

## Gene regulation profile reveals consistent anticancer properties of progesterone in Hormone-independent breast cancer cells transfected with progesterone receptor

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**Absence of estrogen receptor (ER) and progesterone receptor (PR) is the hallmark of most hormone-independent breast cancers. Previous studies demonstrated that reactivation of PR expression in hormone-independent MDA-MB-231 breast cancer cells enabled progesterone to suppress cell growth both *in vitro* and *in vivo*. We determined the whole genomic effect of progesterone in PR-transfected MDA-MB-231 cells. We identified 151 progesterone-regulated genes with expression changes >3-fold after 24 hr treatment. Most are novel progesterone target genes. Real-time RT-PCR analysis of 55 genes showed a 100% confirmation rate. Twenty-six genes were regulated at both 3 and 24 hr. Studies using translation inhibitor suggest that most of the 26 genes are primary progesterone target genes. Progesterone consistently suppressed the expression of genes required for cell proliferation and metastasis and increased the expression of many tumor-suppressor genes. Progesterone also consistently decreased the expression of DNA repair and chromosome maintenance genes, which may be part of the mechanism leading to cell cycle arrest. These data suggest potential usefulness of progestin in combating ER-negative but PR-positive breast cancer and indicate that progesterone can exert a strong anticancer effect in hormone-independent breast cancer following PR reactivation. The identification of many novel progesterone target genes open up new avenues for in-depth elucidation of progesterone-mediated molecular networks.**

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**Key words:** progesterone; gene regulation; breast cancer

Estrogen is a known mitogen for breast cancer. Antiestrogenic therapy is the frontline therapy for hormone-dependent breast cancers that express estrogen receptors (ERs) and progesterone receptors (PRs).<sup>1,2</sup> However, more than one-third of all breast cancer cases are hormone-independent. Absence of ER and PR is the hallmark of most hormone-independent breast cancers.<sup>3</sup> These breast cancers generally exhibit aggressive biologic behavior and poor prognosis, and most fail hormonal therapy. There is currently no specific therapy for hormone-independent breast cancers.

Our previous studies have shown that reactivation of PR expression in ER- and PR-negative MDA-MB-231 breast cancer cells enabled progesterone to strongly inhibit cell growth both *in vivo* and *in vitro*.<sup>4,5</sup> These findings suggest a possible benefit of PR gene therapy for hormone-independent breast cancers. These findings are of realistic significance in light of reports that expression of ER and PR in hormone-independent breast cancer cells can also be reactivated epigenetically by DNA methyltransferase inhibitors and histone deacetylase (HDAC) inhibitors.<sup>6,7</sup>

Although progesterone is strongly growth-inhibitory in PR-transfected hormone-independent breast cancer cells, the exact function of progesterone in the development of breast cancer in general remains unclear. Apart from their reported growth-inhibitory effects,<sup>8,9</sup> progestins stimulate the growth of breast cancer cells,<sup>10,11</sup> depending on the experimental conditions. For PR gene therapy or epigenetic activation of PR gene expression to have practical significance, it is important to determine that progesterone does not have significant adverse effects at the whole-cell level in hormone-independent breast cancer cells receiving PR gene therapy. Since PRs are transcription factors, their molecular

effects are mainly determined by progesterone-regulated gene expression.<sup>12</sup> Our primary objective was to determine the whole-genome effect of progesterone in PR-transfected MDA-MB-231 cells. The progesterone-regulated gene expression profile revealed a strong antiproliferative and antineoplastic function of progesterone in PR-transfected MDA-MB-231 cells, ABC28. This suggests a genomewide benefit of progesterone treatment following PR reactivation in ER- and PR-negative breast cancers.

The inconsistency in reported effects of progestins reflects the complexities in the mechanisms of progesterone action. Although PR is regulated by a number of hormones and growth factors,<sup>13,14</sup> they are normally estrogen receptor-dependent gene products. The action of progesterone requires priming treatment of estrogen to induce PR. It is conceivable that the prior presence of estrogen may significantly confound the assessment of progesterone's effects on growth and other cellular processes in breast cancer cells. Furthermore, there is widely recognized cross-talk between ER and PR signaling pathways, and the nature of the cross-talk may also modify the cellular response to progesterone.<sup>15–17</sup> Thus, our gene expression study using a PR-positive but ER-negative breast cancer cell model allowed identification of potential PR target genes that may otherwise be masked in ER- and PR-positive cells in which PR expression is estrogen-dependent. Indeed, more than two-thirds of the progesterone-regulated genes identified in our study have not been reported as progesterone target genes previously, revealing novel functions of progesterone. Identification of novel progesterone target genes opens up new avenues for in-depth elucidation of progesterone-mediated molecular networks.

### Material and methods

#### Cell lines

MDA-MB-231 cells were obtained from the ATCC (Manassas, VA) in 1995 at passage 28. MDA-MB-231 cells were cloned using 96-well plates by single-cell dilution. Clone 2 (known as MDA-MB-231-CL2) was selected for transfection studies. MDA-MB-231-CL2 cells were transfected with PR expression vectors hPR1 and hPR2, which were generous gifts from Dr. P. Chambon (Institute of Genetics and Molecular and Cellular Biology, Strasbourg, France).<sup>18</sup> Isolation and characterization of the PR-transfected clone ABC28 cells used in the present study have been described in detail previously.<sup>4</sup> ABC28 cells expressed approximately 660 fmol PR/mg protein as determined by enzyme immunoassay (Abbott, Abbott Park, IL). Western blotting analysis showed that ABC28 cells express slightly more PR-B than PR-A using

Grant sponsor: National Medical Research Council of Singapore; Grant sponsor: Ministry of Education of Singapore.

