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ABSTRACT

Estrogen receptor alpha (ERα)-negative breast cancer cells display an aggressive phenotype. We previously showed that adenoviral expression of ERα in ER-negative breast cancer cells leads to an estrogen-dependent down regulation of the proliferation, which could be of interest to control the growth of such cells. In this study, we observed an increase in protein levels of p21 and p27 CDKIs, whereas pRb phosphorylation strongly decreased. Flow cytometry experiments showed that cells were blocked in G2/M-phase. By using cDNA macroarrays, we identified a novel collection of genes regulated by liganded-ERα potentially regulating cell cycle, apoptosis, cell signalling, stress response and DNA-repair.

Key words: estrogen, receptor, proliferation, cDNA array

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1. Introduction

Breast cancer is one of the leading causes of premature death in women in occidental countries [1]. Increased lifetime exposure to estrogens is an established risk factor for development of breast cancer. Estrogen action is mediated by two estrogen receptors ERα and ERβ. ERβ is poorly expressed in breast tumors [2]. A high percentage of early stage mammary tumors are estrogen receptor α (ERα)-positive and about 50% of these patients respond to anti-estrogen or endocrine therapy [3,4]. Later stages of breast cancer are more aggressive and refractory to most therapies and this correlates with the ERα-negative phenotype of these tumors [5]. ERα-positive tumor cells are poorly metastatic in nude mice when compared with some ERα-negative breast cancer cells [6]. In patients, ERα-positive tumors are more differentiated and have lower metastatic potential than ERα-negative tumors [7], suggesting a protective role of the ERα in tumor progression. Introduction of ERα into ER-negative breast cancer cells has been thought to provide a rational basis for converting anti-estrogen resistant cells to hormonal manipulation. However, in contrast to the expected stimulated proliferation of ER-positive cells, the restoration of ERα expression in ER-negative breast cancer cells leads to a ligand-dependent inhibition of proliferation as shown by numerous studies [8-11]. The mechanisms underlying this phenomenon have been poorly investigated and could be of great interest to control the proliferation of these aggressive types of tumor cells. The goal of this study was to discerne events responsible for the ligand-dependent inhibition of breast cancer cells expressing exogenously ERα by analysing possible cell cycle regulation and modulation of gene expression.

2. Materials and Methods

2.1 Recombinant adenovirus construction and propagation.

The complete coding sequence of wild-type hERα cDNA was subcloned in BamHI site of the pACsk12CMV5 shuttle vector. To obtain recombinant viruses, permissive HEK-293 cells (human embryonic kidney cells) were cotransfected with the backbone or recombinant pACsk12CMV5-hER plasmid and with pJM17, which contains the remainder of the adenoviral genome as previously described [11]. *In vivo* recombination of the plasmids generates

infectious viral particles Ad5 (backbone virus) and Ad-hERα). Titered virus stocks were used to infect MDA-MB-231 cells.

2.2 Cell Culture and infection

MDA-MB-231 cells were maintained in Leibowitz L-15 medium supplemented with 10% fetal calf serum (FCS) and gentamycin. To wean the cells off steroids, they were cultured in phenol red-free DMEM/F12 supplemented with 10% CDFCS (charcoal dextran-treated FCS) for 4 days. For infection, cells were cultured in the same medium and infected with Ad5 or Ad-hERα viruses at a multiplicity of infection (MOI) of 100.

