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Selective Estrogen Receptor Modulators: Discrimination of Agonistic *versus* Antagonistic Activities by Gene Expression Profiling in Breast Cancer Cells

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ABSTRACT

Selective estrogen receptor modulators (SERMs) such as tamoxifen are effective in the treatment of many estrogen receptor-positive breast cancers and have also proven to be effective in the prevention of breast cancer in women at high risk for the disease. The comparative abilities of tamoxifen *versus* raloxifene in breast cancer prevention are currently being compared in the Study of Tamoxifen and Raloxifene trial. To better understand the actions of these compounds in breast cancer, we have examined their effects on the expression of ~12,000 genes, using Affymetrix GeneChip microarrays, with quantitative PCR verification in many cases, categorizing their actions as agonist, antagonist, or partial agonist/antagonist. Analysis of gene stimulation and inhibition by the SERMs *trans*-hydroxytamoxifen (TOT) and raloxifene (Ral) or ICI 182,780 (ICI) and by estradiol (E2) in estrogen receptor-containing MCF-7 human breast cancer cells revealed that (*a*) TOT was the most E2-like of the three compounds, (*b*) all three compounds either partially or fully antagonized the action of E2 on most genes, with the order of antagonist activity being ICI > Ral > TOT, (*c*) TOT and Ral, but not ICI, displayed partial agonist/partial antagonist activity on a number of E2-regulated genes, (*d*) several stimulatory cell cycle-related genes were down-regulated exclusively by ICI, (*e*) the estrogen-like activity of Ral nearly always overlapped with that of TOT, indicating that Ral has little unique agonist activity different from that of TOT, and (*f*) some genes were specifically up-regulated by TOT but not Ral, ICI, or E2. Hence, gene expression profiling can discern fundamental differences among SERMs and provides insight into the distinct biologies of TOT, Ral, and ICI in breast cancer.

INTRODUCTION

Selective estrogen receptor modulators (SERMs) are characterized by their diverse range of agonist/antagonist actions on estrogen receptor (ER)-mediated processes. They have the ability to act as either ER antagonists by blocking estrogen action through its receptor, as ER agonists by displaying estrogen-like actions, or as ER partial agonists/antagonists with mixed activity. Frequently, these differences in SERM activity depend upon the target gene promoter, as well as the cell or tissue background (1). Two of the best characterized SERMs are tamoxifen and raloxifene (Ral), which are both considered to act predominantly as estrogen antagonists in breast cancer cells, blocking the effects of estrogens. However, even in breast cancer, SERMs have the potential to act as estrogen-like ER agonists when resistance to these compounds develop or in the case of certain ER mutations in which antiestrogens behave as estrogens (2–4). Despite this potential, tamoxifen has been successfully used for many years as adjuvant therapy for hormone-responsive breast cancer (5). Furthermore, in a large clinical trial, tamoxifen was shown to effectively prevent breast cancer in many women at high risk for the disease (6). In addition, tamoxifen reduced the occurrence of bone fractures in these women; however, some detrimental side effects such as an increased risk of endometrial cancer, stroke, and pulmonary embolism were also associated with tamoxifen treatment (7). Ral was examined in the Multiple Outcomes of Raloxifene Evaluation trial and found to be effective in reducing the incidence of osteoporosis in postmenopausal women, as well as the incidence of breast cancer but, unlike tamoxifen, without the increased risk of endometrial cancer (8, 9). On the basis of the positive outcome of these trials, the Study of Tamoxifen and Raloxifene trial was begun in 1999 to directly compare the effects of these two SERMs, tamoxifen and Ral, in prevention of breast cancer (10, 11).

The beneficial effects of SERMs on breast cancer were originally attributed to their ability to antagonize the actions of endogenous estrogens by competition for ER binding. More recently, X-ray structural work has demonstrated that when different ER ligands such as estradiol (E2), tamoxifen, Ral, and the antiestrogen ICI 182,780 (ICI) interact with the ligand binding domain of the receptor, distinctly different conformations of the receptor are induced (12–15). In addition, introduction of different mutations into the ER ligand binding domain demonstrated that the chemical nature of different ligands can determine which residues of the ligand binding domain can make contact with the ligand, additionally supporting the idea that ligands induce different conformations of the ER (3, 16, 17). It has been suggested that as a result of these different ligand-induced conformations, the ER can thereby recruit different coregulator proteins to target gene promoters and differentially mediate gene transcription (18). Although ER agonists like E2 recruit transcriptional coactivators such as those of the p160 family that can enhance gene transcription, SERMs and ICI generally do not appear to recruit coactivators; rather, they promote the recruitment of corepressors such as NCoR and SMRT that can actively suppress transcription of E2 target genes, thereby additionally antagonizing the action of estrogens (19–22).

SERMs, however, do not always act as ER antagonists, and they can often display estrogen-like agonist activity or mixed agonist/antagonist activity. The mechanisms for these mixed effects of SERMs depend on several factors, including the differing chemical nature of the ligand, the cell background, and the context of the gene promoter (23, 24). For example, tamoxifen has been shown to act either as a partial or full agonist on different DNA regulatory elements such as particular estrogen response elements, Sp1 sites, or activator protein 1 sites (25–29). Several lines of evidence also indicate that SERM agonist activity depends on different regions of the receptor than does E2 for its agonist activity. In particular, the NH_2-terminal transactivation domain activation function-1 is important for tamoxifen agonist activity, whereas the synergistic action of both activation function-1 and activation function-2 is important for estrogen-induced activity (30–33). It is also clear that tamoxifen and E2 use different regions of activation function-1, as was demonstrated with receptor mutations and different peptides that could antagonize either E2 or tamoxifen agonism (32, 34, 35). The agonist activity of SERMs at particular genes may be accompanied by the recruitment of the same (24) or distinct (36) coactivators to the ER as are recruited by E2. It has also been suggested that the availability of coregulators can determine whether tamoxifen will act as an agonist or antagonist. For

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example, in mouse embryonic fibroblast cells from an NCoR knockout model, tamoxifen displayed agonist activity through the ER, whereas it showed antagonist activity in wild-type mouse embryonic fibroblast cells (37). Also, in MCF-7 cells overexpressing SRC-1, tamoxifen but not Ral acted as an agonist on particular genes (24).

Although tamoxifen and Ral prevent E2-induced proliferation of breast cancer cells and are assumed to antagonize many of the actions of estrogens, it is not known globally which genes and gene networks in the cell are altered by SERMs, on which genes SERMs antagonize E2 regulation fully or only partially, and on which genes SERMs exert significant E2-like agonist effects in breast cancer cells. It is also not known to what extent the SERMs or ICI can regulate the expression of genes independently from E2 action, as we observed previously for quinone reductase (38). Furthermore, in contrast to tamoxifen and Ral, the antiestrogen ICI is thought to act as a pure antagonist through the ER, although its effects on gene expression have not been fully explored (39).

With the advent of microarray technology, the effects of SERMs on gene expression can be compared on a very large number of genes without any prior selection bias. In fact, gene expression profiling in breast cancer using microarrays has been carried out in a number of studies investigating genes overexpressed in breast cancer (40) or genes associated with clinical outcome or prognosis (41–43), response to chemotherapy (44), tumor aggressiveness (45), or classification of primary tumors (46–49). Several studies have also investigated gene expression patterns associated with the ER status of breast cancers (50–54). Although there have been several microarray studies examining the actions of SERMs (55–58), these have not directly compared the SERMs tamoxifen and Ral and ICI and their agonistic and/or antagonistic actions on a large set of estrogen-regulated genes. Therefore, in an effort to examine the effects of these three compounds on E2-regulated gene expression in ER-positive breast cancer cells, we carried out gene expression profiling using oligonucleotide microarrays. Our findings indicate that although the regulation of the majority of E2-regulated genes is either partially or fully reversed by the SERMs and by ICI, distinct differences can be observed among these ligands in their balance of agonist, partial antagonist, or full antagonist activities on the spectrum of E2-regulated genes. In addition, a unique subset of genes, encoding proteins that may have beneficial effects, was found to be regulated by the SERMs and/or ICI but not by E2.

