Novel Progesterone Target Genes Identified by an Improved Differential Display Technique Suggest That Progestin-induced Growth Inhibition of Breast Cancer Cells Coincides with Enhancement of Differentiation*

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The steroid hormone progesterone has important and complex effects in female sex organs. In two of its major target organs, the uterus and the mammary gland, it strongly influences proliferation and differentiation. In mouse mammary epithelium, progesterone stimulates proliferation, which eventually culminates in glandular development. In the endometrial epithelium, progesterone inhibits estrogen-mediated growth but also induces differentiation (reviewed in Ref. 1). In line with this, knock-out mice lacking PR have, apart from defects in the ovaries and in sexual behavior, uninhibited proliferation in the endometrium upon estrogen and progestin treatment but severely impaired mammary gland development (2).

Progestins also have strong effects on human hormone-responsive endometrial and breast cancers. Both in endometrial and mammary carcinomas progestins inhibit estrogen-mediated growth, which is, for endometrial carcinoma, associated with increased differentiation. Progestins are therefore used to treat these cancers (reviewed in Ref. 1). The breast tumor-derived cell line T47D exhibits in vitro a similar inhibition of estrogen- or growth factor-mediated growth by progestins, although initially a brief growth stimulation is seen upon progestrone addition (3). The precise mechanism responsible for this biphase response is not known (reviewed in Ref. 1). The influence of progestins on processes essential in tumor progression, like tumor invasion and metastasis, is poorly understood. For endometrial tumor cells, both in vivo and in vitro studies suggest that estrogens stimulate invasion, while progestins inhibit this process (4, 5). For breast tumor cells, estrogens appear to stimulate invasion (Ref. 6 and reviewed in Ref. 7), while an effect of progestins has not been reported.

Progestins bind inside the cell to the PR, which belongs to the nuclear receptor superfamily of transcription factors (reviewed in Ref. 8). This receptor subsequently homodimerizes and activates gene transcription after binding to progestrone response elements (PRE) in promoters (reviewed in Ref. 9). PREs have the same consensus sequence as response elements for the related glucocorticoid, androgen, and mineralocorticoid receptors. PR cannot only activate, but also repress genes, for example by negative cross-talk with transcription factors of the AP1 and NF-κB families (10, 11). To better understand progestrone effects in human breast epithelial cells, we wanted to identify a larger number of progesterone target genes, as only a limited number of established target genes for this hormone in breast tumor cells is known. Negative target genes are the estrogen receptor (ER) (12) and PR itself (13), while strong positive targets are fatty acid synthetase (14), methallothionen-IIα (15), alkaline phosphatase (16), pepsinogen C (17), epidermal growth factor, and the epidermal growth factor receptor (3). Transient induction of c-fos and c-myc has been reported and might be involved in the initial growth stimulatory effect (3). Induction of none of these genes can explain the observed
growth inhibition by prolonged progesterin treatment of these cells. Therefore, we decided to try to identify additional progesterone target genes in this system.

Recently, a new technique for cloning differentially regulated genes has been published, designated as differential display or RNA fingerprinting (18, 19). This technique has substantial advantages over earlier techniques, e.g., subtractive libraries or hybridization, since it does not have such a strong bias toward highly abundant genes and is much more versatile, enabling cloning of down-regulated as well as up-regulated genes and allowing a rapid comparison between multiple samples. For identifying progesterone target genes we used a differential display technique as modified by Van Belzen et al. (2) adding a number of improvements. In this paper we present, apart from 10 new progesterone target genes, a fast procedure to identify the most promising genes. Some of the genes identified suggest that the T47D breast tumor cell line differentiates upon progesterin treatment. Three of these were repressed by E2 treatment and subsequently up-regulated by the progesterin. By means of immunofluorescence detecting three markers for epithelial differentiation, we could also show that E2 addition resulted in delocalization of the marker proteins, while additional progesterin treatment reversed this effect. From this we conclude that E2 dedifferentiates the T47D cells, while progesterones increase the differentiation state of estrogen-treated T47D cells.

