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Progesterone receptors in development and metastasis of endometrial cancer

Progesterone receptors in development and metastasis of endometrial cancer

Progesteronreceptoren tijdens ontwikkeling en metastasering van endometriumkanker

Proefschrift

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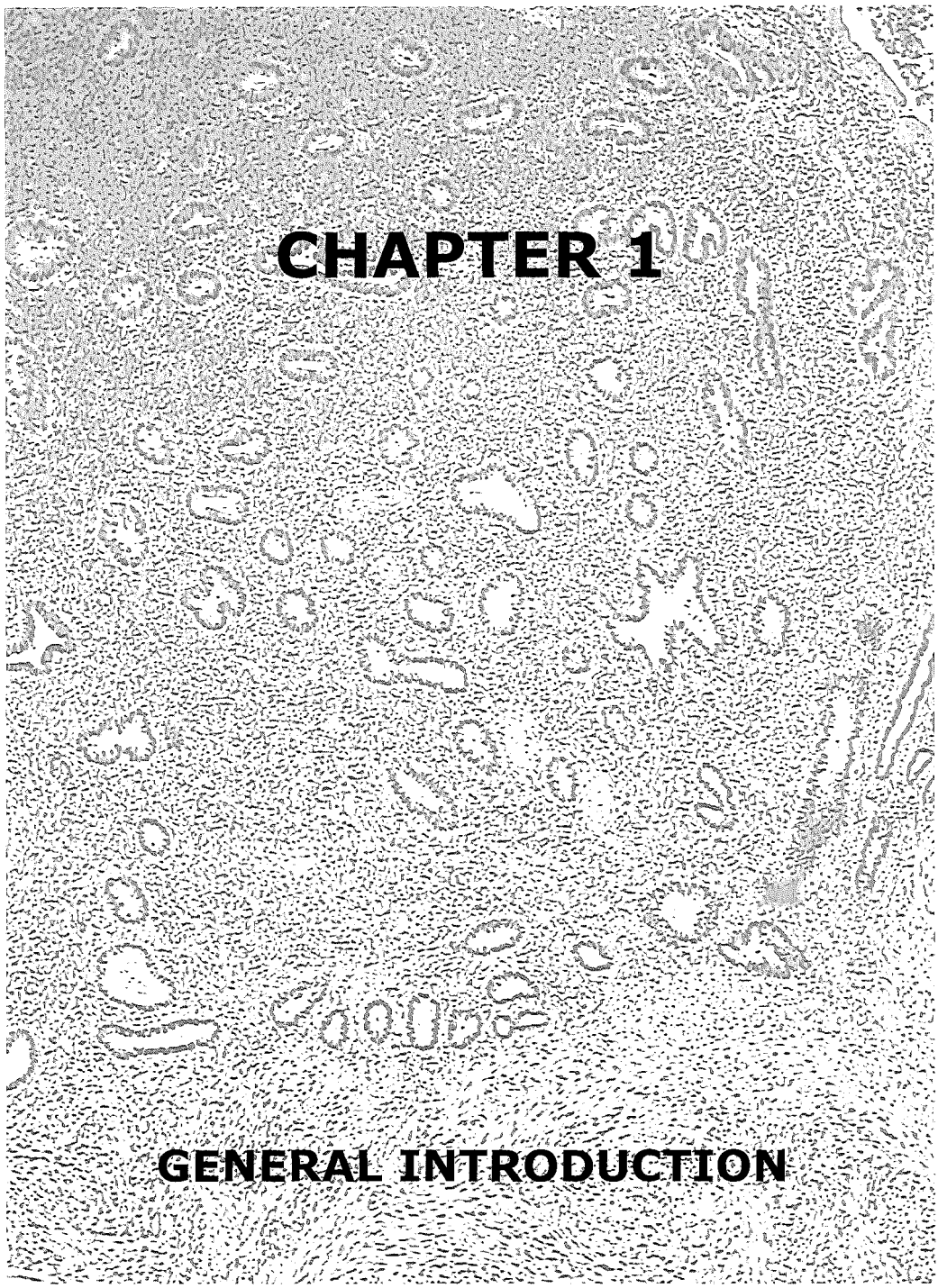

CHAPTER 1

GENERAL INTRODUCTION

1.1. Endometrial cancer

1.1.1. Non-neoplastic endometrium

During the sexual indifferent stage of human embryonal development, the wolffian and müllerian ducts (mesonephric and paramesonephric ducts, respectively) make up the bipotential reproductive tract. In females, the absence of testosterone leads to regression of the wolffian ducts, while the müllerian ducts develop into the fallopian tubes, uterus and upper part of the vagina. Development of the uterus essentially starts in the ninth week, when the caudal tips of the müllerian ducts fuse into a single-lumen tube, the uterovaginal canal (Figure 1). This then fuses with the sinovaginal bulb, resulting in formation of uterus, cervix and vagina (Larsen, 1993). Directly after birth, the uterus of a human baby is enlarged due to stimulation by maternal estrogens (which cross the placenta). Within several weeks after birth, the uterus shrinks again and remains dormant until menarche.

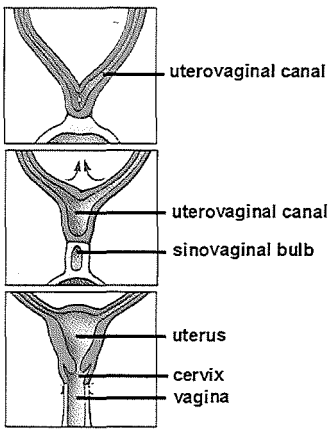

Figure 1. Development of the female genital tract. At nine weeks, the müllerian ducts fuse, forming the uterovaginal canal. The sinovaginal bulb and uterovaginal canal fuse to form the uterus, cervix and vagina. (Figure adapted from Larsen, 1993).

The endometrium makes up the inner layer of the mature uterus (Figure 2), and consists of stromal and epithelial cells. Functionally it can be divided in a basal and a functional layer. The basal layer is closest to the myometrium (the muscular wall of the uterus), and does not change during the menstrual cycle. In contrast, the inner, or functional, layer of the endometrium undergoes extensive changes under the influence of several hormones during the menstrual cycle. During the proliferative phase (from day 4 to 13), proliferation and gland formation are stimulated by high levels of estrogens. Also, the functional endometrium becomes highly vascularized. Luteinizing hormone (LH) induces ovulation (at approximately day 14), at which point estrogen levels drop. After ovulation, the corpus luteum secretes progesterone, which is the start of the secretory phase (days 15-28). Progesterone inhibits growth of the endometrium, but stimulates differentiation. When no pregnancy occurs, the corpus luteum will regress, leading to a drop in

progesterone and estrogen levels. Because of this, the blood vessels in the endometrium will constrict, so that the tissue becomes ischemic. As a result, the functional endometrium disintegrates and is shed from the uterus. When ovulation no longer occurs (menopause), the endometrium no longer undergoes these cyclic changes, and will regress to a layer with a thickness of only a millimeter or less.

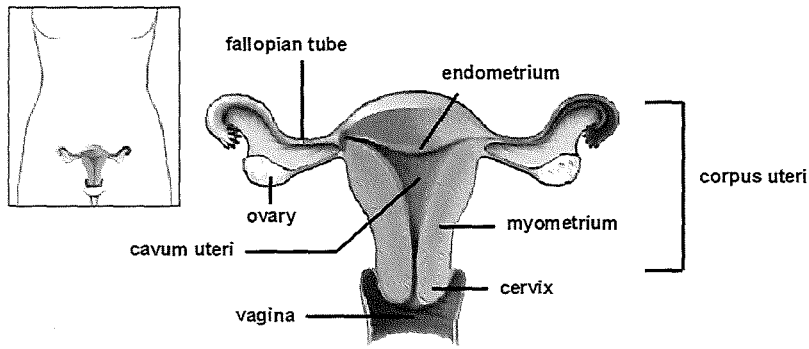

Figure 2. Schematic representation of the human adult female genital tract.

During mammalian organogenesis of the genital tract, locally acting members of the Wnt family of signaling proteins play important roles. Also, during the menstrual cycle, expression patterns of Wnt proteins vary. Especially Wnt4 and Wnt7a are important in female development. Vainio et al. (1999) described that in Wnt4 loss-of-function mutant mice, female development was severely disturbed (Vainio et al., 1999). Gonads of these Wnt4 mutant female mice were masculinized, while male Wnt4 mutant gonads were normal. Wnt4 appeared to be needed for formation of the müllerian ducts both in males and in females, but since these regress in males, altering Wnt4 expression has consequences only in females. In Wnt4 loss-of-function mutant females, the indifferent gonads do not undergo primary sex reversal, and oocyte formation is impaired (Vainio et al., 1999). Wnt7a is important for sex-specific development of müllerian duct derivatives, as is indicated by the observation that Wnt7a heterozygote loss-of-function mutant female mice have poorly developed fallopian tubes and uterus, but normal ovaries (Parr and McMahon, 1998). Furthermore, Wnt7a homozygote loss-of-function mutants do not develop endometrial glands, and the myometrium is disorganized (Miller and Sassoon, 1998).

1.1.2. Endometrial cancer: occurrence and risk factors

Endometrial cancer is one of the most common gynecological cancers in the Western world. Age-standardized yearly incidence numbers vary from < 2.4 per 100.000 women in Western Africa and China to up to 28.9 per 100.000 women in Northern Europe and Northern America (Parkin et al., 2001). In The Netherlands, the yearly incidence is approximately 16 per 100.000 women (www.ikc.nl/vvik). Endometrial cancer is mainly a disease of (post-) menopausal women, with < 5% of cases appearing in women under 40 years of age (WHO World Cancer Report 2003).

The major risk factor for acquiring endometrial cancer is prolonged unopposed estrogen action, either because of endogenous causes (physiologically, such as late onset of menopause, or pathologically, for instance hormone-producing tumors) or through exogenous causes (mainly estrogen-only hormone replacement therapy) (Deligdisch, 2000). Other risk factors include obesity, hypertension, early menarche and late menopause, low parity and polycystic ovary syndrome, which are all factors that are associated with an elevated estrogen level. Use of tamoxifen, a compound that exerts an anti-estrogenic effect in breast and is used in prevention of (recurrence of) breast cancer, is also associated with increased risk for endometrial hyperplasia and cancer in postmenopausal women (reviewed by (Mourits et al., 2001).

Little is known about genetic predisposition for endometrial cancer. In an autosomal dominant cancer susceptibility syndrome, hereditary nonpolyposis colorectal cancer (HNPCC), endometrial cancer is the most common tumor outside the colon (Green et al., 1998; Sumoi et al., 1995). In association with this, in carriers of mutations in the DNA mismatch repair gene *MSH6* (MutS homologue 6) endometrial cancer is the most common clinical manifestation (Wijnen et al., 1999).

1.1.3. Histology and typing

The endometrial epithelium can transform into multiple types of cancer. Endometrial adenocarcinoma is the most abundant type, making up 90% of all endometrial cancers. This type of tumor is characterized by a decrease in stromal tissue between abnormal glands. Other types include papillary serous carcinoma, mucinous adenocarcinoma, clear cell adenocarcinoma, adenosquamous carcinoma and mixed carcinoma. When formulating a diagnosis, the FIGO system is used to assess histological grade and surgical staging.

In addition to conventional pathology- and histology-based classification, the possibility of molecular classification of endometrial cancer is currently being evaluated. Early studies indicate that based on gene expression profiles of

Histopathologic grades (G)	
Gx	Grade can not be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated

Surgical staging classification	
Stage Ia	Tumor is limited to the endometrium
Stage Ib	Invasion < half the myometrium
Stage Ic	Invasion ≥ half the myometrium
Stage IIa	Endocervical glandular involvement only
Stage IIb	Cervical stromal invasion
Stage IIIa	Invasion of serosa of corpus and/or adnexae and/or positive cytology
Stage IIIb	Vaginal metastases
Stage IIIc	Metastases to pelvic and/or para-aortic lymph nodes
Stage IVa	Tumor invasion of bladder and/or bowel mucosa
Stage IVb	Distant metastases

Table 1. Classification of endometrial cancer by the FIGO-guidelines. A complete overview for staging of gynecological cancers is available at http://www.figo.org/content/PDF/staging-booklet.pdf.

endometrial cancer samples, a classification similar to the FIGO classification (Table 1) can be constructed (Smid-Koopman et al., 2004). Also for breast cancer, molecular classification has been shown to help predict clinical outcome of this disease (van 't Veer et al., 2002). With expression profiling becoming more straightforward and less expensive, this highly informative way of evaluating a tumor will become more important in the diagnosis of endometrial cancer and in designing individual treatment for endometrial cancer patients.

1.1.4. Treatment

The most obvious and also most common symptom of endometrial cancer is postmenopausal vaginal bleeding. Bleeding occurs early on in the disease and can be fairly significant. Since this symptom is usually alarming enough for most women to seek medical help, most cases of endometrial cancer are diagnosed at a relatively early stage.

First-line treatment consists mainly of surgical removal of the tumor, if necessary followed by radiotherapy. This treatment, in which generally the entire uterus and the adnexae are removed, is very effective for tumors that are confined to the uterus. This amounts to approximately 75% of cases at time of diagnosis

(Stewart and Kleihues, 2003). In patients with advanced and metastatic disease, prognosis is poor. Chemotherapy for endometrial cancer includes adriamycin, cisplatin, paclitaxel, cyclophosphamide and/or ifosfamide, to which 14-30% of tumors respond with a median progression-free period of approximately four months (www.oncoline.nl and (Burke et al., 1991; Elit and Hirte, 2002; Long et al., 1995). Treatment with progestagens (a term indicating progesterone and compounds with progesterone-like activities) can be used as a temporary treatment in advanced or recurrent endometrial cancer, but response rates and progression-free survival intervals vary (Elit and Hirte, 2002). Other forms of endocrine therapy (which are still in an experimental phase) include the use of selective estrogen receptor modulators (SERMs) and aromatase inhibitors (Rose et al., 2000), again with limited results so far. In the case of young patients with well-differentiated endometrial cancer who want to maintain fertility, prolonged progestagen therapy can be applied with good results (Randall and Kurman, 1997). More details on treatment of endometrial cancer in The Netherlands are provided on the VIKC/Oncoline website (www.oncoline.nl).

Early detection and effective treatment of endometrial cancer result in a low mortality. In The Netherlands, the five-year survival rate of patients with endometrial cancer after diagnosis is 86% (Stewart and Kleihues, 2003).

1.2. Progesterone receptors A and B

1.2.1. General structure

The progesterone receptor is a member of the family of steroid hormone receptors, and can act as a hormone-dependent activator of transcription. The progesterone receptor exists as two isotypes, PRA and PRB, which are transcribed from two different promoters at the same gene on chromosome 11q22.1 (Kastner et al., 1990).

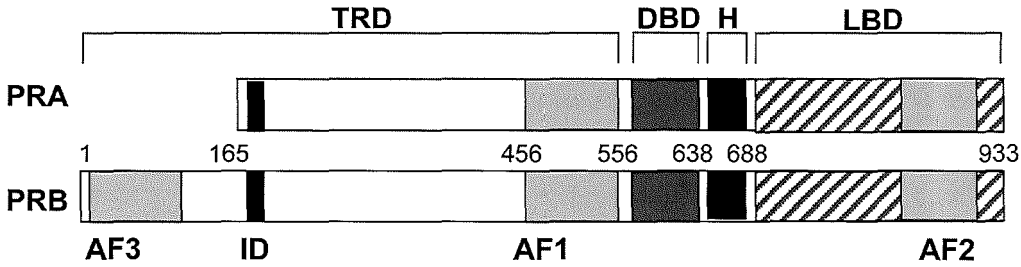

Figure 3. Structure of progesterone receptors A and B. TRD: transcription regulating domain; DBD: DNA-binding domain; H: hinge region; LBD: ligand-binding domain; AF1, AF2, AF3: activation function 1, 2, and 3; ID: inhibitory domain. Numbers indicate amino acid location at the start of domains on PRB.

PRA is a truncated form of PRB, lacking 164 amino acids at the amino terminus. In both PR isotypes several functional regions can be distinguished. The ligand-binding domain (LBD) is located at the carboxy-terminal part of the receptor. The LBD is responsible for binding of ligand, but also has a function in receptor dimerization, heat shock protein (HSP) binding, and transcription activation.

The DNA-binding domain (DBD) contains two zinc finger motifs, which bind specific hormone-response elements (HREs) in the genome. Between the LBD and the DBD lies the hinge region, which contains a nuclear localization signal, and is involved in receptor dimerization. The amino-terminal part of the receptor is described as the transcription-regulating domain and is partly responsible for modulation of transcription. Differences in transcriptional activity between PRA and PRB are discussed in more detail in Section 1.2.3.

1.2.2. General mode of action

The general mode of action of the PRs is depicted in Figure 4. In absence of ligand, PR is associated with heat shock proteins HSP56, HSP70 and HSP90. Upon binding of ligand, a conformational change takes place, leading to dissociation of the HSPs. Receptor-hormone complexes form dimers, both homodimers (PRA-PRA or PRB-PRB) and heterodimers (PRA-PRB). Unliganded PR can be present either in the nucleus or in the cytoplasm. Lim et al. (1999) showed that GFP-bound unliganded PRB is present mainly in the cytoplasm, while GFP-PRA was present in the nucleus in absence of ligand (Lim et al., 1999). Upon binding of ligand, both PRA and PRB localize predominantly in the nucleus (Bergeron et al., 1988). In the nucleus both receptors can bind to progesterone response elements (PREs) on the DNA and form a complex with specific cofactors and general transcription factors to modulate transcription of progesterone-responsive genes.

1.2.3. PRA and PRB have different transcriptional activities

Even though PRA and PRB have similar ligand- and DNA-binding properties, they exhibit different transcriptional activities (Giangrande and McDonnell, 1999). Both isotypes of the progesterone receptor contain two activation domains (AF1 and AF2) and one inhibitory domain (ID). The activation function domains act as sites for interaction with cofactors. Upon binding of hormone to the LBD, the family of SRC cofactors (steroid receptor coactivators or p160) can interact with AF-2 to stimulate transcription. AF-1 can act independently of AF-2, but upon activation by ligand, AF-1 and AF-2 will act synergistically (Giangrande and McDonnell, 1999).

In PRA, the conformation of the receptor allows the ID to prevent transcription activation by AF-1 and AF-2. The additional N-terminal stretch of PRB contains an additional activation domain, AF-3. Since AF-3 is not capable of activating transcription on its own (Sartorius et al., 1994), it is thought that this AF-3

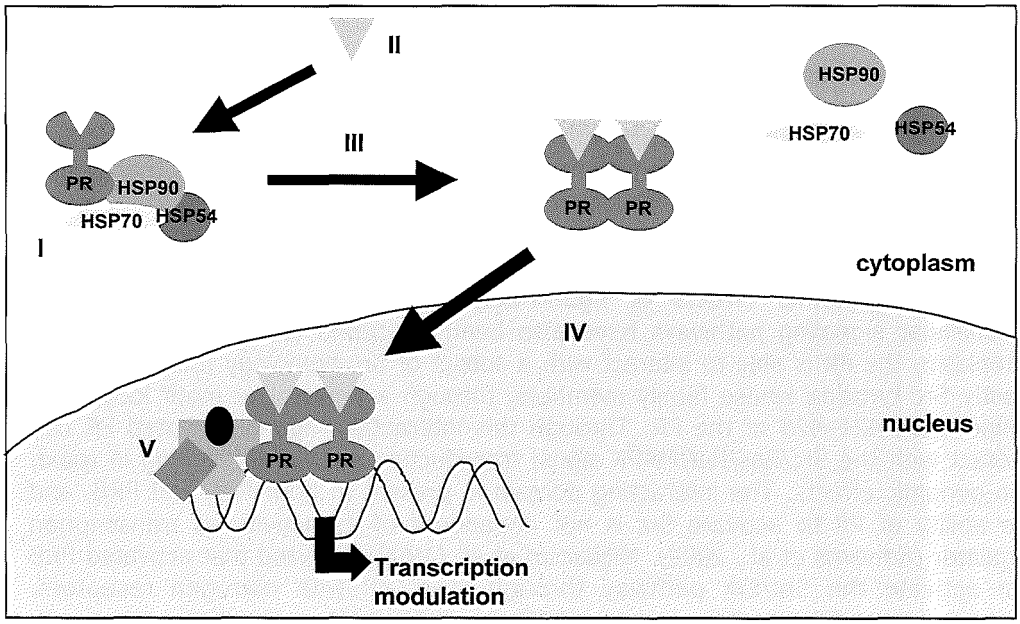

Figure 4. General mode of action of progesterone receptors. In absence of ligand, PR is bound to HSPs (I). Upon binding of ligand (II), the PR-HSP complex dissociates, and PR-dimers are formed (III). These dimers relocate to the nucleus (IV). Cofactors are recruited, and upon binding to progesterone response elements on the DNA, modulation of gene transcription follows (V).

domain acts indirectly through enhancing the activity of AF-1 and AF-2, or through inhibition of the ID of PRB. This renders PRB a stronger activator of transcription than PRA (Giangrande et al., 1997). In PRA, the ID is not inhibited, which is an explanation why PRA is not as transcriptionally active as the PRB, and can sometimes act as a dominant repressor of PRB function (Giangrande et al., 1997). Also, a serine residue in the ID is more readily phosphorylated in PRB than in PRA, which may add to the differences in transcriptional activity (Clemm et al., 2000).

A further explanation for the difference in transcriptional activity of the PR isotypes can be found in recruitment of different cofactors by PRA and PRB. Coactivators interact with components of the general transcription complex to facilitate transcription. They can also acetylate or methylate core histone proteins, which facilitates transcription (Edwards et al., 2003). Corepressors have the opposite effect, resulting in inhibition of gene transcription. Tetel et al. (1999) found that interaction of the N-terminus with the LBD is different for PRA and PRB (Tetel et al., 1999). These interactions could be involved in differences in cofactor binding. More specifically, PRA has a higher affinity for the corepressor SMRT than PRB, while both PRA and PRB interact similarly with the corepressor NCoR. Furthermore, PRA does not recruit the coactivators GRIP1 and SRC-1, while PRB does (Giangrande et al., 2000).

1.2.4. Additional actions of progesterone and PR

Effects of progesterone as a result of transcription activation by PRs will take at least 30 to 60 minutes to develop. However, progesterone can also have much more rapid effects, which may not result from activation of transcription by activated PR. Such non-genomic actions of progesterone may be caused by various triggers that do not involve the PR, such as association of progesterone with G protein-coupled receptors (oxytocin receptor), ion channels (Ca^{2+} influx in spermatozoa), or enzyme-linked receptors in the plasma membrane (reviewed by Simoncini and Genazzani, 2003).

Non-genomic effects of ligand-activated PR through crosstalk with intracellular signaling pathways have also been described (Lange, 2004). In the cytoplasm, the PR is able to interact with a subset of Src homology (SH3) domains, mostly Src tyrosine kinase family members, through a polyproline motif located at amino acids 421–428 of the PR. Through this interaction, ligand-activated PR can interact with the Src/Ras/Raf/MAPK signal transduction pathway, resulting in rapid, non-genomic effects. The interacting domain is present in both PRA and PRB, and the ability of PR to activate Src is not dependent of its activity as transcription regulator (Edwards et al., 2003). Migliaccio et al. (1998) reported that activated PRB can activate the Src/Erk pathway through crosstalk with estrogen receptors. Estrogen receptor interaction domains (ERID-I and ERID-II) on the PRB interact with the LBD of the estrogen receptor α, leading to activation of the Src/Erk pathway, independent of interactions with SH3 domains (Migliaccio et al., 1998). These non-genomic actions of ligand-activated progesterone receptors have implications for cell cycle regulation, which probably contribute to the effects of progesterone on proliferation (Edwards et al., 2003).

In addition to non-genomic effects of ligand-activated PR, ligand-independent functions of the PR have also been described. Jacobsen et al. (2002) described that when PR-negative breast cancer cells were transfected with inducible PR, the gene expression pattern of these cells was affected when expression of PR was induced, even in absence of progesterone. For instance, induced expression of PRA resulted in ligand-independent up-regulation of expression of the cell-cycle regulator p21 (Jacobsen et al., 2002). The ligand-independent activation of gene transcription by PR may be induced by activation of PR by non-steroidal compounds. For instance, the chicken PR (cPR) can be activated by cAMP through phosphorylation (Denner et al., 1990). Epidermal growth factor also can activate cPR, indicating that tyrosine kinases can directly or indirectly activate PR (Zhang et al., 1994). In mice, neurotransmitters like dopamine can activate PR in a hormone-independent manner through crosstalk of dopamine receptors in the plasma membrane with intracellular PR (Mani et al., 2001; Power et al., 1991).

1.2.5. Progesterone-regulated genes

A large set of genes, encoding proteins with a wide variety of functions, is regulated by progesterone through the PR. This set includes the gene encoding the estrogen receptor alpha, but also genes encoding proteases, cyclinD1, VEGF (vascular endothelial growth factor), annexin IV, lactoferrin, NfkappaB inhibitor alpha, ion transporters, monocyte chemoattractants, STAT5A, and many other genes. Regulation of genes by progesterone depends on the presence of cofactors, and ultimate expression of proteins is influenced by many post-translational processes. The availability of cofactors varies between different species, tissues and cell lines, which is why effects of progesterone on different tissues can be very diverse.

Also, within one cell type, different genes are regulated through PRA and PRB, as reported by Smid-Koopman et al. (2003) for endometrial cancer cells (Smid-Koopman et al., 2003), and by Richer et al. (2002) for breast cancer cells (Richer et al., 2002). Smid-Koopman et al. (2003) observed that the expression of six genes (encoding retinoic acid receptor gamma, integrinß4, MAP kinase P97, MTS1, cytokeratin 8, and cyclinD1) was regulated by progesterone through PRA but not PRB, while expression of 2 genes (IGFBP3 and replication protein A) was regulated through PRB but not PRA. Richer et al. (2002) described 94 progesterone-regulated genes in breast cancer cells, 65 of which were regulated specifically by PRB and 4 specifically by PRA. Only 25 out of 94 genes were regulated by both PRA and PRB. For example, STAT5A and C/EBPß are up-regulated by progesterone only through PRB. ESRRA (estrogen-related receptor alpha) and Bcl-XL are up-regulated only through PRA, while TGFβ3 is down-regulated through both PRA and PRB (Richer et al., 2002).

Taking into account the different transcriptional properties of PRA and PRB, it is to be expected that differences in the PRA/PRB ratio will result in formation of different PR dimers, which will affect the overall response of a cell to progesterone. Jacobsen et al. (2002) created an inducible model in T47D breast cancer cells to study the effect of different ratios of PRA and PRB on transcription modulation (Jacobsen et al., 2002). Inducible systems such as this will certainly prove to be of great importance in elucidating the effect of different ratios of PRA and PRB in cells.

1.2.6. PR knockout mouse model

A useful tool in studying biological effects of progesterone and progesterone receptors are the available progesterone receptor knockout mouse models. Female mice lacking PRA (PRAKO), PRB (PRBKO), or both receptor isotypes (PRKO) have been developed (Conneely and Lydon, 2000).

Examination of PRKO mice revealed that these mice do not ovulate. In more detail, follicles with mature oocytes are formed, but follicular rupture does not occur. The PRAKO mouse also shows impaired, but not completely absent, ovarian function. In these mice decidualization is inhibited, which is why pregnancy does not occur,

even though occasionally oocytes are released. Ablation of PRB did not affect ovarian responses to progesterone (Conneely et al., 2002; Conneely et al., 2003b). This indicates that in mice, PRA is mainly responsible for ovarian function, and also for induction of decidualization by progesterone (Conneely et al., 2001).

Not surprisingly, the epithelial component of the endometrium of PRKO mice is hyperplastic due to unopposed estrogen action. The overall architecture of the endometrium is disrupted, and macrophages and neutrophils are abundantly present, in accordance with loss of anti-inflammatory actions through PRs. In PRAKO mice, progesterone adds to the growth-stimulating effect of estrogen, indicating that in mice, PRB can have a proliferative role when PRA is not expressed (Mulac-Jericevic et al., 2000). Uterine responses to progesterone were unaffected in PRBKO mice, indicating that expression of PRA is sufficient and necessary to mediate the anti-proliferative effects of progesterone as well as implantation-associated responses in the mouse uterus (Conneely et al., 2003b).

Also breast tissue is strongly affected by loss of PR. Branching of epithelial ducts and development of terminal end buds was decreased in PRKO and PRBKO mice, but not in PRAKO mice. Also, pregnancy-associated ductal side-branching and lobuloalveolar development was decreased in PRBKO mice only (Conneely et al., 2003b). This indicates that in breast tissue, expression of PRB is sufficient for normal development (Conneely et al., 2003a; Mulac-Jericevic et al., 2000). The phenotypes of the different PR knockout mice thus provide further evidence that PRA and PRB exert different functions in different tissues.

1.2.7. Progesterone receptors A and B in endometrial cancer

In non-neoplastic cycling endometrium, the expression pattern of progesterone receptors varies. The highest level of PR expression is found in the glands of the functional layer during the second half of the proliferative phase, and the lowest level during the luteal phase. PRA expression is relatively constant throughout the cycle, with 5-fold changes in expression level at the most. PRB levels can vary 20-fold, increasing when serum levels of estradiol rise, and decreasing at times of high serum levels of progesterone (Mangal et al., 1997).

A lot of effort has gone into studying expression patterns of PR in endometrial cancer. In general, more poorly differentiated tumors have decreased expression of progesterone receptors, but a major question in these studies has always been whether there is any difference in expression of PRA and PRB, and if so, can this be related to the histopathological features of the tumor or have any implications for the prognosis?

The most extensive study on this was performed by Arnett-Mansfield et al. (2001). They studied expression of PRA and PRB in a large set of endometrial tumors, hyperplasias and non-neoplastic tissues of mainly (87%) post-menopausal patients. From their report it became clear that expression of PR in endometrial cancer, and the implications of this for prognosis, are not straightforward. It was

found that more advanced tumors express less PR compared to hyperplastic and normal endometrial tissue. Of normal tissues, 75% expressed PRA and PRB at equal levels, while the remaining 25% expressed only PRA. Hyperplastic tissues tend to express more PRA: 64% expressed only PRA, 18% more PRA than PRB and 18% expressed equal amounts of PRA and PRB. Non of the hyperplastic tissues showed overexpression of PRB. However, 28% of the tumors expressed only PRB (Arnett-Mansfield et al., 2001).

In tumors that do express PR, expression of PR isotypes varied with histopathological grade. Of grade 1 tumors, approximately 30% expressed equal levels of PRA and PRB; 50% expressed only or predominantly PRA. Grade 2 tumors mainly showed expression of only one isotype: only PRB (60%) or only PRA (30%). Grade 3 tumors again showed a predominant expression of PRA in almost 70% of the cases, with the remaining 30% expressing exclusively PRB (Arnett-Mansfield et al., 2001).

Reports from other groups further complicate the matter. Halperin et al. (2001) reports loss of immunoreactivity for PR in poorly differentiated (G3) tumors, but not in well to moderately differentiated (G1-G2) tumors (Halperin et al., 2001). Fujimoto et al. (1995, 1997) repeatedly reported a relative over-expression of PRB in more advanced endometrial tumors and in distant metastases (Fujimoto et al., 1997; Fujimoto et al., 1995). De Vivo et al. (2002) found a polymorphism in the PRB promoter which leads to increased transcription of the PRB isotype, and was associated with a 2-fold increased risk for endometrial cancer in a population-based study (De Vivo et al., 2002). In contrast, Kumar et al. (1998) speculated that down-regulation of PRB may predict for poorly differentiated endometrial cancers that do not respond to progestagen therapy (Kumar et al., 1998). Sasaki et al. (2001) described selective DNA methylation of the PR gene and subsequent transcriptional silencing of specifically PRB in endometrial cancer (Sasaki et al., 2001). Athanassiadou et al. (1999) reported no relation between expression of PR and five-year survival (Athanassiadou et al., 1999), while in the same year Iwai et al. (1999) showed that loss of PR expression is a prognostic factor for lymph node metastasis (Iwai et al., 1999).

1.3. Wnt signaling

1.3.1. Wnt signal transduction

The Wnt pathway is a signal transduction pathway that is very important in early development of many different organisms, ranging from *Drosophila* to human. Disruptions in this pathway lead to major defects in development, but are also associated with many different types of cancer. Wnt proteins belong to a large family of glycoproteins that are secreted to function in an autocrine or paracrine fashion. In humans, 19 members of the Wnt family have been identified so far (Lustig and Behrens, 2003).

The general cascade of the so-called canonical Wnt pathway is depicted in Figure 5. In short, secreted Wnt protein binds to its receptor in the plasma membrane, frizzled (Fz). Through a cascade of events, including activation of

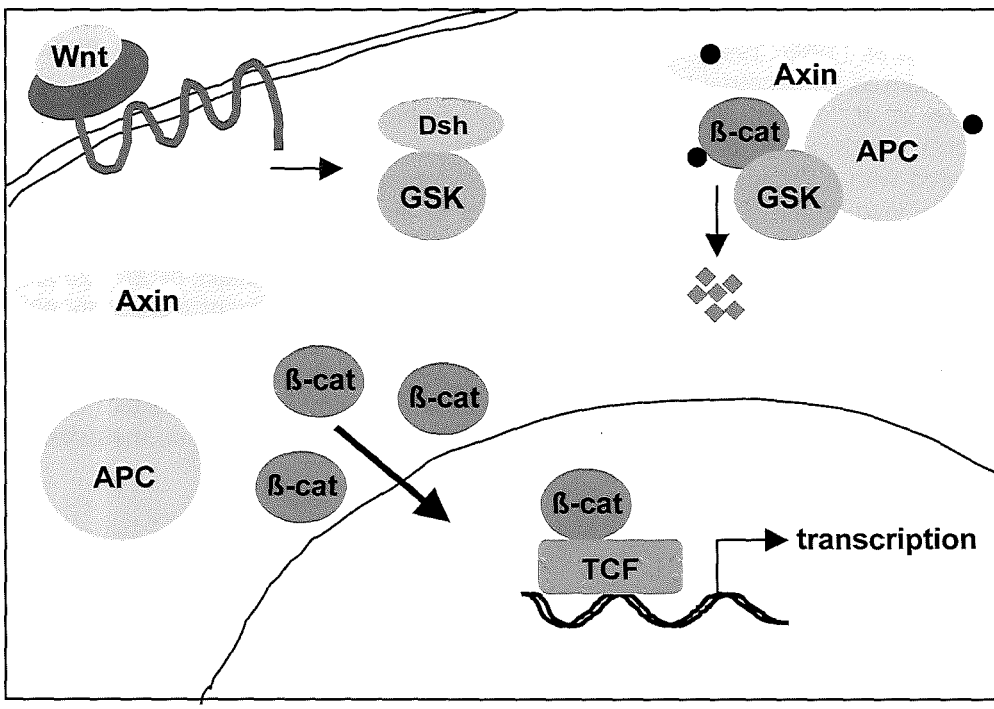

Figure 5. The canonical Wnt signal transduction pathway. In absence of Wnt, ß-catenin is caught in a complex with axin, APC and GSK3ß. Active GSK3ß phosphorylates ß-catenin (black dot), which is subsequently degraded. When Wnt binds to the receptor frizzled in the plasma membrane, this leads to dissociation of the ß-catenin degradation complex. As a result, ß-catenin can be translocated from the cytoplasm to the nucleus, where it acts as a cofactor for TCF/LEF, resulting in transcription of Wnt target genes.

dishevelled (Dsh) and inactivation of GSK3β, the β-catenin degradation complex 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). The key component in canonical Wnt signaling is β-catenin. This molecule has very different functions in the cell. At the plasma membrane, it links E-cadherin to the actin cytoskeleton (as does α-catenin), leading to stabilization of cell-cell adhesion. Disruption of this complex leads to loss of cell-cell adhesion, a status that is strongly associated with tumor cell invasion and metastasis. When free in the cytoplasm, β-catenin is caught in the β-catenin degradation complex. This complex contains APC, axin and GSK3β. In absence of Wnt, GSK3β phosphorylates β-catenin, after which β-catenin is ubiquinated and degraded in the 26S proteasome (Ikeda et al., 2000a).

When Wnt signaling is active, GSK3β is inhibited in its function by Dsh, resulting in free β-catenin being translocated to the nucleus. Exactly how this transport is accomplished is largely unknown, but a role for APC in this process has been proposed (Bienz, 2002). In the nucleus, β-catenin does not function as a transcription activator itself, but it is an essential co-activator for TCF/LEF (Novak and Dedhar, 1999). When Wnt signaling is not active, TCF/LEF is bound to groucho proteins, which are repressors of transcription (Eastman and Grosschedl, 1999).

1.3.2. Wnt in cancer

Disruptions in the Wnt pathway leading to constitutive β-catenin signaling have been found in a wide variety of cancers, including endometrial cancer (Nei et al., 1999). The hallmark feature of these disruptions is nuclear accumulation of β-catenin. These disruptions can be the result of mutations in the gene encoding β-catenin (*CTNNB1*) (Machin et al., 2002; Saegusa and Okayasu, 2001), or mutations in other genes in the pathway, such as *APC*, *FZD* and *GSK3B*. Most common are mutations in APC and β-catenin. In colon cancer, 80% of non-familiar cases have mutations in APC, and several hundred disease-associated mutations in APC have been described. Mutations in *CTNNB1* have been found in many cancers, most notably gastric cancer (up to 76%), melanoma (> 20%), liver cancer (70% in some studies), and ovarian cancer (up to 50%). In endometrial cancer, *CTNNB1* mutations have been described in up to 45% of tumors (Giles et al., 2003).

Increased activity of the Wnt pathway leads to alterations in expression of Wnt target genes. Wnt target genes include genes involved in cell cycle control, apoptosis, and tumor progression, and also growth and transcription factors. In humans, the first genes to be identified as Wnt targets were Myc in 1998 (He et al., 1998) and CyclinD1 in 1999 (Shtutman et al., 1999). Through the proteins encoded by these genes, cell cycle progression can be regulated. Expression of survivin, an anti-apoptotic factor, is down-regulated by Wnt activation (Zhang et al., 2001a). Wnt-regulated growth factors include VEGF (Zhang et al., 2001b) (which is an

important angiogenic factor both in normal and neoplastic tissue), while several transcription factors are either direct (ITF-2 (Kolligs et al., 2002)) or indirect (AF17 (Lin et al., 2001)) targets of active Wnt signaling.

Many Wnt targets are involved in tumor progression. Indications for involvement in tumor invasion first came from the observation that nuclear accumulation of β-catenin was distinct in invasive borders of colon carcinomas, indicating active Wnt signaling at sites of tumor invasion. Indeed, several matrix metalloproteinases (MMPs) including MMP7 (Crawford et al., 1999) are Wnt target genes, as well as cell adhesion modulators such as CD44 (Wielenga et al., 1999).

An up-to-date overview of Wnt target genes is available at www.stanford.edu/~rnusse/wntwindow.html.

1.3.3. Wnt in endometrial cancer

Reports of mutations in the *CTNNB1* gene (Fukuchi et al., 1998; Ikeda et al., 2000b; Machin et al., 2002; Mirabelli-Primdahl et al., 1999) and nuclear localization of β-catenin (Nei et al., 1999; Saegusa and Okayasu, 2001) in endometrial cancer suggest a role for the Wnt pathway in endometrial pathogenesis.

Bui et al. (1997) studied expression of Wnt proteins in non-neoplastic endometrium and endometrial cancer (Bui et al., 1997). Wnt2, Wnt3, Wnt4, Wnt5a, Wnt7a, and Wnt7b proteins, but not Wnt10, were detected in non-neoplastic endometrium. Expression of Wnt proteins does not change throughout the menstrual cycle, which indicates that expression of Wnt proteins is independent of hormonal changes. In endometrial cancer, expression of Wnt4 was significantly less, and also expression of Wnt2, Wnt3, and Wnt5a was decreased. In four endometrial carcinoma cell lines, including Ishikawa, Wnt2 and Wnt3 mRNA was not detected, but Wnt7a and Wnt7b was present in three out of four cell lines (Bui et al., 1997).

In non-neoplastic endometrium, small amounts of β-catenin can be detected in the nucleus of epithelial cells during the proliferative phase of the menstrual cycle. In hyperplastic endometrium, nuclear staining for β-catenin was found to be often very intense, but in endometrial cancer samples the intense staining was observed in only one-third of the tumors investigated (Nei et al., 1999). Searching for mutations in *CTNNB1*, Fukuchi et al. (1998) found mutations in 13% of endometrial cancers (all in exon 3), and 90% of those tumors showed nuclear accumulation of β-catenin (Fukuchi et al., 1998). Moreno-Bueno et al. (2002) found that within tumors with nuclear β-catenin staining, the homogeneity of this staining greatly varied, with 5% to 75% of neoplastic cells staining positive in different cases (Moreno-Bueno et al., 2002).

Nuclear accumulation of β-catenin has been found in endometrial cancer without mutations in *CTNNB1*, suggesting that mutations in other components of the Wnt-pathway or alterations in so-far unrelated factors are also capable of stimulating transcription activation through β-catenin. Mutations in other components have been

found, but non of these were associated with nuclear accumulation of beta catenin (Moreno-Bueno et al., 2002; Schlosshauer et al., 2000).

Endometrial tumors with nuclear β-catenin localization are generally well-differentiated and express ER and PR. Scholten et al. (2003) concluded that nuclear localization of β-catenin is a feature of 'estrogen-related, low-grade endometrioid carcinomas', and they suggest that Wnt-signaling is involved in the genesis of these tumors (Scholten et al., 2003).

