

Targets of 17 β -oestradiol-induced apoptosis in colon cancer cells: a mechanism for the protective effects of hormone replacement therapy?

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Abstract

Epidemiological studies show a strong link between postmenopausal hormone replacement therapy and decreased incidence of colorectal cancer. The colon cancer cell line, COLO 205, develops sensitivity to 17 β -oestradiol (E₂) in apoptosis assays with increasing passage number (>40), and we hypothesised that genes selectively regulated in multiply passaged cells were likely to be important in E₂-related apoptosis. Gene array analysis was used to compare the patterns of genes up- or down-regulated in E₂-sensitive and -insensitive cells. For some genes, changes in mRNA expression were confirmed by protein expression analyses. Changes found in response to E₂ in multiply passaged cells, but not minimally passaged cells, included induction of growth arrest and DNA damage-inducible protein 153 (GADD153), and repression of Kirsten-Ras 2B (K-Ras-2B), metastasis inhibition factor NM23 and vascular endothelial growth factor. A second

group of genes was regulated with E₂ exposure in both cell types, and is unlikely to be critically involved in E₂-associated apoptosis. These included up-regulation of butyrate response factor 1 (BRF1) and down-regulation of c-jun and the breast cancer associated ring domain gene known as BARD1. By comparing control arrays from the two cell populations, cAMP-response element-binding protein (CBP), which is associated with steroid receptor-dependent target gene transcription and the oncoprotein, tyrosine kinase-T3 (TRK-T3), were up-regulated whereas retinoic acid receptor α (RAR α) was down-regulated in multiply passaged cells. This study provides evidence for selective regulation of genes in colon cancer cells by E₂, indicates which of those regulated are likely to be involved in induced apoptosis, and suggests genes likely to be responsible for facilitation.

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Introduction

Epidemiological studies show that postmenopausal hormone replacement therapy (HRT) treatment in women is associated with a decrease in the incidence of colorectal cancer (Calle *et al.* 1995, Herbert-Croteau 1998, Grodstein *et al.* 1999). Consistent with a protective effect of female sex hormones, animal models show that male rats have higher risks of developing colon cancer compared with their female counterparts when exposed to dimethylhydrazine, an experimental carcinogen (Smirnoff *et al.* 1999). The effects of 17 β -oestradiol (E₂) are mediated by two specific, high-affinity receptors, oestrogen receptor α (ER α) and oestrogen receptor β (ER β), which modulate gene expression by interaction with oestrogen response elements or other transcription factors, such as AP1 and Sp1 (Kuiper *et al.* 1997, Muramatsu & Inoue 2000). In colonic epithelial cells, ER β predominates and transformation to the malignant phenotype is accompanied by a loss in ER β (Foley *et al.* 2000, Campbell-Thompson *et al.* 2001). Our previous

study using the colon cancer cell line COLO 205 showed that E₂ induces apoptosis at physiological concentrations through ER β by a genomic pathway (Qiu *et al.* 2002). This may be one of the mechanisms by which E₂ can prevent colon cancer.

Several studies have examined E₂-mediated apoptosis in non-colonic cells. An erythroid transcription factor, GATA-1, essential for survival and maturation, is reported to be decreased in E₂-induced apoptosis in an erythroid cell line (Blobel & Orkin 1996). In E8 CASS breast cancer cells, where E₂ treatment decreases proliferation and increases DNA degradation, E9, a gene homologous to Requiem, plays a central role in E₂-mediated breast cancer regression (Szelei *et al.* 2000). Subsequent studies on E₂-sensitive breast cancer cells showed that FasL expression is increased by E₂ (Song *et al.* 2001), a mechanism also thought to be needed for ER β -induced apoptosis in neuronal cells (Nilsen *et al.* 2000) while activation of p38 mitogen-activated protein kinase is coupled to ER-induced apoptosis in stably transfected ER α -positive HeLa-ER5 cells (Zhang & Shapiro 2000).

E₂ has, however, been linked to both promotion and prevention of cancer (Russo *et al.* 2002) and it is not known whether the targets activated in the prevention of cancer are the same, overlapping or distinct from those affecting promotion or other effects. cDNA array technology provides a rapid and effective method of detecting differential gene expression (Ramsay 1998). We have identified two populations of COLO 205 cells, only one of which is sensitive to *E₂* in apoptosis assays. The populations differ in passage number, the later passage cells being *E₂* sensitive, but they do not differ markedly in growth rate or in morphology. cDNA array was used to identify the genes involved in *E₂*-induced effects in the two populations of COLO 205. The arrays allowed us to distinguish genes involved in *E₂*-mediated apoptosis from other *E₂*-regulated genes. For several genes, Western immunoblotting was used to confirm the cDNA array results. Furthermore, by comparing arrays from unstimulated cells, we have identified genes likely to be involved in the transformation to an *E₂*-sensitive phenotype.

We have provided the first step in identifying the gene pathways by which *E₂* could prevent colon cancer, showing that induction of growth arrest and DNA damage-inducible protein 153 (GADD153) and repression of Kirsten-RAS-2B (K-Ras-2B) oncoprotein and metastasis inhibition factor NM23 (NM23-H1) contribute to *E₂*-induced apoptosis in COLO 205 cells. Additional pathways demonstrate that *E₂* may inhibit pathogenic angiogenesis by inhibition of vascular endothelial growth factor (VEGF) production.

Materials and Methods

Materials

COLO 205 cells, derived from the ascites fluid of a 70-year-old Caucasian male with colorectal cancer, were purchased from the European Animal Cell Culture Collection (EACC, Porton Down, Wilts, UK). In our study, COLO 205 cells between passages 5 and 20 are identified as NCOLO 205 and those from passage > 40 are designated OCOLO 205. HT-29 cells (EACC), established from the primary tumour of a 44-year-old Caucasian, were used as comparators. For experiments used in the arrays, cyclodextrin-encapsulated *E₂* (water-soluble *E₂* from Sigma Chemical Corporation, Poole, Dorset, UK) was dissolved in Milli-Q distilled water (Millipore Ltd, Stonehouse, Glos, UK), at a stock concentration of 10^{-2} M and 2-hydroxypropyl- β -cyclodextrin (Sigma) was used as control for this agent. TRIzol was purchased from Invitrogen Ltd (Paisley, Strathclyde, UK). Atlas human cancer 1.2 cDNA arrays were obtained from BD Biosciences Clontech UK (Oxford, Oxon, UK).