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Received 3 August 2004; Accepted after revision 3 March 2005

DOI 10.1002/ijc.21186

Published online 8 June 2005 in Wiley InterScience (www.interscience.wiley.com).

antibody Ab-8, which recognizes both PR-A and PR-B (Neomarkers, Fremont, CA). Vector-transfected CTC15 cells expressed no PR and were used as a control cell line.

#### Cell culture

All cells were routinely maintained in phenol red-containing DMEM supplemented with 7.5% FCS, 2 mM glutamine and 40 mg/l gentamycin. All experiments with cells were conducted using phenol red-free DMEM supplemented with 2 mM L-glutamine, 40 mg/l gentamycin and 5% dextran-coated charcoal-treated FCS (test medium). FCS was treated with dextran-coated charcoal to remove the endogenous steroid hormones that may complicate the effects of progesterone.

#### Chemicals and reagents

Progesterone was obtained from Sigma (St. Louis, MO). All tissue culture plastics and reagents and most molecular biology reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). Real-time PCR reagents and consumables were from Applied Biosystems (Foster City, CA). Antibody for XRCC3 was a rabbit polyclonal antibody against the N terminus of the protein (Lab Vision, Fremont, CA). Antibody for MCM5 was a mouse monoclonal antibody (clone CRCT5-1, Lab Vision). PR antibody Ab-8, which recognizes both PR-A and PR-B, was from Neomarkers. Antibody to  $\beta$ -actin was also from Neomarkers, and antibody to GAPDH was from Ambion (Austin, TX).

#### Western blotting analysis

Cells ( $1 \times 10^6$ ) were grown on 100 mm Petri dishes in test medium for 48 hr before they were treated with 0.1% ethanol or 0.1  $\mu$ M progesterone for various lengths of time. Treated cells were lysed with 200  $\mu$ l cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5  $\mu$ g/ml pepstatin A, 5  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin, 1 mM PMSF, 100 mM sodium fluoride and 1 mM sodium vanadate, pH 7.5) and left standing on ice for 20 min. Protein supernatants were then collected by centrifugation at 17,950g for 20 min. Protein (20  $\mu$ g) was analyzed by Western blotting with specific antibody against the protein of interest.

#### RNA preparation

To collect RNA for microarray analysis, cells were grown in test medium for 48 hr before they were treated with 0.1  $\mu$ M progesterone from 1,000-fold stock in ethanol. This gave a final concentration of ethanol of 0.1%. Treatment controls received 0.1% ethanol only. Total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD). A second cleanup of total RNA was performed using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). Expression analysis was conducted with total RNA collected from 2 independent experiments conducted 1 month apart to account for variations between experiments.

To determine the primary target genes of progesterone by real-time RT-PCR analysis, ABC28 cells were treated with 10  $\mu$ g/ml cycloheximide (Sigma Aldrich, St Louis, MO) for 30 min before they were treated with control vehicle or 0.1  $\mu$ M progesterone for 5 hr. RNA was then extracted using Trizol reagent (Life Technologies) and reverse-transcribed to cDNA as described below (see Quantitative RT-PCR).

#### Probe labeling and hybridization of Affymetrix GeneChip

The labeling and hybridization procedures were conducted according to the *GeneChip Expression Analysis Technical Manual* (Affymetrix, Santa Clara, CA). Briefly, first-strand cDNA were synthesized from 5  $\mu$ g total RNA using SuperScript II reverse transcriptase (Invitrogen) and T7-oligo(dT) primer. Second-strand cDNA were synthesized using *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I and T4 DNA polymerase. The dsDNA was used as template to generate biotin-labeled cRNA using the Enzo BioArray High Yield Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY). Unincorporated nucleotides were

removed using the RNeasy Mini kit (Qiagen). Labeled cRNA (15  $\mu$ g) was fragmented and added to a hybridization cocktail along with control oligonucleotide B2 and biotinylated control (BioB, BioC, BioD, Cre). An aliquot of each hybridization mixture was first hybridized to an Affymetrix Test 2 Array to determine that the sample quality met the manufacturer's criteria. The hybridization mixture with 10  $\mu$ g labeled cRNA for each sample was then hybridized to GeneChip Human Genome U133A (HG-U133A) arrays. The arrays were stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and scanned using a Hewlett-Packard (Palo Alto, CA) GeneArray Scanner.

#### Data analysis

Scanned images were analyzed using Affymetrix Microarray Suite, version 4.0, and Affymetrix Data Mining Tool, version 3.0, software. Detailed protocols for data analysis of Affymetrix oligonucleotide microarray with extensive documentation of the sensitivity and quantitative aspects of the method have been described.<sup>19</sup> It has been established that a change of the gene expression level by >2-fold using oligonucleotide array is significant and reliable. In this study, progesterone-regulated genes were identified as those with expression changes >3-fold after 24 hr of progesterone treatment in both experiments. Since progesterone-induced expression changes at early time points are generally small, the cut-off point of 2-fold was used to selected progesterone-regulated genes after 3 hr progesterone treatment.