MATERIALS AND METHODS

Cell Culture and RNA Extraction. MCF-7 human breast cancer cells were routinely cultured in MEM (Sigma Chemical Co., St. Louis, MO) supplemented with 5% calf serum (Hyclone, Logan, UT) and antibiotics. Four days before E2 treatment, cells were switched to phenol red-free MEM containing 5% charcoal dextran-treated calf serum. Media were changed on day 2 and day 4 of culture. Cells were treated with 10 nM E2 alone or in the presence of 1 μM ICI, Ral, or *trans*-hydroxy-tamoxifen (TOT) or with 1 μM ICI, Ral, or TOT alone in the absence of E2 for 8 or 48 h. Because multiple combinations of compounds were examined, these time points were chosen based on our earlier time course study of E2 regulation of gene expression in these cells (59). We observed generally similar patterns of gene stimulation at 4 and 8 h, but with more genes down-regulated at 8 *versus* 4 h; hence we selected 8 h as our first time point. Because we observed very similar gene regulations at 24 and 48 h, we selected 48 h as our later time point. Real-time

Table 1 *Primers for real-time PCR used for genes shown in figures*

	Forward primer	Reverse primer
36B4	5′-GTGTTCGACAATGGCAGCAT	5′-GACACCCTCCAGGAAGCGA
BLNK	5′-CCCCTACCCAGCTTTTCATCT	5′-TCACAGGCTCCAGCATACCA
Cal-R	5′-CCGCATACCAAGGAGAAGGTC	5′-ATACTCCAGCCGGTGTGTCAT
CDC2	5′-ACTGGCTGATTTTGGCCTTG	5′-TTGAGTAACGAGCTGACCCCA
Cyclin A2	5′-GAGGCCGAAGACGAGACG	5′-CCAAGGAGGAACGGTGACAT
IGFBP4	5′-AGCTTCAGCCCCTGTAGCG	5′-TCATCTTGCCCCCACTGGT
Mad4	5′-TGGACATAGAGGGCATGGAGT	5′-TGTAGTGGTCGTCCGCGTC
PLC-L2	5′-CAGAACAGGGTGTGGCACATA	5′-GGAGAGCCAGCCCTTTTCC
Rab30	5′-ATCTGGAGACCTCAGCCAAGG	5′-TCACTGATGAGTCGGCATGC
Rab31	5′-GCGGCTGATTCCTTGAAAGA	5′-CAATAGGTGCCATCGTGGTTG
RAP1GAP	5′-GGGCACCAACCACGAAATC	5′-CTCGAGCTTCACCTTGGTTGT

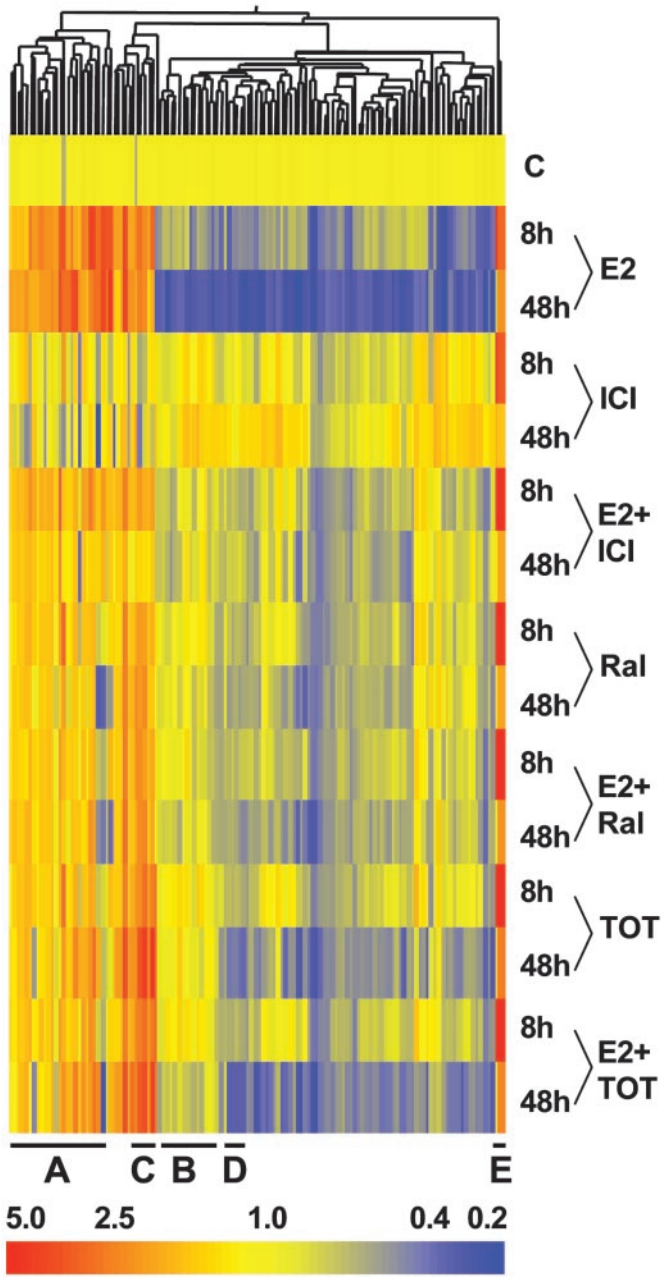

Fig. 1. Gene cluster analysis for estradiol (E2)-regulated gene expression and the effect of selective estrogen receptor modulators and ICI 182,780 (ICI) on genes significantly up- or down-regulated by E2. MCF-7 breast cancer cells were treated with 10 nM E2 and/or 1 μM ICI, raloxifene (Ral), or *trans*-hydroxytamoxifen (TOT) for either 8 or 48 h before cell harvest and Affymetrix gene chip microarray analysis. Gene cluster analysis was performed for the 40 up-regulated and 89 down-regulated genes found to be significantly regulated by E2 using GeneSpring software. Stimulated genes are shown in *red*, inhibited genes in *blue*, and genes not regulated in *yellow*. The color scale corresponding to fold change in gene expression is shown across the bottom. Clusters A and B represent genes that are up- or down-regulated, respectively, by E2 with the E2 regulation reversed by the selective estrogen receptor modulators and ICI. Clusters C and D demonstrate genes that are up- or down-regulated, respectively, by both E2 and TOT but not by Ral or ICI. Cluster E shows two genes that are stimulated by all of the treatments, especially at the 8 h time point.

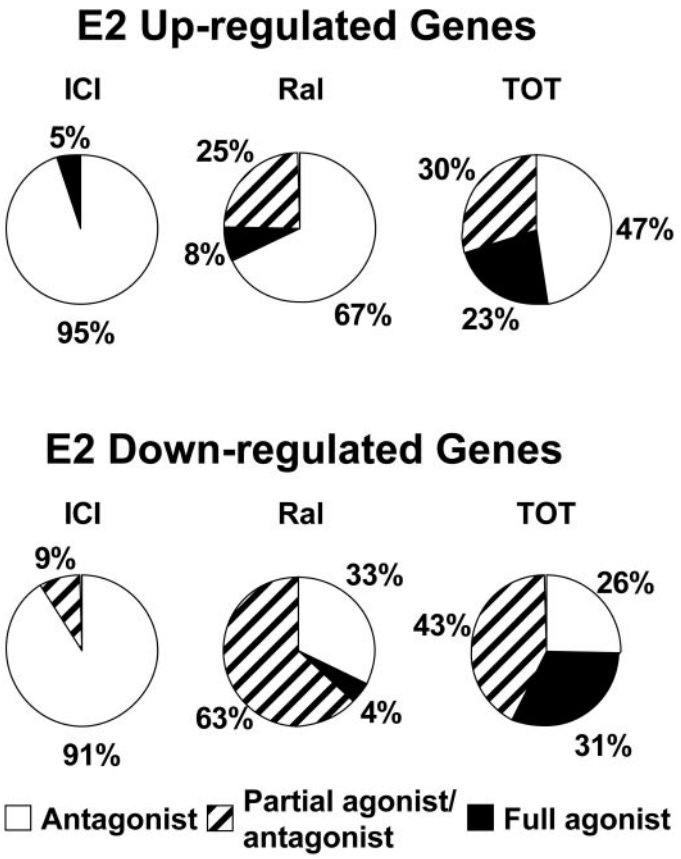

Fig. 2. Selective estrogen receptor modulator and ICI 182,780 (ICI) activity on genes up-regulated ($n = 40$) or down-regulated ($n = 89$) by estradiol (E2). ICI, raloxifene (Ral), or *trans*-hydroxytamoxifen (TOT) activity was defined as antagonistic (□) if it had no E2-like activity alone and it reversed the effect of E2, as fully agonistic (■) if it had >70% of E2's activity alone and did not antagonize the E2 effect, or as partially agonistic/antagonistic (▨) if it had between 35 and 70% of E2's activity alone and partially reversed the effect of E2.

PCR determinations involved three independent experiments and microarray determinations involved two independent experiments. Total RNA was prepared using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was additionally purified using RNeasy columns (Qiagen, Valencia, CA) and treatment with RNase free-DNase I (Qiagen).

GeneChip Microarrays. Total RNA was used to generate cRNA, which was labeled with biotin according to techniques recommended by Affymetrix (Santa Clara, CA). cRNA was then hybridized to Affymetrix Hu95A GeneChips, which contain oligonucleotide probe sets for ~12,000 human genes. After washing, the chips were scanned and analyzed using MicroArray Suite 5.0 software (Affymetrix). Average intensities for each GeneChip were globally scaled to a target intensity of 150. Additional analysis was performed using GeneSpring software V5.0.1 (Silicon Genetics, Redwood City, CA) to obtain fold-change and *P*s for each gene for each treatment relative to the vehicle control. The entire microarray data set will be available through the Gene Expression Omnibus accession no. GSE848.[3]

To identify genes significantly regulated by E2, gene lists were created in GeneSpring using a fold-change cutoff of 2.5 for up-regulated genes and 0.4 for down-regulated genes (*i.e.,* 2.5-fold down-regulation). We then compared this list to one we generated recently using a confidence scoring method in detailed E2 time course gene expression microarray profiling experiments also in MCF-7 cells (59). Only genes that were regulated in both studies were used because this set represents genes that are reproducibly and robustly regulated by E2. The SERMs (TOT and Ral) and ICI were then analyzed for their agonist or antagonist activity on this set of E2-regulated genes.