2.3 Whole cell extract preparation and western blot.

Cell extracts were prepared in NP40 buffer [50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP40, 10µM Sodium fluoride, 0.1mM Sodium orthovanadate, 1mM phenylmethyl sulphonyl fluoride (PMSF), 10 µg/ml aprotinin, leupeptin, pepstatin A and 20nM okadaic acid]. Cells were washed twice in ice-cold PBS, scraped in ice-cold NP40 buffer and lysated for 10 min. Cell lysates were cleared by 15 minutes centrifugation at 4°C, protein content in the supernatant was assayed by Bradford Protein Assay (BIORAD). The cell extracts were diluted 1:1 in 2X Laemmly sample buffer, followed by boiling for 5 min. Equal amounts of protein were loaded and separated for SDS/PAGE gel. Proteins were transferred to cellulose nitrate filters (Schleicher and Schuell, Germany) and blocked 30 min at 37°C in 5% non fat milk in TBST (TBS with 0.1% Tween 20). Membranes were incubated with the primary antibody for 1h at RT in TBST. The primary antibodies were: cdc2 (Ab-4; NeoMarkers)1:500; cdk2 (Transduction Laboratories)1:1000; Cyc A (BF683; Santa Cruz Biotechnology) 1:200; Cyc B1 (GNS1; Santa Cruz Biotechnology)1:200; Cyc D1 (CDS6, Sigma)1:200; Cyc D3 (Transduction Laboratories) 1:1000; Cyc E (M20; Santa Cruz Biotechnology)1:200; ERα (ER-311; [12]) 1:1000; ERK (K23; Santa Cruz Biotechnology) 100µg/ml; Rb (IF8; Santa Cruz Biotechnology)1:500; $p21^{CIP-1}$ (L17; Santa Cruz Biotechnology) 1:100; $p27^{kIP-1}$ (C19; Santa Cruz Biotechnology) 1:100. After washing, horseradish peroxidase-

linked secondary antibodies (Amersham-Pharmacia) were added and target protein bands have been detected using ECL (Amersham-Pharmacia).

2.4 Flow Cytometry Experiments.

To analyze the effects of recombinant viruses on the cell cycle, MCF-7 cells were infected with the adenoviral vectors, and cells were fixed in Ethanol 75% for 2 min. Fixed cells were then stained with PBS containing 40µg/ml propidium iodide and 100 µg/ml RNAse. After a 30 min incubation at 37°C, analysis was performed on an Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA).

2.5 RNA extraction, Northern blot and cDNA Microarrays.

Total RNA was isolated with TRIzol reagent (Invitrogen) as described by the manufacturer. RNA quantity was checked photometrically by absorption at 260 nm and quality was determined by examination of the 28S and 18S rRNA bands in ethidium bromide-stained agarose gels. After two phenol/chloroform extractions, RNA was precipitated. To remove genomic DNA contamination, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNase) I (Clontech, Palo Alto, CA), and was then dissolved in RNase-free H2O and stored at -80°C until analysis. For Northern blot analysis, 20 µg RNA were electrophoresed and then hybridized with the different probes. The Atlas human macroarray (7740-1: 588 genes) was purchased from Clontech. For cDNA probes, 5 µg of total RNA from MDA-MB-231 infected with Ad-hERα and treated or not for 24h with E2 (10-8 M) were used. The Atlas arrays were then hybridized with the probes according to manufacturer instructions. Array images were analyzed using Atlas Image 2.0 software (Clontech, Palo Alto).

3. Results

3.1 Growth inhibition involves G2/M transition blockage

We have previously showed that exogenous expression of ERα in MDA-MB-231 ER-negative breast cancer leads to growth inhibition in the presence of estradiol [11]. To better characterize

the phenomenon involved in growth inhibition, we performed flow cytometry experiments on cells which had been treated for different times with estradiol (Fig. 1). Cell cycle distribution of Ad5 infected remained unchanged upon estrogen treatment. On the other hand, Ad-hERα infected cells cell cycle distribution was strongly affected. The proportion of ERα cells in S phase strongly diminished upon treatment with estradiol (27 to 16%), whereas the proportion of cells in G1 phase was constant and the proportion of cells in G2/M phase increased from 13 to 17%. Importantly, we should also notice the presence of a sub-G1 peak which is characteristic of cells undergoing apoptosis (Fig. 1).