1.4. Tumor invasion and metastasis

The ability to invade into surrounding tissue is the most important distinction between benign and malignant tumors. Benign lesions grow expansively, have sharp borders, and therefore do not truly qualify as cancer. Malignant tumors grow invasively into the surrounding tissue and even into blood vessels, which enables them to metastasize to distant sites in the body, forming secondary tumors. The resulting metastases are the leading cause of death in cancer patients. The process of dissemination is a complex one, in which many factors play a role. In short, cells have to detach from the primary tumor, either singularly or in bulk, travel through the body to secondary sites (often through the bloodstream or the lymphatic system), and at those sites proliferate into secondary lesions. For the scope of this thesis, invasion into and migration through the extracellular matrix (ECM), the first steps in metastasis, will be discussed in some detail. Modulating any of the factors that are involved in these processes can and will affect the invading and disseminating properties of a tumor.

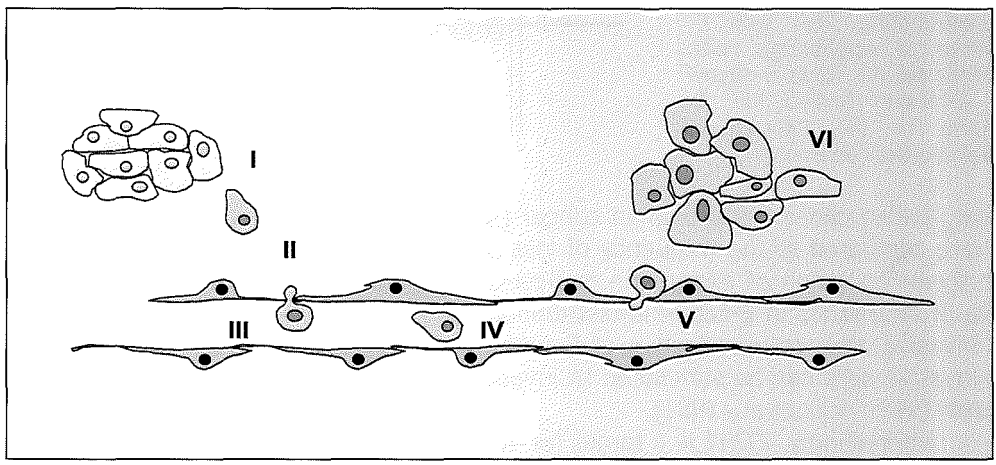

Figure 6. Schematic representation of metastasis. For tumor cells to metastasize to distant parts of the body, they have to detach from the primary tumor (I), migrate through the extracellular matrix (II), intravasate (III) and survive in the circulation (IV). After extravasation (V), the tumor cells will have to proliferate again to form a secondary tumor (VI).

1.4.1. Altered cell-cell interactions

To start invasion into surrounding tissue, tumor cells have to modulate their adhesive properties and interact with the ECM (Mareel and Leroy, 2003). First of all, cell-cell adhesion is altered. Homotypic cell-cell adhesion, i.e., adhesion between cells of the same type, is important for tissue integrity. The major homotypic cell-cell adhesion molecule is E-cadherin, which is present in adherens junctions (reviewed by Beavon, 2000). The extracellular part of this trans-membrane molecule interacts with the extracellular part of E-cadherins of neighboring cells in a Ca^{2+}-dependent way. This adhesion is stabilized by catenins, which link the intracellular part of E-cadherin to the actin cytoskeleton (see Figure 7). Decreased expression or altered distribution of E-cadherin is a common feature of invasive epithelial tumors.

The gene for E-cadherin, *CDH1*, was one of the first genes thought to be an invasion-suppressor gene. With ß-catenin being one of the key components in Wnt signaling, the importance of this cell-cell adhesion complex in inhibiting tumor progression is obvious. Mutations in *CDH1* have been reported in endometrial

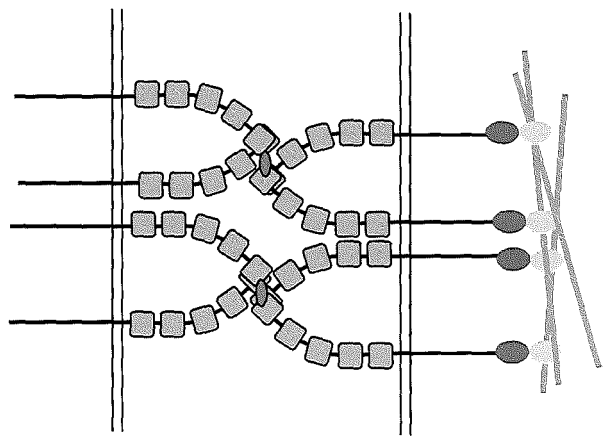

Figure 7. Mechanism of action of E-cadherin. The most N-terminal of the five Ca^{2+}- binding extracellular subdomains of E-cadherin (squares) can, upon binding of Ca^{2+} (small ellips), form trans-interacting sites with E-cadherins of neighboring cells. Inside the cell (far right), ß-catenin and α-catenin (ovals) link E-cadherin to the cytoskeleton, stabilizing cell-cell adhesion.

cancer, breast cancer, and ovarian cancer (Berx et al., 1998), but these mutations are rare compared to the frequency of loss of function of E-cadherin. This suggests that down-regulation of expression or post-translational modifications are the main cause of alterations in E-cadherin distribution. *In vitro*, invasive tumor cells become non-invasive after re-introduction of E-cadherin through transfection. Alternatively, non-invasive cells can be stimulated to invade by inhibiting E-cadherin expression or function (Vleminckx et al., 1991).

Interestingly, *CDH1* is a target for Wnt signaling: expression of E-cadherin is down-regulated by an active Wnt pathway (Jamora et al., 2003). It is appealing to think that constitutively active Wnt signaling could compete with E-cadherin for ß-catenin, implicating that over-active Wnt signaling would inhibit cell-cell adhesion by sequestering ß-catenin, but this does not appear to be necessarily the case. For

instance, Wnt-1 overexpression induced an increase in ß-catenin expression, which subsequently increased stabilization of E-cadherin-mediated cell-cell adhesion in mammalian cells (Hinck et al., 1994). So, Wnt and E-cadherin pathways do converge to some extent, indicating that destabilization of the cadherin-catenin complex and subsequent release of ß-catenin into the cytoplasm could be an alternative way to activate ß-catenin signaling. However, an exact linking mechanism remains to be resolved (Nelson and Nusse, 2004).

While homotypic cell-cell adhesions help to keep a tissue intact, heterotypic cell-cell adhesion can actually stimulate tumor metastasis (Mareel and Leroy, 2003). For example, adhesion of tumor cells to vascular endothelium is needed for both intravasation (entry of tumor cells into the bloodstream) and extravasation ('stepping out' of the bloodstream at secondary sites) of tumor cells, both of which are necessary for blood-borne metastasis.

1.4.2. Invasion into the extracellular matrix

A second early step in tumor cell invasion is alteration of the extracellular matrix (ECM). Non-neoplastic cells depend on interactions with the extracellular matrix for survival. Loss of 'correct' interactions will initiate intracellular signaling sequences that result in apoptosis of the isolated cell (Mareel and Leroy, 2003).

Tumor cells exhibit altered interactions with the ECM, which are pleiotropic and dynamic. Tumor cells secrete proteolytic enzymes that break down components of the ECM, while simultaneously components are deposited into it, resulting in an environment that is more favorable for further growth of the tumor. Well-known matrix disintegrating enzymes are the matrix metalloproteinases (MMPs), which comprise a family of over 20 members in humans, and the expression of which is increased in almost all tumors (Curran and Murray, 1999). In addition to disintegrating basement membrane and ECM components, MMPs can also activate growth factors and promote angiogenesis (Egeblad and Werb, 2002). In addition, many tumor cells secrete other angiogenic factors, such as vascular endothelial growth factor (VEGF), thus stimulating formation of new blood vessels which will provide more nutrients for the growing tumor (Bergers and Benjamin, 2003).

In tumors, expression of cell-matrix interaction molecules on the plasma membrane is generally altered. This enables tumor cells to further modulate their interactions with the ECM. Possibly the most investigated family of cell-matrix interaction molecules are the integrins. This family of transmembrane molecules consists of a collection of α and ß subunits, which co-operate to modulate interactions of cells with components of the ECM, such as fibronectin and collagen (Huttenlocher et al., 1995; Ruoslahti and Giancotti, 1989). Adhesion to matrix components through integrins initiates intracellular signal transduction pathways (Giancotti and Ruoslahti, 1999; Jones and Walker, 1999). Integrin-matrix interactions and subsequent signaling are important in development, apoptosis and many other physiological processes (Danen and Sonnenberg, 2003). As a consequence,

alterations in either matrix composition or integrin expression will alter cell behavior, which can lead to inappropriate cell survival.

Altered adhesive properties of tumor cells to each other and to the ECM results in alternate adhesion and de-adhesion of the tumor cell to its surroundings. Added to the proteolysis of ECM components, this has the effect that tumor cells will invade into, and migrate through, the surrounding tissue.

1.4.3. Invasion and metastasis in endometrial cancer

As with most tumors, endometrial cancer spread can occur through local invasive growth, or through blood- and lymph- borne metastasis.

Through local invasive growth, tumors can spread from the body of the uterus to the cervix and the vagina. When a tumor manages to grow through the myometrium and breach the uterine serosa (the outer lining covering the uterus), direct spread into and through the peritoneal cavity can occur. Spread via the Fallopian tubes to the peritoneal cavity is possible, but this is a rare finding (Southcott, 2001).

Distant metastases, involving either the blood circulation or the lymphatic system, manifest mainly in the lung, but can also occur at other sites, including the bone, adrenals, breast, skin, liver, intestine and brain (Southcott, 2001). Lymphatic dissemination occurs through the pelvic, para-aortic and inguinal lymph nodes, and the presence of tumor cells in the lymph nodes at time of surgery is a strong predictor for recurrence (Feltmate et al., 1999).

A wide variety of growth factors, ECM components, and ECM modulators have been implied in development of an invasive and metastatic phenotype in endometrial cancer. The angiogenic factor VEGF (vascular endothelial growth factor) has been suggested to play a role in progression of endometrial cancer (Seki et al., 2000), and transforming growth factor α (TGFα) and epidermal growth factor (EGF) have been associated with increased tumor cell invasion (possibly through regulation of expression of proteolytic enzymes by these growth factors) (Ueda et al., 1999). In many endometrial tumors, expression of MMPs is up-regulated. MMP8 and MMP9 were found to be secreted by infiltrating immune cells, while expression levels of MMP7 in the cancer cells were associated with clinicopathological features, with an almost 7-fold higher expression of MMP7 in patients with lymph node involvement (Ueno et al., 1999). Di Nezza et al. (2003) reported a correlation of expression of MMPs with increasing histological/pathological grade and invasion. Expression of MMPs in endometrial cancer can be inhibited by progestagens (Di Nezza et al., 2003). Urokinase plasminogen activator (uPA) and PAI-1 (plasminogen activator inhibtor 1), which are also involved in tissue remodeling and invasion-associated ECM changes, have been proposed as independent markers for endometrial cancer invasion (Taponeco et al., 2001). Over-expression of the cell-matrix interacting molecule CD44 in endometrial cancer is associated with increased lymph node involvement (Leblanc et al., 2001), and is therefore associated with recurrence.

1.5. Scope of this thesis

Many women may acquire endometrial cancer during their life. The vast majority of these women will be cured because of early detection of the disease. As in most types of cancer however, the main cause of death lies in metastasis of the primary tumor to other sites in the body. In approximately 25% of endometrial cancer patients, the tumor has spread beyond the uterus at the time of initial surgical treatment.

During endometrial carcinogenesis, the balance between estrogen and progesterone is of great importance. This is indicated by the fact that virtually all risk factors for endometrial cancer are linked to a surplus of estrogenic effects that are not balanced by appropriate progestagenic effects. Also during further development and progression of endometrial cancer, steroid receptor signaling has many important effects.

The major research question of this thesis is summarized as follows: what is the effect of loss of progesterone regulation due to loss of PR expression during development of endometrial cancer? More specifically, the questions that this thesis addresses are:

1) Is loss of PR expression in endometrial cancer linked to development of endometrial cancer to a more advanced stage?
2) What are the effects of changes in expression of PRA and PRB on invasion and metastasis of endometrial cancer?
3) What is the effect of progesterone on invasion and metastasis of endometrial cancer cells that express different PR isotypes?
4) Does any crosstalk exist between PR signaling and Wnt signaling?

In Chapter 2, regulation of gene expression through PRA and PRB together was studied, in order to give a reasonable expectation of the effect of loss of progesterone regulation through loss of PR expression in endometrial cancer. The results of these investigations were translated to the clinic by means of evaluation of clinical samples.

In Chapter 3, a cell line model is applied to study the differences in *in vitro* invasive capacity between endometrial cancer cells that express different isotypes of the progesterone receptors. Also, the effect of progestagens on in vitro tumor cell invasion is studied.

In Chapter 4, a mouse model is presented. This model is applied to study effects of progestagens and the different PR isotypes on metastatic capacity of endometrial cancer cells *in vivo*.

Chapter 5 gives several insights in how progestagens can affect one of the major pathways in development and carcinogenesis, the Wnt-pathway.

In Chapter 6, the Chapters 2 through 5 are discussed and placed in perspective of related research. The research questions as presented above are addressed, and suggestions for follow-up research are given.

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CHAPTER 2

CONSEQUENCES OF LOSS OF PROGESTERONE RECEPTOR EXPRESSION IN DEVELOPMENT OF INVASIVE ENDOMETRIAL CANCER

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Consequences of loss of progesterone receptor expression in development of invasive endometrial cancer

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ABSTRACT

In endometrial cancer, loss of progesterone receptors (PR) is associated with more advanced disease. This study aimed to investigate the mechanism of action of progesterone and the loss of its receptors (PRA and PRB) in development of endometrial cancer.

A 9600-cDNA microarray analysis was performed to study regulation of gene expression in the human endometrial cancer sub-cell line Ishikawa PRAB-36 by the progestagen Medroxyprogesterone Acetate (MPA). Five MPA-regulated genes were selected for further investigation. Expression of these genes was studied by Northern blot and by immunohistochemistry in Ishikawa sub-cell lines expressing different PR isoforms. Additionally, endometrial cancer tissue samples were immunohistochemically stained to study the *in vivo* protein expression of the selected genes.

In the PRAB-36 cell line, MPA was found to regulate the expression of a number of invasion- and metastasis-related genes. Upon further investigation of five of these genes (CD44, CSPG/versican, tenascin-C, fibronectin-1, integrin-β1), it was observed that expression and progesterone regulation of expression of these genes varied in sub-cell lines expressing different PR isoforms. Furthermore, in advanced endometrial cancer, it was shown that loss of expression of both PR and E-cadherin was associated with increased expression CD44 and CSPG/versican .

The present study shows that progestagens exert a modulatory effect on the expression of genes involved in tumor cell invasion. As a consequence, loss of PR expression in human endometrial cancer may lead to development of a more invasive phenotype of the respective tumor.

INTRODUCTION

Endometrial cancer is one of the most common gynecological malignancies in Europe and the United States of America. As many as 70% of the patients can be cured (Southcott, 2001), mostly by surgery (hysterectomy with additional salpingo-oophorectomy), often in combination with radiotherapy. The remaining patients have already developed distant metastases or will develop recurrent disease. For palliative treatment of these patients, and for treatment of premenopauzal patients who want to preserve fertility, high-dose progesterone is prescribed because of the growth-inhibiting effects of this hormone on the endometrium (Koopman et al., 1999; Saegusa and Okayasu, 1998; Southcott, 2001). In 80-90% of patients with more progressed disease, however, this therapy is not beneficial, because the tumor has become unresponsive to progesterone.

Progesterone can act on the endometrium through activation of progesterone receptor A and B (PRA and PRB), which belongs to the family of nuclear receptors. Transcription of the human PR (hPR) gene is under regulation of two different promoters (Kastner et al., 1990). Transcription initiation from these two

promoters results in two distinct mRNAs, which are translated into two distinct proteins: hPRA and hPRB. The hPRA is a truncated form of hPRB, lacking the first 164 amino acid residues at the amino terminus. The hPRA and hPRB can be considered as two independent receptors, which display different transcriptional activities. Vegeto et al. (Vegeto et al., 1993) have described that hPRA is not as transcriptionally active as is hPRB, and that hPRA may have a more important function as a cell- and promoter-specific repressor of hPRB (Giangrande et al., 2000). Furthermore, it has been described that a different set of genes is regulated by progesterone in human breast (Richer et al., 2002) and endometrial (Smid-Koopman, 2003) cancer cells that express different PR isoforms.

Several functional domains can be recognized in hPRA and hPRB. These PR isoforms have in common two transcription activation functions, AF-1 and AF-2, and one inhibitory domain. In hPRB, a third activation function (AF-3) has been defined in the N-terminal amino acid stretch. According to Giangrande et al. (1997) this hPRB-specific AF-3 domain suppresses the activity of the inhibitory domain (Giangrande et al., 1997). While the function of the inhibitory domain in hPRB is neutralized by AF-3, in hPRA the inhibitory domain is still functional in inhibiting transcriptional activity. In support of this view, Giangrande et al. (2000) have shown that in contrast to hPRB, hPRA was unable to recruit co-activators (Giangrande et al., 2000). In addition to the functional difference of both isoforms, hPRA and hPRB are reported to be located in different cellular compartments in absence of ligand. Lim et al. (1999) found that unliganded GFP-labeled hPRA is localized predominantly in the nucleus, while unliganded GFP-labeled hPRB is mainly present in the cytoplasm. Upon activation by ligand, both receptors are exclusively located in the nucleus (Lim et al., 1999).

In general, literature agrees on a loss of PR in endometrial cancer (Arnett-Mansfield et al., 2001; Fujimoto et al., 1997; Fujimoto et al., 1995; Fujimoto et al., 2000; Fukuda et al., 1998; Kumar et al., 1998; Mote et al., 2000; Moutsatsou and Sekeris, 1997; Sakamoto et al., 1999), but reports are conflicting whether this is a consequence of selective down-regulation of hPRA or hPRB, or of both receptors. Fujimoto et al. (1995) reported that in advanced tumors, hPRA could not be detected (Fujimoto et al., 1995). In accordance with this, the same group later described that in distant metastases of endometrial cancer, it is hPRB that is predominantly expressed (Fujimoto et al., 1997). Arnett-Mansfield et al. (2001) found a reduced expression of either one or both of the two PR isoforms in the majority of endometrial tumors, compared to hyperplastic or normal endometrial tissue (Arnett-Mansfield et al., 2001). In addition, hypermethylation of PRB alleles (Sasaki et al., 2001) and expression of exon-deleted PR mRNA (Misao et al., 2000) have been described in endometrial cancer tissue.

To make it possible to differentiate between the effects of the two isoforms of the PR, we previously transfected the well-differentiated human endometrial cancer cell line Ishikawa to stably express either hPRA or hPRB, or both isoforms of the receptor. Making use of this model, we studied differences in expression of metastasis-related genes in the different Ishikawa sub-cell lines. Using endometrial

cancer tissue samples we studied the expression of these genes in relation to the loss of progesterone receptors *in vivo*.

METHODS

Cell culture

Ishikawa endometrial cancer cells (clone 3H12, further referred to as IKpar) were obtained from dr. Masato Nishida (Tsukuba, Japan) and maintained in DMEM/F12 (GibcoBRL/ LifeTechnologies, Carlsbad, CA, USA), supplemented with 10% v/v fetal calf serum (FCS; GibcoBRL) and penicillin/streptomycin. The sub-cell lines which are used are IKpar cells that have been stably transfected with either hPRA (line PRA-1), hPRB (line PRB-1) or both receptors (line PRAB-36), as previously described by Smid-Koopman et al. (Smid-Koopman 2002, submitted). These sub-cell lines were cultured under similar conditions and in similar media as the IKpar cells, but with the addition of neomycin (500 μg/ml, ICN) and hygromycin (250 μg/ml, Invitrogen Corporation, Carlsbad, CA, USA) to maintain selection pressure for the transfected human progesterone receptors. This selection pressure was maintained throughout the experiments. Prior to all experiments, cells were cultured for 72 hours in medium where FCS was substituted by 5% v/v dextran-coated-charcoal-treated fetal calf serum (DCC-FCS). Culture passages 15-25 were used in the experiments.

cDNA microarray

PRAB-36 cells were cultured for 48 hours in DMEM/F12 supplemented with 5% v/v DCC-FCS, either in the presence or absence of progestagen, i.e. 100 nM Medroxyprogesterone Acetate (MPA; Sigma, St. Louis, MO, USA). RNA was isolated according to Auffray and Rougeon (Auffray and Rougeon, 1980), and shipped to Incyte Genomics, Inc. (St Louis, MO, USA), where a 9600 cDNA microarray analysis (Human UniGene1) was performed. Results were analyzed using Incyte GEMTools 2.5.0.

Northern blot

Cells were cultured as described in the presence of 100 nM MPA for 0, 8, 24, 48, or 72 hours. RNA was isolated as described above, separated on a 1.5% w/v agarose gel and blotted onto Hybond membrane (Amersham Biosciences Europe, Roosendaal, The Netherlands). The following ^{32}P-dATP-labeled cDNA probes were hybridized to the blots: CD44: image713145, 549bp; fibronectin-1: image139009, 383bp (both acquired from Dr. G. Jenster, Dept. of Urology, Erasmus MC, Rotterdam, The Netherlands); integrin-ß1: image PRB-118212, 136bp; tenascin-C: image P107816, 437bp; chondroitin sulfate proteoglycan/versican (CSPG/versican): image N038145, 470bp (all acquired from RZPD, Berlin, Germany). In order to verify equal loading, an actin probe (an 1100 bp Pst1 fragment from hamster ß-actin cDNA) was hybridized to the blots.

Collagen gel culture

To study protein expression in the different sub-cell lines, cells were seeded and cultured in collagen gels. Rat tail tendon collagen (final concentration in the gel: 3.0 mg/ml; collagen kindly provided by Dr. H. Nederbragt, Dept. of Veterinary Pathology, Utrecht University, The Netherlands) was mixed with 5x concentrated culture medium (DMEM/F12, supplemented with 5% DCC-FCS) containing 19% v/v 0.11 M NaOH. Mixing was performed on ice to prevent premature polymerization of the gel. To the mixture, cells were added (final concentration in the gel: $2x10^6$ cells/ml), and resuspended to ensure equal distribution of the cells throughout the gel. The collagen/cell mixture was poured in 24-wells plates and the gels were allowed to polymerize in a 37°C incubator, before culture medium was added. Cells were cultured in DCC-FCS-supplemented medium for 72 hours in presence or absence of 100 nM MPA. The gels were fixed in formalin and embedded in paraffin; this to mimic fixation and embedding procedures used in preservation of tissue samples.

Tissue samples

Tissue samples involved formalin-fixed, paraffin-embedded samples of endometrioid adenocarcinoma, obtained from the Department of Pathology from the Erasmus MC, Rotterdam, The Netherlands. Histological typing and grading (according to the modified FIGO staging system (Creasman et al., 2001)), and extent of myometrial invasion, were assessed by the Erasmus MC Department of Pathology.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded tissue samples and of collagen gels containing IKpar, PRA-1, PRB-1 or PRAB-36 cells were deparaffinized and endogenous peroxidase activity was inhibited by treatment with 4% v/v H_2O_2 in methanol for 20 minutes. Antigen retrieval was performed in a microwave oven in 10 mM citric acid buffer, pH 6.0, for 3 x 5 minutes. Slides were allowed to cool to room temperature and washed three times in PBS before being incubated for 20 minutes in PBS containing 10% v/v blocking serum. Normal goat serum (DAKO, Glostrup, Denmark) was used as blocking serum for the mouse monoclonal antibodies, and normal rabbit serum (DAKO, Glostrup, Denmark) was used as blocking serum for the goat polyclonal antibody.

The following antibodies were used: anti-PRAB: hPRa8; anti-PRB: hPRa2 (both acquired from NeoMarkers, Fremont, CA, USA); anti-E-cadherin: G10/sc-8426; anti-CD44: DF1485/sc-7297; anti-integrin-ß1: 4B7R/sc-9970; anti-tenascin-C: N-19/sc-9871 (all acquired from SantaCruz Biotechnology, Santa Cruz, CA, USA); anti-CSPG: CS-56 (Sigma, St. Louis, MO, USA), anti-fibronectin-1: FBN11 (NeoMarkers, Fremont, CA, USA). The primary antibodies were diluted 1:200 in PBS containing 1% v/v blocking serum for tissue slides, and 1:1000 (PRAB antibody) or 1:500 (other) in PBS containing 1% v/v blocking serum for the slides of collagen gels containing the sub-cell lines. Incubation with primary antibody occurred overnight at 4°C. Antibodies were detected indirectly with biotin-labeled goat-anti-mouse or goat-anti-

rabbit antibodies (DAKO, Glostrup, Denmark) 1:400 in PBS containing 1% v/v blocking serum for 30 minutes at room temperature, followed by StreptABComplex (DAKO, Glostrup, Denmark) 1:1:200 in PBS (30 minutes at room temperature). Staining was developed with DAB/concentrated metal complex (Pierce, Rockford, IL, USA). Slides were counterstained with hematoxilin, dehydrated and mounted.

Expression of the proteins was approached semi-quantitatively by visual estimation. Four categories of expression were used: ++: > 80% positive; +: 60-80% positive; +/-: 30-60% positive; -: < 30% positive.

Western blot

PRAB-36 cells were cultured for 0, 8, 24, 48 or 72 hours in presence of 100 nM MPA. Cells were washed twice with PBS, after which the culture flasks were placed at -80° C overnight. Cells were lyzed with RIPA buffer (40 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10% glycerol, 10 mM sodiumphosphate, 10 mM sodiummolybdate, 50 mM NaF, 0.5 mM sodium orthovanadate), containing 10 mM DTT, 1% Triton X-100, 0.08% SDS, 0.5% DOC and 1x complete protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The lysates were centrifuged at 1000 x g for 40 minutes, after which the pellet was discarded.

Equal protein samples were separated on 7% polyacrylamide gels, and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 hour in blocking buffer (PBS/Tween containing 5% non-fat milk powder), and incubated overnight at 4° C with a mouse monoclonal antibody recognizing fibronectin-1 (clone EP5, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:1000 in blocking buffer. The blots were then washed 4 x 10 minutes in PBS/Tween, after which they were incubated with peroxidase-labeled secondary goat-anti-mouse antibody (DAKO, Glostrup, Denmark), diluted 1:1000 in blocking buffer, for 1 hour at room temperature. The blots were washed 6 x 10 minutes in PBS/Tween, before protein bands were visualized using Western Lightning Chemiluminescence reagent (PerkinElmer Life Sciences Inc., Boston, MA, USA), according to the manufacturers manual.

TUMOR PROGRESSION, TISSUE REMODELING, METASTASIS					
-3.2	fibronectin 1	ECM, cell-matrix interactions	3.7	collagen type IV, alpha3	cell-matrix interactions
			3.5	epithelial V-like antigen	placental morphogenesis
-2.4	CSPG2/versican	cell-matrix interactions	3.3	leukocyte cell adhesion molecule	tumor leukocyte recruitment
-2.0	tenascin C (hexabrachion)	tissue remodeling	2.4	antileukoproteinase	placental development
-2.0	B-cell CLL/ lymphoma 1	leukemia	2.3	BTG family 2	cell cycle, differentiation
-1.9	uPA	matrix degradation	2.1	integrinß1	cell-matrix interactions
-1.7	CD44 antigen	cell-matrix interactions			
-1.7	cyclin D1	cell cycle			
-1.7	CYR61	angiogenesis			
-1.7	HGF binding protein	tumor progression/angiogenesis			
-1.6	v-jun	oncogenesis			
-1.6	tissue factor inhibitor 2	vascular remodeling, apoptosis			
-1.6	claudin 1	tight junction, recruiting MMPs			
ION HOMEOSTASIS					
-2.2	salivary proline-rich protein	Ca-homeostasis in saliva	3.0	ferritin, heavy polypeptide 1	iron metabolism
			2.5	AHNAK nucleoprotein	Ca- homeostasis
-1.9	annexin A4	ion channeling	2.4	(desmoyokin)	organic cation transporter
-1.8	solute carrier family 7	organic solute transporter		solute carrier family 22, member 5	

SIGNAL TRANSDUCTION					
-2.7	serum-inducible kinase	serine/threonine protein kinase	3.5	fatty acid-CoA ligase, longchain 2	fatty acid synthesis
-1.8	aldehyde dehydrogenase, A1	drug resistance, proliferation	3.3	methionine adenosyltransferase	s-adenosylmethionine synthesis
-1.6	UDP-glucose ceramide glucosyltransferase	glucosphingolipid synthesis	3.1	FK506-binding protein 5	cell signaling, transcription
			2.7	protein phosphatase 2, cat. subunit ß	protein phosphorylation
-1.6	G protein-coupled rec. kinase 5	β-adrenergic receptor signaling	2.7	serum/glucocorticoid regulated kinase	serine/threonine protein kinase
			2.6	protein phosphatase 1, cat. subunit ß	serine/threonine phosphatase
			2.3	signal recognition particle 54kD	protein targeting to membranes
VARIOUS					
-4.2	IGFBP3	IGF 1 transporter	3.3	IgM	primary antibody response
			2.7	IL-1 receptor, type I	interleukin signaling
			2.2	IFN-γ receptor 1	tumor-immune responses
			2.2	lethal giant larvae homologue 2	cell polarity

Table 1. Progesterone-regulated genes in the PRAB-36 cell line. The Ishikawa sub-cell line PRAB-36 was cultured for 48 hours in presence or absence of 100 nM MPA. Total RNA was hybridized to a 9600 cDNA microarray. The 20 most strongly down-regulated genes (left half of the table), and 20 most strongly up-regulated genes (right half of the table) are listed and grouped by function. Functions of individual genes are summarized briefly. The numbers indicate fold induction (as calculated by the Incyte GEMTools 2.5.0. software), with negative numbers indicating down-regulation and positive numbers indicating up-regulation.

RESULTS

Progesterone-regulation of gene expression in PRAB-36 cells

To make a first selection of genes which are regulated by progesterone in endometrial cancer cells, a Human UniGene 1 microarray analysis (IncyteGenomics Inc, St. Louis, MO, USA) was performed. Using this array, approximately 90 genes were identified that were regulated by the progestagen Medroxyprogesterone Acetate (MPA) in the PRAB-36 sub-cell line (cut-off set at 1.5 fold up- or down-regulation). Table 1 summarizes the 20 most strongly up-regulated and 20 most strongly down-regulated genes, grouped by function. A large panel of these genes encodes for proteins involved in tumor progression, tissue remodeling, and/or metastasis. From this group we selected 5 genes which are involved in cell-matrix interactions, and as such in tumor cell invasion and/or metastasis, for further investigation. These genes are: CD44, CSPG (chondroitin sulfate proteoglycan)/ versican, tenascin-C, integrinβ1 and fibronectin-1.

Analysis of expression of CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1 in Ishikawa sub-cell lines expressing PRA and/or PRB

To confirm the microarray data, and to study expression of the selected genes in the Ishikawa sub-cell lines, analyses of mRNA and protein expression were performed. It was observed that there were large differences in expression of the indicated genes (CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1) between the different PR isoform-expressing sub-cell lines (Figures 1 and 2) in absence of MPA. Furthermore, as described in detail below, expression of the majority of these genes was regulated by MPA.

CD44 and CSPG/versican: The level of expression of both CD44 and CSPG/versican in absence of MPA is lower in the IKpar and PRA-1 sub-cell lines, compared to the PRB-1 and PRAB-36 sub-cell lines (Figure 1A and 1B; Figure 2C and 2D). CD44 and CSPG/versican show similar regulation patterns in the different sub-cell lines. Both genes are down-regulated by MPA in the PRB-1 and PRAB-36 sub-cell lines at the mRNA level (Figure 1A and 1B). At the protein level in PRB-1 and PRAB-36 sub-cell lines, deposition of CD44 and CSPG/versican in the extracellularmatrix is clearly reduced when the cells are cultured in presence of MPA (Figure 2C and 2D). For the PRA-1 sub-cell line, down-regulation by MPA is much less evident. In the IKpar line there is no evidence of MPA-induced regulation of either genes at the mRNA or protein level.

Tenascin-C: For tenascin-C, the Northern blotting data do not correlate well with the microarray data: the Northern blot analysis shows only a very low expression and a weak down-regulation of tenascin-C in the PRAB-36 sub-cell line (Figure 1C). Expression of tenascin-C in absence of MPA is also very low in the PRB-1 cells, and regulation of expression by MPA is not detectable (Figure 1C). However, at the protein level, expression of tenascin-C is high, and is down-regulated by MPA in the PRB-1 and PRAB-36 sub-cell lines (Figure 2E). In the PRA-1 sub-cell line, the basal mRNA expression of tenascin-C is high, and down-regulation is observed, but

at the protein level, expression is low and not regulated by MPA. In the IKpar line, combined mRNA and protein data show no clear regulation (Figure 1C; Figure 2E).

Integrin-ß1: At the mRNA level, basal expression levels of integrin-ß1 expression are similar in all sub-cell lines. Expression of integrin-ß1 is up-regulated by MPA in the PR-expressing sub- cell lines on Northern Blot (Figure 1D). The apparent up-regulation of integrin-ß1 in the IKpar line is due to a difference in the amount of RNA loaded onto the gel (Figure 1F). At the protein level, integrin-ß1 is expressed at high levels in all sub-cell lines. There is no visible regulation of expression by MPA (Figure 2F), but this may be due to the high basal level of expression.

Fibronectin-1: At the mRNA-level, fibronectin-1 was expressed at high levels in the PRB-1 and PRAB-36 lines, and was clearly down-regulated by MPA. In the PRA-1 line, low expression but no regulation was observed. In the IKpar line, expression of fibronectin-1 was detected, but this was not regulated by MPA (Figure 1E). At the protein level, MPA-induced down-regulation of fibronectin-1 could be observed only in the PRAB-36 sub-cell line (Figure 2G). This observation was confirmed by Western blot (Figure 2H).

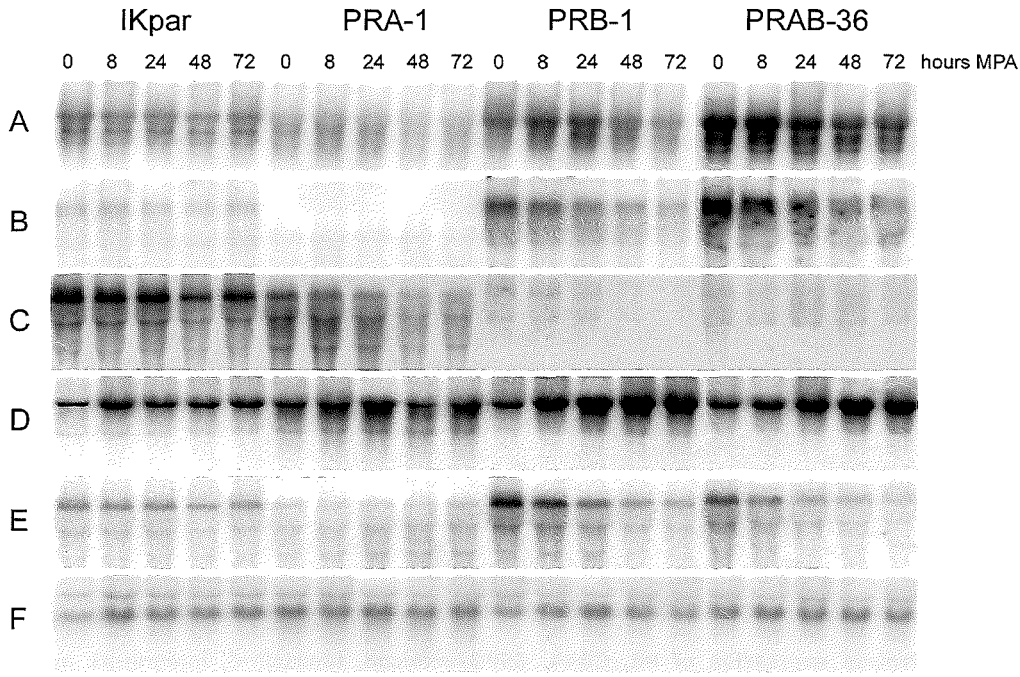

Figure 1. Regulation of expression of CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1 mRNA in the IKpar and PRA and/or PRB-expressing sub-cell lines. IKpar, PRA-1, PRB-1 and PRAB-36 cells were cultured in the presence of 100 nM MPA for 0, 8, 24, 48 and 72 hours. Total RNA was isolated, separated on agarose gel, blotted and hybridized to ^{32}P-labeled cDNA-probes from: CD44 (A), CSPG/versican (B), tenascin-C (C), integrin-β1 (D) and fibronectin-1 (E). Hybridization to a ^{32}P-labeled actin probe (F) was used to verify equal loading in the different lanes of the gel.

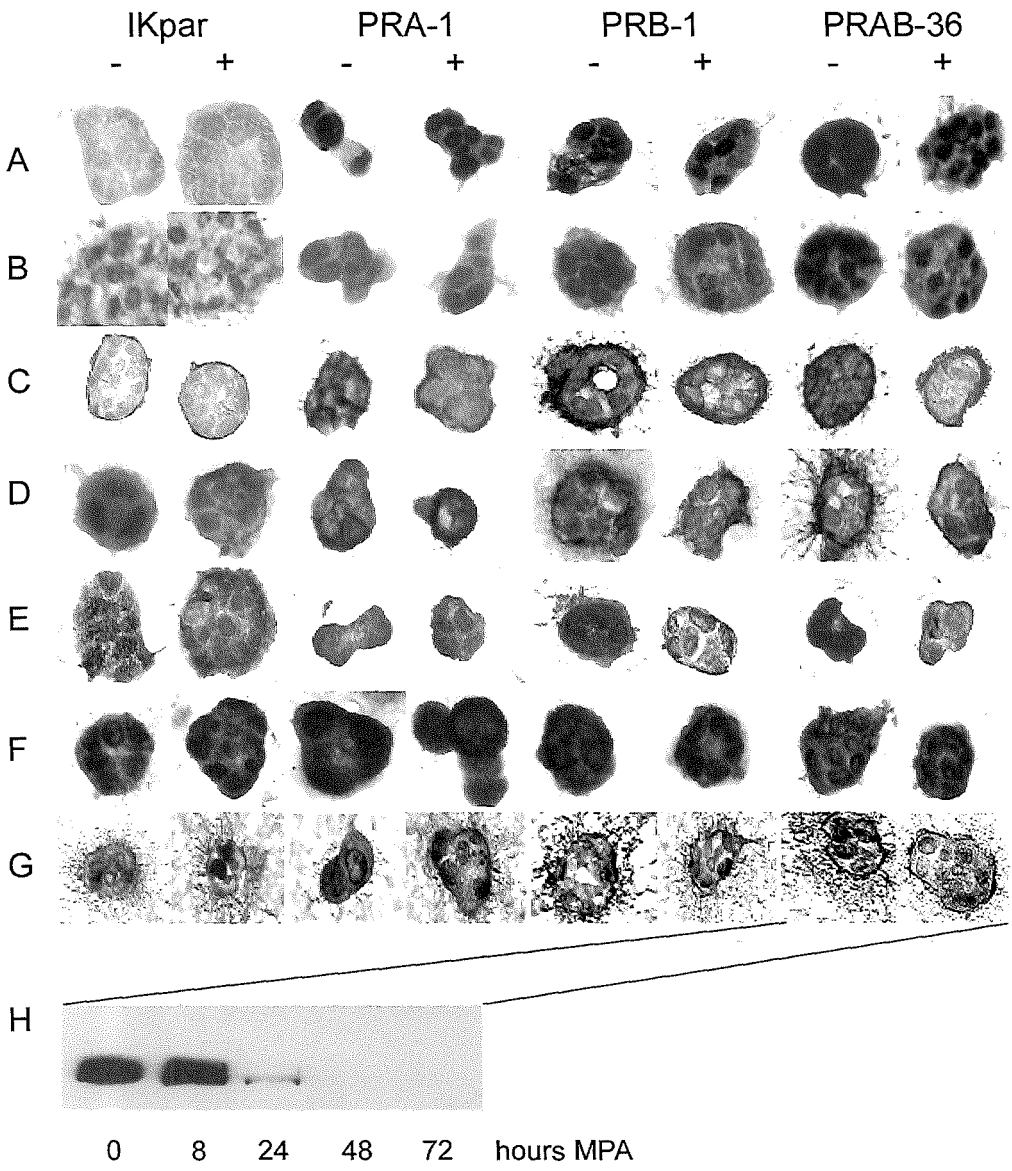

Figure 2. Protein expression of PR, CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1 in the IKpar and PRA- and/or PRB-expressing sub-cell lines. Ishikawa sub-cell lines IKpar, PRA-1, PRB-1 and PRAB-36 were cultured in collagen gels, in absence (-) or presence (+) of 100 nM MPA, for 72 hours. Sections of the formalin-fixed, paraffin-embedded gels were immunohistochemically stained for: hPRA + hPRB (A), hPRB (B), CD44 (C), CSPG/versican (D), tenascin-C (E), integrin-β1 (F) and fibronectin-1 (G). Each panel shows one cluster of cells in collagen gel. Original magnification 400x. Panel H shows Western blot analysis of expression of fibronectin-1 after 0, 8, 24, 48 and 72 hours of culturing in presence of 100 nM MPA.

Expression of CD44, CSPG/versican, tenascin-C, integrinβ1 and fibronectin-1 in endometrial cancer tissue samples.

To study expression of the selected genes in endometrial cancer tissue, consecutive sections of formalin-fixed, paraffin-embedded samples of endometrioid adenocarcinoma were immunohistochemically stained for hPRA + hPRB, CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1. Because loss of expression of E-cadherin in epithelial tumors strongly correlates with increased tumor cell invasion and metastasis (Beavon, 2000) and is also associated with more aggressive endometrial cancer (Leblanc et al., 2001), this marker was studied as an indicator for potential risk for inavsion and metastasis. Staining for E-cadherin in regions with decreased PR expression was less intense, and was also more evenly distributed throughout the whole cell instead of being located at the cell surface (Table 2, Figure 3D). This indicates that during loss of progesterone responsiveness in endometrial cancer, expression of functional E-cadherin is also lost.

