Molecular and Cellular Endocrinology 235 (2005) 39–50

Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Estradiol and medroxyprogesterone acetate regulated genes in T47D breast cancer cells

S. Mrusek, I. Classen-Linke, A. Vloet, H.M. Beier, C.A. Krusche *

Department of Anatomy and Reproductive Biology, RWTH Aachen University, Wendlingweg 2, 52074 Aachen, Germany

Received 23 November 2004; received in revised form 21 December 2004; accepted 17 January 2005

Abstract

Many mammary tumors express estrogen receptors (ER) and progesterone receptors (PR), and there is increasing evidence that progestins influence gene expression of breast tumor cells. To analyse the impact of progestins on breast cancer cells, we compared (a) the expression of two cytokines, involved in tumor progression, and searched (b) for differentially regulated genes by a microarray, containing 2400 genes, on T47D breast cancer cells cultured for 6 days with 17β-estradiol (E_2) or E_2 + medroxyprogesterone acetate (E_2 + MPA). Lower amounts of PDGF and TNFα were found in culture supernatants of E_2 + MPA treated T47D cells. MPA addition induced a 2.8–3.5-fold increase of the mRNA expression of (a) tristetraprolin, which is involved in the posttranscriptional regulation of cytokine biosynthesis, and (b) zinc-α2-glycoprotein and Na, K-ATPase α1-subunit, which both resemble differentiation markers of breast epithelium. In contrast, the mRNA expression of lipocalin 2, which promotes matrixmetalloproteinase-9 activity, was decreased five-fold in E_2 + MPA treated cells.

Our data show that the expression of genes from various functional gene families is regulated differentially by E_2 and E_2 + MPA treatment in T47D cells. This suggests that exogenous progestins applied for therapy and endogenous changes of the progesterone levels during the menstrual cycle both influence breast cancer pathophysiology.

Keywords: Estrogen; Progestins; Breast cancer; T47D Cell line; Gene regulation

1. Introduction

Breast cancer represents 18% of female cancer worldwide and one million new cases occur per year (McPherson et al., 2000). Primarily estrogens were recognized to promote tumor development and progression. Endocrine therapies, aimed to inhibit breast cancer growth, frequently use aromatase inhibitors, antiestrogens (e.g. tamoxifen) and estrogen receptor down-regulators (Pritchard, 2003). Several studies have shown that besides estrogens, progestins seem to influence breast tumor cells, which very often express progesterone receptors (PR) in addition to estrogen receptor α (ER α) (Shi et al., 1993; Lange et al., 1999; Isaksson et al., 2001; Lanari and Molinolo, 2002; Soyal et al., 2002). However, the significance of progestins in the pathophysiology of breast cancer remains largely unknown. It was shown, that progestins impair gene expression of human breast cancer cells cultured in vitro (Richer et al., 2002; Graham et al., 1999; Kester et al., 1997). Following hormone replacement therapy, increased risk of breast cancer was observed when (a) certain synthetic progestins are combined with oral estrogens (DeLignieres, 2002) and (b) estrogens and progestins are administered continuously together for more than 5 years (Weiss et al., 2002). Whether the survival rate of premenopausal women suffering from breast cancer can be increased, when tumor excision is performed during the first half of the luteal phase is still under debate and investigation (Hagen and Hrushesky, 1998; Cooper et al., 1999; Carlson, 2002).

Progestins act via progesterone receptors, which are ligand-activated transcription factors and belong to the nuclear receptor superfamily of transcription factors. Two functionally distinct PR isoforms, the shorter PRA and the PRB isoform, are commonly described, which are transcribed from two promotors located on a single gene (Kastner et al., 1990). In the mouse model, the PR-B seems to be the

* Corresponding author. Tel.: +49 241 8088926; fax: +49 241 8082508.
E-mail address: ckrusche@ukaachen.de (C.A. Krusche).

0303-7207/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2005.01.009

more important isoform for mammary gland differentiation (Brisken et al., 1998), however, both PR isoforms are needed (Conneely et al., 2002). For the full progesterone response of the mammary gland, steroid receptor coregulators are needed. Steroid receptor coactivator-1 (SRC-1, synonym NCOA1) and SRC-3 (synonyms AIB1, NCOA3) are needed for the induction of progestin-activated genes (Rowan and O'Malley, 2000), whereas corepressor complexes, containing proteins like Sin3A, KAP-1 and "silencing mediator of retinoic and thyroid hormone receptor" (SMRT) are involved in progestin-mediated suppression of gene expression (Underhill et al., 2000; Giangrande et al., 2000). The corepressors exert their suppressive effects via histone deacetylases, which are involved in breast cancer pathophysiology and in addition were found to represent attractive druggable targets (Vigushin et al., 2001). Recently, we could show HDAC-1 and -3 expression in a series of 200 breast cancer patients (Krusche et al., in press).

At first, the investigated T47D breast cancer cell line was characterized for expression of ER α and PR as well as six steroid-hormone receptor coregulators and four principal histone deacetylases. Next, the effect of a 6-day lasting treatment of estradiol (E_2) or E_2 + medroxyprogesterone acetate (MPA) on the RNA and protein expression of the cytokines TNFα and PDGF, which both play a pivotal role in breast tumorigenesis, was assessed (Shao et al., 2000; Walker, 2001; Griffin et al., 2002; Duncan and Reed, 1995; Leek et al., 1998; Meng et al., 2001). Furthermore, we used a microarray containing 2400 genes to search for differentially regulated genes in T47D breast cancer cells.

2. Material and methods

2.1. Cell culture experiments

T47D cells were obtained from the American Type Culture Collection in the 76th passage. They were routinely cultured in plastic flasks of 25 cm^2 culturing area (Greiner GmbH, Frickenhausen, Germany) and incubated in 5% CO_2 at 37 °C in a humidified atmosphere. The stock medium contained phenol red-free RPMI 1640 medium (Biochrom AG, Berlin Germany) supplemented with 1% penicillin/streptomycin/fungizone (GibcoBRL, Eggenstein, Germany), 4 mM glutamine (Gibco BRL), 10% steroid-hormone-free (charcoal-treated), heat-inactivated fetal calf serum (FCS; c.c. pro, Neustadt/W., Germany) and 5 μg insulin/ml (Gibco BRL).

For the assessment of hormone-mediated gene expression 3×10^6 T47D cells were seeded in culture flasks. After 2 days of culture in steroid-hormone-free RPMI 1640 stock medium cells were either treated for 6 days with (a) 10^{-8} M 17β-estradiol (E_2) or (b) with 10^{-8} M E_2 and 10^{-6} M medroxyprogesterone acetate (E_2 + MPA). Both steroids were dissolved in 100% ethanol. The ethanol end concentration in the culture medium was <0.1%. The steroid-hormone containing culture medium was replaced on the second and fifth day of treatment. For measuring of cytokine levels, it was collected after each change and on the sixth day, when the culture was discontinued and cells were harvested by trypsinisation. Cell number and viability was determined. Then cells were either homogenized in RNAwiz solution (Ambion, Huntingdon, United Kingdom) for subsequent RNA isolation, or frozen in liquid nitrogen for Western blot and ELISA analysis. Expression studies were performed in T47D cells cultured between the 2nd to 6th and 13th to 19th passage.

2.2. Immunohistochemistry

T47D cells were cultured in four wells plates (1.9 cm^2 culture area, Nunc, Wiebaden, Germany). 10^5 cells per well were incubated as described before: after 2 days in steroid-hormone-free medium, hormones were added for 6 days: (a) E_2, (b) E_2 + MPA. Control cells were grown without steroid hormones. At the end of the culture period, the medium was removed, cells were washed two times with PBS and fixed in 4% neutral buffered formalin at 8 °C for 5 min. Cell membranes were permeabilized with 0.1% Triton X-100 in PBS (3 min). The cells were then subjected to 0.3% H_2O_2 in methanol and blocking solution for 15 min each (Histostain® SP-Kit or Histostain® Broad-Spectrum Kit, both Zymed, San Francisco, CA, USA).

