Consequences of Loss of Progesterone Receptor Expression in Development of Invasive Endometrial Cancer[1]

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ABSTRACT

Purpose: 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.

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

Results: In the PRAB-36 cell line, MPA was found to regulate the expression of a number of invasion- and metastasis-related genes. On additional investigation of five of these genes (*CD44, CSPG/Versican, Tenascin-C, Fibronectin-1,* and *Integrin-β 1*), it was observed that expression and progesterone regulation of expression of these genes varied in subcell 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.

Conclusion: 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. As many as 70% of the patients can be cured (1), 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 premenopausal patients who want to preserve fertility, high-dose progesterone is prescribed because of the growth-inhibiting effects of this hormone on the endometrium (1–3). However, in 80–90% of patients with more progressed disease 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),[3] which belongs to the family of nuclear receptors. Transcription of the hPR gene is under regulation of two different promoters (4). 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 NH_2 terminus. The hPRA and hPRB can be considered as two independent receptors, which display different transcriptional activities. Vegeto *et al.* (5) 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 (6). Furthermore, it has been described that a different set of genes is regulated by progesterone in human breast (7) and endometrial (8) 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 AFs, AF-1 and AF-2, and one inhibitory domain. In hPRB, a third AF (AF-3) has been defined in the NH_2-terminal amino acid stretch. According to Giangrande *et al.* (9) this hPRB-specific AF-3 domain suppresses the activity of the inhibitory domain. Whereas the function of the inhibitory domain in hPRB is neutralized by AF-3, in hPRA the inhibitory domain is still

[3] The abbreviations used are: PR, progesterone receptor; hPRA, human progesterone receptor A; hPRB, human progesterone receptor B; AF, activation function; MPA, medroxy progesterone acetate; CSPG, chondroitin sulfate proteoglycan; DCC-FCS, dextran-coated-charcoal-treated FCS; FIGO, Fédération Internationale des Gynaecologistes et Obstetristes.

functional in inhibiting transcriptional activity. In support of this view, Giangrande *et al.* (6) have shown that in contrast to hPRB, hPRA was unable to recruit coactivators. In addition to the functional difference of both isoforms, hPRA and hPRB are reported to be located in different cellular compartments in the absence of ligand. Lim *et al.* (10) found that unliganded green fluorescent protein-labeled hPRA is localized predominantly in the nucleus, whereas unliganded green fluorescent protein-labeled hPRB is mainly present in the cytoplasm. On activation by ligand, both receptors are exclusively located in the nucleus (10).

In general, literature agrees on a loss of PR in endometrial cancer (11–19), but reports are conflicting whether this is a consequence of selective down-regulation of hPRA or hPRB, or of both receptors. Fujimoto *et al.* (17) reported that in advanced tumors, hPRA could not be detected. In accordance with this, the same group later described that in distant metastases of endometrial cancer, it is hPRB that is expressed predominantly (18). Arnett-Mansfield *et al.* (19) found a reduced expression of either one or both of the two PR isoforms in the majority of endometrial tumors, compared with hyperplastic or normal endometrial tissue. In addition, hypermethylation of PRB alleles (20) and expression of exon-deleted PR mRNA (21) 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.[4] Making use of this model, we studied differences in expression of metastasis-related genes in the different Ishikawa subcell lines. Using endometrial cancer tissue samples we studied the expression of these genes in relation to the loss of progesterone receptors *in vivo*.

MATERIALS AND METHODS

Cell Culture. Ishikawa endometrial cancer cells (clone 3H12, additionally referred to as IKpar) were obtained from Dr. Masato Nishida (Kasumigaura National Hospital, Tsuchiura, Ibarakiken, Japan) and maintained in DMEM/F12 (Life Technologies, Inc., Carlsbad, CA), supplemented with 10% v/v FCS (Life Technologies, Inc.) and penicillin/streptomycin. The subcell lines that are used are IKpar cells that have been stably transfected with hPRA (line PRA-1), hPRB (line PRB-1), or both receptors (line PRAB-36), as described previously by Smid-Koopman *et al.*[4] These subcell 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) to maintain selection pressure for the transfected human progesterone receptors. This selection pressure was maintained throughout the experiments. Before all of the experiments, cells were cul-

tured for 72 h in medium where FCS was substituted by 5% v/v DCC-FCS. Culture passages 15–25 were used in the experiments.