A representative panel of stainings in consecutive sections of a well-differentiated, stage Ia tumor (panel I) and an undifferentiated, stage Ic tumor (panel II) is presented in Figure 3. Results of all stainings are summarized in Table 2. If within one section, regions were present in which the PR was differently expressed, the expression of E-cadherin, CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1 is indicated for these separate regions. It was observed that the expression of PR in these endometrial tumors decreased with dedifferentiation. When PR staining was compared with CD44 staining, a strong inverse correlation was observed: in regions where PR expression was low, expression of CD44 was clearly increased (Figure 3B; Table 2). Similar observations were made for CSPG/versican expression (Figure 3C; Table 2). For tenascin-C, integrin-ß1 and fibronectin-1 immunostaining, there was no clear correlation with PR expression (Table 2).

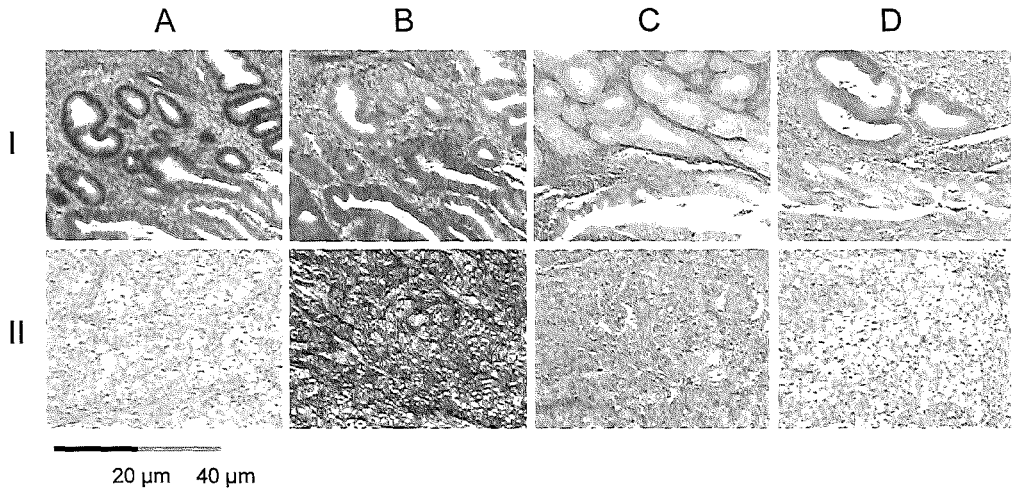

Figure 3. Expression of PR, CD44, CSPG/versican, and E-cadherin in endometrial cancer tissue samples. Sections of formalin-fixed, paraffin-embedded samples of endometrioid adenocarcinoma were immunohistochemically stained for hPRA + hPRB (A), CD44 (B), CSPG/versican (C), E-cadherin (D). A representative set of stainings in semi-consecutive* slides of a well-differentiated (G1, stage Ia) tumor sample (panel I) and an undifferentiated (G3, stage Ic) tumor sample (panel II) is shown. Histopathological typing, grade and stage were assessed by the Erasmus MC Department of Pathology (according to the modified FIGO staging system. See also www.FIGO.org/content/PDF/corpus-uteri_p47-49.pdf). Original magnification 200x.
* Because consecutive slides were stained to detect PRAB, CD44, CSPG/versican, tenascin-C, integrin-ß1, fibronectin-1 and E-cadherin (in this order), the section which was stained for E-cadherin is not directly consecutive.

DISCUSSION

In endometrial cancer, loss of PR-expression is associated with late-stage disease in which the tumor no longer responds to progesterone treatment. In this study, we connect *in vitro* regulation by progesterone of a selected number of genes which are potentially involved in invasion and metastasis, with *in vivo* changes in expression of corresponding proteins during endometrial cancer progression.
Metastasis is a multi-step process, starting with tumor cell invasion. This involves detachment of cells from the primary tumor, and migration of tumor cells through the extracellular matrix (Woodhouse et al., 1997). During invasion of tumor cells, loss of cell-cell adhesion, modulation of cell-matrix interactions and degradation/remodeling of the extracellular matrix are of great importance (Cavallaro and Christofori, 2001). Molecules that have a function in these processes are therefore candidate regulators of invasion and metastasis. When we analyzed which genes were regulated by MPA (which is the progestagen preferably used in the clinic) in the Ishikawa endometrial cancer sub-cell line PRAB-36, it was observed that a large portion of the identified progesterone-regulated genes encoded for

Grade	MI	PR	Ecad	CD44	CS	Ten	Int	Fibro
H	n.a.	++	++	-	-	++	++	+
H	n.a.	++	++	-	-	+	+	+/-
H	n.a.	+	+	-	-	+	++	+
1	0	+	+	+/-	-	+/-	+	+/-
1	<0.5	++	++	-	+	++	++	+
		+/-	+	+/-	+	++	+/-	-
1	<0.5	+	++	++	+/-	+	++	+
		++	+	+/-	+/-	+/-	+	++
1	≈0.5	+/-	+	+	+/-	+	+	+
		+/-	+	+/-	+/-	+	++	+/-
2	<0.5	+/-	+/-	+/-	+	+/-	+/-	+
		+/-	+/-	+/-	+	+	+/-	+
2	>0.5	++	++	-	-	++	+	+/-
2	>0.5	+/-	+	++	-	+/-	+	+
3	≈0.5	+/-	+/-	++	++	+	++	++
		-	-	++	+	+/-	+/-	++
3	≈0.5	-	-	+/-	+	+	+/-	+
3	>0.5	-	+	+	+	++	++	+/-
3	>0.5	-	-	++	+/-	+	++	+/-
3	>0.5	-	+/-	+	++	+	++	-

Table 2. Analysis of expression of PR, CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1 in endometrial cancer samples. Summary of immunohistochemical stainings on formalin-fixed, paraffin-embedded samples of hyperplastic endometrium and endometrioid adenocarcinoma (collected at Erasmus MC 2000-2001). H: hyperplasia, 1: G1 (well-differentiated), 2: G2 (moderately differentiated), 3: G3 (poorly or undifferentiated). MI: myometrial invasion, defined as less (<), more (>) or equal to (≈) half (0.5) of the thickness of the myometrium. Histological typing, grade and stage (according to the modified FIGO staging system, see also www.FIGO.org/content/PDF/corpus-uteri_p47-49.pdf) and extent of myometrial invasion were assessed by the Erasmus MC Department of Pathology. n.a.: not applicable. PR: hPRA + hPRB; Ecad: E-cadherin; CS: CSPG/versican; Ten: tenascin-C; Int: integrin-β1; Fibro: fibronectin-1. Staining was approximated semi-quantitatively at ++: > 80% positive; +: 60-80% positive; +/-: 30-60% positive; -: < 30% positive. If within one section, regions were present in which the PR was differently expressed, the expression of E-cadherin, CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1 is indicated for these separate regions.

proteins which are potentially involved in tumor progression and/or invasion/metastasis. For instance, the progesterone down-regulated genes urokinase plasminogen activator (uPA) and claudin-1 co-operate with matrix metalloproteinases in the process of extracellular matrix degradation (Miyamori et al., 2001; Rabbani and Xing, 1998). Another progesterone down-regulated gene, CYR61, promotes angiogenesis (Babic et al., 1998).

In this study, we focus on a panel of five progesterone-regulated genes that are implicated in cell-matrix interactions and could therefore play a role in tumor cell invasion. These genes are CD44, CSPG/versican, tenascin-C, integrin-β1 and

fibronectin-1. From the microarray data it became clear that CD44, CSPG/versican, tenascin-C and fibronectin-1 were down-regulated by MPA in the PRAB-36 sub-cell line, while integrin-ß1 was up-regulated. The functions of the proteins encoded by these genes are briefly discussed first.

CD44 is a cell surface molecule, which exists in many isoforms and is mainly involved in cell-matrix interaction (Underhill, 1992). CD44 can promote tumor growth and invasiveness (Herrlich et al., 2000), and over-expression of CD44 is associated with increased metastasis in endometrial cancers with increased myometrial invasion (Leblanc et al., 2001). CD44 has been implied in regulation of postpartum uterine involution through association with several matrix metalloproteinases (Yu et al., 2002).

CSPG/versican is a member of the family of proteoglycans, which interacts with CD44. CSPG/versican has been reported to mediate tumor cell invasion (Henke et al., 1996). Also, the CSPG-degrading chondroitinases AC and B inhibit invasion of melanoma cells (Denholm et al., 2001).

Tenascin-C plays a role in tissue remodeling during embryonic development, and is re-expressed in many different tumors (Hanamura et al., 1997; Jones and Jones, 2000a; Jones and Jones, 2000b). Tenascin-C can stimulate proliferation, but growth-inhibiting effects have also been reported (Jones and Jones, 2000a; Jones and Jones, 2000b). In breast cancer, tenascin-C expression correlates with increased invasion and is suggested as a prognostic factor for metastasis (Ishihara et al., 1995; Jahkola et al., 1996).

Fibronectin-1 is a major component of the basal lamina. It has functions in a variety of processes, such as proliferation, adhesion, and migration (Danen and Yamada, 2001; Romberger, 1997). Fibronectin-1 mediates cell-matrix adhesion by interacting with a wide variety of membrane and matrix components, such as the classic fibronectin-1 receptor integrin-α5ß1, collagen and CSPG (Romberger, 1997; Sechler et al., 1998). Recently, Dai et al. (Dai et al., 2002) also showed that fibronectin-1 was down-regulated by progesterone in a poorly differentiated endometrial cancer cell line.

The present observation that MPA down-regulates expression of CD44, CSPG/versican, tenascin-C and fibronectin-1 in the PRAB-36 sub-cell line provides evidence that progesterone could play an inhibiting role in tumor cell invasion.

In contrast to the four down-regulated genes discussed above, integrin-ß1 was found to be up-regulated by MPA in the PRAB-36 sub-cell line. Integrin-ß1 is located in the plasma membrane, where it functions as a complex with integrin-α chains. Integrins mediate cell-matrix interactions that are necessary for cell migration, and are involved in bi-directional cell signaling (Giancotti and Ruoslahti, 1999). Increased expression of integrins in cancer has been associated with increased metastasis through various pathways (Brakebusch et al., 1999; Ossowski and Aguirre-Ghiso, 2000; Ruoslahti, 1994; Yasuda et al., 2001). Up-regulation of integrin-ß1 by MPA in the PRAB-36 sub-cell line therefore seems to be in conflict with the overall invasion- and metastasis-inhibiting effect of MPA in these cells. However, it has been reported that integrin-ß1 can interact with E-cadherin to inhibit tumor cell

detachment and invasion into the extracellular matrix (Celetti et al., 2000; Huttenlocher et al., 1998). In this last setting, the up-regulation of integrin-ß1 by MPA in the PRAB-36 cell line is consistent with the suggested over-all invasion- and metastasis-inhibiting effect of MPA. Recently, expression of integrin-ß1 was reported to be strongly down-regulated in poorly differentiated endometrial cancer cells transiently over-expressing hPRB (Dai et al., 2002).

In the absence of progesterone-regulation, CD44, CSPG/versican, tenascin-C and fibronectin-1 show differential expression in the different Ishikawa sub-cell lines, both at the mRNA level and at the level of protein expression. Particularly CD44 and CSPG/versican, and also fibronectin-1, are expressed at high levels in the sub-cell lines that express the hPRB (PRB-1 and PRAB-36), while in the cell lines lacking hPRB (IKpar and PRA-1) the expression of these genes is much lower. This may indicate that, at least *in vitro*, endometrial cancer cells that express different PR isoforms will have a different invasive capacity in absence of MPA. From the present results, it can be expected that endometrial cancer cells which express hPRB may be more invasive than cells which do not express hPRB.

The observation that MPA down-regulates CD44, CSPG/versican, and fibronectin-1 in the hPRB-expressing sub-cell lines (PRB-1 and PRAB-36), but not (in the case of CSPG/versican and fibronectin-1) or to a lesser extent (in the case of CD44) in the sub-cell line which expresses only hPRA (PRA-1), supports the observations that a different set of genes is regulated by progesterone in cancer cells that express different PR isoforms (Richer et al., 2002); (Smid-Koopman, 2003). From the observed profile of gene regulation by MPA in the Ishikawa sub-cell lines, it is to be expected that progesterone will have an invasion-inhibiting effect on progesterone-sensitive endometrial cancer. This is in agreement with the observations of Dai et al. (Dai et al., 2002), who showed that in poorly differentiated endometrial cancer cells transiently expressing hPRB, invasion through Matrigel could be inhibited by progesterone.

When an endometrial tumor loses expression of PR, it can be expected that this will result in a loss of progesterone-regulation of the genes that are selected in the present study. If this holds true, one would expect a relative up-regulation of CD44, CSPG/versican, tenascin-C and fibronectin-1, and a relative down-regulation of integrin-ß1, in tumors in which expression of PR is absent. The present study indicates that, in advanced tumors, loss of PR is strongly associated with increased expression of CD44 and CSPG/versican. This is in agreement with the findings in the Ishikawa cell line model. For expression of tenascin-C, fibronectin-1, and integrin-ß1, an association with expression of PR was not found.

In many epithelial tumors, a major event in the development of metastasis is the loss of functional E-cadherin (Beavon, 2000). Cadherins are transmembrane glycoproteins that take part in calcium-dependent cell-cell adhesion. (Beavon, 2000; Behrens, 1999; Cavallaro and Christofori, 2001). In metastatic cancer, E-cadherin function is often disturbed, resulting in a loss of cell-cell adhesion. The present study indicates that if in endometrial cancer PR expression is decreased, expression of E-cadherin is also decreased. Additionally, these tumors showed more extensive

myometrial invasion than tumors in which PR is abundantly expressed. This observation seems to indicate that loss of PR is a feature of a more invasive phenotype in endometrial cancer.

We conclude that, in addition to the well-known growth-inhibiting effect of progesterone on endometrial cancer, progesterone and its receptors play an important role in regulating invasive properties of endometrial cancer cells. The genes that were found to be involved in this process are CD44 and CSPG/versican. Loss of PR expression *in vivo*, which results in loss of progesterone-regulation of CD44 and CSPG/versican, may be an early, and possibly initializing event in development of a more invasive phenotype in endometrial cancer.

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CHAPTER 3

DIFFERENCES IN INVASIVE CAPACITY OF ENDOMETRIAL CANCER CELL LINES EXPRESSING DIFFERENT PROGESTERONE RECEPTOR ISOTYPES: POSSIBLE INVOLVEMENT OF CADHERINS

Submitted

Differences in invasive capacity of endometrial cancer cell lines expressing different progesterone receptor isotypes: possible involvement of cadherins

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Submitted

ABSTRACT

Loss of expression of progesterone receptors (PR) in endometrial cancer is related to a more invasive and metastatic phenotype. In this study we aim to investigate whether selective loss of PRA or PRB affects the invasive capacity of endometrial cancer cells. cDNA microarrays were performed to compare gene expression profiles of a set of endometrial cancer sub-cell lines expressing PRA and/or PRB. *In vitro* invasion assays were performed to assess whether differences in gene expression between the lines were reflected by their invasive behavior. It was observed that cell lines that express only PRA express higher levels of cadherins, and show a lower level of invasion compared to cell lines that express PRB. When cadherin function was inhibited in exclusively PRA expressing cell lines, an increase of *in vitro* invasion was observed. In support of these findings, it was observed that in higher grade and more invasive endometrial cancer, expression of E-cadherin decreased. These results indicate that relative loss of PRA during progression of endometrial cancer can have a negative impact on cadherin expression, which may lead to development of a more metastatic phenotype.

INTRODUCTION

Tumor dissemination or metastasis is a complex process, of which the first steps involve dissociation of cells from solid tumors and subsequent invasion and migration of tumor cells into the surrounding extracellular matrix. Interference with one of these processes potentially inhibits metastasis.

For endometrial cancer cells, it has been well-established that progestagens can inhibit *in vitro* invasion: Fujimoto et al. (1996) reported that the progestagen Medroxyprogesterone Acetate (MPA) inhibits estrogen-induced suppression of cell-to-cell aggregation of endometrial cancer cells (Fujimoto et al., 1996). Furthermore, using endometrial cancer cell lines, which were transiently transfected with progesterone receptors (PR), Dai et al. (2002) showed that MPA inhibited *in vitro* invasion of these cells (Dai et al., 2002). In addition to this, our group recently reported that MPA modulates expression of several metastasis-related genes in a set of PR-expressing endometrial cancer sub-cell lines (Hanekamp et al., 2003; Hanekamp et al., 2002).

Progestagens act through the nuclear progesterone receptor (PR). This receptor exists as two isotypes, PRA and PRB, which are transcribed from the same gene by two different promoters (Kastner et al., 1990). PRA lacks 164 amino acids at its N-terminus compared to PRB, and it has become clear that PRA and PRB have different transcriptional activities (Giangrande and McDonnell, 1999; Giangrande et al., 1997; Vegeto et al., 1993). Several groups have reported that PRA and PRB regulate transcription of different genes (Richer et al., 2002; Smid-Koopman et al., 2003). Because of these transcriptional differences it has been hypothesized that

development, differentiation and metastatic potential of endometrial cancer cells can be influenced by the progesterone receptor status of the tumor. Several studies have reported a possible role for both up-regulation and down-regulation of PRB in development and metastasis of endometrial cancer. De Vivo et al. (2002) described a polymorphism in the PRB promoter, which leads to an increased transcription of the PRB isotype. In a population-based study this polymorphism was found to be associated with a 2-fold increased risk for endometrial cancer (De Vivo et al., 2002). Also, Fujimoto et al. (1997) reported that in distant metastases of endometrial cancer, PRB is the dominantly expressed PR isotype (Fujimoto et al., 1997). In contrast, Kumar et al. (1998) speculated that down-regulation of PRB may predict for poorly differentiated endometrial cancers that do not respond to progestagen therapy (Kumar et al., 1998). Arnett-Mansfield et al. (2001) observed that loss of expression of PRA and PRB in advanced endometrial cancer can occur simultaneously, but also loss of only PRA or only PRB was observed (Arnett-Mansfield et al., 2001). This study also reports that well-differentiated tumors (FIGO grade 1) express similar levels of PRA and PRB. Less differentiated tumors (FIGO grade 2 and 3) show dominance of one isotype, with grade 2 tumors expressing more PRB, but grade 3 tumors expressing more PRA.

During the last two years our lab has worked with a model in which endometrial cancer cells were transfected to stably express different PR isotypes. This model can be further applied to gain more insight in those cases of endometrial cancer in which the expression of one or both isotypes of the progesterone receptor is lost. We have reported that the sub-cell lines which express PRB, expressed significantly higher levels of several metastasis-related genes (CD44, CSPG/versican, fibronectin, tenascin-C) than sub-cell lines which express only PRA or no receptors (Hanekamp et al., 2003; Hanekamp et al., 2002) These findings, combined with findings in other reports (Arnett-Mansfield et al., 2001; Fujimoto et al., 1995; Fukuda et al., 1998), led to the hypothesis that endometrial cancers which express different PR isotypes have a different prognosis.

In the present study we compare gene expression patterns of endometrial cancer cell lines that express only PRA, only PRB, or both receptor isotypes, and link differences in gene and protein expression to differences in *in vitro* invasive capacity.

MATERIALS AND METHODS

Cell culture

Ishikawa endometrial cancer cells (clone 3H12, further referred to as IKpar) were obtained from dr M. Nishida (Tsukuba, Japan) and maintained in DMEM/F12 (GibcoBRL/ LifeTechnologies, Carlsbad, CA, USA), supplemented with 10% v/v fetal calf serum (FCS; GibcoBRL) and penicillin/streptomycin. The sub-cell lines which are used are IKpar cells that have been stably transfected with either hPRA (line PRA-1 and PRA-10), hPRB (line PRB-1 and PRB-23) or both receptors (line PRAB-36 and PRAB-18), as previously described by Smid-Koopman et al. (Smid-Koopman et al., 2003). These sub-cell lines were cultured under similar conditions and in similar

media as the IKpar cells, but with the addition of neomycin (500 μg/ml; ICN Biomedicals, Costa Mesa, CA, USA) and hygromycin (250 μg/ml; Invitrogen Corporation, Carlsbad, CA, USA) to maintain selection pressure for the transfected human progesterone receptors.

Microarray analysis

To study differences in gene expression patterns between IKpar and the different sub-cell lines, 6 microarray analyses were performed. The array used is the BEP1200 cDNA microarray, which contains genes involved in breast, endometrium and/or prostate cancer. The 1200 sequence-verified human cDNAs were obtained from Research Genetics (http://www.resgen.com/) via the Leiden Genome Technology Center (http://www.lgtc.nl/index.htm), and from the Image Consortium (Incyte Genomics Inc., C.A.). The BEP1200 cDNA microarray covers about 880 known named genes and 80 ESTs. cDNA amplification was essentially performed as described (http://www.microarrays.org/). cDNAs were spotted in duplicate on Corning CMT-GAPS-II slides by the DNA array facility of Eurogentec S.A. (Belgium). Post-processing of the slides was performed according to the manufacturer's guidelines (Corning, N.Y.). In each analysis, a sample from a pool of total RNA from the IKpar line was used as a reference.

Cells were cultured as described in 10% v/v FCS-supplemented medium for 72 hours, in absence of hormones. RNA was isolated according to Auffray and Rougeon (Auffray and Rougeon, 1980). RNA was labeled using the Micromax TSA Labeling and Detection kit (Perkin Elmer Life Sciences, Inc., Boston, MA, USA), according to the manual provided by the manufacturer. The gene expression patterns obtained were analyzed with Imagene and Genesight computer software (BioDiscovery, Marina del Rey, CA, USA). All signals that were lower than 3 times the standard deviation above the background signal were discarded, as well as the positive and negative control spots. The expression patterns of the remaining 756 genes were entered in the Cluster/Treeview software program for cluster analysis.

Western blotting

Cells were cultured for 72 hours in medium containing 5% v/v DCC-FCS, in absence or presence of 100 nM MPA. Cells were washed twice with PBS, after which the culture flasks were placed at -80° C overnight. Cells were lyzed with lysis buffer (40 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10% v/v glycerol, 10 mM sodiumphosphate, 10 mM sodiummolybdate, 50 mM NaF, 0.5 mM sodium orthovanadate), containing 10 mM DTT, 1% Triton X-100, 0.08% v/v SDS, 0.5% v/v DOC and 1x complete protease inhibitors (Roche, Basel, Switzerland). The lysates were centrifuged at 15000 g for 15 minutes, after which the pellet was discarded. Protein samples (4 μg/lane) were separated on a 7% polyacrylamide gel, and transferred onto nitrocellulose membrane. The membrane was blocked for 1 hour in blocking buffer (PBS/Tween containing 5% w/v non-fat milk powder), and incubated overnight at 4° C with primary mouse monoclonal antibody recognizing E-cadherin (clone G-10; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal antibody

recognizing P-cadherin (clone 12H6; Zymed Laboratories Inc., San Francisco, CA, USA), or mouse monoclonal anti-PR (clone hPRa8; Labvision Neomarkers, Fremont, CA, USA), all diluted 1:1000 in blocking buffer. The blots were then washed 4 x 10 minutes in PBS/Tween, after which they were incubated with peroxidase-labeled secondary goat-anti-mouse antibody (DAKO, Glostrup, Denmark), diluted 1:1000 in blocking buffer for 1 hour at room temperature. The blots were washed 6 x 10 minutes in PBS/Tween, before protein bands were visualized using Western Lightning Chemiluminescence reagent (Perkin Elmer, Boston, MA, USA), according to the manufacturers manual.

Invasion assays

Prior to the invasion experiments, cells were cultured for 72 hours in medium where FCS was substituted by 5% v/v dextran-coated-charcoal-treated fetal calf serum (DCC-FCS). The neomycin/hygromycin selection pressure was maintained throughout the experiments. Culture passages 15-25 were used.

At 70% confluency, cells were detached with trypsin and seeded in the upper well of a modified Boyden chamber (Transwell, 6.5 mm inserts, 8 µm pores; Corning Costar, Cambridge, MA, USA) at $1x10^5$ cells/well. In the lower wells, 600 µl of 5% v/v DCC-FCS-supplemented medium with or without 100 nM MPA was loaded. After 72 hours of culturing, cells that had migrated through the filter into the lower well were counted in quintuplo under a microscope. The experiments were performed in quadruplo, and were repeated 3 times (for the PRA-1, PRA-10, PRB-1, PRB-23 and PRAB-18 sub-cell lines) or 4 times (for the IKpar and PRAB-36 sub-cell lines).

Student's T-test was performed to assess P-values of differences in invasion between sub-cell lines in absence of MPA, and within sub-cell lines in presence or absence of MPA. A P-value < 0.05 was considered significant.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded samples of endometrial cancer tissue and non-neoplastic endometrium (Department of Pathology, Erasmus MC Rotterdam) were deparaffinized and endogenous peroxidase activity was inhibited by treatment with 4% v/v H_2O_2 in methanol for 20 minutes. Antigen retrieval was performed in a microwave oven in 10 mM citric acid buffer, pH 6.0, for 3 x 5 minutes. Slides were allowed to cool to room temperature and washed three times in PBS before being incubated for 20 minutes in PBS containing 10% v/v blocking serum (normal goat serum, DAKO, Glostrup, Denmark). Sections were incubated with a mouse monoclonal antibody recognizing E-cadherin (G-10, Santa Cruz Biotechnology), diluted 1:200 in PBS containing 1% v/v blocking serum. Incubation with primary antibody occurred overnight at 4°C. Incubation with secondary biotin-labeled goat-anti-mouse antibody (DAKO, Glostrup, Denmark) 1:400 in PBS containing 1% v/v blocking serum occurred for 30 minutes at room temperature, followed by StreptABComplex (DAKO, Glostrup, Denmark) 1:1:200 in PBS (30 minutes at room temperature). Staining was developed with DAB/concentrated metal complex (Pierce,

Rockford, IL, USA). Slides were counterstained with hematoxilin, dehydrated and mounted.

Invasion stimulation assays

To study stimulation of invasion of Ishikawa cells, the invasion assays described above were repeated while inhibiting cadherin function. Both in the upper and lower wells of the system, mouse monoclonal antibodies recognizing E-cadherin (clone 36B5; Labvision Neomarkers, Fremont, CA, USA), recognizing P-cadherin (clone N-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or both antibodies, diluted 1:100 in 5% v/v DCC-FCS-supplemented medium, were added when the cells were seeded.

RESULTS

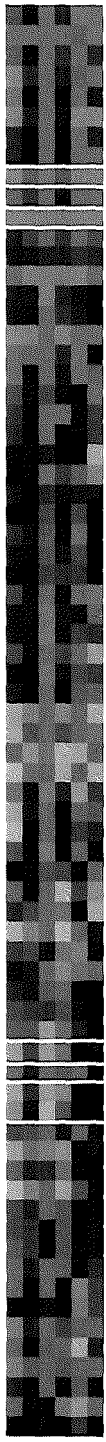
Comparison of gene expression patterns in the different Ishikawa sub-cell lines

mRNA expression patterns of all sub-cell lines were compared. It was observed that gene expression patterns of different cell lines cultured under standard culture conditions differed to some extent. Figure 1A shows a detail, including the genes that are relevant for this study, of the clustering based on the expression of the 756 genes that were loaded into the Cluster/Treeview software program for cluster analysis. Because in all arrays, the IKpar line was used as reference, the patterns obtained represent the gene expression levels in the sub-cell lines that express PRA, PRB, or both, relative to the parental line (which does not express any progesterone receptor). When expression of a gene in a certain sub-cell line is indicated in green, this means that this gene is expressed at a higher level in IKpar than in that sub-cell line. Red indicates that the gene is expressed at a higher level in that sub-cell line than in the IKpar line.

It was found that the sub-cell lines that express only PRA (PRA-1 and PRA-10) cluster away from the sub-cell lines that express either only PRB (PRB-1 and PRB-23) or both receptor isotypes (PRAB-18 and PRAB-36). The PRAB-18 and PRAB-36 sub-cell lines (which express relatively low amounts of PRA) cluster together with the exclusively PRB-expressing lines (PRB-1 and PRB-23).

Figure 1B shows the relative expression of PR, PR ligand binding domain, E-cadherin (two different clones were present on the array) and P-cadherin in the Ishikawa sub-cell lines. The tree that is shown in Figure 1B is the tree that was derived from clustering of the total of 756 genes. In the PRB-expressing lines (PRB-1, PRB-23, PRAB-18 and PRAB-36), expression of the cell-cell adhesion molecules E-cadherin and P-cadherin was decreased, while in the exclusively PRA-expressing lines (PRA-1 and PRA-10) these genes are expressed at the same or at a somewhat higher level than in the IKpar line.

A



STAT6
33.2 1
Keratin 5
Human clone 137308 mRNA, partial cds
Cyclin D2
Progesterone receptor
Nuclear co-repressor 1
NSP1
Progesterone receptor
Mesenchyme homeobox 2
Progesterone receptor ligand-binding domain
Heat shock protein 70
Heat shock protein 70 9B
TNFalfa-induced protein 6
Vimentin
Clusterin
G antigen, family B1
Adenine nucleoide translocator 1
TGF
Ribosomal protein S23
TATA box binding protein
EST/RxR alfa
PDGF- A
V-myb avian myeloblastosis viral oncogene homolog-like 1
Heat shock protein 70 A10
3-phosphoinositide-dependent kinase-1
Protein phosphatase 2, regulatory subunit B56, alpha isoform
Gamma-glutamyl hydrolase
F23
High-mobility group protein 1
CRADD
KIAA0215
APO-1
Glyceraldehyde-3-phosphate dehydrogenase
Alpha-methylacyl-CoA racemase
Collagen 1 alpha1
Carbonic anhydrase II
Transmembrane protease, serine 2
Endothelin 1
Hepatocyte nuclear factor 3, alpha
Cyclin D1
MHC class I promoter binding protein
Cyclin E
P53-binding protein
Ataxia telangiectasia mutated
Deleted in liver cancer 1
TSP-1
Hexabrachion, Tenascin-C
p300-/CBP-associated factor
Caldesmon 1
IL-1 alpha
IGFBP5
E-cadherin
EST. weakly similar to clone 22
P-cadherin
E-cadherin
EST, weakly similar to uncoupling protein-2
Epithelial V-like antigen 1
TGF beta receptor II
Thrombospondin 1
V-erb-b2 avian erythrobastic leukemia viral oncogene homolog 3
Col1A1/PDGF-B fusion transcript
Col1A1/PDGF-B fusion transcript
EPH-related receptor tyrosine kinase ligand 1 precursor
Spleen tyrosine kinase
LIM homeobox gene 2
IGFBP6
Prostate-derived factor
TGF beta
MXT1
TGF beta superfamily protein
EST, weakly similar to RNA polymerase II elongation factor ELL2

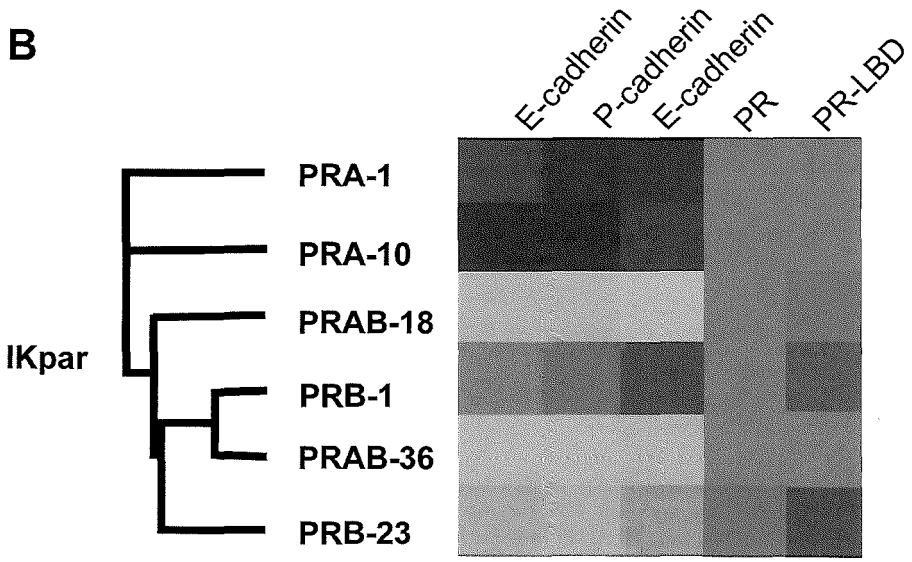


Figure 1. Comparison of gene expression in Ishikawa sub-cell lines. Panel A (previous page): Detail of cluster analysis of gene expression patterns in Ishikawa sub-cell lines expressing PRA and/or PRB compared to IKpar. Columns represent the different sub-cell lines; rows represent the different genes. Gene expression is represented as relative to gene expression in IKpar, with green indicating a higher expression of a gene in IKpar than in the PR-expressing sub-cell line, and red indicating a higher expression of the gene in the PR-expressing sub-cell line than in IKpar. A detail of the original clustering (obtained from the expression pattern of 756 genes) is shown. The yellow blocks indicate the genes that are highlighted in panel B. Panel B (page 53): Relative expression of E-cadherin (two different clones present on the array), P-cadherin, PR and PR ligand binding domain (LBD) in the Ishikawa sub-cell lines. Rows represent the different cell lines, columns the different genes. On the left side of the figure, the cluster-tree-relation between the different sub-cell lines, obtained from the original clustering of 756 genes, is shown.

Protein expression of E-cadherin and P-cadherin in Ishikawa sub-cell lines

To confirm the observation that E-cadherin and P-cadherin are expressed at lower levels in the PRB-expressing lines, Western blot analysis was performed to study the level of protein expression. This showed that in the PRB-expressing lines, expression of E-cadherin is not detectable by Western blot, under the present conditions (Figure 2). In the PRA-1 and PRA-10 lines, E-cadherin is expressed, and expression is somewhat up-regulated by MPA. In the PRB-expressing lines, expression of E-cadherin is not regulated (Figure 2). In absence of progestagens, P-cadherin is expressed at higher levels in the PRA-1 and PRA-10 sub-cell lines than in the PRB-expressing sub-cell lines. MPA increased the expression of P-cadherin in the PRA-1 sub-cell line, while regulation could not be shown in the other sub-cell lines. The figure also shows that the expression of PRA in the PRAB lines is much lower than in the PRA-1 and PRA-10 lines. Therefore the PRAB lines may be more representative of the exclusively PRB-expressing lines rather than representing an intermediate line.

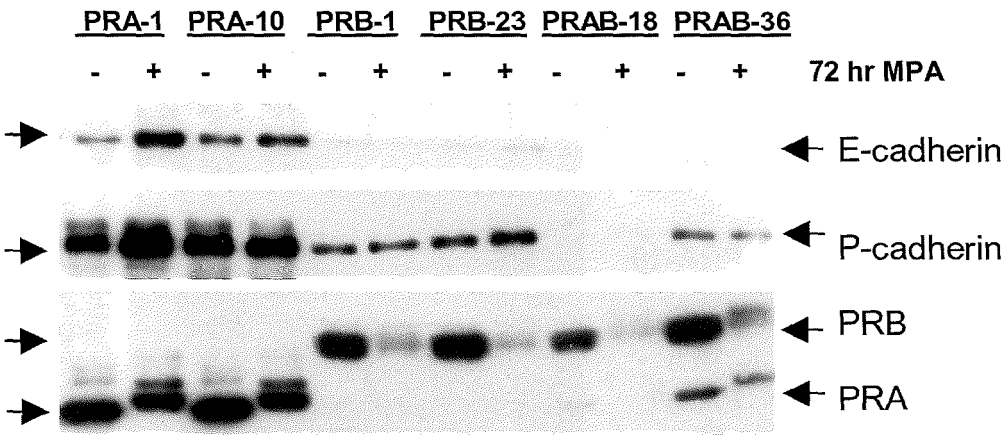


Figure 2. Expression of cadherin protein in Ishikawa sub-cell lines. Western blot showing expression of E-cadherin, P-cadherin and PRA + PRB in the different Ishikawa sub-cell lines, cultured for 72 hours in absence (-) or presence (+) of 100 nM of MPA. Equal quantities of protein (4 μg) were loaded to each lane.

Potential for *in vitro* invasiveness of Ishikawa sub-cell lines

Potential for invasion of Ishikawa cells was studied using a modified Boyden chamber assay. Since the main interest of this experiment is to assess the role of cadherins in this process, the Boyden chambers were not coated with collagen or matrigel. In this way, it was ensured that any differences in invasion were the result of loss of cell-cell interactions, rather than of differences in the ability of cells to dissolve or otherwise alter the artificial extracellular matrix.

When the PRA- and/or PRB-expressing sub-cell lines were tested for their *in vitro* invasive capacity, differences were observed between the different sub-cell lines in absence of MPA (Figure 3A). The sub-cell lines that express exclusively PRB (PRB-1 and PRB-23) showed a significantly higher rate of invasion than the PRA lines ($P< 0.000$). The sub-cell lines expressing both isotypes of the PR (PRAB-36 and PRAB-18) also showed a significantly higher rate of invasion than the PRA lines ($P< 0.000$). There was no significant difference in invasion between the exclusively PRB-expressing lines (PRB-1 and PRB-23) and the lines expressing both PRA and PRB (PRAB-18 and PRAB-36, $P= 0.600$) (Figure 3A).

Additionally, it was observed that 100 nM MPA effectively inhibited invasion in the PRB-1 ($P= 0.001$), PRB-23 ($P= 0.001$), PRAB-18 ($P= 0.008$) and PRAB-36 ($P<0.000$) sub-cell lines (Figure 3B). No significant effect of MPA on invasion was found in the exclusively PRA-expressing lines PRA-1 and PRA-10 (not shown).

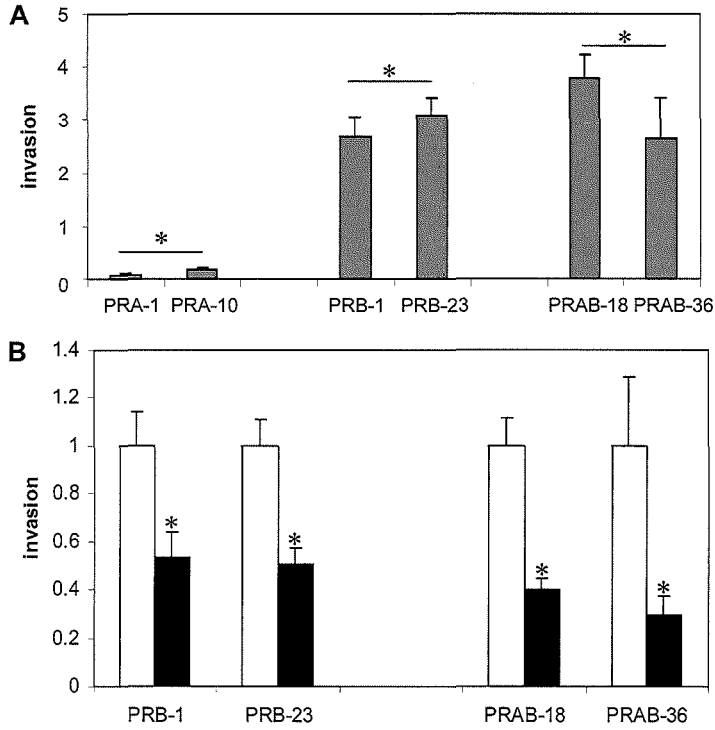


Figure 3. Invasion of Ishikawa sub-cell lines. Panel A represents invasion of the different sub-cell lines, compared to the IKpar line. Invasion of the IKpar line was set at 1, which represents a total of 1.5 x 10^3 cells (or 1.5 % of cells seeded in the upper well). n= 4 (for IKpar and PRAB-36) or n=3 (for PRA-1, PRA-10, PRB-1, PRB-23 and PRAB-18). Error bars represent SE over all experiments. * Indicates a P-value ≤ 0.05 (Student's T-test). Panel B represents the effect of administration of 100 nM MPA (black bars) on invasion of the PRB-expressing sub-cell lines, relative to invasion of cells in absence of MPA (white bars). n=3 (PRB-1, PRB-23 and PRAB-18) or n=4 (PRAB-36). * Indicates a P-value < 0.01 (Student's T-test).

E-cadherin expression in endometrial cancer

During progression of endometrial cancer, in general the metastatic potential of the tumor increases. To study whether this is reflected by alterations of expression of E-cadherin, samples of endometrial tissues of different FIGO grades and with different levels of myometrial invasion, were stained for E-cadherin. Figure 4 shows representative pictures of endometrial cancer samples of increasing grade and increasing myometrial invasion. It is clear that in non-neoplastic tissue (no myometrial invasion: Figure 4A and 4B), E-cadherin is strongly expressed, and also in FIGO grade 1 tumor tissue (little myometrial invasion: Figure 4C and 4D), E-cadherin is present. However, in FIGO grade 3 (Figure 4G and 4H) tumors, there is considerable myometrial invasion and it is clear that the expression of E-cadherin is strongly decreased.

