method only reliable results are generated for samples with an average intensity value of 30 or more, and therefore all values between 0 and 30 were set at 30 (Valk et al., 2004). This procedure affected 46% of all intensity values, of which 95% was flagged as absent or marginal by he GCOS software, while 5% was flagged as present according to the GCOS software, indicating the reliability of this method.

The following steps were undertaken to normalize the data, using Microsoft® Excel software (http://www.microsoft.com). As a first step, per gene, the geometric mean of the hybridisation intensities over all samples was calculated. Secondly, for all samples the level of expression per gene was determined relative to the geometric mean for that gene. And as a last step, the newly generated expression levels were log transformed (on a base 2 scale) to equally ascribe gene-expression levels with similar relative distance to the geometric mean (up- and down regulation relative to the geometric mean). As a result of this, deviation from the geometrical mean reflects differential gene-expression.

Clustering
Using the Omniviz package (http://www.omniviz.com), unsupervised cluster analysis was performed (external information such as tamoxifen-use and pathological classification were not taken into account). Genes (probesets) whose level of expression was indicated as present (using GCOS software) in at least one patient were selected for further analysis (this affected 60% of genes).

Cluster analysis of genes significantly differentially expressed between the tamoxifen-group and the controls was performed with EPSCLUST (Expression Profile data CLUStering and analysis) at http://ep.ebi.ac.uk/EP/EPCLUST.

SAM analysis
Supervised analysis was performed with the use of SAM software provided as a supplement for Excel software (http://www-stat.stanford.edu/~tibs/SAM). Using this analysis gene-expression profiles are related to external variables, in this case tamoxifen-exposure and histological classification. SAM calculates a score per gene based on the change in expression relative to the standard error (SD) of all measurements for that gene. The criteria to identify genes assigned to a cluster were: minimal difference in gene-expression between the assigned cluster and the other samples by a factor 2 ($\log2<-1$, or $\log2>1$), and a q-value of less than 5 percent. The q-value is similar to the p-value and represents the probability of a falsely assigned differential expressed gene between clusters.

Biological classification and pathway analysis
For functional classification of genes significantly differentially expressed between the tamoxifen-group and the controls we used Pathway Assist 2.5 (Ariadne Genomics, Inc., USA). The Pathway assist database contains biological knowledge represented in a formalized form focused on how

proteins, cellular processes and small molecules interact, modify and regulate each other. Pathway Assist provides a method for searching objects individually by keyword, string or attributes. These include, for example, type (protein, enzym), effect (positive, negative, unknown), mechanism (transcription, phosphorylation), tissue type, biological process, belonging to cell structure, and others. The complete databases of Kyoto Encyclopedia of Genes and Genomes (www.genome.ad.jp/kegg), Database of Interacting Proteins (DIP: dip.doe-mbi.ucla.edu), Bimolecular Interaction Network Database (BIND: bind.mshri.on.ca) and Gene Ontology (GO: www.geneontology.org) were imported into the Pathway Assist database.

For building gene expression networks, Ingenuity Pathway analysis was used https://analysis.ingenuity.com/pa/. This database utilizes the Ingenuity Pathway Knowledge Base (IPKB) to computationally analyse datasets to identify networks or pathways.

Quantitative PCR

Validation of micro-array expression data was accomplished by selection of 6 genes. First strand cDNA synthesis was performed using 2µg of total RNA and the Superscript 2 enzyme (Gibco, Carlsbad, CA, USA) according to a standardized protocol (protocol is available on request). Real-time PCR was performed using SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, USA) in the Opticon 2 apparatus (MJ Research, Bio-Rad laboratories Inc., Waltham, MA, USA).
Of each sample 5ng cDNA was used for the PCR reaction. Per reaction, a melting curve analysis was performed following each experiment to ensure the presence of a single amplified product. All PCRs were performed in duplo. Starting quantity for each analyzed gene was determined using the Opticon monitor software. Using this quantity, the expression level of each gene was normalized to the expression level of the reference gene, β-actin. Oneway ANOVA tests were performed to assess p-values of differences between the control-group and tamoxifen-group.

6.3 Results

Patients

Information about the subjects participating in the study is given in Table 1. All patients were postmenopausal and the mean age for the two patients groups was similar (p=0.891). The mean age in the tamoxifen-group was 57.7 +/- 15.3 and in the control group 58.7 +/- 6.9. The median duration of tamoxifen use in the tamoxifen group was 35 months (24-60 months). All patients were referred to the gynaecologist because of vaginal bleeding. The tamoxifen using patients were all treated because of node-positive breast tumours with a positive oestrogen and/or progesterone receptor status and none of the patients had received chemotherapy. The control patients were matched to the tamoxifen using patients, but the control group did not contain breast cancer patients. Furthermore, non of the control patients were using any sort of hormonal treatment which could affect the endometrium (hormone replacement therapy of any sort).

Gene expression profiles are different in tamoxifen-users compared to non-users

Based on histological classification (atrophic/inactive endometrium or polyp) and whether the patients were exposed to tamoxifen, samples were divided into 4 groups (Table 6.1). Group 1 (G1) are samples of atrophic/inactive endometrial tissues from tamoxifen-users, group 2 (G2) are polyps from tamoxifen-users, group 3 (G3) are samples of atrophic/inactive endometrial tissues from non-tamoxifen-users and group 4 (G4) are polyps from non-tamoxifen-users (Table 6.1).

To test whether the generated gene-expression profiles of all samples reflect the classification based on histology and tamoxifen-exposure, unsupervised cluster analysis was performed (Fig. 6.1). In this analysis the samples are clustered into subgroups without taking into account external information. From this analysis it became clear that the samples are clustered into subgroups that reflect their histological classification and exposure to tamoxifen, with the exception of one sample in group 3 (Fig. 6.1). Furthermore, samples of the tamoxifen-user group (G1 and G2) cluster separate from the non-tamoxifen-user group (G3 and G4). This implicates that the factor "tamoxifen-exposure" bares much more weight in clustering of the

subgroups than does histological classification into atrophic/inactive or polypus endometrium. The complete list of genes can be found at our website (http://www2.eur.nl/fgg//rede/gielen/) (expression_data).

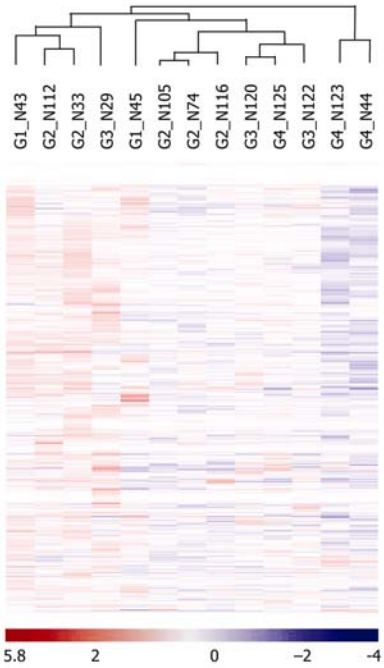


Figure 6.1. **Gene-expression profiles are different between tamoxifen-users and non-users.**
Hierarchical clustering of all genes from all endometrial samples. The expression level of each gene for every patient sample was determined relative to the geometric mean for that gene over all samples. The dendogram at the top illustrates distinction of cases into several arbitrarily groups. For each sample the group (G) (based on pathology and tamoxifen-exposure) is indicated as well as the number of patient (N).

Genes differentially expressed between the tamoxifen-group and the control-group
To identify genes that are related to tamoxifen-exposure, samples in G1 and G2 were defined as the tamoxifen-group, and samples in G3 and G4 as the control-group. SAM analysis was performed between the tamoxifen-group and the control-group, and the so identified differentially regulated genes between the two groups reflect genes whose expression is affected by tamoxifen-exposure.

SAM analysis revealed that the hybridisation signal intensity of 256 genes in the tamoxifen-group were either 2-fold up, or 2-fold down-regulated compared to the control-group. The fold-differences are the average ratios resulting form consistent changes between the tamoxifen-group and the control-group. Unsupervised clustering was performed for these 256 differentially expressed genes (Fig. 6.2), and as expected, two major clusters between the tamoxifen-group and the control group were formed.

Of the 256 genes, 227 genes are known genes, while the others represent expressed sequence tags (ESTs). Some of these genes and their role in endometrial functioning have been described before; HOXB7 and HOXA5 (Yanaihara et al., 2004), SLP1 (Green et al., 1998) and hepatocyte growth factor receptor (Khan et al., 2003; Yoshida et al., 2004), but for most genes this is a new finding. Furthermore, several genes were earlier linked to the estrogen receptor, for example EVA1 and TPD52L1 (Gielen et al., 2005b). The complete list of differentially expressed genes can be found at (http://www2.eur.nl/fgg//rede/gielen/) (SAM analysis).

Biological classification of regulated genes
The discriminative genes identified with SAM analysis may reveal functional pathways that are critical for tamoxifen-induced endometrial pathology. As a first step towards investigating this, we studied which biological processes these genes affect. Using the pathways assist database, which combines a number of other databases (like GO and KEGG databases), several functional categories were identified. Out of the 227 known genes, 85 could be classified and, interestingly,

most of the genes were involved in proliferation (39 genes), apoptosis (27 genes) and/or differentiation (27 genes). The complete list of biological processes can be found at our website (http://www2.eur.nl/fgg//rede/gielen/) (biological classification).

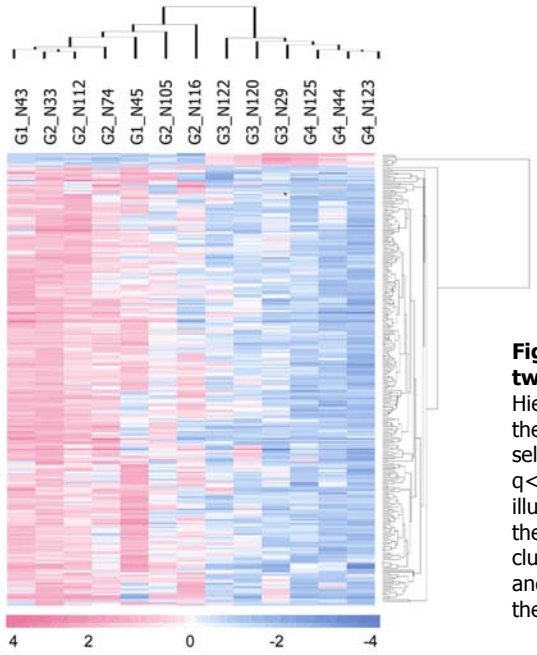


Figure 6.2. Genes differentially expressed between the two groups.
Hierarchical clustering of genes differentially expressed between the tamoxifen-group and the control-group. Genes were selected to be differentially expressed using SAM analysis if $q<0.05$ and fold-induction is > 2. The dendogram at the top illustrates distinction of cases into several arbitrarily groups, and the dendogram at the right site illustrates the different gene-clusters. For each sample the group (G) (based on pathology and tamoxifen-exposure) is indicated as well as the number of the patient (N).

