

Progestins regulate genes that can elicit both proliferative and antiproliferative effects in breast cancer cells

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Abstract. Sex steroid hormone progesterone is known to have profound effects on the growth and differentiation of the normal mammary gland and malignant breast epithelial cells. *In vitro* progesterone and synthetic progesterone-like compounds (progestins) inhibit breast cancer cell growth. Medroxyprogesterone acetate (MPA) is a synthetic hormone widely used in the adjuvant treatment of advanced breast cancer, hormone replacement therapy and in oral contraceptives. It is a paradoxical hormone, since it inhibits breast cancer cell proliferation, but has also been implicated in increased breast cancer risk. To better understand the molecular mechanism by which cell proliferation and differentiation are regulated by progesterone and MPA in human breast cancer, we utilized cDNA microarray and quantitative real-time RT-PCR methods to identify their target genes. This study describes novel progestin/progesterone target genes in breast cancer cells and, notably, novel target genes that elucidate the underlying molecular mechanism of the dual role progestins play in the breast. A cDNA microarray containing 3000 genes showed notable regulation in 30 and 27 genes by MPA and progesterone, respectively. Only 6 out of the 30 genes regulated by MPA are down-regulated, but no progesterone down-regulation was observed. Overlapping in gene regulation by progesterone and MPA occurred, but the majority of genes regulated by these hormones were distinct. Given that progestins both stimulate and inhibit cancer cell growth, we report our findings on novel progestin and progesterone targets, which could explain the paradoxical actions of progestins in the breast.

Introduction

Sex steroid estrogen, progesterone and androgens play a crucial role in the development and differentiation of the normal mammary gland as well as the regulation of breast cancer growth. In the breast cancer cells, progestin has a biphasic action on the cell cycle, since it both stimulates and inhibits breast cancer cell proliferation *in vitro* (1-3). In these cells, progestins initially stimulate G₁ cells to enter the S phase, but the prolonged progestin treatment is marked by the growth inhibition of the cells. In the normal breast progestins stimulate cell proliferation *in vivo*, while in breast tumors and normal breast cells in culture (4) they can inhibit estrogen-stimulated growth, which is often associated with increased differentiation. Progestins, especially medroxyprogesterone acetate (MPA), are therefore used in the adjuvant treatment of advanced breast cancer, hormone replacement therapy (HRT) and in oral contraceptives (5,6).

While the effects of progesterone are mediated by the progesterone receptor (PR) alone, MPA binds to the progesterone, glucocorticoid as well as the androgen receptor (AR) and may exert its antiproliferative effects via different receptors. MPA is a weak AR agonist (7-10), but it activates AR by a mechanism different from other agonists (11). It has been shown that the action of MPA on breast cancer cell proliferation is at least partly due to its intrinsic androgen activity. For example, in an MFM-223 mammary cancer cell line that has high levels of AR, but low levels of PR, its growth is inhibited by DHT or MPA (12). It has recently been shown that decreased AR levels contribute to the failure of response to MPA in patients with metastatic breast cancer (13).

Given that the two hormones mediate their effects partly through different mechanisms, we were interested in finding the specific differences in their gene expression profiles. We compared the gene expression profiles of MPA and progesterone by cDNA microarray analysis in the MCF-7 breast cancer cell line. The human breast cancer cell line MCF-7 possesses androgen, estrogen and progesterone receptors, and is growth-inhibited by MPA and progesterone, thereby offering a good model to study the specific role of each of these steroids in the control of breast cancer growth. There are many different strains of MCF-7 cells and we have tested our cell line with a ribonuclease protection assay for GR, AR, PR, ER and ER,

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Abbreviations: PR, progesterone receptor; MPA, medroxyprogesterone acetate

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(14). Although several expression profiles have been published, none describe progesterone- and MPA-regulated genes in the MCF-7 cell line (15-17).

Our data demonstrate that progesterone and MPA partially regulate the same set of genes. However, the majority of genes regulated by these hormones are distinct. The expression of 6 selected genes (*AQP3*, *SGK1*, *ALCAM*, *FXRD3/MAT-8*, *MUCIN* and *c-MYB*) was analyzed by the quantitative real-time RT-PCR, which confirmed the cDNA microarray results. Furthermore, we describe some novel progesterone/progestin targets that elucidate the underlying molecular mechanism of the dual role progestins play in the breast.

Materials and methods

Chemicals. Dulbecco's modified Eagle's medium with F12 (DMEM/F12), 17 β -estradiol and medroxyprogesterone acetate were provided by Sigma (St. Louis, MO). Fetal bovine serum (FBS), penicillin-streptomycin and insulin were obtained from Gibco/BRL. Progesterone was purchased from Merck (Darmstadt, Germany).

Cell culture and cell growth assay. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium with F12 (DMEM/F12) supplemented with 5% FBS, penicillin-streptomycin and 10 ng/ml insulin. Prior to the experimental studies the cells were cultivated at 2-3 passages in phenol red-free DMEM/F12 supplemented with 5% dextran-coated, charcoal-stripped treated fetal bovine serum, penicillin-streptomycin, 10 ng/ml insulin and 1 nM 17 β -estradiol. The cells were then plated in triplicate in 96-well plates at a density of 3×10^3 cells per well in the experimental medium. The cells were allowed to attach overnight and the medium was replaced. After 24 h, appropriate steroid hormones in 100% ethanol were added. Cells were fixed and the number of cells was measured every 24 h after hormone addition. Briefly, the cells were directly fixed in 96-well plates with 11% glutaraldehyde by shaking at 500 rpm for 15 min, then washed and air-dried. Crystal violet solution (0.1%) was added to stain the fixed cells by shaking for 20 min. Excess dyes were washed away with deionized water and the plates were air-dried before the addition of 10% acetic acid to extract the cell-bound dyes (18). The optical density of the dye extracts in the plates was measured at a wavelength of 590 nm using a Victor 1420 multilabel counter (Wallac).