Assays of DNA fragmentation following treatment with *E₂*

OCOLO 205 and NCOLO 205 cells were serum-starved for 24 h. Following a medium change, *E₂* (10^{-12} M and 10^{-11} M) was added to some wells and incubation was continued for a further 48 h. Vehicle (DMSO) was added to the control wells. Assays of DNA fragmentation on the floating cells were performed as described previously (Qiu *et al.* 2002).

Cell culture and treatment

Cell lines were maintained in phenol-red free RPMI 1640 medium (Sigma) augmented with 10% foetal bovine calf serum (FCS; First Link Ltd, Birmingham, UK), 100 U/ml penicillin G and 100 μ g/ml streptomycin sulphate (Sigma) in a humid atmosphere at 37 °C, with 5% CO₂. Cells were passaged twice a week and plated at a density of 5×10^4 cells/cm² and cultured for 3 days in 10% charcoal-stripped FCS (CSFCS), followed by treatment with *E₂* for the indicated periods of time in serum-free medium in the presence of antibiotics. CSFCS was prepared by mixing 50 ml FCS with 5 g activated charcoal (Sigma) overnight at 4 °C. Following centrifugation to remove the charcoal, another 5 g charcoal was added to the supernatant, incubated for 30 min at 37 °C and removed by centrifugation. This was repeated once.

Preparation of total RNA

Floating and adherent cells were pooled. Total RNA was extracted from cells using TRIzol reagent according to the manufacturer's recommendations and quantified by spectrometry (260 nm). The removal of possible DNA contaminants from extracted RNA was performed with RQ1 RNase-free DNase I (Invitrogen) digestion followed by phenol:chloroform:isoamyl alcohol extraction of RNA. The quality of RNA was assured by gel electrophoresis of 4 μ g extracted RNA on 1% agarose/ethidium bromide gel with clear 28S and 18S bands, and the ratio of 28S to 18S ribosomal RNA was about 2.

cDNA expression array analysis

To identify genes regulated by *E₂*, a human cDNA array that contained 1176 genes was screened using radio-labelled cDNA generated from total RNA extracted from NCOLO 205 (*E₂*-apoptosis resistant) and OCOLO 205 (*E₂*-apoptosis sensitive) cells treated with or without *E₂* (1 nmol/l) for 8 h and for 24 h. Detection of mRNA expression was performed according to the manufacturer's instructions. Briefly, high sensitivity probes were synthesised by *in vitro* transcription with α -[³³P]ATP and the gene-specific CDS primer mix provided, and purified by column chromatography (BD Biosciences Clontech UK). Hybridisation was performed overnight with continuous

agitation at 68 °C, followed by stringent washing to remove non-specific binding. Signals were detected by exposure of the hybridised atlas arrays to a phosphorimager screen for 3–6 days at room temperature, scanned using Molecular Imager FX (BioRad, Hercules, CA, USA) and data were analysed by Atlasimage 1.5 software (BD Biosciences Clontech UK). To ensure accurate comparison between different membranes, signals were normalised to the sum of all of the genes, as suggested by the manufacturer, since most of the genes detected will not be regulated. Gene regulation was taken into account only when genes were up-regulated by more than 2-fold or down-regulated by more than 50%.

Western immunoblotting

Floating and adherent COLO 205 cells were pooled and washed with HBSS twice, and solubilised in lysis buffer (2% SDS, 62.5 mmol/l Tris-HCl, pH 6.8). Protein concentration was assayed using the BioRad Dc protein assay kit (BioRad) with bovine serum albumin (BSA) as standard. Protein (100 µg) was separated by electrophoresis on 15% SDS-PAGE with a 7.5% stacking gel under reducing conditions. The separated proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech Inc., Little Chalfont, Bucks, UK) for 3 h at 450 mA. Non-specific binding was blocked by incubating the membrane in 10% low-fat milk in Tris-buffered saline-Tween 20 (TBS-T; 10 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl and 0.1% Tween 20) for 1 h at room temperature. The blot was incubated with 1:1000 dilution of anti-GADD153 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-K-Ras-2B antibody (Santa Cruz) or 1:500 dilution of antibody against NM23-H1 (Santa Cruz) in TBS-T with 0.25% BSA overnight at 4 °C. After exposure to primary antibody, the blot was washed in TBS-T and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (1:50 000 dilution in TBS-T with 0.25% BSA; Santa Cruz) or HRP-conjugated anti-mouse immunoglobulin (1:20 000) for 1 h at room temperature and washed again. Chemiluminescent substrate (KPL; Insight Biotechnology Ltd, Wembley, London, UK), and Kodak MXB film (GRI; Rayne, Essex, UK) were used for detection of immunoreactive species. For some blots, further confirmation of equal loading was shown by stripping the membranes in 2% SDS, 1% 2-mercaptoethanol and 62.5 mmol/l Tris-HCl, pH 6.8 at 60 °C for 30 min, and, following extensive washing, reprobing with antisera to cytokeratin 8 (the binding site).

ELISA

Cell-conditioned media were collected from OCOLO 205 and NCOLO 205 cells treated with varying doses of E₂ in triplicate wells for 48 h. At collection, FCS (1%