Regulation of signaling pathways

Thus far, we have shown that the genes that are differentially regulated between the tamoxifen-group and the control-group modulate several biological processes. This led us to question whether these genes belonged to the same signaling networks. In other words, what is the interrelationship between the regulated genes, and in which canonical pathways do they fall? To study this question, gene expression network models were constructed using the Ingenuity database. In such a genetic (biological) network, molecules are nodes, and different types of connections represent interactions between the different genes. It should be emphasised that this "network analysis" is an exploratory *in silico* approach and so is only a "model" of a biological pathway and does not indicate that the pathway or network actually exists.
From the 256 genes differentially expressed between the groups, 227 are known genes, and these were subsequently used for this analysis. Using the Ingenuity database, 86 out of these 227 known genes could be clustered into 23 networks. Six of these networks contain more then one regulated gene and figure 3 displays a compilation of these 6 networks, and in table 2 the genes acting in these networks are listed. Three of the 6 networks could be merged and are numbered as network #1 in the figure. This network centres on cyclin-dependent kinase inhibitor 1A (CDKN1A), ß-catenin (CTNNB1) and tumour protein p53 (TP53). Network #2 centres around v-myc myelocytomatosis viral oncogene homolog (MYC) and network #3 around v-rel reticuloendotheliosis viral oncogene homolog A or NF-kappa-B transcription factor subunit p65 (RELA). For network #4 it is less clear, but genes seem to cluster around the epidermal growth factor receptor (EGFR). For reasons of clarity, several expressed, but non-regulated genes were also included in the network analysis (non-coloured blocks). Results from the network analysis are evaluated further in the discussion section.

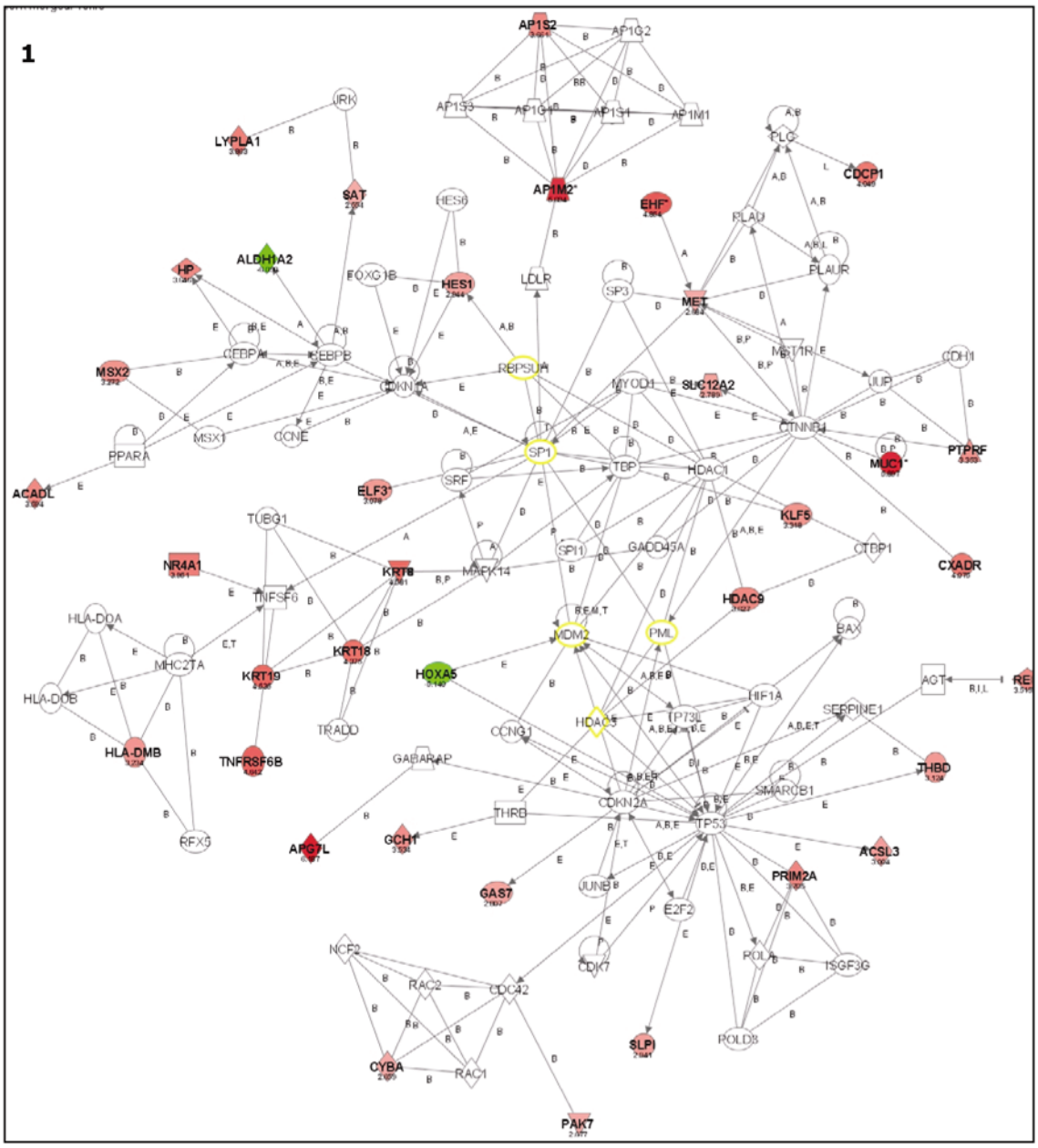
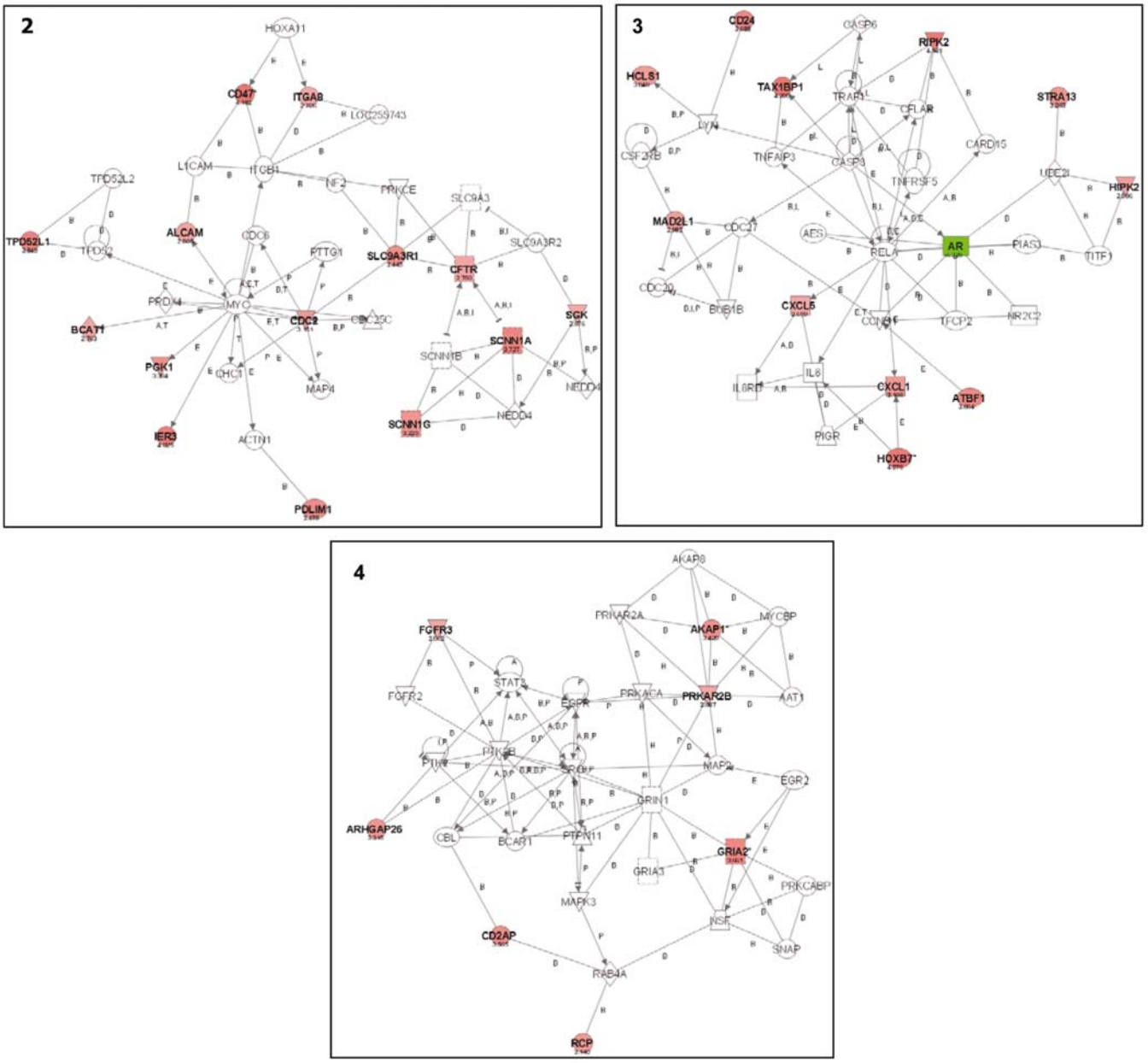


Figure 6.3. Signaling pathways involved in tamoxifen response.
Genetic networks were constructed, using the Ingenuity database, for genes differentially expressed between the tamoxifen-group and the control-group. Off the 23 networks, 6 networks contain more then one regulated gene. Three of the six networks could be merged (1), and center around CDKN1A, CTNNB1 and TP53. The second network (2) centers around MYC, third network (3) around RELA and the forth (4) network around the EGFR.



Two kinds of validations were performed: An analysis of profile reproducibility and a verification of microarray expression data.

Reproducibility of profile generation has been performed on RNA isolated from two postmenopausal endometria of non-treated patients. From both patients two RNA 1µg samples were used. From each 1µg sample, cRNA was synthesized and labelled according to the Affymetrix protocol, following hybridisation to the U133 plus 2.0 GeneChip (Affymetrix, Santa Clara, CA, USA). Generation of cRNA, labelling and hybridisations were all performed independently several weeks apart from each other. Upon reviewing the results, it was observed that the false discovery rates were 1.6% and 1.1%, respectively. These percentages indicate that the technical reproducibility of the microarray experiments is very high.