To identify unique genes, *i.e.,* genes that are regulated only by SERMs or ICI but not by E2, we used the following criteria: (*a*) a fold change for up-regulated genes of ≥2.0 for the SERMs or ICI but <1.3 for E2; (*b*) a fold change for down-regulated genes of ≤0.5 for the SERMs or ICI but >0.78 for E2; (*c*) *P* of <0.1; (*d*) present calls with SERM or ICI treatment; and (*e*) a raw expression level of >20. These cutoffs enable the identification of robust changes in gene expression, as documented in previously published microarray work of our lab and others (59, 60).

Real-Time PCR. Real-time PCR was carried out to verify regulation of gene expression by E2, SERMs, or ICI. One μg of total RNA was reverse transcribed in a total volume of 20 μl using 200 units of reverse transcriptase, 50 pmol random hexamer, and 1 mM deoxynucleotide triphosphates (New England Biolabs, Beverly, MA). The resulting cDNA was then diluted to a volume of 100 μl with sterile water. Each real-time PCR reaction consisted of 1 μl of diluted reverse transcription product, 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and 50 nM forward and reverse primer. Reactions were carried out in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) for 40 cycles (95°C for 15 s and 60°C for 1 min) after an initial 10 min incubation at 95°C. For the genes, the regulation of which is described in detail in this article, the primers used for real-time PCR are listed in Table 1. The fold change in expression of each gene was calculated using the ΔΔCt (threshold cycle) method, with the ribosomal protein 36B4 mRNA as an internal control (61).

RESULTS

Effects of SERMs and ICI on Genes Significantly Regulated by E2. Gene expression profiling was carried out on RNA from MCF-7 cells treated with E2, the SERMs Ral or TOT, or ICI, either alone or in combination with E2 for 8 or 48 h. As shown in Fig. 1 and as we recently reported (59), E2 inhibited the expression of about twice as many genes as E2 stimulated in these breast cancer cells. Two sets of genes, one highly and reproducibly up-regulated by E2 ($n = 40$) and one down-regulated by E2 ($n = 89$), were then identified as described in "Materials and Methods" and used for examining the effects of the SERMs or ICI either alone or in combination with E2. Gene cluster analysis, as shown in Fig. 1, reveals that the up-regulation (in red) and the down-regulation (in blue) of gene expression elicited by E2 were in large part reversed by each of the three compounds. This is clearly visible in the clusters labeled A (Fig. 1) for E2 up-regulated genes and B for E2 down-regulated genes. However, several exceptions to this as well as some additional interesting patterns of regulation can be

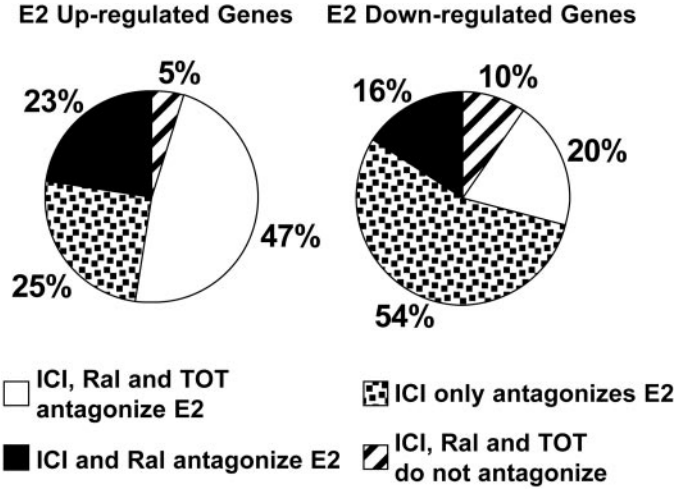

Fig. 3. Comparison of ICI 182,780 (ICI), raloxifene (Ral), and *trans*-hydroxytamoxifen (TOT) as antagonists on estradiol (E2)-regulated genes. □: genes on which all three compounds (ICI, Ral, and TOT; 1 μM) antagonize or reverse E2 (10 nM) activity and show no agonist activity alone. ▨: genes on which ICI only antagonizes E2 action, and Ral and TOT have either partial agonist/antagonist or full agonist activity. ■: genes on which ICI and Ral antagonize E2, and TOT has either full or partial agonist activity. ▨: genes on which ICI, Ral, and TOT all display either partial agonist/antagonist or full agonist activity.

[3] Internet address: http://www.ncbi.nlm.nih.gov/geo.

Table 2 *Genes up-regulated or down-regulated by estradiol (E2; 10 nM) on which ICI, Ral and trans-hydroxytamoxifen (TOT) (1 μM) all act as full or partial antagonists of the E2 response (i.e., reverse the E2 gene stimulation or the E2 gene inhibition) at one or both times analyzed*

Numbers are fold change *versus* vehicle-treated control cells.

			E2		ICI		E2 + ICI		Ral		E2 + Ral		TOT		E2 + TOT	
	Symbol	GenBank	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h
Gene name (E2 up-regulated genes)[a,b]																
Early growth response 3	EGR3	X63741	6.39	12.21	0.71	1.43	**1.77**	**1.51**	1.29	0.57	**1.95**	**0.38**	1.76	0.90	**1.41**	**1.93**
GREB1 protein	GREB1	AB011147	6.14	7.61	0.84	0.67	5.52	**3.49**	2.66	1.57	**2.67**	**2.26**	2.02	1.17	**2.63**	**2.24**
Amphiregulin	AREG	M30704	6.81	7.49	1.18	1.06	**2.82**	**1.96**	0.91	0.36	**0.67**	**0.43**	0.84	0.73	**0.67**	**0.24**
Chemokine ligand 12 (stromal cell-derived factor 1)	CXCL12	L36033	4.28	6.88	0.69	0.49	**1.53**	**1.18**	1.40	1.47	**1.63**	**1.90**	1.37	1.84	**1.59**	**2.05**
Protease, serine, 23	SPUVE	AF015287	6.07	4.38	0.91	1.18	**1.90**	**0.75**	0.95	0.61	**0.82**	**0.76**	0.92	0.81	**0.94**	**0.84**
Solute carrier family 22, member 5	SLC22A5	AF057164	3.21	3.04	0.93	1.01	***2.30***	**1.49**	1.71	1.62	**1.79**	**1.67**	1.39	1.47	**1.46**	**1.29**
Homeobox C4	HOXC4	X07495	3.08	3.03	0.62	0.49	**1.85**	**1.26**	1.62	1.32	**1.50**	**1.47**	1.36	1.30	**1.22**	**1.29**
Seven in absentia homologue 2	SIAH2	U76248	2.60	3.03	0.92	0.87	**1.50**	**1.17**	0.98	0.79	**0.96**	**0.93**	1.00	0.83	**0.89**	**0.69**
γ-adaptin (γ 1 subunit)	AP1G1	AL050025	3.95	2.89	0.95	0.92	**2.26**	**1.38**	1.67	1.51	**1.62**	**1.61**	1.44	1.19	**1.74**	**1.08**
Glycine receptor, β	GLRB	U33267	4.13	2.39	1.51	0.98	***2.76***	1.65	1.68	1.50	**1.76**	1.18	1.72	2.22	**2.56**	3.24
Carbonic anhydrase XII	CA12	AF037335	4.49	2.30	0.63	0.58	***3.38***	2.05	2.18	1.71	**2.29**	2.14	2.07	1.61	**2.29**	1.02
c-fos	FOS	V01512	3.14	2.08	0.96	1.00	***1.37***	1.14	1.11	0.94	**1.15**	1.04	1.12	1.05	**1.19**	1.35
CDC6 cell division cycle 6 homologue	CDC6	U77949	2.63	1.82	1.08	0.27	**1.51**	1.02	1.34	1.38	**1.22**	1.60	1.36	1.98	**1.30**	2.31
Ret/Ptc	RET	HG4679-HT5104	3.54	1.74	1.15	0.96	***2.48***	1.12	1.71	0.85	**1.04**	1.60	1.50	2.49	**1.69**	1.78
Ret/Ptc2	RET	HG4677-HT5102	2.71	1.37	1.12	1.00	***1.95***	1.39	1.17	1.49	**1.55**	1.42	1.52	2.97	**1.25**	2.90
WNT1 inducible signaling pathway protein 2	WISP2	AF100780	2.39	4.90	0.86	0.95	1.38	**1.08**	1.08	0.83	1.38	**0.94**	1.21	1.56	1.32	**1.96**
Adenylate cyclase 9	ADCY9	AF036927	1.83	2.53	0.98	0.81	1.64	**1.46**	1.19	1.57	1.47	**1.76**	1.44	1.09	1.14	**1.01**
Prostaglandin E receptor 3 (EP3)	PTGES	D86096	1.42	2.62	1.18	1.54	1.33	**1.22**	1.05	1.07	1.12	**1.43**	1.02	1.18	1.27	**1.13**
Gene name (E2 down-regulated genes)[a,c]																
Inhibin, β B	INHBB	M31682	0.73	0.18	0.83	1.07	0.77	**0.70**	0.83	0.94	0.99	**0.81**	1.06	0.94	1.19	**0.76**
Immediate early response 3	IER3	S81914	0.48	0.29	1.15	1.62	0.79	**0.75**	1.29	1.02	1.26	**1.04**	4.53	5.05	5.18	**4.49**
Erythropoietin receptor	EPOR	M60459	0.81	0.30	1.43	1.87	0.95	**1.17**	1.13	1.02	1.27	**0.89**	1.20	1.01	1.22	**0.77**
Phosphoribosyl pyrophosphate synthetase 1	PRPS1	X15331	0.60	0.32	0.86	0.84	0.60	***0.52***	0.84	0.87	0.92	**0.83**	1.05	0.95	1.05	**0.87**
Transforming growth factor, β 2	TGFB2	M19154	0.64	0.34	1.87	2.15	1.11	**0.82**	0.89	0.93	0.94	**0.91**	0.92	0.79	1.14	**0.70**
Myosin IB	MYO1B	AJ001381	0.73	0.34	1.31	2.14	1.03	**0.83**	1.12	1.18	0.90	**1.08**	1.49	0.96	1.26	**0.78**
T-cell receptor γ locus	TRG	M30894	0.73	0.35	0.89	0.85	0.67	**0.71**	1.01	1.03	0.93	**0.78**	1.09	0.99	1.05	**0.73**
Folate hydrolase 1	FOLH1	M99487	0.65	0.36	1.41	1.42	1.03	**1.13**	1.08	0.84	0.84	**0.94**	1.29	0.80	1.09	***0.67***
Bone morphogenetic protein 4	BMP4	M22490	0.55	0.36	0.91	0.90	0.78	**0.76**	0.99	0.84	0.91	**0.85**	0.94	0.91	0.88	***0.61***
Serum-inducible kinase	SNK	AF059617	0.62	0.36	1.21	1.29	1.22	**1.23**	1.15	1.06	0.84	**1.01**	1.07	1.04	1.11	**0.90**
Cathepsin L	CTSL	X12451	0.65	0.39	1.17	1.73	0.91	**0.99**	0.93	1.10	1.03	**1.02**	1.11	0.96	0.90	**0.76**
Myoglobin	MB	X00371	0.72	0.40	0.84	1.08	0.80	**0.83**	0.86	0.86	1.02	**0.80**	1.03	0.98	0.87	**0.80**
Stanniocalcin 1	STC1	U25997	0.80	0.40	1.26	1.68	1.35	**1.04**	1.40	1.04	1.44	**1.14**	1.91	0.86	1.62	***0.63***
ATP binding cassette, subfamily G (WHITE), member 1	ABCG1	X91249	0.35	0.47	1.08	1.25	**0.69**	0.95	0.87	0.91	**0.79**	0.75	0.95	0.62	**0.73**	0.43
Monocyte to macrophage differentiation-associated	MMD	X85750	0.26	0.29	1.69	1.86	**1.00**	**1.26**	1.26	1.34	**0.98**	**1.48**	1.30	1.10	**0.98**	**0.79**
HIF-1-responsive RTP801	RTP801	W29115	0.36	0.28	0.98	1.44	**0.93**	**0.78**	1.02	0.93	**0.96**	**0.92**	0.97	0.89	**1.11**	**0.74**