#### Quantitative RT-PCR

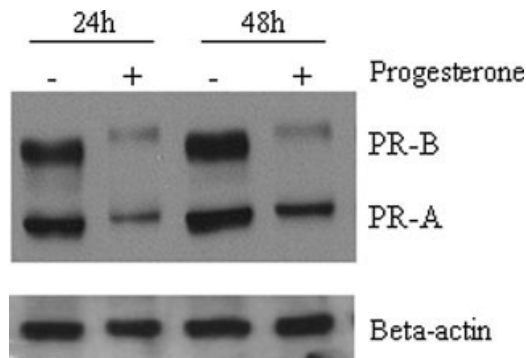
cDNA was synthesized from 5  $\mu$ g total RNA using random primer and SuperScript II reverse transcriptase. Real-time PCR was performed using SYBR green PCR reagents on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the Hot Start AmpliTaq Gold DNA polymerase. PCR product lengths ranged 180–250 bp. PCR for each gene fragment was performed in triplicate, and each primer set was repeated 2 or 3 times. Melting curves were generated after amplification to check PCR specificity. Amplicon size and reaction specificity were further confirmed by electrophoresis on a 1% agarose gel, where a single PCR product of the expected size should be observed. The changes in fluorescence of the SYBR green I dye in each cycle were monitored by the ABI 7700 system, and the threshold cycle (Ct), which is defined as the cycle number at which the amount of amplified target reaches a fixed threshold, was obtained for each gene. The relative amount of PCR product generated from each primer set was determined on the basis of the Ct value. Primer sets for the *36B4* gene, which codes for human acidic ribosomal phosphoprotein PO, were included in each experiment as controls for normalizing the quantity of cDNA used. More than 200 assays were conducted using primers for *36B4*. The average intraassay coefficient of variation for the triplicate Ct values of *36B4* was 1.36%. The average interassay variation of the 2 or 3 assays for the 55 genes was 16.1%.

The expression difference for each gene between control and progesterone-treated samples was calculated by normalizing with *36B4* gene expression according to the following formula: Fold change =  $2^{[Ct(\text{control gene } X) - Ct(\text{control } 36B4)] - [Ct(\text{progesterone gene } X) - Ct(\text{progesterone } 36B4)]}$ .

#### Statistical analysis

Correlation between expression fold changes obtained by microarray analysis and by quantitative real-time RT-PCR analysis was tested by regression analysis. The intraassay variation of quantitative real-time RT-PCR was determined for triplicate Ct values of *36B4* in 24 randomly selected assays. The average interassay variation was based on expression fold changes in 2 or 3 determinations for all 73 genes analyzed by quantitative real-time RT-PCR.

To make a statistical inference about the validation rate by real-time RT-PCR for genes of 2-fold expression change obtained by microarray analysis, a confidence interval was calculated accord-



**FIGURE 1** – Expression of PR protein in PR-transfected MDA-MB-231 cells ABC28. Cells were treated with control vehicle (–) or 0.1 μM progesterone (+) for 24 and 48 hr before whole-cell lysates were collected. Total protein (20 μg) was analyzed by Western blotting using specific PR antibody, which recognized both PR-A (90 kDa) and PR-B (120 kDa). β-Actin antibody was used as a loading control.

ing to the following formula: where  $Sp = \sqrt{pq/n}$ ,  $p$  = percentage of genes validated,  $q = 1 - p$ ,  $\mu_{0.05} = 1.96$ ,  $n$  = total population size (number of 2-fold genes identified in microarray study).

## Results and discussion

ABC28 cells are MDA-MB-231 cells transfected with PR cDNA coding for PR isoform A (PR-A) and PR-B. Figure 1 shows that ABC28 cells express slightly more PR-B than PR-A. Treatment with 0.1 μM progesterone markedly reduced the level of PR-B and, to a much lesser extent, the level of PR-A. There were also upshifts of both PR-A and PR-B bands, suggesting that both receptor isoforms are phosphorylated.<sup>20</sup> ABC28 cells were studied for progesterone-regulated gene expression using Affymetrix GeneChip HG-U133A, which contains oligo probes for 14,593 genes.

### Progesterone-regulated genes in ABC28 cells and verification of microarray results by real-time RT-PCR analysis

RNA samples extracted from ABC28 cells in 2 independent experiments were analyzed for progesterone-regulated gene expression after 24 hr treatment. An average of 50% ( $\pm 0.78$ ) of the gene probes on the microarray chips detected the presence of RNA transcripts in the samples. In both experiments, 151 genes were identified with expression changes  $>3$ -fold after 24 hr of progesterone treatment. Of the 151 genes, 68 were upregulated and 83 were downregulated (Table I).

Of the 151 progesterone-regulated genes, 55 were verified for gene expression by quantitative real-time RT-PCR. The analysis confirmed that the expression changes of all 55 genes (shown in bold in Table I) after 24 hr progesterone treatment were  $>3$ -fold. Regression analysis of fold changes showed that the RT-PCR result was significantly ( $p = 2.08E-11$ ) correlated with the microarray result, with a correlation coefficient of 0.77. The 100% validation rate for 55 genes gave us solid ground to infer that the 151 genes identified in the microarray analysis can be verified by real-time RT-PCR analysis.

Our microarray analysis revealed a total of 387 genes with expression changes  $>2$ -fold after 24 hr progesterone treatment (data not shown). RT-PCR analysis confirmed the microarray results in 70 of the 71 genes analyzed (98.6%). The confidence interval for all the 387 genes to be verifiable was  $0.974 \leq p \leq 0.998$ . Hence, we are 95% confident that 97.4–99.8% of the genes with 2-fold change can be validated by real-time RT-PCR. This is the basis for the discussion of the cell proliferation genes with  $>2$ -fold expression changes below.

### Identification of primary progesterone target genes

The duration of 24 hr treatment allows effective identification of downstream target genes in the progesterone-mediated molecular pathway. With the exception of immediate early genes, the expression changes of most genes at early time points are generally of small magnitude. However, genes identified at early time points are more likely to be primary progesterone target genes. For this reason, microarray analysis of progesterone-regulated gene expression after 3 hr treatment was also conducted. Forty-six genes were identified with expression changes  $>2$ -fold. Of the 46 genes, 26 were among those identified after 24 hr progesterone treatment (Table II).