EXPERIMENTAL PROCEDURES

Cell Culture—A phenol red-free 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (DF) was obtained from Life Technologies, Inc. Trypsin and the EDTA used for cell culture were bought from Flow Laboratories (Irvine, Scotland). Fetal calf serum (FCS) was purchased from Integro (Linz, Austria), E2, cycloheximide, dexamethasone, bFGF, human transferrin, and bovine were from Sigma. Org2058 was kindly provided by Organon (Oss, The Netherlands). Ham’s T12–18 was kindly provided by Dr. A. J. M. van den Eijnden-van Raaij. DCC-FCS was prepared by treating FCS with DCC as described (22). RNA was isolated from cells that were treated with 10–8 M E2 (48 h before harvesting) and 10–8 M Org2058 or vehicle (24 h before harvesting). 20 μg of total RNA was digested with RNase-free RQ1 DNAase according to the manufacturer’s protocol (Promega), then phenol-extracted and precipitated (22). Cells were plated at a density of 4 × 104 cells/175 cm2 dish or 1.5 × 105/60 cm2 dish in DF medium containing 5% (v/v) FCS, 20 mM HEPES (pH 7.0), and an agarose gel, bands were excised, purified with glassmax (Life Technologies, Inc.), and cloned in pGEM-T (Promega). If necessary, the PCR was repeated.

Procedure for Elimination of False Positive Clones—DNA was isolated from 6 to 8 colonies per excised band using the cetyltrimethylammonium bromide miniprep procedure (23). DNA was cut with PstI, ApaI, Rsal, and TaqI and analyzed on agarose gel. Clones with different restriction patterns were sequenced and reamplified from the plasmid by standard PCR with the primers used in the differential display. The PCR mix was dot-blotted in duplicate on two Hybond C extra filters (Amersham Corp., Little Chalfont, United Kingdom; Ref. 24). Clones identified with the same primer were spotted on the same two filters. With this primer, two differential display PCR’s were carried out with RNA from progesterone treated and untreated cells. These reactions were performed with 5 μCi of [32P]dCTP instead of 1 μCi of [32P]dATP, purines, and phosphates with ampicillin and ethidium bromide as a probe on the two filters. Filters were hybridized and washed as described (25) in 2-ml Eppendorf reaction vials. A schematic outline of the strategy is depicted in Fig. 1.

Northern Hybridization—Northern blotting was carried out as described (22). The inserts were cut from pGEM-T with ApaI–PstI or SalI–PstI, run on agarose gel, and purified. Labeling was performed with the Rediprime random primer labeling kit and 50 μCi of [32P]dCTP (Amersham), hybridization, and washing was as described (25).

Immunofluorescence Microscopy—Cells were plated at a density of 4 × 104 cells for (3-day inductions) or 1 × 105 cells for (6-day inductions) per coverslip and allowed to attach for 24 h. Medium was refreshed, and hormones were added at the concentrations indicated. After 3 days, medium and hormones were refreshed. Cells were fixed using 2% paraformaldehyde and immunolabeled following standard procedures. As anti-E-cadherin antibody EMCa-1, a kind gift from Dr. R. Kemler (Freiburg, Germany) was used (1:200); anti-a-catenin was from Sigma (1:2000). As second antibody, species-specific CY-3-labeled antibodies from Jackson (West Grove, PA) were used in a dilution of 1:250.

RESULTS

Differential Display—To clone PR target genes we used cDNA prepared from total RNA isolated from control T47D cells in parallel with cells treated for 24 h with Org2058 (a synthetic progesterin). In both cases cells were grown in the presence of E2 for 48 h before harvest to obtain maximal PR induction (26). For each primer the differential display was performed in duplicate with cDNA derived from two different batches of RNA per treatment. In total, 34 primers were used. We isolated 57 clones with different inserts, derived from 38 differentially regulated bands. Most of them represented up-regulation in the presence of the progesterin. All clones were partially sequenced and compared against EMBL-44, EMBL-NEW11, and UGenBank 91_44 data bases, while apparent open reading frames were compared against PIR 46 and Swissprot 31 using IGSetite software. Initially a number of clones was analyzed on Northern blots using total RNA and poly(A)+ RNA Northern blots. Although some clones were found to be up-regulated, several clones gave no signal on Northern blots. In addition, very frequently different clones were isolated from the same band, indicating that the DNA fragments present in the excised gel slices were impure. Therefore, we designed an assay that could potentially discriminate false positives and contaminations from genuine progesterone targets. In Fig. 1 the strategy is illustrated. Because hybridization and washing can be performed in 2-ml Eppendorf tubes, all steps permit simultaneous handling of