[a] Fold change values represent the average of two independent microarray experiments.

[b] All genes listed are up-regulated at least 2.5-fold by E2 at either 8 h, 48 h, or both time points (underlined), with this stimulation being reversed at one or both time points by all three compounds (ICI, Ral, and TOT). Compounds are considered full antagonists if the E2 up-regulation is reversed by ≥50% (indicated in bold) or as partial antagonists if reversed by 30–50% (indicated in bold italics).

[c] All genes listed are down-regulated at least 2.5-fold (*i.e.,* ≤0.4 of control level) by E2 at either 8 h, 48 h, or both time points (underlined), with this down-regulation being reversed at one or both time points by all three compounds. Compounds are considered full antagonists if the E2 down-regulation is reversed by ≥50% (in bold) or as partial antagonists if the reversal is by 30–50% (in bold italics).

observed. Cluster C shows several genes stimulated more robustly by E2 and TOT than by Ral or ICI, and cluster D contains genes down-regulated by both E2 and TOT. Cluster E highlights two genes encoding the drug-metabolizing enzymes Cyp1A1 and Cyp1B1 that were stimulated by all four ligands, especially so at the 8 h time point.

SERM and ICI Agonistic and/or Antagonistic Activity on E2-Regulated Genes. To examine what effect the SERMs or ICI might have on E2-regulated gene expression, the activity of each compound was first assessed on those genes found to be significantly up- or down-regulated by E2 (Fig. 2). We defined a compound as either having no agonistic activity (≤35% of E2 activity), partial agonistic activity (if the compound alone evoked activity > 35% but ≤70% of E2 activity), or full agonistic activity (if the compound evoked >70% of E2 activity). As expected, on those genes where SERMs displayed full agonistic activity, no antagonism of E2 action by the SERM was observed. Also, on those genes where SERMs displayed partial agonistic activity, the SERM generally acted as a partial antagonist, reducing E2 activity to the level seen with the SERM alone (see below). On those genes where the compound alone displayed no E2-like agonistic activity, we defined the compound as a full antagonist if the effect of E2 could be reversed by the compound by ≥50% or as a partial antagonist if the compound reversed the E2 effect by 30–50%. If reversal of E2 activity by the compound was <30%, we considered this to be no antagonism. In every case where the compound had no agonist activity, we found that it acted as either a full or partial antagonist of E2 action.

As shown in Fig. 2, ICI had the greatest antagonist activity among the 3 compounds evaluated. ICI antagonized E2 action on 95% of E2 up-regulated genes and 91% of E2 down-regulated genes. Ral acted as an antagonist on 67% of E2 up-regulated genes and 33% of E2 down-regulated genes, whereas TOT antagonized E2 action on only 47% of E2 up-regulated genes and 26% of E2 down-regulated genes.

Both Ral and TOT displayed partial agonist/antagonist activity on a larger proportion of the genes than did ICI, with this being 25% for Ral and 30% for TOT on E2 up-regulated genes. On E2 down-regulated genes, Ral and TOT displayed more partial agonist/antagonist activity, with Ral showing partial agonist/antagonist activity on 63% of the genes and TOT displaying partial agonist/antagonist activity on 43% of these genes. Only TOT acted as a full agonist on a substantial percentage of genes, 23% of the E2 up-regulated genes, and 31% of the E2 down-regulated genes. In contrast, both Ral and ICI had full agonist activity on ≤8% of E2 up- or down-regulated genes.

We next examined whether TOT, Ral, or ICI were acting as agonists, partial agonist/antagonists, or antagonists on the same genes, or whether each of the compounds was regulating expres-

Table 3 *Genes up-regulated or down-regulated by estradiol (E2, 10 nM) on which ICI (1 μM) acts as an antagonist, but Ral and trans-hydroxytamoxifen (TOT; 1 μM) act as either partial agonists/antagonists or full agonists at one or both times analyzed*

Numbers are fold change *versus* vehicle-treated control cells.