As a second step to verify our micro-array data, expression of a number of genes was measured using real time PCR. The genes KRT18, AR, TGFB1, CTSD, and MUC-1 all showed expression corresponding to our original micro-array data (Fig. 2). In the RT-PCR data,

expression of AREG is increased in the tamoxifen-group compared to the control-group, while in the micro-array data no difference is seen between the two groups.

	Micro-array			RT-PCR		
gene	control-group	tam-group	p-value	control-group	tam-group	p-value
KRT18	1 (+/-0.23)	2.21(+/-0.9)	0.009	1 (+/-0.56)	4.9 (+/-3.4)	0.018
AR	1 (+/-0.29)	0.5 (+/-0.14)	0.004	1 (+/-1.2)	0.25 (+/-0.31)	0.153
AREG	1 (+/-0.26)	1.21 (+/-0.53)	0.419	1 (+/-0.7)	4.7 (+/-3)	0.01
TGFB1	1 (+/-0.4)	0.8 (+/-0.4)	0.444	1 (+/-1.1)	0.8 (+/-0.6)	0.672
CTSD	1 (+/-0.5)	1.5 (+/-0.8)	0.223	1(+/-0.6)	0.9 (+/-0.65)	0.789
MUC1	1(+/-0.3)	2.8(+/-0.9)	0.001	1(+/-0.7)	3.6(+/-2)	0.01

Table 6.3. Validation of differential gene-expression by quantitative RT-PCR. Data represent the average (+/-SE) levels per group. Differences between control and treatments were considered significant (*) at $p<0.05$.

6.4 Discussion

When tamoxifen-induced cancers are matched (for grade and stage) to non-tamoxifen associated endometrial cancers, no differences are observed in gene-expression profile (Ferguson et al., 2004). It is only when developing tumours are followed in time that it becomes clear that tamoxifen-induced tumours are growing more aggressively than non-tamoxifen associated endometrial tumours (Cohen, 2004). The hypothesis that we have chosen to start to investigate was that tamoxifen specifically induces early endometrial changes leading to a more aggressive cancer phenotype. Therefore, as a first step, gene-expression profiles were generated of early benign endometrial pathology samples of women using tamoxifen, and were compared to gene expression profiles measured in the same kind of tissue-pathology from women not using tamoxifen.

Unsupervised clustering of all genes in all samples revealed that samples of patients that had used tamoxifen clustered together and away from samples of women that had never used tamoxifen. This was somewhat surprising because in both groups atrophic/inactive as well as endometrial polyps were included. It might have been expected that initially the polyps would cluster together and away from the atrophic/inactive samples regardless of tamoxifen-treatment..

In order to further investigate the working mechanism of tamoxifen, differentially expressed genes between the tamoxifen-group and the control-group were identified using SAM analysis. Using this method, 256 genes were found specifically regulated by tamoxifen in our patient population. Interestingly, of these 256 genes most were expressed higher in the tamoxifen-group. Since genes can have either a suppressing or an inducing effect on a biological process, this does not necessarily mean that cells in the samples of the tamoxifen-group are more biological active.

Our next step was to test if and to what extend the mechanism of action of tamoxifen differs from that of oestrogen. Therefore, the 256 tamoxifen-regulated genes were compared to oestrogen-regulated genes identified from a similar patient population using the exact same micro-array. The patient details are described in Klaassens et al. (Klaassens et al., 2005), data on oestrogen-regulated genes are from Hanifi-Moghaddam et al. (Hanifi-Moghaddam et al., 2005). In short, in these studies postmenopausal women were treated for three weeks with oestrogen and compared to matched controls (Klaassens et al., 2005). Again using SAM analysis, 746 genes were found differentially expressed between the control-group and the oestrogen-treated group. Interestingly, of those 746 genes, only 11 overlap with 256 genes induced by tamoxifen. As indicated before (Fig.1), this seems to point to a distinct working mechanism of tamoxifen. In cancer cell lines of breast (Frasor et al., 2004), bone (Kian Tee et al., 2004) and endometrium (Gielen et al., 2005a) the unique gene expression profile after treatment with tamoxifen has been described before, however, to our knowledge this is the first report of this finding in human benign endometrial samples.

When we subsequently evaluated the function of the tamoxifen-regulated genes, most of these genes were found to be involved in proliferation, apoptosis and differentiation. Upon more

thoroughly analysing the biological functions of the tamoxifen regulated genes, it was observed that 69 tamoxifen-regulated genes could be connected with each other in 4 gene expression networks. This implies an interrelationship between the regulated genes, and could indicate that several different pathways are orchestrated by tamoxifen signalling in the human endometrium.

An interesting question, which can then be asked, is whether these 4 gene expression networks point to a similar physiological function of tamoxifen signalling or whether these networks represent different cellular entities. Since four well known genes are at the centre of 3 of the networks (ß-catenin, TP53, MYC and RELA) and the fourth network more diffusely focuses on growth factor signalling centred on the EGF receptor, the discussion that follows focuses on signalling in the endometrium around these five genes: ß-catenin, TP53, MYC, RELA and EGFR.

ß-catenin

ß-catenin (network #1) has a dual function in the cell. Together with α-catinin it links the cell-cell adhesion molecule E-cadherin to the cytoskeleton, and thus stabilizes cell-cell adhesion (Beavon, 2000). The other function of ß-catenin is in the canonical Wnt signal transduction pathway (Bienz, 2002). In short, Wnt signaling has a central function in the maintenance and control of stem cell compartments where the fine balance between proliferation (Wnt-on) and differentiation (Wnt-off) is regulated (Giles et al., 2003; Logan and Nusse, 2004). In this study, ß-catenin itself is not differentially expressed, but this can be explained by the fact that activation through translocation of ß-catenin to the membrane or the nucleus will not be detected in the method used in this study.

The finding that tamoxifen enhances the expression of MUC1 (increased in breast cancer metastasis (Schroeder et al., 2003), PTPRF (enhanced in metastatic breast cancer) (Levea et al., 2000) and CXADR (inhibitor of cancer cell migration) (Walters et al., 2002), and the fact that all three genes can bind to ß-catenin, could indicate a specific role of tamoxifen in cell-cell adhesion. The finding that MET expression was enhanced by tamoxifen seems to indicate that Wnt signalling is enhanced by tamoxifen (c-MET has recently been identified as a Wnt regulated gene) (Boon et al., 2002). If it is true that the Wnt signal transduction pathway is activated, this is of interest because its central role in homeostasis of adult stem cell niches is reflected by the frequent association of Wnt signalling activating defects in different cancer types including endometrial cancer (Giles et al., 2003; Saegusa et al., 2001).

Balance between proliferation and apoptosis via the MYC, RELA and TP53 network

The balance between cell proliferation and cell death is important in epithelial homeostasis. Interestingly, three of the generated gene expression networks centre on proteins (MYC, RELA and TP53) involved in this balance between proliferation and apoptosis. TP53 is a transcription factor and induces a G1 arrest in the cell cycle, creating extra time for DNA-repair mechanisms. If DNA-repair fails TP53 initiates apoptosis via activation of members of the Bax/Bcl-2 family (Lane and Fischer, 2004). Initiation of apoptosis via activation of Bax/Bcl-2 family is inhibited by RELA (p65) in complex with p50 (NFκB complex) (Shukla and Gupta, 2004). The MYC protein is a transcription factor that regulates the cell cycle via regulation of E2F, cyclin D1 and p27 (progression of the cell cycle), or via activation of TP53 (inhibition of the cell cycle through induction of apoptosis) (Patel et al., 2004). In the current investigations, several tamoxifen regulated genes were found connected to TP53, MYC and RELA signalling, suggesting that these networks may be of specific importance for regulation of the endometrial response to tamoxifen. Furthermore, as TP53, MYC and RELA are also themselves interconnected, a putative tamoxifen-induced imbalance in these pathways could play a determining role in endometrial carcinogenesis in tamoxifen-users.

EGF receptor signalling.

Earlier work of our group indicated that EGF receptor signalling in the ECC-1 endometrial carcinoma cell line was very important for estrogen as well as tamoxifen signalling. Furthermore, the EGF receptor ligand amphiregulin (AREG) turned out to be up-regulated by estrogens as well

as tamoxifen while AREG was also shown to be able to induce growth of the endometrial carcinoma cell line (Gielen et al., 2005a). Also, in the current RT-PCR data the EGF receptor ligand AREG was observed to be more highly expressed in the tamoxifen-group compared to the control-group (Fig.4). The fact that we did not extract this finding from the micro-array experiments may be due to the low expression of AREG in the endometrial samples (RT-PCR is more sensitive then micro-array). This further strengthens the earlier observation that AREG activation of the EGF receptor may play a role in tamoxifen induced endometrial pathology. Furthermore, as was also observed earlier in the cell line experiments (Gielen et al., 2005a), tamoxifen seems to have its own specific effect on some other proteins involved in EGF receptor signalling (as indicated in network # 4).