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Multiple samples. This allowed us to analyze all samples in one experiment. In this assay, ten positive clones (Ptg-1 to Ptg-10, for progesterone target gene) could be easily distinguished by visual comparison and quantification (Fig. 1). After verification by Northern blot analysis, all of these genes were found to be strongly up-regulated by the progestin (Fig. 2A). Ptg-1, Ptg-5, and Ptg-10 were also clearly up-regulated after 6 h by the progestin in the presence of cycloheximide, a protein synthesis inhibitor. This indicates that these genes are direct progesterone receptor targets, since protein synthesis is not required. To check if PR target genes had been missed with this selection procedure, 18 from the 47 remaining clones were checked on Northern blots. Five gave no signal at all, ten appeared to be false positives, and three (clones Ptg-11 to Ptg-13) were slightly regulated by the progestin (Fig. 2B). In conclusion, with this assay we did not obtain false positives. Analysis of the remaining clones suggested that only some weakly regulated clones were missed (3 out of 13 giving a signal on Northern blots). Therefore, this method is a very effective selection procedure, particularly for strongly induced mRNAs.

**Novel Progesterone Target Genes**—The ten clones identified in the Dot blot assay, Ptg-1 to Ptg-10, were derived from seven different genes: CD-9/MRP-1, a gene known to have effects on tumor metastasis and cell motility (27, 28); CD-59/protectin, a gene coding for a protein that protects cells from lysis by complement factors, acting as a ligand for CD-2 and which has been shown to bind to Gi (29–31); TSC-22, a gene encoding a putative transcriptional repressor (32, 33); desmoplakin, a desmosomal protein; FKBP51, an immunophilin (34, 35); Na/K-ATPase subunit a1 (cloned three times), and a fragment with no homology to known genes (cloned two times) (see Table I).
(56% amino acid identity) to the arginine-serine domain of SR proteins, although also other arginine-serine-rich proteins have high scores (like protamines and viral DNA-binding proteins). The only clone that was found to be down-regulated (Ptg-13), was annexin-VI. This gene encodes a calcium-dependent phospholipid binding protein possibly involved in intracellular endosomal trafficking (37, 38).

The expression pattern of four genes (FKBP51, Na⁺/K⁺-ATPase α1, CD-9, and desmoplakin) was further characterized (Fig. 3). Time-dependent expression in the presence of the progesterin and E₂ was investigated to determine whether the response was transient or sustained. All four genes were still strongly up-regulated after 48 h. Furthermore, regulation of these genes by the progesterin in the additional presence of different growth stimuli (E₂ and bFGF) was examined, and in the mammary carcinoma cell line MCF-7, which is also strongly responsive to glucocorticoids and the growth inhibitor TGFβ, FKBP51 was strongly up-regulated by both the progesterin and the synthetic glucocorticoid dexamethasone under all conditions tested, agreeing with the assumption that FKBP51 is a direct target gene.

The three other genes showed more complex expression patterns. The Na⁺/K⁺-ATPase α1 was up-regulated by the progesterin in T47D cells under all conditions tested (Fig. 3A); in MCF-7 cells, the gene was up-regulated by the progesterin but down-regulated by dexamethasone, which is not to be expected for a direct progesterone target gene (Fig. 3B). Also the regulation by TGFβ was complex with up-regulation in the presence of E₂ and down-regulation in its absence. CD-9 was only strongly up-regulated in T47D cells by the progesterin in the presence of E₂ (Fig. 3A), while in MCF-7 cells, this gene was activated constitutively (Fig. 3B). Desmoplakin was clearly induced in T47D cells by the progesterin in the absence of additional hormones or in the presence of estradiol, but not in the presence of bFGF (Fig. 3A). In MCF-7 cells, this gene was only slightly up-regulated by the progesterin, dexamethasone, and TGF-β in the presence of estradiol (Fig. 3B). Interestingly, we noted that Na⁺/K⁺-ATPase α1, CD-9, and desmoplakin were all down-regulated in T47D cells by E₂, while the progesterin clearly reversed the effect (Fig. 3A).