			E2		ICI		E2 + ICI		Ral		E2 + Ral		TOT		E2 + TOT	
	Symbol	GenBank	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h
Gene name (E2 up-regulated genes)[a,b]																
Calcitonin receptor	CALCR	L00587	7.45	7.91	1.19	1.77	4.15	2.14	***4.06***	***4.23***	4.09	4.96	***4.14***	**6.76**	4.15	6.95
a-myb	MYBL1	S75881	6.66	5.00	2.98	0.52	1.69	1.11	***5.38***	***2.74***	2.07	1.52	**4.66**	***2.86***	2.09	3.32
Insulin-like growth factor binding protein 4	IGFBP4	M62403	4.94	4.11	0.65	0.45	2.87	1.23	***2.80***	***2.87***	3.57	3.54	**3.44**	**6.40**	4.03	6.22
Clone IMAGE: 3140802		AL050002	3.39	3.56	0.73	0.86	2.25	1.18	***2.58***	**3.46**	2.76	3.89	***4.86***	**8.01**	4.67	6.82
RAS guanyl releasing protein 1	RASGRP1	AF081195	3.81	2.35	0.94	3.04	1.94	1.44	***2.36***	3.85	3.51	2.44	**3.05**	3.25	3.63	3.59
KIAA0869 protein	KIAA0869	AB020676	2.67	1.28	1.10	1.32	2.06	1.14	***1.70***	1.52	1.86	1.56	**2.65**	3.66	2.86	2.91
Topoisomerase (DNA) II alpha	TOP2A	L47276	1.47	3.21	1.86	0.49	2.13	1.79	1.93	***1.78***	1.18	1.91	2.09	***2.26***	1.80	2.25
Chromosome 11 open reading frame 8	C11orf8	U57911	2.42	3.08	0.54	0.57	1.96	0.97	1.37	***1.83***	1.53	1.55	1.53	**2.77**	1.79	2.24
Clone FEBRA-2000874		W26466	2.47	3.06	1.18	1.40	1.78	0.99	1.73	***1.96***	1.88	1.71	2.20	**3.04**	2.20	4.67
Replication factor C4	RFC4	M87339	1.78	2.51	1.39	0.69	1.84	1.49	1.54	***1.65***	1.25	1.46	1.60	***1.63***	1.54	1.79
Gene name (E2 down-regulated genes)[a,c]																
Interleukin 1 receptor, type I	IL1R1	M27492	0.22	0.04	1.37	1.35	0.61	0.80	***0.75***	***0.96***	0.65	0.82	***0.83***	***0.64***	0.71	0.63
BCL2-interacting killer	BIK	U34584	0.33	0.21	0.86	1.14	0.47	0.54	***0.56***	**0.58**	0.42	0.39	**0.53**	**0.37**	0.48	0.42
KIAA0922 protein	KIAA0922	AB023139	0.35	0.30	0.96	1.15	0.69	0.81	***0.75***	***0.60***	0.71	0.66	***0.72***	**0.44**	0.68	0.37
Dual specificity phosphatase 1	DUSP1	X68277	0.37	0.31	0.79	0.89	0.48	0.50	***0.73***	***0.55***	0.53	0.52	***0.73***	***0.58***	0.66	0.54
Dual specificity phosphatase 4	DUSP4	U48807	0.38	0.32	0.75	1.27	0.72	0.74	***0.65***	***0.61***	0.66	0.64	0.86	***0.56***	0.67	0.53
RAP1, GTPase activating protein 1	RAP1GA1	M64788	0.39	0.32	0.70	0.79	0.57	0.61	***0.67***	***0.63***	0.69	0.61	***0.74***	***0.54***	0.69	0.50
IFN regulatory factor 6	IRF6	AL022398	0.37	0.33	1.03	0.96	0.63	0.54	***0.58***	**0.51**	0.62	0.34	***0.73***	**0.41**	0.64	0.41
B-cell linker	BLNK	AF068180	0.36	0.40	1.11	1.64	0.63	0.84	***0.72***	***0.65***	0.67	0.59	0.98	**0.55**	0.77	0.50
L1 cell adhesion molecule	L1CAM	U52112	0.64	0.07	0.79	1.31	0.71	0.42	0.72	***0.40***	0.66	0.36	0.79	**0.32**	0.80	0.29
Keratin, hair, basic, 1	KRTHB1	X81420	0.75	0.09	0.75	0.98	0.70	0.52	0.68	***0.59***	0.76	0.58	0.77	***0.51***	0.74	0.41
Parvalbumin	PVALB	X63578	0.66	0.13	0.74	1.04	0.57	0.41	0.64	***0.53***	0.65	0.63	0.78	**0.26**	0.83	0.21
Transmembrane 4 superfamily member 1	TM4SF1	AI445461	0.41	0.16	0.92	1.36	0.84	0.96	0.60	***0.51***	0.55	0.67	0.56	**0.31**	0.57	0.32
Laminin, β 1	LAMB1	M61916	0.60	0.17	0.75	1.23	0.79	0.75	0.72	***0.52***	0.74	0.61	0.84	***0.45***	0.71	0.33
Keratin 7	KRT7	AJ238246	0.72	0.21	0.80	1.28	0.93	0.67	0.94	***0.52***	0.66	0.49	1.07	***0.55***	0.92	0.63
H2A histone family, member O	H2AFO	AI885852	0.80	0.22	0.88	1.34	0.85	0.73	0.84	***0.70***	0.92	0.83	0.96	***0.59***	0.89	0.51
Retinoic acid-induced 3	RAI3	AF095448	0.53	0.22	0.85	1.24	0.66	0.80	0.67	***0.63***	0.77	0.50	0.64	**0.39**	0.65	0.37
Matrilin 2	MATN2	U69263	0.54	0.25	1.00	1.19	0.87	0.63	0.94	***0.57***	0.75	0.50	0.94	**0.39**	0.94	0.38
MAD, mothers against decapentaplegic homologue 3	MADH3	U68019	0.49	0.26	0.87	1.15	0.71	0.61	0.51	***0.64***	0.78	0.57	0.58	***0.57***	0.57	0.55
Clusterin	CLU	M25915	0.79	0.26	0.82	0.95	0.67	0.57	0.72	***0.66***	0.81	0.70	0.78	***0.62***	0.81	0.54
Kynureninase (L-kynurenine hydrolase)	KYNU	AI148772	0.55	0.27	1.04	1.16	0.65	0.88	0.72	***0.57***	0.76	0.57	0.71	**0.42**	0.66	0.37
CD24 antigen	CD24	L33930	0.74	0.27	1.03	1.32	1.10	1.01	1.01	***0.71***	0.73	0.67	1.14	***0.57***	1.11	0.46
Slit homologue 2 (Drosophila)	SLIT2	AB017168	0.52	0.27	0.88	1.25	0.59	0.67	0.69	***0.68***	0.80	0.63	0.72	***0.66***	0.82	0.49
Annexin A3	ANXA3	M20560	0.71	0.28	1.07	1.59	0.88	0.76	0.92	***0.71***	0.73	0.69	1.01	***0.66***	0.91	0.54
Flavin-containing monooxygenase 5	FMO5	Z47553	0.44	0.28	0.64	1.31	0.79	0.55	0.79	***0.65***	0.66	0.61	0.73	**0.49**	0.66	0.26
Adipose-specific 2	APM2	AI381790	0.80	0.29	0.76	1.23	0.75	0.45	0.59	***0.58***	0.66	0.65	0.72	***0.64***	0.71	0.51
Enoyl Coenzyme A hydratase 1, peroxisomal	ECH1	AF030249	0.67	0.29	0.88	1.05	0.62	0.56	0.71	***0.68***	0.76	0.60	0.83	***0.54***	0.74	0.49
Melanoma-associated gene	D2S448	D86983	0.62	0.31	0.75	0.91	0.60	0.61	0.71	***0.74***	0.75	0.72	0.78	***0.56***	0.72	0.43
Chromobox homologue 6	CBX6	H10776	0.69	0.31	1.03	1.25	0.87	0.84	0.58	**0.34**	0.89	0.46	0.68	***0.55***	1.06	0.47
Core promoter element binding protein	COPEB	AF001461	0.52	0.32	0.87	1.05	0.78	0.77	0.64	***0.65***	0.79	0.70	0.66	**0.48**	0.64	0.35
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	PPP1R3C	Y18207	0.63	0.32	0.82	0.85	0.63	0.77	0.63	***0.67***	0.61	0.71	0.56	***0.53***	0.47	0.42
H2B histone family, member Q	H2BFQ	X57985	0.78	0.33	1.00	1.35	0.81	0.74	1.00	***0.75***	0.86	0.70	0.97	***0.72***	0.91	0.58
Glucosamine (*N*-acetyl)-6-sulfatase	GNS	Z12173	0.84	0.34	0.83	0.99	0.60	0.58	0.45	***0.67***	0.66	0.66	0.77	***0.55***	0.69	0.47
Cathepsin H	CTSH	X16832	0.78	0.34	0.95	1.08	0.85	0.74	0.79	***0.70***	0.80	0.71	0.84	**0.54**	0.81	0.49
N-myc downstream-regulated gene 1	NDRG1	D87953	0.49	0.35	1.02	0.96	0.73	0.82	0.72	***0.68***	0.67	0.64	0.74	**0.48**	0.72	0.37
IFN γ-inducible protein 30	IFI30	J03909	0.76	0.35	0.88	1.14	0.66	0.63	0.68	***0.59***	0.82	0.55	0.90	**0.50**	0.87	0.45
Breast carcinoma-amplified sequence 1	BCAS1	AF041260	0.81	0.36	1.17	1.94	1.25	0.82	1.05	***0.61***	0.67	0.59	1.13	***0.63***	1.02	0.36
Drebrin 1	DBN1	D17530	0.69	0.36	0.79	1.08	0.76	0.65	0.71	***0.58***	0.79	0.61	0.95	**0.54**	0.84	0.46
MAX dimerization protein 4	MXD4	AF040963	0.41	0.36	0.96	1.16	0.58	0.79	0.60	***0.73***	0.71	0.60	0.80	**0.49**	0.66	0.43
Selenium binding protein 1	SELENBP1	U29091	0.60	0.37	0.77	0.88	0.65	0.67	0.66	***0.67***	0.77	0.63	0.67	***0.65***	0.69	0.60
Aldehyde dehydrogenase 4, member A1	ALDH4A1	U24266	0.64	0.38	0.78	1.00	0.73	0.73	0.72	***0.70***	0.68	0.69	0.75	**0.52**	0.73	0.50
Laminin, β 2 (laminin S)	LAMB2	X79683	0.68	0.38	0.75	0.89	0.69	0.64	0.67	***0.69***	0.73	0.53	0.76	**0.51**	0.76	0.48
LPS-induced TNF-α factor	PIG7	AF010312	0.51	0.38	0.87	0.85	0.63	0.66	0.60	***0.74***	0.64	0.71	0.70	***0.61***	0.77	0.57
Phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A	PIB5PA	U45975	0.61	0.39	0.83	0.95	0.73	0.78	0.82	***0.73***	0.74	0.73	0.74	**0.57**	0.80	0.57
v-erb-b2	ERBB2	M11730	0.47	0.40	0.67	0.83	0.57	0.61	0.61	**0.52**	0.58	0.55	0.57	**0.50**	0.70	0.47
Transforming growth factor, β 3	TGFB3	M60556	0.53	0.40	0.86	0.87	0.59	0.72	0.67	***0.75***	0.79	0.66	0.69	***0.68***	0.74	0.67

[a] Fold change values represent the average of two independent microarray experiments.