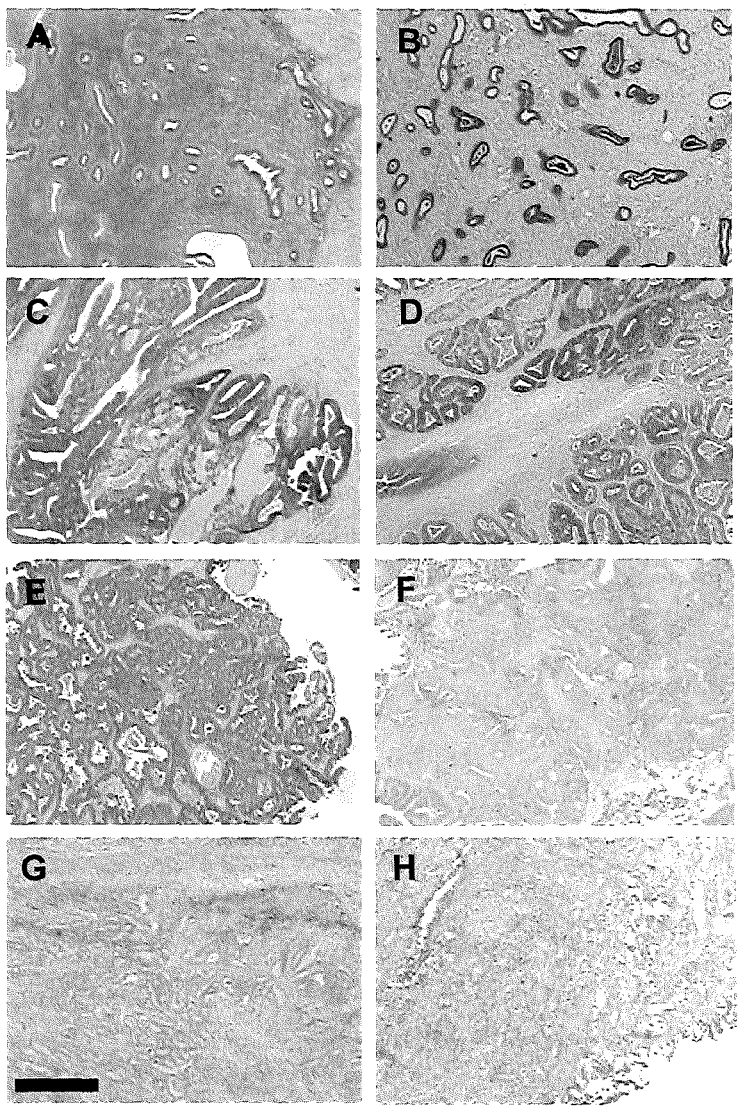


Figure 4. Expression of E-cadherin in endometrial cancer tissue. Immunohistochemical detection of E-cadherin in samples of non-neoplastic human endometrial tissue (A-B) and endometrial cancer (C-H). Histological typing and grade (according to the modified FIGO staging system, see also www.FIGO.org/content/PDF/corpus-uteri_p47-49.pdf) and extent of myometrial invasion (MI; defined as less (<), more (>) or equal to (≈) half (0.5) of the thickness of the myometrium) were assessed by the Erasmus MC Department of Pathology. C: grade 1, MI ≈ 0.5; D: grade 1, MI < 0.5; E: grade 2, MI < 0.5; F: grade 2, MI ≈ 0.5; G: grade 3, MI >0.5; H: grade 3, MI > 0.5. Bar indicates 0.5 mm.

Anti-cadherin antibodies stimulate invasion of Ishikawa cells

To investigate whether cadherins are involved in inhibiting the invasive capacity of the exclusively PRA-expressing sub-cell lines, we studied the effect of inhibiting cadherin function on invasion. The invasion assays were repeated with anti-E-cadherin and/or anti-P-cadherin antibodies added to the culture medium to block cell-cell adhesion. Addition of anti-E-cadherin and/or anti-P-cadherin antibodies increased the invasion of PRA-1 and PRA-10 cells (Figure 5). Invasion of PRB-expressing cells was not further stimulated by inhibiting E-cadherin or P-cadherin (not shown).

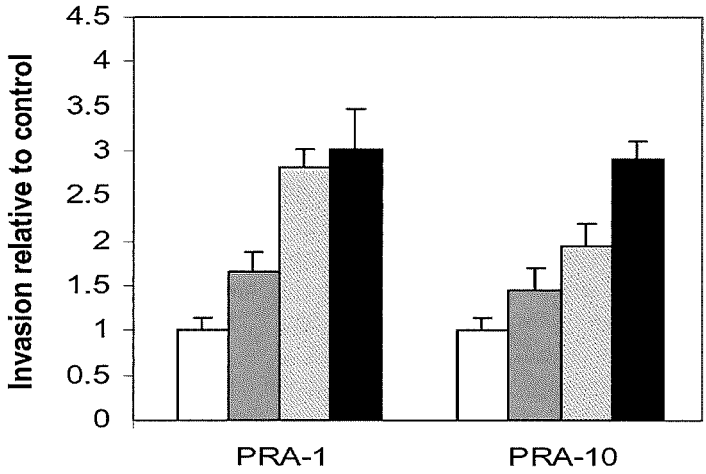


Figure 5. Invasion of cadherin-expressing cells is stimulated by inhibiting cadherin function. *In vitro* invasion in a modified Boyden chamber assay of IKpar, PRA-1 and PRA-10 cells, in absence of anti-cadherin antibodies (white bars) or presence of antibodies recognizing E-cadherin (dark grey bars), P-cadherin (light grey bars) or a combination of both antibodies (black bars). Antibodies were diluted 1:100 in DMEM culture medium. Invasion in absence of antibodies (white bars) is set at 1. Error bars represent SD within the experiment.

DISCUSSION

Previous work, performed by us (Hanekamp et al., 2003; Hanekamp et al., 2002) and by others (Arnett-Mansfield et al., 2001; Dai et al., 2002; De Vivo et al., 2002; Fujimoto et al., 1997; Fujimoto et al., 1995), suggested that progesterone receptor status and the presence or absence of progestagens strongly influences development, differentiation and metastatic potential of endometrial cancer cells. In the present study, we further investigated the effect of the different progesterone receptor isotypes on gene expression and on the capacity of endometrial cancer cells to invade.

The results show that Ishikawa sub-cell lines that express exclusively PRA express more E-cadherin and P-cadherin than sub-cell lines that express exclusively PRB or both receptor isotypes. Furthermore, PRA-expressing cell lines show low invasive capacity while PRB- and PRAB-expressing cell lines show high invasive capacity. Consequently, it was observed that high E- and P-cadherin expression levels negatively correlate to the invasive capacity of these cell lines.

From the results of the microarray analysis and Western blots it became clear that in the lines that express PRB, expression of E-cadherin and P-cadherin is decreased compared to the PRA-1 and PRA-10 sub-cell lines. Cadherins are a family of Ca^{2+}-dependent cell-cell adhesion molecules that play a crucial role in maintaining integrity of epithelial tissues (Beavon, 2000; Behrens, 1999). In tumors of epithelial origin, impaired E-cadherin function is associated with a more invasive and metastatic tumor. When invasive epithelial tumor cells are transfected to over-express E-cadherin, the invasive capacity of these cells is decreased (Frixen et al., 1991; Vleminckx et al., 1991). P-cadherin is a member of the cadherin family that was first described for the placenta, but is also present at a relatively high level in the endometrium, cervix, skin and peritoneum (van der Linden et al., 1994) Chen *et al.* 2002), as well as in breast and colon cancer (Peralta Soler et al., 1999), (Hardy et al., 2002). In endometrial cancer, decreased expression of E-cadherin is associated with local recurrence, with lymph node metastasis (Leblanc et al., 2001), and with dedifferentiation and increased myometrial invasion (Sakuragi et al., 1994). P-cadherin is expressed in endometriotic lesions and is thought to mediate endometrial-peritoneal cell interactions in this condition (Chen et al., 2002).

In the present study we show that in cells that express both E-cadherin and P-cadherin (PRA-1 and PRA-10), antibodies directed against E-cadherin or P-cadherin stimulate invasion. Invasion of sub-cell lines that do not express E-cadherin was not stimulated by the addition of anti-E-cadherin antibodies. Anti-P-cadherin antibodies also did not stimulate invasion in PRB-1 or PRAB-36 cells, which do express some P-cadherin. This suggests that in Ishikawa cells, decreased function of specifically E-cadherin results in an increase in invasive capacity. Inhibition of P-cadherin function increased the invasive capacity only in cells that also express E-cadherin, suggesting that P-cadherin alone is not enough to provide a stable enough cell-cell adhesion to prevent detachment of these cells from each other. A more extensive study will be needed to provide more insight in the precise action of the different cadherins in endometrial cancer cells. Our results indicate that the decreased expression of cadherins in the Ishikawa sub-cell lines that express PRB could at least partly be responsible for their relatively increased invasive capacity.

In a recent study by Dai et al. (2002), a difference in invasion between PRA and PRB-expressing cells was not found: exclusively PRA- and PRB-expressing Hec50 endometrial cancer cells both were invasive (Dai et al., 2002). These experiments, however, were different from the present study in several ways. The most important difference probably concerns the cell lines that were used. The Hec50 endometrial cancer cell line represents a poorly differentiated endometrial cancer, which was already highly invasive before transfection with PR (Dai et al., 2002). The IKpar cell

line used in the present study represents a well to moderately differentiated tumor that shows a limited invasive capacity (Nishida, 2002). Furthermore, Dai et al. (2002) used transiently transfected cells in the invasion assay, while in the present study, stable cell lines were implemented. Both approaches have their advantage: while the use of cells that have been transfected immediately before the experiment ensures that the different cells lines are as similar as possible, the use of stable cell lines enables one to study invasion in cells that have adapted to long-term effects of expression of PR. We have chosen for the latter option, since we feel that the use of stable cell lines more strongly reflects the *in vivo* situation.

The invasion shown by the PRAB-18 and PRAB-36 sub-cell lines was not significantly different from that of the PRB-1 and PRB-23 sub-cell lines. Furthermore, the microarray gene expression data showed that the PRB-1, PRB-23, PRAB-18 and PRAB-36 sub-cell lines are grouped in the same cluster, while both PRA-1 and PRA-10 are assigned to a separate branch of the tree. In the Ishikawa sub-cell lines PRAB-18 and PRAB-36, PRB is expressed at a higher level than PRA (Figure 2). When activated by progestagens, PRA and PRB form hetero- and/or homodimers, which subsequently act as transcription factors. It is likely that the predominance of PRB will result in PRAB lines that respond to progestagens similar to the exclusively PRB-expressing cell lines. However, the arrays performed in the present study are all performed in the absence of hormone. On the basis of our observations, we hypothesize that in the lines that stably express PRB, unliganded cytoplasmic PRB has a function in a signaling pathway that stimulates loss of cell-cell adhesion and subsequent invasion. Relocalization of the receptor to the nucleus following binding of ligand would render the PRB unavailable for this function, resulting in decreased invasive capacity. This could also explain why PRB-expressing cells, in which MPA does not up-regulate expression of cadherins, show decreased invasion in the presence of MPA. Mechanistically, this would be a novel way for MPA to inhibit invasion.

In conclusion, it was found that Ishikawa endometrial cancer sub-cell lines that express PRA express more cadherins than sub-cell lines that express PRB. This is reflected by an increased invasive capacity of sub-cell lines expressing PRB. These results suggest that in endometrial cancer, loss of PRA, resulting in relative over-expression of PRB, contributes to progression to a potentially more metastatic tumor.

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CHAPTER 4

PROGESTERONE RECEPTORS IN ENDOMETRIAL CANCER INVASION AND METASTASIS: DEVELOPMENT OF A MOUSE MODEL

Steroids 68 (10-13): 795-800 (2003)

PROGESTERONE RECEPTOR A AND B EXPRESSION AND PROGESTAGEN TREATMENT IN GROWTH AND SPREAD OF ENDOMETRIAL CANCER CELLS IN NUDE MICE

Endocrine-Related Cancer, in press

Progesterone receptors in endometrial cancer invasion and metastasis: development of a mouse model

Eline E. Hanekamp[1], Susanne C.J.P. Gielen[2], Susanna A. van Oosterhoud[1], Curt W. Burger[2], J. Anton Grootegoed[1], Frans J. Huikeshoven[2], and Leen J. Blok[1].

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Progesterone receptor A and B expression and progestagen treatment in growth and spread of endometrial cancer cells in nude mice

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ABSTRACT

Progestagens inhibit growth of endometrial cancer cells *in vivo* and *in vitro*, and also are reported to inhibit endometrial cancer cell invasion. The progesterone receptor isotypes PRA and PRB have different transcriptional activity. There are indications that relative over-expression of PRB could lead to development of a more invasive phenotype in endometrial cancer. To study the effect of progestagens and the two PR isotypes on tumor dissemination, *in vitro* and *in vivo* models should be applied. The Ishikawa endometrial cancer cell line (clone 3H12) was transfected to stably express a high level of hPRB, which resulted in the PRB-1 sub-cell line. Ovariectomized athymic NMRI nu/nu mice were injected intraperitoneally with these PRB-1 cells. After three, five and ten weeks, the animals were sacrificed. Spread of PRB-1 cells in and outside the peritoneal cavity was studied macroscopically and microscopically, and also by PCR detection. After ten weeks, the PRB-1 cells had formed extensive tumor mass in the peritoneal cavity. Also, cells could be detected outside the peritoneal cavity, indicating metastatic ability of these cells. The present study describes an *in vivo* model that can provide a valuable tool in studying the influence of progestagens and the two PR isotypes on endometrial cancer cell invasion and metastasis.

INTRODUCTION

Progesterone controls growth of the human endometrium by antagonizing the proliferative activity of estradiol through induction of differentiation of endometrial epithelium and stromal cells (Martin et al., 1973). In clinical practice, progesterone is used, with limited results, in a palliative setting as treatment of advanced and recurrent endometrial cancer.

In addition to exerting a growth-inhibiting effect on endometrial cancer cells, progestagens appear to have an effect on tumor integrity. It has been reported that the progestagen Medroxyprogesterone Acetate (MPA) can inhibit in vitro invasion of endometrial cancer cells (Dai et al., 2002; Ueda et al., 1996). Furthermore, Fujimoto et al. (1996) reported that MPA could inhibit estrogen-induced suppression of cell-to-cell aggregation of endometrial cancer cells (Fujimoto et al., 1996), while the progestagen R5020 inhibited anchorage-independent growth of endometrial cancer cells transiently transfected to express PRA or PRB (Dai et al., 2001). In addition to this, our group recently reported that MPA inhibits expression of several metastasis-related genes in a set of endometrial cancer sub-cell lines (Hanekamp et al., 2002).

The human progesterone receptor (PR) is present in two isotypes, PRA and PRB. These isotypes are translated from the same gene, but transcription is initiated from different promoters. PRA is a truncated form of PRB, lacking the first 164 amino acid residues at the N-terminus. Even though both forms have similar DNA and ligand binding affinities, PRA and PRB do exhibit different activating properties. Several functional domains can be recognized in PRA and PRB. They have in common two transcription activation functions, AF-1 and AF-2, and one inhibitory domain. In PRB, a third activation function (AF-3) has been defined in the N-terminal amino acid stretch. Giangrande et al. (1997) proposed that this AF-3 domain suppresses the activity of the inhibitory domain of PRB (Giangrande et al., 1997). Vegeto et al. (1993) showed that, in cells in which PRA was not transcriptionally active, PRA could repress PRB activity (Vegeto et al., 1993). Several years later, Giangrande et al. (2000) showed that differential cofactor binding caused the opposing transcriptional activities of PRA and PRB (Giangrande et al., 2000).

In the normal human endometrium, expression of PRA predominates in stromal cells throughout the menstrual cycle, whereas in epithelial cells, a shift occurs from PRA to PRB during the early secretory phase (Mote et al., 1999). The varying ratio of the two isotypes in different target cells and under different physiological circumstances suggests that the differential expression level of PRA and PRB may determine the cellular response to progesterone. In endometrial cancer, literature agrees on a down-regulation of PR in general (Fukuda et al., 1998; Kumar et al., 1998; Mote et al., 2000; Moutsatsou and Sekeris, 1997; Sakamoto et al., 1999), but reports are conflicting whether this is a consequence of selective down-regulation of PRA or PRB, or of both receptors. Fujimoto et al. (Fujimoto et al., 1995) reported that in advanced tumors, PRA was lost. In accordance with this, the same group later described that in distant metastases of endometrial cancer, PRB is predominantly expressed (Fujimoto et al., 1997). Arnett-Mansfield et al. (Arnett-

Mansfield et al., 2001) found a reduced expression of either one or both of the two PR isotypes in the majority of endometrial tumors, compared to hyperplastic or normal endometrial tissue. In addition to this, hypermethylation of PRB alleles (Sasaki et al., 2001) and expression of exon-deleted PR mRNA (Misao et al., 2000) have also been described in endometrial cancer tissue. De Vivo et al. (De Vivo et al., 2002) described a polymorphism in the promoter of the PR gene that increased the production of hPRB. Women carrying this mutation were found to have a two-fold higher risk for endometrial cancer. Recently, our group described that in a model in which endometrial cancer cells were transfected to stably express different PR isotypes, the sub-cell lines that express PRB expressed several metastasis-related genes (CD44, CSPG/versican, fibronectin, tenascin-C) at a higher level than sub-cell lines that express only PRA or no PR (Hanekamp et al., 2002).

Because of these findings, it is of interest to study the invasive capacity of endometrial cancer cell lines that express different amounts of PRA, PRB, or both receptors. Modified Boyden chamber assays are a valuable tool to study *in vitro* tumor cell invasion (Lam et al., 1981). However, little work has been done to study the invasive and metastatic potential of endometrial cancer cells *in vivo*. In the present study we describe a model which can be used, and will in the future be exploited, to study the *in vivo* metastatic capacity of different PR-expressing endometrial cancer cells.

MATERIALS AND METHODS

Animals

Ten eight-week-old female athymic NMRI nude/nude mice (Harlan, Horst, The Netherlands) were used for this study. The animals were housed in Individually Ventilated Cage units (five animals per unit). Food and water were provided ad libitum.

Cells

Ishikawa clone 3H12 was transfected to stably express high levels of human PRB (hPRB), as described by Smid-Koopman et al. (Smid-Koopman, 2003), which resulted in the sub-cell line PRB-1. Ishikawa clone 3H12, the PRB-1 cells and T47D cells were routinely maintained in DMEM-F12 culture medium supplemented with 10% v/v fetal calf serum (GibcoBRL/ LifeTechnologies, Carlsbad, CA, USA) and penicillin/streptomycin. Furthermore, neomycin (500 μg/ml, ICN) and hygromycin (250 μg/ml, Invitrogen Corporation, Carlsbad, CA, USA) were supplemented for the PRB-1 sub-cell line in order to maintain selection pressure for the transfected hPRB.

Western blot

Ishikawa 3H12, PRB-1 and T47D cells were washed twice with PBS, after which the culture flasks were placed at -80° C overnight. Cells were lysed with RIPA buffer (40

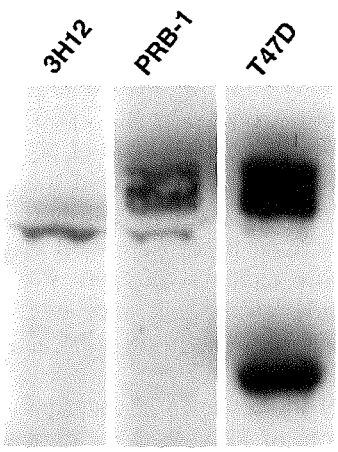

Figure 1. Western blot analysis of PR expression in the Ishikawa cancer cell line clone 3H12, the PRB-1 sub-cell line, and the T47D breast cancer cell line.

mM Tris-HCl (pH 7.4), 5 mM EDTA, 10% v/v glycerol, 10 mM sodiumphosphate, 10 mM sodiummolybdate, 50 mM NaF, 0.5 mM sodium orthovanadate), containing 10 mM DTT, 1%v/v Triton X-100, 0.08% w/v SDS, 0.5% v/v DOC and 1x complete protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The lysates were centrifuged at 350,000 x g at 4° C for 10 minutes, after which the pellet was discarded. Western blot analysis was performed as described by Blok et al. (Blok LJ, 2003).

Experimental design

The mice were ovariectomized, and, after one week, exposed to 5 Gy of total body irradiation 24 hours prior to injection with tumor cells. Irradiation was performed to improve attachment of tumor cells to peritoneal surfaces. At t = 0, the mice were intraperitoneally injected with 5 x 10^6 PRB-1 cells in a volume of 300 μl of PBS. At three weeks (two animals), five weeks (three animals), or ten weeks (two animals), the animals were sacrificed. The peritoneal cavity was opened and examined macroscopically. Samples of all abdominal organs, as well as samples of the lungs, heart, brain and upper hind leg muscle were snap frozen in liquid nitrogen for DNA extraction. The remaining tissue was washed in sterile PBS to remove blood and body fluids, after which it was formalin-fixed and processed for routine histological examination (hematoxylin-eosin staining) and immunohistochemistry.

Two animals died as a result of the ovariectomy, before inoculation of PRB-1 cells; these animals were examined as controls. At week seven, one animal was euthanized when a 1 cm-large lump appeared on its left flank. After examination, this lump turned out to be scar tissue and adipose tissue that probably developed as a result of the ovariectomy. Therefore, this animal was also examined and included as a week seven time-point.

PCR detection

PCR detection of PRB-1 cells is based on amplification of the hygromycin resistance (HR) gene. This gene is present in the vector containing the hPRB, which was transfected into the parental Ishikawa 3H12 clone. DNA was extracted using the QiaAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Polymerase chain reaction was performed using forward primer B06: GTGGATATGTCCTGCGGGTAA and reverse primer E04: TCACTGCATTCTAGTTGTGG (annealing temperature 63°C, 30 cycles), followed by a nested PCR with forward primer B07: CGCAAGGAATCGGTCAATACA and reverse primer E02:

TCGTCCGATCCGGAGGAGCC (annealing temperature 63°C, 30 cycles) (primers from Invitrogen, Breda, The Netherlands). This resulted in amplification of a 494 bp fragment of the hygromycin resistance gene (HRG). The reaction product was visualized on 1.5 % agarose gel with ethidium bromide.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples of internal organs were sliced into 7 μm thin sections. Every fifth of these sections was fixed on PLL-coated slides and routinely stained with hematoxylin and eosin and examined microscopically. When tumor mass was detected, consecutive sections were again fixed on PLL-coated slides for immunohistochemical staining. Slides were deparaffinized and endogenous peroxidase activity was inhibited by treatment with 4% v/v H_2O_2 in methanol for 20 minutes. Antigen retrieval was performed in a microwave oven in 10 mM citric acid buffer, pH 6.0, for 3 x 5 minutes. Slides were allowed to cool down to room temperature and washed three times in PBS before being incubated for 20 minutes in PBS containing 10% v/v normal goat serum (DAKO, Glostrup, Denmark).

Incubation with rabbit polyclonal antibody recognizing PR (clone C20, SantaCruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 in PBS containing 1% normal goat serum occurred overnight at 4°C. Negative control slides were incubated with PBS containing 1% normal goat serum. Antibodies were detected indirectly with biotin-labeled goat-anti-rabbit antibodies 1:400 in PBS containing 1% normal goat serum for 30 minutes at room temperature, followed by StreptABComplex (DAKO, Glostrup, Denmark), according to the manufacturer's protocol. Staining was developed with DAB/concentrated metal complex (Pierce, Rockford, IL, USA). Slides were counterstained with hematoxylin, dehydrated and mounted.

RESULTS

Expression of hPR in the PRB-1 sub-cell line

Western blot analysis showed that, while the Ishikawa clone 3H12 does not express any hPR, the PRB-1 sub-cell line expressed a high level of hPRB (Figure 1). The T47D breast cancer cell line was used as a positive control, since this cell line is known to express high levels of both hPRA and hPRB.

Macroscopic and microscopic detection of tumor mass

At week three, a few very small tumors could be detected in the peritoneal cavity. Some small foci could be seen on the parietal peritoneum and mesenterium (not shown). At weeks five (Figure 2A) and seven (Figure 2B and 2C), tumor growth on the parietal peritoneum and mesenterium was more pronounced. At week ten, there was extensive tumor growth in the peritoneal cavity, again mainly on the parietal peritoneum and mesentery, but also in the abdominal fat (Figure 2D) and on the diaphragm (Figure 2E). Microscopical analysis showed that the nodules consisted of

a cell mass with an appearance consistent with that of an undifferentiated endometrial carcinoma (Figure 2A-C). Even though macroscopically no tumor growth into any organs was observed, microscopical analysis showed extensive growth of tumor mass into the pancreas of both animals at week ten (Figure 2D and 2E). Also, in one animal at week ten, a small tumor nodule growing on the left kidney was detected (Figure 3F).

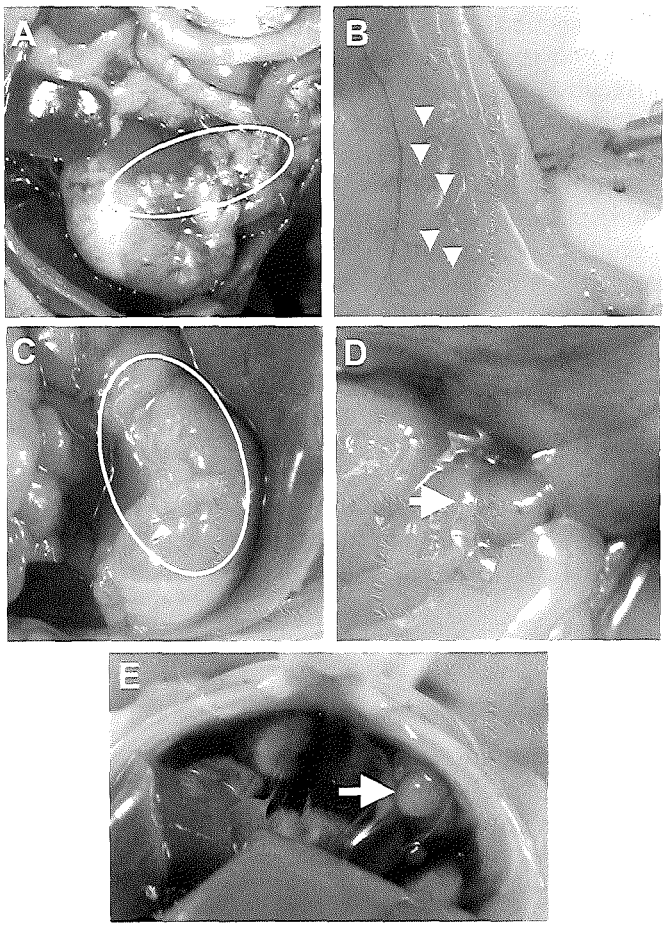

Figure 2. Macroscopic investigation of abdominal tumor growth. Panel A shows tumor growth on the peritoneum covering the stomach after 5 weeks. After 7 weeks, tumor growth was clear on the peritoneum covering the abdominal wall (B) and again covering the stomach (C). After 10 weeks, additional large tumor nodules were present in the abdominal fat (D) and on the diaphragm (E). Tumor nodules are encircled (A and C) or indicated by arrowheads (B) or arrows (D and E).

Immunohistochemical analysis

When the formalin-fixed, paraffin-embedded samples of tumor nodules were immunohistochemically stained to detect hPR, it was observed that the nodules stained heterogeneously positive for hPR. Some nodules showed a region in which the hPR was homogeneously expressed adjacent to a region in which hPR was not expressed (Figure 3G), while other nodules showed an over-all heterogeneous hPR expression (Figure 3I).

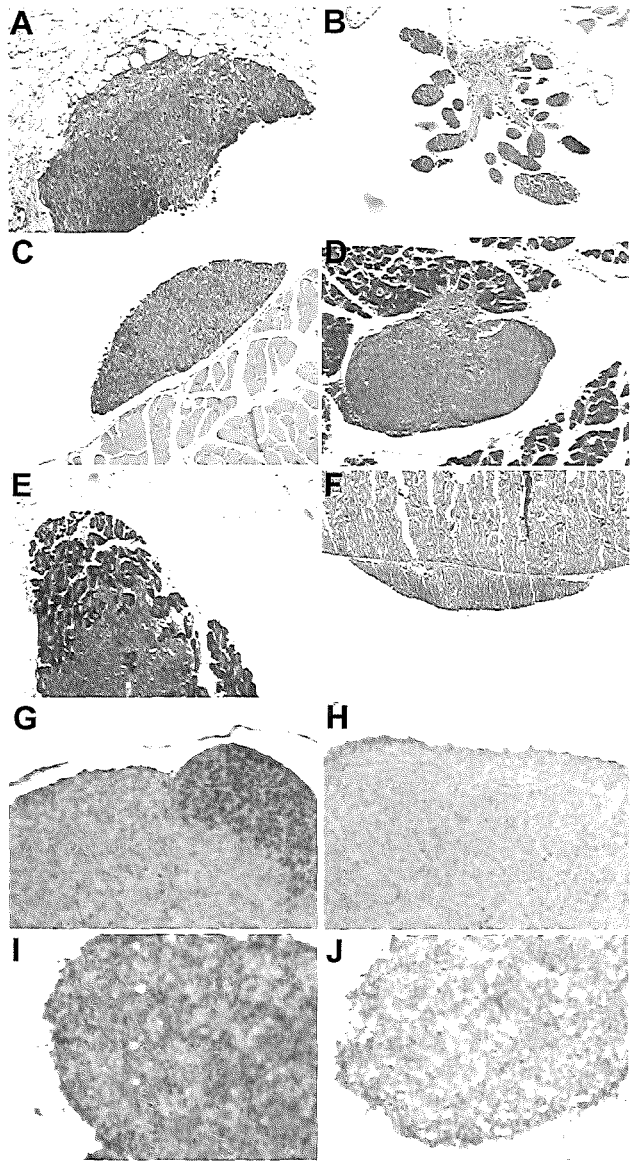

Figure 3. Microscopic analysis of tumor foci. Hematoxylin-eosin staining of nodules growing in the abdominal fat (A), on the mesenterium (B), and on the peritoneum (C) is shown. After ten weeks, tumor was growing into the pancreas of both animals (D and E) and on the left kidney of one animal (F). Immunohistochemical analysis was performed to detect expression of hPR (G and I, with H and J as respective negative controls). Original magnification 50 x (A-F) and 100 x (G-J).

Detection of PRB-1 cells by PCR

PCR was performed on DNA extracts from whole tissue samples from organs from all animals to detect the hygromycin resistance (HR) gene. This HR gene is present on the vector with which the parental Ishikawa cell line was transfected to stably express hPRB. Since during culturing the transfected cells were maintained under selection of hygromycin, only the hPRB-expressing PRB-1 cells should contain this gene. As expected, the HR gene was not detected when PCR was performed on tissue from control animals (results not shown).

At week three, the HR gene was detected in several organs of both mice. At week five however, the HR gene could not be amplified from any of the animals. At week seven, the HR gene could be amplified from the uterus, while at week ten, the HR gene could be detected in several tissues of both animals sacrificed at that time. For an overview, see Table 1.

Time point	Mouse	Organs in which the HR gene was detected
3 weeks	00	liver; pancreas
	01	lung
5 weeks	02	-
	05	-
	06	-
7 weeks	07	uterus
10 weeks	08	uterus; pancreas
	09	kidney; pancreas; lung

Table 1. Detection of HR gene in mouse organs. A PCR reaction to amplify the HR gene was performed on DNA extracted from mouse organs. The table summarizes in which organ the gene could be detected. If the HR gene could not be detected in any organ, this is indicated with " - ".

DISCUSSION

Animal models are powerful tools to study the *in vivo* behavior of tumor cells. Jordan et al. (1989) described that athymic mice could be used to study the influences of steroid hormones on growth of human endometrial cancer xenografts (Jordan et al., 1989). Since then, several studies used subcutaneous xenograft models to study the behavior of endometrial cancer cells (Dardes et al., 2002; Greenberger et al., 2001; Horvath et al., 1993; Legro et al., 2001). The main topic of our interest is the metastatic behavior of endometrial cancer. We therefore chose to inject tumor cells in a well-described, restricted cavity of the body close to the uterus, in order to be able to observe the ability of these cells to spread outside this cavity. To the best of our knowledge this is the first model that studies spread of

endometrial cancer cells in this manner. In the present study we tested the feasibility of this *in vivo* model, injecting PRB-expressing endometrial cancer cells intraperitoneally in mice. We used athymic NMRI nu/nu mice to avoid a host-anti-graft reaction, and irradiated the mice 24 hours prior to inoculation to improve attachment of tumor cells to surfaces in the peritoneal cavity (Huang et al., 1996). Since progestagens have been reported to inhibit endometrial cancer cell proliferation and invasion, the mice were ovariectomized one week prior to injection of PRB-1 cells in order to minimize endogenous progesterone activity. Furthermore, because several reports indicated that a relatively high expression of hPRB in endometrial cancer would lead to a more invasive or even metastatic phenotype, an endometrial cancer sub-cell line that expresses exclusively hPRB was used.

The current experiments show that PRB-1 cells survive when injected into the peritoneal cavity of NMRI nu/nu mice. Furthermore, the cells proliferate and form solid tumor masses on the peritoneum covering the abdominal organs, abdominal wall, diaphragm and mesenterium. Moreover, PRB-1 cells were also shown to grow invasively into retroperitoneal organs, as indicated by the tumor growth in the pancreas of both animals sacrificed at ten weeks. Expression of hPRB was conserved over the ten-week period of the study. Additionally, PRB-1 cells appeared to be able to leave the abdominal cavity. This is indicated by PCR detection of the HR gene in the lung. In order to reach the lung, cells would either have to enter the blood or lymph circulation, or alternatively invade through the diaphragm into the thorax. Either way, PRB-1 cells are obviously able to form secondary colonies outside the peritoneal cavity in which they were inoculated.

Interestingly, the PRB-1 cells could be detected in several organs after three, seven and ten weeks, but not after five weeks. An explanation could be that the PRB-1 cells initially are quite effective in entering the circulation, so that the HR gene can be detected at three weeks, but that they are not very efficiently homing into different organs, and thus no PRB-1 cells can be detected at five weeks. Eventually, a few cells will begin to grow in distant organs, so that at seven and ten weeks the HR gene signal is back.

By injecting Ishikawa cells expressing different hPR isotypes we will be able to study the effect of both isotypes on invasive and metastatic potential of endometrial cancer cells *in vivo*. Treatment of animals with different progestagens or antiprogestagens will give an insight into the effects of these steroid hormones on endometrial cancer invasion and metastasis. It appears that ten weeks is an appropriate time-span for these experiments, since the animals do not appear to suffer any discomfort, while at the same time tumor growth is extensive, and dissemination of tumor cells into different organs can be detected. We feel that this model will provide a useful experimental setting in which to study the effect of progestagens and expression of the different progesterone receptor isotypes on endometrial cancer invasion and metastasis.

Progesterone receptor A and B expression and progestagen treatment in growth and spread of endometrial cancer cells in nude mice

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ABSTRACT

In endometrial cancer, decreased expression of progesterone receptor isotypes A and B (PRA and PRB) is a feature of poorly differentiated tumors. In distant metastases, PRB is the predominantly expressed isotype, and endometrial cancer cells that express PRB have been observed to be more invasive. Furthermore, PRB-associated *in vitro* invasion is markedly inhibited by progestagens.

In the present study, ovariectomized mice were injected intraperitoneally with Ishikawa endometrial cancer cells that express only PRA, only PRB, both PRA and PRB, or no PR. Half of the mice was substituted with Medroxyprogesterone Acetate (MPA). After ten weeks, growth and spread of the cancer cells was examined macroscopically, microscopically, and by PCR detection. Without MPA substitution, cells that express only PRB were found to be the most proliferative and migrative, while cells that express only PRA, both receptor isotypes, or no PR, showed minimal growth and spread. MPA appeared to inhibit growth and spread of PR-positive cells. Surprisingly, when mice that were inoculated with PR-negative cells were substituted with MPA, this resulted in massive abdominal tumor growth.

These results provide further evidence that overexpression of PRB in endometrial cancer contributes to the development of a more aggressive phenotype. MPA inhibits tumor growth and spread of PR-positive cells, but also can have an indirectly stimulating effect on PR-negative tumor cells, probably through a host-mediated response.

INTRODUCTION

In normal human endometrium, the growth-stimulating effects of estrogens are counterbalanced by progestagens, which exert a differentiating and growth-inhibiting effect. Early, non-invasive stages of endometrial cancer can be inhibited in growth by progesterone treatment, but generally this does not cure the patient (Creasman et al., 2001; Southcott, 2001).

Progesterone exerts its effect through the progesterone receptors A and B (PRA and PRB), which can act as transcription factors upon activation by ligand. Although both PR isotypes have similar DNA- and ligand-binding affinities, PRA and PRB do exhibit different activating properties (Giangrande and McDonnell, 1999), and mediate transcription of a different set of genes in endometrial cancer cells (Smid-Koopman et al., 2003).

During progression of endometrial cancer, expression of PR is decreased (Arnett-Mansfield et al., 2001; Fujimoto et al., 1997; Fujimoto et al., 1995; Fukuda et al., 1998; Kumar et al., 1998; Sakamoto et al., 1999). Although it remains unclear whether this is a consequence of down-regulation of only one PR isotype or both, several studies suggest that relative over-expression of PRB is associated with more aggressive tumor growth. Fujimoto et al. (Fujimoto et al., 1995) reported that in advanced tumors PRA was lost, and that in distant metastases of endometrial cancer, PRB was predominantly expressed (Fujimoto et al., 1997). De Vivo et al. (De Vivo et al., 2002) described a polymorphism in the promoter of the PR gene that selectively increased the expression of PRB, which was found to be associated with a two-fold higher risk for endometrial cancer. In contrast, Sasaki et al. (2001) reported inactivation of only PRB alleles through hypermethylation (Sasaki et al., 2001) in endometrial cancer tissue.

Several studies report that in addition to growth inhibition, progestagens have an effect on tumor integrity in endometrial cancer. Progestagens have been shown to inhibit estrogen-induced suppression of cell-to-cell aggregation of well-differentiated endometrial cancer cells (Fujimoto et al., 1996), and progestagens were also shown to inhibit anchorage-independent growth of poorly differentiated Hec50 endometrial cancer cells (Dai et al., 2001). Our group recently reported that Medroxyprogesterone Acetate (MPA) inhibits expression of several metastasis-related genes in a set of endometrial cancer sub-cell lines expressing different PR isotypes (Hanekamp et al., 2003a). Additionally, MPA has been shown to inhibit *in vitro* invasion of endometrial cancer cells (Dai et al., 2002; Ueda et al., 1996). Recently, we observed (Hanekamp et al., submitted) that endometrial cancer cells that express PRB are more invasive *in vitro* than cells that do not express PRB.

In the present study we set out to investigate the *in vivo* metastatic potential of these cells, in comparison to PR-negative cells and to cells expressing only PRA or both PRA and PRB, as well as to examine the effect of progestagens on *in vivo* metastasis.

MATERIALS AND METHODS

Animals

Female athymic NMRI nude/nude mice (Harlan, Horst, The Netherlands) were used for this study. The animals were housed at the Erasmus MC animal facility (EDC) in Individually Ventilated Cage units (five animals per unit). Food and water were provided ad libitum.

Cells

Ishikawa clone 3H12 (IKpar) was transfected to stably express high levels of human PRA, PRB, or both PRA and PRB (Blok et al., 2003; Hanekamp et al., 2003b; Smid-Koopman et al., 2003). This resulted in the sub-cell lines PRA-1, PRB-1 and PRAB-36. A sub-cell line stably transfected with empty vector (EV3) was used as control. These cell lines do not express estrogen receptors. Cells were routinely maintained in DMEM-F12 culture medium supplemented with 10% v/v fetal calf serum (GibcoBRL/ LifeTechnologies, Carlsbad, CA, USA), penicillin/streptomycin, neomycin (500 μg/ml, ICN) and hygromycin (250 μg/ml, Invitrogen Corporation, Carlsbad, CA, USA).

Western blot

Cells were lyzed as described (Hanekamp et al., 2003b). Equal amounts of protein were separated on a polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking in 5% w/v non-fat milk powder in PBS the membrane was incubated with a mouse monoclonal antibody recognizing PRA and PRB (hPRa8, Labvision Neomarkers, Fremont, CA, USA) and a secondary peroxidase-labeled goat-anti-mouse antibody (Sigma). Bands were visualized using Western Lightning ECL reagent (Pierce, Rockford, IL, USA).

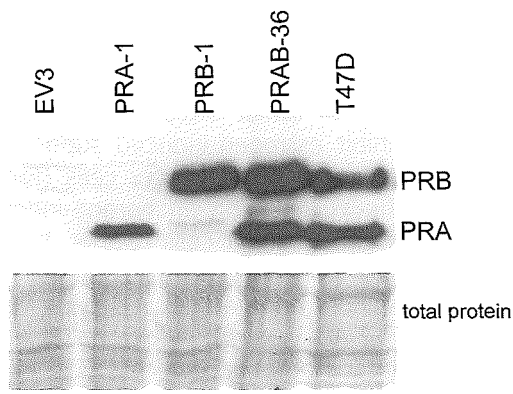

Figure 1. Western blot, showing expression of PRA and PRB in Ishikawa sub-cell lines. T47D breast cancer cells are shown as positive control. Lower panel: total protein (Ponceau-S staining).

Experimental design

The experiments were performed according to protocol 124-01-08, as described by Hanekamp et al. (Hanekamp et al., 2003b). The mice (n=40) were bilaterally ovariectomized, and half of them were substituted with a subcutaneous placed 90-day time-release Medroxyprogesterone Acetate (MPA) pellet. After one week, the mice were exposed to 5 Gy of total body irradiation six hours prior to injection with

tumor cells. Irradiation was performed to improve attachment of tumor cells to peritoneal surfaces (Strobel et al., 1997). One mouse had died before this procedure. At t = 0, mice were intraperitoneally injected with 5 x 10^6 EV3, PRA-1, PRB-1 or PRAB-36 cells (n= 8 for each group, of which 4 with MPA substitution) in a volume of 300 μl of PBS. Seven animals were injected with 300 μl PBS only (of which 4 with MPA substitution).

Two mice died without any obvious reason, one at week 6 (EV3, no MPA substitution) and one at week 7 (AB-36, no MPA substitution) and were lost for further analyses. Also at week 7, one mouse was euthanized because of discomfort (PRAB-36 no substitution). This mouse was examined, but no signs could be found that its discomfort was caused by the experimental procedures. This animal was included in the final analyses.