RNA isolation and cDNA microarray hybridization. After treatment for 48 h with, MPA, progesterone or vehicle, the cells were harvested from 150 cm² plates and total RNA was extracted using a TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. A cDNA microarray was performed according to the manufacturer's instructions. In brief, 20 μ g of RNA sample from MPA-, progesterone- and vehicle-treated cells was labelled with CyTM 5-dUTP (25 nM) by reverse transcription under an oligo(dT)₍₁₂₋₁₈₎ primer direction. Concomitantly, an equal amount of RNA sample from the untreated cells was labelled with CyTM 3-dUTP (25 nM) as a control. The RNA labelling reactions were performed at 42°C for 80 min. After the labelling reaction, RNA was removed from synthesized

cDNA by the addition of NaOH solution (1 M) followed by neutralisation with Tris-HCl (1 M, pH 7.5). Cy3- and Cy5-cDNA were combined together in microcon column (Millipore Corporation, Bedford, USA) and washed four times in TE buffer (pH 7.4) by centrifugation. In the final washing step, COT-1 DNA, polyA and yeast tRNA were added to the washing buffer and centrifuged to make the final volume of the labelled cDNA mixture <10 μ l. For hybridization, a Human2-1 Glass chip containing 3000 cDNA probes (Turku Centre for Biotechnology, Turku, Finland) was pre-treated by succinic anhydride. The labelled cDNA mixture was hybridized with the chip in a humid chamber at 65°C overnight. After hybridization, the chip was washed four times by shaking and dried by centrifugation. The fluorescence intensities of Cy3 and 5 were measured using a ScanArray 4000 laser confocal scanner (GSI Lumonics, Billerica, MA), the hybridization images were analyzed using QuantArray microarray analysis software v3.0 (Packard BioScience) and the data were finally normalized to median by using Excel date normalization macro.

Quantitative real-time RT-PCR analysis. Reverse transcription (RT) and real-time PCR were performed separately. The total RNA from each sample was reverse-transcribed using a high capacity cDNA archive kit (Applied Biosystems, USA) and PCR reactions were performed in MicroAmp optical 96-well reaction plates using a SYBR-Green PCR master mix kit (Applied Biosystems) following the manufacturer's instructions. Briefly, for reverse transcription, 5 μ g of RNA dissolved in 50 μ l PCR-compatible buffer was mixed with 50 μ l of 2x RT master mix [10 μ l of 10x reverse transcription buffer, 4 μ l of 25x dNTPs, 10 μ l of 10x random primers, 5 μ l of multiscribe reverse transcriptase (50 U/ μ l) and 21 μ l of nuclease-free H₂O], RT was performed at 25°C for 10 min followed by 37°C for 2 h in the programmable thermal controller (MJ Research, Inc., Watertown, USA). For real-time PCR, 30 ng of cDNA was combined with the primers and 2x SYBR-Green PCR master mix to the final volume of 30 μ l per reaction. Real-time PCR was performed at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min on an ABI PRISM 7000 Detection System (Applied Biosystems). The PCR product was examined by dissociation curve analysis to verify the specific products.

The data were analyzed with ABI PRISM 7000 SDS software and normalized to the human acidic ribosomal phosphoprotein P0 (RPLP0) as the endogenous control to verify uniform RNA loading in the experiments. The final results were expressed as the N-fold difference in the gene expression between the treated and control sample as described (19). The values used for calculating the formula were obtained from the corresponding standard curve. The standard curves of the endogenous controls and target genes were made by a series of dilutions of the treated samples.

All primers used were designed by using Primer Express v2.0 software (Applied Biosystems). To avoid the amplification of any genomic DNA, the forward and reverse primers for each gene were chosen from different exons. BLASTN searches were performed to confirm the specificity of the primer sequences. The primers for selected genes are listed in Table I.

 SPANDIDOS genes and their primer sequences used in the real-time RT-PCR.^a

Gene	Forward primer	Reverse primer
MUC1	5'-tgccttggtctgtctcagt-3'	5'-cccgggctggaaagatgt-3'
AQP3	5'-gccggcatctttgctacct-3'	5'-tgtgcctatgaactggtggtaaagaa-3'
ALCAM	5'-tctgctcttctgctcttgatct-3'	5'-tatgctgaatttacagtataccatccaa-3'
SGK1	5'-ctccccctttaacccaaatg-3'	5'-gggacaggctcttcggtaaac-3'
c-MYB	5'-agacaatgttctcaaaagcatttacagta-3'	5'-aggatgcagggtcccaggta-3'
FXYD3/MAT-8	5'-ccacaactgccagcaatcct-3'	5'-cggcctaagcaaacctcact-3'
RPLP0	5'-aatctccagggcaccatt-3'	5'-cgctggtcccactttgt-3'

^aThe forward and reverse primers for each gene were chosen from different exons. BLASTN searches were performed to confirm the specificity of the primer sequences.