[b] All genes listed are up-regulated at least 2.5-fold by E2 at either 8 h, 48 h, or both time points (underlined). On these genes, both Ral and TOT acted as either partial agonist/antagonists (>35% of E2 stimulation, indicated in bold italics) or as full agonists (>70% of E2 stimulation, indicated in bold), whereas ICI had no agonist activity alone and acted to reverse E2 gene stimulation.

[c] All genes listed are down-regulated at least 2.5-fold (*i.e.*, ≤0.4 of control level) by E2 at either 8 h, 48 h, or both time points (underlined). On these genes, both Ral and TOT acted as either partial agonist/antagonists (>35% of E2 inhibition, indicated in bold italics) or as full agonists (>70% of E2 inhibition, indicated in bold), whereas ICI had no agonist activity and acted to reverse gene down-regulation by E2.

sion of different subsets of E2-regulated genes. To address this question, we compared the activities of ICI, Ral, and TOT on each gene and identified four major combinations of activities. As shown in Fig. 3, all three compounds (white area) acted as antagonists of E2 action (*i.e.*, reversed partially or fully the stimulation or the suppression of gene expression by E2) on 47% of the genes up-regulated by E2 and on 20% of the genes down-regulated by E2. These genes, on which all of the compounds acted as antagonists of E2 action, are given in Table 2. On 25% of E2 up-regulated genes and 54% of E2 down-regulated genes, only ICI antagonized

Table 4 *Genes up-regulated or down-regulated by estradiol (E2, 10 nM) on which ICI and Ral (1 μM) act as antagonists (i.e., reverse the stimulation or suppression of gene expression by E2), but trans-hydroxytamoxifen (TOT; 1μM) acts as a partial agonist/antagonist or a full agonist at one or both times analyzed*

Numbers are fold change *versus* vehicle-treated control cells.

			E2		ICI		E2 + ICI		Ral		E2 + Ral		TOT		E2 + TOT	
	Symbol	GenBank	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h
Gene name (E2 up-regulated genes)[a,b]																
RAB31	RAB31	U59877	7.22	4.95	0.94	1.11	2.62	1.36	1.92	1.79	2.10	2.48	2.20	**5.31**	2.84	4.83
Trefoil factor 1	TFF1	AA314825	4.74	4.64	0.59	0.19	1.69	1.33	0.78	0.32	0.76	0.52	0.77	***2.29***	0.80	3.38
Nuclear receptor-interacting protein 1	NRIP1	X84373	3.27	3.81	1.20	1.14	2.32	1.85	1.78	1.54	1.46	1.59	***1.86***	***2.01***	2.17	1.97
Erythrocyte membrane protein band 4.1-like 3	EPB41L3	AB023204	7.99	3.77	1.06	1.39	4.20	1.41	1.87	1.54	1.79	3.05	2.46	**3.09**	3.49	2.85
PDZ domain containing 1	PDZK1	AF012281	5.13	2.12	1.21	0.72	2.67	1.47	2.16	1.70	2.39	2.37	**4.11**	9.69	4.32	8.24
Syndecan 2	SDC2	J04621	2.50	2.41	1.08	1.81	1.97	1.35	1.42	1.80	1.33	1.80	***1.67***	2.68	1.84	2.62
Paternally expressed 10	PEG10	AB028974	2.16	7.37	1.37	1.08	2.14	1.68	1.95	1.60	1.38	1.65	2.12	***3.83***	1.70	4.14
Microtubule-associated protein [τ]	MAPT	J03778	1.82	2.86	0.41	0.21	0.87	0.32	0.68	0.92	0.96	1.62	0.73	***2.13***	0.97	1.72
LIV-1 protein, estrogen regulated	LIV-1	U41060	1.60	2.58	1.17	1.13	2.02	1.89	1.59	1.32	1.34	1.54	1.74	***1.84***	1.59	1.55
Gene name (E2 down-regulated genes)[a,c]																
UDP-N-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase	GNE	AJ238764	0.26	0.25	1.96	1.94	0.74	1.22	0.84	1.50	0.98	1.09	0.89	***0.52***	0.76	0.51
NEL-like 2	NELL2	D83018	0.75	0.20	1.35	1.16	1.15	1.19	1.08	0.79	0.97	0.59	1.14	**0.31**	1.15	0.29
ATP-binding cassette, sub-family C (CFTR/MRP), member 5	ABCC5	U83661	0.42	0.26	1.13	1.61	0.75	0.85	0.97	0.98	1.01	0.89	0.98	***0.68***	0.92	0.55
Ankyrin repeat and SOCS box-containing 9	ASB9	AL080091	0.58	0.26	1.15	1.60	0.87	0.99	1.00	0.99	0.95	0.74	1.15	***0.57***	1.07	0.41
Ectodermal-neural cortex (with BTB-like domain)	ENC1	AF059611	0.49	0.28	1.09	1.39	0.84	0.75	0.87	0.79	0.85	0.77	0.97	**0.47**	0.80	0.43
Ephrin-A1	EFNA1	M57730	0.52	0.29	1.24	1.39	0.82	0.78	0.88	0.85	0.89	0.82	0.97	**0.46**	0.81	0.29
Insulin induced protein 2	LOC51141	AL080184	0.49	0.32	1.53	1.79	0.84	1.10	0.99	1.04	0.92	0.83	1.02	***0.63***	0.74	0.56
Syntaxin binding protein 1	STXBP1	AF004563	0.62	0.37	1.00	1.38	0.72	0.76	0.86	0.78	0.86	0.56	1.05	***0.69***	0.90	0.43
Nuclear factor related to κ B binding protein	NFRKB	U08191	0.75	0.37	0.87	1.40	0.88	0.69	0.93	0.89	0.73	0.65	0.71	***0.64***	0.69	0.83
Aryl-hydrocarbon receptor nuclear translocator 2	ARNT2	AB002305	0.55	0.37	1.19	1.54	0.70	0.80	0.84	1.06	0.80	0.81	0.96	***0.65***	0.89	0.45
Collagen, type IV, α 5 (Alport syndrome)	COL4A5	M58526	0.67	0.38	1.11	1.58	1.11	1.06	1.09	1.10	0.83	0.72	1.03	***0.60***	1.11	0.34
Lipoma HMGIC fusion partner-like 2	LHFPL2	D86961	0.80	0.39	1.27	1.18	1.04	1.02	1.19	0.98	1.08	0.99	1.04	**0.54**	1.05	0.57
ras homologue gene family, member E	ARHE	S82240	0.78	0.40	1.41	1.18	1.37	1.09	1.44	1.04	1.06	0.80	1.20	***0.59***	1.01	0.55

[a] Fold change values represent the average of two independent microarray experiments.

[b] All genes listed are up-regulated at least 2.5-fold by E2 at either 8 h, 48 h, or both time points (underlined), with this stimulation being reversed by ICI and Ral. On this set of genes, TOT acted as either a partial agonist/antagonist (35–70% of E2 stimulation in bold italics) or as a full agonist (>70% of E2 stimulation in bold).

[c] All genes listed are down-regulated at least 2.5-fold (*i.e.*, ≤0.4 of control level) by E2 at either 8 h, 48 h, or both time points (underlined), with this down-regulation being reversed by ICI and Ral. TOT acted as either a partial agonist/antagonist (evoking down-regulation 35–70% of E2 activity in bold italics) or a full agonist (activity >70% of E2 activity in bold).

E2 action (stippled area, Fig. 3), whereas TOT and Ral acted as either full or partial agonists on these genes (Table 3). On 23% of E2 up-regulated genes and 16% of E2 down-regulated genes (black area, Fig. 3), both ICI and Ral acted as antagonists, whereas only TOT acted as a partial or full agonist (Table 4). On only a small percentage of genes (5% of up-regulated and 10% of down-regulated, striped area, Fig. 3), all three compounds acted as either full or partial agonists (Table 5).

Distinct patterns of gene regulation by the SERMs were verified by real-time PCR, as demonstrated in Figs. 4 and 5. On several E2 up-regulated genes, both Ral and TOT but not ICI displayed agonist activity; this is seen for the calcitonin receptor (Fig. 4*A*) and insulin-like growth factor binding protein-4 (Fig. 4*B*). Of the three compounds, only TOT displayed substantial E2-like activity on Rab31 (Fig. 4*C*) and phospholipase C-like 2 (PLC-L2) gene expression (Fig. 4*D*). For E2 down-regulated genes, including MAX dimerization protein 4 (MAD 4), B-cell linker (BLNK), and RAP1 GTPase activating protein 1 (RAP1GAP) (Fig. 5), both TOT and Ral displayed partial agonist/antagonist activity, whereas ICI alone had no E2-like down-regulating activity and instead displayed antagonist activity, largely reversing the effect of E2.