At ten weeks, the remaining animals (n=36) were sacrificed. Upon decapitation, blood samples were taken. Subsequently, the peritoneal cavity was opened and examined macroscopically. Samples of all abdominal organs, as well as samples of the lungs, heart, brain and upper hind leg muscle were snap frozen in liquid nitrogen for DNA extraction. The remaining tissue was formalin-fixed and processed for routine histological examination (hematoxylin-eosin staining) and immunohistochemistry.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples of internal organs were sliced into 8 μm sections. Every tenth of these sections was routinely stained with hematoxylin and eosin, and examined microscopically. When tumor mass was detected, consecutive sections were fixed on polylysin-coated slides for immunohistochemical staining. Endogenous peroxidase activity was inhibited and a 10 mM citric acid/ microwave pre-treatment was applied. PBS containing 10% v/v normal swine serum (DAKO, Glostrup, Denmark) was used as a blocking buffer, followed by an additional avidin/biotin blocking step according to the manufacturers protocol.

Primary antibodies used were directed against PR (hPRa8; LabVision Neomarkers, Fremont, CA, USA) and against CD44 (DF1485; Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:200 in 1% blocking buffer. Negative control slides were incubated with 1% blocking buffer only. Antibodies were detected indirectly with biotin-labeled swine Multilink antibodies 1:400 in blocking buffer, followed by StreptABComplex (DAKO, Glostrup, Denmark), according to the manufacturer's protocol. Staining was developed with DAB/concentrated metal complex (Pierce, Rockford, IL, USA). Slides were counterstained with hematoxylin.

PCR detection

PCR detection of tumor cells is based on amplification of the hygromycin resistance (HR) gene, which is present on the vector that was transfected into the parental Ishikawa 3H12 clone, and was performed as described (Hanekamp et al., 2003b). The HR gene was amplified by nested PCR (forward primer: GTGGATATGTCCTGCGGGTAA, reverse primer: TCACTGCATTCTAGTTGTGG; nested

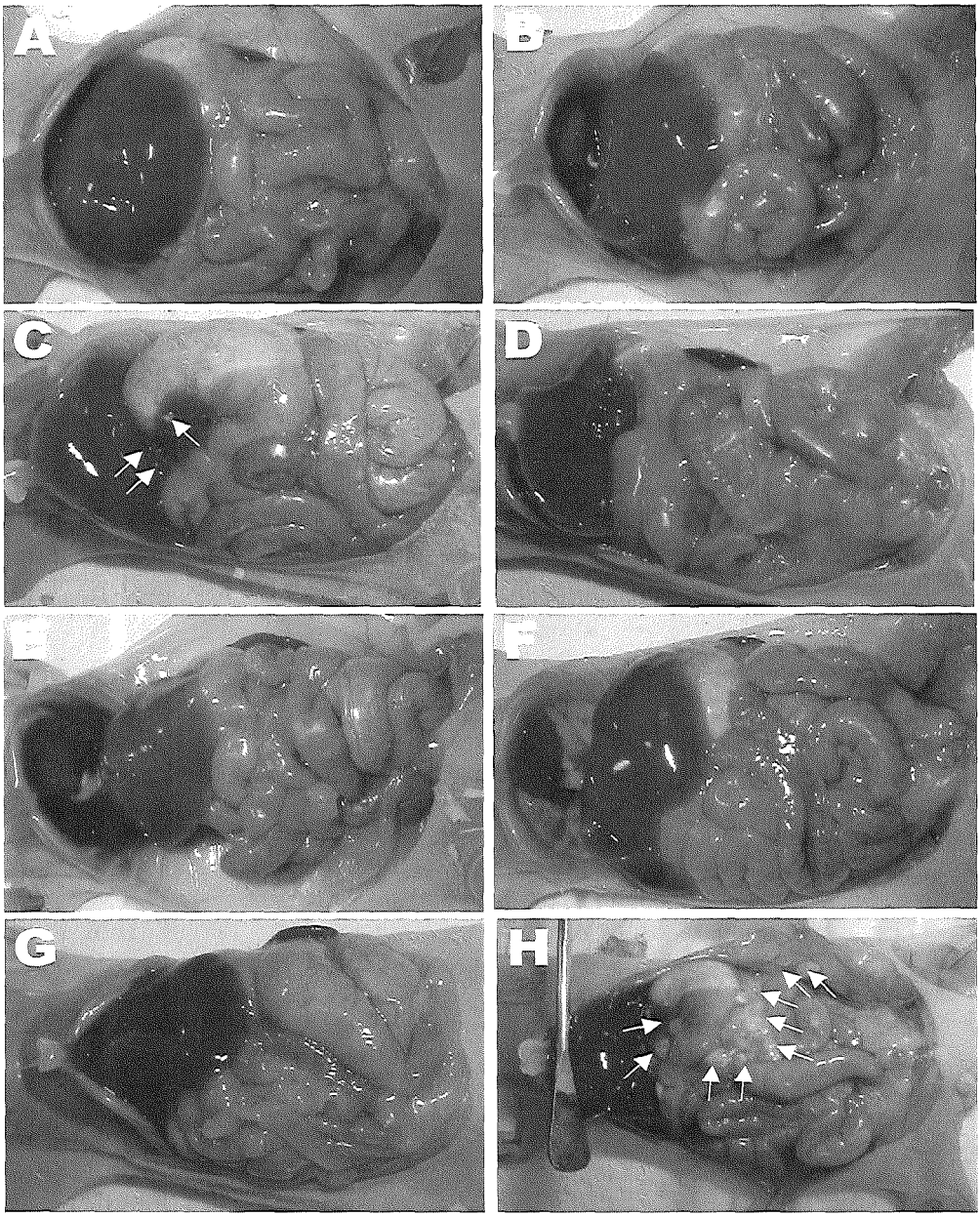

Figure 2. Macroscopic anatomy of mice representative of their group. A and B: PRA-1; C and D: PRB-1; E and F: PRAB-36; G and H: EV3. Mice without MPA substitution are shown on the left (panel A, C, E, G); mice with MPA substitution on the right (panel B, D, F, H). The massive abdominal tumor growth in the EV3 mice that were substituted with MPA is clearly visible (H, arrows). Small tumor spots are also visible in the PRB-1 without MPA (C, arrows). No tumor is visible in PRA-1 and PRAB-36 mice (A, B, E, F).

forward primer: CGCAAGGAATCGGTCAATACA, nested reverse primer: TCGTCCGATCCGGAGGAGCC). PCR detection was statistically analysed (Students T-test) using SPSS software. P-values <0.05 were considered significant.

RESULTS

Cell lines

Western blot confirmed the PR status of the cell lines that were used, at the time of injection (Figure 1). EV3 does not express PR, PRA-1 expresses only PRA, PRB-1 only PRB, and PRAB-36 expresses both PR isotypes (more PRB than PRA).

Macroscopic and microscopic detection of tumor mass

Upon macroscopic investigation of mice injected with the different cell lines, PRB-1 cells clearly produced the highest tumor load in absence of MPA substitution (Figure 2C, Table 1). Most tumor spots had a diameter of approximately 1 mm, but on the diaphragm of one mouse a tumor mass of approximately 4 mm was found. When PRB-1 injected mice were substituted with progestagens, tumor could only be detected in two animals (Figure 2D, Table 1). When mice were injected with PRA-1 or PRAB-36 cells, only in one animal distinct tumor mass could be detected. Substitution with MPA did not have a marked effect (Figure 2, Table 1).

For the mice that were injected with EV3 cells (which do not express PR), in the absence of progestagens tumor load was virtually undetectable (Figure 2G, Table 1), while substitution with MPA dramatically increased tumor burden (Figure 2H, Table 1). The EV3 tumors were larger than those in the other groups, with a diameter of approximately 3 mm on average. Also, these tumors appeared to be cystic, while PRA-1, PRB-1 and PRAB-36 tumors had a solid appearance (Figure 3A, inserts).

Histological findings

In general, tumor masses were detected growing loosely connected by stalk-like structures (Figure 3B, panels I, II, IV) and attached to abdominal surfaces (Figure 3B, panels II-VI). All tumors consisted of a non-differentiated mass (Figure 3A), with occasional regions that appeared more organized; virtually all tumors (originating from all four cell lines) contained blood vessels and lymphocytic infiltrations. The EV3 and PRB-1 tumors were found to be capable of invading into the pancreas (Figure 3C) and most EV3-tumors showed necrosis, leading to the formation of fluid-filled cavities (Figure 3A), which give these tumors a cystic appearance.

The expression of PR was conserved in the PRA-1, PRB-1 and PRAB-36 tumors (Figure 4 panels B, E, H), but was not homogeneous. This is illustrated in Figure 5, where three tumors from the same mouse (PRB-1, no MPA) were stained to detect expression of PR. Some tumors expressed PR in all cells (Figure 5A), but also heterogeneous staining throughout the tumor (Figure 5C), or regions of cells expressing PR next to regions that did not express PR (Figure 5E) were observed. All

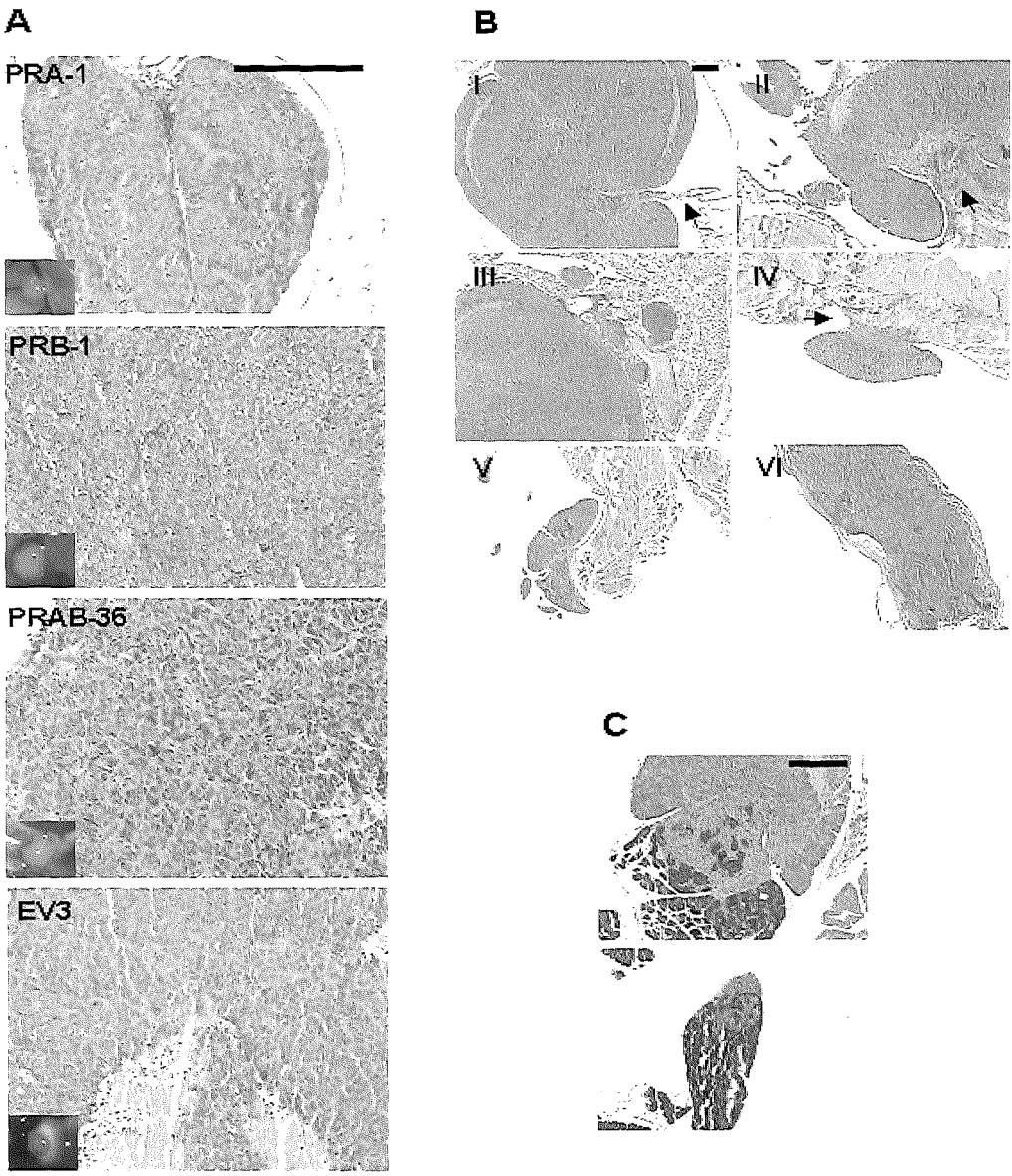

Figure 3. Histological comparison of tumor spots. Panel A: Hematoxylin-eosin stained sections of tumor spots originating from the different cell lines. Small inserts show the macroscopic appearance of the spot, microscopic pictures are (black bar represents 0.05 mm). Panel B: tumors were found growing from stalk-like structures (I and II, EV3 tumors), and attached to abdominal surfaces: III: EV3 tumor attached to fatty tissue; IV: EV3 tumor growing on ventral abdominal wall; V: PRB-1 tumor growing on ventral abdominal wall; VI: PRB-1 tumor growing on the diaphragm. Black bar represents 0.05 mm. Panel C: tumors originating from EV3 (upper panel) and PRB-1 (lower panel) cells were found to grow invasively into the pancreas. Black bar represents 0.05 mm.

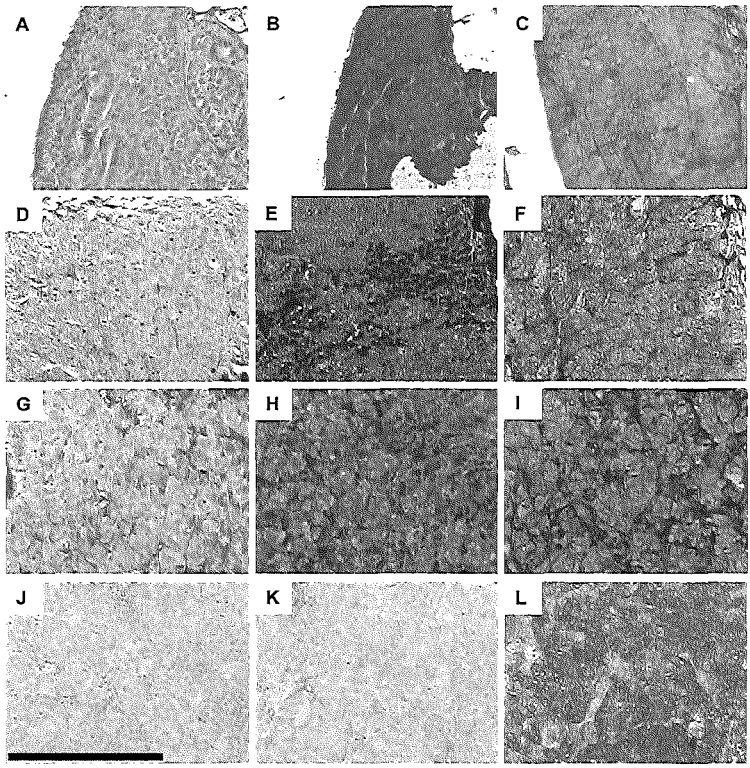

Figure 4.Progesterone receptor expression in tumors originating from different cell lines.
Panels A, B, C: EV3; Panels D, E, F: PRA-1; Panels G, H, I: PRB-1; Panels J, K, L: PRAB-36.
Immunohistochemical staining for both PRA and PRB to assess PR-status (middle panels) or CD44 to detect human cells (right panels). Negative control slides (no primary antibody) are shown on the left (left panels). Black bar represents 0.05 mm.

tumors expressed CD44 (Figure 4 C, F, I, L), which was used to make a distinction between mouse and human cells (using the current antibody, CD44 could be detected in the four human cell lines, but not in any mouse tissues).

Detection of circulating tumor cells

Detection of tumor cells by PCR was possible in all groups (Figure 6). Tumor cells could be detected predominantly in tissues of mice injected with PRB-1 cells without MPA substitution, and in mice injected with EV3 cells with MPA substitution. The number of tissue samples in which tumor cells could be detected was significantly higher in the group of animals injected with PRB-1 cells ($p < 0.05$, Figure 6E). While macroscopically and microscopically virtually no tumor mass could be detected in PRA-1 or PRAB-36 mice, tumor cells could be detected by PCR. Not surprisingly, tumor cells could be detected mostly in de abdominal tissues (panels A and B), but also outside the abdomen (panel C). In contrast, no tumor growth outside the abdominal cavity was observed macroscopically. Tumor cells were detected most often in samples of the diaphragm and the pancreas, but also in the lower intestine, the stomach and the mesentery, and less frequent in the spleen, upper intestine,

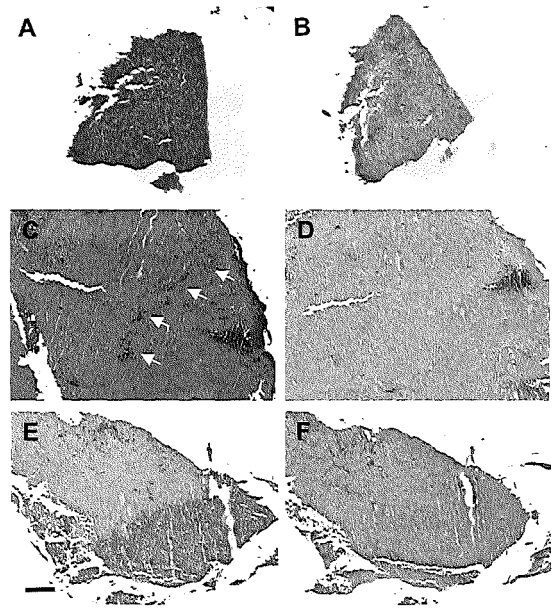

Figure 5. Variation in homogeneity of PR expression within different tumors in the same mouse. Patterns showing overall high expression of PR (panel A), overall low expression with regions of high expression (panel C, arrows indicate regions with high PR expression) or only partial expression of PR (panel E) in tumors taken from one PRB-1 mouse which was not substituted with MPA. Negative control slides are shown on the right (B, D, F). Black bar represents 0.05 mm.

uterus and even kidney. The different cell lines could generally be detected in the same tissues, with no targeting of a specific tissue by specific cell lines.

MPA inhibited spread of PRB-1 tumor cells ($p= 0.002$, Figure 6E), but this was not observed for PRA-1 or PRAB-36 cells. One possible explanation is that the number of positive tissues in the PRA-1 and PRAB-36 injected mice is already very low in the absence of MPA. Substitution of the mice with MPA did not lead to spread of tumor cells to other tissues than when these mice were not substituted with MPA. In mice injected with EV3 cells, MPA has a significant ($p= 0.001$, Figure 6E) tumor cell growth-stimulating effect in the peritoneal cavity: tumor cells could only be detected in the diaphragm of one out of three mice that were not substituted with MPA, while tumor cells could be detected in total 12 tissues from 3 out of 4 mice substituted with MPA (Figure 6). In all mice, no tumor cells could be detected in the blood ten weeks post injection (Figure 6D). Previously, we found that tumor cells could be detected in tissues at 3 and 10 weeks post injection, but not at 5 weeks (19). This suggests that early on, the cells are spread through the body but that most of these do not survive. Later on, the cells that did survive give rise to a secondary tumor that is large enough to be detected. The absence of circulating tumor cells in the blood confirms that the spread of tumor cells throughout the body is an event that occurs shortly after inoculation. Also, absence of tumor cells in the blood indicates that detection of cells in tissues is not due to contamination of these tissues with blood-borne tumor cells.

Table 1 summarizes in which tissues tumor mass was detected, either macroscopically, microscopically, or by PCR. To complete the overview, also macroscopically detected single spots and tumor clusters are included in Table 1. The macroscopically detected tumor masses were confirmed to be of Ishikawa origin

either by microscopical analysis or by PCR detection. Clusters of tumor (as indicated by the arrows in Figure 2H) were found only in the group of animals injected with EV3 cells which were treated with MPA. In several cases, tumor could be detected by PCR when macroscopically or microscopically no tumor was found, indicating the higher sensitivity of PCR detection.

DISCUSSION

In the present study we describe that when endometrial cancer cells that express different PR isotypes are injected intraperitoneally into nude mice, cells that express exclusively PRB produce the highest tumor load in absence of progestagens. Upon substitution with MPA, tumor outgrowth from PRB-expressing cells is inhibited, while PR-negative cells give rise to a massive abdominal tumor load.

Several studies have investigated the effect of progestagens on *in vitro* endometrial cancer cell invasion. It was shown that progestagens stimulated cell-to-cell aggregation (Fujimoto et al., 1996) and inhibited anchorage-independent growth (Dai et al., 2001) of endometrial cancer cells. Dai et al. (2002) also showed that MPA could inhibit invasion of Hec50 cells that had been virus-infected to transiently express PRA or PRB (Dai et al., 2002). This study did not demonstrate any difference in invasive capacity between cells that expressed different PR isotypes. In contrast, Hanekamp et al. (submitted) described that endometrial cancer cells that express PRB are more invasive *in vitro* than cells that express only PRA. In support of this, Fujimoto et al. (1997) reported that distant metastases of endometrial cancer predominantly expressed PRB.

Based on these findings, we expected tumor cells that express PRB to show more extensive growth and spread *in vivo* than cells that express exclusively PRA or no PR. Cells that express exclusively PRB indeed showed the most extensive macroscopically detectable abdominal tumor outgrowth when injected into ovariectomized nude mice. Upon closer examination of different tissues (microscopy, PCR detection), this finding could be further extended. These observations are in agreement with reports that distant metastases of endometrial cancer express predominantly PRB (Fujimoto et al., 1997), and with our own observations that PRB-1 cells are highly invasive *in vitro* (Hanekamp et al., submitted). As expected, growth and spread of PRB-1 cells was clearly inhibited by MPA substitution. This confirms previous findings in which progestagens inhibit growth and invasion of endometrial cancer cells (Dai et al., 2001; Dai et al., 2002; Fujimoto et al., 1996; Ueda et al., 1996). Injection of PRA-1 cells only resulted in minimal tumor growth and spread, which is also consistent with its minimal *in vitro* invasive capacity (Hanekamp et al., submitted). The PRAB-36 cell line expresses more PRB than PRA, and has an *in vitro* invasive capacity similar to that of PRB-1 cells. Therefore, we expected that this cell line would behave more like the exclusively PRB-expressing cell line than the exclusively PRA-expressing line.

EV3
PRA-1
PRB-1
PRAB-36
no MPA
MPA
no MPA
MPA
no MPA
MPA
no MPA
MPA
A
peritoneum
diaphragm
mesentery
B
stomach
upper intestine
lower intestine
liver
spleen
pancreas
uterus
kidney
C
lung
heart
brain
D
blood

E

	EV3	PRA-1	PRB-1	PRAB-36	no MPA vs MPA
EV3		0.308	0.000	0.284	0.001
PRA-1	0.308		0.005	0.893	0.322
PRB-1	0.000	0.005		0.014	0.002
PRAB-36	0.284	0.893	0.014		0.160

Figure 6. PCR detection of tumor cells in tissues by PCR amplification of the hygromycin resistance gene. The tissues have been divided into different groups as indicated on the left side of the figure. A: peritoneal membranes; B: abdominal organs; C: extra-abdominal organs; D: blood. During processing of tissues, 6 samples were lost. These are indicated by the gaps in the figure. Panel E: Statistical analysis of spread of tumor cells as detected by PCR. Numbers of positive tissues per group were compared using Students T-test. P-values are shown of differences between different groups in absence of MPA treatment, and of the effect of MPA treatment within each group.

However, injection of PRAB-36 cells did not result in tumor growth and spread similar to PRB-1 cells, but similar to PRA-1 cells. While this behavior of PRAB-36 cells was not as predicted, the observed minimal tumor growth and spread does represent the behavior of such an endometrial cancer in patients: endometrial cancers that express both PRA and PRB are often well-differentiated and stay primarily confined to the uterus.

In the present study, inoculation of PR-negative endometrial cancer cells in MPA substituted mice resulted in massive abdominal tumor growth. This is in contrast to the in vitro situation, where it was observed that these PR-negative cells did not show any response to MPA stimulation. On the basis of this observation, it is thought that MPA acts on the PR-negative cells through the tumor-bearing host. MPA treatment of the mice probably results in a favorable environment for attachment and growth of the injected cells. Because no stimulation of PRA-1, PRB-1 or PRAB-36 cells was observed in the mice, the inhibiting effect of MPA on these cells is most likely dominant over any secondary stimulating effect of MPA through the host. Based on these observations, the hypothesis is the following: On the one hand, MPA provides a favorable environment for attachment and growth of endometrial cancer cells via the host, but on the other hand, it inhibits growth of PR-positive tumor cells directly.

An intriguing question becomes how the present findings relate to the situation in the clinic. Since endometrial cancer is predominantly a disease of post-menopausal women, patients generally have very low endogenous levels of circulating progestagens. In clinical practice many women with advanced or recurrent endometrial cancer are treated with progestagens on the basis that this will inhibit growth of the cancer and will generally improve well-being of the patient.

Table 1

	Macroscopy	Microscopy	PCR
EV3 (n=3)		peritoneum (1/3)	peritoneum (1/3)
EV3 + MPA (n=4)	peritoneum (3/4) diaphragm (3/4) mesentery (3/4) pancreas (2/4) spleen (1/4) single spots (4/4)[1] clusters of tumor (2/4)[1]	peritoneum (3/4) diaphragm (3/4) mesentery (3/4) pancreas (2/4)	peritoneum (1/4) diaphragm (2/4) mesentery (2/4) pancreas (3/4) spleen (1/4) lower intestine (1/4) uterus (2/4)
PRA-1 (n=4)	peritoneum (1/4) mesentery (1/4)	 mesentery (1/4)	peritoneum (1/4) diaphragm (1/4) stomach (1/4) brain (1/4)
PRA-1 + MPA (n=4)	peritoneum (1/4)	peritoneum (1/4) mesentery (2/4)	 mesentery (1/4) pancreas (1/4) liver (1/4) lower intestine (2/4) uterus (1/4)
PRB-1 (n=4)	peritoneum (2/4) diaphragm (1/4) mesentery (3/4) spleen (1/4) single spots (4/4)[1]	peritoneum (3/4) diaphragm (2/4) mesentery (2/4) pancreas (1/4) spleen (1/4) lymphe node (1/4)[2]	peritoneum (1/4) diaphragm (3/4) mesentery (2/4) pancreas (2/4) spleen (1/4) stomach (1/4) liver (1/4) upper intestine (1/4) uterus (1/4) kidney (1/4) lung (1/4)
PRB-1 + MPA (n=4)	peritoneum (1/4)	peritoneum (1/4) pancreas (1/4)	peritoneum (1/4) pancreas (1/4) stomach (3/4) upper intestine (1/4) heart (1/4)
PRAB-36 (n=4)	peritoneum (1/4)	 diaphragm (1/3)[2]	peritoneum (1/3) pancreas (1/3) spleen (1/3) lower intestine (1/3)
PRAB-36 + MPA (n=3)	diaphragm (2/4)	diaphragm (1/4)	diaphragm (1/4) pancreas (1/4) lower intestine (2/4) kidney (1/4)

Table 1. (previous page) Mouse tissues in which tumor was detected. Tumor was detected either on macroscopical investigation, on microscopical investigation or by PCR detection. Numbers in brackets indicate in how many of the animals tumor was found in that tissue, out of the total number of animals in that group. Single spots are defined as small (<1 mm), but distinct tumor patches on the serosa covering intraperitoneal organs. Clusters of tumor are defined as a collection of multiple interconnected tumors (on average 3 mm in diameter). These were found only in mice injected with EV3 cells which were treated with MPA (see Figure 2H, white arrows).
[1] Single spots and clusters of tumor do not qualify as mouse tissue. However, to complete the overview of tumor growth, also these macroscopically detected single spots and tumor clusters have been included in this table.
[2] These tumor masses were detected only after microscopy. In the corresponding tissue that was processed for PCR detection, no further tumor mass was detected.

In fact, 20% of patients with advanced or recurrent endometrial cancer do respond to MPA therapy (Elit and Hirte, 2002). The results of our study lead to the question whether the remaining 80% of these tumors really show no response, or if tumor growth may in fact be stimulated by progestagen therapy? At this moment, we cannot answer this question. However, if indeed progestagens indirectly stimulate growth of PR-negative endometrial neoplasias, the practice of administering progestagens to endometrial cancer patients regardless of their progesterone receptor status should be re-evaluated.

In summary, the results of this study support the idea that loss of expression of especially PRA is associated with a more aggressive type of endometrial cancer. Furthermore, treatment with MPA seems to inhibit growth and spread of PR-positive tumors. However, when expression of both PRA and PRB is lost, treatment with MPA may provide a favorable environment for attachment and growth of endometrial cancer cells. Because of possible implications for patient treatment, the dynamics of this phenomenon need to be further investigated, preferably with an *in vivo* monitoring system.

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CHAPTER 5

MEDROXYPROGESTERONE ACETATE INHIBITS WNT SIGNALING IN ENDOMETRIAL CANCER CELLS

Medroxyprogesterone Acetate inhibits Wnt signaling in endometrial cancer cells

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ABSTRACT

Wnt signal transduction plays a major role during development in mammals, and is also implicated in carcinogenesis. In endometrial cancer, nuclear localization of ß-catenin, which is a hall-mark of active Wnt signaling, and abnormalities in Wnt signaling have been described. Progestagens have a anti-proliferative effects on endometrial cancer. In the present study, we have investigated the effect of the progestagen Medroxyprogesterone Acetate (MPA) on Wnt signaling in endometrial cancer cells. It was observed that MPA regulates a number of Wnt target genes in progesterone-responsive endometrial cancer cells, in a way that is consistent with inhibition of Wnt signaling. By utilizing the TOPflash/FOPflash reporter system, it was found that MPA inhibited TCF/ß-catenin-mediated transcription activation. Inhibition of proteoglycan synthesis was found to inhibit TCF/ß-catenin signaling. Our results indicate that in progesterone-responsive endometrial cancer cells, MPA inhibits Wnt signaling through modulation of gene transcription, and that possibly proteoglycans can play a role in this process.

INTRODUCTION

Endometrial cancer is one of the most common cancers in women. One of the features of more advanced endometrial cancer is loss of expression of progesterone receptors (PR). This renders the tumor less responsive to the growth-inhibiting effects of progestagens, and results in development of a more aggressive tumor (Southcott, 2001). The PR exists as two isotypes, PRA and PRB. PRA is a truncated from of PRB, lacking 164 amino acids at the N-terminus. Upon binding of ligand, PRA and PRB can regulate transcription of the same and of different genes (Giangrande and McDonnell, 1999). Additionally, PRA has been described to be a repressor of PRB-mediated transcription activation (Giangrande et al., 1997). Besides directly modulating the transcription of certain genes, the PR has also been reported to induce non-genomic effects. Through SH3 domains, PR can interact with the Src/Ras/Raf/MAPK signal transduction pathway (Boonyaratanakornkit et al., 2001; Edwards et al., 2003). Additionally, Migliaccio et al (1998) reported that PRB can activate the Src/p21^{ras}/Erk pathway through crosstalk with estrogen receptors (Migliaccio et al., 1998). In contrast to the genomic effects, these interactions are thought to take place in the cytoplasm rather than in the nucleus.

The Wnt pathway is one of the most important signal transduction pathways during development of a wide variety of organisms, ranging from *Drosophila* to *Homo sapiens*. In short, the classical Wnt pathway is described as follows. Secreted Wnt protein binds to its receptor frizzled (Fz) in the plasma membrane. Through a cascade of events, including activation of disheveled (Dsh) and inactivation of GSK3ß, ß-catenin is freed from its degradation complex (Aberle et al., 1997). Instead of being degraded, ß-catenin can now translocate to the nucleus, through

mechanisms that are as yet not clear. In the nucleus, ß-catenin functions as a co-factor for the transcription factor TCF/LEF, resulting in transcription of Wnt target genes (Novak and Dedhar, 1999). Nuclear accumulation of ß-catenin is considered a hallmark of active Wnt signaling (Giles et al., 2003).

Aberrant Wnt signaling has been implicated in carcinogenesis and tumor progression in mammals (Barker and Clevers, 2000; Giles et al., 2003; Lustig and Behrens, 2003). Mutations in components of the Wnt pathway have been detected in endometrial cancer, and nuclear accumulation of ß-catenin can be observed in early stages of endometrial cancer (Ashihara et al., 2002; Fujimoto et al., 1998; Fukuchi et al., 1998; Machin et al., 2002; Moreno-Bueno et al., 2001; Moreno-Bueno et al., 2002; Schlosshauer et al., 2000). Nei et al. (1999) studied localization of ß-catenin in normal and neoplastic endometrium (Nei et al., 1999). Their results indicated that although Wnt signaling is often active in endometrial carcinogenesis, it can also be activated under physiological conditions. Moreno-Bueno et al. (2002) studied abnormalities in Wnt signaling in endometrial cancer, and found mutations in the ß-catenin gene *CTNNB1* in 14.9% of a set of endometrial carcinomas (Moreno-Bueno et al., 2002). Mutations in *CTNNB1* are found mainly in exon 3, and these mutations lead to stabilization of the ß-catenin protein (Fukuchi et al., 1998). All tumors that contained *CTNNB1* mutations demonstrated nuclear localization of ß-catenin, but this was also found in tumors that did not contain *CTNNB1* mutations (Moreno-Bueno et al., 2002). This indicates that there are additional mechanisms by which ß-catenin can accumulate in the nucleus. While several reports mention that nuclear localization of ß-catenin can be found in endometrial hyperplasias (Ashihara et al., 2002; Moreno-Bueno et al., 2001; Nei et al., 1999), mutations in *CTNNB1* have been found only in endometrial cancers, and not in associated hyperplasias. This indicates that mutations of *CTNNB1* occur early in carcinogenesis (Ashihara et al., 2002).

Recently, we have shown that the progestagen Medroxyprogesterone Acetate (MPA) down-regulates a panel of Wnt target genes in a progesterone-responsive endometrial cancer cell line (Hanekamp et al., 2003). This observation suggests that MPA inhibits Wnt signaling in these cells. Furthermore, we observed that endometrial cancer cells that express PRB are more invasive *in vitro* than cells that express only PRA or no PR (unpublished results), and that invasion can be inhibited by MPA. In the present study, we set out to test the hypothesis that Wnt signaling is activated by unliganded PRB and can be inhibited by progestagen action.

MATERIALS AND METHODS

Cell culture

Ishikawa endometrial cancer cells (clone 3H12, further referred to as IKpar) were obtained from Dr M. Nishida (Nishida et al., 1996), and maintained in DMEM/F12 (GibcoBRL/ LifeTechnologies, Carlsbad, CA, USA), supplemented with 10% v/v fetal calf serum (FCS; GibcoBRL) and penicillin/streptomycin. The cell lines that are used are IKpar cells that have been stably transfected with either hPRA (lines PRA-1, PRA-10), hPRB (lines PRB-1, PRB-23), both receptors (lines PRAB-18, PRAB-36), or empty vector (line EV8), as previously described by Smid-Koopman et al. (Smid-Koopman et al., 2003) These cell lines were maintained under constant selection pressure through addition of neomycin (500 μg/ml; ICN Biomedicals, Costa Mesa, CA, USA) and hygromycin (250 μg/ml; Invitrogen Corporation, Carlsbad, CA, USA; not for the EV8 line) to the media.

Compounds

Medroxyprogesterone Acetate (MPA), 4-methylumbelliferyl 7-ß-D-xyloside (xyloside), and chondroitinase ABC (CHase) were obtained from Sigma Chemical Co (St. Louis, MO, USA). RU486 was a gift from Roussel Uclaf (Romainville, France), and Org31489 was provided by Organon NV (Oss, The Netherlands).

Northern blot

Cells were cultured as described, in the presence of 100 nM MPA for 0, 24, or 48 hours. RNA was isolated as described by Auffray and Rougeon (Auffray and Rougeon, 1980), separated on a 1.5% w/v agarose gel and blotted onto Hybond membrane (Amersham Biosciences Europe, Roosendaal, The Netherlands). The following ^{32}P-dATP-labeled cDNA probes were hybridized to the blots: CD44: image713145; MMP7: image3545760; claudin-1: image4857809; VEGF: image34778; follistatin: image124543. Clones were obtained from RZPD (Heidelberg, Germany). Equal loading was verified by ethidium bromide staining.

Western blot

Cells were cultured for the indicated times, in culture medium containing 5% v/v dextran charcoal treated FCS (DCC-FCS), in absence or presence of 100 nM MPA. Cells were lyzed with lysis buffer (40 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10% v/v glycerol, 10 mM sodiumphosphate, 10 mM sodiummolybdate, 50 mM NaF, 0.5 mM sodium orthovanadate), containing 10 mM DTT, 1% v/v Triton X-100, 0.08% w/v SDS, 0.5% v/v DOC and 1x Complete protease inhibitors (Roche, Basel, Switzerland). The lysates were centrifuged at 13000 rpm for 15 minutes, after which the pellet was discarded. Protein samples were separated on polyacrylamide gels, and transferred onto nitrocellulose membrane. For detection of APC, lysates were separated on 3% w/v agarose gels (low melting point agarose in TBE/0.1% SDS), and transferred to nitrocellulose membrane by downward capillary transfer. The blots were incubated with the following antibodies: ß-catenin: E-5 (mouse); axin: H-

98 (rabbit); GSK3ß, 0011-A (mouse); E-cadherin, G-10 (mouse); jun: H-79 (rabbit); cyclinD1: A-12 (mouse; all obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA); APC: AB-1 (mouse; Oncogene Research Products, San Diego, CA, USA); PR, hPRa8 (mouse; Labvision Neomarkers, Fremont, CA, USA).

Immunohistochemical staining

Cells were cultured on glass coverslips. Monolayers were fixed in methanol at –20°C for 20 minutes, after which the cell layers were allowed to dry at room temperature. For immunohistochemistry, the coverslips were pretreated with 4% v/v H_2O_2 in methanol for 20 minutes, before being preincubated with 10% w/v bovine serum albumine in PBS. Incubation with primary antibody (diluted 1:200 in PBS/ 1% w/v BSA) occurred at 4°C overnight. Primary antibodies used were: ß-catenin: Ab-1 (rabbit, Labvision Neomarkers); axin: H-98 (rabbit); APC: C-20 (rabbit); GSK3ß: 0011-A (mouse) (all from Santa Cruz Biotechnology). Excess primary antibody was washed away, followed by a second preincubation with 10% v/v normal goat serum in PBS. The coverslips were then incubated with secondary antibody (either biotin-labeled goat-anti-mouse or FITC-labeled goat-anti-mouse or goat-anti-rabbit (DAKO, Glostrup, Denmark) for 1 hour at room temperature. After repeated washing in PBS, coverslips were counterstained and mounted (cell-side down) on glass slides with Vectashield/DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

Co-immunoprecipitation

Monolayers were washed twice with PBS, and cells were scraped in ice-cold PBS and centrifuged at 800 rpm at 4°C. The pelleted cells were resuspended in 120 µl buffer A (0.1% NP40, 1 mM EDTA, 20 mM Tris, 25% v/v glycerol, 0.3 M NaCl, 20 mM $Na_2MoO4.2H_2O$), and lyzed by repeated freezing in liquid nitrogen and thawing. Lysates were centrifuged for 15 min at 13,000 rpm at 4°C. Anti-mouse or anti-rabbit agarose beads were coated with the precipitating antibody by rotating incubation with 1 µl antibody per 50 µl beads and 150 µl PBS for 3 hours at 4°C. Antibodies used were: ß-catenin: Ab-1 (rabbit, Labvision Neomarkers); PR: hPRa8 (mouse, Labvision Neomarkers); axin: H-98 (rabbit, Santa Cruz Biotechnology). Coated beads were washed with PBS, and incubated with 50 µl lysate in 150 µl buffer B (0.1% v/v NP40, 1 mM EDTA, 20 mM Tris, 25% v/v glycerol, 20 mM $Na_2MoO4.2H_2O$) overnight at 4°C. The beads were subsequently washed 5 times with washing buffer (0.1% v/v NP40, 1 mM EDTA, 20 mM Tris, 25% v/v glycerol, 0.1 M NaCl, 20 mM $Na_2MoO4.2H_2O$). Precipitated protein was extracted from the beads by boiling in Laemmli sample buffer for 2 minutes. When cell culture conditions included MPA, this ligand was also added to all steps during co-immunoprecipitation. Precipitated proteins were detected on Western blot as described.

TCF reporter assay

Wnt signaling activity was estimated using the TOPflash reporter assay. The TOPflash vector contains three TCF binding sites upstream of a minimal promoter. The FOPflash vector contains mutated TCF binding sites and functions as a control

for the TOPflash vector. The TOPflash/FOPflash signal ratio is a measure for Wnt signaling activity (Korinek et al., 1997). Ishikawa EV8 cells were seeded in 48-wells plates 24 hours prior to transfection. Cells were transfected with 150 ng/well TOPflash or FOPflash reporter (Upstate, Waltham, MA, USA). Simultaneously, cells were transfected with varying amounts of pcDNA3.1 vectors containing either PRA or PRB, or empty vector, using FuGene transfection reagent (Roche Diagnostics, Almere, The Netherlands), according to the manufacturers' protocol. After 24 hours, hormones were added. Reporter luciferase activity was measured 72 hours post transfection with SteadyGlo reagents (Promega, Leiden, The Netherlands), according to the manufacturers' protocol.

