Interplay of Genes Regulated by Estrogen and Diindolylmethane in Breast Cancer Cell Lines

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Diindolylmethane (DIM), a biologically active congener of indole-3-carbinol (I3C) derived from cruciferous vegetables, is a promising agent for the prevention of estrogen-sensitive cancers. Both DIM and estrogen affect transcription of genes by binding receptors, such as aryl hydrocarbon receptor (AhR) or estrogen receptors (ER). Gene regulation by DIM and estradiol (E2) can be very complex. While DIM typically binds the AhR, this complex can directly associate with the ER, recruit co-activators that bind to estrogen-responsive promoters, and activate transcription. Alternately, DIM can bind the ER directly. In this study, we have analyzed gene expression using microarray profiling and quantitative real time–polymerase chain reaction in MCF7 breast cancer cells treated with E2 (1 nM) or DIM (25 μM) alone or in combination for 16 h. The interplay of E2 and DIM was reflected in the expression of a subset of genes (<90) in which the combination of E2 and DIM acted either additively or antagonistically to alter gene expression.

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INTRODUCTION

I3C, DIM, and Estrogen-Enhanced Cancers

Indole-3-carbinol (I3C) and its chief condensation product, diindolylmethane (DIM), are naturally occurring phytochemicals from cruciferous vegetables that stimulate a number of cellular responses that are proapoptotic, anti-proliferative, and anti-estrogenic, i.e., processes incompatible with tumor development (1-3). Conversely, estrogen-initiated activity can lead to increased replication (4-7) and inhibition of apoptosis (8), processes that are amenable with the development of tumors. Many animal studies indicate that I3C has anti-tumor efficacy for breast, cervical, and endometrial cancers (9-12), indicating that I3C holds promise for the prevention of estrogen-enhanced cancers. Both I3C and DIM abrogate estrogen-enhanced cell proliferation (1,2), and the amount of apoptosis depends on the relative amount of I3C versus stradiol (E2) (13). Studies indicate that I3C and DIM affect estrogen, for example, by inducing enzymes that modulate estrogen metabolism (14-16). I3C induces the expression of the tumor suppressor gene BRCA1 (17), which inhibits ER α-regulated gene expression (18). I3C and DIM bind to estrogen receptors (ER) (19) and can compete with estrogen in reporter gene assays to inhibit estrogen dependent gene expression (20).

Estrogen, I3C, DIM, and Gene Expression

Estrogen, I3C, and DIM regulate multiple genes, and this regulation is not always mutually exclusive. 17β-Estradiol (E2) regulates genes by binding to either ER-α or ER-β, forming a complex, which binds the estrogen-responsive elements (ERE) in the promoter of estrogen-dependent genes (21,22). I3C or DIM binding to the aryl hydrocarbon receptor (AhR) activates it, resulting in nuclear translocation and complex formation with the basic helix loop helix region of the aryl hydrocarbon nuclear translocator protein (ARNT). The AhR/ARNT complex serves as a transcriptional unit, binding to highly conserved enhancer sequences termed xenobiotic response elements in promoters of relevant genes (23). However, I3C and DIM can also bind the ER acting as agonist (18,19) as well as competing with E2 for this binding (24). DIM-dependent AhR/ARNT-complex associates with and co-activates unliganded ER, modulating the estrogen-driven transcriptional signature, and thus, may act to

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enhance estrogenic activity (25). In contrast, DIM or its precursor I3C, have been found to inhibit estrogen-induced genes (26), carcinogen-induced rat mammary tumor formation, as well as the growth of estrogen-dependent tumors in a mouse xenograft model (27,28,11).

In this paper, we report the interplay between DIM and E2 upon the gene expression profile of the E2-responsive breast cancer cell line MCF-7.

MATERIALS AND METHODS

Reagents

17β-Estradiol and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO, USA). DIM was a gift from Dr. M. Zeligs, BioResponse, Boulder, CO, USA.

Cell Lines and Cultures

The breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained as monolayer cultures at 37°C in 5% CO_2 and were grown in Dulbecco's modified Eagle's medium (DMEM) that contained 4.5 g glucose and bicarbonate/L (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 110 mg of sodium pyruvate/L, 200 mmol glutamine/L, 100 mL of fetal bovine serum/L, and 100,000 U each of penicillin and streptomycin/L.

Microarray

The experiment was performed on estrogen responsive breast cancer MCF7 cells treated with combinations of DIM and E2 for 16 h. Four different sets of culture conditions were used on 1×10^5 MCF7 cells, and the cultures were done in triplicate. Cells grown in culture supplemented with 1 nM E2, cells supplemented with 25 μM DIM, and cells supplemented with 1 nM E2 and 25 μM DIM combination. As E2 and DIM were solubilized with DMSO, it served as vehicle control.

Microarray profiling

Total RNA was prepared from MCF-7 cells treated in the 4 culture conditions using the Qiagen RNeasy Kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA). Total RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and the 260/280 ratio was determined using the BioSpec-1601 Spectrophotometer (Shimadzu, Columbia, MD, USA). cDNA and cRNA were processed following the Ambion message AMP cRNA kit (Ambion, Austin, TX, USA) employing a T-7oligo-dT primer according to the manufacturer's instructions. Biotinylated 16-UTP (Roche-Boehringer-Mannheim, Palo Alto, USA) and 11-CTP (Perkin Elmer, Wellesley, MA, USA) were used during the in vitro transcription process, and the cRNA was fragmented and hybridized to Affymetrix Human Genome U133A chips (Affymetrix, Santa Clara, CA, USA), which contains over 20,000 known genes. The hybridized chips were stained with phycoerythrin-streptavidin and washed to remove nonspecific signal according to the Affymetrix protocol. The chips were scanned using a laser confocal scanner manufactured by Hewlett Packard. The expression levels were calculated by Affymetrix's Microarray Suite 5.0, and the overall intensity values were all scaled to the same value, 1500.