2.2.1. ER α, PR and KI67 immunohistochemistry

The first antibody diluted in PBS/1.5% BSA (bovine serum albumin, fraction V, Sigma, Deisenhofen, Germany) was added (see Table 1 for antibody sources and dilution factors) for 1 h. Addition of the second, biotin-conjugated goat anti-mouse antibody for 20 min (Histostain® SP-Kit) followed before the incubation with streptavidin-peroxidase conjugate (Histostain® SP-Kit) for 15 min. After each individual antibody incubation step the cells were washed with PBS/0.1% Tween 20. Cells were then covered with aminoethyl carbazole (AEC)/H_2O_2 solution (Zymed, San Francisco, CA, USA) to detect the peroxidase–antibody–antigen complex. Color development was stopped with distilled water and cells were finally mounted with glycerol gelatine.

2.2.2. HDAC-1 and -3 immunohistochemistry

The first antibody (anti-HDAC-1 or anti-HDAC-3; Cell signalling technology, Frankfurt, Germany both diluted 1:50 in PBS/3% BSA) was incubated for 2 h. Thereafter, cells were treated with the biotin-conjugated second antibody (30 min, Histostain®-Plus Broad Spectrum-Kit) and with streptavidin-peroxidase conjugate (30 min, Histostain®-Plus Broad Spectrum-Kit). The reaction was detected with AEC/H_2O_2 (Zymed, San Francisco, CA, USA). Between incubation steps, slides were washed with PBS/0.1% Tween 20.

Two negative controls were performed: (a) the first antibody was omitted and (b) the first antibody was replaced by non-immune IgG at the same concentration. None of the

Table 1
Antibodies used to characterize the hormone receptor status and proliferation of T47D cells

Antigen	Clone	Supplier	Dilution
Human progesterone receptor (A and B)	10A9	Coulter Immunotech	1:30
Human progesterone receptor (A and B; Western blot)	PGR Ab8	Neo Markers	1:200
Human estrogen receptor α	1D5	Coulter Immunotech	1:30
Ki67 proliferating antigen	MIB1	DAKO	1:40
HDAC-1	Polyclonal	Cell Signaling	1:100
HDAC-2 (Western blot)	Polyclonal	Santa Cruz	1:5000
HDAC-3	Polyclonal	Cell Signaling	1:100

controls revealed a positive staining. Each marker was assessed at least in four independent experiments.

2.3. Western blot to detect the two PR isoforms and HDAC-2

The SDS-disc polyacrylamide gel electrophoresis was performed under reducing conditions (5% mercaptoethanol). Ten percent gels were used to separate the T47D cell proteins (20 μg per lane) according to their molecular weight. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Hamburg, Germany, pore diameter 0.45 μm) by a semidry electroblotting procedure (2 mA/cm^2) for 40 min. The membrane was blocked in TBS (5 mM Tris-buffered saline; pH 7.6) supplemented with 5% milk powder and 0.1% Tween (1 h). The detection of PR isoforms and the HDAC-2 protein was performed by incubating a blocked membrane overnight at 4 °C with the antibody PGRAb8 (Labvision Cooperation, California, USA) diluted 1:200 or the HDAC-2 antibody (Santa Cruz Biotechnologies, California, USA) diluted 1:5000 in TBS/1% milk powder/0.1% Tween, respectively. After washing with TBS/0.1% Tween membranes were incubated for 1 h with an HRP-conjugated secondary antibody. The detection of PGRAb8 was performed using goat anti-mouse IgG (Santa Cruz, CA, USA) diluted 1:5000 in PBS/1% milk powder/0.1% Tween. For the detection of the HDAC-2 antibody goat anti-rabbit immunoglobulin-HRP (DAKO Cytomatics) diluted 1:5000 in PBS/1% milk powder/0.1% Tween was used. Immunoreactive proteins were detected with the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions. Four different culture experiments were assessed.

2.4. RNA isolation for array hybridisation, semiquantitative RT-PCR and Northern blotting

After 6 days of hormone treatment 5×10^6 T47D cells were homogenized in 1 ml RNAwiz solution before the addition of 100 μl chloroform. After centrifugation (10 600 × *g*, 4 °C, 10 min) the supernatant was mixed with an equal volume of 70% ethanol. RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Finally, RNA was eluted from the columns with 60 μl DEPC-treated double distilled water. RNA concentration and purity was determined by $OD_{260\,nm}$ and $OD_{280\,nm}$ spectrophotometric values. RNA integrity was evaluated by gel electrophoresis and subsequent visualization of 28S and 18S rRNA bands.

2.5. Array hybridisation

The array hybridisation was performed with the MICROMAX Human cDNA System I-Direct labeling-kit (PerkinElmer Life Sciences, Boston, USA) using direct cDNA labeling chemistry. 2400 cDNAs from known genes with an average length of 2200 bp are spotted onto a glass slide. One hundred and twenty micrograms of total cellular (tc) RNA isolated from E_2 treated T47D and 120 μg tc RNA isolated from E_2 + MPA treated T47D cells were labeled with Cyanine 5 (Cy5)-dUTP or Cyanine 3 (Cy3)-dUTP during cDNA synthesis, respectively. Prior to the cDNA labeling reaction according to the manufacturer's recommendation the RNA from E_2 and E_2 + MPA treated T47D cells was spiked with equal amounts of three plant RNAs supplied with the kit to control equal incorporation of Cy3- and Cy5-dUTP. These three plant cDNAs were spotted four-fold on the array.

The Cy5- and Cy3-dUTP labeled cDNA was pooled and cleaned from unincorporated NTPs, buffers and degraded RNA by ultrafiltration using the Microcon YM-100 centrifugal filter unit (Millipore, Eschborn, Germany) according to the manufacturer's instructions. The purified labeled cDNA was dried in a Savant Speed Vac and resuspended in 40 μl of hybridisation buffer. Equal RNA input into the Cy3- and Cy5-labeling reactions as well as equal labeling efficiency was confirmed by hybridising a test slide before the microarray containing the 2400 genes was hybridised over night at 65 °C. Stringency washes were performed with washing solutions filtered through a 0.22 μm filter. The microarray was washed for 5 min in 0.5× SSC/0.01% SDS, another 5 min with 0.06× SSC/0.01% SDS and finally 5 min with 0.06× SSC. The manufacturer performed laser scanning of the processed microarray.

2.6. Semiquantitative RT-PCR

Five micrograms of RNA from T47D cells either treated with E_2 or E_2 + MPA were used in the cDNA synthesis reaction with the Ready to Go™ You Prime First Strand

Table 2
Primer used to characterize the steroid receptor and steroid receptor cofactor expression of the investigated T47D cell line by RT-PCR

Gene	Accession number	Forward-primer	Reverse-primer	Cycles	Annealing (°C)
Progesterone receptor (PR)	AF016381	2483–2502	2910–2896	32	59
Estradiol receptor (ER α)	XM045967	1410–1429	1936–1919	32	59
SRC-1	NM_003743	1324–1344	2145–2124	29	67
SRC-2	NM_006540	4246–4267	4676–4656	35	67
SRC-3	AF012108	4391–4411	5032–5012	32	61
SIN3A	AY044430	807–827	1326–1307	35	67
SMRT	XM_045602	5603–5622	6124–6103	35	61
KAP-1	U78773	1319–1340	1840–1819	35	62
HDAC-1	NM_004964	846–866	1264–1245	31	67
HDAC-2	XM_004370	809–830	1678–1657	29	67
HDAC-3	XM_048959	461–480	1032–1011	32	63
HDAC-6	NM_006044	1351–1372	1722–1701	35	67

Bead Kit (Amersham Pharmacia Biotech, Freiburg, Germany) containing 1.6 μg oligo-$(dT)_{15}$ primer according to the manufacturer's protocol. After each cDNA reaction 4 μl cDNA was analysed on a 1.2% agarose gel containing ethidium bromide to assess RNA integrity. The optical density of the 18S rRNA band was measured to control equal RNA input in the cDNA synthesis reaction. Differential gene expression of four genes was studied by a semiquantitative RT-PCR approach. PCR-primers were generated with GeneFisher software (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). Primer position is given in relation to the entire published sequence in Tables 2 and 3.