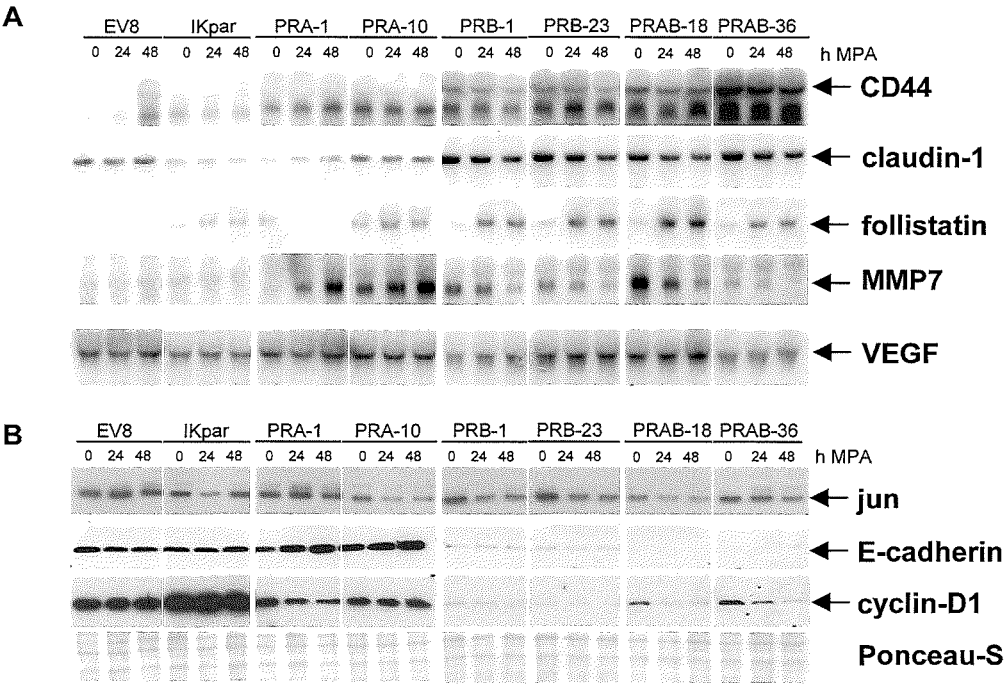

Figure 1. Regulation of expression of Wnt target genes by MPA in Ishikawa cells. Cells expressing no PR (EV8, IKpar), only PRA (PRA-1, PRA-10), only PRB (PRB-1, PRB-23), or PRA and PRB (PRAB-18, PRAB-36) were cultured in presence of 100 nM MPA for 0, 24 or 48 hours. Panel A: Northern blots showing expression of CD44, MMP7, VEGF, follistatin, and claudin-1 mRNAs. Panel B: Western blots showing expression of jun, E-cadherin and cyclin-D1 proteins. Loading is verified by total protein staining (Ponceau-S).

RESULTS

MPA regulates transcription of Wnt target genes in Ishikawa sub-cell lines

To study the regulation of Wnt target genes by MPA in Ishikawa cells, expression levels of cyclin-D1 (Shtutman et al., 1999), MMP7 (Crawford et al., 1999), CD44 (Wielenga et al., 1999), claudin-1 (Miwa et al., 2000), follistatin (Willert et al., 2002), VEGF (Zhang et al., 2001), jun (Mann et al., 1999), and E-cadherin (Jamora et al., 2003) were examined in eight Ishikawa sub-cell lines. Northern and Western blotting showed down-regulation by MPA of CD44, claudin-1, jun, and cyclin-D1 (Figure 1) in PR-expressing cells. E-cadherin was up-regulated by MPA in PRA-1 and PRA-10 cells (Figure 1B), while regulation of expression of MMP7 was different through PRA and PRB: MPA up-regulates expression of MMP7 through PRA, but down-regulates expression of MMP7 through PRB (Figure 1A). Expression of follistatin was induced by MPA, while regulation of VEGF could not be detected (Figure 1A). Over all, MPA appears to inhibit Wnt signaling (Figure 1).

Subcellular localization of ß-catenin in Ishikawa sub-cell lines

Nuclear accumulation of ß-catenin is considered a hallmark of active Wnt signaling. Therefore, we measured the influence of MPA on subcellular ß-catenin localization. Immunohistochemical staining of cells stably expressing PRA, or PRB, or both receptor isotypes showed that ß-catenin was accumulated in the nucleus in cells expressing PRB (PRB-1 and PRAB-36), in absence of MPA (Figure 2A).

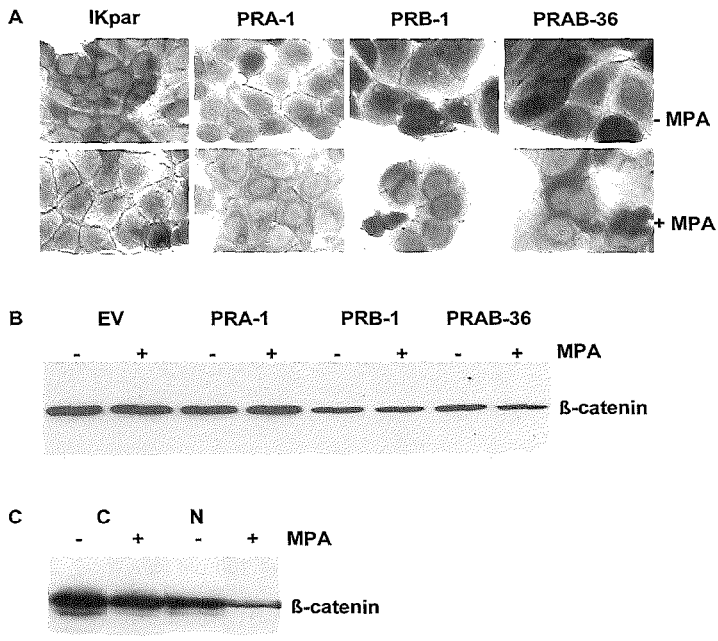

Figure 2. Localization of ß-catenin in Ishikawa cell lines. Panel A shows immunohistochemical detection of ß-catenin in Ishikawa cell lines IKpar, PRA-1, PRB-1 and PRAB-36. Cells were cultured for 72 hours in presence of 100 nM of MPA, or vehicle (ethanol) only. Panel B: Western blot, indicating that total protein expression of ß-catenin in these cells is not influenced by MPA. Panel C represents detection of ß-catenin on a Western blot of cytosolic (C) and nuclear (N) protein fractions in PRB-1 cells.

Upon stimulation with MPA, ß-catenin moved out of the nucleus. Nuclear accumulation of ß-catenin was not clear in cells expressing only PRA (PRA-1) or no PR (IKpar). Figure 2B shows that total ß-catenin expression in all sub-cell lines is not influenced by MPA, and in Figure 2C the MPA-induced decrease in ß-catenin expression in the nuclear fraction of PRB-1 cells is shown on Western blot.

MPA does not influence interaction of PRB with components of the ß-catenin degradation complex

Figure 2 indicates that MPA influences subcellular ß-catenin localization in PRB-expressing cells. A possible mechanism may involve a physical interaction of PR with components of the Wnt signaling pathway.

First we measured whether MPA affected expression of components of the ß-catenin degradation complex. No regulation of total protein levels of APC, axin or GSK3ß by MPA could be shown (Figure 3). Also, no alterations in subcellular localization of these components could be detected (Figure 3B). This indicated that any effect of MPA on Wnt signaling does not occur through an effect on ß-catenin degradation by a direct effect on expression or localization of components of the ß-catenin degradation complex.

In view of our working hypothesis that unliganded PR has a function as stimulator of cytoplasmic components of the Wnt signaling cascade, we performed experiments to show binding of PR to components of the ß-catenin degradation

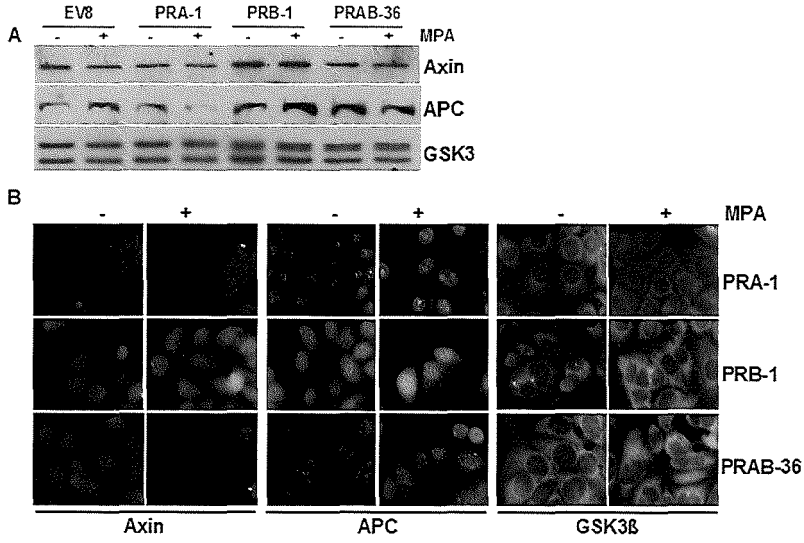

Figure 3. Expression of APC, Axin and GSK3ß in Ishikawa cells is not influenced by MPA. Panel A: Detection of APC, axin, and GSK3ß on Western blots of total cell lysates of Ishikawa cells cultured for 72 hours in absence or presence of 100 nM MPA. Panel B: immunofluorescent detection of APC, axin, and GSK3ß in Ishikawa cells cultured on glass coverslips for 72 hours in absence or presence of 100 nM MPA.

complex. Immunoprecipitation of PR and associated proteins, followed by immunodetection of ß-catenin, did not show a direct binding of PR with ß-catenin (Figure 4A). Also, binding of PR with axin, APC or GSK3ß could not be detected (not shown). We did find co-immunoprecipitation of E-cadherin with ß-catenin in IKpar and PRA-1 cells, but not in PRB-1 cells (Figure 4B). This indicates that in IKpar and PRA-1 cells, ß-catenin is participating in cell-cell adhesion, but that this is not the case in PRB-expressing cells.

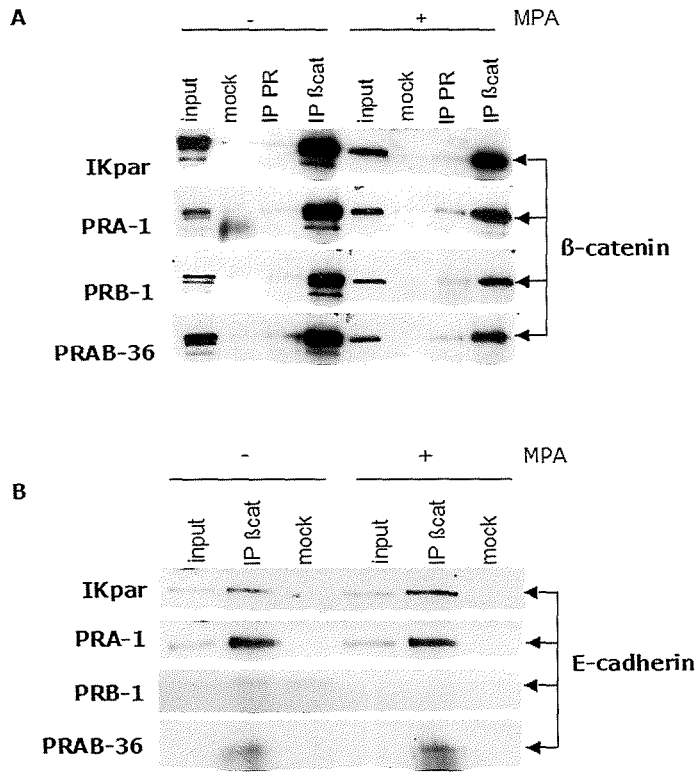

Figure 4. Co-immunoprecipitation assay in Ishikawa cell lines. Panel A: immunoprecipitation of PR or ß-catenin (positive control) from total protein lysates of Ishikawa cells, followed by detection of ß-catenin on Western blot. Panel B: Immunoprecipitation of ß-catenin, followed by detection of E-cadherin on Western blot. Control immunoprecipitation with anti-mouse agarose beads that were not coated with primary antibody is indicated with: mock.

Effect of MPA on TCF/ß-catenin signaling in Ishikawa cells

MPA regulates a number of Wnt target genes (Figure 1). To study the effect of MPA on Wnt signaling in a more direct manner, the TOPflash/FOPflash reporter system was used (Korinek et al., 1997). Effective transfection of TOPflah and FOPflash vectors into the stable cell lines PRB-1 and PRAB-36 was unsuccessful, and consequently we have no data about any effects of MPA on the reporter system in these cells. Therefore, PR constructs were transiently introduced into EV8 cells, simultaneously with the TOPflash/FOPflash reporter constructs.

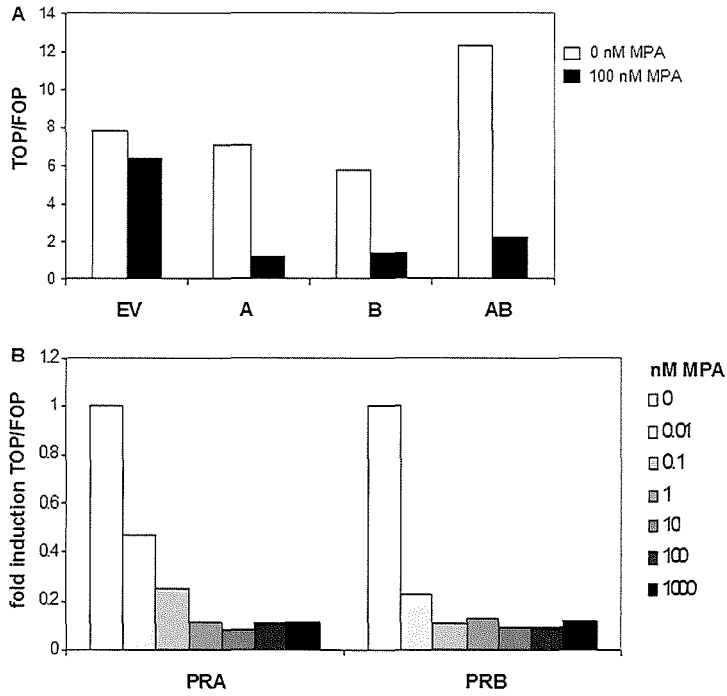

Figure 5. Effect of MPA on TOPflash reporter signal. In all assays, 150 ng/well reporter was transfected. Reporter signal was measured 72 hours post transfection. Panel A: EV8 cells were transfected with 50 ng/well empty vector (EV), PRA (A), PRB (B), or PRA + PRB (AB). Cells were treated with 100 nM MPA (black bars) or vehicle (ethanol, white bars). Y-axis represents the ratio of TOPflash reporter and FOPflash control signal. Panel B: EV8 cells were transfected with 10 ng/well PRA or PRB, and treated with increasing concentrations of MPA (gray bars). Y-axis represents fold induction of the TOPflash/FOPflash ratio compared to vehicle alone (white bars).

Introduction of PR-encoding constructs did not substantially alter the TCF/ß-catenin reporter signal in absence of ligand (Figure 5a). MPA inhibited TCF/ß-catenin-mediated reporter transcription via progesterone receptors. However, we did not see any difference in effect of 100 nM MPA in cells transfected with only PRA, or only PRB, or with both receptor isotypes (Figure 5A). At lower concentrations of MPA, inhibition of TCF/ß-catenin signaling is somewhat more pronounced in cells transfected with PRB than with PRA (Figure 5B).

Effect of PR antagonists on TCF/ß-catenin signaling in Ishikawa cells

PR antagonists bind the PR, which results in nuclear localization of the PR, but this will not result in gene transcription activation. If inhibition of Wnt by MPA is a result of relocalization of PRs, PR antagonists should also inhibit the TCF/ß-catenin reporter signal. On the other hand, if inhibition of Wnt by MPA is a result of modulation of gene transcription, incubation with PR antagonists should not inhibit the TCF/ß-catenin reporter signal.

It was observed that incubation with the PR antagonists RU468 and Org31489 resulted in relocalization of PR to the nucleus (Figure 6B), but did not inhibit the TCF/ß-catenin reporter signal in cells transfected with PRA (Figure 6A). In cells transfected with PRB, a slight decrease in the reporter signal was observed, but this effect was not nearly as strong as the effect of MPA (Figure 6A). These data indicate that inhibition of Wnt signaling is not the result of relocalization of PR, but is the result of modulation of gene transcription.

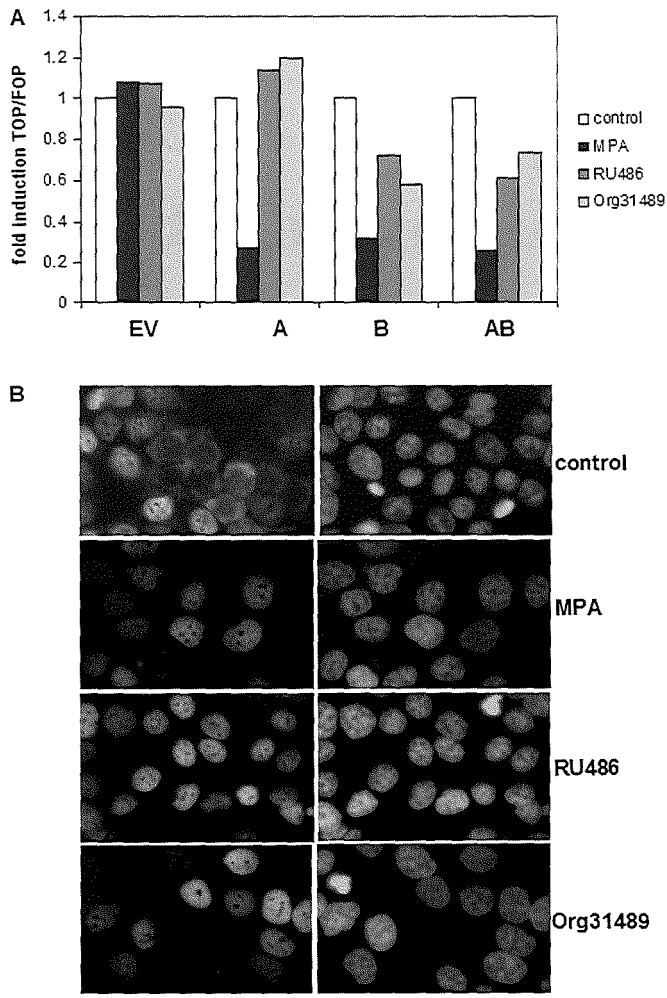

Figure 6. Effect of antiprogestins on TCF/ß-catenin signaling. In all assays, 150 ng/well reporter was transfected. Reporter signal was measured 72 hours post transfection. Panel A: EV8 cells were transfected with 10 ng/well empty vector (EV), PRA (A) or PRB (B), or both (AB), and treated with MPA, RU486 or Org31489 (all 100 nM). Y-axis represents fold induction of the TOPflash/FOPflash ratio compared to vehicle alone (white bars). Panel B: incubation of PRB-1 cells for only one hour with MPA, RU486 or Org31489 (all 100 nM) resulted in rapid translocation of PRB from the cytosol to the nucleus. Left panels: PRB; right panels: DAPI nuclear stain.

Effect of inhibiting proteoglycan expression on TCF/ß-catenin signaling

In *Drosophila* and in mouse, proteoglycans have been shown to play a role in activating Wnt signaling (Alexander et al., 2000; Tsuda et al., 1999). Furthermore, we have shown that MPA down-regulates expression of several proteoglycans in Ishikawa cells (Hanekamp et al., 2003). If in these cells proteoglycans are involved in Wnt signaling, the observed inhibition of Wnt signaling by MPA could be explained, at least in part, by MPA-induced inhibition of proteoglycan synthesis.

When cells in which the TOPflash reporter system was transfected were treated with xyloside, an inhibitor of proteoglycan synthesis that prevents assembly of glycosaminoglycan side chains to the proteoglycan protein core, the reporter

signal decreased (Figure 7A). This indicates that Wnt signaling can be affected by proteoglycans in these cells. Treatment with xyloside decreased the TOPflash signal in a concentration-dependent manner. Additional treatment with chondroitinase ABC (an enzyme which breaks down specifically chondroitin sulfate proteoglycans) did not have an additional inhibiting effect (Figure 7B). These results indicate that proteoglycans can play a role in Wnt signaling in Ishikawa cells, and inhibition of proteoglycan synthesis by MPA might be a mechanism by which MPA inhibits Wnt signaling.

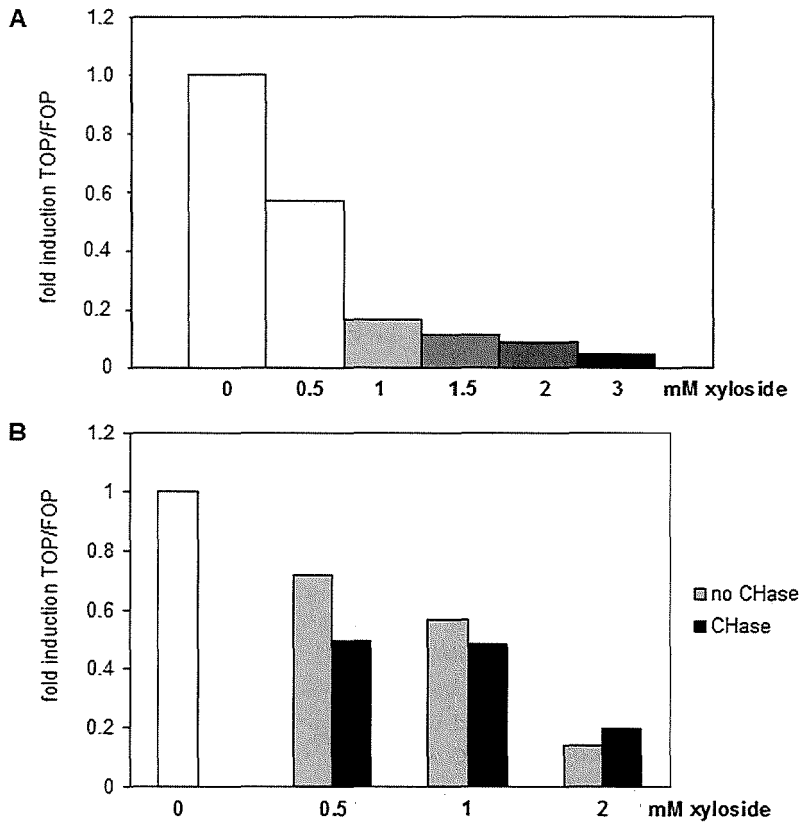

Figure 7. Effect of changes in proteoglycan synthesis on TCF/ß-catenin signaling. EV8 cells were transfected only with TOPflash or FOPflash vectors (150 ng/well). Reporter signal was measured 72 hours post transfection. Panel A: cells were treated with increasing concentrations of xyloside (gray bars). Y-axis represents fold induction of the TOPflash/FOPflash ratio compared to vehicle (DMSO) alone (white bar). Panel B: 24 Hours post transfection, 20 µl of 2 units/ml chondroitinase ABC (CHase, 0.04 units in total) was added, followed after 2 hours by 0.5, 1 or 2 mM xyloside. Y-axis represents fold induction of the TOPflash/FOPflash ration compared to vehicle (DMSO) alone (white bar).

DISCUSSION

MPA affects expression of Wnt target genes

Modifications in Wnt signaling play an important role in cancer development, including endometrial carcinogenesis. We have previously observed that the progestagen MPA inhibits expression of a panel of Wnt target genes in the Ishikawa endometrial cancer cell line PRAB-36 (Hanekamp et al., 2003). Because of the importance of Wnt signaling in endometrial cancer, we further investigated this effect of MPA on Wnt signaling.

In the present study, MPA was found to regulate expression of Wnt target genes. CD44, claudin-1, cyclinD1, and jun are down-regulated by MPA, while E-cadherin is up-regulated in cells expressing PRA. This is compatible with a down-regulation of Wnt signaling. In contrast, MPA up-regulated expression of follistatin. Interestingly, MPA down-regulates MMP7 through PRB, which is in agreement with inhibition of Wnt signaling, but an up-regulation of MMP7 in cells expressing only PRA was observed. So, even though observations on regulation of Wnt target genes by MPA in endometrial cancer cells are in some aspects inconsistent, the overall pattern points to inhibition of Wnt signaling by MPA.

MPA inhibits TCF/ß-catenin signaling

In Ishikawa cells, MPA induces relocalization of ß-catenin from the nucleus to the cytoplasm in cells that stably express PRB. In exclusively PRA-expressing cells and in PR-negative cells, the level of ß-catenin in the nucleus is much lower in absence of MPA. This indicates that in PRB-expressing cells, Wnt signaling is more active than in cells that express exclusively PRA, and that Wnt signaling in PRB expressing cells is inhibited by MPA.

Besides from being a key component of Wnt signaling, ß-catenin links the cell-cell adhesion molecule E-cadherin to the cytoskeleton, and thus stabilizes cell-cell adhesion (Beavon, 2000). Absence or destabilization of E-cadherin complexes, and subsequent release of ß-catenin, could be a way to activate Wnt signaling (Nelson and Nusse, 2004). Total levels of ß-catenin are similar in all the Ishikawa sub-cell lines used in the present study (Figure 2B), but PRB-expressing Ishikawa cells express little E-cadherin protein compared to cells expressing exclusively PRA or no PR (Figure 1). Therefore, it is possible that in PRB-1 and PRAB-36 cells, more ß-catenin will be available to mediate Wnt signaling. This could explain the nuclear accumulation of ß-catenin in these cells.

Another explanation why Wnt signaling is more active in PRB-expressing cells may be found in interaction of PR with components of the Wnt signaling pathway in the cytoplasm. In absence of ligand, PRB is present in both cytoplasm and nucleus, while PRA is located only in the nucleus (Lim et al., 1999). In absence of ligand, interaction of cytoplasmic PRB with Wnt signal transduction components in the cytoplasm may inhibit degradation of ß-catenin, resulting in nuclear accumulation of ß-catenin. Since PRA is always located in the nucleus, it cannot perform this cytoplasmic function. Therefore, in this explanation, ß-catenin does not accumulate

in the nucleus of cells that express only PRA as it does in cells that express PRB. Upon binding of ligand, PRB translocates to the nucleus, which will restore the ß-catenin degradation complex and thus inhibit transport of ß-catenin to the nucleus.

To further explore effects of MPA on Wnt signaling, we applied the TOPflash/FOPflash reporter system. Since we did not succeed in introducing the TOPflash system effectively into the stably PRB-expressing Ishikawa cells, we performed these experiments with EV8 cells that were transiently expressing PR. In this way, it was observed that MPA inhibits TCF/ß-catenin signaling through PRB, but also through PRA. It has previously been described that both PRA and PRB have the ability to interact with cytoplasmic signal transduction pathways, but that only PRB actually does so, since *in vivo* PRA is not present in the cytoplasm (Boonyaratanakornkit et al., 2001). If transiently expressed PRA is also present in the cytoplasm in EV8 cells, it could participate in cytoplasmic signal transduction, similar to PRB. This may explain the discrepancy between Wnt signaling as detected by nuclear localization of ß-catenin in the stable cell lines, where we found inhibition mainly in PRB-expressing cells, and by the TOPflash reporter assay in the transiently transfected cells, where we found inhibition through both PRA and PRB.

MPA inhibits TCF/ß-catenin signaling via modulation of gene expression

To investigate whether the observed inhibition of TCF/ß-catenin activation by MPA is mediated through interaction of PR with components of the Wnt signal transduction pathway, we attempted to show binding of PR with APC, axin or GSK3ß. Interaction of PR with any of these components could interfere with formation of the ß-catenin degradation complex, resulting in inhibition of degradation of ß-catenin. However, we have not detected binding of PR with any of these Wnt signaling components, indicating that this is probably not the mechanism through which MPA inhibits Wnt signaling.

Antiprogestagens bind PR, which then translocates to the nucleus, but transcription modulation is not induced as it is by MPA. When inhibition of ß-catenin signaling by MPA occurs through relocalization of PR to the nucleus, also antiprogestagens should inhibit the TOPflash reporter signal. However, when transiently transfected cells were treated with antiprogestagens, this did not inhibit the TOPflash reporter signal. The observation that RU486 and Org31489 do not inhibit the TOPflash signal indicates that the effect of MPA on Wnt signaling is not mediated through interactions of PR with components of Wnt signaling in the cytoplasm, but rather by modulation of gene expression.

MPA may inhibit TCF/ß-catenin signaling by inhibition of proteoglycan expression

We show that when proteoglycan assembly is inhibited in Ishikawa cells, ß-catenin signaling is decreased. This indicates that in endometrial cancer cells, proteoglycans can play a role in Wnt signaling. In Ishikawa cells, MPA inhibits expression of several proteoglycans (Hanekamp et al., 2003).

Proteoglycans are a very large family of proteins that are coated with long sugar polymers, called glycosaminoglycans. Proteoglycans are important components of the extracellular matrix, but are also abundant on the cell surface. A large variety of different proteoglycans exists, and they exert many different functions in development, tissue remodeling, growth factor signaling and many other processes (Iozzo, 1998) (Wegrowski and Maquart, 2004). Cell-surface proteoglycans are important in morphogenesis in *Drosophila* and in mammals (Selleck, 2000), and more specifically, the cell-surface heparan sulphate proteoglycan dally ('division abnormally delayed') has been shown to be able to function as co-receptor for the *Drosophila* Wnt homologue Wg (wingless) (Reichsman et al., 1996; Tsuda et al., 1999). In mice, the heparan/chondroitin sulphate proteoglycan syndecan-1 has been shown to be essential for development of Wnt1-induced mammary cancer (Alexander et al., 2000). Possibly, inhibition of proteoglycan synthesis is a mechanism by which MPA mediates the observed inhibition of ß-catenin signaling.

Because of the large variety of proteoglycans and functions of proteoglycans (Iozzo, 1998; Selleck, 2000; Wegrowski and Maquart, 2004), there is still much to learn about these molecules. To confirm that proteoglycans can function in modulation of Wnt signaling by MPA in endometrial cancer cells, first one needs to specify which proteoglycans are regulated by MPA, and second, experiments are required to elucidate how specific inhibition of these proteoglycans would affect Wnt signaling. This should lead to further studies on mechanisms by which proteoglycans affect Wnt signaling in endometrial cancer.

Conclusions

In summary, we have described that MPA can inhibit Wnt signaling in Ishikawa endometrial cancer cells. This inhibition occurs through modulation of gene expression, rather than through cytoplasmic interaction of PR with components of the Wnt signaling cascade. It remains to be determined which MPA target genes are responsible for the ultimately observed inhibition of Wnt, and a role for proteoglycans cannot be excluded.

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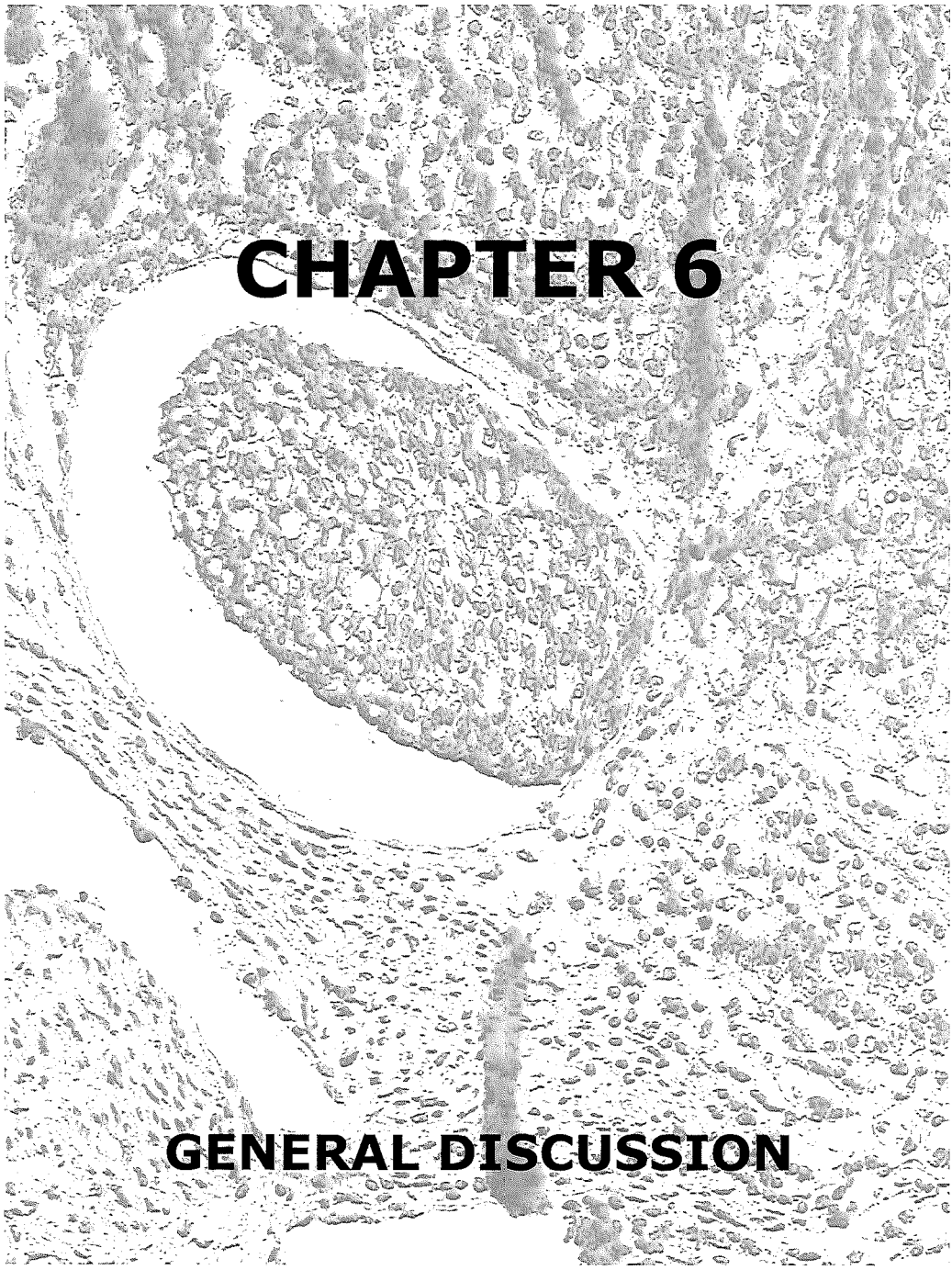

CHAPTER 6

GENERAL DISCUSSION

Women in The Netherlands have a risk of 1.4% of developing endometrial cancer before the age of 74 (Netherlands Cancer Registry 2000). This makes endometrial cancer the fourth most common form of cancer in women in The Netherlands, after breast, lung, and colon cancer. Most risk factors that are associated with endometrial cancer are related to prolonged unopposed estrogen action. During life, or during use of oral contraceptives or hormone replacement therapy, estrogen action on the uterus is usually counteracted by progestagens, which have differentiating properties. However, during development from early to more progressed endometrial cancer, expression of progesterone receptors (PRs) is often lost, which renders the tumor unresponsive to the growth-inhibiting effect of progesterone. In the General Introduction of this thesis, our major research question was summarized as follows: what is the effect of loss of progesterone regulation due to loss of PR expression during development of endometrial cancer? Four more detailed questions were formulated, which will be addressed in this General Discussion.

6.1. Is loss of PR expression in endometrial cancer linked to development of endometrial cancer to a more advanced stage?

During development of endometrial cancer, expression of PR is often decreased or lost. Several studies have reported selective loss of PRA, of PRB, or of both PR isotypes (Arnett-Mansfield et al., 2001; Fujimoto et al., 1997; Fujimoto et al., 1995; Fujimoto et al., 2000; Fukuda et al., 1998; Halperin et al., 2001; Leslie et al., 1997). As described in Chapter 2, gene expression data indicate that the progestagen MPA regulates expression of a set of metastasis-related genes in endometrial cancer cells. Loss of progesterone responsiveness through loss of expression of PR in endometrial cancer could therefore result in a change of expression of metastasis-related genes. When expression of these MPA-regulated genes was examined in tumor samples of endometrial cancer patients, it became clear that expression of CD44 and CSPG/versican was increased when expression of PR was decreased. Increased expression of CD44 in endometrial cancer is associated with increased lymph node involvement (Leblanc et al., 2001), which in turn is a strong predictor for recurrence (Feltmate et al., 1999). CSPG/versican is a matrix proteoglycan, which can interact with CD44 and can modulate tumor cell invasion (Henke et al., 1996). The observation that expression of CD44 and CSPG/versican is increased when expression of PR (PRA and PRB) is decreased, indicates that a decrease in expression of PR could be an initiating event in development of more advanced endometrial cancer.

6.2. What are the effects of changes in expression of PRA and PRB on invasion and metastasis of endometrial cancer?

Since PRA and PRB have different transcriptional activities and regulate expression of different genes in endometrial cancer cells (Giangrande and McDonnell, 1999; Smid-Koopman et al., 2003), it is to be expected that alterations in expression of PRA will have different effects than alterations in expression of PRB. In PRA knockout (PRAKO) mice, it has been shown that progesterone can have a growth-stimulating effect on the endometrium through PRB (Mulac-Jericevic et al., 2000). Even though in endometrial cancer proliferation of cells that express exclusively PRB is inhibited by MPA (Smid-Koopman et al., 2003), the PRAKO mouse phenotype is an indication that selective loss of PRA, rather than PRB, in endometrial cancer may be sufficient for the tumor to escape from the growth-inhibiting effects of progestagens.

Interestingly, in the absence of progestagens, cells that express different PR isotypes exhibit distinct properties. Expression of CD44 and CSPG/Versican was found to be higher in PRB-1 and PRAB-36 cells, compared to expression in PRA-1 and IKpar cells, and in all *in vitro* experiments PRB-1 and PRAB-36 cells showed similar expression patterns. Furthermore, gene expression patterns in IKpar and exclusively PRA-expressing cells were similar to each other, but different from PRB-expressing cells (Chapter 2 and Chapter 3). Striking is the observed difference in expression of cell-cell adhesion molecules: cells that express exclusively PRA or no PR express high levels of E-cadherin and P-cadherin, while expression of cadherins in cells expressing PRB was much lower, or even undetectable. Considering these gene expression patterns, it was not surprising to find that PRB-expressing cells showed more extensive *in vitro* invasive capacity (Chapter 3).

PR status	Relative expression of CD44	Relative expression of E-cadherin	In vitro invasiveness (- MPA)	Behavior in mice (- MPA)
no PR	Low	High	Low	Non-aggressive
PRA	Low	High	Low	Non-aggressive
PRB	High	Low	High	Aggressive
PRA + PRB	High	Low	High	Non-aggressive

Table 1. Overview of phenotype of Ishikawa endometrial cancer cells, as defined by expression of a metastasis-promoting gene (CD44), a metastasis-inhibiting gene (E-cadherin), *in vitro* invasiveness, and behavior of the cells in mice.

When injected into the peritoneal cavity of nude mice, only PRB-1 cells gave rise to macroscopically distinct tumor growth, in absence of progestagenic influences. In the same mouse model, injection of PRAB-36 cells resulted in only minimal tumor growth. This means, that while PRAB-36 cells are very similar to PRB-1 cells with respect to their gene expression pattern and *in vitro* invasive capacity, they behave more like well-differentiated, non-invasive, PRAB-positive endometrial tumor cells in this *in vivo* model. Why exactly these PRAB-36 cells behave so differently *in vitro* and *in vivo* cannot readily be explained. Obviously, the effect of the host is of great importance for growth and spread of tumor cells. The host environment differs greatly from the *in vitro* culture conditions, which in all probability contributes to the observed difference in behavior of PRAB-36 cells.

Our results indicate that endometrial cancer cells that express different PR isotypes behave very differently, also in absence of progestagens. This argues for different, ligand-independent, effects of PRA compared to PRB. Differential ligand-independent transcription regulation by PRA and PRB has also been described for breast cancer cells (Jacobsen et al., 2002). Even though the results obtained with the PRAB-36 cells are confusing, it has become clear that relative over-expression of PRB, caused by either loss of expression of PRA, or by increased PRB expression, may result in development of a more aggressive endometrial cancer.

A point of discussion raised by reviewers of different manuscripts has been the cell lines that have been used throughout the experiments. The Ishikawa sub-cell lines are stable cell lines, and therefore one must always be aware that any differences in behaviour can be the result of clonal differences.

A way to avoid clonal differences would be the use of an inducible system. Ideally, such a system would consist of a cell line in which one can induce expression of PRA and PRB separately, resulting in a single cell line expressing only PRA, only PRB, or both PRA and PRB. Uninduced, this cell line would function as PR-negative, and thus clonal differences between cell lines are prevented.

The current system of stable cell lines is more prone to clonal disturbances, but also has advantages. Continued culturing will allow the stable cell lines to adapt to their PR status better than when PR expression is transiently induced, and will thus result in a more pronounced and stable phenotype. This phenotype will resemble the *in vivo* situation more closely than an inducable system, since *in vivo* a tumor will also adapt to changes in receptor status over time. Since receptor status proved stable even in absence of antibiotic selection, the Ishikawa stable cell lines could also be used in *in vivo* experiments in mice. As a final point, the set of Ishikawa cell lines currently used consists of at least two cell lines of each type (PRA, PRB, PRAB, PR-negative). Each type of cell line behaves similarly as a group, but distinctly different from the other groups, as far as gene expression regulation by MPA and *in vitro* invasion is concerned. This is a strong indication that the phenotype and behavior of these cells can be explained by the differences in receptor status, rather than being the result of clonal differences.

6.3. What is the effect of progesterone on invasion and metastasis of endometrial cancer cells that express different PR isotypes?

Results from the *in vitro* invasion assays presented in Chapter 3 showed that MPA inhibits invasion of endometrial cancer cells that express PRB. This is consistent with previously reported data (Dai et al., 2002; Fujimoto et al., 1996). Since cells that express only PRA did not show any invasion, an inhibiting effect of MPA on invasion of these cells could not be detected. In mice, MPA inhibited growth and spread of PRB-1 cells, which is consistent with the earlier observed inhibiting effect of MPA on both growth (Smid-Koopman et al., 2003) and *in vitro* invasion (Chapter 3) of PRB-expressing Ishikawa cells. No effect of MPA on growth and spread of tumors originating from PRA-1 cells or from PRAB-36 cells could be shown, but since injection of these cell lines resulted in very limited tumor growth even in absence of progestagens, this is not surprising (Chapter 4).