Statistical Analysis

Microarray data analysis with Genespring (Agilent Technologies) was done by importing Affymetrix MAS5.0 data. Principle component analysis with the different treatment types indicated two predominant patterns showing additive up- and down-regulation of genes in cells treated with E2 + DIM (E + D) compared with E2 or DIM alone. Genes of these two expression patterns were filtered on flags and on confidence with a *P* value of less than 0.05. This list was further filtered with a fold change cut off of 1.5 for E + D group for both up- and down-regulation. This gave a final list of 32 additively up-regulated genes and 46 additively down-regulated genes for E + D group. Important genes from these two lists were confirmed by quantitative polymerase chain reaction (QPCR).

Pathway Analysis

Pathway analysis was conducted using Pathway Studio Central version 1.1 (Ariadne Genomics, MD, USA). We imported the Genespring list of up- or down-regulated genes to initiate database mining. The software retrieves the most relevant networks that are differentially perturbed in a disease or provides insights into the common regulatory mechanisms of the set of genes. The networks are graphically displayed and allow validation by referral to the original abstracts or articles the facts were drawn from. The database can be queried for all known interaction or pathways that involve a specific protein or target molecules as well as for common relationships among a group of proteins. The software uses ResNet, a comprehensive molecular network database compiled by MedScan containing more than 500,000 events of regulation, interaction, and modification among thousands of proteins, cell processes, and small molecules. This is displayed as a global network of molecular interactions with pathways being sub-networks. This allows the building of individual as well as interplays among pathways.

Quantitative real time (RT)-PCR

RT-PCR was performed using TaqMan chemistry. TaqMan primers and probes were designed using Primer Express software version 1.5 (Applied Biosystems, Foster City, CA, USA) and synthesized at Feinstein Institute core facility. The probes were labeled with FAM at the 5′ end and TAMRA at the 3′ end. The relative expression of the various genes was determined using the Eurogentec RTqPCR mastermix (Eurogentec, Belgium) and ABI PRISM 7700 Sequence Detection System. The PCR mix contained 1 X master mix and 0.125 μL of Euroscript + RT and Rnase Inhibitor (RT-0.125 U/μL and Rnase Inhibitor 0.05 U/μL). Optimal concentrations of primers, probes, and the RNA were standardized. The final concentrations of forward and reverse primers and probes for PGDH, LDLR, CXCR4, and IFIP were 500 nm and 200 nm, respectively. The concentration of B-actin primers and probe, served as the internal housekeeping gene control were 500 nm and 100 nm, respectively. Fifty nanograms of total RNA were used per 25 μL reaction with all samples run in duplicate. The thermal cycler conditions were 48°C for 30 min, 95°C for 10 min, and 45 cycles of 95°C for 0.15 min, and 60°C for 1 min. Data was analyzed using Sequence Detection System (SDS) software version 1.9.1. Results were obtained as Ct (threshold cycle) values. Ct is inversely proportional to the starting template copy number. Relative expression in all samples was calculated in comparison with untreated control samples using *delta delta Ct method*. Results were expressed as change with respect to the experimental control.

Primers and Probes

Gene	ASNS
FP	5′TGGTTAAATATCATCACTGTCGGG
RP	5′AAC CTG GAA AGA GTT TCT CCA CAT
Probe	5′TGA ACC CCT GCA CGC CCT CTA TGA-TAMRA

Gene	CXCR 4
FP	5′TGAGAAGCATGACGGACAAGTAC
RP	5′GGGAAGCGTGATGACAAAGAG
Probe	5′CTGCACCTGTCAGTGCCGACCT

Gene	PGDH
FP	5′CCTGAAGAATGCTGGGAACTG
RP	5′ACATCCGCCTGCTTGGAA
Probe	5′CTAAGCCCCGCAGTCATTGTCG

Gene	CYP1A1
FP	5′AGCGGAAGTGTATCGGTGAGA
RP	5′AATTCCACCCGTTGCAGC
Probe	5′CATTGCCCGCTGGGAGGTCTTTCT

Gene	CYP1B1
FP	5′TTTCGGCTGCCGCTACA
RP	5′CGAACTCTTCGTTGTGGCTG
Probe	5′CGACGACCCCGAGTTCCGTGAG

Gene	H-SCD (solute carrier family 7)
FP	5′GAGTACCGCTGGCACATCAA
RP	5′ATGGCGGCCTTGGAGACT
Probe	5′CCGCCCTCGGTCTGGCCTATG

Gene	P8 (P8 protein-candidate of metastasis)
FP	5′CGCTGAGACAGAGCTGGAGAT
RP	5′CTCCGCAGTCCCGTCTCTATT
Probe	5′AGGCCAGACCATGGACACTACACCCA

Gene	TGFb1 (Camurati-Engelmann disease)
FP	5′CCCTGCCCCTACATTTGGA
RP	5′GCCCGGGTTATGCTGGTT
Probe	5′ACACGCAGTACAGCAAGGTCCTGGC

RESULTS

Microarray Analysis of Cell Lines

MAS5 analysis. We were interested in genes whose expression was modulated by treatment with E2 and DIM when compared with control treatment with DMSO. A list of genes was compiled, and these same genes were examined for additive increases or decreases in expression levels (fold changes) when E2 and DIM were used in combination. We found four different sets of gene lists: (a) genes changed by E2, but not by DIM or the combination; (b) genes changed by DIM but not by E2 or the combination; (c) genes changed by E2 or DIM and the expression of the genes were enhanced by the combination of both E2 and DIM; (d) genes modulated by E2 or DIM and the expression dampened by the combined effect. We have focused on genes where we found additive effects when E2 and DIM were present together in the same culture, as in sets three and four (data not shown).