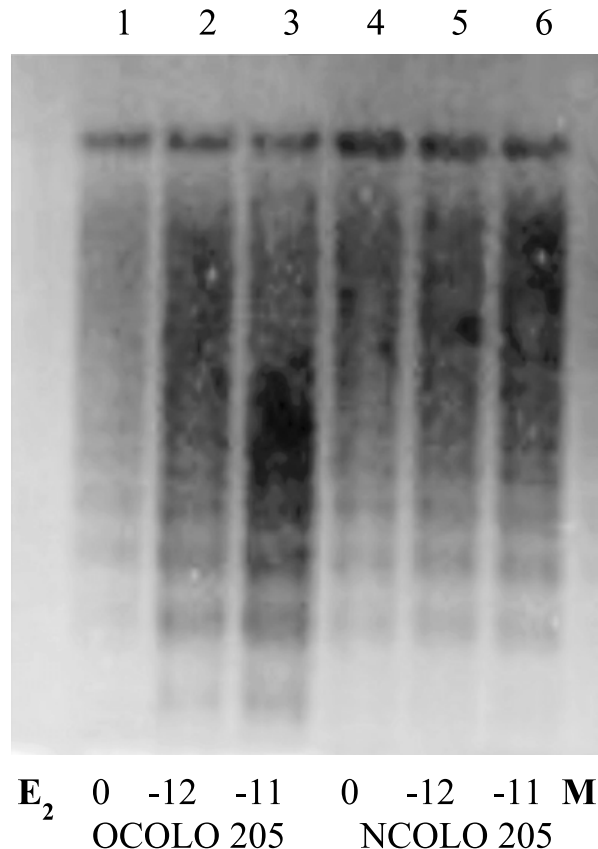


Figure 1 Effect of E₂ on DNA fragmentation in OCOLO 205 and NCOLO 205 cells. Lanes 1–3 are from OCOLO 205 cells and lanes 4–6 are from NCOLO 205 cells. Lanes 2 and 5 were from cells challenged with 10⁻¹² M E₂ and lanes 3 and 6 from cells challenged with 10⁻¹¹ M E₂.

final concentration) was added to prevent loss of VEGF and samples were frozen at -20 °C until use. ELISA was performed as suggested by the manufacturer (R&D Systems Europe Ltd, Abingdon, Oxon, UK). Secreted VEGF was corrected for protein content of the cell layer.

Results

Effect of E₂ on DNA fragmentation in OCOLO 205 and NCOLO 205 cells

The effects of E₂ on DNA fragmentation in OCOLO 205 (>40 passages) and NCOLO 205 cells (5–20 passages), prepared as described in the Materials and Methods are shown in Fig. 1. OCOLO 205 cells showed increased DNA fragmentation with E₂ treatment whereas NCOLO 205 cells were E₂ insensitive.

Effects of E₂ on gene expression in OCOLO 205 and NCOLO 205 cells

Out of 1176, 35–107 (3–9%) genes were detected with ³³P-labelled probes on the cDNAs. Non-specific

hybridisation was shown to be minimal since no plasmid or bacteriophage DNAs (M13 mp18(+) strand DNA, λ DNA and pUC18 DNA) or genomic DNA included in the array were positive.

Genes regulated by E_2 treatment (1 nmol/l) in OCOLO 205 but not in NCOLO 205 cells were considered likely to be involved in E_2 -induced apoptosis and are listed in Table 1. Using this selection criterion, 28 of 1176 (2.4%) genes were identified. Of these, seven genes were up-regulated, 18 genes down-regulated and three genes regulated bi-directionally when signals from two time-points (8 and 24 h) were analysed. Genes previously shown to be regulated by E_2 are indicated with an asterisk.

The gene encoding growth arrest and DNA damage-inducible protein (GADD153), a stress- and apoptosis-associated gene, showing induction or overexpression associated with endoplasmic reticulum stress (Ubeda & Habener 2000, Maytin *et al.*) or apoptosis induced by u.v. light (Luethy *et al.* 1990, Gujuluva *et al.* 1994, Kawahara *et al.* 2001) was increased 5.3-fold. Other genes that were up-regulated included those encoding TRAP1 (15-fold), sentrin (8-fold) and SHMT (3.8-fold).

In contrast, the gene encoding K-Ras-2B, a G protein involved in cell proliferation, was decreased by 80% with E_2 treatment in OCOLO 205 cells, NM23-H1 was decreased by 26% at 8 h and by 78% at 24 h and VEGF, an angiogenesis factor, was also decreased. Other genes repressed by E_2 include those encoding ERBB-3 receptor protein-tyrosine kinase precursor (90% inhibition), myc (60%), GRP 78, bax (66%) as well as the P68 TRK-T3 oncoprotein.

Target verification by Western immunoblotting analysis and ELISA

Confirmation that the mRNA expression changes were translated to changes in protein expression was obtained by Western immunoblotting using specific antisera or ELISA measurement. GADD153, K-Ras-2B, NM23-H1 and VEGF were examined. These genes were chosen somewhat arbitrarily because the primary reason to do this was to check whether the changes in mRNA expression seen in the array were reflected in similar changes in protein expression, but we also selected these genes because we considered them of interest as described in the Discussion. GADD153 protein was dose-dependently increased with E_2 treatment in OCOLO 205 cells (Fig. 2a), while expression of oncoprotein K-Ras-2B (Fig. 2b) and NM23-H1 (Fig. 2c) were down-regulated. In NCOLO 205 cells, these genes were not regulated by E_2 treatment (Fig. 2d and e). Similarly, in another colon cancer cell line, HT-29, which did not apoptose in response to E_2 , neither GADD153 (Fig. 2f) nor K-Ras-2B (Fig. 2g) was regulated by E_2 .

Inhibition of VEGF secretion by E_2

Figure 3 shows the effect of E_2 on VEGF secretion from OCOLO 205 cells. E_2 treatment produced

dose-dependent reduction in VEGF secretion in OCOLO 205 cells that was significant at 1 nM E_2 . No significant changes in VEGF expression were found in NCOLO 205 cells. Comparable amounts of VEGF were secreted by NCOLO 205 and OCOLO 205 control cells.

Identification of E_2 -responsive genes common to OCOLO 205 and NCOLO 205 cells

Significant and equivalent up-regulation of four genes and down-regulation of nine genes were detected in both NCOLO and OCOLO 205 cells (Table 2). These include BRF1 and ELF1 which were increased, and the breast cancer associated ring domain gene known as BARD1 and c-jun which were decreased.

Differences between untreated OCOLO 205 and NCOLO 205 cells detected with cDNA array

By analysing the differences in cDNA array between OCOLO 205 and NCOLO 205 cells not treated with E_2 , we were able to identify genes likely to be involved in the increased sensitivity of OCOLO 205 cells. These data are shown in Table 3. OCOLO 205 cells were found to express 5-fold more cAMP-response element (CREB)-binding protein (CBP) than NCOLO 205 cells when incubated in serum-free medium for 24 h. Expression levels of the oncoprotein TRK-T3 were 10-fold greater whereas RAR α mRNA was decreased in multiply passaged cells.