3.2 Analysis of cell cycle modulators expression

To dissect the mechanisms underlying this inhibition of proliferation, we analyzed at the protein level the expression of a collection of known cell cycle modulators. A time course of estradiol treatment was performed on Ad5 or Ad-hERα infected cells and whole cell extracts were analyzed by western blot (Fig. 2). ERα was only detected in Ad-hERα infected cells. Of particular note is the appearance of a slower migrating band upon estradiol treatment which could correspond to a phosphorylated form of ERα. ERK phosphorylation did not show any modification whereas pRb phosphorylation was strongly diminished. Cyclins D1 and E were up-regulated in a ligand-independent manner, whereas cyclin A and D3 expression remained constant. CDKI p21 and p27 showed a nice up-regulation following estradiol treatment. Finally, cdc2 and cdk2 levels were stable. In summary, these data show a complex regulation of cyclins and CDKI involved in G1 to S phase transition.

3.3 Liganded-ERa induces a complex pattern of gene regulation

To have a better sense of the mechanisms underlying the growth inhibitory effect of liganded-ERα, we compared the expression of 588 genes between ERα-infected cells treated or not with E2, by using cDNA macroarrays. A total of 12 genes were up-regulated by E2 (Table 1), whereas 27 genes were down-regulated by E2 (Table 2). The genes identified were potentially involved in oncogenesis or tumor suppression (RAF, c-myc, c-fms, c-jun, Fra-1, Axl), cell cycle

(cyclin G2, TOB, p21, STK-1), apoptosis (BNIP3, Gadd45, Gadd153, DAP-3), transcription (TAFII31, ATF-2, ATF-4, HIP116, CNBP, Y box), cell signalling (TGFα, G-CSF, Thrombomodulin, BMP-4, IL1β, Thrombin receptor, Macmarcks, CTGF), cell adhesion (Integrin α3 chain, integrin β1 subunit), stress response (HSP60, Glutathione reductase, Glutathione peroxidase), DNA synthesis and repair (TopoIIα, HHR23A, HHR23B, DNA-PK, DBI). This suggests that exogenous expression of ERα and subsequent activation by E2 leads to major changes in gene regulation of the cells affecting multiple aspects of cell life.

3.4 Confirmation of the regulations by northern blot

To confirm the regulations identified by cDNA array screening, we analyzed the expression of 13 genes by northern blot (Fig. 3). pS2 gene (not present on the array) was used as a positive control of estrogen regulation and it was induced only in Ad-ERα infected cells treated with E2. We observed that the levels of Fra-1, Gadd45, DAP-3 and TopoIIα and DNA-PK were effectively down-regulated by liganded-ERα, whereas cyclin G2, G-CSF, IL1β, Macmarcks, BNIP3, p21/CIP-1 and TGFα levels were up-regulated, which validates our screen.

4. Discussion

To identify the mechanisms underlying cell growth inhibition triggered by liganded-ERα in ER-negative breast cancer cells, we have analyzed cell cycle distribution and gene expression changes. Our results suggest that cycle cycle blockage in G2/M phase occurs, which is concomittant with an up-regulation of p21, p27, TOB and cyclin G2 levels, a down-regulation of STK1 expression and a dephosphorylation of pRb. p21, p27, TOB and cyclin G2 have been shown to inhibit proliferation [13-15], whereas STK1 overexpression could increase proliferation [16]. The down regulation of Gadd45 which expression is frequently increased in growth-arrested cells and regulated by BRCA-1 [17] might be explained by the down-regulation of BRCA-1 we observed previously in such infected cells [18]. On the other hand, apoptosis is also likely to occur as shown by appearance of a subG1 peak, induction of pro-apoptotic BNIP3 [19]