Table II. Genes showing the expression ratio >2 or <2 in MCF-7 cells treated by MPA for 48 h.^a

Accession no.	Gene name	Description	-Fold
R32848	S100P	S100 calcium-binding protein P	18.5
R92737	AQP3	Aquaporin 3	4.4
AA126009	FXYD3/MAT-8	FXYD domain-containing ion transport regulator 3	3.8
AA459100	TPD52	Tumor protein D52	3.6
H23187	CA2	Carbonic anhydrase II	3.5
T77840	Dlg5	Disc large homolog 5	3.5
AA488073	MUC1	Mucin 1	3.4
AA486082	SGK1	Serum/glucocorticoid-regulated kinase	3.3
R93124	2- α -HSD	Aldo-keto reductase family 1, member C1	3.0
AA070226	SEPP1	Selenoprotein P	3.0
AA434373	ELF3	E74-like factor 3	2.8
R62817	EPB72	Erythrocyte membrane protein band 7.2	2.6
H77597	MT1	Metallothionein 1H	2.5
R39862	ALCAM	Activated leucocyte cell adhesion molecule	2.5
H53340	MT1G	Metallothionein 1G	2.4
H50323	FAS	Fatty acid synthase	2.3
R63065	GST5	Glutathione S-transferase M3	2.3
N80129	MT1XL	Metallothionein 1L	2.3
AA425746	RUNX1	Runt-related transcription factor 1	2.1
H45668	KLF4	Kruppel-like factor 4	2.1
H72028	GSN	Gelsolin	2.1
T73556	ACSL1	Fatty-acid-coenzyme A ligase, long-chain	2.1
N20475	CTSD	Cathepsin D	2.0
AA496576	NFE2L	Nuclear factor (erythroid-derived 2)-like 1	2.0
AA434397	ITGB5	Integrin, β 5	-3.3
N49284	c-MYB	V-myb avian myeloblastosis viral oncogene homolog	-2.5
AA027012	KDR/VEGFR2	Kinase insert domain receptor	2.5
AA463257	ITGA2/CD49B	Integrin, α -2	-2.5
R43817	NPY1R	Neuropeptide Y receptor Y1	-2.0
AA464532	THBS1	Thrombospondin 1	-2.0

^aThe cDNA chip contained each gene in triplicate. Results are the average fold changes of triplicates in two independent experiments. The genes are listed in descending order.

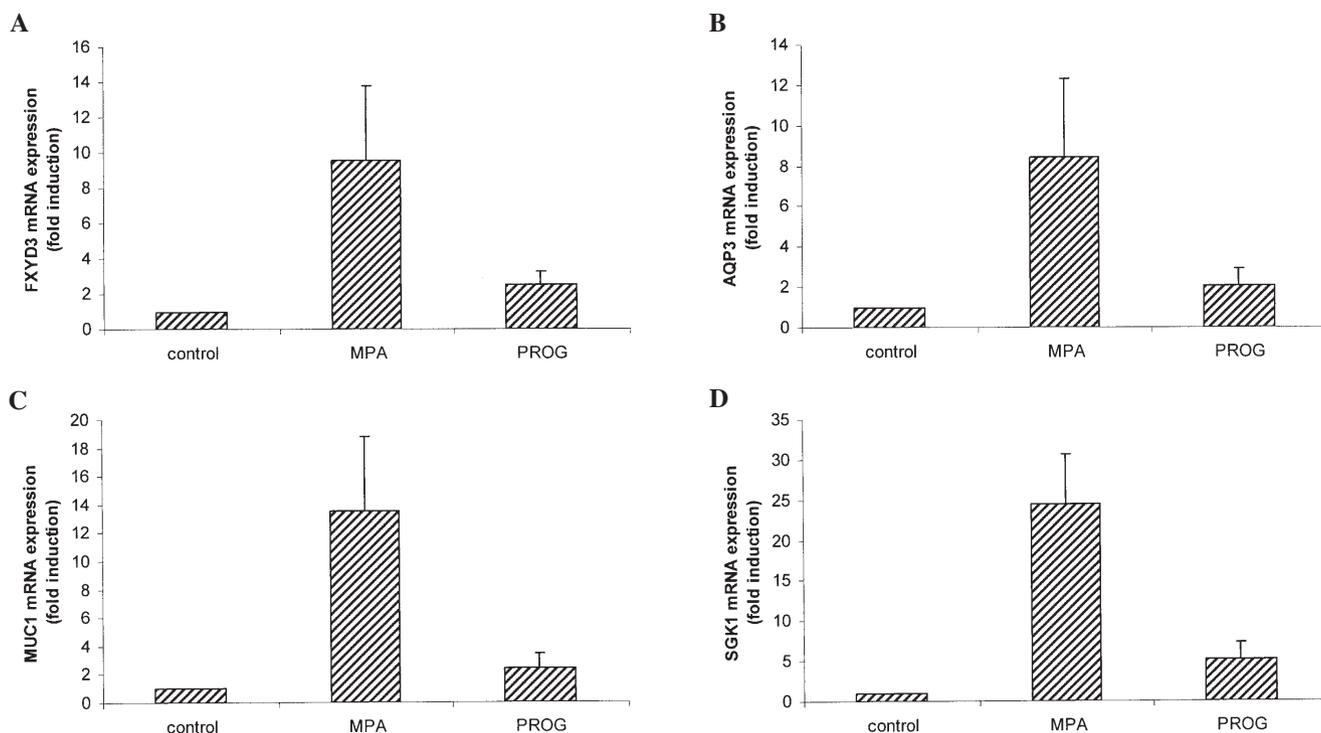


Figure 1. A quantitative real-time RT-PCR analysis of selected MPA and progesterone up-regulated genes. MCF-7 cells cultured in the insulin-supplemented serum in the presence of 1 nM 17 β -estradiol were treated with ethanol vehicle, 10 nM MPA or 10 nM progesterone for 48 h. The cells were then harvested and RNA was used for quantitative real-time RT-PCR. Results are expressed as the means (\pm SD) of two independent experiments performed in triplicate. (A) *FXYD3/MAT-8*, (B) *AQP3*, (C) *MUCIN1* and (D) *SGK1*.