Discussion

By comparing findings in OCOLO 205 and NCOLO 205 colonic cancer cells which respectively respond and do not respond by apoptosis to physiologic concentrations of E_2 , we have been able to show that there is evidence of selective up- and down-regulation of genes either predisposing to or protecting from apoptosis in the cells which are responsive. Some of these are genes previously identified as E_2 responsive in non-colonic cancer cells, but others have not been so identified. We have sought for and found that there are parallel and appropriate responses in OCOLO 205 but not in NCOLO 205 cells in protein expression for some of these genes. This confirmatory evidence of appropriate changes in protein expression establishes the reliability of the cDNA array data although it must be appreciated that the arrays were not performed to the same exacting rigour as that recently described by Lobenhofer *et al.* (2002). Our study aimed to establish the principle of this approach and to confirm the array data at the protein expression level. Unresponsiveness of HT-29 colon cancer cells to E_2 in the expression of target genes further confirmed that these genes are likely involved in apoptosis induction by E_2 .

Table 1 Apoptosis-associated genes: genes regulated by E₂ in OCOLO 205 but not NCOLO 205 cells. Gene regulation was considered significant when the increase is >2-fold or the decrease is >50%

	Accession number	Fold induction		Classification
		8 h	24 h	
Genes up-regulated				
Tumour necrosis factor type 1 receptor-associated protein (TRAP1)	U12595		15.81	Death receptor-associated proteins and adaptors
Growth arrest and DNA damage-inducible protein 153 (GADD153)	S40706		5.32	Stress response proteins, apoptosis-associated proteins
Ubiquitin-like protein SMT3C (sentrin)	U83117		8.55	Enzymes involved in protein turnover, apoptosis-associated proteins
KIAA0078	D38551		14.9	DNA damage repair proteins and ligases
40S ribosomal protein S16	M60854		2.04	Ribosomal proteins
Cytosolic serine hydroxymethyltransferase (SHMT)	L11931	3.75		Amino acid, nucleotide and lipid metabolism
BIGH3/keratoepithelin protein	M77349	2.18		Microfilament proteins
		% Reduction		
Genes down-regulated				
*myc	V00568		62	Transcription activators and repressors
*Nucleoside diphosphate kinase A (NDKA); metastasis inhibition factor NM23 (NM23-H1)	X17620		78	Apoptosis-associated proteins, transcription activators
*ERBB-3 receptor protein-tyrosine kinase precursor; epidermal growth factor receptor	M29366		92	Growth factor and chemokine receptors
ADP/ATP carrier protein; fibroblast adenine nucleotide translocator 2 (ANT2)	J02683 P05141		86	Symporters and antiporters
Ras-related C3 botulinum toxin substrate 2; p21-rac2; small G protein	M64595		71	G proteins
Ras-related C3 botulinum toxin substrate 1; p21-rac1; ras-like protein TC25	M29870	55	88	G proteins
RhoGDP dissociation inhibitor 1 (Rho-GDI1); Rho-GDI α (RDIA1); ARHGDI A	X69550		93	GTP/GDP exchangers and GTPase activity modulators
*78 kDa glucose-regulated protein precursor (GRP 78)	M19645		95	Immune system proteins, heat shock protein
*Bone morphogenetic protein 4 (BMP4)+bone morphogenetic protein 2B (BMP2B)	D30751		77	Growth factors, cytokines and chemokines
Macrophage inhibitory cytokine 1 (MIC1)	AF019770		65	Growth factors, cytokines and chemokines
*Erythroid potentiating activity (EPA); fibroblast collagenase inhibitor; TIMP1	X03124 P01033		72	Protease inhibitors
TRAMP protein	X63679		66	Trafficking proteins
*v-ki-RAS2B proto-oncogene (KRAS2)	M54968		83	Oncogenes and tumour suppressors, G proteins
RBP2 retinoblastoma binding protein	S66431		89	Transcription factors, cell cycle proteins
*Apoptosis regulator bax	L22474		66	Bcl family proteins
trk-T3; P68 TRK-T3 oncoprotein	X85960		98	Growth factor and chemokine receptors
CENP-F kinetochore protein	U19769		84	DNA-binding and chromatin proteins
*VEGF precursor; vascular permeability factor (VPF)	M32977 M27281	57		Growth factors, cytokines and chemokines
Genes regulated bi-directionally				
Early growth response protein 1 (EGR1)	X52541	2.4	78	Transcription activators and repressors
Cartilage-specific proteoglycan core protein (CSPCP)	M55172 P16112	67	99	Extracellular matrix proteins
*Bone morphogenetic protein 2A (BMP2A)	M22489	67	57	Growth factors, cytokines and chemokines

*Genes previously shown in the literature to be regulated by E₂.