cDNA Microarray. PRAB-36 cells were cultured for 48 h in DMEM/F12 supplemented with 5% v/v DCC-FCS, either in the presence or absence of progestagen, *i.e.* 10^{-7} M MPA (Sigma, St. Louis, MO). RNA was isolated according to Auffray and Rougeon (22), and shipped to Incyte Genomics, Inc. (St. Louis, MO), 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 10^{-7} M MPA for 0, 8, 24, 48, or 72 h. 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, 549-bp; Fibronectin-1: image139009, 383-bp (both acquired from Dr. Guido Jenster, Department of Urology, Erasmus MC, Rotterdam, the Netherlands); Integrin-β1: image PRB-118212, 136-bp; Tenascin-C: image P107816, 437-bp; and CSPG/Versican: image N038145, 470-bp (all acquired from RZPD, Berlin, Germany). To verify equal loading, an actin-probe (an 1100-bp *Pst*I fragment from hamster β-actin cDNA) was hybridized to the blots.

Collagen Gel Culture. To study protein expression in the different subcell 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. Bert Nederbragt, Department of Veterinary Pathology, Utrecht University, Utrecht, the Netherlands) was mixed with 5× 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: 2×10^6 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 h in the presence or absence of 10^{-7} M MPA. The gels were fixed in formalin and embedded in paraffin 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. Histological typing and grading (according to the modified FIGO staging system; Ref. 23) 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 min. Antigen retrieval was performed in a microwave oven in 10 mM citric acid buffer (pH 6.0) for 3 × 5 min. Slides were allowed to cool to room temperature and washed three times in PBS before being incubated for 20 min in PBS containing 10% v/v blocking

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[4] E. Smid-Koopman, E. C. M. Kuhne, E. Hanekamp, S. Gielen, P. E. De Ruiter, J. A. Grootegoed, T. J. M. Helmerhorst, C. W. Burger, A. O. Brinkmann, F. J. Huikeshoven, and L. J. Blok. Progesterone regulation of growth and gene expression in human endometrial carcinoma cell lines, submitted for publication.

serum. Normal goat serum (DAKO, Glostrup, Denmark) was used as blocking serum for the mouse monoclonal antibodies, and normal rabbit serum (DAKO) 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); anti-E-cadherin: G10/sc-8426; anti-CD44: DF1485/sc-7297; anti-Integrin-β1: 4B7R/sc-9970; anti-Tenascin-C: N-19/sc-9871 (all acquired from SantaCruz Biotechnology, Santa Cruz, CA); anti-CSPG: CS-56 (Sigma); and anti-Fibronectin-1: FBN11 (NeoMarkers). 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 subcell lines. Incubation with primary antibody occurred overnight at 4°C. Antibodies were detected indirectly with biotin-labeled goat-antimouse or rabbit-antigoat antibodies (DAKO) 1:400 in PBS containing 1% v/v blocking serum for 30 min at room temperature, followed by StreptABComplex (DAKO) 1:1:200 in PBS (30 min at room temperature). Staining was developed with 3,3′-diaminobenzidine/concentrated metal complex (Pierce, Rockford, IL). Slides were counterstained with hematoxilin, dehydrated, and mounted.

Expression of the proteins was approached semiquantitatively by visual estimation. Four categories of expression were used: ++: > 80% positive; +: 60–80% positive; ±: 30–60% positive; and −: < 30% positive.