Gene Symbol	Gene Title	Genbank ID	UniGene ID	Fold Change	q-value
ACADL	acyl-Coenzyme A dehydrogenase, long chain	AI367275	Hs.430108	2.11	0.03
ACSL3	acyl-CoA synthetase long-chain family member 3	AL525798	Hs.268012	2.03	0.04
AKAP1	A kinase (PRKA) anchor protein 1	BC000729	Hs.78921	2.37	0.04
ALCAM	activated leukocyte adhesion molecule	AA156721	Hs.150693	0.49	0.04
ALDH1A2	aldehyde dehydrogenase 1 family, member A2	NM_003888	Hs.435689	-3.00	0.02
AP1M2	adaptor-related protein complex 1, mu 2 subunit	NM_005498	Hs.18894	2.36	0.02
AP1S2	adaptor-related protein complex 1, sigma 2 subunit	AA205444	Hs.121592	0.44	0.02
APG7L	APG7 autophagy 7-like (S. cerevisiae)	BC000091	Hs.38032	2.02	0.02
AR	androgen receptor	AF162704	Hs.99915	-2.12	0.02
ARHGAP26	Rho GTPase activating protein 26	AI768563	Hs.132942	2.46	0.03
ATBF1	AT-binding transcription factor 1	NM_006885	Hs.108806	2.36	0.03
BCAT1	branched chain aminotransferase 1, cytosolic	AL390172	Hs.438993	2.91	0.05
CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	M58664	Hs.375108	2.13	0.03
CD2AP	CD2-associated protein	NM_012120	Hs.374340	2.08	0.03
CD47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	BG230614	Hs.446414	2.35	0.02
CDC2	cell division cycle 2, G1 to S and G2 to M	D88357	Hs.334562	2.10	0.04
CDCP1	CUB domain-containing protein 1	NM_022842	Hs.146170	2.15	0.02
CFTR	cystic fibrosis transmembrane conductance regulator	NM_000492	Hs.411882	2.83	0.04
CXADR	coxsackie virus and adenovirus receptor	NM_001338	Hs.79187	2.17	0.03
CXCL1	chemokine (C-X-C motif) ligand 1	NM_001511	Hs.789	7.18	0.04
CXCL5	chemokine (C-X-C motif) ligand 5	AK026546	Hs.89714	9.88	0.04
CYBA	cytochrome b-245, alpha polypeptide	NM_000101	Hs.68877	2.01	0.04
EHF	Ets homologous factor	AI763378	Hs.200228	2.22	0.02
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	U73844	Hs.67928	2.24	0.04
FGFR3	fibroblast growth factor receptor 3	NM_000142	Hs.1420	2.07	0.04
GAS7	growth arrest-specific 7	BC001152	Hs.462214	2.08	0.04
GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)	NM_000161	Hs.86724	2.01	0.03
GRIA2	glutamate receptor, ionotropic, AMPA 2	BE219628	Hs.335051	5.05	0.02
HCLS1	hematopoietic cell-specific Lyn substrate 1	NM_005335	Hs.14601	2.25	0.04
HDAC9	histone deacetylase 9	NM_014707	Hs.487662	2.36	0.03
HES1	hairy and enhancer of split 1, (Drosophila)	NM_005524	Hs.250666	2.09	0.04
HIPK2	Homeodomain interacting protein kinase 2	BF218115	Hs.397465	2.54	0.04
HLA-DMB	major histocompatibility complex, class II, DM beta	NM_002118	Hs.1162	2.39	0.04
HOXA5	homeo box A5	NM_019102	Hs.37034	-4.82	0.03
HOXB7	homeo box B7	S49765	Hs.436181	2.17	0.03
HP	haptoglobin	NM_005143	Hs.403931	3.06	0.03
IER3	immediate early response 3	NM_003897	Hs.76095	4.07	0.03
ITGA8	integrin, alpha 8	AI193623	Hs.171025	2.03	0.04
KLF5	Kruppel-like factor 5 (intestinal)	AF132818	Hs.84728	2.28	0.03
KRT18	keratin 18	NM_000224	Hs.406013	2.21	0.02
KRT19	keratin 19	NM_002276	Hs.309517	3.05	0.02
KRT8	keratin 8	U76549	Hs.356123	2.12	0.02
LYPLA1	lysophospholipase I	BG288007	Hs.446676	2.30	0.03
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	NM_002358	Hs.79078	2.22	0.04
MET	met proto-oncogene (hepatocyte growth factor receptor)	BG170541	Hs.419124	2.22	0.04
MSX2	msh homeo box homolog 2 (Drosophila)	D89377	Hs.89404	2.60	0.04
MUC1	mucin 1, transmembrane	NM_002456	Hs.89603	2.79	0.02
NR4A1	nuclear receptor subfamily 4, group A, member 1	D85245	Hs.1119	2.23	0.03
PAK7	p21(CDKN1A)-activated kinase 7	AB040812	Hs.32539	2.98	0.04
PDLIM1	PDZ and LIM domain 1 (elfin)	BC000915	Hs.75807	2.01	0.03
PGK1	phosphoglycerate kinase 1	AA069778	Hs.78771	2.34	0.03
PRIM2A	primase, polypeptide 2A, 58kDa	NM_000947	Hs.440603	2.17	0.03
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	NM_002736	Hs.77439	2.03	0.04
PTPRF	protein tyrosine phosphatase, receptor type, F	NM_002840	Hs.75216	2.16	0.03
RCP	RAB11 family interacting protein 1 (class I)	NM_025151	Hs.96125	0.46	0.03
REN	renin	NM_000537	Hs.3210	14.95	0.03
RIPK2	receptor-interacting serine-threonine kinase 2	AF027706	Hs.103755	2.10	0.03
SAT	spermidine/spermine N1-acetyltransferase	BE971383	Hs.28491	2.26	0.05
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	NM_001038	Hs.130989	2.40	0.03
SCNN1G	sodium channel, nonvoltage-gated 1, gamma	AI985987	Hs.145645	2.50	0.03
SGK	serum/glucocorticoid regulated kinase	NM_005627	Hs.296323	3.08	0.04
SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporters), member 2	AK025062	Hs.110736	2.15	0.05
SLC9A3R1	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1	NM_004252	Hs.396783	4.06	0.03
SLPI	secretory leukocyte protease inhibitor (antileukoproteinase)	NM_003064	Hs.251754	2.32	0.04
STRA13	stimulated by retinoic acid 13	U95006	Hs.37616	0.35	0.04
TAX1BP1	Tax1 (human T-cell leukemia virus type I) binding protein 1	AI935415	Hs.5437	2.08	0.02
THBD	thrombomodulin	NM_000361	Hs.2030	2.19	0.04
TNFRSF6B	tumor necrosis factor receptor superfamily, member 6b	NM_003823	Hs.434878	2.54	0.02
TPD52L1	tumor protein D52-like 1	AF208012	Hs.16611	2.43	0.03

Table 6.2. **Genes differentially expressed between the tamoxifen-group and control-group and assigned to the generated networks.** All other genes can be found on our website (http://www2.eur.nl/fgg//rede/gielen/).

Concluding remarks:
Tamoxifen seems to exert a specific effect on the non-malignant human endometrium that is different from the effect of estrogens. As is shown in the current exploratory study, tamoxifen-treatment seems to affect several genes involved in proliferation, cell survival, apoptosis, differentiation and cell-cell adhesion of normal endometrial cells. It is of interest to note that the currently available parameters (such as vaginal bleeding, endometrial thickness and dose and duration of tamoxifen-use) are not fully associated with endometrial pathology in tamoxifen-users (Cohen, 2004). Therefore, beginning to understand the molecular mechanism of tamoxifen-induced stimulation of the endometrium is important and may be a starting point to provide further insights into the early detection of endometrial aberrations that may eventually result in tumour formation. Additional research including higher patient numbers, however, is needed into those early endometrial changes that may lead to tumour formation.

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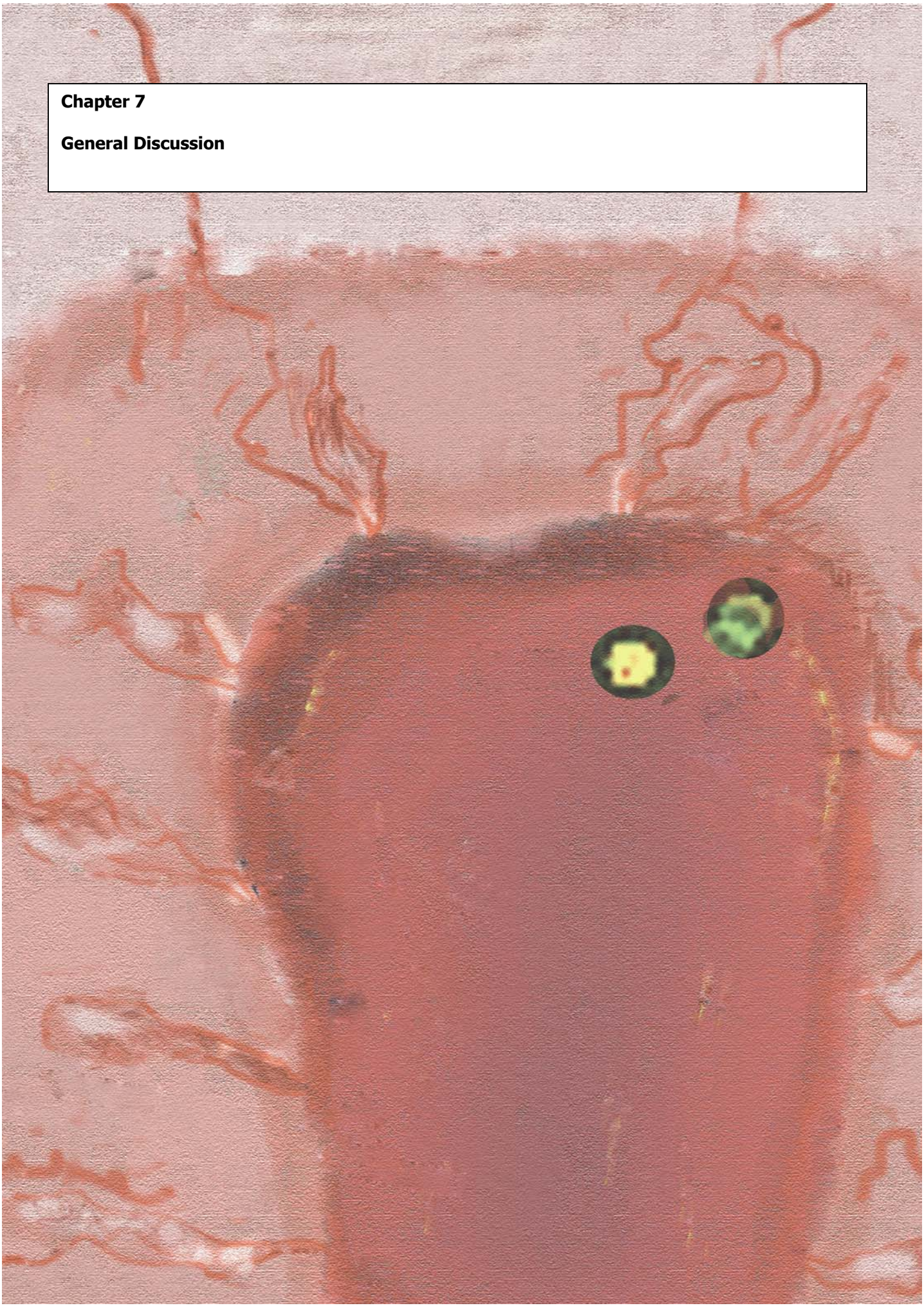
Chapter 7

General Discussion



Endometrial cancer is the fourth most common form of cancer in women in The Netherlands (www.ikcnet.nl). Most risk factors associated with the development of endometrial cancer are related to prolonged unopposed estrogen action, which can be either endogenous (such as late onset of menopause or estrogen-producing tumors) or exogenous (mainly estrogen-only hormone replacement therapy) (Akhmedkhanov et al., 2001; Hale et al., 2002; Schottenfeld, 1995). Normally, during fertile life, the proliferative properties of estrogens are counterbalanced by progesterone, which has differentiating properties (Persson et al., 1989). After menopause, a decline in serum levels of estrogen and progesterone is observed because of the absence of follicle recruitment in the ovary. This consequently results in an atrophic/inactive state of the endometrium. However, in some women increased levels of estrogens (endogenous or exogenous) are present, which will stimulate the endometrium and may result in endometrial cancer induction.