To allow semiquantitative evaluation of gene expression, the log-phase of each PCR reaction was determined in a separate set of experiments for each performed RT reaction. The number of PCR cycles used for assessment of differential gene expression is given in Tables 2 and 3. The log-phase of the PCRs was controlled after each cDNA synthesis reaction.

An equivalent to 60 ng total cellular RNA were used as template to amplify specific products under the following conditions: denaturation at 94 °C for 2 min; 23–35 cycles of 94 °C (1 min), 55–62 °C (1 min), 72 °C (1 min); then 72 °C (3 min). The expression of the two housekeeping genes L23 ribosomal protein and S26 ribosomal protein served as control. The 50 μl PCR reaction consisted of 5 μl 10× Taq reaction buffer (Mg^{2+} end concentration 1.5 mM), 2 μl of 10 mM dNTP and 2U Taq-DNA polymerase (Roche, Mannheim, Germany) and 10 pM of the appropriate forward and reverse primer. Twenty picomolars of forward and reverse primer were used in PCR reactions performed to detect the PDGF receptor A, PDGF receptor B and SMRT.

PCR generated fragments were analysed by electrophoresis on a 1.2% agarose gel. The optical densities (ODs) of the PCR products were determined by the Kodak Image Station 440 CF using the 1D Image analysis software, Version 3 (PerkinElmer Life Sciences). The OD values of the specific PCR product bands were normalized against the OD value of the S26 and L23 housekeeping gene product band. The target/housekeeping gene ratio of the E_2 treated cells was then set to "1" and the target/housekeeping gene ratio of the corresponding E_2 + MPA treated cells was set into relation. The values are given as arbitrary expression units.

The specificity of each PCR product was confirmed by nucleotide sequencing of representative PCR product bands (SeqLab, Göttingen, Germany).

2.7. Cytokine assessment in culture supernatants by ELISA

Commercially available ELISA kits (R&D Systems, Minneapolis, USA) were used to detect PDGF-AA (cat no. DAA00; sensitivity 2 pg/ml; intra-assay variation coefficient

Table 3
Primer pairs used to assess mRNA expression of genes found to be strongly regulated in T47D cells by array hybridisation, functionally related genes and housekeeping genes by semiquantitative RT-PCR

Gene	Accession number	Forward-primer	Reverse-primer	cycles	Annealing (°C)
Lipocalin 2 (NGAL)	X83006	58–79	387–366	30/32	58
Tristetrapolin (TTP)	M63625	1006–1026	1496–1476	24/27	57
Na, K-ATPase α1-subunit (ATPase)	X04297	1424–1444	1895–1874	29/27	61
Zn-α2-glycoprotein	X59766	211–231	796–776	28/26	58
C-sis/PDGF B	M12783	1128–1148	1583–1563	28	62
PDGF A	X06374	316–335	792–773	35	64
PDGF receptor A (PDGFRA)	NM_006206	672–693	1438–1417	35	64
PDGF receptor B (PDGFRB)	NM_002609	1003–1024	1833–1812	35	64
TNFα	X01394	429–450	1049–1029	35	62
Ribosomal protein L23a (L23)	U37230	57–78	432–411	23/22	60
S26 ribosomal protein	NM001029	47–66	354–373	25/23	56

<7.2%; intra-assay variation coefficient <7.9%), PDGF-AB (cat no. DHD00B; sensitivity 1.7 pg/ml; intra-assay variation coefficient <4.9%; inter-assay variation coefficient <9%), and TNFα (cat no. HSTA00C; sensitivity: 0.12 pg/ml; intra-assay variation coefficient <8.8%; inter-assay variation coefficient <16.7%) in culture supernatants of T47D breast cancer cells.

The ELISA kits have been validated for serum-, plasma- and culture supernatant samples. To exclude that the culture medium (RPMI + 10% FCS) crossreacts with the ELISAs, the unincubated medium was used as control in each ELISA experiment. The unincubated medium did not react with the PDGF-AA and TNFα ELISA. However, a slight color development was observed in the PDGF-AB ELISA. The obtained value of 9.9 pg PDGF-AB/ml culture medium was subtracted from the values measured in the culture supernatants.

Cytokine levels were measured in the culture supernatants of cells that were cultured between the 13th and 19th passage. We investigated T47D cells from our original cell line and a subline. This subline resembles cells from our original cell line that were passaged four times and then stored again in liquid nitrogen until the presented study was performed. The subline had the same characteristics as the original cell line. All culture supernatants were assessed in duplicate.

2.8. Statistical analysis

To test whether gene regulation, protein release into the culture medium, protein content or Ki67-immunostaining were significantly different between E_2 and E_2 + MPA treated T47D cells the paired *t*-test was applied. $P < 0.05$ was considered to be statistically significant.

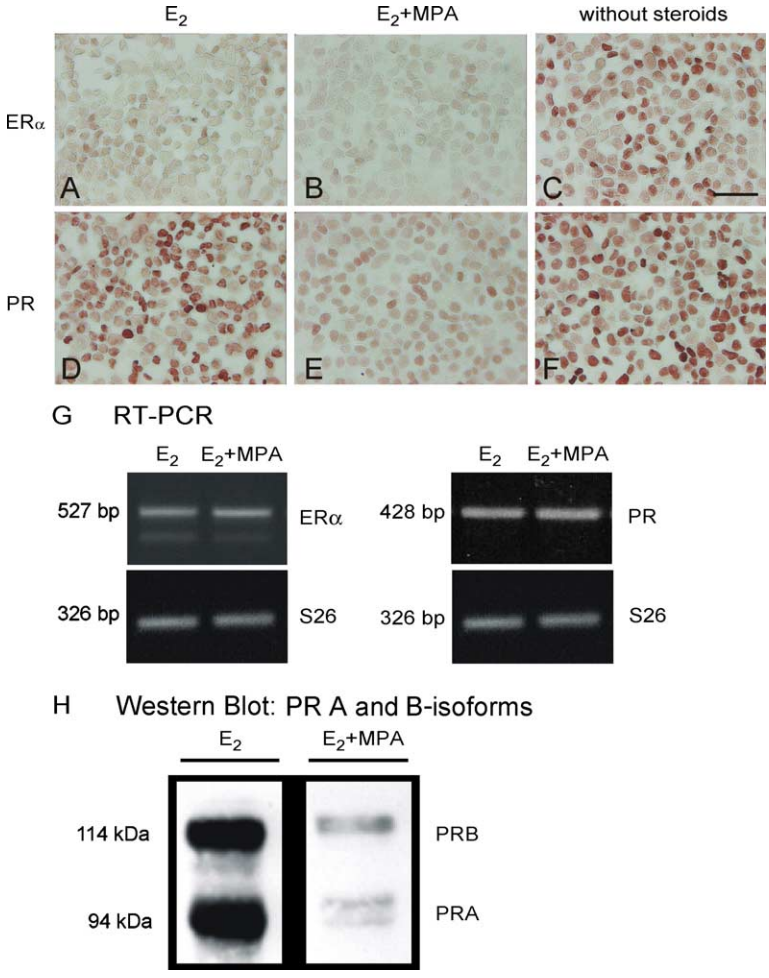

Fig. 1. Estrogen receptor α and progesterone receptor A and B expression. Immunohistochemical detection of estrogen receptor α (ER α; A–C) and progesterone receptor (PR; D–F) in T47D cells cultured in multiwells for 6 days with 10^{-8} M E_2 and 10^{-8} M E_2 + 10^{-6} M MPA. Bar represents 50 μm. (G) shows the results of the semiquantitative RT-PCR analysis of ER α and PR mRNA expression and (H) the assessment of the two PR isoforms in T47D cells cultured with E_2 or E_2 + MPA by Western blot.