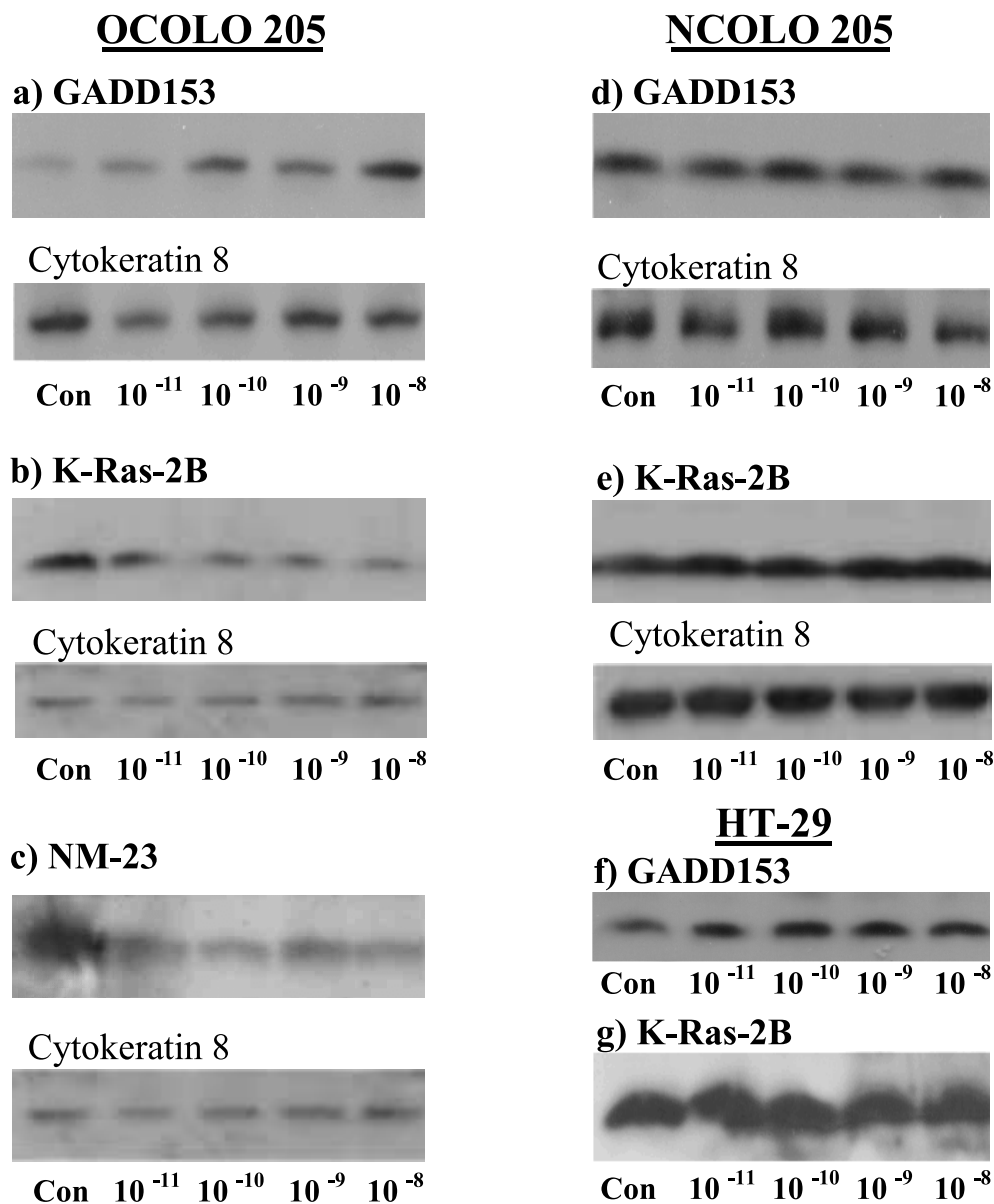


Figure 2 Western blots showing expression of (a) GADD153, (b) K-Ras-2B and (c) NM23-H1 (NM-23) expression in OCOLO 205 cells following E_2 treatment. Expression of (d) GADD153 and (e) K-Ras-2B in NCOLO 205 cells and (f) GADD153 and (g) K-Ras-2B in HT-29 cells following E_2 treatment are shown. (a–e) Blots were stripped and reprobed with cytokeratin 8 as further confirmation of equal loading and these blots are shown in the lower blots of each pair. Cells were cultured in 10% CSFCS for 3 days before treatment with various concentrations of E_2 for 24 h in serum-free media. Protein was extracted, equal amounts were loaded onto SDS-PAGE and Western immunoblotting was performed. Representative images are shown. Con, control.

E_2 treatment in OCOLO 205 cells induced GADD153, a leucine zipper transcription factor able to heterodimerize with members of the CEBP family of transcription factors (Ron & Habener 1992). It was originally identified based on its induction following treatment of cells with growth-arresting and DNA-damaging agents. GADD153 has been

correlated with the onset of apoptosis through down-regulation of Bcl-2 (McCullough *et al.* 2001), p38 and other mechanisms (Russo *et al.* 2002). Studies in GADD153 knockout mice suggest a role in apoptosis during the endoplasmic reticulum stress response in kidney cells (Bruhat *et al.* 1997). In a study using gene array and

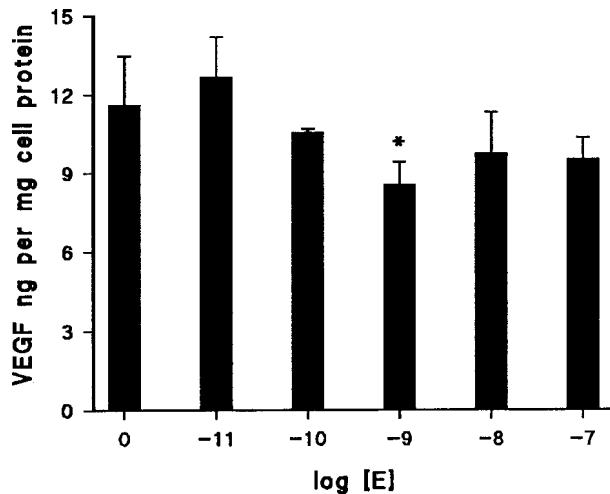


Figure 3 Effect of E₂ treatment on secretion of VEGF measured by ELISA. Conditioned media were collected from triplicate wells of OCOLO 205 cells treated with E₂ for 48 h. The amount of VEGF secreted was corrected for mg cell protein and plotted against E₂ concentration. Values are means ± s.d. (n=3). *P < 0.05 (ANOVA and Dunnett's post test).

HT-29 colon cancer cells, GADD153 expression was increased following activation of peroxisome proliferator-activated receptor γ which induced apoptosis in these cells (Shimada *et al.* 2002).

TRAP1, which binds to the intracellular domain of the tumour necrosis factor receptor, was also significantly increased. TRAP1 is postulated to be a direct myc target, as part of a pathway that leads to increased apoptosis in myc-overexpressed cells (Coller *et al.* 2000). Up-regulation of cytosolic serine hydroxymethyltransferase (SHMT) by E₂ was unexpected. SHMT is a key enzyme involved in folate metabolism (Stover *et al.* 1997). Much epidemiological and experimental evidence is in accord with such protection from colon cancer by folate (Choi & Manon 2000) and our result suggests that there may be mechanistic features in common between E₂ and folate protection.

E₂ treatment also led to down-regulation of 78 kDa glucose-regulated protein (GRP 78) in OCOLO 205 cells. GRP 78 is a chaperone that guides proteins through the folding process, and the up-regulation in response to endoplasmic reticulum stress increases the cell's capacity to cope with the accumulation of immature, misfolded proteins in the endoplasmic reticulum. If GRP 78 induction is prevented, cell survival diminishes greatly following treatment with agents that stress the endoplasmic reticulum (Jamora *et al.* 1996).