 Estradiol and Progesterin Have Opposing Effects on Differentiation of T47D Cells—Interestingly, five of the genes identified suggested that the T47D cells differentiate upon progesterin treatment. These are: desmoplakin, which is a marker for epithelial differentiation and is expressed higher in well differentiated mammary carcinoma cell lines (39); CD-9, which is expressed higher in well differentiated leukemias and lower in highly metastatic breast tumors (28, 40); CD-59, which is expressed higher in more differentiated colorectal carcinomas (41); Na⁺/K⁺-ATPase α1, which is a very important protein in secretory epithelial cells (42); and the negative target gene annexin-VI, which is down-regulated in developing mammary gland secretory epithelium (43–45). Note that all three positive

| Clone | Homology | Northern, fold induction 6 hrs Org2058 + Ch | Northern, fold induction 24 hrs Org2058 | Function (putative) |
|-------|-----------|---------------------------------------------|-----------------------------------------|-------------------|
| Ptg-1 | =Human EST, Ptg-3 | 3.3 | 13 | Leucin zipper, transcriptional regulator |
| Ptg-2 | =TSG-22 | 1.2 | ND | |
| Ptg-3 | =Human EST, Ptg-1 | ND | 6.9 | |
| Ptg-4 | =CD-9/MRP-1 | 1.7 | 4.9 | Metastasis, binds integrins |
| Ptg-5 | =Na⁺/K⁺-ATPase α1 | 2.5 | 5.4 | Sodium/potassium pump |
| Ptg-6 | =Desmoplakin | 1.6 | 8.0 | Cell-cell interaction |
| Ptg-7 | =CD59/protectin | 1.5; 1.5 | 2.1; 3.1 | Protects cells against complement, binds Gαs |
| Ptg-8 | =Na⁺/K⁺-ATPase α1 | ND | 2.8 | Sodium/potassium pump |
| Ptg-9 | =Na⁺/K⁺-ATPase α1 | ND | ND | |
| Ptg-10 | =FKBP51 | 2.9 | 13 | Immunophilin |
| Ptg-11 | 56% aa S. cerevisiae SC35 | ND | 2.1 | 5 × RRHSRSR |
| Ptg-12 | 56% aa C. elegans ZK686.4 | ND | 2.9; 3.1 | Putative C₂H₂ zinc finger |
| Ptg-13 | Annexin-VI | ND | 0.4 | Endosomal protein |

**Fig. 3. Expression level of FKBP51, Na⁺/K⁺-ATPase α1, CD-9, and desmoplakin upon hormone treatment of two breast tumor cell lines.** A, regulation in T47D cells by progesterin Org2058 (10⁻⁸ M), E₂ (10⁻⁸ M), and bFGF (10 ng/ml) treatment. Superscripts top row: fold induction, corrected for GAPDH expression (lanes compared indicated by line). For desmoplakin (DP) both transcripts were quantified. Middle row, addition to DCC media 48 h before harvest, = no addition. Bottom row, hours of progesterin addition before harvest, GAP = GAPDH signal. B, regulation in MCF-7 cells upon different hormonal stimuli. Superscripts top row and middle row: see A. Bottom row: P = progesterin (10⁻⁸ M Org2058); T = 2 ng/ml TGFβ1; D = 10⁻⁷ M dexamethasone, added 24 before harvest. GAP = GAPDH signal. N.Q. = not quantifiable. For all Northern blots, 20 μg of total RNA was used.
progesterone target genes that were repressed by E₂ as compared with no hormonal treatment belong to this group.