Western Blot. PRAB-36 cells were cultured for 0, 8, 24, 48, or 72 h in the presence of 10^{-7} M MPA. Cells were washed twice with PBS, after which the culture flasks were placed at −80°C overnight. Cells were lysed with RIPA buffer [40 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10% glycerol, 10 mM sodium phosphate, 10 mM sodium molybdate, 50 mM NaF, and 0.5 mM sodium orthovanadate], containing 10 mM DTT, 1% Triton X-100, 0.08% SDS, 0.5% sodium deoxycholate, and 1× complete protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The lysates were centrifuged at $1000 \times g$ for 40 min, 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 h in blocking buffer (PBS/Tween containing 5% nonfat milk powder) and incubated overnight at 4°C with a mouse monoclonal antibody recognizing Fibronectin-1 (clone EP5; Santa Cruz Biotechnology) diluted 1:1000 in blocking buffer. The blots were then washed four times for 10 min in PBS/Tween, after which they were incubated with peroxidase-labeled secondary goat-antimouse antibody (DAKO) and diluted 1:1000 in blocking buffer for 1 h at room temperature. The blots were washed six times for 10 min in PBS/Tween before protein bands were visualized using Western Lightning Chemiluminescence reagent (Perkin-Elmer Life Sciences Inc., Boston, MA) according to the manufacturer's manual.

RESULTS

Progesterone Regulation of Gene Expression in PRAB-36 Cells. To make a first selection of genes that are regulated by progesterone in endometrial cancer cells, a Human UniGene 1 microarray analysis (IncyteGenomics Inc.) was performed. Using this array, ~90 genes were identified that were regulated by the progestagen MPA in the PRAB-36 subcell line (cutoff 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 that are involved in cell-matrix interactions, and as such in tumor cell invasion and/or metastasis, for additional investigation. These genes are: *CD44, CSPG /Versican, Tenascin-C, Integrin*β *1,* and *Fibronectin-1.*

Analysis of Expression of CD44, CSPG/Versican, Tenascin-C, Integrin-β 1, and Fibronectin-1 in Ishikawa Subcell Lines Expressing PRA and/or PRB. To confirm the microarray data and to study expression of the selected genes in the Ishikawa subcell 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*β *1,* and *Fibronectin-1*) between the different PR isoform-expressing subcell lines (Figs. 1 and 2) in the absence of MPA. Furthermore, as described in detail below, expression of the majority of these genes was regulated by MPA. The level of expression of both CD44 and CSPG/Versican in the absence of MPA is lower in the IKpar and PRA-1 subcell lines compared with the PRB-1 and PRAB-36 sub-cell lines (Fig. 1, *A* and *B*; Fig. 2, *C* and *D*). CD44 and CSPG/Versican show similar regulation patterns in the different subcell lines. Both genes are down-regulated by MPA in the PRB-1 and PRAB-36 sub-cell lines at the mRNA level (Fig. 1A and 1B). At the protein level in PRB-1 and PRAB-36 subcell lines, deposition of CD44 and CSPG/Versican in the extracellular matrix is clearly reduced when the cells are cultured in the presence of MPA (Fig. 2, *C* and *D*). For the PRA-1 subcell 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. 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 subcell line (Fig. 1*C*). Expression of Tenascin-C in the absence of MPA is also very low in the PRB-1 cells, and regulation of expression by MPA is not detectable (Fig. 1*C*). However, at the protein level, expression of Tenascin-C is high, and is down-regulated by MPA in the PRB-1 and PRAB-36 subcell lines (Fig. 2*E*). In the PRA-1 subcell 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 (Fig. 1*C*; Fig. 2*E*). At the mRNA level, basal expression levels of Integrin-β1 expression is similar in all of the subcell lines. Expression of Integrin-β1 is up-regulated by MPA in the PR-expressing subcell lines on Northern blot (Fig. 1*D*). The apparent up-regulation of Integrin-β1 in the IKpar line is because of a difference in the amount of RNA loaded onto the gel (Fig. 1*F*). At the protein level, Integrin-β1 is expressed at high levels in all of the subcell lines. There is no visible regulation of expression by MPA (Fig. 2*F*), but this may be because of the high basal level of expression. 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

Table 1 Progesterone-regulated genes in the PRAB-36 cell line

The Ishikawa subcell line PRAB-36 was cultured for 48 h in presence or absence of 10^{-7} M 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.