and down-regulation of DAP-3 [20]. It is also interesting to note that several oncogenes such as c-myc, c-fms, c-jun, Fra-1, Axl, or transcription factors such as ATF-2, ATF4, HIP116, CNBP or Y box are down regulated. All these proteins are generally promoting tumorigenesis [21-26]. Abnormal expression of c-fms, Axl, members of AP-1 family, CNBP (which stimulates c-myc promoter activity) or Y-box protein by malignant cells is correlated with poor prognosis [21-25,27]. Down regulation of Topoisomerase IIα, HHR23A, HHR23B, DBI/ACBP and to a lesser extent of DNA-PK suggests that reintroduction of ERα in MDA-MB-231 cells leads to a decreased efficiency of DNA repair [28-30], which could eventually lead to apoptosis. In addition to the weaker ability of the cells, to defend themselves against DNA damage, it is likely that the cells are also losing their ability to respond to stress signals as shown by down-regulation of HSP60, glutathione peroxidase and glutathione reductase [31,32]. Changes in integrin and BMP-4 [33,34] could also account for the previously observed inhibition of invasion observed after reintroduction of ERα [9]. Other events such as down regulation of thrombin receptor, up-regulation of thrombomodulin could also account for the decreased invasiveness of these cells [35-37]. In addition, the down regulation of CTGF, which is promoting angiogenesis [38], suggests that ERα exogenous expression could reduce the angiogenesis events during tumor formation. It is also interesting to note that, the majority of the genes regulated by E2 that we discovered, have not been previously identified as potential targets of ER, confirming the importance of our screen.

In conclusion, our data suggest that reintroduction of ERα in ER-negative breast cancer cells could be a valuable strategy to limit their growth and their invasion. This is also concomitant to a reduced ability to repair damaged DNA and to response to stress signals, which further suggests that the cells are more likely to lose their advantages over normal cells.

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Footnote

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Legends to Figures

Fig. 1. ERa blocks cell cycle

Ad5 or Ad-hERα infected MDA-MB 231 cells which had been treated for 0, 4, 24 or 48h with E2 10^{-8} M were analyzed by FACS for cell cycle distribution. **A.** Representative profiles of FACS analysis. **B.** Quantification of cell cycle distribution.

Fig. 2. Modulation of cell cycle regulatory factors by ERa

WCE from Ad5 or Ad-hERα infected MDA-MB 231 cells which had been treated for 0 to 48h with E2 (10^{-8} M) were used for determination of the expression of ERα, Erk, cyclin D1, cyclin D3, cyclin E, cyclin A, $p21^{CIP-1}$, $p27^{Kip-1}$, cdk2, cdc2 and pRb by western blot.

Fig. 3: Analysis of a subset of genes regulated by ERa in MDA-MB-231 cells

The expression of Cyclin G2 DNA-PK, Fra-1, Gadd45, G-CSF, IL1β, DAP-3, Macmarcks, BNIP3, $p21^{CIP-1}$, TGFα, TopoIIα and pS2 was analyzed by northern blot using RNA form Ad5 or Ad-hERα infected MDA-MB-231 cells, treated or not for 48h with E2.