3. Results

3.1. Steroid-hormone receptor and cofactor expression of the investigated T47D cell line

As the T47D breast cancer cell line is a genetically unstable cell line, which may develop hormone resistance (Reddel et al., 1988; Graham et al., 1989), we first assessed the steroid-hormone receptor status in our T47D cell line. The protein expression of ER α and PR was investigated by immunohistochemistry and Western blot analysis, whereas the mRNA expression was demonstrated by RT-PCR. The nuclear expression of ER α and PR protein is shown in Fig. 1A–F. The ER α protein was found to be strongly expressed in the nuclei of cells cultured without hormones (Fig. 1C), whereas the staining intensity was clearly reduced after 6 days of culture with E_2 or E_2 + MPA (Fig. 1A and B). We found a strong expression of the PR protein in the nuclei of T47D cells cultured without hormones or with E_2 (Fig. 1D and F). However, PR staining intensity decreased after 6 days of culture with E_2 + MPA (Fig. 1E). Interestingly, when performing RT-PCR (Fig. 1G) the ER α and PR mRNAs were expressed constitutively in T47D cells being treated with E_2 or E_2 + MPA. Western blot analysis verified immunohistochemical results (Fig. 1H) and demonstrated that the isoforms PR-A and PR-B are present in the cytosolic, soluble cell fraction of T47D cells.

In a next step, the mRNA expression of the main steroid receptor coactivators SRC-1, SRC-2 and SRC-3 as well as the mRNA expression of the fundamental corepressors SIN3A, SMRT and KRAB-associated protein 1 (KAP-1) were analysed by RT-PCR and found to be expressed in our T47D breast cancer cell line (Fig. 2). Additionally, histone deacetylase-1, -2, -3 and -6 mRNA expression (HDAC-1, -2, -3, -6) was detected in T47D cells treated with E_2 or E_2 + MPA (Fig. 3A). The mRNA expression of all these factors showed no obvious difference between the two hormonal treatments. HDAC-1 protein was located in the nuclei of nearly all T47D cells without any significant difference in staining intensity between cells cultured with E_2 or E_2 + MPA (Fig. 3B). Similar results were obtained for HDAC-3 (Fig. 3B). HDAC-2 protein expression was equally strong in cells cultured with E_2 or E_2 + MPA when analysed by Western blot (Fig. 3B).

3.2. Effects of E_2 and E_2 + MPA treatment on cytokine expression of T47D cells

We studied PDGF A and B mRNA expression by RT-PCR as well as the concentration of the isoforms PDGF-AA, -AB and -BB in cell culture supernatants by ELISA. We also searched for the mRNA expression of the PDGF receptor A and B. Our data show that the mRNAs of PDGF A and B were expressed constitutively (Fig. 4A). The PDGF isoforms could be detected in cell culture supernatants by ELISA (PDGF-AA: 14.95–162 pg/ml, PDGF-AB: 12.9–94.2 pg/ml, PDGF-BB: barely detectable). There was a differential expression of the PDGF-AA and PDGF-AB protein with a significant protein reduction in T47D cells treated for 5 days with E_2 + MPA compared to those treated with E_2 (Fig. 4A). Similar results were found when cell culture supernatants were assessed after 6 days of hormonal treatment (data not shown).

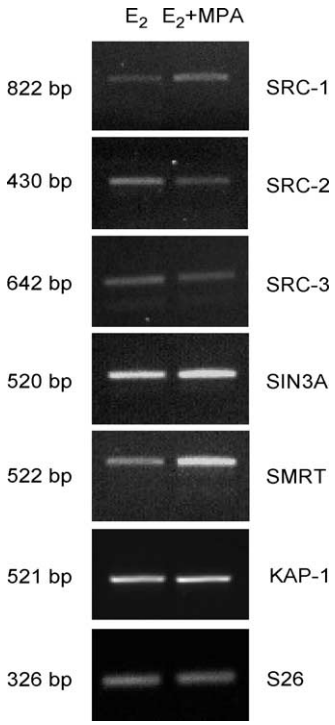

Fig. 2. mRNA expression of principal coactivators and corepressors in T47D cells. Representative RT-PCR analysis (n = 3–4) demonstrating mRNA expression of the steroid receptor coactivators (SRC)-1, -2 and -3 and three principal steroid-hormone receptor corepressors Sin3A, SMRT and KAP-1 in T47D cells cultured for 6 days with 10^{-8} M E_2 and 10^{-8} M E_2 + 10^{-6} M MPA. S26 ribosomal protein mRNA expression served as control.

We could neither find PDGF receptor A nor B chain mRNA in our T47D cells, whereas in the control tissues, both PDGFR chain mRNAs were clearly detectable by RT-PCR (Fig. 4B).

As TNFα is known to be another important mediator in breast cancer pathology, we analysed whether TNFα mRNA and protein were expressed in our T47D cells (Fig. 5). TNFα mRNA was constitutively expressed in T47D cells and the TNFα protein was found in low amounts in culture supernatants after 5 days of hormonal treatment. The TNFα protein concentration was lower in supernatants of T47D cells cultured with E_2 + MPA (0.32 ± 0.05 pg/ml) than in supernatants of cells cultured with E_2 only (0.54 ± 0.12 pg/ml). When the relative amount of TNFα in culture medium of E_2 and E_2 + MPA treated cells was compared, TNFα protein concentration was statistically significant lower in E_2 + MPA treated cells (Fig. 5).

The reduction of PDGF isoforms as well as TNFα in the cell culture supernatants was not caused by differences in the amount of cells per culture flask. Equal numbers of cells were seeded per flask and neither cell proliferation (E_2: 58.33 ± 6.06% Ki67 positive cells; E_2 + MPA:

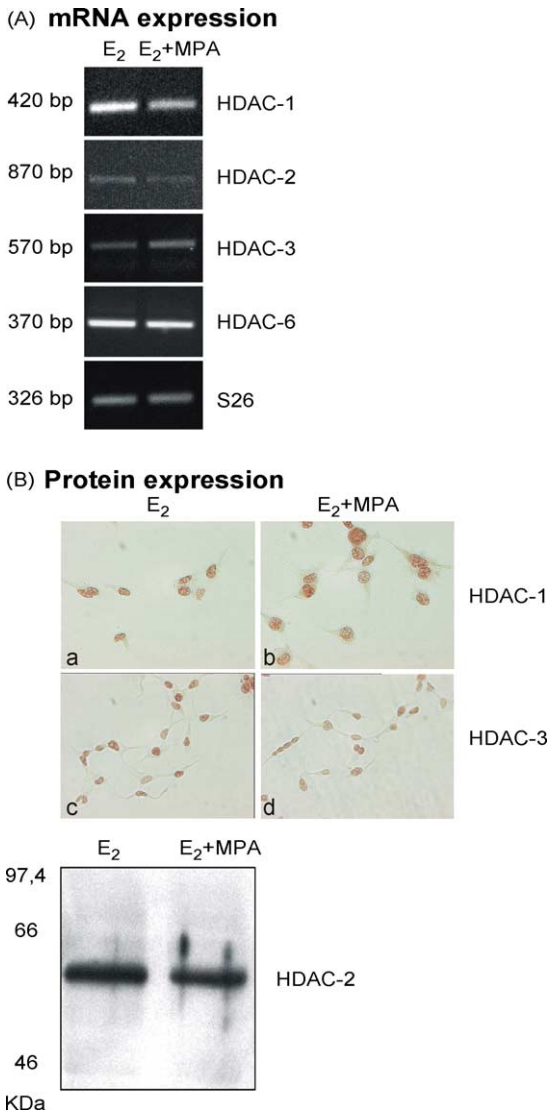

Fig. 3. Expression of histone deacetylases in T47D breast cancer cells. HDAC-1, -2, -3 and -6 mRNA (A) and protein expression (B) in T47D cells cultured for 6 days with 10^{-8} M E_2 and 10^{-8} M $E_2 + 10^{-6}$ M MPA. Representative results of three (mRNA analysis) or four (analysis on the protein level) experiments are shown.