Tumor progression/tissue remodeling/metastasis

−3.2	fibronectin 1	ECM component, cell-matrix interactions	3.7	collagen type IV, alpha3	cell-matrix interactions
−2.4	chondroitin sulfate proteoglycan 2 (versican)	cell-matrix interactions	3.5	epithelial V-like antigen	placental morphogenesis
−2.0	tenascin C (hexabrachion)	tissue remodeling	3.3	leucocyte cell adhesion molecule	tumor leukocyte recruitment
−2.0	B-cell CLL/lymphoma 1	leukemia	2.4	antileukoproteinase	placental development
−1.9	urokinase plasminogen activator	matrix degradation	2.3	BTG family, 2	cell cycle, differentiation
−1.7	CD44	cell-matrix interactions	2.1	integrin, beta 1	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 avian sarc. virus 17 homologue	oncogenesis			
−1.6	tissue factor pathway inhibitor 2	vascular remodeling, apoptosis			
−1.6	claudin 1	tight junction, recruiting MMPs			

Signal transduction

−2.7	serum-inducible kinase	serine/threonine protein kinase	3.5	fatty-acid-Coenzyme A ligase, long-chain 2	fatty acid synthesis
−1.8	aldehyde dehydrogenase 1 family, A1	drug resistance, proliferation	3.3	methionine adenosyltransferase II, alpha	s-adenosylmethionine synthesis
−1.6	UDP-glucose ceramide glucosyltransferase	glucosphingolipid synthesis	3.1	FK506-binding protein 5	cell signaling, transcription
−1.6	G protein-coupled receptor kinase 5	β-adrenergic receptor signaling	2.7	protein fosfatase 2, cat. subunit β isoform	protein phosphorylation
			2.7	serum/glucocorticoid regulated kinase	serine/threonine protein kinase
			2.6	protein fosfatase 1, cat. subunit, β isoform	serine/threonine phosphatase
			2.3	signal recognition particle 54kD	protein targeting to membranes

Ion homeostasis

−2.2	salivary proline-rich protein	Ca-homeostasis in saliva	3.0	ferritin, heavy polypeptide 1	iron metabolism
−1.9	annexin A4	ion channeling	2.5	AHNAK nucleoprotein (desmoyokin)	Ca-homeostasis
−1.8	solute carrier family 7	organic solute transporter	2.4	solute carrier family 22, member 5	organic cation transporter

Various

−4.2	IGFBP3	IGF 1 transporter	3.3	IgM	primary antibody response
			2.7	IL-1 receptor, type 1	interleukin signaling, innate immunity
			2.2	IFN-γ receptor 1	co-ordination tumor-immune responses
			2.2	lethal giant larvae homologue 2	cell polarity

expression but no regulation was observed. In the IKpar line, expression of Fibronectin-1 was detected, but this was not regulated by MPA (Fig. 1*E*). At the protein level, MPA-induced down-regulation of Fibronectin-1 could be observed only in the PRAB-36 subcell line (Fig. 2*G*). This observation was confirmed by Western blot (Fig. 2*H*).