To test if the 26 genes are primary target genes for progesterone, 14 were randomly selected to determine if their regulation by progesterone requires *de novo* protein synthesis. Gene expression was analyzed by quantitative real-time RT-PCR. Table III shows that all 14 genes were progesterone-regulated for  $>2$ -fold after 5 hr of progesterone treatment. Progesterone-induced fold changes in 13 of the 14 genes were  $>2$ -fold in the presence of the translation inhibitor cycloheximide, suggesting that they are primary target genes of progesterone. Based on these data, we speculate that most of the 26 genes in Table II are primary target genes of progesterone.

Vector-transfected CTC15 control cells were also tested for progesterone-regulated gene expression using the genes listed in Table III. Progesterone had no effect on the expression of these genes in control cells. This is in accordance with our previous reports<sup>4,21</sup> that progesterone inhibited cell proliferation and induced focal adhesion in ABC28 cells but had no effect in CTC15 control cells.

### Most of the genes identified are novel progesterone target genes

Twenty-six genes were regulated by progesterone by  $>2$ -fold after both 3 and 24 hr treatments (Table II). Thirteen of the 26 genes in Table II are not in Table I, which only includes genes with expression change  $>3$ -fold. The total number of genes in Tables I and II is, therefore, 164. An extensive search of the literature suggests that 10 of the 26 genes in Table II (shown in bold) and 26 of the 151 genes in Table I (shown in bold) were reported to be progesterone-regulated genes.<sup>22–45</sup> Therefore, our study revealed 133 putative novel progesterone target genes, a majority of which are of known function. These genes may not necessarily be regulated by progesterone in a given breast cancer cell line, such as MCF-7 or T47D. However, they are potential progesterone target genes and may be regulated under various physiologic or pathologic conditions and during different developmental stages. The progesterone-regulated genes may serve as markers for studying progesterone-mediated molecular networks under different experimental paradigms.

Progesterone is known to induce varied cellular responses in different breast cancer cell lines with different hormone receptor status. In ER- and PR-positive MCF-7 cells with estrogen-dependent PR expression, the effect of progesterone on cell proliferation is marginal.<sup>9,10</sup> The ER-positive T47D cell line and its derivatives, T47D-YA and T47D-YB, express estrogen-independent PR. The effect of progesterone on these cells is biphasic; *i.e.*, progesterone causes growth stimulation during the first 24–48 hr of treatment, followed by inhibition.<sup>9,46</sup> The PR-positive but ER-negative ABC28 cells used in our study respond to progesterone with marked growth inhibition and focal adhesion.<sup>4,21</sup> It is interesting to compare how the similarities and differences in cellular response to progesterone are reflected in progesterone-regulated gene expression among the cell lines. There have been 3 reports describing microarray analysis of progesterone-regulated gene expression in the T47D cell line and its sublines.<sup>30–32</sup> All studies were done 6 hr after progesterone treatment. It is expected that the gene regulation profile of progesterone in T47D cells would be different from that in ABC28 cells as progesterone stimulated the proliferation of T47D cells during the first cell cycle when the