Use of tamoxifen, as adjuvant therapy in breast cancer patients, is associated with an increased risk for the development of endometrial pathologies, including endometrial cancer (Cohen, 2004). This is caused by the estrogen-agonistic mode of action that tamoxifen displays in the endometrium of postmenopausal women.

A significant portion of endometrial cancer research focuses on the role of steroid receptor signaling, and molecular genetic research plays an essential role herein. The experiments described in this thesis focus on three aspects of endometrial carcinogenesis.

1. The effects of estrogen and progesterone on gene-expression during modulation of proliferation and differentiation of endometrial cancer cells (chapter 2 and 3).
2. Involvement of IGF receptor signaling and EGF receptor signaling during steroid-induced modulation of endometrial cancer (chapter 3, 4 and 5).
3. Molecular mode of action of tamoxifen on the human endometrium (chapter 4, 5, and 6).

7.1 Estrogen and progesterone signaling in endometrial cancer

Estrogen, the main risk factor for the development of endometrial cancer, exhibits functional activity via binding to the estrogen receptor (ER). In the human endometrium both ERs, ERα and ERβ, are expressed at a ratio of 100:1. As normal endometrium, also endometrial cancer is, to some extend, responsive to progesterone. The differentiative properties of progesterone are accomplished via activation of the progesterone receptor (PR) signaling pathway. During endometrial carcinogenesis signaling via ER and PR plays a central role. The molecular mechanisms involved in estrogen-induced growth induction and progesterone-induced differentiation of the human endometrium and endometrial cancer are largely unknown. Regarding these mechanisms, the following question was posed:

- What are the molecular mechanisms underlying estrogen-induced growth stimulation and progesterone-induced growth inhibition of endometrial cancer cells?

In chapter 2, gene-expression data are presented from ECC-1 cells cultured for 72 hours in the presence of estrogen, and PRAB-36 cells cultured for 48 hours in the presence of MPA. The ECC-1 cell line is a well-differentiated endometrial cell line that expresses both ERs and is stimulated in growth by estrogen. The PRAB-36 cell line expresses both PR isoforms and is inhibited in growth by MPA (MPA is the progestagen used in the clinic to treat endometrial cancer patients). Biological classification of the regulated genes revealed that most genes are involved in "cell growth and maintenance". Within this group of regulated genes only 5 genes were regulated by estrogen as well as progesterone. The unique gene-expression profile for estrogen and progesterone can not be explained by the different background of the cell lines used; when micro-arrays are performed on ECC-1 and PRAB-36 cells grown under control conditions, more than 95% of expressed genes are expressed by both cell lines at comparable levels (Hanifi-Moghaddam et al., 2005).

In the ECC-1 cell line, expression of progesterone receptor is regulated by estrogen. Micro-array analysis revealed that RNA of the progesterone receptor is enhanced upon treatment with estrogen (chapter 4) and western blot analysis showed that also on the protein level, expression of the PR (mainly PRB) is increased by estrogen (chapter 2). Analysing the effects of progesterone treatment on expression of ER showed a decrease of ERα protein upon treatment (Hanifi-Moghaddam et al., in preparation). Interestingly, also during the menstrual cycle estrogen increases expression of the PR (Mote et al., 1999). Expression of ERα is higher in the proliferative phase compared to the secretory phase of the menstrual cycle, probably due to an increase of progesterone synthesis (Vienonen et al., 2004). Combining these data suggests that the cross-talk between estrogen signaling and progesterone signaling mainly occurs via direct modulation of the expression of receptors which, subsequently, regulate different sets of genes within the same functional families.

7.2 Overlap between IGF and EGF receptor signaling and Estrogen receptor signaling

Neoplastic transformation of cells involves deregulation of several genes and pathways. For many cancers, and also for endometrial cancer, activation of growth factor signaling pathways plays a role during carcinogenesis. Furthermore, for the normal endometrium it is known that estrogen and progesterone mediate their biological activity via activation of growth factor systems, like the IGF and EGF receptor pathway. From micro-arrays performed to study estrogen and progesterone induced growth modulation (chapter 2), we identified several members of the IGF and EGF receptor pathways to be involved herein. On the basis of this, the following question was put forward:

- Does activation of the ER signaling pathway result in activation of IGF or EGF signaling, and, *vice versa*, does activation of the IGF and EGF receptor signaling pathways result in activation of ER signaling?

In experiments described in chapter 3, it is shown that estrogen-induced proliferation is partly modulated via the IGF receptor signaling pathway. It was determined that estrogen-induced proliferation is decreased upon inhibition of IGF receptor signaling (co-incubation with a specific IGF1 receptor antibody). Since expression and direct activation of the IGF receptor is not regulated by estrogen nor does treatment with estradiol results in increased of expression of the ligands of the IGF1 receptor (IGF1 or IGF2; chapter 6), this mechanism of growth regulation is not easily explained. Regulation of expression of the major receptor substrate IRS2 might be involved in this process.

From experiments described in chapter 6 it became clear that IRS2 is increased by estradiol after treatment for 1hr as well as after treatment for 24 hrs and 48 hrs (chapter 6). Cui et al. (Cui et al., 2003) have shown that in breast cancer cells estrogen as well as progesterone induce expression of IRS2 and enhance proliferation of cells (Cui et al., 2003; Lee et al., 1999). Furthermore, if cells were pre-incubated with progesterone for 24 hrs and subsequently treated for 10 minutes with IGF1, enhanced phosphorylation of IRS2 was measured, while pre-treatment alone did not induce phosphorylation of IRS2 (Cui et al., 2003). Downstream from IRS2, progesterone also enhances the IGF1 triggered association of IRS2 with other proteins (like P85 and GRB2) leading to activation of the RAS/ERK and PI3K/Akt signaling pathways. These results indicate that progesterone-induced proliferation of breast cancer cells is at least partly due to activation of the IGF1 receptor pathway. It is therefore speculated that in ECC-1 cells estrogen-induced up-regulation of IRS2 sensitizes the response to locally produced IGF1. This hypothesis is further strengthened by the fact that treatment of cells with estrogen or IGF1 results in co-regulation of expression of 407 genes (chapter 5). Interestingly, within this group of regulated genes several target genes of RAS/ERK and PI3K/Akt signaling pathways were identified.

For both estrogen and tamoxifen, activation of EGF receptor signaling also seems to play a role. Treatment of cells for 1hr with estrogen or tamoxifen results in activation of several early

EGF receptor responsive genes, like FOS and EGR3 (chapter 5). More evidence that EGF receptor signaling is important in mediating effects of estrogen and tamoxifen is given in chapter 4 where upon construction of a genetic network of estrogen and tamoxifen regulated genes, it was observed that the largest possible network centred on EGF receptor signaling (chapter 4). Furthermore, the EGF receptor ligand amphiregulin (AREG) was found up-regulated by estrogen and tamoxifen (chapter 4). Interestingly, AREG can also induce phosphorylation of the EGF receptor, and activate early EGF receptor responsive genes (chapter 4 and 5). Furthermore, as expected, stimulation with AREG increased proliferation of ECC-1 cells (chapter 4).

To test further whether activation of the EGF receptor plays a role in estrogen and tamoxifen mediated signaling, a new set of experiments was performed in which cells were treated for various periods of time with either estrogen, tamoxifen or AREG (chapter 5). 305 genes were co-regulated by estrogen and AREG and 168 genes were co-regulated by tamoxifen and AREG (chapter 5), indicating that EGF receptor signaling may indeed play a role in ER signal transduction. Interestingly, gene-expression profiles generated from benign endometrial samples of tamoxifen-users compared to matched controls (matched on pathological classification), revealed that a subset of the discriminative genes between these two groups could be linked to EGF receptor signaling (chapter 6). This indicates that also in vivo EGF receptor signaling is intertwined with ER signaling.

So far we have shown that ER signaling is partly dependent on IGF1 signaling and shows considerable overlap with EGF signaling. The question that remains is whether IGF and EGF signaling depends on ER signaling. In ECC-1 cells, IGF1 induced proliferation is independent of the ER since blocking ER signaling by treatment with the pure anti-estrogen ICI182780 did not influence IGF1 mediated proliferation. Whether AREG induced proliferation is independent of ER signaling remains to be resolved. Interestingly, micro-array analysis performed with ECC-1 cells, indicated that treatment with IGF1 and AREG increased the expression of the ER responsive genes cathepsin D (CTSD) and trefoil factor 1 (TFF1) (chapter 5). Using deletion analysis experiments with the promotor of the CTSD gene it was shown that the IGF1 induced increase in expression of CTSD was ER dependent, while EGF activates the CTSD gene independent of the ER (Wang et al., 2000). Interestingly, for the TFF1 gene both IGF1 and EGF were reported to increase the expression of the TFF1 gene, and blocking ER signaling with the anti-estrogen ICI164384 inhibited expression of TFF1 gene (El-Tanani and Green, 1997; Stoica et al., 2000). These results indicate that for some genes IGF and EGF regulation is completely dependent of ER signaling, while for other genes this is not the case.

7.3 Estrogen, SERMs and anti-estrogen signaling in endometrial cancer

Aberrant exposure to estrogen and estrogen-like compounds is associated with an increased incidence of hormone-dependent cancers, like breast and endometrial cancer (Akhmedkhanov et al., 2001). Furthermore, a decline in estrogen production after menopause results in side effects like osteoporosis and hot flushes. These estrogen-related effects have inspired the development of a new class of drugs, the Selective Estrogen Receptor Modulators (SERMs). These SERMs exhibit, depending on the tissue type, estrogenic or anti-estrogenic properties.

Treatment of breast cancer patients with tamoxifen, for example, reduces the chance for breast cancer recurrence but at the same time results in an increased incidence of endometrial pathologies, including endometrial cancer (Bergman et al., 2000; Cohen, 2004). Tissue specific effects are generally explained by differences in conformational change of the ER upon ligand binding, and subsequent recruitment of different sets of co-activators and co-repressors to the ER-ligand complex (McDonnell, 2004). Since distinct regulatory effects of SERMs on gene-expression are largely unknown, the following question was raised:

- Which genes are regulated by estrogen, tamoxifen, raloxifene and the anti-estrogen ICI182780 in endometrial cancer cells, and do these four ER-ligands regulate similar genes, in the same cellular processes or pathways?