We analyzed gene expression profiles from three replicates in which MCF-7 cells were treated for 16 h with concentrations of E2 (1 nM) or DIM (25 μM) or the combination of both E2 and DIM. We imported our data as a tab-delimited file into GeneSpring software, where we were able to filter for genes of interest, i.e., genes whose expression reflected interplay of E2 and DIM.

Genes Significantly Up- or Down-regulated by Treatment with E2 and DIM but Not E2 or DIM Alone

Table 1 is a list of genes whose expression was significantly increased in the E2 + DIM combination when compared with the DMSO control, but not significantly changed in E2 or DIM alone when compared with the DMSO control. The up-regulated genes were 32 in number, and the fold change ranged from 3.93 for NM_000104, Cytochrome P450, CYP1B1 to 1.5 for AF070587.1 deleted in liver cancer 1. The genes whose expression were significantly decreased by the combination of E2 + DIM but not by E2 or DIM alone are shown in Table 2. We identified 46 down-regulated genes whose fold changes ranged from 2.21 for NM_006156 NEL to about 2 to 1.5 for NM_015950 mitochondrial ribosomal protein L2.

Increased Gene Expression by Combination Treatment with E2 + DIM or Inhibition by E2 + DIM Confirmation by Quantitative Real Time-Reverse Transcriptase PCR (Q-RT-PCR)

We selected genes of interest from the microarray data that were highly expressed or suppressed in the E2 + DIM combination and showed a minor or no change in the E2 or DIM only samples. We have confirmed these microarray results by Q-RT-PCR analysis with an aliquot of the RNA extracted from the MCF7 cells treated under four experimental conditions (DMSO [control], E2 [1 μM] alone, DIM [25 μM] alone, or the combination of E2 and DIM) and used in the processing for the microarray analysis. We selected six genes that showed increased expression with the combined treatment of E2 and DIM: CYP1A1, CYP1B1, PGDH, AS, P8, Caldesmon, and SCD. We also looked at three genes that

Table 1. fold change of genes whose expression is increased by the combination of E2 + DIM

Gene title	Genbank	EvsC ± SE	DvsC ± SE	E+D ± SE
Cytochrome P450 CYP1B1	NM_000104	1.389 ± 0.12	3.101 ± 0.22	3.93 ± 0.25
Cytochrome P450, CYP1B1	NM_000104.2	1.436 ± 0.15	2.738 ± 0.26	3.32 ±0.32
Cytochrome P450, CYP1B1	NM_000104.2	1.581 ± 0.13	2.617 ± 0.53	2.86 ± 0.66
Cytochrome P450 CYP1A1	NM_000499	0.773 ± 0.13	2.605 ± 0.61	2.84 ± 0.51
Stearoyl-CoA desaturase (delta-9-desaturase)	AF116616.1	1.533 ± 0.14	1.96 ± 0.34	2.40 ± 0.28
Caldesmon 1	NM_018495	1.708 ± 0.32	1.75 ± 0.33	2.37 ± 0.31
Solute carrier family 7/ member 11	AB040875.1	1.444 ± 0.17	1.566 ± 0.19	1.90 ± 0.13
p8 protein (candidate of metastasis 1)	AF135266.1	1.118 ± 0.12	1.395 ± 0.45	1.92 ± 0.27
Interferon-induced protein/ tetratricopeptide repeats 1	NM_001548	0.93 ± 0.17	1.438 ± 0.13	1.88 ± 0.20
Nucleophosmin/nucleoplasmin, 3	NM_006993	1.72 ± 0.22	1.775 ± 0.16	1.85 ± 0.16
Amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)	X06989.1	1.717 ± 0.39	1.624 ±0.31	1.84 ± 0.32
Zinc finger protein 557	NM_024341	1.324 ± 0.12	1.559 ± 0.21	1.83 ± 0.20
Like Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial precursor	NM_025001	1.263 ± 0.13	1.055 ± 0.20	1.81 ± 0.23
Asparagine synthetase	NM_001673	1.267 ± 0.16	1.303 ± 0.32	1.81 ± 0.25
GTP bp overexpressed in skeletal muscle	NM_005261	1.666 ± 0.12	1.133 ± 0.19	1.78 ± 0.21
TBC1 domain family, member 5	NM_014744	1.486 ± 0.11	1.395 ± 0.16	1.76 ± 0.12
Fatty acid binding protein 5 (psoriasis-assoc)	NM_001444	1.564 ± 0.12	1.33 ± 0.12	1.75 ± 0.12
Clathrin, heavy polypeptide (Hc)	NM_014127	1.406 ± 0.24	1.43 ± 0.29	1.75 ± 0.85
Solute carrier family 33 (acetyl-CoA transporter)1	NM_004733	1.338 ± 0.15	1.348 ± 0.13	1.69 ± 0.21
Hypothetical protein FLJ21148	NM_024860	1.469 ± 0.17	1.424 ± 0.17	1.68 ± 0.16
Thyroid receptor interacting protein 15	AA496247	1.088 ± 0.15	1.226 ± 0.13	1.68 ± 0.20
FK506 binding protein 14, 22 kDa	NM_017946	1.165 ± 0.15	1.247 ± 0.21	1.65 ± 0.16
Stromal antigen 2	BC001765.1	1.359 ± 0.43	1.553 ± 0.81	1.65 ± 1.29
Kelch-like 9 (Drosophila)	AW138594	1.495 ± 0.17	1.38 ± 0.13	1.64 ± 0.26
Phosphoglycerate dehydrogenase	NM_006623	1.167 ± 0.10	1.399 ± 0.17	1.64 ± 0.13
Diacylglycerol kinase, epsilon 64kDa	NM_003647	1.119 ± 0.25	1.53 ± 0.16	1.62 ± 0.24
KIAA0746 protein	AB018289.1	1.227 ± 0.10	1.361 ± 0.19	1.62 ± 0.16
Aldo-keto reductase family 1, member C3	NM_003739	0.901 ± 0.13	1.309 ± 0.18	1.62 ± 0.13
Insulin induced gene 1	NM_005542	1.395 ± 0.11	1.41 ± 0.27	1.56 ± 0.14
Esterase D/formylglutathione hydrolase	AU145746	1.306 ± 0.15	1.078 ± 0.16	1.55 ± 0.15
Transducin (beta)-like 1X-linked receptor 1	NM_030921	1.42 ± 0.15	1.341 ± 0.13	1.53 ± 0.14
Ataxin 1	AW235612	1.361 ± 0.36	1.361 ± 0.35	1.53 ± 0.24
Similar to 0610010D24Rik protein	AF070587.1	1.121 ± 0.12	1.337 ± 0.18	1.52 ± 0.12
Deleted in liver cancer 1	AF026219.1	1.361 ± 0.15	1.108 ± 0.12	1.50 ± 0.14