The importance of the host in tumor cell behavior has been mentioned above in relation to the behavior of PRAB-36 cells, but became even more pronounced when mice were injected with PR-negative tumor cells. In mice that were not treated with MPA, injection of PR-negative cells resulted in only minimal tumor growth. However, in mice that were substituted with MPA, injection of PR-negative cells resulted in massive abdominal tumor growth. Since these PR-negative cells do not respond to MPA themselves, an explanation for the observed stimulation

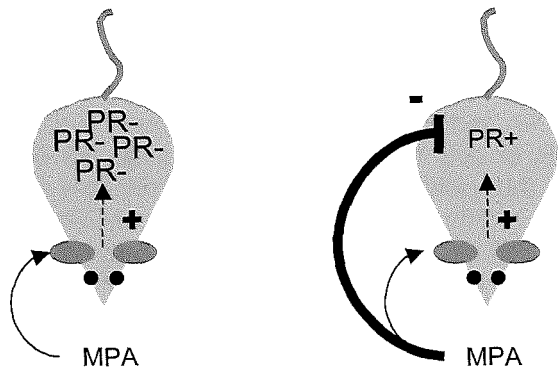

Figure 1. Schematic representation of the suggested effect of MPA on endometrial cancer cells injected in mice. Mice may respond to MPA treatment (curved arrow) by secreting factors that stimulate abdominal growth and spread of endometrial cancer cells (dashed arrows). However, MPA has a direct growth-inhibiting effect on PR-positive cells (fat blocking arrow), which overrules the indirect host-mediated effect. Therefore, in presence of MPA, PR-negative cells will rapidly grow and spread (left mouse), while PR-positive cells will not (right mouse).

of tumor growth should be sought in the possibility of a secondary host effect (which is initiated by MPA). We have proposed that the host responds to MPA substitution by producing unspecified growth factors, which render the abdominal cavity a favorable environment for growth of these endometrial cancer cells (Chapter 4). Since PR-positive cells are directly inhibited in their growth and invasive capacity by MPA (Smid-Koopman et al., 2003; Chapter 3), any host-mediated effect to stimulate growth and spread of PR-positive cells is obstructed by this direct inhibiting effect of MPA on the cells (Figure 1). The mechanism of such a host-mediated stimulation of

PR-negative tumor growth by MPA, and which growth factors are involved, remains to be elucidated.

It needs to be established whether a similar secondary host-mediated effect can also occur in humans. In the clinic, many patients with advanced or recurrent endometrial cancer are treated with progestagens. This practice is based on the experience that most women will generally feel better as a result of progesterone treatment, and on the observation that 20% of advanced endometrial cancers do respond positively to this therapy. However, when the results as presented in Chapter 4 are taken into account, the question arises whether the remaining 80% of advanced endometrial cancer patients really are non-responders, or might actually have some undetected negative response (i.e., stimulation of tumor growth). To our knowledge, no data are available in the literature on studies in which treatment of endometrial cancer patients with MPA is compared to no treatment at all. Such data are urgently needed to address this very important question.

There is also little data available on effects of MPA on levels of other hormones or growth factors in the human body. The vast majority of studies in which MPA is used in post-menopausal women concern hormone replacement therapy, and in these studies MPA is administered to subjects mainly as an addition to estrogens. Even so, it is clear that MPA induces many responses. Administration of MPA results in a decrease in serum levels of LH and FSH, which would result in growth inhibition of endometrial tissue, but stimulates IGF-1 production, which may stimulate proliferation of endometrial cancer cells (Saaresranta et al., 2002), and MPA can modulate hepatic endocrine function (Nugent et al., 2003). Early studies, in which MPA was administered to women with inoperable breast cancer, have shown that MPA inhibits adrenal function in postmenopausal women (van Veelen et al., 1985), but how this might affect endometrial cancer cells is not clear.

Before drawing any definite conclusions on the effect of MPA on growth and spread of PR-negative endometrial cancer, the experiments as performed in Chapter 4 should be repeated, preferably with a set of several different PR-negative endometrial cancer cell lines, with application of available *in vivo* monitoring systems, and with coverage of a selected number of serological parameters. Only then it may be possible to give a well-founded advice on treatment of PR-negative endometrial cancer patients with MPA.

6.4. Does any crosstalk exist between PR signaling and Wnt signaling?

Several studies have reported alterations in components of the Wnt signaling pathway in endometrial cancer. Nei et al. (1999) found nuclear staining of ß-catenin in 14 out of 20 (70%) endometrial hyperplasias and in 24 out of 30 (80%) endometrial cancers (Nei et al., 1999). It should be noted, that of these 24 positive endometrial cancers, only 9 had strong nuclear ß-catenin staining, while in hyperplasias, all positive samples exhibited strong nuclear staining. In a study where catenin expression was evaluated in a limited number of endometrial cancers, no

correlation was found between nuclear ß-catenin staining and clinicopathological features, such as histological grade, myometrial invasion and microsatellite instability (Palacios et al., 2001), but in a more recent study in which 233 endometrial cancer samples were evaluated, 90% of the tumors with nuclear ß-catenin staining were classified as well-differentiated tumors (Scholten et al., 2003).

Moreno-Bueno et al. (2002) studied molecular alterations of ß-catenin, APC and axin in 94 cases of endometrial cancer. They found mutations in exon 3 of *CTNNB1* (14.9%), but none in the *APC* gene. All tumors with a mutation in *CTNNB1* exhibited nuclear localization of ß-catenin. In contrast, only 46.6% of tumors with nuclear localization of ß-catenin had *CTNNB1* mutations, indicating that other mechanisms leading to nuclear accumulation of ß-catenin are present. Hypermethylation of the APC promoter 1-A was observed in almost half of the cases, but this was not associated with nuclear localization of ß-catenin (Moreno-Bueno et al., 2002). Matias-Guiu et al. (2001) also reported mutations in exon 3 of *CTNNB1*, and also in that study nuclear localization of ß-catenin could not always be explained by *CTNNB1* mutations (Matias-Guiu et al., 2001). Additionally, the described mutations in *CTNNB1* were generally not associated with other molecular changes that are associated with endometrial cancer, such as microsatellite instability and mutations in *PTEN* and *KRAS2* (Matias-Guiu et al., 2001). These studies indicate that Wnt signaling plays a role early in development of endometrial cancer, but that the role of Wnt signaling is independent from other molecular changes that are found in endometrial cancer.

In the early stages of endometrial cancer, progesterone signaling is important in limiting growth and progression of the tumor. In the study by Nei et al. (1999), nuclear staining of ß-catenin did not correlate with nuclear expression of PR (Nei et al., 1999). However, Scholten et al. (2003) have found a significant correlation between nuclear ß-catenin staining and PR expression: 27% of PR-positive tumors showed nuclear ß-catenin staining, versus 8.7% of PR-negative tumors (Scholten et al., 2003). No data are given on serum progesterone levels of the patients in these studies. We have observed that introduction of PR into endometrial cancer cells did not lead to alterations in TCF/ß-catenin reporter signal in absence of progestagens, but that subsequent treatment with MPA inhibited TCF/ß-catenin signaling. In stably PR-expressing cells, nuclear localization of ß-catenin was only found in PRB-expressing cells in absence of progestagens, and ß-catenin moved out of the nucleus in response to MPA (Chapter 5). This indicates that, *in vivo,* inhibition of ß-catenin signaling by progestagens may only occur in PRB-expressing cells. In the most extensive study on expression of PR-isotypes in endometrial cancer, 28% of PR-positive tumors expressed only PRB (Arnett-Mansfield et al., 2001). This percentage is tantalizingly close to the 27% of PR-positive tumors with nuclear ß-catenin localization observed in the study by Scholten et al. (2003). Unfortunately, the antibody used by Scholten et al. (2003) to detect PR does not differentiate between PRA and PRB, so it is not clear whether the 27% of PR-positive tumors with nuclear ß-catenin represent tumors that are expressing only

PRB, and therefore show some functional similarity to our PRB-expressing Ishikawa cells.

Tulac et al. (2003) investigated regulation of Wnt signaling in human endometrium during the menstrual cycle (Tulac et al., 2003). They describe that during the secretory phase, expression of dickkopf1 (Dkk1) is significantly up-regulated (234-fold, $p < 0.001$). Dkk1, which binds to LRP5/6 (low-density-lipoprotein receptor-related protein 5/6) (Mao et al., 2001), is an inhibitor of Wnt signaling in *Xenopus* (Glinka et al., 1998) and in mammals (Fedi et al., 1999). The mechanism by which Dkk is thought to inhibit Wnt signaling is described in Figure 2. Wnt binds both Fz and LRP5/6. Through Fz, GSK3ß is inactivated, while LRP5/6 interacts with axin in the cytoplasm. Consequently, the ß-catenin degradation complex disintegrates and ß-catenin can translocate to the nucleus. When secreted Dkk1 binds the LRP5/6 receptor, LRP5/6 can no longer interact with Wnt or axin. This has as result that Wnt signaling is not initiated, even when Wnt is bound to Fz (Figure 2) (Nusse, 2001).

The observation that expression of Dkk1 is up-regulated during the secretory phase of the menstrual cycle (Tulac et al., 2003), when progesterone levels are high, is indicative for induction of Dkk1 by progesterone. Additionally, Kao et al. (2002, 2003) found up-regulation of Dkk1 during the window of implantation (when the levels of E2 and P are high) compared to the late proliferative phase (when only the level of E2 is high) (Kao et al., 2002) (Kao et al., 2003). To our knowledge, the three papers cited here are the only published reports that address Dkk1 in relation to the endometrium or progesterone. Up-regulation of Dkk1 by progesterone could be a mechanism for the observed inhibition of ß-catenin signaling by MPA. Hence, a more extensive study on possible regulation of Dkk1 by progesterone in endometrium and endometrial cancer cells is warranted.

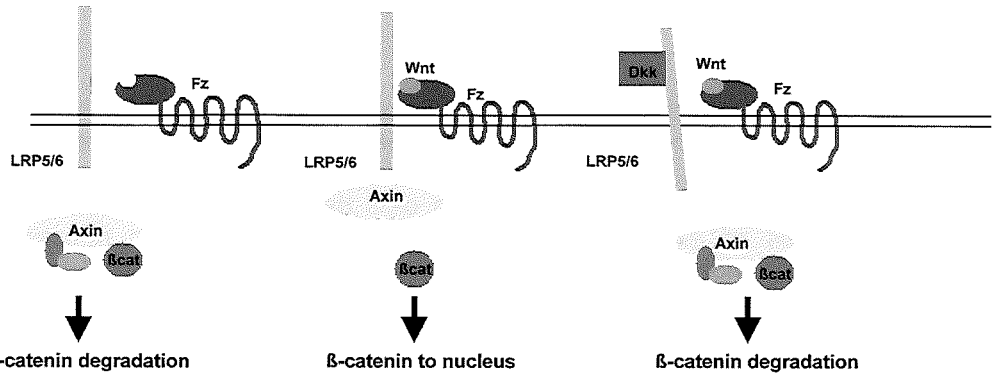

Figure 2. Schematic representation of action of Dkk. In the absence of Wnt, ß-catenin is degraded. Upon binding of Wnt to Fz and LPR5/6, ß-catenin is released from its degradation complex and can translocate to the nucleus. When Dkk is bound to LPR5/6, Wnt cannot interact with LPR5/6. As a result, ß-catenin is degraded, regardless of binding of Wnt to Fz.

Proteoglycans are very large molecules that consist of a protein core, to which glycosaminoglycan side chains are attached. The glycosaminoglycan side chain composition attached to the protein core can vary, and many posttranslational modifications are possible. This means that many different proteoglycans exist, and both matrix and cell associated proteoglycans can have many different functions (Iozzo, 1998; Selleck, 2000; Wegrowski and Maquart, 2004). In Chapter 5, regulation of proteoglycan synthesis is pointed out as a possible mechanism by which MPA inhibits Wnt signaling. Synthesis of proteoglycans in endometrial tissue is influenced by steroid hormones (Inki, 1997; Tellbach et al., 2002; Wu et al., 2000). Expression of vascular heparan sulphate proteoglycans is decreased in the endometrium of users of progestagen-only contraception (Hickey et al., 1999). We have observed that in Ishikawa cells, expression of several proteoglycans could be influenced by MPA (Chapter 2). In *Drosophila*, the cell-surface proteoglycan dally can function as co-receptor for the Wnt homologue Wg (Tsuda et al., 1999). In mice, a similar proteoglycan, syndecan-1, is required for Wnt1-induced breast cancer development (Alexander et al., 2000). We have observed that in Ishikawa endometrial cancer cells, Wnt signaling could be inhibited by inhibiting proteoglycan synthesis (Chapter 5). Modification of cell-surface proteoglycans might be a mechanism through which MPA inhibits Wnt signaling in endometrial cancer cells (Chapter 5).

The results discussed in Chapter 5, combined with data in literature, indicate that crosstalk between progesterone signaling and Wnt signaling could be of importance during development of endometrial cancer. At which point in the signaling cascade this crosstalk occurs, remains to be established, but possible targets for further research would be progestagenic regulation of expression of Dkk1 and of cell-associated proteoglycans.

6.5. Concluding remarks

In this thesis, data have been presented that indicate that loss of PR expression can lead to development of a more aggressive endometrial cancer. However, endometrial cancer cells that express only PRB are more invasive *in vitro*, and are more aggressive when injected into nude mice than cells that express only PRA. This indicates that loss of expression of specifically PRA might have negative consequences for patients. MPA can inhibit growth of PR-positive endometrial cancer cells, and also inhibits *in vitro* invasion and spread of PR-positive cells in nude mice. MPA can also inhibit Wnt signaling in PR-positive endometrial cancer cells. Crosstalk between PR signaling and Wnt signaling during development of endometrial cancer deserves to be studied in more detail, and could provide interesting new insights in the functions and regulations of these high-profile signal transduction pathways.

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SUMMARY

SAMENVATTING

SUMMARY

Endometrial cancer is one of the most common gynecological malignancies in Europe and the USA. While many patients can be cured by surgical treatment, the tumor has already spread beyond the uterus at time of diagnosis in approximately 25% of the patients. As with many types of cancer, the prognosis for patients with disseminated endometrial cancer is poor. Progesterone inhibits growth of endometrial epithelial cells, and can also inhibit growth of endometrial cancer. However, during progression of endometrial cancer, expression of progesterone receptors (PR) often is decreased. Tumors in which little or no PR is expressed will not be inhibited in their growth by progestagens, resulting in poor response to progesterone administration.

This thesis describes the results of investigations that aim to answer the main research question that was posed in the General Introduction: What is the effect of loss of progesterone regulation due to loss of PR expression during development of endometrial cancer?

In Chapter 1, an introduction to the topics that will be encountered while reading this thesis is given. Section 1.1. gives a general overview of human non-neoplastic and neoplastic endometrium, including endometrial cancer classification and treatment. In section 1.2., properties of PRA and PRB are described, and progesterone-regulated genes are discussed. In addition, the PRKO mouse models are discussed. Section 1.2.7. discusses altered expression of PR in endometrial cancer. Section 1.3. gives an introduction on Wnt signaling, in particular with regard to cancer. General mechanisms of tumor invasion and metastasis are discussed in section 1.4.

In Chapter 2, regulation of gene expression via PR was studied in an endometrial cancer cell line to give a reasonable expectation of the effect of loss of progesterone regulation through loss of PR expression in endometrial cancer. This showed that progesterone down-regulates a panel of metastasis-related genes. On closer examination, cells that express PRB show higher expression of several metastasis-related genes than cancer cells that do not express PRB. The results of these investigations were translated to the clinic by means of evaluation of clinical samples. This showed that when tumors lose expression of PR, the loss of progesterone-regulation results in up-regulation of CD44 and CSPG/versican. Therefore, loss of PR expression in endometrial cancer may lead to development of a more invasive tumor.

The differences in expression of metastasis-related genes between Ishikawa cells that express PRA, or PRB, or both receptors, indicated that these cells may have different invasive capacities. In Chapter 3, the *in vitro* invasive capacity of endometrial cancer cells that express different isotypes of the progesterone receptor was investigated. Ishikawa cells that express PRB were observed to be more invasive *in vitro* than cancer cells that do not express PRB. Furthermore, invasion can be inhibited by MPA. Analysis of gene expression in the absence of progestagens showed that cancer cells that express no PRB express high levels of cadherins.

Invasion of these cells can be stimulated by inhibiting cadherin function, which indicates that the differences in cadherin expression between the different PR-expressing cell lines contributes to the differences in *in vitro* invasive capacity.

In Chapter 4, a mouse model was presented. Ishikawa cells were injected into mice to study growth and spread of cells that express different PR isotypes. In this model, cells that express only PRB showed the most extensive growth and spread through the body, in absence of progestagens. Upon treatment with MPA, growth and spread of these cells decreased. However, while PR-negative cells did not grow or spread to any substantial extent in absence of progestagens, treatment with MPA induced massive abdominal tumor growth in mice injected with PR-negative cells. This indicates that MPA can have a secondary, growth-stimulating effect on PR-negative tumor growth.

From the initial data on gene transcription regulation by MPA, it became clear that MPA regulated a panel of genes that are known to be targets of the Wnt signal transduction pathway. In Chapter 5, we set out to further investigate regulation of Wnt signal transduction by progestagenic influences. In cells that stably express PRB, ß-catenin was located in the nucleus. This is considered a hallmark of active Wnt signaling. Upon treatment with MPA, ß-catenin moved from the nucleus, indicating inhibition of Wnt signaling. Implementing the TOPflash reporter system, it became clear that MPA can indeed inhibit the endpoint of Wnt signaling, i.e., TCF/ß-catenin mediated transcription activation. This effect of MPA appears to result from gene expression modulation.

In Chapter 6, the findings that are presented in this thesis are discussed, and relations between findings in different chapters are explained. In addition, suggestions for further lines of research are proposed.

SAMENVATTING

Endometriumkanker (kanker van de binnenbekleding van de baarmoeder) is een van de meest voorkomende vormen van kanker bij vrouwen in Europa. Het grootste deel van de patiëntes kan effectief worden behandeld door de gehele baarmoeder operatief te verwijderen, maar in ongeveer 25% van de gevallen heeft de tumor zich ten tijde van diagnose, al tot buiten de baarmoeder uitgebreid. In een dergelijk geval is er sprake van een zeer slechte prognose, zoals bij de meeste gevallen van uitgezaaide kanker. Groei van het endometrium kan worden geremd door het vrouwelijke geslachtshormoon progesteron. Dit hormoon kan ook de groei van endometriumkanker remmen. Echter, naarmate de kanker verder vordert wordt vaak een afname van progesteronreceptor expressie gevonden. Dientengevolge zullen deze tumoren niet langer in de groei geremd worden door progestagenen.

In dit proefschrift zijn de resultaten beschreven van onderzoek naar de rol van progesteron en progesteronreceptoren in endometriumkanker. De voornaamste onderzoeksvraag wordt gesteld in Hoofdstuk 1: Wat is het effect van het verlies van progesteron-regulatie, door verlies van expressie van progesteronreceptoren, tijdens de progressie van endometriumkanker?

In Hoofdstuk 1 wordt een algemene inleiding gegeven op onderwerpen die men tegen zal komen tijdens het lezen van dit proefschrift. Sectie 1.1. geeft een algemeen overzicht van het normale humane endometrium en van endometriumkanker. De officiële classificatie van endometriumkanker volgens de internationale FIGO-standaard wordt omschreven, en mogelijkheden voor therapie worden genoemd. In sectie 1.2. worden eigenschappen van de beide progesteronreceptoren (PRA en PRB) omschreven, alsmede welke genen door progesteron gereguleerd worden. Sectie 1.2.7. vertelt over de veranderingen in expressie van progesteronreceptoren in endometriumkanker. Een belangrijk signaaltransductiepad (het Wnt-pad) in de embyonale ontwikkeling en tijdens de ontwikkeling van kanker wordt geïntroduceerd in sectie 1.3. Algemene mechanismen van het uitzaaien van kanker worden besproken in sectie 1.4.

In Hoofdstuk 2 wordt regulatie van genexpressie via PRA en PRB bestudeerd in een endometriumkankercellijn. Op deze manier kan men een redelijk beeld krijgen van het te verwachten effect van het verlies van progesteronregulatie tijdens progressie van endometriumkanker. Uit deze experimenten bleek dat progesteron een aantal genen reguleert die te maken hebben met het uitzaaien van kanker. Ook werd duidelijk dat cellen die PRB tot expressie brengen deze metastase-gerelateerde genen sterker tot expressie brengen dan cellen zonder PRB. De resultaten van deze experimenten werden vertaald naar de kliniek door patiëntenmateriaal te onderzoeken. Hieruit bleek dat in tumoren welke geen progesteronreceptoren meer tot expressie brengen, het verlies van progesteronregulatie resulteert in een sterkere expressie van de metastase-gerelateerde genen CD44 en CSPG/versican. Dit is een aanwijzing dat verlies van expressie van progesteronreceptoren in endometriumkanker kan leiden tot de ontwikkeling van een meer agressieve vorm van kanker.

De verschillen in expressie van metastase-gerelateerde genen tussen endometriumkankercellen die verschillende vormen van de progesteronreceptor tot expressie brengen, duidden op een mogelijk verschil in invasiviteit tussen deze cellen. In Hoofdstuk 3 werd de *in vitro* invasiviteit van cellen, welke verschillende vormen van de progesteronreceptor tot expressie brengen, bestudeerd. Cellen welke PRB tot expressie brengen zijn meer invasief dan cellen welke alleen PRA tot expressie brengen. Deze invasie kan worden geremd met het progestageen MPA. Verdere analyse van genexpressie in deze cellen gaf aan dat cellen zonder PRB veel cadherines (belangrijke cel-cel adhesie moleculen) tot expressie brengen. Invasie van deze cellen kan worden gestimuleerd door de functie van deze cadherines te remmen met behulp van antilichamen. Deze resultaten geven aan dat het verschil in cadherine-expressie bijdraagt tot het verschil in *in vitro* invasiviteit tussen cellen welke verschillende vormen van progesteronreceptoren tot expressie brengen.

In Hoofdstuk 4 wordt een muismodel gepresenteerd. Endometriumkanker cellen werden in muizen geinjecteerd om groei en verspreiding van cellen, welke verschillende vormen van progesteronreceptoren tot expressie brengen, te bestuderen. In dit model laten cellen die alleen PRB tot expressie brengen de sterkste uitgroei en verspreiding door het muizenlichaam zien. Door de muizen met MPA te behandelen wordt deze uitgroei sterk verminderd. progesteronreceptor-negatieve cellen gaven vrijwel geen uitgroei in muizen in afwezigheid van progestagene invloeden, maar in muizen welke met MPA waren behandeld groeiden deze cellen juist explosief uit. Dit betekent dat in dit muizenmodel MPA een indirect groeistimulerend effect kan hebben op progesteronreceptor-negatieve endometriumtumoren.

Uit de oorspronkelijke analyse van het effect van MPA op genexpressie in endometriumkankercellen bleek dat MPA een aantal genen reguleert waarvan bekend is dat zij doelwit zijn van Wnt signaaltransductie. In Hoofdstuk 5 is verder onderzocht wat het effect van MPA is op Wnt signaaltransductie in endometriumkankercellen. In cellen die stabiel PRB tot expressie brengen bevindt zich ß-catenine in de celkern, wat een teken is van een actieve Wnt signaaltransductie. Na behandeling met MPA verdween ß-catenine uit de kern, wat op een remming van Wnt signaaltranductie wijst. Met behulp van het TOPflash reporter systeem werd duidelijk dat MPA een late stap van de Wnt signaaltransductie cascade, te weten TCF/ß-catenine-gemedieërde activatie van gentranscriptie, kan remmen. Dit effect van MPA bleek te verlopen door modulatie van genexpressie via progesteronreceptoren.

In Hoofdstuk 6 worden de bevindingen uit de voorgaande hoofdstukken van dit proefschrift in relatie tot elkaar besproken. De onderzoeksvragen welke in Hoofdstuk 1 werden gesteld worden beantwoord, en suggesties voor vervolgonderzoek worden gegeven.

ABBREVIATIONS

and

FEATURED GENES

ABBREVIATIONS

AF : activation function
APC : adenomatous polyposis coli
BSA : bovine serum albumine
cAMP : 3', 5'-cyclic adenosine monophosphate
CHase : chondroitinase
CSPG : chondroitin sulfate proteoglycan
DBD : DNA binding domain
DCC : dextran coated charcoal
Dkk : dickkopf protein
DMEM : Dulbecco's modified Eagle's medium
DNA : desoxyribonucleic acid
DOC : deoxycholine
Dsh : disheveled protein
DTT : dithiothreitol
E-cad : epithelial cadherin
ECM : extracellular matrix
EDTA : ethylene diamine tetraacetic acid
EGF : epidermal growth factor
ER : estrogen receptor
ERID : estrogen receptor interaction domain
ESRRA : estrogen-related receptor alpha
EV : empty vector
FCS : fetal calf serum
FIGO : International Federation of Gynecology and Obstetrics
FSH : follicle-stimulating hormone
Fz : frizzled protein (human and *Drosophila*)
GFP : green fluorescent protein
Grg : groucho protein
GRIP-1 : glucocorticoid receptor interacting protein
GSK3ß : glycogen synthetase kinase 3ß protein
Gy : Gray
h (prefix) : human
HNPCC : hereditary nonpolyposis colorectal cancer
HR : hygromycin resistance
HRE : hormone response element
HRT : hormone replacement therapy
HSP : heat shock protein
ID : inhibitory domain
i.e. : id est (in other words, which means)
IGF : insulin-like growth factor
IGFBP : insulin-like growth factor binding protein
IP : immunoprecipitation

ITF-2 : immunoglobulin transcription factor
kDa : kilodalton
LBD : ligand binding domain
LH : luteinizing hormone
LRP : low-density-lipoprotein receptor-related protein
MAPK : mitogen-activated protein kinase
MI : myometrial invasion
MMP : matrix metalloproteinase
MPA : medroxyprogesterone acetate
MSH6 : MutS homologue 6
NCoR : nuclear receptor corepressor
NGS : normal goat serum
NMRI : Naval Medical Research Institute
PAI-1 : plasminogen activator inhibitor-1
PBS : phosphate-buffered saline
PCR : polymerase chain reaction
PR : progesterone receptor
PRA : progesterone receptor A
PRAKO : progesterone receptor A knockout
PRB : progesterone receptor B
PRBKO : progesterone receptor B knockout
PRE : progesterone response element
PRKO : progesterone receptor knockout
RNA : ribonucleic acid
SDS : sodium dodecyl sulfate
SERM : selective estrogen receptor modulator
SH : Src homolgy
SMRT : silencing mediator for retinoid/ thyroid-hormone receptors
SRC : steroid receptor coactivator
STAT : signal transducer and activator of transcription
TCF/LEF : T-cell factor/lymphocyte enhancing factor
TGF : transforming growth factor
TRD : transcription regulating domain
VEGF : vascular endothelial growth factor
WHO : World Health Organization
Wg : wingless
Wnt : Wnt protein (human) (named from fusing the names of the *Drosophila* gene *Wingless* and the mouse gene *Int-1*)

FEATURED GENES

The following human genes were studied in this thesis:

Protein	**Gene**	**Location**	**GeneID**
APC	*APC*	5q21-q22	324
Axin	*AXIN1*	16p13.3	8312
ß-catenin	*CTNNB1*	3p21	1499
CD44	*CD44*	11p13	960
Claudin-1	*CLDN1*	3q28-q29	9076
CyclinD1	*CCND1*	11q13	595
E-cadherin	*CDH1*	16q22.1	999
Fibronectin	*FN1*	2q34	2335
Follistatin	*FST*	5q11.2	10468
GSK3ß	*GSK3ß*	3q13.3	2932
Integrinß1	*ITGB1*	10p11.2	3688
Jun	*JUN*	1p32-31	3725
MMP7	*MMP7*	11q21-q22	4316
P-cadherin	*CDH3*	16q22.1	1001
PR	*PGR*	11q22-q23	5241
Tenascin-C	*TNC*	9q33	3371
VEGF	*VEGF*	6p12	7442
Versican	*CSPG2*	5q14.3	1462

(More information can be obtained from
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene)

LIST OF PUBLICATIONS

This thesis:

EE Hanekamp, ECM Kühne, E Smid-Koopman, PE de Ruiter, S Chadha-Ajwani, AO Brinkmann, CW Burger, JA Grootegoed, FJ Huikeshoven, and LJ Blok. Loss of progesterone receptor may lead to an invasive phenotype in human endometrial cancer. Eur J Cancer 2002 38 Suppl 6, S71-2.

EE Hanekamp, SCJP Gielen, E Smid-Koopman, ECM Kühne, PE de Ruiter, S Chadha-Ajwani, AO Brinkmann, JA Grootegoed, CW Burger, FJ Huikeshoven, and LJ Blok. Consequences of loss of progesterone receptor expression in development of invasive endometrial cancer. Clin Cancer Res 2003 9, 4190-9.

EE Hanekamp, SCJP Gielen, SA van Oosterhoud, CW Burger, JA Grootegoed, FJ Huikeshoven, and LJ Blok. Progesterone receptors in endometrial cancer invasion and metastasis: development of a mouse model. Steroids 2003 68, 795-800.

EE Hanekamp, ECM Kühne, JA Grootegoed, CW Burger, and LJ Blok. Progesterone Receptor A And B Expression And Progestagen Treatment In Growth And Spread Of Endometrial Cancer Cells In Nude Mice. Endocrine-Related Cancer, in press

EE Hanekamp, SCJP Gielen, PE de Ruiter, S Chadha-Ajwani, FJ Huikeshoven, CW Burger, JA Grootegoed, and LJ Blok. Differences in invasive capacity of endometrial cancer cell lines expressing different progesterone receptor isotypes: possible involvement of cadherins. Submitted.

EE Hanekamp, ECM Kühne, CW Burger, JA Grootegoed, and LJ Blok. Progesterone inhibits Wnt signaling in endometrial cancer cells. In preparation.

Other:

LJ Blok, PE de Ruiter, ECM Kühne, **EE Hanekamp**, JA Grootegoed, E Smid-Koopman, SCJP Gielen, ME de Gooyer, HJ Kloosterboer, CW Burger. Progestogenic effects of tibolone on human endometrial cancer cells. J Clin Endocrinol Metab. 2003 88(5):2327-34.

IJ Nijman, M Otsen, EL Verkaar, C de Ruijter, **EE Hanekamp**, JW Ochieng, S Shamshad, JE Rege, O Hanotte, MW Barwegen, T Sulawati, JA Lenstra. Hybridization of banteng (Bos javanicus) and zebu (Bos indicus) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites. Heredity 2003 90(1):10-6.

EL Verkaar, IJ Nijman, M Beeke, **EE Hanekamp**, JA Lenstra. Maternal and paternal lineages in cross-breeding bovine species. Has wisent a hybrid origin? Mol Biol Evol. 2004 Jan 22 (Epub ahead of print).

SCJP Gielen, **EE Hanekamp**, LJ Blok, FJ Huikeshoven, CW Burger. Steroid induced growth modulation of human endometrial carcinoma cell lines: any role for IGF signaling? J. Soc. Gynecol. Invest, in press.

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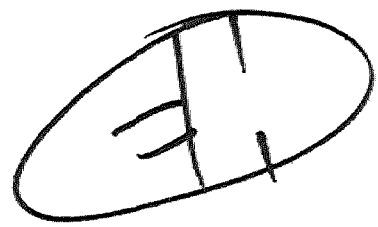
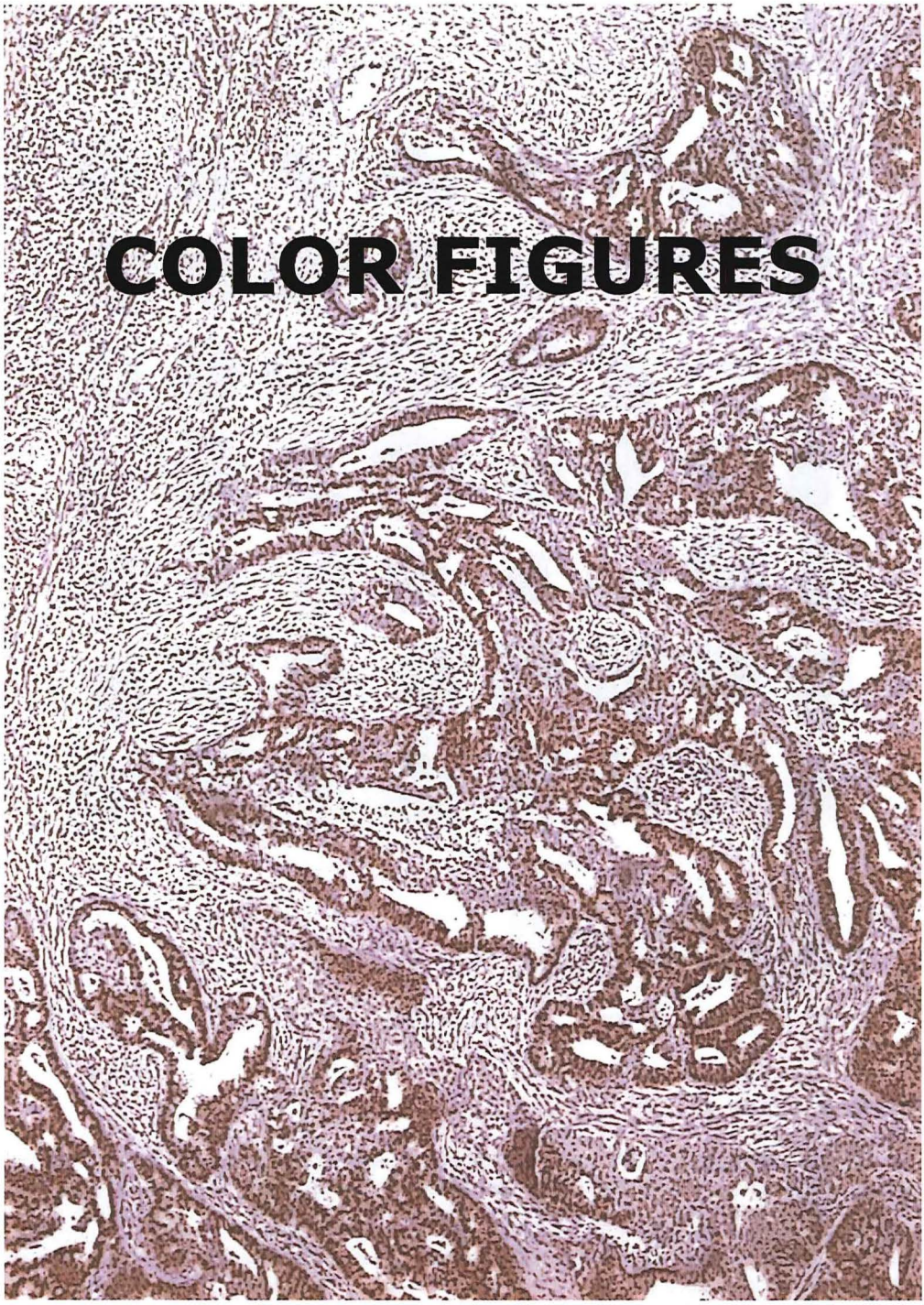
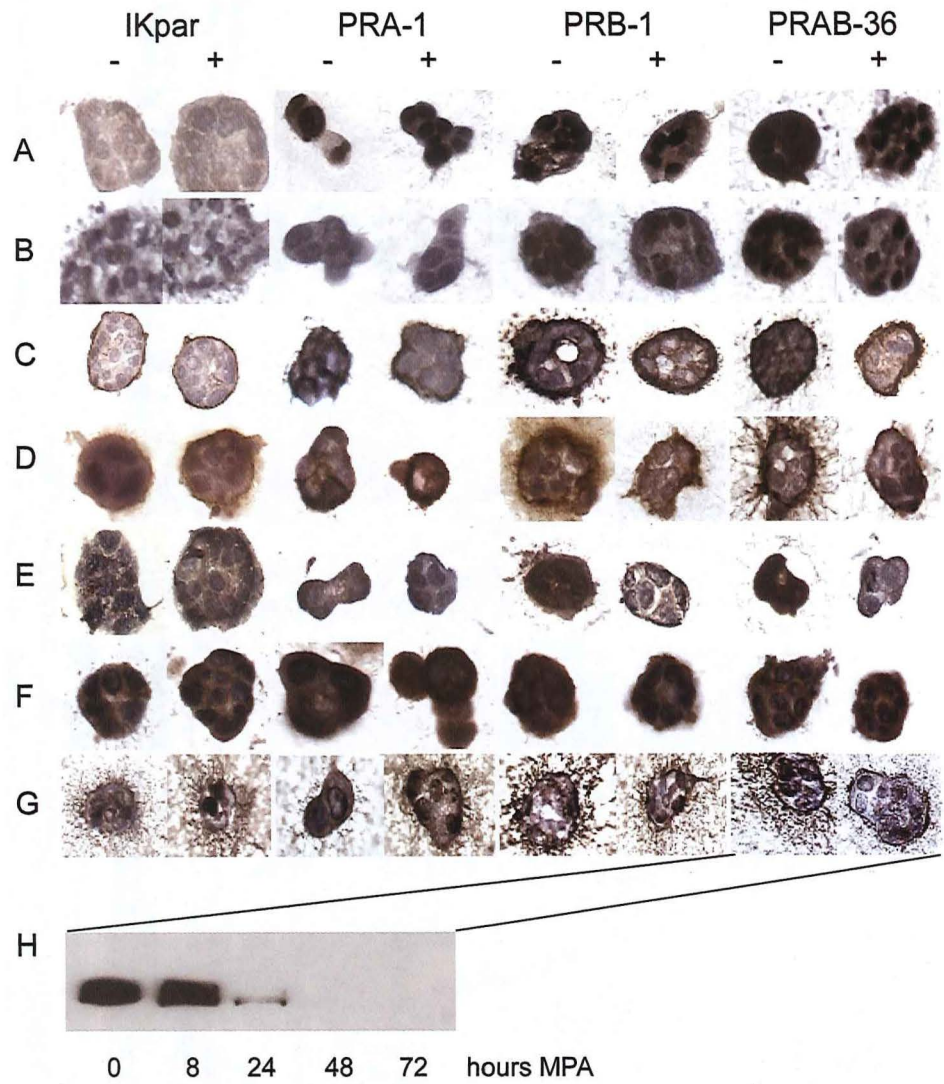
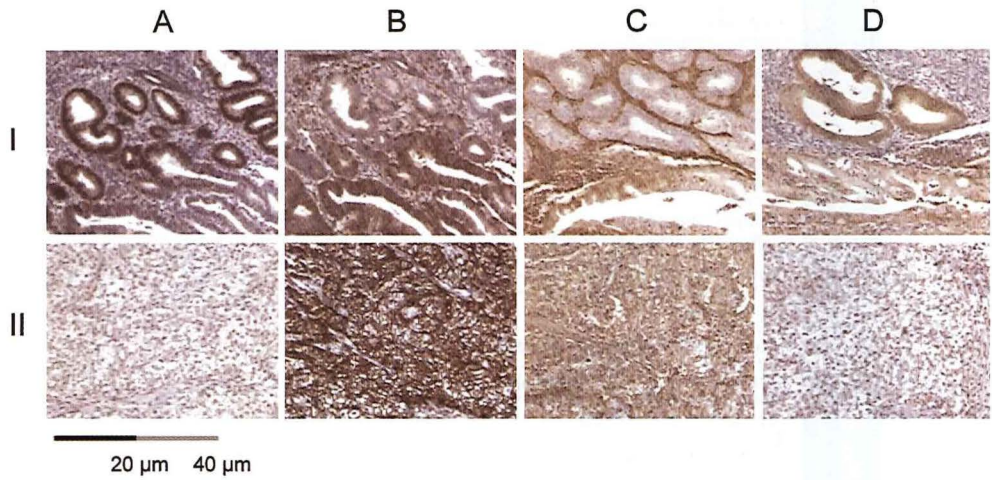



COLOR FIGURES



Chapter 2, Figure 2.

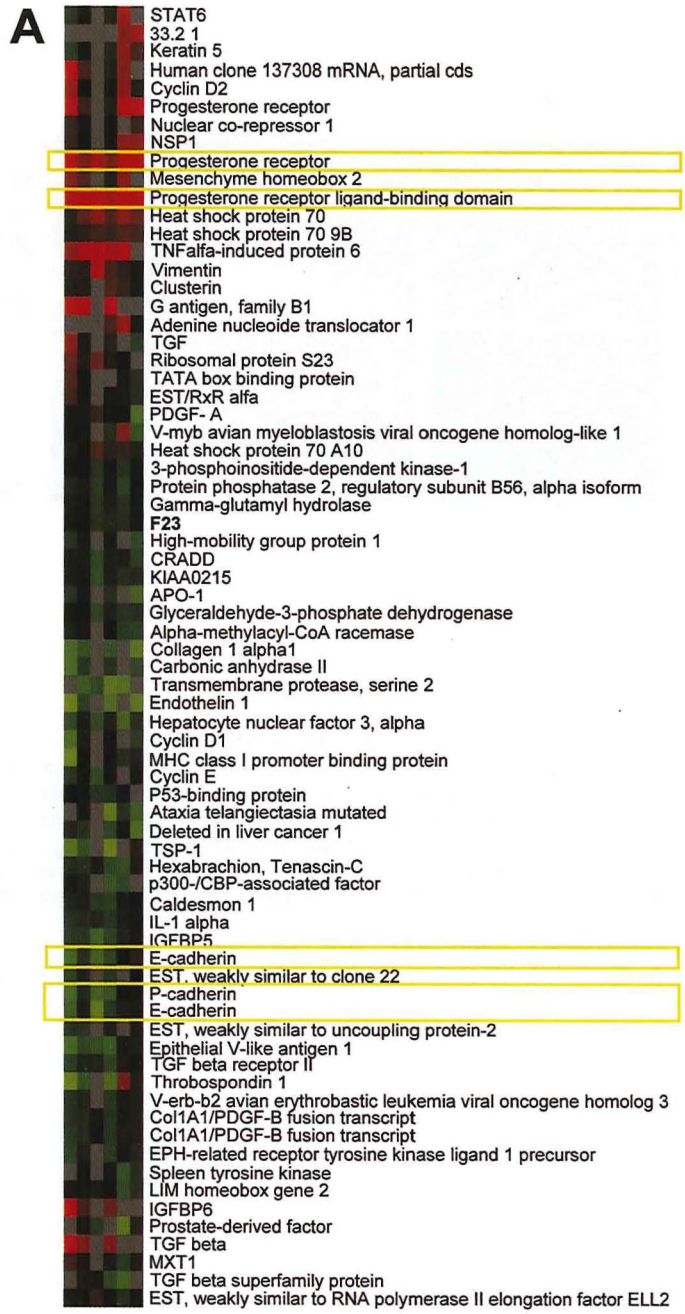
Protein expression of PR, CD44, CSPG/versican, tenascin-C, integrin-β1 and fibronectin-1 in the IKpar and PRA- and/or PRB-expressing sub-cell lines. Ishikawa sub-cell lines IKpar, PRA-1, PRB-1 and PRAB-36 were cultured in collagen gels, in absence (-) or presence (+) of 100 nM MPA, for 72 hours. Sections of the formalin-fixed, paraffin-embedded gels were immunohistochemically stained for: hPRA + hPRB (A), hPRB (B), CD44 (C), CSPG/versican (D), tenascin-C (E), integrin-β1 (F) and fibronectin-1 (G). Each panel shows one cluster of cells in collagen gel. Original magnification 400x. Panel H shows Western blot analysis of expression of fibronectin-1 after 0, 8, 24, 48 and 72 hours of culturing in presence of 100 nM MPA.