Expression of CD44, CSPG/Versican, Tenascin-C, Integrinβ 1, 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-β 1, and Fibronectin-1. Because loss of expression of E-cadherin in epithelial tumors strongly correlates with increased tumor cell invasion and metastasis (24), and is also associated with more aggressive endometrial cancer (25), this marker was studied as an indicator for potential risk for invasion 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
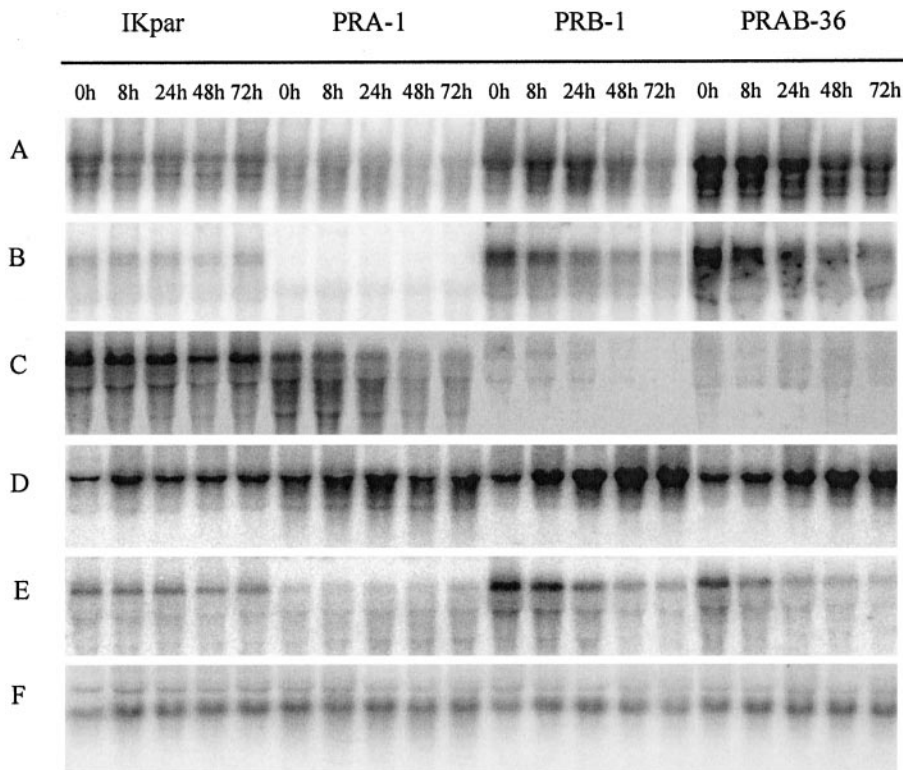
Fig. 1 Regulation of expression of CD44, CSPG/Versican, Tenascin-C, Integrin-β 1, and Fibronectin-1 mRNA in the IKpar and PRA and/or PRB-expressing subcell lines. IKpar, PRA-1, PRB-1, and PRAB-36 cells were cultured in the presence of 10^{-7} M MPA for 0, 8, 24, 48, and 72 h. Total RNA was isolated, separated on agarose gel, blotted, and hybridized to ^{32}P-labeled cDNA-probes from: CD44 (*A*), CSPG/Versican (*B*), Tenascin-C (*C*), Integrin-β 1 (*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.

cell surface (Table 2; Fig. 3*D*). 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 Fig. 3. Results of all of the 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-β 1, 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 (Fig. 3*B*; Table 2). Similar observations were made for CSPG/Versican expression (Fig. 3*C*; Table 2). For Tenascin-C, Integrin-β1, and Fibronectin-1 immunostaining, there was no clear correlation with PR expression (Table 2).

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 multistep process, starting with tumor cell invasion. This involves detachment of cells from the primary tumor and migration of tumor cells through the extracellular matrix (26). 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 (27). Therefore, molecules that have a function in these processes are 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 subcell line PRAB-36, it was observed that a large portion of the identified progesterone-regulated genes encoded for proteins that are potentially involved in tumor progression and/or invasion/metastasis. For instance, the progesterone down-regulated genes *Urokinase plasminogen activator* and *Claudin-1* cooperate with matrix metalloproteinases in the process of extracellular matrix degradation (28, 29). Another progesterone down-regulated gene, *CYR61,* promotes angiogenesis (30). 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*β *1,* 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 subcell line, whereas *Integrin-β1* was up-regulated. The functions of the proteins encoded by these genes are discussed briefly first.