Because several of the target genes identified were in line with the induction of differentiation by progestins, we studied if this could be confirmed by studying other markers of differentiated epithelia. We observed that upon hormonal treatment clear morphological changes of the T47D cells were apparent. Upon E₂ treatment, cells become more pointed and lamellipodia are visible, already after 24 h (Fig. 4). These lamellipodia clearly contain, as determined by immunocytochemistry, paxillin (data not shown), a cell-matrix adhesion protein commonly associated with lamellipodia (46). In the presence of the progestin, with or without E₂, hardly any lamellipodia are induced, and cells become much more rounded, consistent with a more differentiated phenotype. We also investigated the localization of two adherens junction proteins, E-cadherin and α-catenin. The expression of these genes is often reduced in tumor cells and correlates with differentiation state (39, 47). After 6 days in the presence of E₂, E-cadherin and α-catenin localization was clearly reduced at cell borders and much was present in lamellipodia (Fig. 4). The localization of these proteins in lamellipodia is already visible after 24 h and does not occur in the presence of cycloheximide, indicating that changes in protein expression are required (data not shown). This situation is completely reversed when a progestin is added. In that case these proteins were again localized at the cell borders, which is consistent with the localization in fully differentiated epithelial cells (42). These results confirm that the progestin induces differentiation in T47D cells.

DISCUSSION

Cloning of Genes Using an Improved Differential Display Protocol—Differential display is a very useful technique for identifying regulated genes. However, a major problem with this technique is the occurrence of a large number of false positives, contaminations, and inefficient probes for detection on Northern blots, as has been described for the Liang and Pardee method (48–50). Hence, we devised a powerful strategy to identify the most promising clones rapidly and efficiently. During the preparation of this manuscript a comparable technique was published (51). There are some differences with our procedure. Vogel-Lange et al. (51) recommend to pool multiple reaction mixtures of previous differential display reactions for use as a probe, while in our procedure, fresh differential display reactions are performed. Using the same reaction mixture has the disadvantage that when a false positive band is cloned, again a false positive signal will be generated in the Dot blot procedure. Moreover, we use 32P instead of 33P as label in the Dot blot procedure, which gives a stronger signal and circumvents the need to pool reactions.

In the present case we have identified mostly up-regulated genes. Possibly, there are more genes up-regulated than down-regulated after progestin treatment in T47D cells. A different explanation could be that genes which are up-regulated show larger differences in expression level upon progestin treatment and therefore are identified more easily. Clones derived from the positive target Na⁺/K⁺-ATPase α1 were even collected independently three times. Probably this is due to the fact that the expression of this gene upon stimulation is very high. One
other gene was selected twice (Pg-1 and Pg-3), but this is presumably not a result of abundance of the mRNA. Both clones had the same primer incorporated at the same site of the gene at one end, while at the other end the primer had annealed at different sites. The clones were therefore of different size and cut out twice. All the genes identified here have never been reported to be progesterone targets, although the Na\(^+\)/K\(^+\)-ATPase subunit $\alpha_1$ and TSC-22 have been shown to be up-regulated by dexamethasone (32, 36), a synthetic activator of the glucocorticoid receptor (note that the glucocorticoid response element is identical to the PRE). This could suggest that there are still more progesterone target genes to be identified.

**Novel Progesterone Target Genes**—The genes that were identified can be divided into two groups. The first group consists of the four genes that might play a role in regulating gene expression. These are TSC-22, which is a putative transcriptional regulator, Pg-12, possibly encoding a zinc finger protein, Pg-11, which has homology with members of the SR protein family of splicing factors, and FKBP51, an immunophilin. The immunophilins FKBP52, FKBP54, and CYP40 have been shown to bind to steroid hormone receptor complexes (52–54), and FKBP52 is required for nuclear transport of the glucocorticoid receptor (55). FKBP51 might have a similar function and therefore directly influence transcriptional activity of steroid hormone receptors. Very clear was the strong up-regulation of this gene by the ligand-bound PR (also in the presence of cycloheximide) and glucocorticoid receptor, which could indicate the presence of one or more PRE glucocorticoid response elements in its promoter.