TABLE 1 – PROGESTERONE-REGULATED GENE EXPRESSION WITH &gt;3-FOLD CHANGES AFTER 24 HR OF TREATMENT

Public ID number	Gene symbol	Mean fold change at 24h		Public ID number	Gene symbol	Mean fold change at 24h	
		MA	RT-PCR			MA	RT-PCR
CELL CYCLE REGULATORS				CELL ADHESION AND MOTILITY			
DNA replication				BE251211	LOXL2	-14.2	
NM_018248	FLJ10858	-3.8		NM_001195	BFSP1	-7.6	-7
NM_003686	EXO1	-3.7		NM_002275	KRT15	-6.8	
<b>NM_000234</b>	<b>L1G1</b>	<b>-3.3</b>		<b>NM_000094</b>	<b>COL7A1</b>	<b>-6.1</b>	
NM_001067	TOP2A	-3		AL356504	FLG	-5.9	
Cell cycle				<b>NM_002658</b>	<b>PLAU</b>	<b>-5.4</b>	<b>-10.6</b>
AL031680	PARD6B	-12.6	-6.3	<b>NM_002421</b>	<b>MMP1</b>	<b>-4.2</b>	<b>-4.9</b>
NM_016343	CENPF	-4		NM_000362	TIMP3	-4	-5.1
NM_018365	MNS1	-3.4		NM_005965	MYLK	-3.9	
<b>NM_001262</b>	<b>CDKN2C</b>	<b>-3.3</b>		NM_001109	ADAM8	-3.9	-22.3
NM_004523	KIF11	-3.3	-11.3	AF031824	CST7	-3.8	
<b>NM_004702</b>	<b>CCNE2</b>	<b>-3.1</b>	<b>-4.2</b>	Z54367	PLEC1	-3.7	-6.1
AL524035	<b>CDC2</b>	<b>-3</b>		<b>AK023795</b>	<b>ADAMTS1</b>	<b>-3.3</b>	
AL567411	CDK5R1	3.3		NM_005573	LMNB1	-3.2	
Cancer-related protein				AF154005	FIIR	3.4	13.8
BC000069	RARRES2	-4.0		<b>BC000915</b>	<b>PDLIM1</b>	<b>3.5</b>	
NM_006101	KNTC2	-3.1	-4.6	BE877796	COL8A1	4.3	
NM_006997	TACC2	2.9		NM_019114	EPB41L4B	4.3	
BC005047	DUSP6	3.2		AF153882	PDLIM4	4.8	
NM_005418	ST5	3.3		NM_002705	PPL	5	5.3
NM_004833	AIM2	3.5		NM_004572	PKP2	5.2	14
NM_004417	DUSP1	7.2	22	NM_002345	LUM	5.4	11.3
DNA repair				NM_003710	SPINT1	6	4.7
AK022829	XRCC3	-5.2	-3.9	AA761181	CD24	8	
NM_003579	RAD54L	-4.5	-8.1	J03223	PRG1	18.9	39.8
NM_006739	MCM5	-3.7	-4.1	GENES INVOLVED IN METABOLISM			
NM_000107	DDB2	-3.6	-7.2	Carbohydrate and lipid metabolism			
BE966146	RAD51AP1	-3.5	-6.8	NM_000691	ALDH3A1	-3.5	
NM_022346	HCAP-G	-3.5	-6.8	AB046692	AOX1	3	
NM_004526	MCM2	-3.1	-3.8	NM_003105	SORL1	3.4	
TRANSCRIPTION REGULATORS				<b>NM_003033</b>	<b>SIAT4A</b>	<b>4.4</b>	<b>4.6</b>
NM_012153	EHF	-10.9	-8.6	BC000474	TP5313	5.4	
<b>NM_002146</b>	<b>HOXB4</b>	<b>-7.8</b>		NM_003570	CMAH	5.8	
NM_012087	GTF3C5	-7.6		<b>NM_000196</b>	<b>HSD11B2</b>	<b>6.1</b>	<b>9.3</b>
AF176039	HMGA1	-4.9		Nucleotide metabolism			
BG251266	FOSL1	-3.4		BC001051	ARL7	-4.3	-7.1
NM_005978	S100A2	-3.2		BC000879	KYNU	-4.3	-6.4
NM_012081	<b>ELL2</b>	<b>3.3</b>	<b>5.4</b>	NM_001034	RRM2	-3.9	
NM_012429	SEC14L2	4.6	4.7	NM_002526	NT5E	-3.4	
CARRIER PROTEINS				NM_004121	GGTLA1	8.4	
NM_016354	SLC04A1	-4.8		Protein metabolism			
AF098951	ABCG2	4.1	4.1	NM_024642	GALNT12	-3.1	-6
R06655	MT1K	4.7		<b>NM_004199</b>	<b>P4HA2</b>	<b>3.2</b>	
NM_003982	SLC7A7	6.5		NM_000050	ASS	3.3	
GENES RELATED TO NEURAL FUNCTION				AA584297	LRP4	3.7	
NM_002518	NPAS2	-7.7		AA923354	MAOA	4.2	
AC004010	AMIGO2	-6	-12.5	<b>NM_000031</b>	<b>ALAD</b>	<b>5.1</b>	
NM_003020	SGNE1	-6		<b>NM_000963</b>	<b>PTGS2</b>	<b>8.2</b>	<b>8.9</b>
NM_014903	NAV3	-3.6		HORMONES AND RECEPTORS/MEMBRANE PROTEINS			
<b>NM_002506</b>	<b>NGFB</b>	<b>-3.2</b>	<b>-8.5</b>	NM_014210	EV12A	-8.2	
SIGNAL TRANSDUCERS				NM_002261	KLRC3	-5.6	
D30751	BMP4	-23.9	-10.2	BC003179	BENE	-4.8	
AL514445	RGS4	-6.7		<b>NM_005544</b>	<b>IRS1</b>	<b>-4.4</b>	<b>-6.7</b>
<b>U63917</b>	<b>GPR30</b>	<b>-5.7</b>		NM_001423	EMP1	-3.6	-5.6
NM_014264	PLK4	-3.6		NM_013390	TMEM2	3.1	
U77917	PTPRR	-3.4		NM_002820	PTHLH	3.5	
AB011446	AURKB	-3.3	-5.3	AI537887	STOM	3.7	
<b>NM_003897</b>	<b>IER3</b>	<b>-3.3</b>	<b>-6.8</b>	<b>NM_001124</b>	<b>ADM</b>	<b>3.9</b>	<b>6.3</b>
AL137654	BUB1	-3.2		<b>A1610869</b>	<b>MUC1</b>	<b>5.3</b>	<b>18.1</b>
NM_003151	STAT4	3.2	7.3	AB022177	CALCR	5.4	
NM_016594	FKBP11	3.4	4.9	NM_000916	OXTR	6	
NM_004657	SDPR	3.6		NM_003862	FGF18	6.6	
NM_003407	ZFP36	3.6	5	NM_001400	EDG1	7.1	39.9
AF338650	PDZK3	3.7		CYTOKINES AND CHEMOKINES			
NM_002220	ITPKA	3.7		NM_021805	SIGIRR	-9.3	
<b>AV655640</b>	<b>CEBPD</b>	<b>4</b>		<b>NM_000584</b>	<b>IL8</b>	<b>-6.5</b>	<b>-26.2</b>
<b>NM_014737</b>	<b>RASSF2</b>	<b>4.8</b>		AF015524	CCRL2	-3.3	
NM_014214	IMPA2	4.9		NM_000313	PROS1	5.1	11.3
BC004399	Clorf34	5.9					
<b>NM_004117</b>	<b>FKBP5</b>	<b>12.6</b>	<b>9.2</b>				

TABLE I – PROGESTERONE-REGULATED GENE EXPRESSION WITH &gt;3-FOLD CHANGES AFTER 24 HR OF TREATMENT (CONTINUED)