From the experiments described in chapter 4, it became clear that estrogen and tamoxifen activate the promotor of the known estrogen-responsive genes TFF1 and C3, while raloxifene had no effect and the pure anti-estrogen ICI182780 inhibited the activity of these genes (chapter 4). Micro-array analysis under these conditions revealed the expression of several estrogen, tamoxifen, raloxifene and ICI182780 regulated genes (chapter 4). Interestingly, if estrogen in combination with either tamoxifen, or raloxifen or ICI182780 is added to the cells, the activity of TFF1 and C3 genes (luciferase-assays) as well estrogen-mediated transcription measured by micro-array analysis, is clearly inhibited. This corresponds to the situation in endometrial tissues of premenopausal women, (who have high estrogen levels) where tamoxifen acts as an estrogen-antagonist (Chang et al., 1998; Mourits et al., 2001).

Analysis of the gene-expression profiles generated for estrogen, tamoxifen, raloxifene and ICI182780 showed that the four ligands influence the same biological processes, but do so via regulation of largely different sets of genes. This may be surprising since all of these ligands act via binding to the ER, but it has been shown by others that these ligands have a differential effect because of their ability to induce different conformational changes of the receptor upon binding (Brzozowski et al., 1997; Katzenellenbogen and Katzenellenbogen, 2002; Kong et al., 2003; Pike et al., 1999; Shiau et al., 1998).

In the experiments in chapter 4, results of analysis of gene-expression profiles are described. Hypothetically, it is possible that treatment for 48 hrs is not optimal for the ligands used. A higher similarity between estrogen and tamoxifen regulated genes may be observed if cells are treated at several time points, and analysis are made between the different ligands over these different time points. To test this hypothesis, cells were treated for 1, 6, 12, 24, 48 and 72 hrs with estrogen or tamoxifen and a full-genome micro-array analysis was performed (chapter 5). Unsupervised cluster analysis of these samples, however, again showed that estrogen and tamoxifen regulate different sets of genes (chapter 5).

It should be noted here that in the currently described experiments, treatment of cells affects regulation of direct ER target genes but also regulation of genes via activation of other transcription factors. For the endometrium, it has been reported that tamoxifen acts as a full agonist on AP-1 consensus sites, while the effect on ERE consensus sites is still under debate. Analysing the promoters of the newly identified estrogen and tamoxifen responsive genes in order to determine regulation via known consensus sites (like ERE, AP-1 and Sp1) and identification of new consensus sites, will help to expand our knowledge on the transcription machinery induced by estrogen and tamoxifen. In literature several databanks are described that are helpful to determine consensus sites in promotor sequences.

Carcinogenicity of estrogens. A question which can be asked concerning the association between enhanced estrogen levels and the increased incidence of endometrial cancer is whether estrogens induce cancer or are simply involved in stimulating proliferation of already transformed cells. This question is not easily answered. Experiments with bacteria and mammalian cells failed to identify estrogen as a potent mutagenic agent. Liehr et al. (Liehr et al., 2000) has reviewed literature and indicated that the truth could be somewhere in the middle: conversion of estradiol into 4-hydroxyestradiol and further activation into reactive semiquinone/quinone intermediates can cause DNA damage with a low frequency. Tumor formation is then caused by induction of hormone-receptor-mediated proliferation of these damaged cells.

7.4 Effect of tamoxifen *in vivo*

The advantage of performing research on hormonal regulation of gene-expression in cancer cell lines is that it is possible to study direct relations between the ligand used with respect to the genes regulated. Furthermore, the experiments are usually well controlled and therefore very reproducible. However, the major disadvantage is that conclusions drawn from these experiments

are difficult to translate to the *in vivo* situation. Therefore additional experiments on tamoxifen signaling were performed in human endometrial tissues.

From literature it is known that gene-expression profiles generated from tamoxifen-induced endometrial cancer are similar to gene-expression profiles from matched controls (matched for grade, stage and histology) (Ferguson et al., 2004). However, tamoxifen-induced endometrial tumors do tend to have a less favorable stage and grade, and histological appearance upon diagnose (Cohen, 2004). It was therefore speculated that tamoxifen specifically induces this more aggressive phenotype at an early stage of endometrial pathology. In order to investigate this, the following question was asked:

- Which genes are regulated in benign endometrial tissues of tamoxifen-users compared to non-users, and can we, based on the generated gene-expression profiles, elucidate which pathways are activated by tamoxifen during the early changes which may lead to endometrial cancer formation.

To answer this question, we have collected benign endometrial samples of tamoxifen-users and matched control samples (chapter 6). The pathological classification of all samples from both groups was revised and included atrophic/inactive endometrium and endometrial polyps. Furthermore, the percentage of endometrium (both stroma and glands) was determined and only those samples that contained 100% pure endometrium were used. Unsupervised clustering revealed that samples of tamoxifen-users were, irrespective of pathological classification, similar and consequently formed a subgroup distinct from the matched controls. Going one step further, using SAM analysis (a statistical method to select genes differentially expressed between groups) 256 genes could be selected that were significantly differentially expressed between the tamoxifen- and the control-group. Interestingly, comparing these genes with estrogen-regulated genes, identified under similar circumstances, revealed that 95% of the differentially expressed genes were tamoxifen specific. This distinct effect of tamoxifen signaling compared to estrogen signaling is similar to the earlier described differences in gene-expression profiles determined in cell lines (chapter 3 and 4).

Network analysis of the tamoxifen-regulated genes implied that 69 genes are centred around 5 well-known genes; TP53, RELA, MYC, EGF receptor and ß-catenin. This might indicate that the pathways in which these 5 genes act are important in tamoxifen-induced signaling in the endometrium. Inactivation or hyperactivation through mutations and deletions of these genes leading to an imbalance in function of the pathways in which they act, might therefore play a determining role in tamoxifen-induced endometrial carcinogenesis.

As mentioned before, tamoxifen-use increases the incidence of endometrial cancer, however, most women using tamoxifen will not develop any (Cohen, 2004). So far, it has been impossible to predict beforehand, or while using tamoxifen, which patient will develop endometrial cancer. The currently used parameters such as vaginal bleeding, increased endometrial thickness and dose and duration of tamoxifen-use, do not accurately predict chance of finding endometrial cancer in tamoxifen-users.

Using micro-array analysis we identified 256 differentially expressed genes between endometrial-tissues of tamoxifen-users and matched controls (chapter 6). An imbalance in regulation of some of these genes, and the pathways they act in, might play a role in endometrial carcinogenesis in tamoxifen-users. Using these tamoxifen-regulated genes and performing additional micro-array experiments it should be possible to predict which tamoxifen-using patient runs an unacceptable high risk to develop endometrial cancer.

In order to investigate this further, we are currently in the process of evaluating the expression of the 256 tamoxifen-specific genes identified in patients in paraffin embedded benign endometrial tissues from two patient groups:

- Women with benign endometrial changes while using tamoxifen, who developed endometrial cancer while or after treatment with tamoxifen.

- Women with benign endometrial changes while using tamoxifen who did not develop endometrial cancer while or after treatment with tamoxifen.

It is expected that some of these genes will show a differential regulation between the two groups. These differentially expressed genes then are marker for tamoxifen-induced carcinogenesis and need to be evaluated further.

Is there a future for tamoxifen? Up until now, tamoxifen has been the standard care for adjuvant endocrine therapy for breast cancer patients. Recent data from the ATAC trial (tamoxifen *versus* anastrozole) suggest that in postmenopausal women with early breast cancer treatment with aromatase inhibitors is more beneficial in terms of disease-free survival and time to recurrence, significantly reduced time to distant metastasis and is superior in overall tolerability (Baum et al., 2002; Howell et al., 2005). Furthermore, treatment with anastrozole is associated with a decreased incidence of endometrial cancer, thromboembolic events, ischaemic cerebrovascular events, hot flushes and vaginal bleeding and discharge compared to tamoxifen (Baum et al., 2002; Howell et al., 2005). Aromatase inhibitors suppress plasma estrogen levels by inhibiting or inactivating aromatase, which is the enzyme responsible for conversion of androgenic substrates into estrogens (Smith and Dowsett, 2003). These compounds, however, can only be used in postmenopausal women since in premenopausal women the reduced feedback of estrogen to the hypothalamus and pituitary gland results in an increased secretion of gonadotropines, which will stimulate the ovary and lead to an increase in androgen and aromatase production (Miller, 2004). It is expected that in the near future anastrozole will replace tamoxifen as first choice adjuvant therapy. Tamoxifen, however, may still have a role in treatment in premenopausal women and patients that become resistant to aromatase inhibitors.

7.5 Concluding remarks

In this thesis we aimed to discuss molecular and cellular mechanisms involved in hormone-controlled endometrial cancer growth.

In chapters 2 and 3, it was observed that estrogen and progesterone regulate different sets of genes. The balance between estrogen-induced proliferation and progesterone-induced growth inhibition is not accomplished by contra regulation of similar genes but rather by regulating different genes involved in shared biological processes. Furthermore, activation of the IGF receptor pathway seems to play a role in mediating these effects.

In chapter 4 and 5, mechanisms putatively involved in endometrial carcinogenesis of tamoxifen-users were evaluated. It is suggested that the workingsmechanism of tamoxifen is rather different from the mechanism of estrogen signaling, however, similarities in both signal transduction pathways are found. Activation of the EGF receptor pathway, either through direct activation of the EGF receptor or through up-regulation of the EGF receptor ligand amphiregulin, seems to play a significant role.

In chapter 6, effects of tamoxifen on the non-malignant endometrium were tested *in vivo*. It was concluded that tamoxifen, as in cell line experiments, induces a specific gene expression profile, which is rather different from the gene expression profile of women treated with estrogen. Furthermore, in network analysis tamoxifen-regulated genes centre around five well-known genes; TP53, RELA, MYC, EGF receptor and ß-catenin.

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Samenvatting
Endometriumkanker (kanker van de binnenbekleding van de baarmoeder) is de meest voorkomende gynaecologische kanker in Europa en de Verenigde Staten. Tijdens de vruchtbare levensfase is het endometrium onderhevig aan cyclische veranderingen die ontstaan door de productie van zowel oestrogenen als progesteron in groeiende follikels in de eierstok (het ovarium). Na de overgang is de concentratie van oestrogenen en progesteron laag door het uitblijven van follikelrijping. Hierdoor ondergaat het endometrium geen cyclische veranderingen meer en krijgt het een atrofisch voorkomen. Bij sommige vrouwen is er na de overgang nog een verhoogde hoeveelheid oestrogenen aanwezig door een verhoogde inname (exogeen), of door een verhoogde eigen productie (endogeen). Onder invloed hiervan kan het endometrium weer gestimuleerd worden tot groei. Deze oestrogeen geïnduceerde groei kan resulteren in ongecontroleerde groei en kan uiteindelijke zelfs leiden tot endometriumkanker.