demonstrated decreased expression when treated with the combination of E2 and DIM, CXCR4, TGFb-1, and BCLL-6. All genes chosen from the microarray experiment and subsequently confirmed by QPCR were analyzed for correlation between the two gene expression technologies. The correlation coefficient is shown in each figure for comparison. The results of this analysis are shown in Figure 1.

Pathway analysis

We imported our Genespring lists of additively up-regulated genes in cells treated with a combination of DIM and E2 into PathwayStudio. We sought to analyze the relationships and biological interactions among the up-regulated genes identified by combination treatment. Figure two displays the pathways retrieved from the PathwayStudio software when we put in three of our up-regulated genes.

In a like manner, we also analyzed the common regulators and/or biological interactions among our incrementally suppressed or down-regulated genes by querying the relationships between several of the genes showing the most significantly diminished gene expression, data not shown.

DISCUSSION

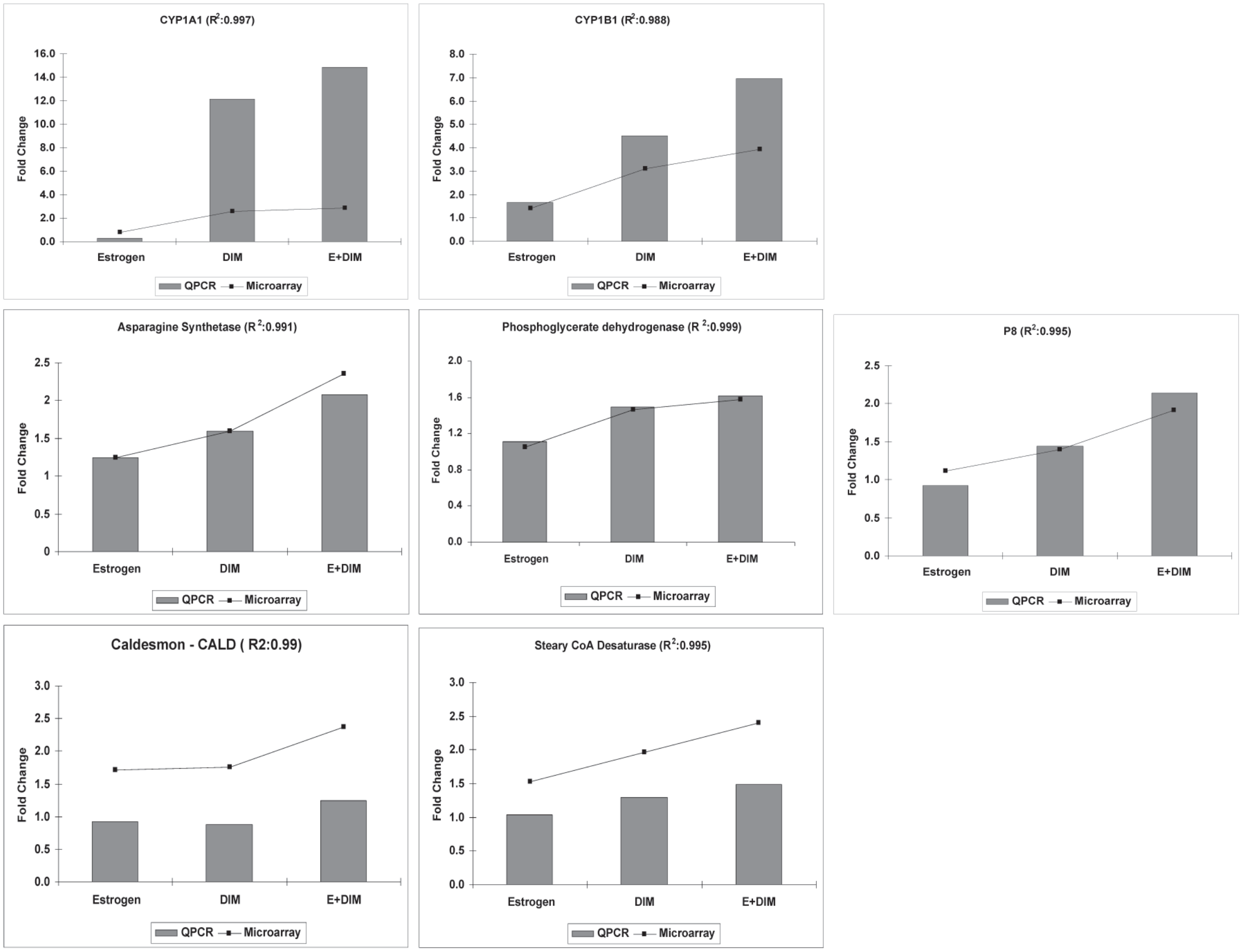
These results indicate that there are a subset of genes in MCF7 whose expression can be modulated by the combination of E2 and DIM. The expression of 32 genes was significantly up-regulated or enhanced by combination treatment with E2 and DIM. Expression of 46 genes was down-regulated by the combination of E2 and DIM in the MCF7 cultures. The subset of genes analyzed by QRT-PCR confirmed the results of microarray profiling. Clearly, the expression of many genes is interdependent on both E2 and DIM in cells that respond to estrogen.