CD44 is a cell surface molecule, which exists in many isoforms and is mainly involved in cell-matrix interaction (31).
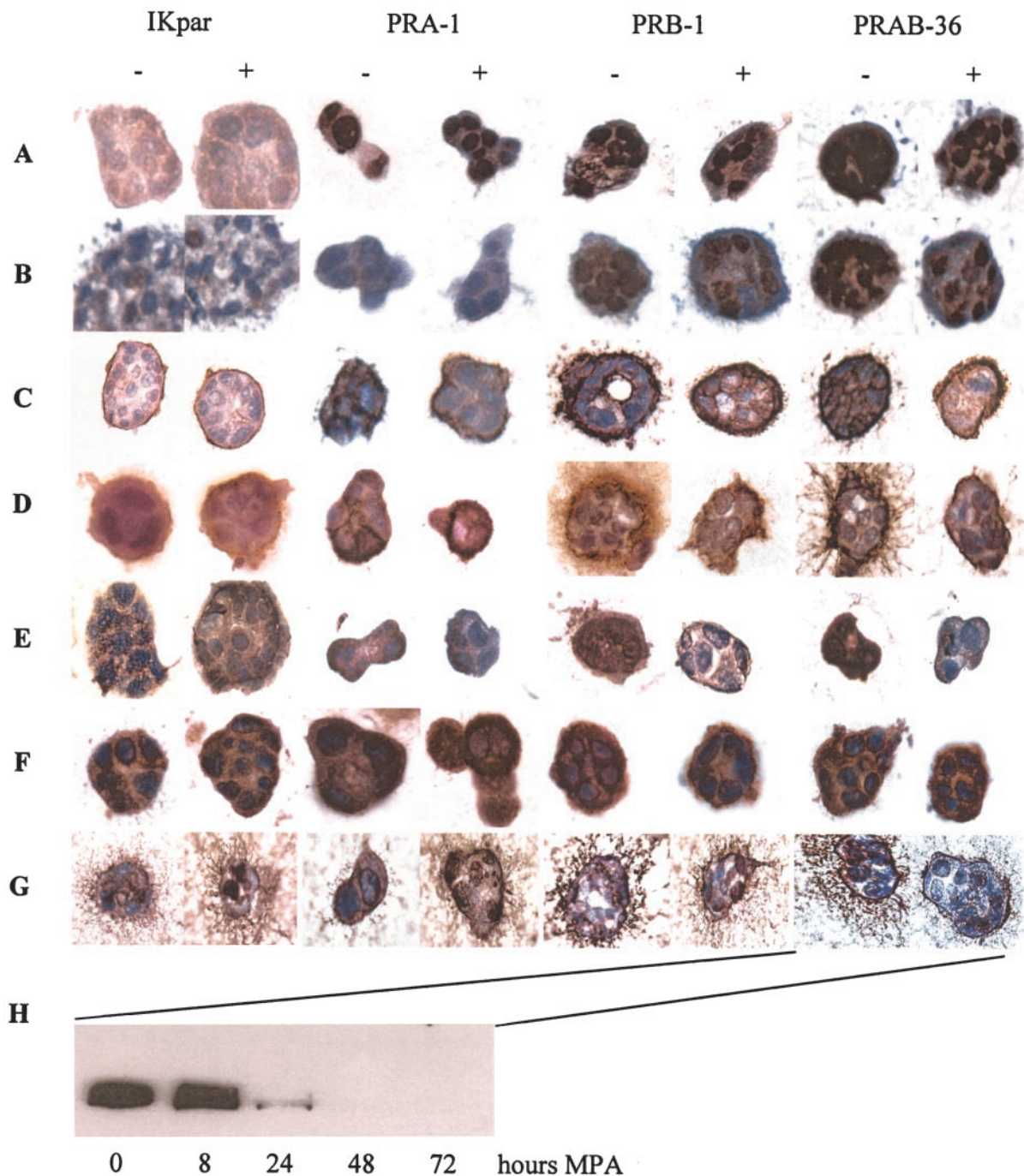
Fig. 2 Protein expression of PR, CD44, CSPG/Versican, Tenascin-C, Integrin-β 1, and Fibronectin-1 in the IKpar, and PRA- and/or PRB-expressing subcell lines. Ishikawa subcell lines IKpar, PRA-1, PRB-1, and PRAB-36 were cultured in collagen gels, in absence (−) or presence (+) of 10^{-7} M MPA, for 72 h. 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-β 1 (*F*), and Fibronectin-1 (*G*). Each panel shows one cluster of cells in collagen gel. Original magnification ×400. *H* shows Western blot analysis of expression of Fibronectin-1 after 0, 8, 24, 48, and 72 h of culturing in presence of 10^{-7} M MPA.

CD44 can promote tumor growth and invasiveness (32), and over-expression of CD44 is associated with increased metastasis in endometrial cancers with increased myometrial invasion (25). CD44 has been implied in regulation of postpartum uterine

involution through association with several matrix metallopro-teinases (33).