Activating Ras mutation occurs in 50% of colon cancer and 90% of these are K-Ras mutations associated with the early stage of colon cancer development (Gryfe *et al.* 1997). Two isoforms generated by alternative splicing of K-Ras exist, i.e. K-Ras-2A, which is relatively rare, and

K-Ras-2B. Sequencing of the COLO 205 K-Ras-2B gene (data not shown) showed that it is mutated in codon 6 leading to a leucine to proline change but whether this is an activating mutation is not known. Down-regulation of K-Ras-2B by treatment with E₂ was confirmed by both cDNA array analysis and Western immunoblotting consistent with an E₂-mediated reduction in the proliferative and anti-apoptotic properties of K-Ras-2B.

Evidence is accumulating that ER α and ER β function differently and our findings support this. We found down-regulation of both NM23-H1 and VEGF in ER β -expressing cells by E₂ in OCOLO 205 cells whereas, in three ER α -dominant breast cancer cell lines, an increase in NM23-H1 expression was seen (Lin *et al.* 2002) and, in MCF-7 cells which express ER α , VEGF was increased by E₂ treatment (Bogin & Degani 2002). NM23-H1 was first identified as a metastatic inhibitor in a murine melanoma cell line (Steege *et al.* 1988). Expression of the human NM23 homologue is also lower in human breast cancers of high metastatic potential than in breast cancers of low metastatic potential (Bevilacqua *et al.* 1989). However, Haut *et al.* (1991) reported that, in the colon, no difference in the expression of NM23-H1 was found among colon tumours with high and low metastatic potential (Haut *et al.* 1991). Furthermore, in addition to its correlation with metastasis, NM23-H1 expression is also directly related to cell proliferative activity (Keim *et al.* 1992). Further clarification of its role in cell cycle control is clearly needed.

Neovascularisation is a critical requirement for tumour growth and metastasis. A connection between E₂ exposure and inhibition of angiogenesis is suggested by inhibition of VEGF mRNA and protein secretion. Higher concentrations of E₂ (1 nmol/l) were required to decrease VEGF secretion than to induce apoptosis (10 pmol/l) in the cells but the effects of VEGF are paracrine, to act on the endothelial cells, and not involved in the apoptosis effected by E₂. Several studies have suggested that VEGF is the angiogenic factor most closely associated with induction and maintenance of the neovasculature in human colon cancer (Takahashi *et al.* 1995, 1997, Ellis *et al.* 1996, Warren *et al.* 1996). In primary tumours, the expression of VEGF mRNA is increased in tumours relative to histologically normal bowel mucosa (Berse *et al.* 1992, Brown *et al.* 1993) and further analyses have implicated VEGF expression in tumour progression and metastasis (Warren *et al.* 1995).

If the genes which we have identified as up- or down-regulated by E₂ in apoptosis-sensitive cells are causally associated, then overlap with findings in other cell types is to be expected. Ten (36%) of the identified genes have been previously reported to be targets of E₂. The other genes, not previously linked with E₂-associated apoptosis outside the colon therefore deserve further study. Among proteins known to be involved in apoptosis but not previously shown to be regulated by E₂ are TRAP1,

Table 2 E₂-responsive but not apoptosis-related genes: genes regulated by E₂ in both OCOLO 205 and NCOLO 205 cells. Gene regulation was considered significant when the increase is >2-fold or the decrease is >50%

	Accession number	Fold induction by 24 h		Classification
		NCOLO 205	OCOLO 205	
Genes up-regulated				
Endonuclease III homolog 1 (HNTH1) (OCTS3)	U79718	3.71	14.02	DNA damage repair proteins and ligases
Putative regulatory protein TGFβ-stimulated clone 22 homologue (TSC22)	U35048	10.92	9.88	Transcription activators and repressors
TIS11B protein; EGF response factor 1 (ERF1); butyrate response factor 1 (BRF1)	X79067	34	16.52	Transcription activators and repressors
ets-related transcription factor E74-like factor 1 (ELF1)	M82882	#	8.96	Transcription activators and repressors
		% reduction by 24 h		
Genes down-regulated				
*c-jun proto-oncogene; *transcription factor AP-1	J04111	52	56	Oncogenes and tumour suppressors Transcription activators and repressors
*APC interacting protein (EB1) protein	U24166	61	79	Intracellular transducers, effectors and modulators Oncogenes and tumour suppressors
Synapse-associated protein 102 (SAP102); neuroendocrine-DLG (NE-DLG; DLG3; human homologue of <i>Drosophila</i> discs large (DLG)	U49089	85	71	Intracellular transducers, effectors and modulators Oncogenes and tumour suppressors
retinoic acid activated CDNA1 (RATS1)	U37688	99	99	Other apoptosis-associated proteins Cell cycle-related proteins Oncogenes and tumour suppressors
BRCA1-associated ring domain protein	X82200	98	100	Transcription activators and repressors
V(D)J recombination activating protein 1 (RAG1)	M29474	97	100	Transcription activators and repressors
rac-α serine/threonine kinase (rac-PKα); *protein kinase B (PKB); c-akt; akt1	M63167	90	71	Recombination proteins Death kinases, intracellular kinase network members, Oncogenes and tumour suppressors
fibroblast growth factor homologous factor (FHF-1)	U66197	82	77	Transcription proteins
Ku 70 kDa subunit; ATP-dependent DNA helicase II 70 kDa subunit; lupus ku autoantigen protein P70; thyroid-lupus auto-antigen (TLAA); CTC box binding factor 75 kDa subunit (CTC75)	M32865 S38729	83	82	DNA damage repair proteins and ligases DNA polymerases, replication factors and topoisomerases Stress response proteins

*Genes previously reported to be regulated by E₂.

#There was no expression in control cells; TGF, transforming growth factor; EGF, epidermal growth factor.

Table 3 Genes showing a greater than 5-fold difference between OCOLO 205 and NCOLO 205 cells (O:N) by cDNA array. Analyses were performed on mRNAs isolated from cells incubated in serum-free conditions for 8 and 24 h

Name of gene	GeneBank no.	O:N at 8 h	O:N at 24 h
(1) Placental calcium-binding protein; calvasculin; S100 calcium-binding protein A4; MTS1 protein	M80563	– 0.31	– 0.81
(2) Retinoic acid receptor α 1	X06538; X06614+M73779		– 0.84
(3) CREB-binding protein C12m	U47741		5.11
(4) trk-T3; P68 TRK-T3 oncoprotein	X85960		10.22
(5) Tenascin precursor; hexabrachion; cytotactin; neuronectin; motendinous antigen; glioma-associated extracellular matrix antigen	X78565; M55618	– 0.81	– 0.79
(6) Elongation factor 2	X51466		– 0.91
(7) human transforming growth factor β induced gene product (BIGH3)	M77349		– 0.88

sentrin, 40S ribosomal protein S16, and RAD21 which were all increased. Genes down-regulated, not previously shown to be E₂ sensitive, include retinoblastoma binding protein 2 which has been shown to potentiate nuclear hormone receptor-mediated transcription (Chan & Hong 2001) and the tissue inhibitor of metalloprotease 1 (TIMP1).