Table 2. Fold change of genes whose expression is diminished by the combination of E2 + DIM

Gene Title	Genbank	EvsC + SE	DvsC + SE	E+D + SE
Mitochondrial ribosomal protein L2	NM_015950	0.758 ± 0.09	1.035 ± 0.22	0.666 ± 0.10
Tumor protein p53 (Li-Fraumeni disease)	NM_000546	0.873 ± 0.11	0.889 ± 0.15	0.663 ± 0.10
HERV-H LTR-associating 3	NM_007071	0.942 ± 0.12	0.929 ± 0.16	0.662 ± 0.14
Lipin 2	U55968	0.983 ± 0.09	0.82 ± 0.16	0.659 ± 0.10
ATP-binding cassette, D (ALD), 1	NM_000033	0.894 ± 0.09	0.911 ± 0.13	0.653 ± 0.10
Cytoplasmic FMR1 interacting protein 2	NM_030778	0.903 ± 0.09	0.844 ± 0.17	0.653 ± 0.11
Endothelin converting enz 1	BF969352	0.858 ± 0.11	0.723 ± 0.19	0.648 ± 0.11
Aldehyde dehydrogenase 3 B1	BC002553.1	0.84 ± 0.09	0.718 ± 0.15	0.648 ± 0.12
ATP-binding cassette,G 1	U34919.1	0.754 ± 0.12	0.809 ± 0.14	0.648 ± 0.11
Kelch domain containing 3	BF063121	0.949 ± 0.16	1.11 ± 0.14	0.639 ± 0.11
Erythropoietin receptor	M34986.1	0.782 ± 0.09	0.788 ± 0.15	0.636 ± 0.12
Tripartite motif-containing 3	AA114843	0.764 ± 0.10	0.743 ± 0.15	0.633 ± 0.12
GPI-anchored metastasis-associated proteinhomolog	NM_014400	0.693 ± 0.09	0.88 ± 0.13	0.631 ± 0.10
DEAD box polypeptide 54	NM_024072	1.185 ± 0.17	0.863 ± 0.25	0.619 ± 0.18
Distal-less homeobox 4	NM_001934	0.777 ± 0.11	0.944 ± 0.15	0.618 ± 0.12
Slingshot homolog 3 (Drosophila)	NM_018276	0.861 ± 0.11	0.936 ± 0.13	0.617 ± 0.14
Synaptobrevin 2	BC002737.1	0.675 ± 0.10	1.09 ± 0.26	0.617 ± 0.12
Ribosomal protein S6 kinase, 90kDa, polypeptide 4	NM_003942	0.813 ± 0.12	0.912 ± 0.19	0.615 ± 0.10
Chromodomain helicase DNA binding protein 3	BE379542	0.751 ± 0.15	0.848 ± 0.12	0.615 ± 0.10
TGFB1-induced anti-apoptotic factor1	NM_004740	0.858 ± 0.09	0.915 ± 0.17	0.612 ± 0.12
Agrin	AK021586.1	0.849 ± 0.10	1.187 ± 0.18	0.607 ± 0.12
Kynurenine 3-monooxygenase	AI074145	0.744 ± 0.11	0.697 ± 0.13	0.601 ± 0.11
Chr 20 ORF 149	NM_024299	0.796 ± 0.14	1.169 ± 0.31	0.596 ± 0.11
SH3 domain binding glutamic-acid-rich protein like	NM_003022	0.787 ± 0.13	0.748 ± 0.14	0.594 ± 0.11
EST	AI560951	0.697 ± 0.10	0.655 ± 0.12	0.594 ± 0.10
ATPase, Ca++ transporting, ubiquitous	NM_005173	0.816 ± 0.12	0.777 ± 0.14	0.591 ± 0.12
KIAA0912 protein	AK025247.1	0.843 ± 0.13	0.837 ± 0.17	0.588 ± 0.14
E74-like factor 5 (ets like)	AF115403.1	0.77 ± 0.11	0.748 ± 0.12	0.586 ± 0.11
Tyr 3-/Trp 5-monooxygenase activation protein, ?	U28936.1	0.864 ± 0.11	0.965 ± 0.20	0.585 ± 0.16
TGFbeta 1	BC000125.1	0.725 ± 0.10	0.863 ± 0.16	0.583 ± 0.10
Homeo box A6	NM_024014	0.615 ± 0.11	0.712 ± 0.16	0.575 ± 0.13
Agrin	AF016903.1	0.86 ± 0.11	0.87 ± 0.13	0.572 ± 0.12
Chondroitin polymerizing factor	NM_024536	0.837 ± 0.12	1.068 ± 0.15	0.569 ± 0.10
B-cell CLL/lymphoma 6	NM_001706	0.707 ± 0.09	0.739 ± 0.14	0.568 ± 0.10
CCR4-NOT transcription complex, 3	NM_014516	0.803 ± 0.09	1.044 ± 0.22	0.562 ± 0.12
Collagen, type VII, alpha 1	NM_000094	0.786 ± 0.09	0.912 ± 0.13	0.552 ± 0.10
Aldehyde oxidase 1	NM_001159	0.631 ± 0.15	0.664 ± 0.28	0.552 ± 0.12
Solute carrier family 4	NM_003040	1.021 ± 0.10	0.835 ± 0.16	0.551 ± 0.15
Oxidised low density lipoprotein R1	AF035776.1	0.614 ± 0.12	0.76 ± 0.16	0.546 ± 0.15
B-cell CLL/lymphoma 3	NM_005178	0.754 ± 0.17	0.853 ± 0.16	0.532 ± 0.12
TCR gamma variable 9	M13231.1	0.703 ± 0.11	0.569 ± 0.13	0.525 ± 0.11
Actin, gamma 2, smooth muscle	NM_001615	0.946 ± 0.10	0.619 ± 0.13	0.503 ± 0.10
Neurofibromin 1	D12625.1	0.655 ± 0.16	0.576 ± 0.14	0.497 ± 0.17
Keratin 15	NM_002275	0.657 ± 0.10	0.642 ± 0.12	0.486 ± 0.17
SH3-domain GRB2-like endophilin B2	NM_020145	1.109 ± 0.18	0.917 ± 0.16	0.482 ± 0.11
NEL-like 2 (chicken)	NM_006159	0.814 ± 0.09	0.723 ± 0.13	0.451 ± 0.10