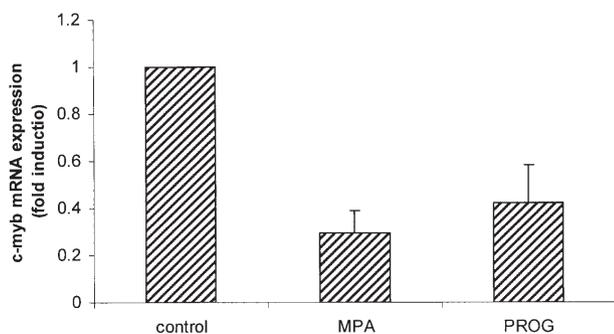


Figure 2. *c-MYB* proto-oncogene is down-regulated by progesterone and MPA. MCF-7 cells cultured in the insulin-supplemented serum in the presence of 1 nM 17 β -estradiol were treated with ethanol vehicle, 10 nM MPA or 10 nM progesterone for 48 h. The cells were then harvested and RNA was used for quantitative real-time RT-PCR. Results are expressed as the means (\pm SD) of two independent experiments performed in triplicate.

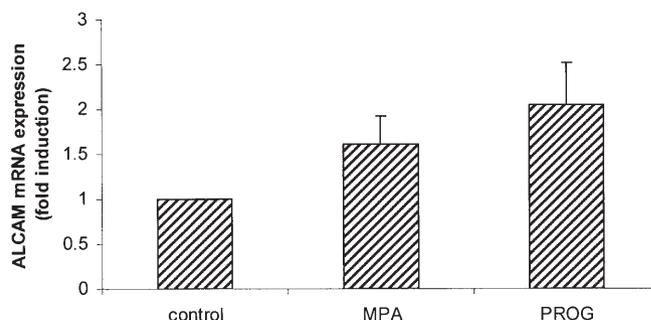


Figure 3. *ALCAM* is regulated by progesterone but not by MPA. MCF-7 cells cultured in the insulin-supplemented serum in the presence of 1 nM 17 β -estradiol were treated with ethanol vehicle, 10 nM MPA or 10 nM progesterone for 48 h. The cells were then harvested and RNA was used for quantitative real-time RT-PCR. Results are expressed as the means (\pm SD) of two independent experiments performed in triplicate.

Results

Growth inhibition of MCF-7 cells by MPA and progesterone.

The growth of MCF-7 cells was inhibited by 10 nM MPA and progesterone. MCF-7 cells were treated with 10 nM MPA, 10 nM progesterone or vehicle in the presence of 1 nM estradiol. The growth-inhibitory effect by MPA and progesterone after 6 days was 20 and 10% respectively (20,21).

Identification of MPA and progesterone-regulated genes (cDNA microarray hybridization). To examine the molecular

mechanism of the synthetic progestin MPA and progesterone, we compared the gene expression profiles of the MPA and progesterone by cDNA microarray analysis in the MCF-7 breast cancer cell line. Total RNA was isolated from the cells cultured in the presence of 1 nM estrogen treated with vehicle, progesterone (10 nM) and MPA (10 nM) for 48 h.

Tables II and III list genes that were regulated at least 2-fold by MPA and progesterone, respectively. The cDNA chip used in this study contained each gene in triplicate. Fold changes are the average of the triplicates in two independent experiments.



SPANDIDOS PUBLICATIONS Genes showing an expression ratio of >2 in MCF-7 cells treated by progesterone for 48 h.^a

Accession no.	Gene name	Description	-Fold
N20475	CTSD	Cathepsin D	5.5
R32848	S100P	Calcium-binding protein P	4.5
AA434115	CHI3L1	Chitinase-3-like protein 1 precursor	4.0
AA485959	KRT7	Keratin 7	3.6
N54596	IGF2	Insulin-like growth factor 2	3.5
AA126009	FXYD3/MAT-8	FXYD domain-containing ion transport regulator 3	3.3
R39862	ALCAM	Activated leucocyte cell adhesion molecule	3.0
N38990	GYPE	Glycophorin 3	3.0
T77840	DLG5	Discs, large homolog 5	3.0
N75028	PSPHL	Phosphoserine phosphatase-like	2.9
H23187	CA2	Carbonic anhydrase II	2.9
N71003	PDCD4	Programmed cell death 4	2.8
AA410429	VASP	Vasodilator-stimulated phosphoprotein	2.7
AA411554	SLC25A16	Solute carrier family 25 member 16	2.7
R10973	PAPPA	Pregnancy-associated plasma protein A	2.6
R01638	CTDSPL	Small CTD phosphatase 3	2.5
AA434373	ELF3	E74-like factor 3	2.5
W93472	CNGA1	Cyclic nucleotide gated channel α 1	2.5
AA402960	RNF5	Ring finger protein 5	2.4
AA668595	TP53I3	Tumor protein p53 inducible protein 3	2.4
H14343	CDC25B	Cell division cycle 25B	2.3
AA425853	SFPQ	PTB-associated splicing factor	2.3
N66852	ORC2L	Origin recognition complex subunit 2	2.2
AA452848	AAMP	Angio-associated, migratory cell protein	2.2
AA453293	PDE4B	Phosphodiesterase 4B	2.1
AA598659	NUMA1	Nuclear mitotic apparatus protein 1	2.1
N98524	F10	Coagulation factor X	2.1

^aA cDNA chip contained each gene in triplicate. Results are the average fold changes of the triplicates in two independent experiments. The genes are listed in descending order.