CSPG/Versican is a member of the family of proteoglycans, which interacts with CD44. CSPG/Versican has been reported to

Table 2 Analysis of expression of PR, CD44, CSPG/Versican, Tenascin-C, Integrinβ1, 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: G_1 (well-differentiated), 2: G_2 (moderately differentiated), 3: G_3 (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; Ref. 23; see also website.[6]) and extent of myometrial invasion were assessed by the Erasmus MC Department of Pathology.

	MI	*stage*	E-cad	PR	CD44	CSPG	Ten	Int	Fibr
H	0	n.a.[a]	++[b]	++	−	−	++	++	+
H	0	n.a.	++	++	−	−	+	+	+/−
H	0	n.a.	+	+	−	−	+	++	+
1	0	1a	+	+	+/−	−	+/−	+	+/−
1	<0.5	1b	++	++	−	+	++	++	+
			+	+/−	+/−	+	++	+/−	−
1	<0.5	11a	++	+	++	+/−	+	++	+
			+	++	+/−	+/−	+/−	+	++
1	≈0.5	1c	+	+/−	+/−	+/−	+	++	+/−
2	<0.5	1b	+/−	+/−	+/−	+	+/−	+/−	+
2	>0.5	1c	++	++	−	−	++	+	+/−
2	>0.5	1c	+	+/−	++	−	+/−	+	+
3	≈0.5	1b	+/−	+/−	++	++	+	++	++
			−	−	++	+	+/−	+/−	++
3	≈0.5	1b	−	−	+/−	+	+	+/−	+
3	>0.5	1c	+	−	+	+	++	++	+/−
3	>0.5	1c	−	−	++	+/−	+	++	+/−
3	>0.5	1c	+/−	−	+	++	+	++	−

[a] n.a., not applicable; PR, hPRA + hPRB; E-cad, E-cadherin; CSPG, CSPG/Versican; Ten, Tenascin-C; Int, Integrin-β1; Fibr, Fibronectin-1.
[b] Staining was approximated semiquantitatively 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.

mediate tumor cell invasion (34). Also, the CSPG-degrading chondroitinases AC and B inhibit invasion of melanoma cells (35).

Tenascin-C plays a role in tissue remodeling during embryonic development and is re-expressed in many different tumors (36–38). Tenascin-C can stimulate proliferation, but growth-inhibiting effects have also been reported (37, 38). In breast cancer, Tenascin-C expression correlates with increased invasion and is suggested as a prognostic factor for metastasis (39, 40).

Fibronectin-1 is a major component of the basal lamina. It has functions in a variety of processes, such as proliferation, adhesion, and migration (41, 42). 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 α5β1, collagen, and CSPG (41, 43). Dai *et al.* (44) also showed recently 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, Tenascin-C, and Fibronectin-1 in the

PRAB-36 subcell line provides evidence that progesterone could play an inhibiting role in tumor cell invasion.

In contrast with the four down-regulated genes discussed above, Integrin-β1 was found to be up-regulated by MPA in the PRAB-36 subcell line. Integrin-β1 is located in the plasma membrane, where it functions as a complex with Integrinα chains. Integrins mediate cell-matrix interactions that are necessary for cell migration and are involved in bidirectional cell signaling (45). Increased expression of integrins in cancer has been associated with increased metastasis through various pathways (46–49). Therefore, up-regulation of Integrin-β1 by MPA in the PRAB-36 subcell line 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-β1 can interact with E-cadherin to inhibit tumor cell detachment and invasion into the extracellular matrix (50, 51). In this last setting, the up-regulation of Integrin-β1 by MPA in the PRAB-36 cell line is consistent with the suggested overall invasion- and metastasis-inhibiting effect of MPA. Expression of Integrin-β1 was reported recently to be strongly down-regulated in poorly differentiated endometrial cancer cells transiently overexpressing hPRB (44).