In general, the effect of the combination treatment of E2 and DIM on MCF7 cells was antagonistic, reflected in the greater number of down-regulated genes observed in our microarray analysis. This finding is consistent with previous reports that showed interactions that were primarily inhibitory in studies both in vitro and in vivo (27,28). Many of the changes are subtle, but show an effect over a wide variety of affected genes, including transcription factors as well as numerous metabolic gene products whose expression is diminished in comparison to the findings of treatment with E2, or for that matter DIM alone. Where the changes in gene expression are modest, it is important to note that, while in-

Up-regulated genes



Down-regulated genes

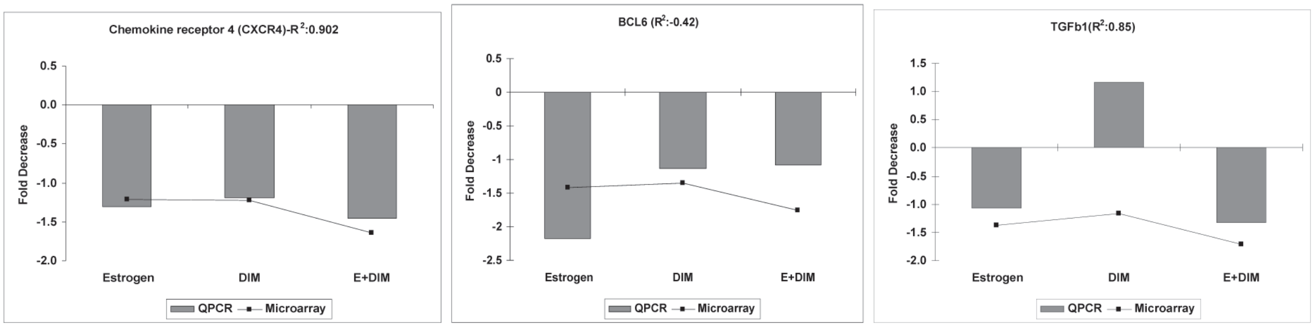


Figure 1. Validation of microarray data by quantitative real time PCR with the corresponding correlation coefficients. The figure is comprised of two sets of data, up-regulated genes, indicated by the heading showing seven genes with increased fold changes for cells treated with E2 + DIM, and down-regulated genes heading three genes whose expression is decreased with treatment of E2 + DIM. The vertical bars indicate relative mRNA expression in E2, DIM, and E2 + DIM. The expression levels were normalized against DMSO treated MCF-7 cells. Microarray fold change is shown as a line along the bars. The one-step QRT-PCR was performed by TaqMan chemistry. B-actin was used as endogenous control.