58.73 ± 2.9% Ki67 positive cells; $P = 0.938$) nor the amount of proteins isolated from the harvested cells (E_2 treated cells: 332 ± 34 μg/flask versus E_2 + MPA treated cells 352 ± 41 μg/flask; $P = 0.053$, $n = 4$) showed statistical significant differences.

3.3. Assessment of differential gene expression in the T47D cells by microarray hybridisation and semiquantitative RT-PCR

Fig. 6 shows the false color overlay of the hybridised microarray (Fig. 6A) and the scatter plot analysis (Fig. 6B). The majority of hybridisation signals were located around the diagonal line on the scatter plot with the mean value of the 52 housekeeping genes of the array being 1.06 ± 0.33. This result and the Cy3/Cy5 ratio of the three control plant RNAs, which were spiked into the RNA samples of T47D cells (1.09 ± 0.3, 0.65 ± 0.12 and 1.08 ± 0.11), prove an efficient Cy3- and Cy5-labeling reaction.

The mRNA expression of four genes, tristetraprolin, zinc-α2-glycoprotein, and Na, K-ATPase α1-subunit and lipocalin 2, appeared to be strongly differentially regulated in the two cell samples (Fig. 6A). Their expression was therefore assessed by semiquantitative RT-PCR using RNA isolated from T47D cells cultured with E_2 or E_2 + MPA between the 2nd and 6th and also between the 13th and 19th passage. Furthermore, the housekeeping genes ribosomal protein S26, ribosomal protein L23a (L23), β-actin and cytosolic malate dehydrogenase (cMD) were analysed by RT-PCR and found to be expressed constitutively under E_2 or E_2 + MPA treatment (data not shown).

In Fig. 7 the results of the semiquantitative RT-PCR assessment are summarized. These four genes were confirmed to be regulated between E_2 and E_2 + MPA treatment independent of culture passage. Na, K-ATPase α1-subunit was found to be upregulated by 3.74 ± 0.88-fold, which confirms data published by Kester et al. (1997). Tristetraprolin (TTP) and Zn-α2-glycoprotein mRNA expression was increased by 3.55 ± 0.71- and 2.27 ± 0.33-fold, respectively. In contrast, lipocalin 2 mRNA expression decreased by 4.16 ± 1.21-fold in E_2 + MPA treated cells compared to cells treated with E_2 alone.

4. Discussion

Progestins are suggested to be involved in breast cancer development. However, on the other hand, they are used in anticancer treatment and excision of breast tumors during the first half of progesterone-dominated luteal phase of the menstrual cycle has resulted in an increased survival time of premenopausal women suffering from breast cancer (Carlson, 2002; Weiss et al., 2002; Hagen and Hrushesky, 1998).

To establish T47D cells as an in vitro model to study the effects of progestins, we characterized ER α and PR as well as PR cofactor expression. As expected, T47D cells cultured without steroid hormones expressed ER α and PR mRNA and protein. E_2 and E_2 + MPA treatment suppressed ER α protein expression and PR protein expression was suppressed by E_2 + MPA. The corresponding mRNAs were expressed equally strong under both hormonal treatments, which suggests, that ER α and PR protein expression is under translational control in T47D cells. In addition, we demonstrated mRNA expression of steroid receptor coactivators SRC-1, -2 and -3 and the corepressors SMRT, Sin3A and KAP-1 in E_2 and E_2 + MPA treated T47D cells. The expression status of these cofactors is of importance, because changes in cofactors expression lead to hormone resistance or dysregulation of cell differentiation (Rowan and O'Malley, 2000; Liao et al., 2002; Hudelist et al., 2003). In further studies, it now

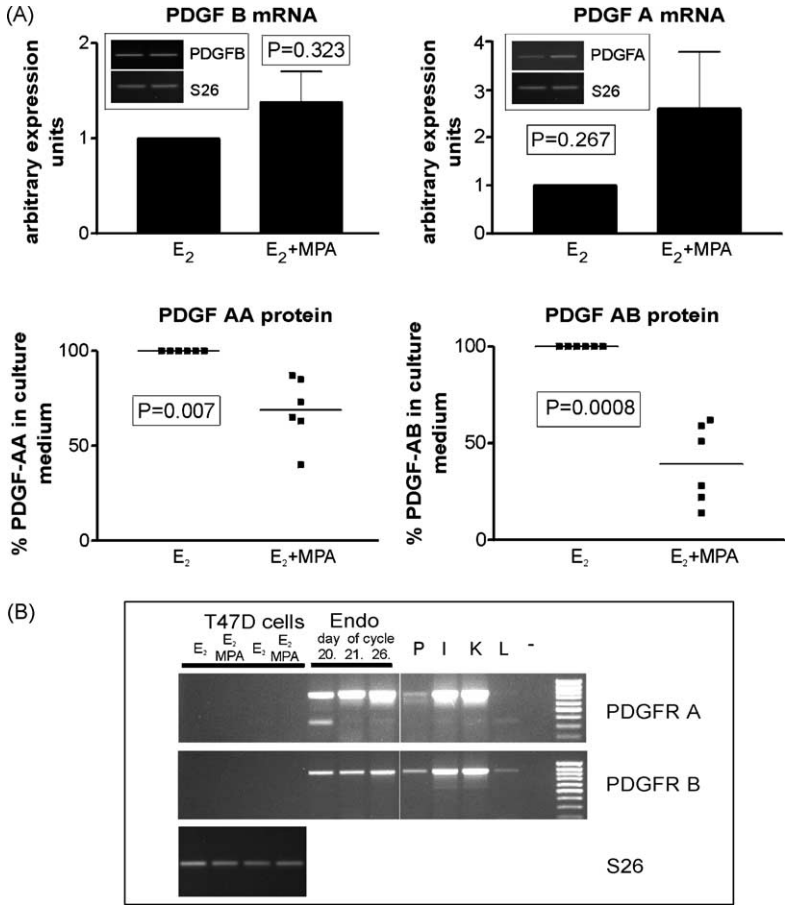

Fig. 4. PDGF A and B mRNA and protein expression in T47D cells. (A) Semiquantitative assessment of PDGF-A and -B mRNA expression by RT-PCR ($n = 4$; mean ± S.E.M.) after 6 days of hormonal with 10^{-8} M E_2 and 10^{-8} M $E_2 + 10^{-6}$ M MPA and quantitative assessment of the PDGF-AA and -AB isoform in culture supernatants after 5 days of hormonal treatment using ELISA technique ($n = 6$; mean ± S.E.M.). In (B) RT-PCR analysis of the PDGFA and PDGFB receptor gene expression in T47D cells and positive control tissues is demonstrated (Endo, endometrium; P, placenta; I, intestine; K, kidney; L, lung; –, water). The S26 ribosomal protein PCR product band demonstrates the integrity of T47D cell RNA/cDNA.

has to be analysed, if and how these cofactor proteins are recruited to the promoters of steroid-regulated genes in T47D cells. Recently, we have shown HDAC-1 and -3 protein expression in human breast tumors (Krusche et al., in press). Here we provide evidence that HDAC-1, -2 and -3, which are involved in hormonal regulation, differentiation and carcinogenesis (Thiagalingam et al., 2003), are expressed on the mRNA and protein level without obvious differences between both hormonal treatments. In addition, we found HDAC-6 mRNA expression, which is overexpressed in breast tumors with poor prognosis (Yoshida et al., 2004), in T47D cells. Therefore, our T47D cell line could represent an interesting model system for studying the impact of histone deacetylase in breast cancer pathology.