In the absence of progesterone-regulation, CD44, CSPG/ Versican, Tenascin-C, and Fibronectin-1 show differential
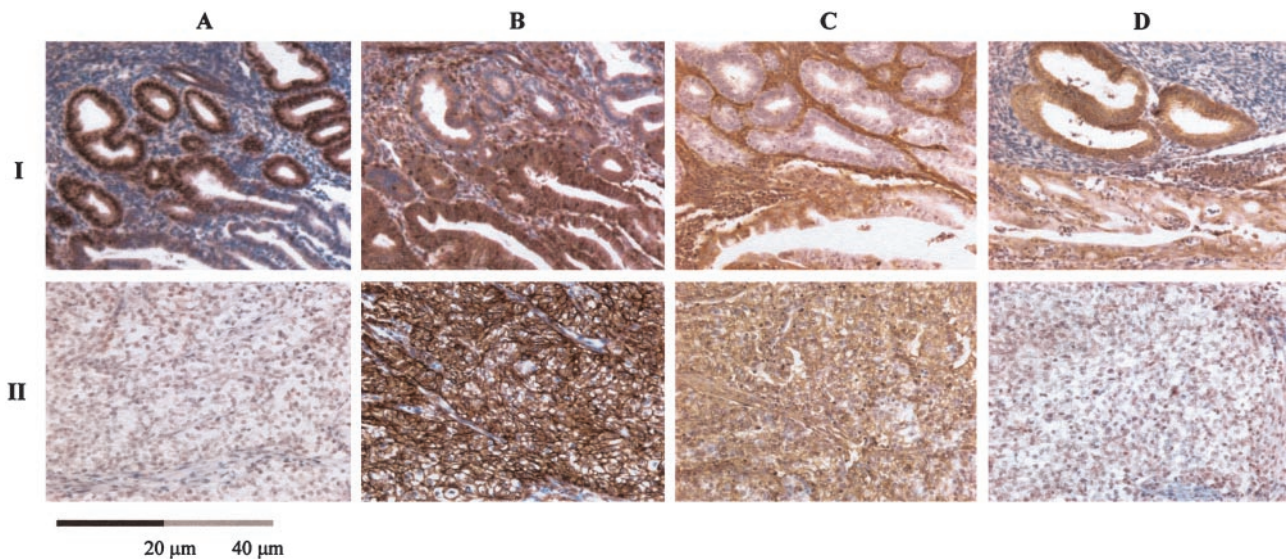
Fig. 3 Expression of PR, CD44, CSPG/Versican, and E-cadherin in endometrial cancer tissue samples. Sections of formalin-fixed, paraffin-embedded samples of endometrioid adenocarcinoma were immunohistochemically stained for hPRA + hPRB (*A*), CD44 (*B*), CSPG/Versican (*C*), and E-cadherin (*D*). A representative set of stainings in semiconsecutive[5] slides of a well-differentiated (G_1, stage Ia) tumor sample (*I*) and an undifferentiated (G3, stage Ic) tumor sample (*II*) is shown. Histopathological typing, grade, and stage were assessed by the Erasmus MC Department of Pathology (according to the modified FIGO staging system; Ref. 23). See also website.[6] Original magnification ×200.

expression in the different Ishikawa subcell 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 subcell lines that express the hPRB (PRB-1 and PRAB-36), whereas 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 the absence of MPA. From the present results, it can be expected that endometrial cancer cells that express hPRB may be more invasive than cells that do not express hPRB.

The observation that MPA down-regulates CD44, CSPG/Versican, and Fibronectin-1 in the hPRB-expressing subcell 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 subcell 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 (7, 8). From the observed profile of gene regulation by MPA in the Ishikawa subcell 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.* (44), 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-β1, 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-β1, 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 (24). Cadherins are transmembrane glycoproteins that take part in calcium-dependent cell-cell adhesion (24, 27, 52). 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

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[5] Because consecutive slides were stained to detect PRAB, CD44, CSPG/Versican, Tenascin-C, Integrinβ1, Finbronectin-1, and E-cadherin (in this order), the section that was stained for E-cadherin is not directly consecutive.

an early, and possibly initializing event in the development of a more invasive phenotype in endometrial cancer.

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