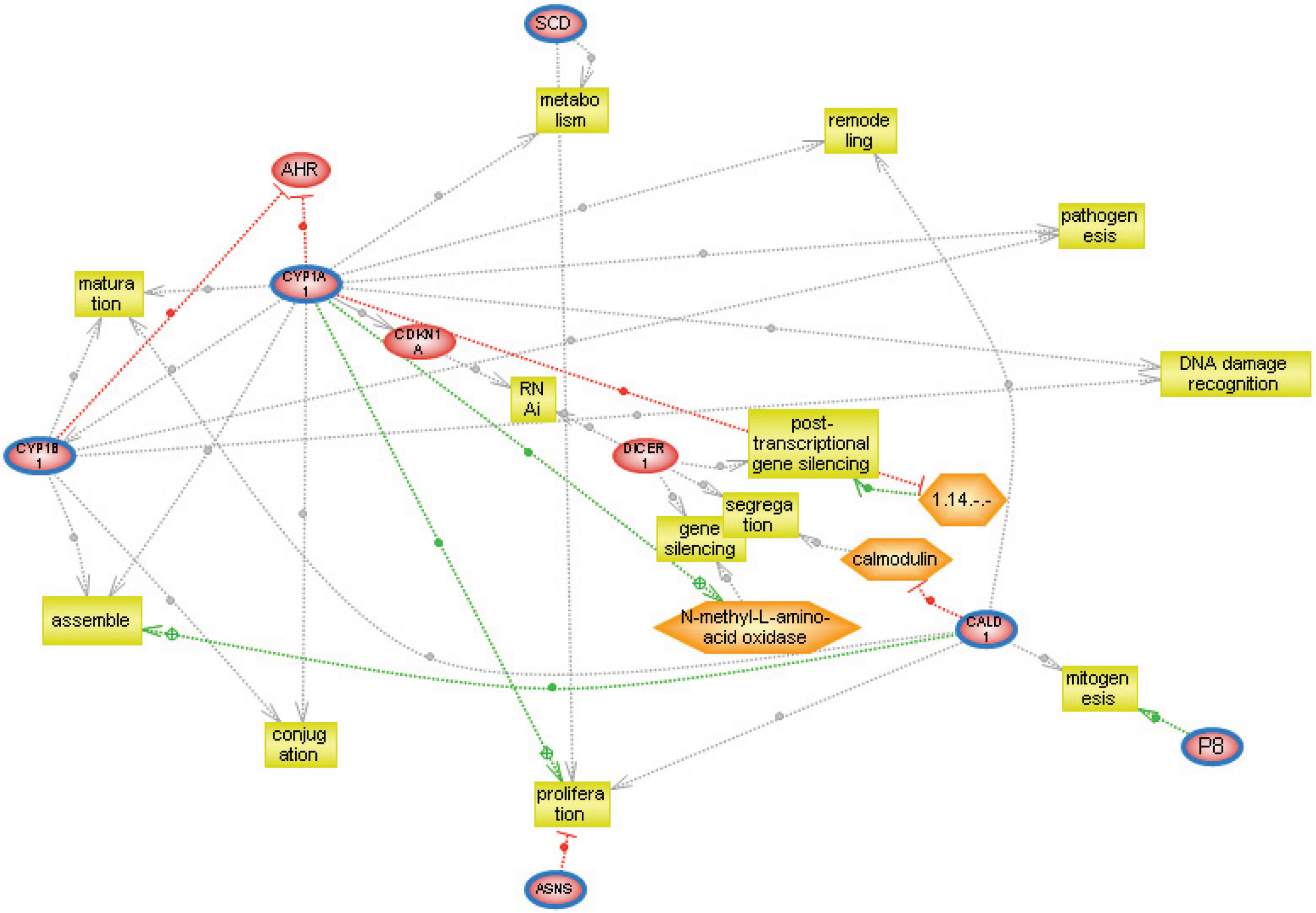


Figure 2. Pathway analysis was performed using PathwayStudio software by importing three of the 32–up-regulated genes in our list. The key to color identification of network components is as follows: yellow, cell processes; orange, functional class; red, protein; red with blue border, genes up-regulated; red connection lines, negative regulation; green connection lines, positive regulation; grey lines, unknown regulation type.

triguing, these gene modulations are only possibilities and will need additional studies to determine whether or not they significantly impact the behavior of the cell culture.

Previous studies by Chen et al. (26) using subtractive hybridization indicated that DIM could decrease the expression of a number of genes up-regulated by E2 and hypothesized cross-talk between AhR and ER. Our studies support these findings, but additionally showed that expression of a number of genes up-regulated in the presence of 25 μM DIM could be modulated in the presence of 1 nM E2, with expression either dampened by E2 or enhanced by E2. Alternately, some genes whose expression was up-regulated by E2 could be further enhanced by DIM. Finally, the expression of some genes was only detectable when the cells were exposed to the combination of E2 and DIM. These observations dramatically increase the complexity of the interplay by E2 and DIM and how they might affect the microenvironment of a cell. The study raises questions as to imbalances of the relative amount of these compounds. It is clear that the risk of breast cancer is inversely proportional to the amount of cruciferous vegetables in diet (the natural source of DIM) (29), and that more estrogen increases risk of breast cancer (30). Relative amounts of these compounds in combination may prove crucial to the protective or preventative benefits of DIM (or cruciferous vegetables).

As one example, DIM induces the expression of CYP1A1 and CYP1B1 members of the P450 superfamily. This study indicated that a further enhancement of expression of these genes occurred in the presence of E2. CYP1A1, a phase I enzyme, can be the first step in the detoxification of number of carcinogens. Conversely, it is known to convert many procarcinogens to carcinogens. Importantly, CYP1A1 increases 2-hydroxylation of estrone leading to 2-hydroxyestrone (not estrogenic), and which is rapidly

O-methylated into compounds that are anti-proliferative, pro-apototic, and anti-angiogenic. However, induction of CYP-1B1 shifts metabolism toward 4-hydroxyestrone, which can be carcinogenic. An imbalance of estrogen metabolism is indicated in breast (31), cervical (32), and endometrial (unpublished results) cancers, which are all estrogen-enhanced cancers where I3C and DIM appear to be preventative. Systemic lupus erythematosis and rheumatoid arthritis, diseases predominantly affecting women, also have abnormal estrogen metabolism (33). An animal study indicated that a diet rich in I3C ameliorated the lupus disease and changed estrogen metabolism (34).

An interesting aspect of altered estrogen metabolism is found in the up-regulation of aldo-keto reductase family 1, member 3 (AKR1C3), an isomer of the AKR superfamily that is found most prominently in prostate and mammary glands, in samples treated with the combination of E2 and DIM. This enzyme has the ability to interconvert testosterone with 4-androstene-3-17-dione but inactivate 5a-DHT and, therefore, eliminate active androgens from the prostate. In the mammary gland, AKR1C3 may function predominantly as a reductase to produce testosterone from 4-androstene-3-17-dione in an intracrine manner and to reduce estrone to estradiol (35). Recently, it has been shown that levels of these steroid-metabolizing genes are diagnostic of tumor versus normal breast tissue (36). Levels of AKR1C3 are reduced in tumor versus normal tissue, which reflect our findings in E2 treatment alone compared with treatment with both E2 and DIM. The induction of this gene, which has been correlated well with its enzyme activity, suggests a rebalancing to normal steroid-metabolism homeostasis.

One can predict numerous effects of how genes affected by DIM and E2 change the microenvironment of a cell. For instance, the DEAD box polypeptide 4 is a member of a family of genes characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD). These proteins are putative RNA helicases that mediate nucleoside triphosphate-dependent unwinding of double-stranded RNA. They are thought to be involved in a variety of cellular processes that involve modification of RNA secondary structure, i.e., translation initiation, nuclear and mitochondrial splicing, ribosome and spliceosome assembly. Members of this family are alleged to be involved in embryogenesis, spermatogenesis, and cellular growth and division.

It is of interest that the combination of DIM and E2 leads to a decrease in expression of genes such as Bcl-6, a zinc finger nuclear phosphoprotein, normally expressed in the germinal center B cells and some intrafollicular T cells. This gene codes for a DNA-binding transcriptional repressor that exerts an important role in the development of normal germinal centers. Its constitutive expression has been associated with suppression of p53 expression as well as phenotypic changes in germinal center cells by affecting differentiation and/or apoptosis. Mature germinal center B cells that leave the germinal center environment generally down-regulate Bcl-6 expression. A block in normal down-regulation of Bcl-6 has been postulated to cause genetic instability in the germinal center cells, and subsequently leads to neoplastic transformation (37). Additionally the down regulation of Bcl3 in cells treated in combination with E2 and DIM disrupts a signaling pattern observed in MCF7 cell (38) where Bcl3 complexes with phosphorylated Bcl10 and translocates to the nucleus, where it alters transcription.