The second group consists of genes that are suggestive for differentiation of T47D cells upon progesterin treatment. Low expression of CD-9/MRP-1 (motility-related protein 1) correlates in leukemias with poor differentiation (40), in lung cancer with poor prognosis (56), and in breast cancer with stronger metastatic potential and poor prognosis (28, 57). CD-59/protectin is strongly expressed in well and moderately differentiated colorectal carcinomas but to a lower extent in metastasizing tumors (41). A third gene that suggests differentiation to be induced by progesterone is desmoplakin. The product of this gene connects intermediate filaments with the desmosomes, and in this way a continuous filamentous network is formed between cells (58). Desmoplakin markers are key in epithelial differentiation. The expression of desmoplakin, along with other epithelial markers, in mammary carcinoma cell lines has been correlated negatively with invasiveness and positively with the differentiation state (39). In normal breast tissue, progesterone stimulates glandular development (1). This is consistent with the induction of the Na\(^+\)/K\(^+\)-ATPase $\alpha_1$ subunit. This gene is likely to be important for vectorial transport in epithelial cells (42) and is indeed up-regulated during development of the intestinal epithelium (59). Furthermore, we identified the negative target gene annexin VI, which is down-regulated in developing mammary gland secretory epithelium (43–45). Three positive target genes of this group all showed not only an up-regulation upon progesterin treatment in the presence of $E_2$, but also an estrogen-induced down-regulation. Not many negative estrogen receptor target genes are known so far, and the ER is mostly considered as a positive transcription factor.

**Progesterins Reverse Dedifferentiating Effects of Estrogens**—Apart from opposing $E_2$-induced suppression of gene expression, progesterins oppose morphological changes (cell shape, appearance of lamellipodia) induced by $E_2$. This is also clear from our findings concerning the localization of α-catenin and E-cadherin. A vast literature is available on the significance of expression and distribution of E-cadherin during differentiation and invasion of carcinoma cells. E-cadherin expression is high in well differentiated breast or other tumors, is considered to be an invasion suppressor, both in vivo and in vitro, and correlates with good prognosis (60–63). α-Catenin expression is often reduced in breast tumors, and this is probably also important in invasion (47, 64).

Our data suggest that the human mammary carcinoma line T47D dedifferentiates after estrogen treatment, while a differentiation program is activated upon adding the progesterin. Progestins can be used in breast cancer treatment and can induce regression of tumors through unknown mechanism (reviewed in Ref. 65). In contrast, estrogens are strong stimulants of breast cancer proliferation (reviewed in Ref. 66). Possibly, the influence of estrogens and progestins on breast tumor cell differentiation plays an important role in growth modulation of breast tumors.

In general, a higher state of differentiation is linked to a lower metastasizing capacity (reviewed in Ref. 67). The dedifferentiating action of $E_2$ correlates well with the reported stimulation of invasion by estrogens (6, 68). A clear picture for progestins has not emerged yet. In literature, progestins have been shown to up-regulate invasion-associated proteins, like laminin receptors and proteinases (7, 17, 69). However, to our knowledge in in vitro or in vivo invasion assays no stimulatory effect of progestins has been reported. Our data rather suggest that progesterin-treated cells will be less invasive, at least in the presence of $E_2$. This is based on the progestin-induced expression of the invasion suppressors E-cadherin, CD-9, and α-catenin and from the effects on lamellipodia, which are instrumental in locomotion and tumor cell migration.

The mechanism by which the progestin antagonizes $E_2$ in our experiments is not clear. The PR has been shown to down-regulate ER activity in many ways. As possible mechanism down-regulation of ER levels, the induction of $E_2$ degrading enzymes, or competition for corepressors that bind to both receptors can be envisaged (reviewed in Ref. 1; Ref. 70). Although this down-regulation of ER activity seems to play a role in progesterin action in T47D cells, some of the differentiation-associated genes are already up-regulated by the progestin in the absence of $E_2$. Therefore, the progестin on its own has a clear effect on differentiation. The influence of steroid hormones on breast tumor differentiation and the role of this differentiation in growth inhibition and metastasis clearly represents an important area for further study.

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