Of the 30 genes regulated by MPA only 6 were down-regulated. Progesterone up-regulated 27 genes 7 (*E74-like factor 3/elf3*, *carbonic anhydrase II*, *ALCAM*, *cathepsin D*, *S100P*, *Dlg5* and *FXYD3/MAT-8*) of which were also regulated by MPA. As expected, progesterone was a less potent regulator than MPA. Several genes found in this study have previously been reported to be progesterone- or progestin-regulated, confirming the reliability of our data. The genes are: *S100P*, *Dlg5*, *tumor protein D52*, *cathepsin D*, *neuropeptide Y receptor Y1* and *carbonic anhydrase II* (16,17,21). Many, however, were not known to be progestin-responsive and therefore represent novel progestin targets.

Quantitative real-time RT-PCR analysis. To validate the expression results of the cDNA microarray hybridization, the quantitative real-time RT-PCR analyses were utilized. The selected genes and their primer sequences (6 genes for MPA and progesterone) are listed in Table I. The selected genes were

confirmed to be regulated either by MPA and progesterone, or progesterone alone as suggested by cDNA microarray analysis (Figs. 1, 2 and 3). *FXYD3/MAT-8*, *AQP3*, *MUCIN* and *SGK1* are all up-regulated by MPA and to a lesser extent by progesterone (Fig. 1A-D). Of the 6 genes tested, *SGK1* shows the most remarkable regulation under MPA treatment (~24-fold). The stimulation by progesterone is less pronounced, but notable (~5-fold). *MUCIN* and *FXYD3/MAT-8* are also strongly stimulated by MPA (~13.5 - and ~9.5-fold), but progesterone stimulation is much weaker. *c-MYB* is down-regulated by the two hormones (~3.5-fold by MPA and ~2.3-fold by progesterone) (Fig. 2). *ALCAM* is specifically up-regulated by progesterone (Fig. 3).

Discussion

To investigate the molecular basis for progestin- and progesterone-induced inhibition of breast cancer cell

proliferation, we used cDNA microarray to detect the MPA- and progesterone-induced alterations of the gene expression in MCF-7 cells. The cDNA microarray of 3000 genes (each in triplicate) showed notable regulation in 30 and 27 genes by MPA and progesterone, respectively. Only 6 out of the 30 genes regulated by MPA are down-regulated and no progesterone down-regulation was observed.

To validate the microarray results, genes from each category were chosen for further analysis: four MPA up-regulated, one MPA down-regulated and one progesterone up-regulated. The regulation of *FXYD3/MAT-8*, *AQP3*, *MUCIN*, *SGK1*, *c-MYB* and *ALCAM* were individually tested by quantitative real-time RT-PCR. These genes were selected because of their obvious or possible relevance to breast cancer or to known pathways which may explain the dual role of progestins. *FXYD3/MAT-8*, *AQP3*, *MUCIN* and *SGK1* are all induced in response to the treatment of cells with MPA and to a lesser extent by progesterone, while *c-MYB* is down-regulated by the two hormones and *ALCAM* is up-regulated only by progesterone. All the selected genes from the MPA category were tested by quantitative real-time RT-PCR for progesterone and MPA regulation, although the initial expression profiling with microarray suggested the genes be regulated only by MPA. Based on the quantitative RT-PCR results, all of the selected genes were found to be regulated not only by MPA but also by progesterone. This is not unexpected, since quantitative RT-PCR is a more accurate method of identifying quantitative differences in the gene expression than microarray profiling, especially when the genes have low expression levels. A recent study showed that quantitative RT-PCR often fails to validate differences in gene expression observed by gene expression profiling (15).

MPA up-regulated genes. The first gene tested, *FXYD3/MAT-8*, is a mammary tumor 8 kDa transmembrane protein, that is expressed in primary human breast tumors and in human breast tumor cell lines and serve as a Na,K-ATPase regulator (22,23). *Mat-8* is a marker of a specific cell type preferentially transformed by the action of Neu and Ras, but not Myc oncoproteins during murine mammary carcinogenesis (24). It is up-regulated in prostate cancer and its siRNA-mediated inhibition of the expression decreases the proliferation of human prostate carcinoma cells (25). *FXYD3/MAT-8* is therefore an example of the genes that are induced by progestins and are potentially proliferation-related. *FXYD3/MAT-8* up-regulation (along with *MUC1*, see below) could point to the proliferative facet of the dual role progestins play in the breast.

The second gene analyzed, the epithelial cancer-associated antigen *MUCIN 1* (*muc1/cd227*), is a membrane-associated glycoprotein expressed by many types of ductal epithelia, is expressed in a wide variety of tumors and is considered to function as an anti-adhesion molecule which inhibits cell-to-cell interactions (26-28). Several studies have demonstrated the regulation of *MUC1* expression by steroid hormones, including estrogens, glucocorticoids and androgens (29,30). Although *MUC1* is known to be progesterone-regulated (stimulated or repressed) in some organ systems, only one report exists suggesting that the expression of cellular and secreted *MUC1* is stimulated by progesterone in the breast

cancer cell line (31-33). To our knowledge, however, the effect of progestins in *MUC1* mRNA expression has yet to be demonstrated.

Our finding that the *MUC1* mRNA level is elevated as late as 48 h after progestin treatment may partly explain the harmful actions of progestins in the breast. Considering the role of *MUC1* as an anti-adhesive molecule, its up-regulation by progesterone in breast cancers may enhance cell proliferation and/or metastatic spread. Our finding is supported by the fact that *MUC1* is overexpressed in breast cancer (34,35).