TABLE 1. PROGESTERONE-REGULATED GENE EXPRESSION WITH ≥5-FOLD CHANGES AFTER 24 HR OF TREATMENT (CONTINUED)							
Public ID number	Gene symbol	Mean fold change at 24h		Public ID number	Gene symbol	Mean fold change at 24h	
		MA	RT-PCR			MA	RT-PCR
GENES OF UNKNOWN FUNCTION							
NM_022770	FLJ13912	−6		NM_014705	DOCK4	−3	
BF062629	RIS1	−4		AW575493	NS3TP2	3.1	
NM_022842	CDCP1	−3.8		NM_017935	BANK1	3.4	
NM_018410	DKFZp762E13	−3.7		AK023042	LASS6	3.5	
BG403615	FLJ10719	−3.7		AA631242	RAB15	3.7	
NM_016448	RAMP	−3.6		NM_018212	ENAH	3.9	
AF225416	SPC25	−3.3		NM_017791	C14orf58	4	
NM_014783	ARHGAP11A	−3.3		NM_014181	HSPC159	4.1	
NM_024629	MLFIIP	−3.2		AK026720	LOC283537	4.3	6
AF070641	LOC221810	−3.2		AL117523	SAMD4	4.5	3.7
AW205215	KIAA0286	−3.1		AI992251	RPS6KA2	4.7	26.3
NM_018186	FLJ10706	−3.1		NM_024633	C14orf139	6.5	12.8
NM_018154	ASF1B	−3		NM_024119	LGP2	7.1	4.6
NM_018136	ASPM	−3					

Results are the average fold changes of 2 independent experiments of microarray analysis (MA) and real-time RT-PCR analysis. Genes in bold are previously reported progesterone-regulated genes.

TABLE II – PROGESTERONE-REGULATED GENES OF &gt;2-FOLD CHANGES AT BOTH 3 AND 24 HR

Gene symbol	Mean fold change	
	MA (3 hr)	MA (24 hr)
<i>BMP4</i>	−12.6	−23.9
<b><i>IL8</i></b>	<b>−6.6</b>	<b>−6.5</b>
<b><i>ADAMTS1</i></b>	<b>−5.9</b>	<b>−3.3</b>
<b><i>CSF2</i></b>	<b>−5.8</b>	<b>−2.3</b>
<b><i>IER3</i></b>	<b>−5.7</b>	<b>−3.3</b>
<i>PARD6B</i>	−4.6	−12.6
<i>PLK2</i>	−3.7	−2.2
<b><i>NGFB</i></b>	<b>−3.0</b>	<b>−3.2</b>
<b><i>SOX9</i></b>	<b>−3.0</b>	<b>−2.8</b>
<b><i>TSC22</i></b>	<b>−3.0</b>	<b>−2.0</b>
<i>FOSL1</i>	−2.9	−3.4
<i>PHLDA1</i>	−2.9	−2.4
<b><i>IRS1</i></b>	<b>−2.7</b>	<b>−4.4</b>
<i>AMIGO2</i>	−2.4	−6.0
<i>NAV3</i>	−2.3	−3.6
<i>DAAM1</i>	−2.2	−2.4
<i>ARL7</i>	−2.1	−4.3
<i>BAG2</i>	2.2	2.1
<b><i>STEAP</i></b>	<b>2.3</b>	<b>2.8</b>
<i>SIAT10</i>	2.5	2.8
<i>TACC2</i>	2.9	2.9
<i>C18orf11</i>	2.9	2.7
<i>DUSP6</i>	3.0	3.2
<i>SMPDL3A</i>	3.0	2.5
<i>ZFP36</i>	3.4	3.6
<b><i>FOXC1</i></b>	<b>4.5</b>	<b>2.3</b>

Results are the average fold changes of 2 independent experiments of microarray analysis (MA). Genes in bold are reported progesterone-regulated genes.

gene expression study was conducted, whereas it consistently inhibited the growth of ABC28 cells throughout its treatment period. Nonetheless, 11 genes (*IER3*, *CEBPD*, *RASSF2*, *FKBP5*, *PDLIM1*, *LIG1*, *SIAT4A*, *HSD11B2*, *ELL2*, *STEAP* and *FOXC1*) were shown to be progesterone-regulated in both T47D cells and ABC28 cells.<sup>30–32</sup> This suggests some common genomic effect of progesterone in the 2 cell models despite its different effects on cell proliferation. Two of the 11 genes (*LIG1*, *IER3*) were downregulated in this study but upregulated by progesterone in T47D cells. Interestingly these 2 genes are involved in DNA replication and cell proliferation.<sup>47–49</sup> Differences in progesterone effect on cell growth and gene regulation between ABC28 and T47D family cell lines may be a reflection of their hormone receptor status, as outlined earlier in this paragraph. There is accumulating evidence to suggest that the function of progesterone is governed by complex cross-talk between ER $\alpha$  and PR.<sup>15–17</sup> For example, the pres-

TABLE III – PROGESTERONE REGULATION DOES NOT REQUIRE *DE NOVO* PROTEIN SYNTHESIS IN 13 OF THE 14 GENES TESTED

Gene symbol	RT-PCR fold change (CTC15)	Mean RT-PCR fold change (ABC28)	
	Progesterone	Progesterone	Progesterone+CHX
<i>CSF2</i>	−1.171	−11.3	−6.8
<i>IER3</i>	−1.089	−9.2	−7.4
<i>BMP4</i>	1.115	−9.2	−3.2
<i>NGFB</i>	1.047	−8.8	−8.6
<i>FOSL1</i>	1.045	−8.1	−8.2
<i>AMIGO2</i>	1.023	−7.2	−3.2
<i>PHLDA1</i>	1.294	−5.7	−4.0
<i>NAV3</i>	−1.387	−5.7	−2.5
<i>IRS1</i>	1.341	−5.6	−2.6
<i>ARL7</i>	−1.111	−4.8	−5.9
<i>BAG2</i>	1.047	2.7	2.2
<i>ZFP36</i>	−1.089	3.9	1.2
<i>TACC2</i>	1.107	5.4	2.0
<i>IL8</i>	1.062	6.5	11.5