The mixture of E2 and DIM treatment dampens the expression of cytoplasmic FMR1 interacting protein 2, which associates with FMRP (Fragile X mental retardation protein) as well as FMRP-related proteins FXRIP and FXR2P. The protein is cytoplasmically colocalized with FMRP and ribosomes, and is thought to interact with RAC1. RAC1 is a small GTPase that stimulates actin polymerization toward lamellopodia formation, whose overexpression in tumor cells has been associated with invasion and metastasis in human tumor cells (39,40).

Intriguingly, CXCR4 is also down-regulated by the combination treatment with E2 and DIM. CXCR4 appears to be necessary for breast cancer metastasis (41). This interesting gene was significantly suppressed in one gene list (data not shown), but not in the other, yet bears mention. CXCR4, a cytokine, is the cognate receptor for stromal cell derived factor 1, and the expression of this complex in breast cancer cells is associated with significant increases in invasiveness and faster migration of these cells to the lymph nodes (42). Silencing CXCR4 gene expression with siRNA blocks in vitro invasion and in vivo metastasis of breast cancer cells in animal models (41,43). Also of note is the down-regulation of CCR4-NOT complex 3 in the presence of both E2 and DIM, suggesting a suppressive effect on global transcription through regulation of transcription factor TFIID. As a master switch, dampening the expression of this factor would result in myriad gene effects both positive and negative (44).

Many of the noted gene changes and effects are subtle in form, with regulation modulated rather than radically altered. These findings suggest interesting perturbations in a biological system in the presence of physiologic concentrations of hormone and low concentrations of bioactive chemicals present in the environment. As much of the effects of DIM and E2 shift metabolism toward a proapoptotic, moderated, proliferative state, our results are consistent with DIM offering an effective preventive adjuvant in a healthy nutritional regime.

We have examined the overlap between lists of genes identified by each of the analysis programs. Using MAS5 and dChip (45,46), we identified 17 genes whose expression was increased or up-regulated when the cells were treated with a combination of E2 + DIM. This is in comparison to 32 genes that were identified using our filtering criterion with GeneSpring. Between these two groups, there are six genes that are found on both lists. The six overlapping genes are among the largest fold changes on the dChip list while they are found throughout the list with GeneSpring. However, the list does completely overlap with the clustering data identifying enhanced gene expression when treated with the combination of E2 + DIM (data not shown). In a similar manner, we identified 14 genes whose expression was suppressed or inhibited by treatment with E2 + DIM using dChip and the same filtering criterion as for gene induction, contrasted with 46 genes identified with GeneSpring. Between these two groups were six genes in common.

The pathway analysis is consistent with our in vitro observations as well as our expression analysis of treatment of MCF7 cells with E2 or DIM or both. Among the up-regulated gene pathway we found interrelated cell processes, which contain other genes found in our up-regulated list but not included in our query, such as nuclear receptors, cyclin dependent kinase inhibitor, and dicer (a ribonuclease essential for RNA interference of small temporal RNA pathways which represses gene expression). These genes are in keeping with a program focused on increasing cell differentiation, through cytoskeletal development and assembly, detection of DNA damage and cell cycle arrest, as well as apoptosis, post translation, and gene silencing. These results suggest there may be common regulators or signal transduction pathways among these genes.

When we examined cell systems affected by the expression of BCL-6 and TGF-b, the expression of both is down-regulated in treatment of cells with E2 and DIM, we find a multitude of processes that are common targets of these molecules but not necessarily overlapping in their modulation. In fact, there are almost an equal number of processes, which are coordinately regulated by these genes as those that receive opposite and antagonistic signals from these powerful molecules. The net result may be due to the relative concentrations of each protein.

We noted common regulators between TGF-b and BCL6. These include MAPK1 and 3, ABL-1, FOXO 3a (belonging to the forkhead family of transcription factors, which may function as a trigger for apoptosis), tumor suppressor gene EP300, a cyclin D–related transcription factor, as well as cyclin D itself. In many ways, the paradoxical effects of the regulated genes we found with the E2 + DIM–treated cells mirror the complicated physiologic findings of estrogens and exposure to toxins such as TCDD and dioxin.

Joint effects of DIM and E2 are complicated. One possibility is coordinate gene regulation due to the binding of DIM to its cognate AhR receptor as well as unliganded ER complex. The binding of AhR/ARNT complex to its response elements functions as a *cis*-acting enhancer in the regulatory domains of its target genes, a representative group identified as the AhR gene battery (47). The presence of proximal regulatory elements to AhR binding sites within discrete chromosomal locals suggests a mechanism by which this complex can activate a number of other transcription factors (not AhR targets) and clusters of genes as a secondary effect of binding (48). The juxtaposition of ligands dependent AhR-ER binding may have a concerted effect on the activation of additional transcription factors with both induction as well as repression of gene expression. The concentration of receptors as well as the concentration of DIM and E2 would matter and determine how much E2 or DIM bind to their cognate receptors (DIM binding to AhR and E2 binding to ERs) or compete for binding to the opposing receptors.

This work focused on the combined activity of E2 and DIM on gene expression, i.e., how the combination would be different than individual effects. It is well established that most activities of I3C and DIM are not related to estrogen per se. Nonetheless, this study confirms that there is a strong interplay of estrogen and DIM, which is reflected in gene expression changes when both compounds are present.

Note added in proof: A recent paper by Liu et al. (49) showed AhR agonists directly activate ERa in MCF-7 breast cancer cells.

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