PDGFs and TNFα can enhance tumor progression by supporting the desmoplastic reaction, promoting angiogenesis and enhancing estradiol production of the tumor (Shao et al., 2000; Walker, 2001; Griffin et al., 2002; Duncan and Reed, 1995). PDGFA and B mRNA expression did not differ significantly between E_2 and E_2 + MPA treated T47D cells, whereas the secretion of PDGF-AA and -AB isoforms decreased, when cells were treated with E_2 + MPA. The PDGF-BB isoform was barely detectable, showing selective isoform production by T47D breast cancer cells. Neither PDGF receptor A nor B chain mRNA expression was detected in T47D cells using RT-PCR, which is in accord with the in vivo situation (De Jong et al., 1998; Kommoss et al., 1990).

TNFα was thought to be delivered to breast tumor tissue exclusively by macrophages (Duncan and Reed, 1995; Leek et al., 1998), until Meng et al. (2001) showed TNFα mRNA expression in T47D cells. In vivo, TNFα was recently found to be expressed in epithelial breast cancer cells (Leung et al., 2003). We confirmed TNFα mRNA expression in T47D breast cancer cells. Furthermore, we showed release of low amounts of TNFα protein by T47D cells into the culture medium. Since TNFα is a major enhancer of breast cancer malignancy, it is important to notice, that reduced amounts of TNFα were found in culture supernatants of E_2 + MPA treated cells. These results are in accord with the overall in-

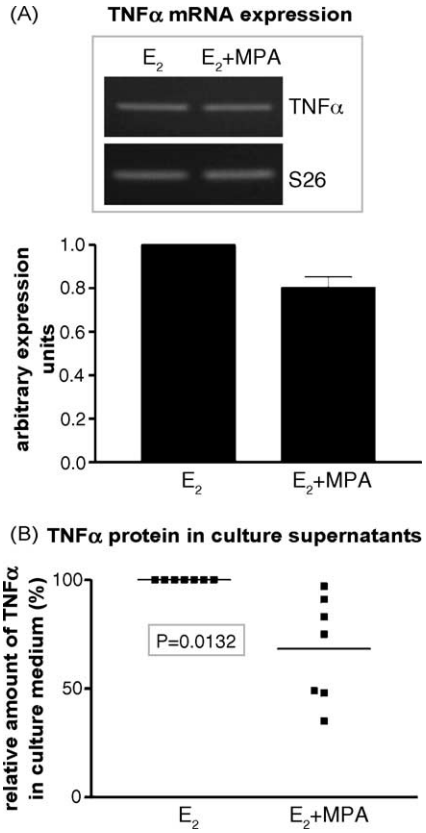

Fig. 5. TNFα mRNA and protein expression in T47D breast cancer cells. (A) Semiquantitative RT-PCR analysis of TNFα mRNA expression ($n = 6$; mean ± S.E.M.) after 6 days of treatment with 10^{-8} M E_2 and 10^{-8} M $E_2 + 10^{-6}$ M MPA. S26 ribosomal protein mRNA expression served as control. In (B) quantitative assessment of TNFα protein in cell culture supernatants after 5 days of hormonal treatment using a supersensitive ELISA ($n = 7$; mean ± S.E.M.).

hibitory effect of progestins on protein release in T47D cells (Chalbos and Rochefort, 1984).

By microarray hybridisation and subsequent semiquantitative RT-PCR analysis, we identified four genes that were differentially regulated between E_2 and E_2 + MPA treatment in T47D cells. ZAG and Na, K-ATPase α1 subunit mRNA expression was found to be upregulated by E_2 + MPA treatment. Whereas increased Na, K-ATPase α1 subunit mRNA expression in E_2 + MPA treated T47D cells is in accord with previously published data (Kester et al., 1997), ZAG expression was not found to be under progestin regulation by Lopez-Boado et al. (1994). This discrepancy is possibly due to the usage of different T47D cell subclones and/or differences in culture conditions.

However, our results support the hypothesis that progestins induce differentiation of breast cancer cells (Alkhalaf et al., 2002; Lin et al., 2003): ZAG and Na, K-ATPase α1 subunit are differentiation markers, that are upregulated during lobulo-alveolar development of the mammary gland and apocrine differentiation of the breast epithelium (Hovey et al., 2002; Shennan and Peaker, 2000; Bundred et al., 1987).

Another differentially regulated gene, identified here is lipocalin 2, belongs to a family of proteins, which comprises extracellular carriers of lipophilic molecules and immunomodulators (Bratt, 2000). Lipocalin 2 directly interacts with and enhances activity of MMP-9 (Yan et al., 2001). These data correspond with the observation that breast tumors with high lipocalin 2 expression are associated with poor prognosis (Stoesz et al., 1998). Here we show that lipocalin 2 expression is suppressed by E_2 + MPA compared to E_2 treatment. Should progestins also suppress the expression of lipocalin 2 with a subsequent reduction of local MMP-9 activity in vivo, it is possible that a reduction in invasive potential would be detected.

Tristetraprolin (synonyms TTP, NUP475, G0S24, TIS11d) mRNA expression and its increase by E_2 + MPA

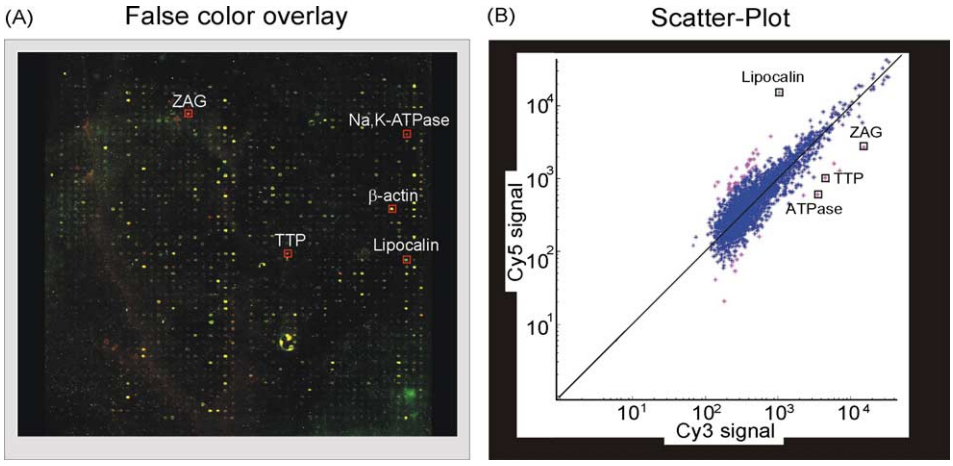

Fig. 6. Assessment of differential gene expression by microarray analysis. False color overlay of the microarray (A) and Scatter plot of the assessed microarray (B). In the scatter plot the distribution of spots based on their intensity values in the Cyanine 3 and Cyanine 5 channels are depicted. A shift from the diagonal line is proportional to a difference in expression levels between T47D cells treated for 6 days either with 10^{-8} M E_2 (Cyanine 5-dUTP labeled) or 10^{-8} M $E_2 + 10^{-6}$ M MPA (Cyanine 3-dUTP labelled).