The third gene tested, a serum and glucocorticoid-inducible serine/threonine protein kinase (*SGK1*), was originally identified as an immediate early gene induced by glucocorticoids (36). Emerging evidence thereafter has established *SGK1* as a vital regulatory molecule in coordinating extracellular signals (steroid hormones, protein growth factors and other environmental cues) and intracellular processes in controlling cell proliferation and survival. An original study by Webster *et al* reports on the *SGK1* induction by glucocorticoids and serum, but not by progesterone in Con 8 rat mammary tumor cells (36). We showed herein that in human MCF-7 mammary tumor cells, *SGK1* is markedly induced by MPA and progesterone (Fig. 1D). This is expected, since glucocorticoid and progesterone response elements are identical and progestins are supposed to regulate *SGK1* expression via PR. MPA can also act via the glucocorticoid receptor. The function of *SGK1* is poorly understood, but it is now recognized that *SGK1* has a very important role in cross-talk that can couple anti-proliferative and proliferative cell signalling pathways (37). *SGK1* is transcriptionally regulated in rat mammary tumor cells by serum under proliferative conditions or by glucocorticoids that induce a G₁ cell cycle arrest (38) and *SGK1* actively shuttles between the nucleus (in S and G₂/M) and the cytoplasm (in G₁) in synchrony with the cell cycle (37). Therefore, it has been proposed that *SGK1* could maintain the cells in a growth-suppressed state while localized to the cytoplasm and facilitate proliferation as a result of its nuclear-cytoplasmic shuttling during the cell cycle in mammary tumor cells (37,39). The mechanism whereby progestins affect cell growth through *SGK1* calls for further investigation. Whether the progestins cause *SGK1* localization to the cytoplasm or nucleus can be an important determinant as to which of the pathways (stimulatory or inhibitory) is activated. This finding clarifies the capacity progestins have in breast either to increase or decrease growth depending on the cell context and other milieu.

MPA down-regulated genes. Six MPA down-regulated genes were identified in this study: *thrombospondin 1* (*THBS1*), *integrin α -2* (*ITGa2*), *vascular endothelial growth factor receptor 2*, (*VEGFR2/KDR*), *integrin β -5* (*ITG β 5*), *neuropeptide Y receptor Y1* (*NPY1-R*) and *MYB proto-oncogene* (*c-MYB*). Of these, neuropeptide Y receptor Y1 and *c-MYB* are known progestin targets (17) and *thrombospondin 1* is overexpressed in breast carcinomas (40).

c-MYB is clearly down-regulated by MPA and progesterone (Fig. 2), and is an interesting progestin target. The *myb* gene family consists of three members: A, B and *c-MYB*, which encode nuclear proteins that function as transcriptional trans-

 SPANDIDOS PUBLICATIONS. The human *c-MYB* proto-oncogene is the cellular

homolog of the viral *v-MYB* oncogene. The expression of *c-MYB* correlates with proliferation and decreases during differentiation. Several studies have shown that *c-MYB* down-regulation is needed for terminal differentiation, constitutive *c-MYB* expression blocks differentiation and antisense oligonucleotides targeting *c-MYB* block the proliferation of human hematopoietic cells (2,41-43). *MYB* oncogene amplification has been observed in hereditary BRCA1 breast cancer (44) and there is a strong association between *c-MYB* and estrogen receptor expression in breast cancer (45). Notably, *c-MYB* is also induced in response to estrogen treatment in the MCF-7 cells (46). As estrogens are strong proliferation stimulants, it appears that opposing the estrogen induced effect in *c-MYB* expression, progestins could potentiate differentiation and decrease the proliferation of breast cancer cells.

Progesterone-regulated genes. Progesterone up-regulated 27 genes by >2-fold, but no down-regulation was observed, as assessed by cDNA microarray analysis. In general, there is overlapping in gene regulation by progesterone and MPA, but the majority of genes regulated by these hormones are distinct. For those genes that are regulated by the two hormones, progesterone regulation is less pronounced.

We selected one progesterone-regulated gene for further analysis. The activated-leukocyte cell-adhesion molecule (ALCAM) is a glycoprotein of the immunoglobulin superfamily. *ALCAM* is involved with cellular adhesion, proliferation and tumor progression, and is specifically induced by progesterone, but not MPA, as verified by quantitative RT-PCR (Fig. 3). Studies have shown that *ALCAM* decreases the proliferation and adhesion of breast cancer cells to each other and this reduced expression indicates a more aggressive phenotype and poor prognosis (47). Its up-regulation by progesterone is consistent with the anti-proliferative effects of progesterone *in vitro*.

Multifaceted gene regulation by progestins. Certain conclusions can be drawn at this stage. A vast majority of MPA and/or progesterone target genes found in this study are up-regulated. This phenomenon has also been observed in previous studies (16,17,48). It has been proposed that genes which are up-regulated show larger differences in the expression level upon progestin treatment and are therefore identified more easily (48). While this may be partially true, it appears however, that down-regulated genes are uncommon. Relatively few progesterone down-regulated genes have been identified thus far. In studies by Richer *et al* only 12 such genes were identified using chips consisting of 5,600 full-length human genes (16). Using the same chip, Wan and Nordeen identified 34 down-regulated genes, when using the more potent PR agonist, the synthetic progestin R5020 (17). Our data are therefore consistent with previously published data. Using the chip containing 3000 cDNA probes, we found 6 MPA down-regulated genes, but no progesterone-induced gene down-regulation was observed.

Previous studies have shown that at early time points (2 and 6 h), progestins induce certain genes associated with cell cycle progression and/or down-regulate growth-suppressive

genes (16,49). Based on what we know about the biphasic role of progestins, that is to be expected since during the first 24 h progestins stimulate cell growth. Of particular note, therefore, is our finding that certain genes that can potentially exacerbate harmful effects of progestins, are still up-regulated after 48 h. Evidence indicates that under differing conditions, the balance between growth promoting and inhibiting factors, determine whether a stimulatory or inhibitory pathway will predominate.