We have identified several genes that were regulated by E₂ in both NCOLO 205 and OCOLO 205 cells. This suggests that if these genes are involved in apoptosis, then either they are not critical, or they lie on alternative pathways not facilitated completely by E₂. BRF1 was markedly increased in both cell strains. BRF1 is an immediate early gene which is induced rapidly and usually transiently in response to growth factor or hormone stimulation. In HT-29 cells this gene was down-regulated by butyrate which induces differentiation in these cells (Maclean *et al.* 1998). The transcription factor ELF1 was also increased in both cell types. C-jun which acts with c-fos to form the transcription factor AP1 was down-regulated as was breast cancer type I susceptibility protein 1 (BRCA1)-associated ring domain protein (BARD1). Half of familial breast cancer cases are found to have mutations, usually resulting in truncations, in the breast cancer susceptibility gene, BRCA1. Significant although lesser excesses were also observed for colon cancer (Ford *et al.* 1994). BARD1 is one of several partners that can interact with BRCA1. In mammary epithelial cells, reductions in BARD1 with antisense RNAs induced a premalignant phenotype, suggesting that it could function as a tumour suppressor. In the colon cancer cells, we found that physiological concentrations of E₂ induced marked reductions in BARD1 at 24 h. Whether the cells express BRCA1, what the function of either protein is, and whether the cells express mutated forms of either protein awaits clarification. That BARD1 may have importance in colon carcinogenesis is indicated in a study which showed that autoantibodies to cleaved BARD1 have antitumoral activity against colon cancers (Gautier *et al.* 2000).

Possible explanations for the difference in responsiveness to E₂ between OCOLO 205 and NCOLO 205 were also provided by the gene arrays. OCOLO 205 cells were found to express 5-fold more CBP than NCOLO 205 cells when incubated in serum-free and E₂-free medium for 24 h. CBP has been discovered to bind specifically to the protein kinase A-phosphorylated form of the CREB and to enhance steroid receptor-dependent target gene transcription (Shibata *et al.* 1997). Greater expression of CBP in OCOLO 205 cells, which apoptose upon E₂ treatment, indicates that this gene may importantly affect responsiveness to E₂. The decreased expression of the mRNA for RAR α in the OCOLO 205 cells may also be significant in this regard. RAR α is an important mediator of apoptosis in many cancer cells. The relationship between the increased expression of TRK-T3 oncogene, which is a constitutively activated version of the nerve growth factor receptor, and ER β sensitivity is hard to explain, although TRK-T3 activates many signalling pathways, not all of which co-operate to increase cell transformation (Roccatto *et al.* 2002).

We conclude that use of apparently similar cell lines which respond differently to a specific stimulus is a valuable way of obtaining insight into underlying mechanisms. Our data based on such studies suggest that there are specific apoptotic pathways activated by E₂ in colon cancer cells.