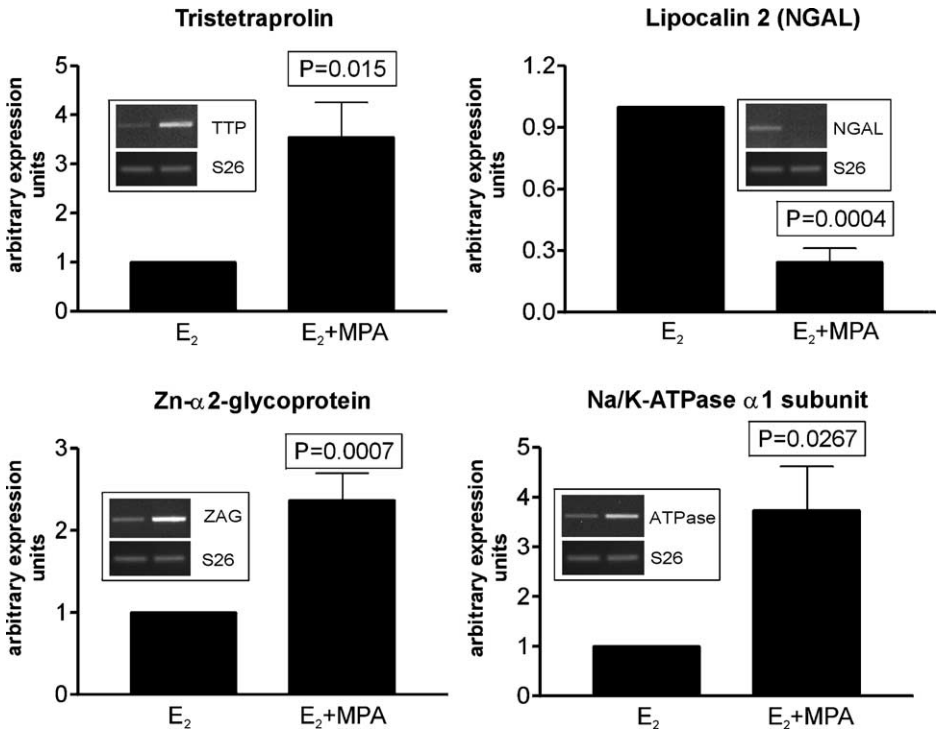

Fig. 7. Differentially expressed genes in T47D breast cancer cells. Validation of selected genes of T47D cells found to be more than 3.5-fold up- or down-regulated by 10^{-8} M E_2 vs. 10^{-8} M E_2 + 10^{-6} M MPA treatment. Representative RT-PCR results are shown. Primer sequences and PCR conditions are given in Table 2. All obtained products were of appropriate size and the correct identity was confirmed by DNA sequencing. Expression of the S26 ribosomal protein housekeeping gene served as control. The mRNA expression of a studied gene (ratio of the OD of the specific gene divided by the OD of the housekeeping gene) in cells treated with 17β-estradiol was set to "1". Then the specific gene/housekeeping gene ratio of the corresponding cells cultured with E_2 and E_2 + MPA was set into relation. The bar resembles mean ± S.E.M. (n = 5–6).

treatment, to the best of our knowledge is described for the first time in T47D cells. TTP is a zinc finger protein that controls TNFα-, interleukin-3, GM-CSF and c-fos biosynthesis at the posttranscriptional level by binding to AU-rich elements in the 3′UTR of these mRNAs (Carballo et al., 1997; Raghavan et al., 2001). The existence of a causal relation between the above described decreased TNFα protein concentration in culture supernatants with the increased tristetraprolin mRNA expression in E_2 + MPA treated T47D cells needs further investigation.

In summary, we describe steroid-hormone receptor cofactor mRNA expression and expression of four histone deacetylases involved in gene regulation and carcinogenesis in T47D cells. We also show that mRNA expression of ZAG and Na, K-ATPase α1 subunit indicating cell differentiation is up-regulated and that expression of genes, such as lipocalin 2, PDGF and TNFα which are involved in the enhancement of tumorigenesis are suppressed by E_2 + MPA compared to E_2 treatment. The presented in vitro data fit into the therapeutical concept of progestin administration as second, third or fourth line therapy in premenopausal women with metastatic breast cancer. They also support the observed potential benefits in long term survival (Hagen and Hrushesky, 1998), when breast tumor excision is performed in the first half of the luteal phase. However, functional assays are needed for a better understanding of the role of the identified progestin regulated genes in the pathogenesis of breast tumor development and for a proper assessment of the overall effect of progestins, progesterone receptor modulators or substances modulating the activity of PR cofactors on angiogenesis, invasion and metastasis.

Acknowledgement

We thank Sabine Eisner, Sabina Hennes-Mades, Rick Kamps and Diana Seelis-Schmidt for excellent technical assistance. This work was supported by the START programme "Molekulare Endokrinologie" (TP6, 1/2000-12/2003) of the School of Medicine, RWTH University of Aachen.

References

Alkhalaf, M., El-Mowafy, A., Karam, S., 2002. Growth inhibition of MCF-7 human breast cancer cells by progesterone is associated with cell differentiation and phosphorylation of Akt protein. Eur. J. Cancer Prev. 11, 481–488.

Bratt, T., 2000. Lipocalins and cancer. Biochim. Biophys. Acta 1482, 318–326.

Brisken, C., Park, S., Vass, T., Lydon, J.P., O'Malley, B.W., Weinberg, R.A., 1998. A paracrine role for the epithelial progesterone receptor in mammary gland development. Proc. Natl. Acad. Sci. U.S.A. 95, 5076–5081.

Bundred, N.J., Miller, W.R., Walker, R.A., 1987. An immunohistochemical study of the tissue distribution of the breast cyst fluid protein, zinc alpha2 glycoprotein. Histopathology 11, 603–610.

Carballo, E., Gilkeson, G.S., Blackshear, P.J., 1997. Bone marrow transplantation reproduces the tristetraprolin-deficiency syndrome in recombination activating gene-2 (−/−) mice. Evidence that monocyte/macrophage progenitors may be responsible for TNFalpha overproduction. J. Clin. Invest. 100, 986–995.

Carlson, R.W., 2002. Sequencing of endocrine therapies in breast cancer—integration of recent data. Breast Cancer Res. Treat. 75, S27–S32.

Chalbos, D., Rochefort, H., 1984. Dual effects of the progestin R5020 on protein release by the T47D human breast cancer cells. J. Biol. Chem. 259, 1231–1238.

Conneely, O.M., Mulac-Jericevic, B., DeMayo, F., Lydon, J.P., O'Malley, B.W., 2002. Reproductive functions of progesterone receptors. Recent Prog. Horm. Res. 57, 339–355.

Cooper, L.S., Gillett, C.E., Patel, N.K., Barnes, D.M., Fentiman, I.S., 1999. Survival of premenopausal breast carcinoma patients in relation to menstrual cycle timing of surgery and estrogen receptor/progesterone receptor status of the primary tumor. Cancer 15, 2053–2058.

De Jong, J.S., van Diest, P.J., van der Valk, P., Baak, J.P., 1998. Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I. An inventory in search of autocrine and paracrine loops. J. Pathol. 184, 44–52.

DeLignieres, B., 2002. Effects of progestrogens on the postmenopausal breast. Climacteric 5, 229–235.

Duncan, L.J., Reed, M.J., 1995. The role and proposed mechanism by which oestradiol 17 beta-hydroxysteroid dehydrogenase regulates breast tumor oestrogen concentrations. J. Steroid Biochem. Mol. Biol. 55, 565–572.

Giangrande, P.H., Kimbrel, E.A., Edwards, D.P., McDonnell, D.P., 2000. The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. Mol. Cell. Biol. 20, 3102–3115.

Graham, J.D., Hunt, S.M.N., Tran, N., Clarke, C.L., 1999. Regulation of the expression and activity by progestins of a member of the SOX gene family of transcriptional modulators. J. Mol. Endocrinol. 22, 295–304.

Graham, M.L., Dalquist, K.E., Horwitz, K.B., 1989. Simultaneous measurement of progesterone receptors and DNA indices by flow cytometry: analysis of breast cancer cell mixtures and genetic instability of the T47D line. Cancer Res. 49, 3943–3949.

Griffin, R.J., Williams, B.W., Wild, R., Cherrington, J.M., Park, H., Song, C.W., 2002. Simultaneous inhibition of the receptor kinase activity of vascular endothelial, fibroblast, and platelet-derived growth factors suppresses tumor growth and enhances tumor radiation response. Cancer Res. 62, 1702–1706.