Our results also show that all those MPA-regulated genes whose expression was verified by quantitative real-time RT-PCR, are also progesterone-regulated, but to a lesser extent. This was to be expected, since MPA is a more potent progestin and quantitative RT-PCR is a more accurate method of detecting the expression differences. It is not known, however, whether this is true for all MPA-regulated genes. The study by Bray *et al* suggests that differentially regulated genes by different progestins through PR are rare at least in the T47DCo cell line (15).

In summary, we have identified several novel progestin (MPA) and progesterone target genes in the MCF-7 breast cancer cell line. The majority of progestin-regulated genes are up-regulated. Of particular note for this study is that certain up-regulated genes are growth-associated, which may explain the harmful effects progestins elicit in the breast. Our data therefore point to the capacity progestins possess at a transcriptional level either to stimulate or inhibit cell growth. It is probably the balance between growth promoting and inhibiting factors that determine whether proliferative or antiproliferative effects take place.

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References

1. Schoonen WG, Joosten JW and Kloosterboer HJ: Effects of two classes of progestagens, pregnane and 19-nortestosterone derivatives, on cell growth of human breast tumor cells: I. MCF-7 cell lines. *J Steroid Biochem Mol Biol* 55: 423-437, 1995.
2. Sutherland RL, Hall RE, Pang GY, Musgrove EA and Clarke CL: Effect of medroxyprogesterone acetate on proliferation and cell cycle kinetics of human mammary carcinoma cells. *Cancer Res* 48: 5084-5091, 1988.
3. Musgrove EA, Lee CS and Sutherland RL: Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor alpha, epidermal growth factor receptor, c-fos, and c-myc genes. *Mol Cell Biol* 11: 5032-5043, 1991.
4. Gompel A, Malet C, Spritzer P, Lalardrie JP, Kuttent F and Mauvais-Jarvis P: Progestin effect on cell proliferation and 17 beta hydroxysteroid dehydrogenase activity in normal human breast cells in culture. *J Clin Endocrinol Metab* 63: 1174-1180, 1986.
5. Haller DG and Glick JH: Progestational agents in advanced breast cancer: an overview. *Semin Oncol* 13: 2-8, 1986.
6. Sedlacek SM and Horwitz KB: The role of progestins and progesterone receptors in the treatment of breast cancer. *Steroids* 44: 467-484, 1984.
7. Bardin CW, Brown T, Isomaa VV and Janne OA: Progestins can mimic, inhibit and potentiate the actions of androgens. *Pharmacol Ther* 23: 443-459, 1983.