Table 1: Genes up-regulated by E2 in Ad-hERa infected MDA-MB-231 cells

Table 2: Genes down-regulated by E2 in Ad-hERa infected MDA-MB-231 cells

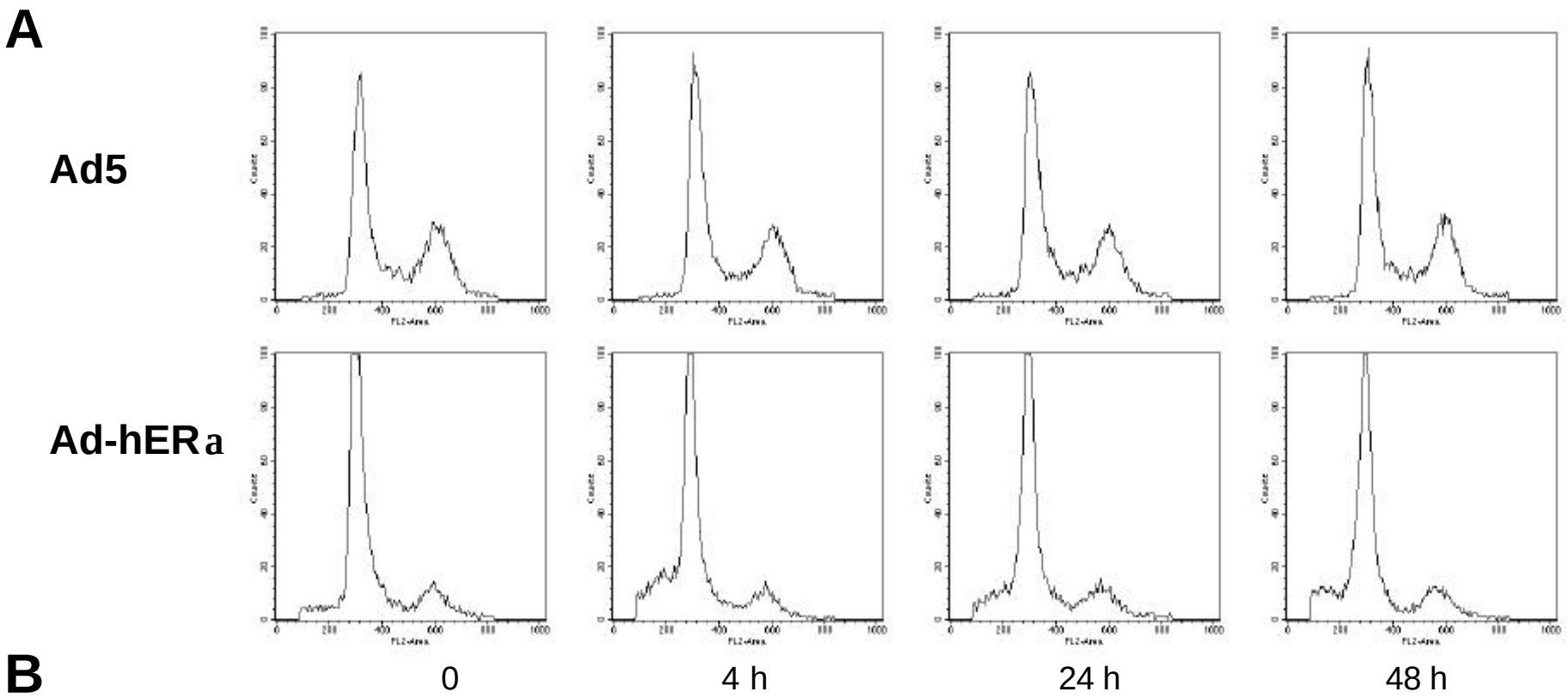

Fig. 1

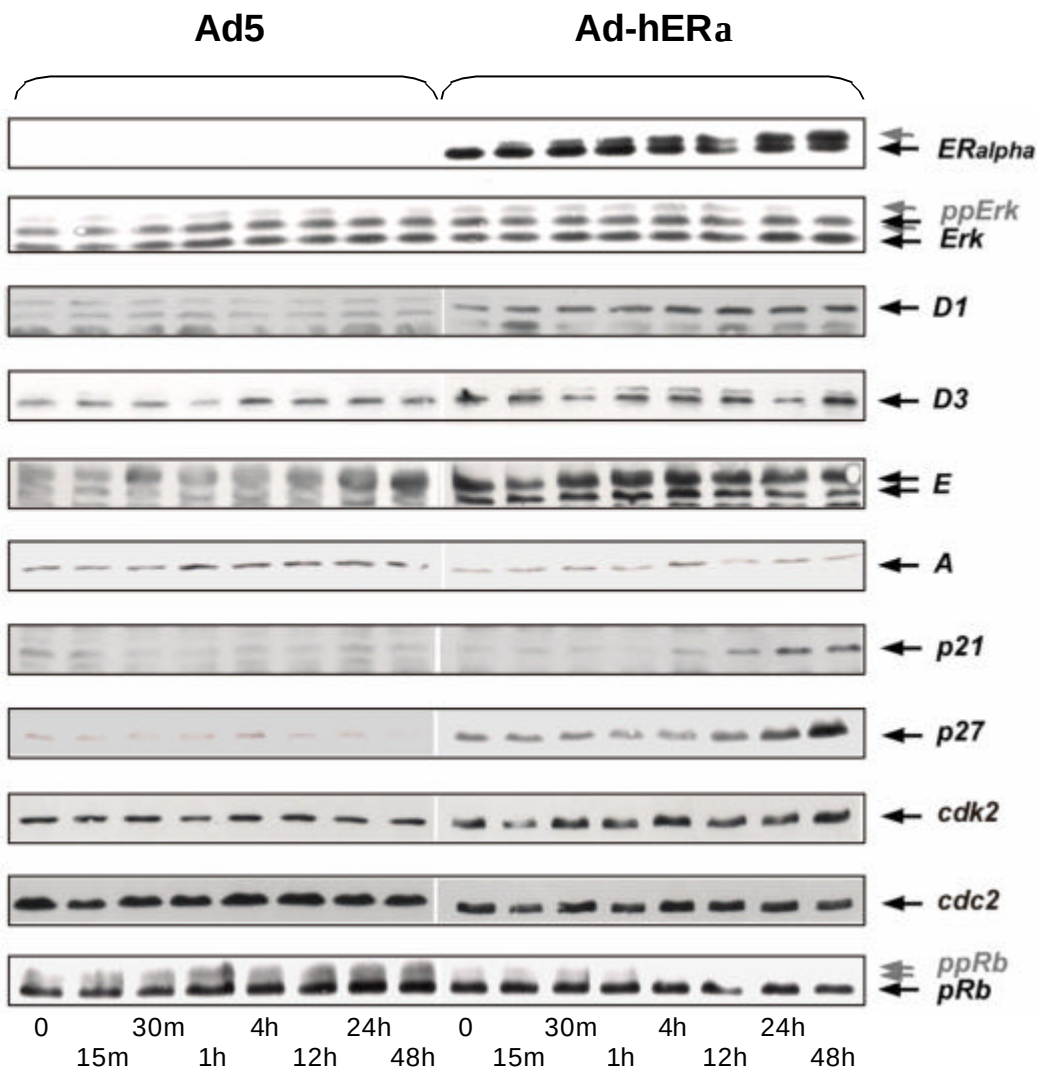

Fig. 2

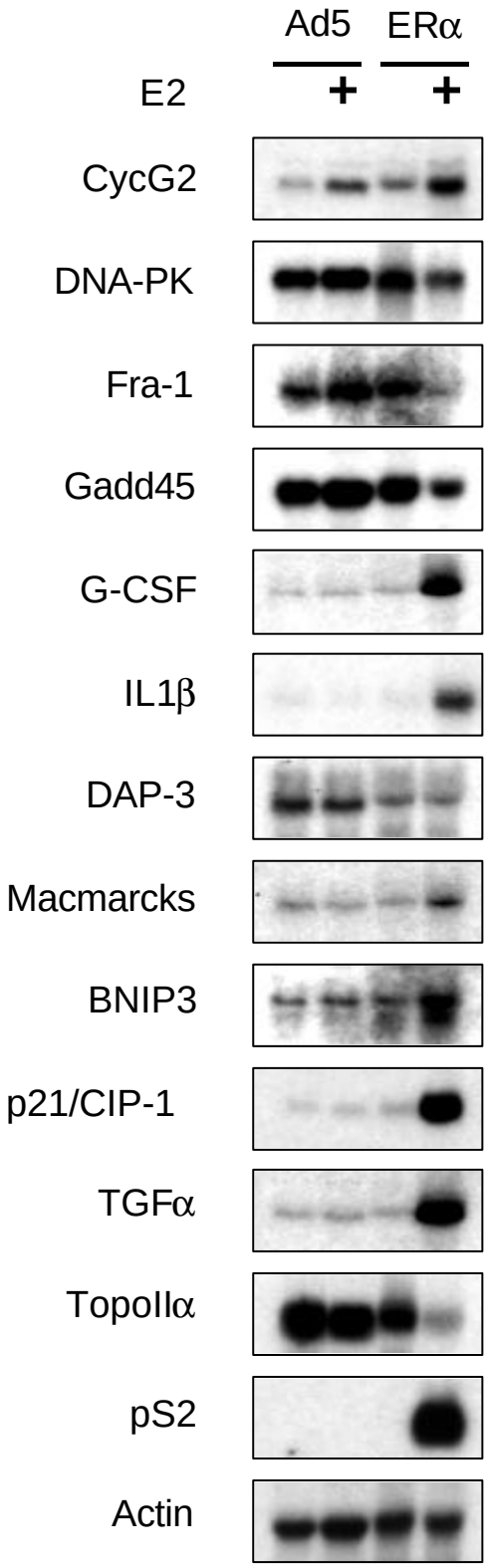

Fig. 3

Table 1: Genes induced by E2

GenBank accession number	Gene Name	Putative Function
X03484	RAF	Oncogenes - Tumor suppressors / Intracellular Kinase network members
U47414	CYCLIN G2	Cell Cycle / Cyclins
D38305	TOB	Cell Cycle / Adaptators and receptor- associated proteins
U15174	BNIP3	Apoptosis / BCL2 family proteins
S40706	GADD153	Apoptosis / DNA damage
U09579	p21/CIP-1	Cell Cycle / CDK inhibitors
U30504	TAFII31	Transcription / RNA Polymerase
K03222	TGFα	Cell signalling – extracellular communication proteins / Growth Factors
X03438	G-CSF	Cell signalling – extracellular communication proteins / Cytokines
M16552	Thrombomodulin	Cell signalling – extracellular communication proteins / Growth Factors
D30751	BMP-4	Cell signalling – extracellular communication proteins / Growth Factors