Hagen, A.A., Hrushesky, W.J.M., 1998. Menstrual timing of breast cancer surgery. Am. J. Surg. 104, 245–261.

Hovey, R.C., Trott, J.F., Vonderhaar, B.K., 2002. Establishing a framework for the functional mammary gland: from endocrinology to morphology. J. Mammary Gland Biol. Neoplasia 7, 17–38.

Hudelist, G., Czerwenka, K., Kubista, E., Marton, E., Pischinger, K., Singer, C.F., 2003. Expression of sex steroid receptors and their cofactors in normal and malignant breast tissue: AIB1 is a carcinoma-specific co-activator. Breast Cancer Res. Treat. 78, 193–204.

Isaksson, E., von Schoultz, E., Odlind, V., Soderqvist, G., Csemiczky, G., Carlstrom, K., Skoog, L., von Schoultz, B., 2001. Effects of oral contraceptives on breast epithelial proliferation. Breast Cancer Res. Treat. 65, 163–169.

Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., Chambon, P., 1990. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J. 9, 1603–1614.

Kester, H.A., van der Leede, B.M., van der Saag, P.T., van der Burg, B., 1997. 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. J. Biol. Chem. 272, 16637–16643.

Kommoss, F., Colley, M., Hart, C.E., Franklin, W.A., 1990. In situ distribution of oncogene products and growth factor receptors in breast carcinoma: c-erbB-2 oncoprotein, EGFr, and PDGFr-beta-subunit. Mol. Cell Probes 4, 11–23.

Krusche, C.A., Wuelfing, P., Kersting, C., Vloet, A., Boecker, W., Kiesel, L., Beier, H.M., Alfer, J., in press. Histone deacetylase-1 and -3 protein expression in human breast cancer: a tissue microarray analysis. Breast Cancer Res. Treat.

Lanari, C., Molinolo, A.A., 2002. Progesterone receptors—animal models and cell signalling in breast cancer: diverse activation pathways for the progesterone receptor: possible implications for breast biology and cancer. Breast Cancer Res. 4, 240–243.

Lange, C.A., Richer, J.K., Horwitz, K.B., 1999. Hypothesis: progesterone primes breast cancer cells for cross-talk with proliferative or antiproliferative signals. Mol. Endocrinol. 13, 829–836.

Leek, R.D., Landers, R., Fox, S.B., Ng, F., Harris, A.L., Lewis, C.E., 1998. Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. Br. J. Cancer 77, 2246–2251.

Leung, G., Tsao, S.W., Wong, Y.C., 2003. Sex hormone-induced mammary carcinogenesis in female Noble rats: detection of differentially expressed genes. Breast Cancer Res. Treat. 77, 49–63.

Liao, L., Kuang, S.Q., Yuan, Y., Gonzalez, S.M., O'Malley, B.W., Xu, J., 2002. Molecular structure and biological function of the cancer-amplified nuclear receptor coactivator SRC-3/AIB1. J. Steroid Biochem. Mol. Biol. 85, 3–14.

Lin, V.C.L., Jin, R., Tan, P.H., Aw, S.E., Woon, C.T., Bay, B.H., 2003. Progesterone induces cellular differentiation in MDA-MB-231 breast cancer cells transfected with progesterone receptor complementary DNA. Am. J. Pathol. 162, 1781–1787.

Lopez-Boado, Y.S., Diez-Itza, I., Tolivia, J., Lopez-Otin, C., 1994. Glucocorticoids and androgens up-regulate the Zn-α2-glycoprotein messenger RNA in human breast cancer cells. Breast Cancer Res. Treat. 29, 247–258.

McPherson, K., Steel, C.M., Dixon, J.M., 2000. ABC of breast diseases. Breast cancer epidemiology, risk factors, and genetics. Br. Med. J. 321, 624–628.

Meng, L., Zhou, J., Sasano, H., Suzuki, T., Zeitoun, K.M., Bulun, S.E., 2001. Tumor necrosis factor α and interleukin 11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively down-regulating CCAAT/enhancer binding protein α and peroxisome proliferator-activated receptor γ: mechanism of desmoplastic reaction. Cancer Res. 61, 2250–2255.

Pritchard, K.I., 2003. Endocrine therapy of advanced disease: analysis and implications of existing data. Clin. Can. Res. (Suppl.) 9, 460s–467s.

Raghavan, A., Robison, R.L., McNabb, J., Miller, C.R., Williams, D.A., Bohjanen, P.R., 2001. HuA and tristetraprolin are induced following T cell activation and display distinct overlapping RNA binding specificities. J. Biol. Chem. 276, 47958–47965.

Reddel, R.R., Alexander, I.E., Koga, M., Shine, J., Sutherland, R.L., 1988. Genetic instability and the development of steroid hormone insensitivity in cultured T47D human breast cancer cells. Cancer Res. 48, 4340–4347.

Richer, J.K., Jacobsen, B.M., Manning, N.G., Abel, M.G., Wolf, D.M., Horwitz, K.B., 2002. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J. Biol. Chem. 277, 5209–5218.

Rowan, B.G., O'Malley, B.W., 2000. Progesterone receptor coactivators. Steroids 65, 545–549.

Shao, Z.M., Nguyen, M., Barsky, S.H., 2000. Human breast carcinoma desmoplasia is PDGF initiated. Oncogene 19, 4337–4345.

Shennan, D.B., Peaker, M., 2000. Transport of milk constituents by the mammary gland. Physiol. Rev. 80, 925–951.

Shi, Y.E., Torri, J., Yieh, L., Sobel, M.E., Yamada, Y., Lippman, M.E., Dickson, R.B., Thompson, E.W., 1993. Expression of 67 kDa laminin receptor in human breast cancer cells: regulation by progestins. Clin. Exp. Metast. 11, 251–261.

Soyal, S., Ismail, P.M., Li, J., Mulac-Jericevic, B., Conneely, O.M., Lydon, J.P., 2002. Progesterone's role in mammary gland development and tumorigenesis as disclosed by experimental mouse genetics. Breast Cancer Res. 4, 191–196.

Stoesz, S.P., Friedl, A., Haag, J.D., Lindstrom, M.J., Clark, G.M., Gould, M.N., 1998. Heterogenous expression of the lipocalin NGAL in primary breast cancers. Int. J. Cancer 79, 565–572.

Thiagalingam, S., Cheng, K.H., Lee, H., Mineva, N., Thiagalingam, A., Ponte, J.F., 2003. Histone deacetylases: unique players in shaping the epigenetic histone code. Ann. NY Acad. Sci. 983, 84–100.

Underhill, C., Qutob, M.S., Yes, S.P., Torchia, J., 2000. A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. J. Biol. Chem. 51, 40463–40470.

Vigushin, D.M., Ali, S., Pace, P.E., Mirsaidi, N., Ito, K., Adcock, I., Coombes, R.C., 2001. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. Clin. Cancer Res. 7, 971–976.

Walker, R.A., 2001. The complexities of breast cancer desmoplasia. Breast Cancer Res. 3, 143–145.

Weiss, L.K., Burkman, R.T., Cushing-Haugen, K.L., Voigt, L.F., Simon, M.S., Daling, J.R., Norman, S.A., Bernstein, L., Ursin, G., Marchbanks, P.A., et al., 2002. Hormone replacement therapy regimens and breast cancer risk. Obstet. Gynecol. 100, 1148–1158.

Yan, L., Borregaard, N., Kjeldsen, L., Moses, M.A., 2001. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). J. Biol. Chem. 276, 37258–37265.

Yoshida, N., Omoto, Y., Inoue, A., Eguchi, H., Kobayashi, Y., Kurosumi, M., Saji, S., Suemasu, K., Okazaki, T., Nakachi, K., Fujita, T., Hayashi, S., 2004. Prediction of prognosis of estrogen receptor-positive breast cancer with combination of selected estrogen-regulated genes. Cancer Sci. 95, 496–502.