8. Labrie F, Poulin R, Simard J, *et al*: Interactions between estrogens, androgens, progestins and glucocorticoids in ZR-75-1 human breast cancer cells. *Ann NY Acad Sci* 595: 130-148, 1990.
9. Raynaud JP, Bouton MM, Moguilewsky M, *et al*: Steroid hormone receptors and pharmacology. *J Steroid Biochem* 12: 143-157, 1980.
10. Mowszowicz I, Bieber DE, Chung KW, Bullock LP and Bardin CW: Synandrogenic and antiandrogenic effect of progestins: comparison with nonprogestational antiandrogens. *Endocrinology* 95: 1589-1599, 1974.
11. Kempainen JA, Langley E, Wong CI, Bobseine K, Kelce WR and Wilson EM: Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. *Mol Endocrinol* 13: 440-454, 1999.
12. Hackenberg R, Hawighorst T, Filmer A, Nia AH and Schulz KD: Medroxyprogesterone acetate inhibits the proliferation of estrogen- and progesterone-receptor negative MFM-223 human mammary cancer cells via the androgen receptor. *Breast Cancer Res Treat* 25: 217-224, 1993.
13. Buchanan G, Birrell SN, Peters AA, *et al*: Decreased androgen receptor levels and receptor function in breast cancer contribute to the failure of response to medroxyprogesterone acetate. *Cancer Res* 65: 8487-8496, 2005.
14. Sarvilinna N, Eronen H, Miettinen S, Vienonen A and Ylikomi T: Steroid hormone receptors and coregulators in endocrine-resistant and estrogen-independent breast cancer cells. *Int J Cancer* 118: 832-840, 2006.
15. Bray JD, Jelinsky S, Ghatge R, *et al*: Quantitative analysis of gene regulation by seven clinically relevant progestins suggests a highly similar mechanism of action through progesterone receptors in T47D breast cancer cells. *J Steroid Biochem Mol Biol* 97: 328-341, 2005.
16. Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM and Horwitz KB: Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J Biol Chem* 277: 5209-5218, 2002.
17. Wan Y and Nordeen SK: Overlapping but distinct gene regulation profiles by glucocorticoids and progestins in human breast cancer cells. *Mol Endocrinol* 16: 1204-1214, 2002.
18. Kueng W, Silber E and Eppenberger U: Quantification of cells cultured on 96-well plates. *Anal Biochem* 182: 16-19, 1989.
19. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.
20. Ahola TM, Purmonen S, Pennanen P, Zhuang YH, Tuohimaa P and Ylikomi T: Progestin upregulates G-protein-coupled receptor 30 in breast cancer cells. *Eur J Biochem* 269: 2485-2490, 2002.
21. Purmonen S, Ahola TM, Pennanen P, *et al*: HDLG5/KIAA0583, encoding a MAGUK-family protein, is a primary progesterone target gene in breast cancer cells. *Int J Cancer* 102: 1-6, 2002.
22. Crambert G, Li C, Claeys D and Geering K: FXyD3 (Mat-8), a New Regulator of Na,K-ATPase. *Mol Biol Cell* 16: 2363-2371, 2005.
23. Morrison BW, Moorman JR, Kowdley GC, Kobayashi YM, Jones LR and Leder P: Mat-8, a novel phospholemman-like protein expressed in human breast tumors, induces a chloride conductance in *Xenopus oocytes*. *J Biol Chem* 270: 2176-2182, 1995.
24. Morrison BW and Leder P: Neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors. *Oncogene* 9: 3417-3426, 1994.
25. Grzmil M, Voigt S, Thelen P, Hemmerlein B, Helmke K and Burfeind P: Up-regulated expression of the MAT-8 gene in prostate cancer and its siRNA-mediated inhibition of expression induces a decrease in proliferation of human prostate carcinoma cells. *Int J Oncol* 24: 97-105, 2004.
26. Braga VM, Pemberton LF, Duhig T and Gendler SJ: Spatial and temporal expression of an epithelial mucin, Muc-1, during mouse development. *Development* 115: 427-437, 1992.
27. Gendler SJ: MUC1, the renaissance molecule. *J Mammary Gland Biol Neoplasia* 6: 339-353, 2001.
28. Makiguchi Y, Hinoda Y and Imai K: Effect of MUC1 mucin, an anti-adhesion molecule, on tumor cell growth. *Jpn J Cancer Res* 87: 505-511, 1996.
29. Evangelou A, Letarte M, Marks A and Brown TJ: Androgen modulation of adhesion and antiadhesion molecules in PC-3 prostate cancer cells expressing androgen receptor. *Endocrinology* 143: 3897-3904, 2002.
30. Gollub EG, Waksman H, Goswami S and Marom Z: Mucin genes are regulated by estrogen and dexamethasone. *Biochem Biophys Res Commun* 217: 1006-1014, 1995.
31. McGuckin MA, Quin RJ and Ward BG: Progesterone stimulates production and secretion of MUC1 epithelial mucin in steroid-responsive breast cancer cell lines. *Int J Oncol* 12: 939-945, 1998.
32. Hey NA, Graham RA, Seif MW and Aplin JD: The polymorphic epithelial mucin MUC1 in human endometrium is regulated with maximal expression in the implantation phase. *J Clin Endocrinol Metab* 78: 337-342, 1994.
33. Braga VM and Gendler SJ: Modulation of Muc-1 mucin expression in the mouse uterus during the estrus cycle, early pregnancy and placentation. *J Cell Sci* 105: 397-405, 1993.
34. Hilkens J, Vos HL, Wesseling J, *et al*: Is episialin/MUC1 involved in breast cancer progression? *Cancer Lett* 90: 27-33, 1995.
35. Zaretsky JZ, Barnea I, Aylon Y, Gorivodsky M, Wreschner DH and Keydar I: MUC1 gene overexpressed in breast cancer: structure and transcriptional activity of the MUC1 promoter and role of estrogen receptor alpha (ERalpha) in regulation of the MUC1 gene expression. *Mol Cancer* 5: 57, 2006.
36. Webster MK, Goya L, Ge Y, Maiyar AC and Firestone GL: Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol Cell Biol* 13: 2031-2040, 1993.
37. Buse P, Tran SH, Luther E, Phu PT, Aponte GW and Firestone GL: Cell cycle and hormonal control of nuclear-cytoplasmic localization of the serum- and glucocorticoid-inducible protein kinase, Sgk, in mammary tumor cells. A novel convergence point of anti-proliferative and proliferative cell signaling pathways. *J Biol Chem* 274: 7253-7263, 1999.
38. Goya L, Maiyar AC, Ge Y and Firestone GL: Glucocorticoids induce a G1/G0 cell cycle arrest of Con8 rat mammary tumor cells that is synchronously reversed by steroid withdrawal or addition of transforming growth factor-alpha. *Mol Endocrinol* 7: 1121-1132, 1993.
39. Firestone GL, Giampaolo JR and O'Keeffe BA: Stimulus-dependent regulation of serum and glucocorticoid inducible protein kinase (SGK) transcription, subcellular localization and enzymatic activity. *Cell Physiol Biochem* 13: 1-12, 2003.
40. Bertucci F, Houlgatte R, Benziane A, *et al*: Gene expression profiling of primary breast carcinomas using arrays of candidate genes. *Hum Mol Genet* 9: 2981-2991, 2000.
41. Oh IH and Reddy EP: The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* 18: 3017-3033, 1999.
42. Patel G, Kreider B, Rovera G and Reddy EP: v-Myb blocks granulocyte colony-stimulating factor-induced myeloid cell differentiation but not proliferation. *Mol Cell Biol* 13: 2269-2276, 1993.
43. Gewirtz AM and Calabretta B: A c-myc antisense oligodeoxynucleotide inhibits normal human hematopoiesis *in vitro*. *Science* 242: 1303-1306, 1988.
44. Kauraniemi P, Hedenfalk I, Persson K, *et al*: MYB oncogene amplification in hereditary BRCA1 breast cancer. *Cancer Res* 60: 5323-5328, 2000.
45. Guerin M, Sheng ZM, Andrieu N and Riou G: Strong association between c-myb and oestrogen-receptor expression in human breast cancer. *Oncogene* 5: 131-135, 1990.
46. Hodges LC, Cook JD, Lobenhofer EK, *et al*: Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. *Mol Cancer Res* 1: 300-311, 2003.
47. King JA, Ofori-Acquah SF, Stevens T, Al-Mehdi AB, Fodstad O and Jiang WG: Activated leukocyte cell adhesion molecule in breast cancer: prognostic indicator. *Breast Cancer Res* 6: R478-R487, 2004.
48. Kester HA, van der Leede BM, van der Saag PT and van der Burg B: 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, 1997.
49. Wan Y and Nordeen SK: Identification of genes differentially regulated by glucocorticoids and progestins using a Cre/loxP-mediated retroviral promoter-trapping strategy. *J Mol Endocrinol* 28: 177-192, 2002.