Overall, these findings indicate that TOT is capable of acting as a full agonist on more E2-regulated genes than either Ral or ICI. In contrast, ICI's activity is primarily as an antagonist of the E2 effect on gene expression, with little agonistic activity. Of note, Ral had no agonistic effects on any genes different from those on which TOT was an agonist, such that all of Ral's agonistic activity overlapped with that of TOT.

Table 5 *Genes up-regulated or down-regulated by estradiol (E2, 10 nM) on which ICI, Ral, and trans-hydroxytamoxifen (TOT; 1 μM) act as either partial agonists or as full agonists (i.e., partially or fully mimic the effect of E2)*

Numbers are fold change *versus* vehicle treated control cells.

			E2		ICI		Ral		TOT	
	Symbol	GenBank	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h
Gene name (E2 up-regulated genes)[a,b]										
Cytochrome P450, Cyp1A1	CYP1A1	K03191	19.31	0.79	**21.19**	2.09	**98.95**	1.16	**175.58**	1.35
Cytochrome P450, Cyp1B1	CYP1B1	U03688	5.23	3.17	**5.98**	1.67	**19.07**	***2.31***	**24.01**	**4.24**
Gene name (E2 down-regulated genes)[a,c]										
KIAA0227 protein	KIAA0227	D86980	0.24	0.17	***0.70***	***0.63***	***0.47***	***0.44***	***0.47***	**0.31**
BCL2-antagonist/killer 1	BAK1	U16811	0.28	0.29	***0.66***	***0.66***	**0.46**	**0.40**	**0.49**	**0.27**
Solute carrier family 16, member 5	SLC16A5	AA705628	0.33	0.48	***0.76***	1.18	***0.69***	0.83	***0.66***	0.56
Retinoblastoma-like 2 (p130)	RBL2	W25828	0.40	0.60	***0.67***	1.29	***0.72***	1.29	**0.54**	0.91
Synuclein, γ (breast cancer-specific protein 1)	SNCG	AF044311	0.69	0.26	0.85	***0.74***	0.69	***0.61***	0.84	**0.37**
Sphingomyelin phosphodiesterase 1, acid lysosomal	SMPD1	X59960	0.44	0.32	0.70	***0.71***	0.55	***0.58***	0.62	**0.49**
Mucin 1, transmembrane	MUC1	J05581	0.43	0.36	0.54	***0.63***	0.48	**0.48**	0.59	**0.38**
v-erb-b2 homologue 3	ERBB3	H06628	0.54	0.40	0.70	***0.77***	0.50	***0.64***	0.61	**0.53**

[a] Fold change values represent the average of two independent microarray experiments.

[b] Genes listed are up-regulated at least 2.5-fold by E2 at either 8 h, 48 h, or both time points (underlined), with ICI, Ral, and TOT all acting as partial agonists (35–70% of E2 stimulation in bold italics) or as full agonists (>70% of E2 activity in bold).

[c] Genes listed are down-regulated at least 2.5-fold (*i.e.*, ≤0.4 of control level) by E2 at either 8 h, 48 h, or both time points (underlined), with this down-regulation being mimicked by all three compounds either partially (35–70% of E2 activity in bold italics) or fully (>70% of E2 activity in bold).

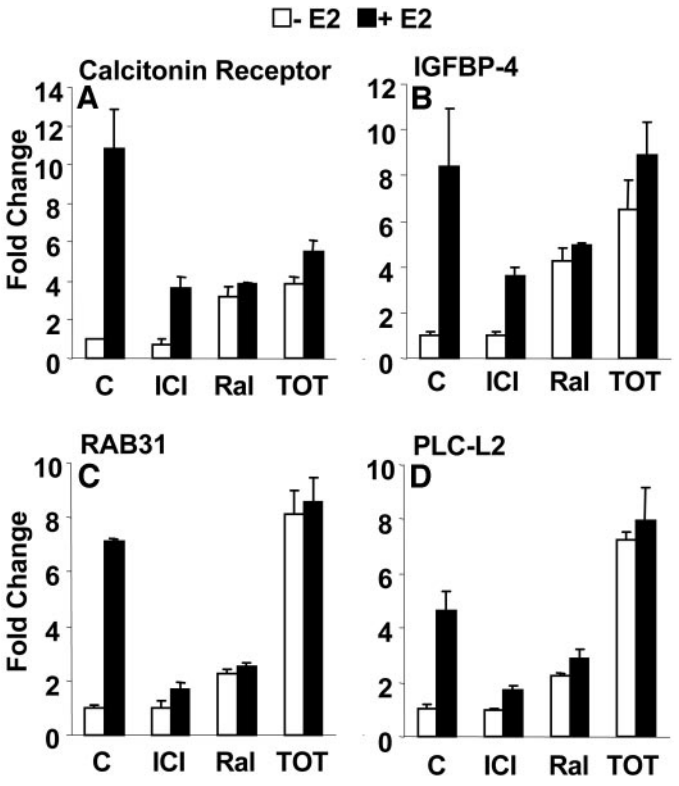

Fig. 4. Real-time PCR analysis of genes stimulated by estradiol (E2) on which ICI 182,780 (ICI), raloxifene (Ral), and *trans*-hydroxytamoxifen (TOT) have distinctly different activities. Real-time PCR was carried out as described in "Materials and Methods" for calcitonin receptor (*A*) and insulin-like growth factor binding protein-4 (IGFBP-4; *B*) on which ICI acts to largely antagonize E2 action, whereas Ral and TOT have partial to full agonist activity. Analyses for Rab31 (*C*) and phospholipase C-like 2 (PLC-L2; *D*) show that TOT has greater E2-like activity than Ral on these two genes.

Unique Gene Expression Regulation by SERMs and ICI but not E2. Using the technique described in "Materials and Methods," we identified 24 genes that were up-regulated and 51 genes that were down-regulated by the SERMs or ICI that were not significantly regulated in the same manner by E2. Gene cluster analysis was performed for these genes to identify any major patterns of regulation, and the results are shown in Fig. 6. Cluster A demonstrates up-regulated genes that appear to be stimulated to a greater extent by the three compounds than by E2. In contrast, cluster B demonstrates that most of the down-regulated genes were down-regulated specifically by ICI, with only a few being down-regulated by Ral or TOT but not E2. Also, a set of genes that is up-regulated specifically by TOT but not by E2, Ral, or ICI is shown in cluster C. The identity of these SERM- and ICI-regulated genes and their fold change in gene expression in response to these ligands are given in Table 6. Immediate early response 3, also called IEX-1, represents an additional gene found to be highly and specifically induced by TOT but not Ral or ICI, but it is also significantly down-regulated by E2 (Table 2). Real-time PCR was performed to verify regulation by the SERMs or ICI. Rab30 is an example of a gene stimulated by TOT but not by E2, Ral, or ICI (Fig. 7*A*). Additional studies demonstrated that regulation of Rab30 by TOT is mediated by the ER because 100-fold excess of E2 could reverse the effect of TOT (Fig. 7*B*). The genes cyclin A2 and cdc2 have their expression markedly down-regulated by ICI only and this is reversed by E2 (Fig. 7, *C* and *D*). Interestingly, these two genes and the majority of genes down-regulated by ICI (Table 6) are associated with the control of cell cycle progression.