Tamoxifen, een selectieve oestrogeen receptor modulator, is standaard aanvullende behandeling voor vrouwen met oestrogeen receptor positieve borstkanker. Tamoxifen werkt hier als een remmer van oestrogeen activiteit, een zogenaamde oestrogeen-antagonist. In het endometrium, echter, werkt tamoxifen als een oestrogeen-agonist. Gebruik van tamoxifen lijkt op gebruik van oestrogenen en is daarom geassocieerd met een verhoogde kans op de ontwikkeling van afwijkingen in het endometrium, waaronder ook endometriumkanker.

Uit onderzoek is bekend dat in het endometrium, naast de hiervoor beschreven effecten van oestrogenen, progesteron en hormoonachtige stoffen als tamoxifen, ook groeifactoren een rol spelen. Samenspraak tussen IGF-1 en EGF receptor signaaltransductie en effecten op de activatie van oestrogeen receptor signaaltransductie zijn beschreven voor zowel het endometrium als endometriumkanker.

In dit proefschrift zijn de resultaten beschreven van onderzoek naar het moleculaire werkingsmechanisme van de oestrogeen receptor (ER) tijdens stimulatie van het endometrium, en de rol van deze receptor in de ontwikkeling van endometriumkanker.

Op basis van het voorgaande komen in dit proefschrift de volgende vragen aan de orde:
1. Wat zijn de moleculaire mechanismen van oestrogeen gestimuleerde groei en progesteron geïnduceerde groeiremming?
2. Leidt activatie van ER signaaltransductie tot activatie van IGF en/of EGF receptor signaaltransductie, en *vice versa* leidt activatie van IGF en EGF receptor signaaltransductie tot activatie van ER signaaltransductie?
3. Welke genen worden gereguleerd door oestradiol, tamoxifen, raloxifene en het puur anti-oestrogeen ICI182780? Stimuleren deze stoffen, die allen werken via binding aan de ER, dezelfde genen en signaaltransductie paden, en activeren ze dezelfde biologische processen?
4. Welke genen worden door tamoxifen gereguleerd in het normale endometrium van vrouwen die tamoxifen gebruiken als therapie tegen borstkanker? Kunnen we op basis van deze genen bestuderen welke signaaltranductie paden worden geactiveerd door tamoxifen?

In **hoofdstuk 1** wordt een algemene inleiding gegeven op onderwerpen beschreven in dit proefschrift. Sectie 1.1 geeft een algemeen overzicht van de functie en werking van het normale endometrium. De risicofactoren gerelateerd aan de ontwikkeling van endometriumkanker worden uiteen gezet. Tevens wordt de officiële classificatie van endometriumkanker volgens de internationale FIGO standaard beschreven, en mogelijkheden voor therapie worden genoemd. In sectie 1.2 worden de effecten van tamoxifen op het endometrium beschreven. In sectie 1.3 worden eigenschappen van de ER en progesteron receptor (PR) beschreven, en in sectie 1.4 wordt de rol van groeifactoren in de ontwikkeling van endometriumkanker uiteengezet. Als laatste wordt in sectie 1.5 een korte beschrijving van de micro-array techniek gegeven.

In **hoofdstuk 2** worden resultaten beschreven van onderzoek naar genregulatie door oestradiol en progesteron. Hiervoor werden twee endometriumkanker cellijnen gebruikt: de oestrogeen gevoelige ECC-1 cellijn en de progesteron gevoelige IKPRAB-36 cellijn. Met behulp van de micro-array techniek werden 148 oestrogeen gereguleerde genen en 148 progesteron gereguleerde genen geïdentificeerd, die op basis van hun functie in de cel, biologisch werden geclassificeerd. Van alle gereguleerde genen konden er 81 gegroepeerd worden als genen die betrokken zijn bij "groei en homeostase". Binnen deze biologische subgroep reguleren oestradiol en progesteron verschillende genen, die echter wel vallen binnen gelijke functionele families (bijvoorbeeld genen die betrokken zijn bij de celcyclus).

In **hoofdstuk 3** zijn resultaten beschreven van experimenten naar de rol van IGF-1 signaaltransductie in oestrogeen gestimuleerde groei en progesteron geïnduceerde groeiremming van het endometrium. Hiervoor werden dezelfde twee endometriumkanker cellijnen gebruikt als in hoofdstuk 2: de oestrogeen gevoelige ECC-1 cellijn en de progesteron gevoelige IKPRAB-36 cellijn. In beide cellijnen komen de IGF-1 receptor en de IGF-2 receptor tot expressie. Echter, oestrogeen noch progesteron reguleren de expressie van deze receptoren.

ECC-1 cellen worden gestimuleerd in groei door zowel IGF-1 als IGF-2. Wanneer in de ECC-1 cellen IGF-1 signaaltransductie wordt geblokkeerd, is oestradiol minder goed in staat om groei te stimuleren. Dit betekent dat een deel van de oestradiol geïnitieerde groei kan worden verklaard door activatie van het IGF-1 signaaltransductie pad. Wanneer echter, de ER geblokkeerd wordt, heeft dit geen invloed op de IGF-1 gestimuleerde groei. IGF-1 kan dus onafhankelijk van de ER groei stimuleren.

In de cel wordt de biologische activiteit van de IGFs gemoduleerd door de IGFBPs; IGFs binden aan deze eiwitten en deze binding kan ervoor zorgen dat de IGFs actiever zijn, of dat juist de activiteit geremd wordt. Wij hebben gevonden dat in ECC-1 cellen, de expressie van IGFBP-4 wordt verhoogd door oestradiol. In de IKPRAB-36 cellijn is gevonden dat de expressie van IGFBP-3 en IGFBP-6 wordt verlaagd door progesteron. Dit kan betekenen dat naast de invloed van oestradiol op IGF-1 gestimuleerde groei, ook regulatie van expressie van de IGFBPs door oestradiol en progesteron een rol speelt in de groeimodulatie van endometriumkanker.

In **hoofdstuk 4** zijn resultaten beschreven van experimenten naar oestrogeen-agonistische (stoffen die werken als oestrogenen) en oestrogeen-antagonische (stoffen die de werking van oestrogenen blokkeren) effecten van verschillende ER liganden. Uit onderzoek is bekend dat oestrogenen, selectieve oestrogeen receptor modulatoren (SERMs) en anti-oestrogenen per orgaan een ander effect kunnen hebben. Zo wordt in de borst groei gestimuleerd door oestrogenen, en wordt dit weer geremd door tamoxifen (oestrogeen-antagonistisch effect). Tamoxifen is dan ook standaardtherapie voor vrouwen met borstkanker. In het endometrium zien we een ander effect van deze stoffen: in deze cellen stimuleren zowel oestrogenen als tamoxifen groei (tamoxifen heeft hier een oestrogeen-agonistisch effect). Het gebruik van tamoxifen door borstkanker patiënten is als gevolg hiervan geassocieerd met een verhoogd risico op ontwikkeling van endometriumkanker.

Om de effecten van oestrogene en antioestrogene stoffen op het endometrium te bestuderen zijn ECC-1 cellen behandeld met oestradiol, tamoxifen, raloxifen en ICI182780 en is met behulp van micro-array experimenten gekeken welke genen gereguleerd worden door deze stoffen. Uit de analyse van deze genexpressie profielen blijkt dat deze stoffen dezelfde biologische processen beïnvloeden, maar dit doen door regulatie van verschillende genen.

Binnen de groepen van gereguleerde genen (bijvoorbeeld oestrogeen-gereguleerde genen, tamoxifen-gereguleerde genen) is met behulp van genetische netwerk analyse de onderlinge relatie tussen de gereguleerde genen onderzocht. Hieruit blijkt dat voor genen gereguleerd door oestradiol, tamoxifen en ICI182780 het grootste netwerk zich concentreert om de EGF receptor. Uit de microarray experimenten bleek verder dat de expressie van het EGF receptor ligand amphireguline (AREG) wordt verhoogd door oestradiol en tamoxifen en dat de expressie van AREG wordt verlaagd door het anti-oestrogeen ICI182780. Deze regulatie is dus

gelijk aan de effecten die oestradiol, tamoxifen en ICI182780 hebben op het endometrium *in vivo*. Het was verder ook mogelijk te laten zien dat AREG ook een biologisch heeft in de ECC-1 cellen: de EGF receptor wordt geactiveerd door AREG, de expressie van bekende EGF receptor gereguleerde genen wordt verhoogd en behandeling van cellen met AREG stimuleert groei. Als conclusie kunnen we zeggen dat activatie van EGF receptor signaaltransductie een belangrijke rol lijkt te spelen in de regulatie van oestrogeen-agonistische groei van het endometrium.

In **hoofdstuk 5** worden resultaten beschreven van meer gedetailleerde experimenten naar effecten van oestradiol en tamoxifen op het endometrium. Hiervoor zijn micro-array experimenten gedaan met ECC-1 cellen die voor 1, 6, 12, 24, 48 of 72 uur behandeld zijn met oestradiol of tamoxifen. Uit analyse van deze experimenten blijkt opnieuw dat effecten op genregulatie anders zijn voor oestradiol dan voor tamoxifen. Daarnaast blijkt dat effecten op genregulatie heel anders zijn na kortstondige behandeling (1 uur) in vergelijking met langdurige behandeling (24, 48 en 72 uur).

Binnen de groep van genen die gereguleerd worden na 1 uur stimulatie worden veel genen gevonden die ook betrokken zijn bij IGF en/of EGF receptor signaaltransductie. Uit deze gegevens blijkt dat de snelle respons (niet-genomische respons) van het endometrium op behandeling met oestradiol of tamoxifen lijkt te verlopen door activatie van EGF en IGF receptor signaaltransductie.

Uit analyse van genen, die worden gereguleerd na een langdurige behandeling door oestradiol of tamoxifen, en van genen die worden gereguleerd door IGF-1 of AREG, blijkt ook dat de trage respons (genomische respons) van het endometrium op behandeling met oestrogeen en tamoxifen deels komt door activatie van EGF of IGF-1 receptor signaaltransductie. Tamoxifen effecten lijken met name te lopen via activatie van het EGF pad, terwijl voor de effecten van oestrogeen activatie van zowel het EGF receptor pad als het IGF-1 receptor pad gebruik wordt gemaakt.