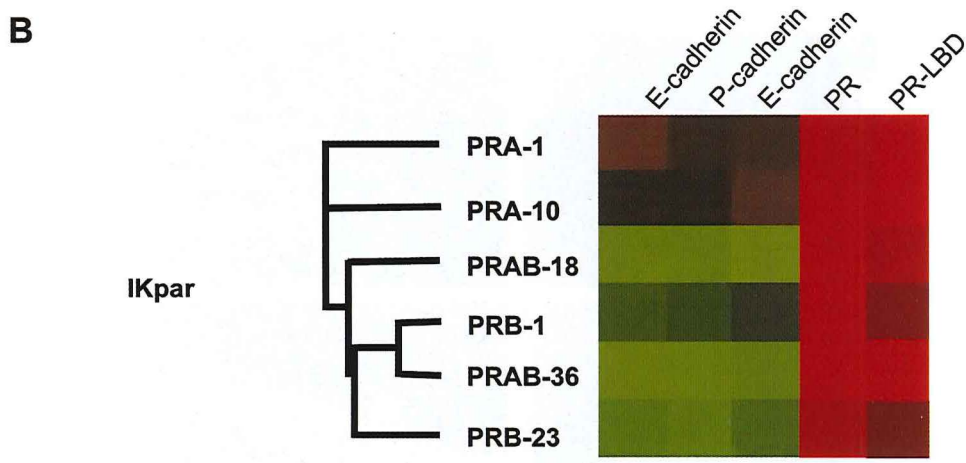


Chapter 2, Figure 3.

Expression of PR, CD44, CSPG/versican, and E-cadherin in endometrial cancer tissue samples. Sections of formalin-fixed, paraffin-embedded samples of endometrioid adenocarcinoma were immunohistochemically stained for hPRA + hPRB (A), CD44 (B), CSPG/versican (C), E-cadherin (D). A representative set of stainings in semi-consecutive* slides of a well-differentiated (G1, stage Ia) tumor sample (panel I) and an undifferentiated (G3, stage Ic) tumor sample (panel II) is shown. Histopathological typing, grade and stage were assessed by the Erasmus MC Department of Pathology (according to the modified FIGO staging system. See also www.FIGO.org/content/PDF/corpus-uteri_p47-49.pdf). Original magnification 200x.

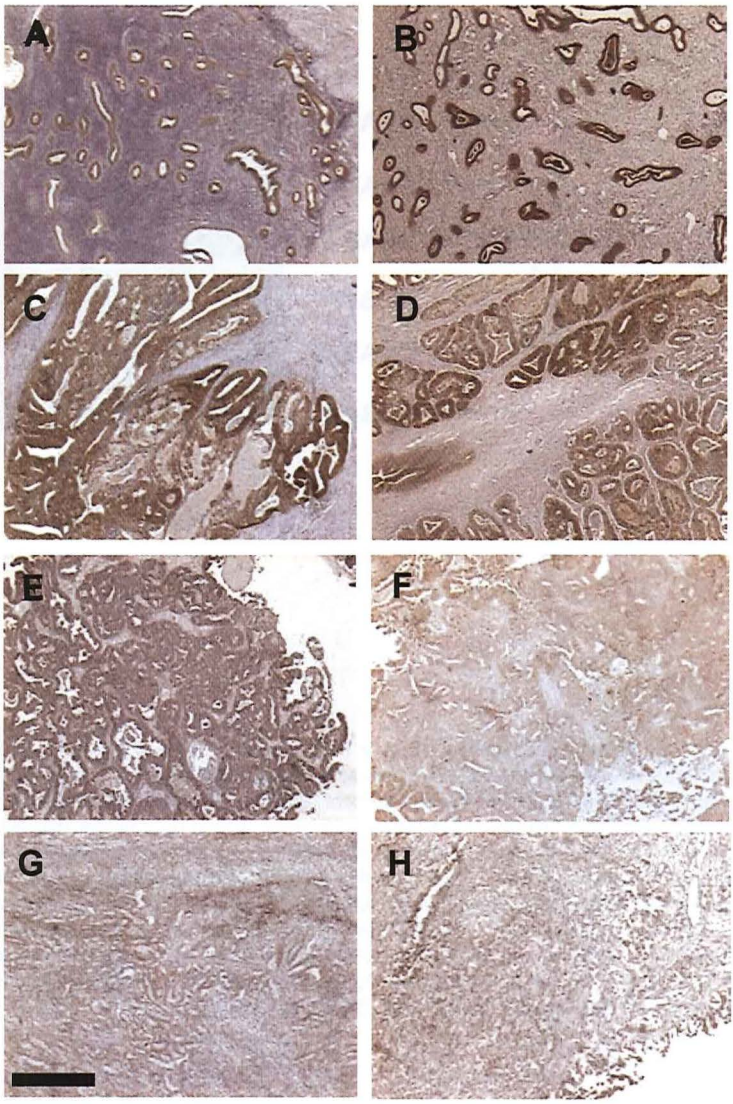
* Because consecutive slides were stained to detect PRAB, CD44, CSPG/versican, tenascin-C, integrin-ß1, fibronectin-1 and E-cadherin (in this order), the section which was stained for E-cadherin is not directly consecutive.


A
STAT6
33.2 1
Keratin 5
Human clone 137308 mRNA, partial cds
Cyclin D2
Progesterone receptor
Nuclear co-repressor 1
NSP1
Progesterone receptor
Mesenchyme homeobox 2
Progesterone receptor ligand-binding domain
Heat shock protein 70
Heat shock protein 70 9B
TNFalfa-induced protein 6
Vimentin
Clusterin
G antigen, family B1
Adenine nucleoide translocator 1
TGF
Ribosomal protein S23
TATA box binding protein
EST/RxR alfa
PDGF- A
V-myb avian myeloblastosis viral oncogene homolog-like 1
Heat shock protein 70 A10
3-phosphoinositide-dependent kinase-1
Protein phosphatase 2, regulatory subunit B56, alpha isoform
Gamma-glutamyl hydrolase
F23
High-mobility group protein 1
CRADD
KIAA0215
APO-1
Glyceraldehyde-3-phosphate dehydrogenase
Alpha-methylacyl-CoA racemase
Collagen 1 alpha1
Carbonic anhydrase II
Transmembrane protease, serine 2
Endothelin 1
Hepatocyte nuclear factor 3, alpha
Cyclin D1
MHC class I promoter binding protein
Cyclin E
P53-binding protein
Ataxia telangiectasia mutated
Deleted in liver cancer 1
TSP-1
Hexabrachion, Tenascin-C
p300-/CBP-associated factor
Caldesmon 1
IL-1 alpha
IGFBP5
E-cadherin
EST, weakly similar to clone 22
P-cadherin
E-cadherin
EST, weakly similar to uncoupling protein-2
Epithelial V-like antigen 1
TGF beta receptor II
Thrombospondin 1
V-erb-b2 avian erythrobastic leukemia viral oncogene homolog 3
Col1A1/PDGF-B fusion transcript
Col1A1/PDGF-B fusion transcript
EPH-related receptor tyrosine kinase ligand 1 precursor
Spleen tyrosine kinase
LIM homeobox gene 2
IGFBP6
Prostate-derived factor
TGF beta
MXT1
TGF beta superfamily protein
EST, weakly similar to RNA polymerase II elongation factor ELL2



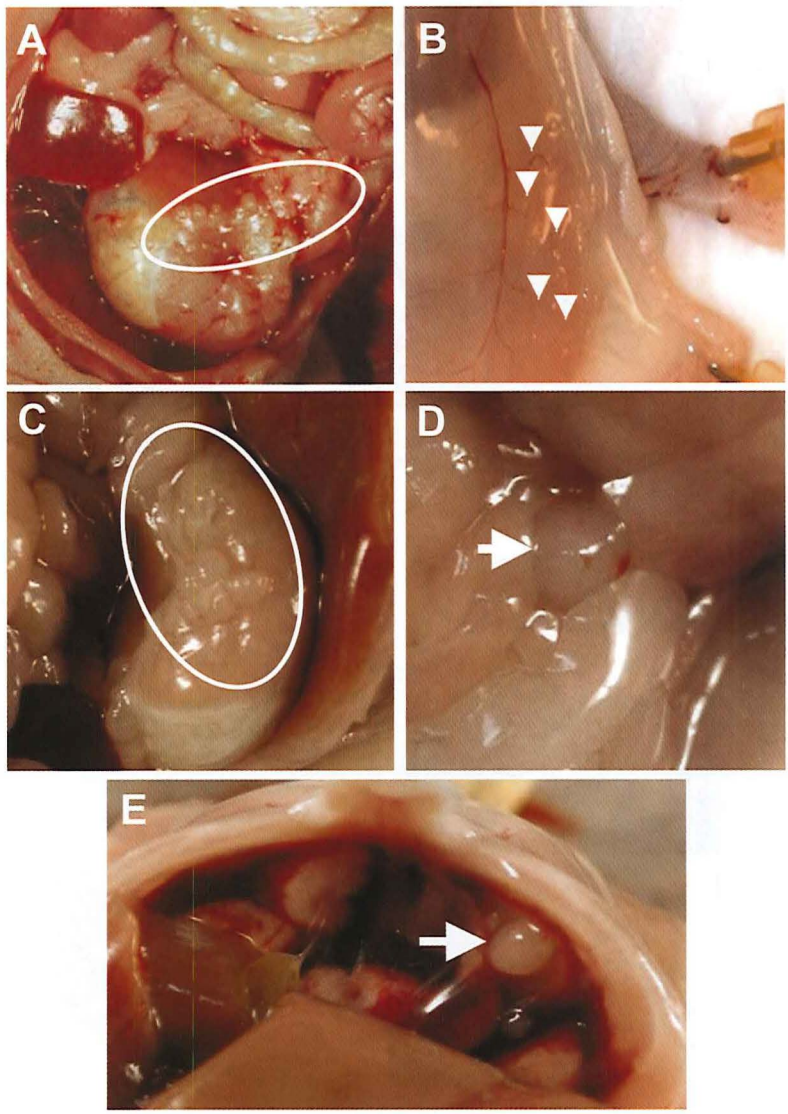
Chapter 3, Figure 1.

Comparison of gene expression in Ishikawa sub-cell lines. Panel A (previous page): Detail of cluster analysis of gene expression patterns in Ishikawa sub-cell lines expressing PRA and/or PRB compared to IKpar. Columns represent the different sub-cell lines; rows represent the different genes. Gene expression is represented as relative to gene expression in IKpar, with green indicating a higher expression of a gene in IKpar than in the PR-expressing sub-cell line, and red indicating a higher expression of the gene in the PR-expressing sub-cell line than in IKpar. A detail of the original clustering (obtained from the expression pattern of 756 genes) is shown. The yellow blocks indicate the genes that are highlighted in panel B. Panel B: Relative expression of E-cadherin (two different clones present on the array), P-cadherin, PR and PR ligand binding domain (LBD) in the Ishikawa sub-cell lines. Rows represent the different cell lines, columns the different genes. On the left side of the figure, the cluster-tree-relation between the different sub-cell lines, obtained from the original clustering of 756 genes, is shown.



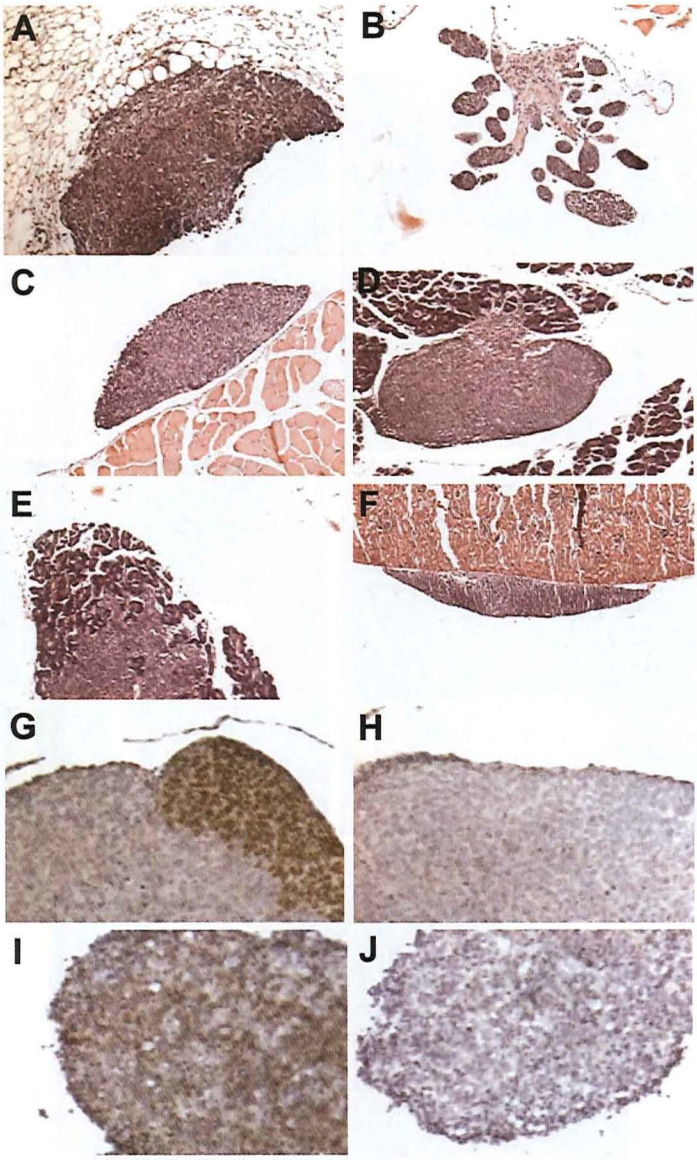
Chapter 3, Figure 4.

Expression of E-cadherin in endometrial cancer tissue. Immunohistochemical detection of E-cadherin in samples of non-neoplastic human endometrial tissue (A-B) and endometrial cancer (C-H). Histological typing and grade (according to the modified FIGO staging system, see also www.FIGO.org/content/PDF/corpus-uteri_p47-49.pdf) and extent of myometrial invasion (MI; defined as less (<), more (>) or equal to (≈) half (0.5) of the thickness of the myometrium) were assessed by the Erasmus MC Department of Pathology. C: grade 1, MI ≈ 0.5; D: grade 1, MI < 0.5; E: grade 2, MI < 0.5; F: grade 2, MI ≈ 0.5; G: grade 3, MI >0.5; H: grade 3, MI > 0.5. Bar indicates 0.5 mm.



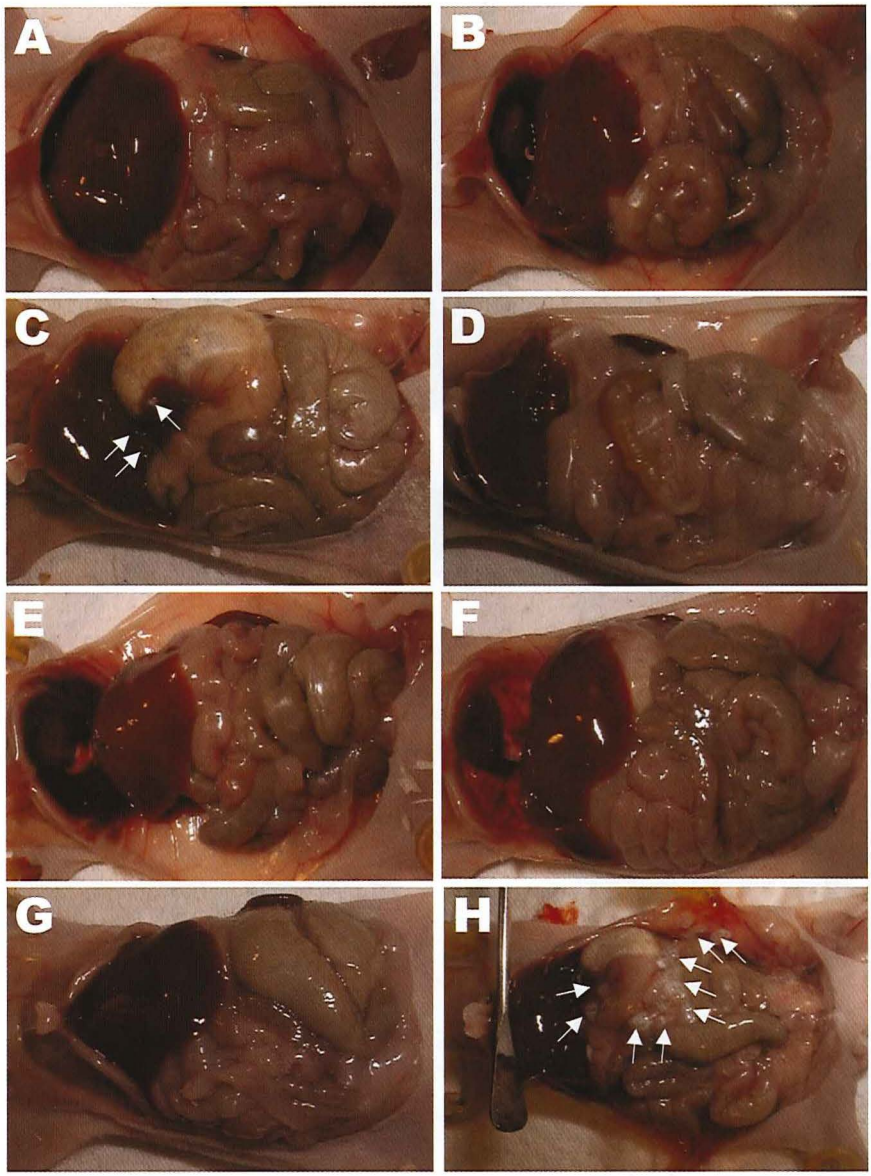
Chapter 4, Paper 1, Figure 2.

Macroscopic investigation of abdominal tumor growth. Panel A shows tumor growth on the peritoneum covering the stomach after 5 weeks. After 7 weeks, tumor growth was clear on the peritoneum covering the abdominal wall (B) and again covering the stomach (C). After 10 weeks, additional large tumor nodules were present in the abdominal fat (D) and on the diaphragm (E). Tumor nodules are encircled (A and C) or indicated by arrowheads (B) or arrows (D and E).



Chapter 4, Paper 1, Figure 3.

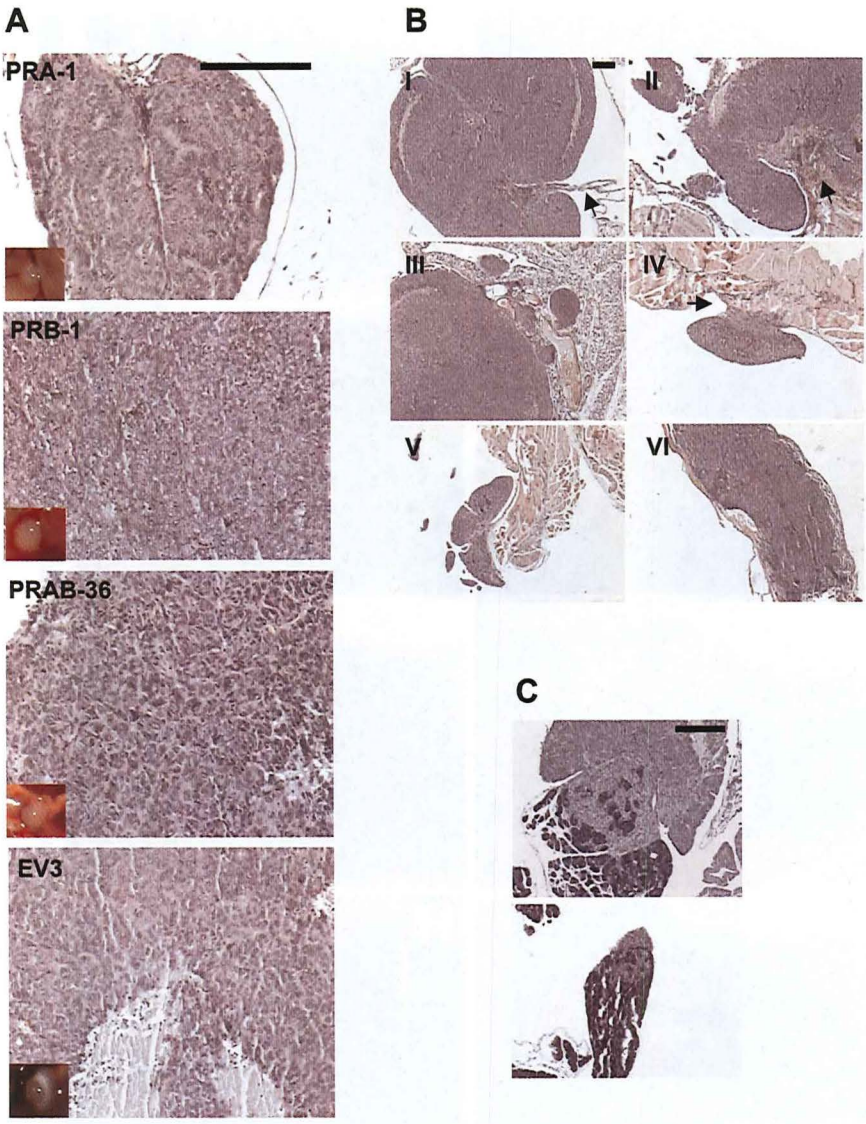
Microscopic analysis of tumor foci. Hematoxylin-eosin staining of nodules growing in the abdominal fat (A), on the mesenterium (B), and on the peritoneum (C) is shown. After ten weeks, tumor was growing into the pancreas of both animals (D and E) and on the left kidney of one animal (F). Immunohistochemical analysis was performed to detect expression of hPR (G and I, with H and J as respective negative controls). Original magnification 50 x (A-F) and 100 x (G-J).



Chapter 4, Paper 2, Figure 2.

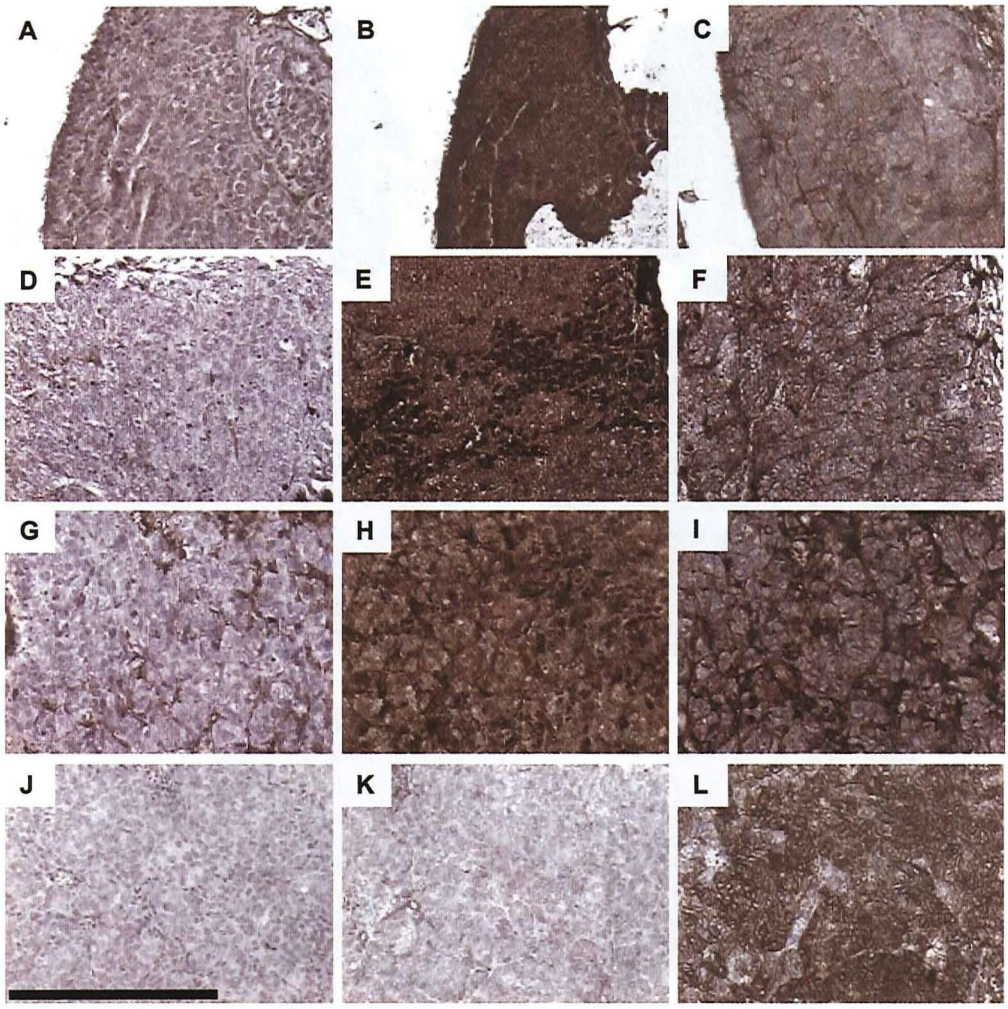
Macroscopic anatomy of mice representative of their group. A and B: PRA-1; C and D: PRB-1; E and F: PRAB-36; G and H: EV3. Mice without MPA substitution are shown on the left (panel A, C, E, G); mice with MPA substitution on the right (panel B, D, F, H). The massive abdominal tumor growth in the EV3 mice that were substituted with MPA is clearly visible (H, arrows). Small tumor spots are also visible in the PRB-1 without MPA (C, arrows). No tumor is visible in PRA-1 and PRAB-36 mice (A, B, E, F).

Color figures



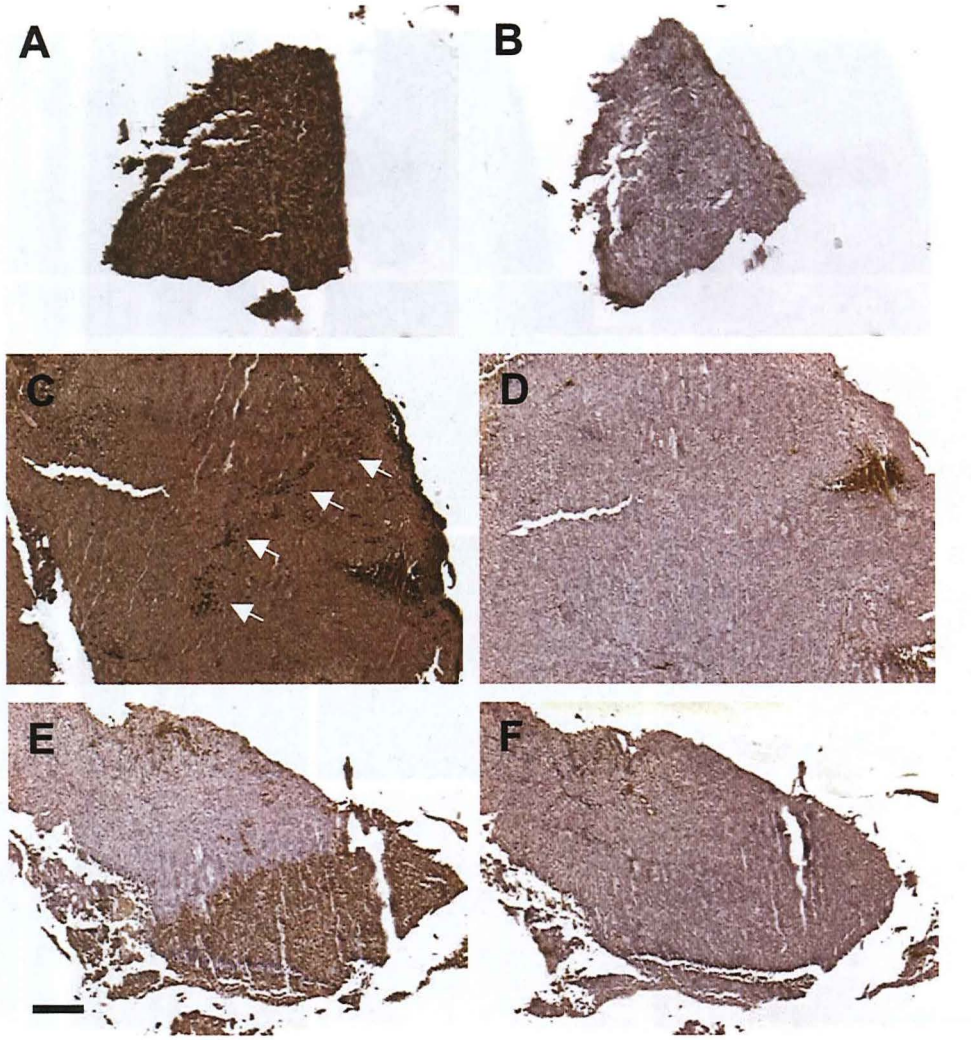
Chapter 4, Paper 2, Figure 3.

Histological comparison of tumor spots. Panel A: Hematoxylin-eosin stained sections of tumor spots originating from the different cell lines. Small inserts show the macroscopic appearance of the spot, microscopic pictures are (black bar represents 0.05 mm). Panel B: tumors were found growing from stalk-like structures (I and II, EV3 tumors), and attached to abdominal surfaces: III: EV3 tumor attached to fatty tissue; IV: EV3 tumor growing on ventral abdominal wall; V: PRB-1 tumor growing on ventral abdominal wall; VI: PRB-1 tumor growing on the diaphragm. Black bar represents 0.05 mm. Panel C: tumors originating from EV3 (upper panel) and PRB-1 (lower panel) cells were found to grow invasively into the pancreas. Black bar represents 0.05 mm.



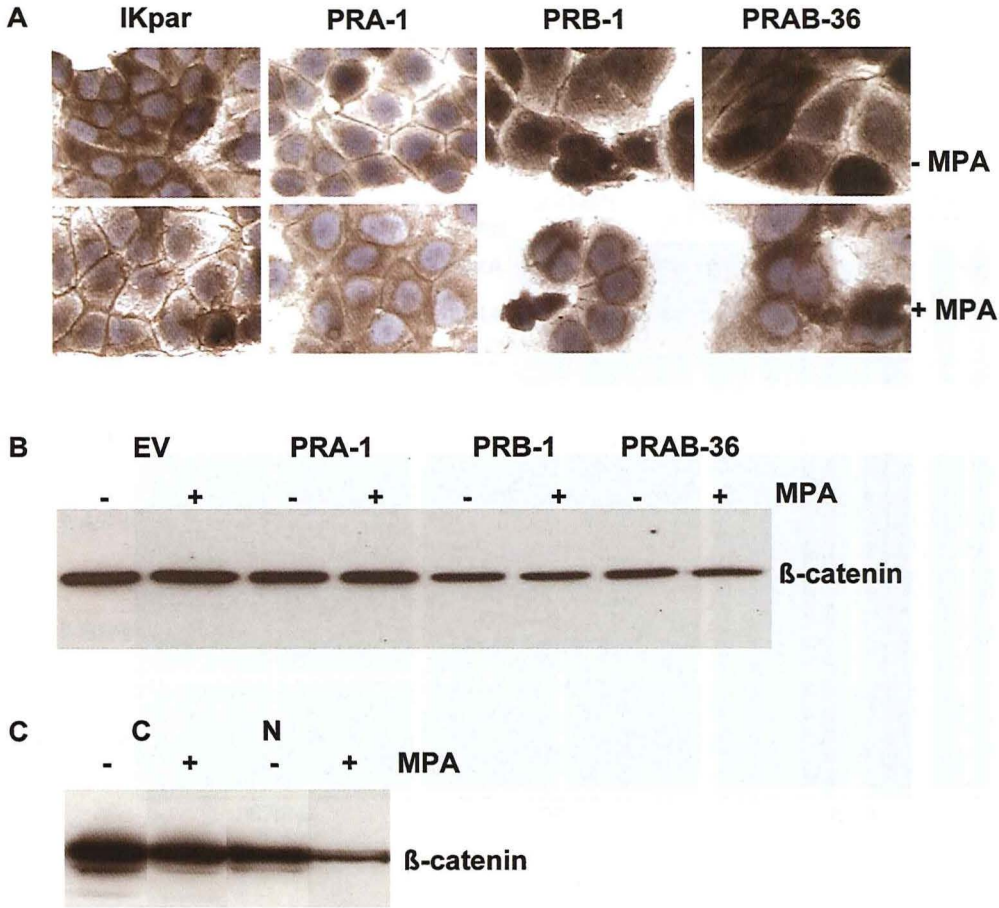
Chapter 4, Paper 2, Figure 4.

Progesterone receptor expression in tumors originating from different cell lines. Panels A, B, C: PRA-1; Panels D, E, F: PRB-1; Panels G, H, I: PRAB-36; Panels J, K, L: EV3. Immunohistochemical staining for both PRA and PRB to assess PR-status (middle panels) or CD44 to detect human cells (right panels). Negative control slides (no primary antibody) are shown on the left (left panels). Black bar represents 0.05 mm.



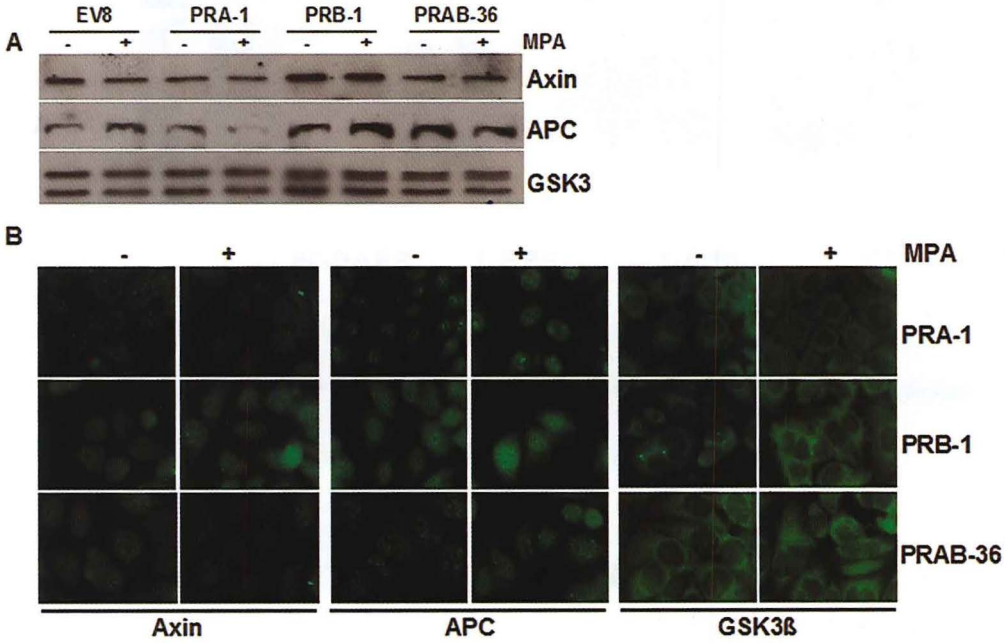
Chapter 4, Paper 2, Figure 5.

Variation in homogeneity of PR expression within different tumors in the same mouse. Patterns showing overall high expression of PR (panel A), overall low expression with regions of high expression (panel C, arrows indicate regions with high PR expression) or only partial expression of PR (panel E) in tumors taken from one PRB-1 mouse which was not substituted with MPA. Negative control slides are shown on the right (B, D, F). Black bar represents 0.05 mm.



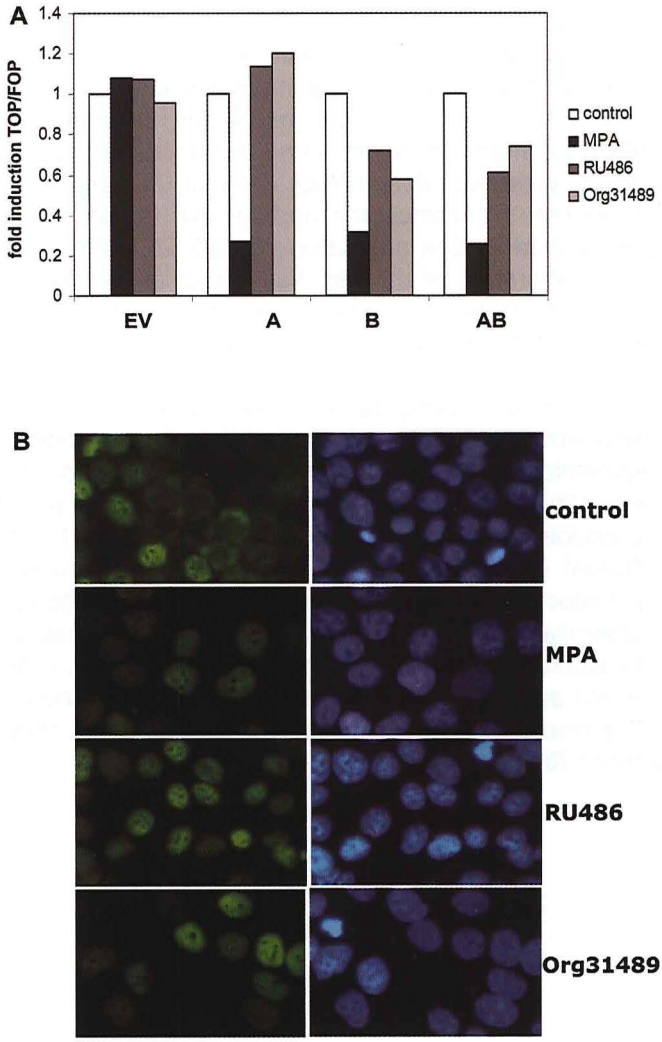
Chapter 5, Figure 2.

Localization of ß-catenin in Ishikawa cell lines. Panel A shows immunohistochemical detection of ß-catenin in Ishikawa cell lines IKpar, PRA-1, PRB-1 and PRAB-36. Cells were cultured for 72 hours in presence of 100 nM of MPA, or vehicle (ethanol) only. Panel B: Western blot, indicating that total protein expression of ß-catenin in these cells is not influenced by MPA. Panel C represents detection of ß-catenin on a Western blot of cytosolic (C) and nuclear (N) protein fractions in PRB-1 cells.



Chapter 5, Figure 3.

Expression of APC, axin and GSK3ß in Ishikawa cells is not influenced by MPA. Panel A: Detection of APC, axin, and GSK3ß on Western blots of total cell lysates of Ishikawa cells cultured for 72 hours in absence or presence of 100 nM MPA. Panel B: immunofluorescent detection of APC, axin, and GSK3ß in Ishikawa cells cultured on glass coverslips for 72 hours in absence or presence of 100 nM MPA.



Chapter 5, Figure 6.

Effect of antiprogestins on TCF/ß-catenin signaling. In all assays, 150 ng/well reporter was transfected. Reporter signal was measured 72 hours post transfection. Panel A: EV8 cells were transfected with 10 ng/well PRA or PRB, and treated with MPA, RU486 or Org31489 (all 100 nM). Y-axis represents fold induction of the TOPflash/FOPflash ratio compared to vehicle alone (white bars). Panel B: incubation of PRB-1 cells for only one hour with MPA, RU486 or Org31489 (all 100 nM) resulted in rapid translocation of PRB from the cytosol to the nucleus. Left panels: PRB (green); right panels: DAPI nuclear stain (blue).

Curriculum Vitae

Eline Erika Hanekamp was born June 28, 1975 in Waalwijk, The Netherlands.
Following her graduation from the Dr. Mollercollege in Waalwijk in 1993, she started her study Medical Biology at the Faculty of Medicine of the University of Utrecht. During het study, she did a six-month research period at the laboratory of dr. Hans Lenstra at the Department of Veterinary Bacteriology (Utrecht University), where she studied polymorphisms on the Y chromosome and mtDNA of Bovini, and their possible use as phylogenetic marker. She graduated October 26, 1998, after a one-year research period at the laboratory of dr. Bert Nederbragt at the Department of Veterinary Pathology (Utrecht University), where she studied the role of TGFß-mediated chondroitin sulfate proteoglycans in induction of collagen gel contraction and tumor cell invasion.
After her graduation, she started working Januari 1999 as a PhD student at the laboratory of prof. dr. Peter Rottier at the Department of Veterinary Virology (Utrecht University), studying epidemiology of and development of diagnostic assays for Feline Infectious Peritonitis Virus infection. After realizing that she was not made for a career in virology, she decided it would be better to change course. In September 2000, she started a different PhD project at the department of Reproduction and Development (formerly Endocrinology and Reproduction) at the Erasmus MC in Rotterdam. Under the supervision of dr. Leen Blok, prof. dr. Anton Grootegoed, and prof. dr. Curt Burger, she worked within the Endometrial Cancer Group, investigating the role of progesterone and progesterone receptors in development and metastasis of endometrial cancer. The results of this research are presented in this thesis, which she hopes to defend October 20, 2004.