K02770	IL-1β	Cell signalling – extracellular communication proteins / Cytokines

Table 2: Genes repressed by E2

GenBank accession number	Gene Name	Putative Function
V00568	c-myc	Oncogenes - Tumor suppressors / Transcription activators and repressors
X03663	c-fms	Oncogenes - Tumor suppressors / Intracellular transducers
J04111	c-jun	Oncogenes - Tumor suppressors / Transcription activators and repressors
X16707	FRA-1	Oncogenes - Tumor suppressors / Transcription activators and repressors
M76125	Axl	Oncogenes - Tumor suppressors / Intracellular transducers
U02687	STK-1	Cell cycle / Intracellular transducers
M62424	Thrombin Receptor	Intracellular transducers / Growth factor receptors
M31630	ATF-2	Transcription activators and repressors / Intracellular transducers
X70326	Macmarcks	Intracellular transducers / Kinase activators and inhibitors
M34664	HSP60	Stress response proteins / Heat shock proteins
X15722	Glutathione Reductase	Stress response proteins / Xenobiotic transporters
M21304	Glutathione Peroxidase (GPX1)	Stress response proteins / Xenobiotic transporters
U18321	DAP-3	Apoptosis
J04088	TopoIIα	DNA synthesis – Recombination and repair / Topoisomerases
D21235	HHR23A	DNA synthesis – Recombination and repair / Nucleotide excision repair
D21090	HHR23B	DNA synthesis – Recombination and repair / Nucleotide excision repair
M60974	Gadd45	Apoptosis / DNA synthesis – Recombination and repair
U35835	DNA-PK	DNA synthesis – Recombination and repair / Stress response proteins
L34673	HIP116	Transcription / Basic transcription factors
D90209	ATF-4	Transcription / Transcription activators and repressors
M28372	CNBP	Transcription / Basic transcription factors
M83234	Y-box protein	Transcription / Basic transcription factors
M59911	Integrin α-3 chain	Cell adhesion proteins / Cell-cell adhesion receptors
X07979	Integrin β1 subunit	Cell adhesion proteins / Cell-cell adhesion receptors
M92934	CTGF	Cell signalling – extracellular communication proteins / Growth factors
M14200	DBI/ACBP	Metabolism / Nucleotide metabolism
M31159	IGFBP-3	Cell signalling – extracellular communication proteins / Hormones