In **hoofdstuk 6** zijn resultaten beschreven van experimenten naar effecten van tamoxifen op het humane endometrium *in vivo*. Micro-array experimenten zijn uitgevoerd met RNA geisoleerd uit endometriumweefsels (normaal atrofische endometrium of endometrium poliepen) van vrouwen die tamoxifen gebruikten als behandeling voor borstkanker en de resulterende expressiepatronen zijn vergeleken met de expressiepatronen verkregen uit soortgelijke endometriumweefsels van vrouwen die geen tamoxifen gebruikt hebben. Uit analyse van deze genexpressie profielen blijk dat 256 genen anders tot expressie komen in de tamoxifengroep vergeleken met de controlegroep. Wanneer deze tamoxifen gereguleerde genen vergeleken worden met oestradiol gereguleerde genen blijkt er nauwelijks overlap te zijn. Hieruit blijkt opnieuw dat, net als uit experimenten die gedaan zijn in de ECC-1 cellijn, tamoxifen anders werkt dan oestrogeen.

Uit netwerk analyses van de 256 tamoxifen-gereguleerde genen blijkt dat 69 van deze genen gerelateerd zijn aan 5 bekende genen; TP53, RELA, MYC, EGF receptor en ß-catenine. Het is daarom goed mogelijk dat deze genen belangrijk zijn bij tamoxifen geïnduceerde groei van het endometrium. Een veranderde biologische functie van deze genen en van de signaaltransductie paden waartoe ze behoren kan daarom een belangrijke stap zijn in de ontwikkeling van endometriumkanker bij tamoxifengebruikers.

In **hoofdstuk 7** worden de bevindingen uit voorgaande hoofdstukken van dit proefschrift in relatie tot elkaar besproken. De onderzoeksvragen die in hoofdstuk 1 werden gesteld worden beantwoord en suggesties voor vervolgonderzoek worden gegeven.

Summary
Endometrial cancer is the most common gynecological malignancy in Europe and the USA. In the normal endometrium, growth and differentiation is controlled by the ovarian hormones estrogen and progesterone. After menopause, the absence of follicle recruitment in the ovary results in a decline in serum levels of estrogen and progesterone, and consequently results in an atrophic/inactive state of the endometrium. However, in some women increased levels of estrogen (either endogenous or exogenous) are present, which will stimulate the endometrium. This estrogen-induced growth of the endometrium may result in uncontrolled growth, which can eventually develop into cancer. As in the normal endometrium, progesterone inhibits growth of endometrial cancer cells and is therefore used in the clinic as adjuvant therapy.

Tamoxifen, a selective estrogen receptor modulator (SERM), is standard adjuvant therapy for patients with estrogen receptor positive (ER+) breast cancer (estrogen-antagonistic effect). In the endometrium, however, tamoxifen displays an estrogen-agonistic effect, and use of tamoxifen is therefore associated with an increased risk for development of endometrial pathologies, including endometrial cancer.

For the endometrium, but also for many other organs, growth factors and growth factor receptors play a central role in mediating the effects of steroid hormones. Growth factors like IGF-1 and EGF mediate estrogen receptor signaling and are therefore also involved in the regulation of proliferation of the endometrium and endometrial cancer.

The emphasis of this thesis is on the molecular mechanisms of estrogen receptor controlled proliferation of the human endometrium and subsequent induction of endometrial cancer.

We postulated and addressed the following questions in this thesis:

1. What are the molecular mechanisms underlying estrogen-induced growth stimulation and progesterone-induced growth inhibition of endometrial cancer cells?
2. Does activation of the ER signaling pathway result in activation of IGF and/or EGF signaling, and vice versa, does activation of the IGF and EGF signaling pathways result in activation of ER signaling?
3. Which genes are regulated by estrogen, tamoxifen, raloxifene and the anti-estrogen ICI182780 in endometrial cancer cells, and do the four ER-ligands regulate similar genes, in the same cellular processes or pathways?
4. Which genes are regulated in endometrial tissues of tamoxifen-users compared to non-users, and can we, based on the generated gene-expression profiles, elucidate which pathways are activated by tamoxifen during the early changes which may lead to endometrial cancer formation?

In **Chapter 1,** an introduction is given. Section 1.1 gives a general overview of endometrial (cancer) development and maintenance, including endometrial cancer classification and treatment. In section 1.2 the effects of tamoxifen on the endometrium are described. Section 1.3 describes the properties of estrogen receptor (ER) signaling and progesterone receptor (PR) signaling, and section 1.4 highlights the role of growth factor signaling in endometrial carcinogenesis. In section 1.5 the micro-array technique and analysis are discussed.

In **Chapter 2**, results of investigations into regulation of gene-expression by estrogen and progesterone using the micro-array approach are described. The estrogen-responsive ECC-1 cell line and progesterone-responsive IKPRAB-36 cell line were used for the experiments. 148 estrogen-responsive and 148 progesterone-responsive genes were identified, which could be categorized into several biologically relevant groups. Of the regulated genes, 81 genes were clustered within the biological group of "cell growth and maintenance". Within this subgroup estrogen and progesterone regulate different genes that belong to the same functional families. It was therefore speculated that the cross-talk between estrogen and progesterone signaling

does not occur by counter regulation of single genes, but rather exists at the level of differential regulation of different genes within the same functional families.

In **Chapter 3**, the role of IGF-1 signaling in estrogen-induced proliferation and progesterone-induced growth inhibition of the endometrium was investigated. The estrogen-responsive ECC-1 cell line and progesterone-responsive IKPRAB-36 cell line were used. The expression of the IGF-1 and IGF-2 receptor was determined and it was observed that expression of these receptors was not regulated by either estrogen or progesterone. The ECC-1 cells are stimulated in growth by both IGF-1 and IGF-2. Blocking the IGF-1 signaling pathway resulted in a decrease in estrogen-induced proliferation, indicating that estrogen-induced stimulation of proliferation is partly achieved via IGF signaling. IGF signaling is, however, independent of ER signaling since blocking ER signaling did not inhibit IGF-1 induced proliferation. The biological activity and availability of IGFs is modulated through binding of IGFs to the insulin-like growth factor binding proteins (IGFBPs). In the current set-up three IGFBPs were found regulated; IGFBP-4 is up-regulated by estrogen, while IGFBP-3 and IGFBP-6 are down-regulated by progesterone. This could indicate that regulation of expression of these proteins also plays a role in modulation of endometrial cancer cell proliferation.

In **Chapter 4**, estrogen-agonistic effects and estrogen-antagonistic effects of ER ligands were studied. In different tissues, estrogens, selective estrogen receptor modulators (SERMs) and anti-estrogens exert different biological activities. For the endometrium, estradiol and tamoxifen induce proliferation, and because of this, tamoxifen-treatment of breast cancer patient's results in a 2-7 fold increased risk for development of endometrial cancer. In ECC-1 cells, gene-expression profiles were generated for estrogen, tamoxifen, raloxifene and the pure anti-estrogen ICI182780, to gain insights into the molecular mechanism of action of these ligands. Analysis of these profiles revealed that the four ligands influence the same biological processes, but do so via regulation of different sets of genes. Construction of genetic networks of the regulated genes showed that for estrogen, tamoxifen and ICI182780 regulated genes, the largest possible network centred on EGF receptor signaling. Interestingly, expression of the EGF receptor ligand amphiregulin (AREG) is regulated by estrogen, tamoxifen and ICI182780 and regulation of expression of AREG coincides with the *in vivo* effects these ligands have on the endometrium. Furthermore, it was shown that AREG directly activates the EGF receptor signaling pathway; stimulation of cells with AREG increased phosphorylation of the EGF receptor, enhanced expression of the EGF responsive genes FOS and EGR1 and increased proliferation rate of the cells. It was therefore concluded that EGF receptor signaling could be an important player in estrogen-agonistic growth of the endometrium.

In **Chapter 5**, the effects of estrogen and tamoxifen on the endometrium were studied in more detail; micro-arrays were performed with RNA from ECC-1 cells cultured for 1, 6, 12, 24, 48 and 72 hours in the presence or absence of estrogen and tamoxifen. Cluster analysis of these samples showed that the 1hr samples cluster separate and away from samples treated for longer time periods. This indicates that early, non-genomic effects are very different for these ligands as compared to the late, genomic effects. Within the group of early response genes, a high number of genes could be linked to the IGF or the EGF receptor signaling pathway.

Comparing estrogen and tamoxifen regulated genes (24, 48 or 72 hr of treatment) with genes regulated by IGF-1 and AREG revealed that also the late genomic effects of estrogen and tamoxifen signaling were partly mediated via activation of IGF and EGF receptor signaling pathways. Several distinct gene clusters were identified from the regulated genes and it was concluded that tamoxifen signaling was partly achieved via activation of the EGF receptor pathway, while estrogen signaling was partly achieved via activation of both the EGF receptor as well as the IGF receptor signaling pathway.

In **Chapter 6**, the results of investigations into the mechanism of action of tamoxifen in benign human endometrial tissues are described. Gene-expression profiles were generated from benign endometrial samples of tamoxifen-users and compared to matched controls. Using cluster and SAM analysis, 256 genes were identified that were differentially regulated between the tamoxifen-group and the control-group. Comparing these 256 genes with estrogen-regulated genes, 95% of the differentially expressed genes turned out to be tamoxifen specific. Network analysis of the differentially expressed genes, revealed that 69 genes centred on 5 well-known genes; TP53, RELA, MYC, EGF receptor and ß-catenin. These genes could be important for tamoxifen-controlled proliferation of the endometrium, and an imbalance of the pathways in which these genes function could maybe play a determining role in endometrial carcinogenesis in tamoxifen-users.

In **Chapter 7**, the findings that are presented in this thesis are discussed, and relations between findings from different chapters are explained. In addition, suggestions for further research are described.

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Curriculum Vitae

Susanne Gielen was born June 8, 1973 in Etten-Leur, the Netherlands. Following graduation from the K.S.E. in Etten-Leur in 1992 (VWO), she attended Medical School at the University of Maastricht. During her study, she did a three-month research period at the Department of Gynecology and Obstetrics, University of Durban in South Africa and a six-week internship at the Department of Gynecology and Obstetrics, University of Karachi in Pakistan.
After her graduation, she started to work as a resident (AGNIO). At first, from January 2000 till December 2000 at the Department of Surgery of Spaarne Hospital in Haarlem, followed by a residency Obstetrics and Gynecology (January 2001 till August 2001) at the Erasmus University Medical Center Rotterdam (head: Prof. dr. Th.J.M. Helmerhorst).
In September 2001 she started a PhD project at the Department of Gynecologic Oncology (head: Prof.dr. C.W. Burger) in collaboration with the Department of Reproduction and Development (head: Prof.dr. J.A. Grooetegoed). She worked within the Endometrial Cancer Group, supervised by Dr.ir. L.J. Blok, Prof.dr. C.W. Burger and Dr.F.J. Huikeshoven (from September 2001 till June 2003), performing the studies described in this thesis. In May 2005 she started her AGIO residency Obstetrics and Gynecology at Sint Fransciscus Gasthuis in Rotterdam (head; Dr. A.Th. Alberda).