Progesterone does not effect gene expression in vector-transfected cells, CTC15, after 5 hr of 0.1  $\mu$ M progesterone treatment compared to control treated cells. ABC28 cells were treated with control vehicle, control vehicle plus 10  $\mu$ g/ml cycloheximide (CHX), 0.1  $\mu$ M progesterone or 0.1  $\mu$ M progesterone plus CHX for 5 hr. Gene expression was analyzed by quantitative real-time RT-PCR using gene-specific primers. The fold changes induced by progesterone and progesterone plus CHX are relative to those induced by control vehicle and control vehicle plus CHX, respectively. Results are mean fold changes relative to vehicle-treated controls in 2 or 3 experiments.

ence of ER $\alpha$  can facilitate progesterone-mediated activation of the c-Src/Erk pathway in COS-7 cells by direct interaction with PR.<sup>17</sup> Lack of a prominent effect of progesterone in MCF-7 cells with estrogen-dependent PR compared to its marked effect in T47D and ABC28 cells also suggests an intricate interrelationship between the ER and PR systems. How different hormone/receptor milieus determine the function of progesterone in breast cancer is largely unknown. Progesterone-regulated gene expression profiles in the absence of estrogen/ER provide useful markers for further understanding the functional interaction between ER $\alpha$  and PR.

#### Progesterone-regulated gene expression and cancer development

We reported previously that reactivation of PR expression in hormone-independent breast cancer cells rendered progesterone strongly able to inhibit cell proliferation. Our primary goal was to determine if the overall gene regulation pattern of progesterone opposes cancer development. With the exception of *CDK5R1*, all the cell cycle genes and DNA replication genes in Table I were downregulated by progesterone. Table IV shows that progesterone

**TABLE IV – CELL PROLIFERATION PROMOTERS THAT ARE REGULATED BY PROGESTERONE BY >2-FOLD IN MICROARRAY ANALYSIS (MA)**

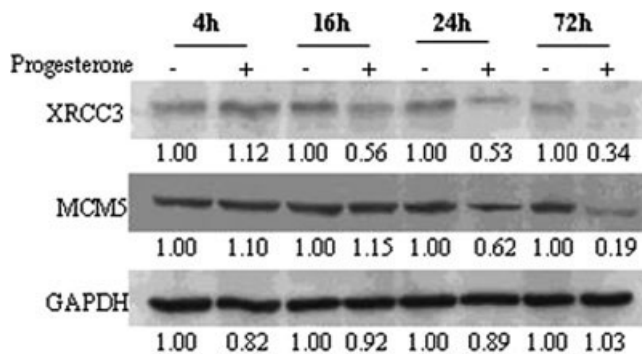
Accession number	Gene symbol	MA fold change at 24 hr
<b>Cell cycle-regulator</b>		
BG251266	<i>FOSL1</i>	-3.4
NM 001262	<i>CDKN2C</i>	-3.3
NM 004702	<i>CCNE2</i>	-3.1
AL524035	<i>CDC2</i>	-3.0
NM 002592	<i>PCNA</i>	-2.8
AL365505	<i>RBL1</i>	-2.7
AU132185	<i>MK167</i>	-2.7
NM 001237	<i>CCNA2</i>	-2.5
NM 001761	<i>CCNF</i>	-2.4
NM 016195	<i>MPHOSPH1</i>	-2.4
BC001425	<i>DDA3</i>	-2.3
NM 003810	<i>TNFSF10</i>	-2.3
BC000076	<i>CCND1</i>	-2.3
NM 004701	<i>CCNB2</i>	-2.2
NM 016426	<i>GTSE1</i>	-2.1
NM 003672	<i>CDC14A</i>	2.2
AL567411	<i>CDK5R1</i>	3.3
<b>DNA replication</b>		
NM 003686	<i>EXO1</i>	-3.7
NM 000234	<i>LIG1</i>	-3.3
NM 001067	<i>TOP2A</i>	-3.0
BC000323	<i>FEN1</i>	-2.8
BC000149	<i>RFC3</i>	-2.7
BC001866	<i>RFC5</i>	-2.6
D26018	<i>POLD3</i>	-2.6
AF321125	<i>CDT1</i>	-2.5
NM 002692	<i>POLE2</i>	-2.4
NM 002916	<i>RFC4</i>	-2.3
D42046	<i>DNA2L</i>	-2.2
AW138827	<i>TAF5</i>	-2.2
NM 002945	<i>RPA1</i>	-2.1
NM 002912	<i>REV3L</i>	2.4
<b>Mitosis</b>		
NM 016343	<i>CENPF</i>	-4.0
NM 004523	<i>KIF11</i>	-3.3
AF179308	<i>KIF4A</i>	-2.8
NM 018492	<i>TOPK</i>	-2.7
NM 002497	<i>NEK2</i>	-2.6
NM 012291	<i>ESPL1</i>	-2.6
NM 006461	<i>SPAG5</i>	-2.5
BC000712	<i>KIFC1</i>	-2.4
AY026505	<i>KIF2C</i>	-2.4
NM 002358	<i>MAD2L1</i>	-2.3
NM 004856	<i>KIF23</i>	-2.3
NM 005733	<i>KIF20A</i>	-2.0

Results are the average of 2 experiments.

inhibited the expression of 40 of the 43 genes involved in DNA replication, cell cycle progression and mitosis. This gene expression profile suggests a consistent antiproliferative function of progesterone in ABC28 cells.