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References

- Berse B, Brown LF, Van de Water L, Dvorak HF & Senger DR 1992 Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Molecular Biology of the Cell* **3** 211–220.
- Bevilacqua G, Sobel ME, Liotta LA & Steeg PS 1989 Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential. *Cancer Research* **49** 5185–5190.
- Blobel GA & Orkin SH 1996 Estrogen-induced apoptosis by inhibition of the erythroid transcription factor GATA-1. *Molecular and Cellular Biology* **16** 1687–1694.
- Bogin L & Degani H 2002 Hormonal regulation of VEGF in orthotopic MCF7 human breast cancer. *Cancer Research* **62** 1948–1951.
- Bruhat A, Jousse C, Wang XZ, Ron D, Ferrara M & Fafournoux P 1997 Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels. *Journal of Biological Chemistry* **272** 17588–17593.
- Calle EE, Miracle-McMahill HL, Thun MJ & Heath CW Jr 1995 Estrogen replacement therapy and risk of fatal colon cancer in a prospective cohort of postmenopausal women. *Journal of the National Cancer Institute* **87** 517–523.
- Campbell-Thompson M, Lynch I J & Bhardwaj B 2001 Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. *Cancer Research* **61** 632–640.
- Chan SW & Hong W 2001 Retinoblastoma-binding protein 2 (Rb2) potentiates nuclear hormone receptor-mediated transcription. *Journal of Biological Chemistry* **276** 28402–28412.
- Choi SW & Mason JB 2000 Folate and carcinogenesis an integrated scheme. *Journal of Nutrition* **130** 129–132.
- Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN & Golub TR 2000 Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *PNAS* **97** 3260–3265.
- Ellis LM, Liu W & Wilson M 1996 Down-regulation of vascular endothelial growth factor in human colon carcinoma cell lines by antisense transfection decreases endothelial cell proliferation. *Surgery* **120** 871–878.
- Foley EF, Jazaeri AA, Shupnik MA, Jazaeri O & Rice LW 2000 Selective loss of estrogen receptor beta in malignant human colon. *Cancer Research* **60** 245–248.
- Ford D, Easton DF, Bishop DT, Narod SA & Goldgar DE 1994 Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Lancet* **343** 692–695.
- Gautier F, Irminger-Finger I, Gregoire M, Meflah K & Harb J 2000 Identification of an apoptotic cleavage product of BARD1 as an autoantigen: a potential factor in the antitumoral response mediated by apoptotic bodies. *Cancer Research* **60** 6895–6900.
- Grodstein F, Newcomb PA & Stampfer MJ 1999 Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. *American Journal of Medicine* **106** 574–582.
- Gryfe R, Swallow C, Bapat B, Redston M, Gallinger S & Couture J 1997 Molecular biology of colorectal cancer. Ras and colon cancer. *Current Problems in Cancer* **21** 233–300.
- Gujuluva CN, Baek JH, Shin KH, Cherrick HM & Park NH 1994 Effect of UV-irradiation on cell cycle, viability and the expression of p53, gadd153 and gadd45 genes in normal and HPV-immortalized human oral keratinocytes. *Oncogene* **9** 1819–1827.
- Haut M, Steeg P S, Willson J K & Markowitz SD 1991 Induction of nm23 gene expression in human colonic neoplasms and equal expression in colon tumors of high and low metastatic potential. *Journal of the National Cancer Institute* **83** 712–716.
- Hebert-Croteau N 1998 A meta-analysis of hormone replacement therapy and colon cancer in women. *Cancer Epidemiology, Biomarkers and Prevention* **7** 653–659.
- Jamora C, Dennert G & Lee AS 1996 Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10 ME. *PNAS* **93** 7690–7694.
- Kawahara, K, Oyadomari S, Gotoh T, Kohsaka S, Nakayama H & Mori M 2001 Induction of CHOP and apoptosis by nitric oxide in p53-deficient microglial cells. *FEBS Letters* **506** 135–139.
- Keim D, Hailat N, Melhem R, Zhu X X, Lascu I, Veron M, Strahler J & Hanash SM 1992 Proliferation-related expression of p19/nm23 nucleoside diphosphate kinase. *Journal of Clinical Investigation* **89** 919–924.
- Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138** 863–870.
- Lin KH, Wang WJ, Wu YH & Cheng SY 2002 Activation of antimetastatic Nm23-H1 gene expression by estrogen and its alpha-receptor. *Endocrinology* **143** 467–475.
- Lobenhofer EK, Bennett L, Cable PL, Li L, Bushel PR Afshari CA 2002 Regulation of DNA replication fork genes by 17 beta-estradiol. *Molecular Endocrinology* **16** 1215–1229.
- Luethy JD, Fargnoli J, Park JS, Fornace AJ Jr & Holbrook NJ 1990 Isolation and characterization of the hamster gadd153 gene. Activation of promoter activity by agents that damage DNA. *Journal of Biological Chemistry* **265** 16521–16526.
- Maclean KN, McKay IA & Bustin SA 1998 Differential effects of sodium butyrate on the transcription of the human TIS11 family of early-response genes in colorectal cancer cells. *British Journal of Biomedical Science* **55** 184–191.
- McCullough KD, Martindale JL, Klotz LO, Aw TY & Holbrook NJ 2001 Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Molecular and Cellular Biology* **21** 1249–1259.
- Maytin EV, Ubeda M, Lin JC & Habener JF 2001 Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. *Experimental Cell Research* **267** 193–204.
- Muramatsu M & Inoue S 2000 Estrogen receptors: how do they control reproductive and nonreproductive functions? *Biochemical and Biophysical Research Communications* **270** 1–10.
- Nilsen J, Mor G & Naftolin F 2000 Estrogen-regulated developmental neuronal apoptosis is determined by estrogen receptor subtype and the Fas/Fas ligand system. *Journal of Neurobiology* **43** 64–78.
- Qiu Y, Waters CW, Lewis AE, Langman MJS & Eggo MC 2002 Oestrogen-induced apoptosis in colonocytes expressing oestrogen receptor β . *Journal of Endocrinology* **174** 369–377.
- Ramsay G 1998 DNA chips state-of-the art. *Nature Biotechnology* **16** 40–44.
- Roccatto E, Miranda C, Ranzi V, Gishizki M, Pierotti MA & Reco A 2002 Biological activity of the thyroid TRK-T3 oncogenes requires signalling through Shc. *British Journal of Cancer* **87** 645–653.
- Ron D & Habener JF 1992 CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes and Development* **6** 439–453.
- Russo J, Tahin Q, Lareef MH, Hu YF & Russo IH 2002 Neoplastic transformation of human breast epithelial cells by estrogens and chemical carcinogens. *Environmental and Molecular Mutagenesis* **39** 254–263.
- Shibata H, Spencer TE, Onate SA, Jenster G, Tsai SY, Tsai MJ & O'Malley BW 1997 Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Progress in Hormone Research* **52** 141–164.
- Shimada T, Kojima K, Yoshiura K, Hiraishi H & Terano A 2002 Characteristics of the peroxisome proliferator activated receptor gamma (PPARgamma) ligand induced apoptosis in colon cancer cells. *Gut* **50** 658–664.
- Smirnov P, Liel Y, Gnainsky J, Shany S & Schwartz B 1999 The protective effect of estrogen against chemically induced murine

- colon carcinogenesis is associated with decreased CpG island methylation and increased mRNA and protein expression of the colonic vitamin D receptor. *Oncology Research* **11** 255–264.
- Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J & Santen RJ 2001 Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 beta-estradiol. *Journal of the National Cancer Institute* **93** 1714–1723.
- Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA & Sobel ME 1988 Evidence for a novel gene associated with low tumor metastatic potential. *Journal of the National Cancer Institute* **80** 200–204.
- Stover PJ, Chen LH, Suh JR, Stover DM, Keyomarsi K & Shane B 1997 Molecular cloning, characterization, and regulation of the human mitochondrial serine hydroxymethyltransferase gene. *Journal of Biological Chemistry* **272** 1842–1848.
- Szelei J, Soto AM, Geck P, Desronvil M, Prechtel NV, Weill BC & Sonnenschein C 2000 Identification of human estrogen-inducible transcripts that potentially mediate the apoptotic response in breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **72** 89–102.
- Takahashi Y, Kitadai Y, Bucana CD, Cleary KR & Ellis LM 1995 Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Research* **55** 3964–3968.
- Takahashi Y, Tucker SL, Kitadai Y, Koura AN, Bucana CD, Cleary KR & Ellis LM 1997 Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. *Archives of Surgery* **132** 541–546.
- Ubeda M & Habener JF 2000 CHOP gene expression in response to endoplasmic-reticular stress requires NFY interaction with different domains of a conserved DNA-binding element. *Nucleic Acids Research* **28** 4987–4997.
- Warren RS, Yuan H, Matli MR, Gillett NA & Ferrara N 1995 Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *Journal of Clinical Investigation* **95** 1789–1797.
- Warren RS, Yuan H, Matli MR, Ferrara N & Donner DB 1996 Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. *Journal of Biological Chemistry* **271** 29483–29488.
- Zhang CC & Shapiro DJ 2000 Activation of the p38 mitogen-activated protein kinase pathway by estrogen or by 4-hydroxytamoxifen is coupled to estrogen receptor-induced apoptosis. *Journal of Biological Chemistry* **275** 479–486.

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