Progesterone also increased the expression of many putative tumor suppressors (*TACC2*, *AIM2*, *ST5* and *DUPS1*) and inhibited the expression of *HEC* protooncogenes. Furthermore, the genes *LOXL2*, *PLAU*, *MMP1*, *ADAM 8* and *ADAMTS1*, which are known to promote invasion and metastasis, are downregulated by progesterone (Table I), suggesting that progesterone may act against cancer cell invasion and metastasis. A possible role of progesterone in metastasis was suggested in early studies, where surgery performed during the luteal phase of the menstrual cycle, when progesterone levels are higher, results in a higher rate of overall survival.<sup>50,51</sup> It has also been reported in rats inoculated with mammary adenocarcinoma cells that susceptibility to metastatic development was significantly lower during metestrus and diestrus than during proestrus and estrus, when progesterone level is low.<sup>52</sup> Our findings provide molecular evidence for the antime-tastatic role of progesterone in cancer.

In contrast, a microarray study in T47D-YA and T47D-YB cells revealed that progesterone aggravates the harmful gene expression



**FIGURE 2 – Progesterone mediates the downregulation of XRCC3 and MCM2 proteins in PR-transfected MDA-MB-231 cells ABC28.** Cells were treated with control vehicle (-) or 0.1  $\mu$ M progesterone (+) for 4, 16, 24 and 72 hr before whole cell lysates were collected. Total protein (20  $\mu$ g) was analyzed by Western blotting using specific antibody against each protein.

trend seen in breast cancer.<sup>31</sup> Recall that the study was done at the 6 hr time point, at which progesterone actually exhibited a stimulatory effect on cell proliferation.<sup>9</sup> Therefore, progesterone-mediated gene expression profile reflects well its effect on cell proliferation at this time point, although it remains to be determined if progesterone-regulated gene expression profile is antineoplastic at the phase when progesterone is growth-inhibitory in T47D cells. The genomic effect of progesterone in breast cancer may vary considerably depending on hormone receptor status and some other intrinsic properties of cancer cells.

#### Progesterone inhibits expression of DNA repair machinery

Progesterone inhibited the expression of all DNA repair genes identified in this study (Table I). The downregulation of these DNA repair genes has been confirmed by real-time RT-PCR analysis. Furthermore, 2 genes (*XRCC3* and *MCM2*) were randomly selected to test if this inhibition is also reflected at the protein level. Western blotting analysis showed that after 24 and 72 hr of progesterone treatment, protein levels of both XRCC3 and MCM2 are 2- to 5-fold lower in progesterone-treated cells compared to vehicle-treated controls (Fig. 2). Of note is that the fold changes in protein levels after 24 hr of progesterone treatment are lower than those in mRNA in both genes possibly due to the time lag of the 2 events. The changes in protein levels appear to have caught up with that of mRNA after 72 hr treatment.

At present, we can only speculate on the significance of progesterone-mediated inhibition of these DNA repair genes. Cells with unrepaired DNA could give rise to a transformed phenotype leading to cancer. Compromising the DNA repair machinery of the target cells may, therefore, reflect the negative effect of progesterone. However, body cells have a built-in mechanism to halt the cell cycle or induce apoptosis until the damaged DNA is repaired. In that sense, inhibition of the DNA repair genes may be just part of the proliferation-inhibitory mechanism. This notion is supported by evidence that increased level of XRCC3 is positively correlated with melphalan resistance in epithelial tumor cell lines.<sup>53</sup> This study suggests that with melphalan-induced DNA damage effectively repaired by XRCC3, the cancer cells will continue to divide. Hence, inhibition of DNA repair machinery by progesterone may be a mechanism leading to growth interruption. It has been reported that pregnant women have increased chromosome breakage in response to  $\gamma$  irradiation compared to nonpregnant women,<sup>54</sup> and there is a very strong correlation between the amount of progesterone and the increase in radiosensitivity. Thus, progesterone-mediated inhibition of DNA repair genes may be the molecular basis for the increased radiosensitivity in pregnant women. The physiologic significance of this effect of progesterone remains to be elucidated.

### Concluding remarks

The roles of progesterone in breast cancer development have long been a topic of controversy, and there is evidence to suggest that the function of progesterone in breast cancer depends on ER and PR status and whether PR is estrogen-dependent. The effect of progesterone on cell proliferation is minimal in ER- and PR-positive MCF-7 cells, whereas progesterone was shown to have a prominent biphasic effect in T47D cells that are ER- and PR-positive; however, PR is constitutively expressed independent of estrogen. PR-transfected MDA-MB-231 cells ABC28 provide an ER-negative but PR-positive model for elucidating the function of progesterone independent of estrogen and ER, and progesterone exhibited a strong growth-inhibitory effect in these cells. This genomewide

study of progesterone-regulated gene expression revealed over 100 new progesterone target genes that are components of progesterone-mediated genomic pathways. These genes are useful markers for studying the mechanism of progesterone action. Our study also shows that the whole-genome effect of progesterone in ER-negative but PR-positive ABC28 cells is consistently antiproliferative and antineoplastic. The findings suggest potential usefulness of progestin in treating ER-negative but PR-positive breast cancer, which accounts for as much as 12% of ER-negative breast cancer cases.<sup>55,56</sup> Our study also indicates that progesterone can exert a strong anticancer effect in hormone-independent breast cancer following PR reactivation and that PR gene therapy offers a potential therapeutic approach to ER- and PR-negative breast cancers.

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