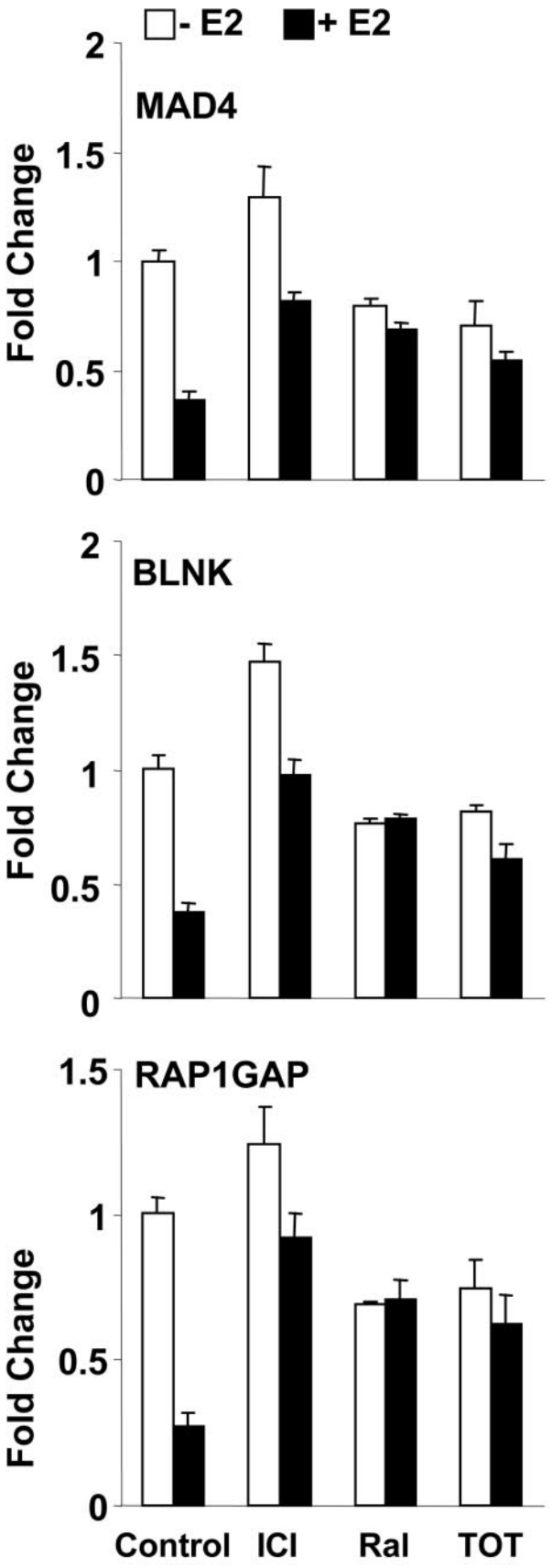

Fig. 5. Real-time PCR analysis of genes down-regulated by estradiol (E2) on which raloxifene (Ral) and *trans*-hydroxytamoxifen (TOT), but not ICI 182,780 (ICI), have partial E2-like activity. Real-time PCR was carried out for MAD4, BLNK, and RAP1GAP, all of which were down-regulated by E2, as well as by Ral and TOT to varying degrees, but were not down-regulated by ICI.

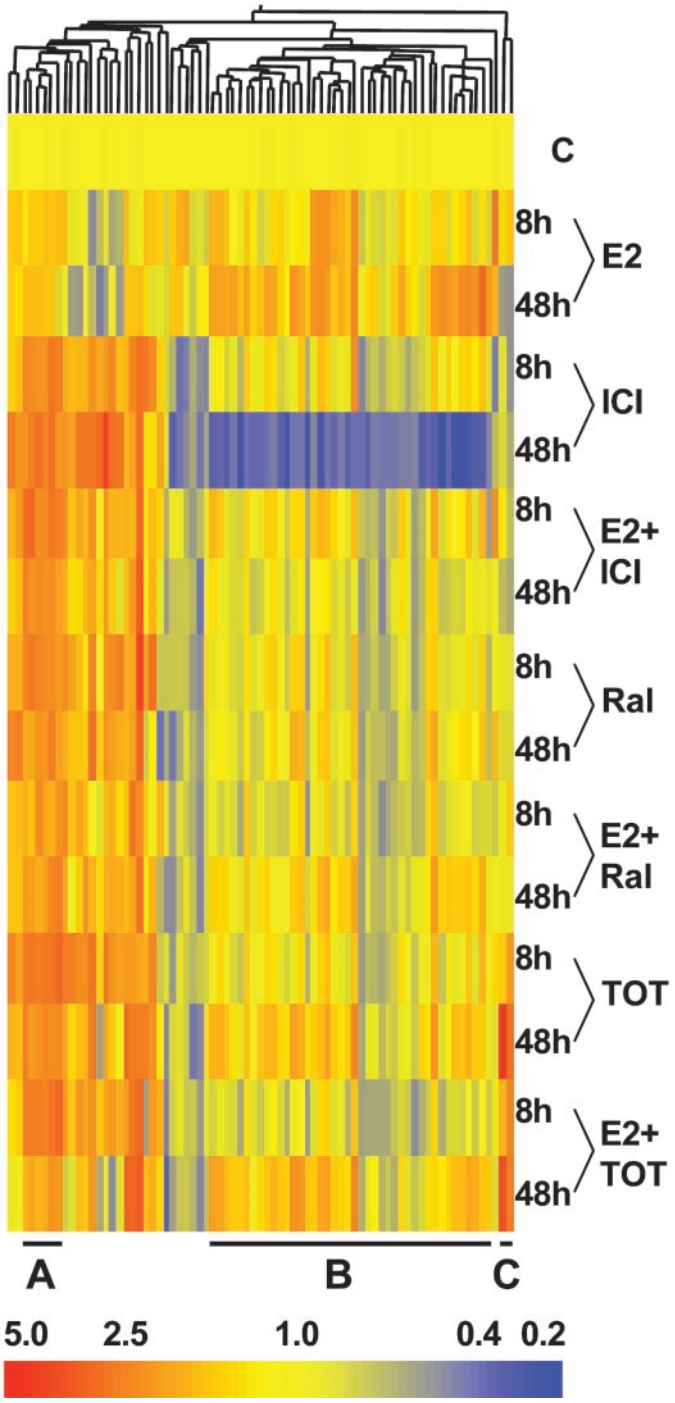

Fig. 6. Gene cluster analysis for ICI 182,780 (ICI) and selective estrogen receptor modulator-selective regulated gene expression. Gene cluster analysis was performed for the 24 up-regulated and 51 down-regulated genes found to be significantly regulated by ICI, raloxifene (Ral), and/or *trans*-hydroxytamoxifen (TOT) but not by estradiol (E2) using GeneSpring software. Stimulated genes are shown in *red*, inhibited genes in *blue*, and genes not regulated in *yellow*. The color scale corresponding to fold change in gene expression is shown across the bottom. Cluster A represents genes that are more highly stimulated by ICI, Ral, and TOT than by E2. Cluster B shows a large subset of genes specifically down-regulated by ICI and not the other ligands. Cluster C shows two genes that are stimulated specifically by TOT only.

DISCUSSION

Tamoxifen and Ral are of significant value in breast cancer treatment and prevention and are currently under investigation in the Study of Tamoxifen and Raloxifene trial (4–11), but the genes that are regulated by these SERMs have not been broadly examined. Gene expression profiling using oligonucleotide microarrays can provide this information. Indeed, our findings indicate that in the MCF-7 breast cancer cell line, where TOT and Ral and the antiestrogen ICI all act as physiological antagonists of E2 action in terms of cell proliferation, each of these ER ligands evokes a very different pattern of gene regulation. Overall, ICI, Ral, and TOT acted as either partial or full antagonists on the majority of E2 up- and down-regulated genes. However, in contrast to ICI, which displayed primarily antagonistic activity only, both Ral and TOT displayed partial agonist/antagonist behavior on a substantial proportion of E2 up- and down-regulated genes. Furthermore, TOT had more estrogen-like activity that either Ral or ICI, with full agonist behavior on 23% of E2 up-regulated and 31% of E2 down-regulated genes. Interestingly, any agonist activity that was seen with Ral overlapped with that of TOT. In addition to these effects on E2-regulated genes, TOT, Ral, and ICI were observed to regulate a small subset of genes that were not regulated by E2, but which appear to be mediated by ER because a 100-fold excess of E2 reversed the SERM effect.

SERMs and ICI as Antagonists of Estrogen Action through the ER. As might be expected, ICI, the pure antiestrogen, antagonized E2 action on >95% of E2-regulated genes. Similarly, Ral acted as an antagonist on >90% of E2 regulated genes, whereas TOT antagonized E2 action on fewer genes (~70%). Despite the anticipated nature of these results, two interesting points can be made. First, on all genes where the SERMs and ICI did not have any partial or full agonist activity, they always antagonized E2 action to some extent, and second, very little of the antagonism by ICI was accompanied by partial agonism, but for Ral and TOT, partial agonist/antagonist activity was more frequent.

Also of note is the functional nature of the genes on which all of the SERMs acted as antagonists (Table 2). As we have previously demonstrated, E2 up-regulates a number of genes that would have stimulatory effects on cell proliferation such as cell cycle-associated genes, growth factors, and transcription factors, as well as down-regulating numerous genes that would inhibit cell proliferation (59). On the basis of the results reported here in this study, it is apparent that many of the genes on which the SERMs act as antagonists could affect cell proliferation. For example, all three of the compounds antagonized the E2 up-regulation of the transcription factor c-fos, the DNA synthesis regulator CDC6, and the growth stimulatory growth factors amphiregulin and chemokine ligand 12, the last of which is also known as SDF-1 and has previously been shown to be antagonized by ICI (62). Similarly, all three compounds antagonized the E2 down-regulation of growth inhibitory factors such as transforming growth factor β2, inhibin βB, and IEX-1, the last of which is also known as IER3 and has been shown to inhibit breast cancer cell growth (63). These findings suggest that although the SERMs show agonist activity on some E2-regulated genes in MCF-7 cells (as discussed below), their ability to block the E2 stimulation of cell proliferation suggests that the genes they antagonize are those that are essential for the stimulatory effect of E2 on cell proliferation.

Agonist Activities of SERMs and ICI. Although ICI activity was almost always antagonistic to E2 action, TOT and Ral displayed a fairly high degree of partial agonist/antagonist activity, whereas only TOT displayed any substantial full agonist activity, indicating that these ligands have very different natures. On the other hand, any agonistic activity seen with Ral nearly always overlapped with that of TOT, which suggests that the agonist activity of Ral may be through a similar mechanism as TOT.

Perhaps one of the more interesting findings revealed by this study is that TOT had full agonist activity on a number of genes on which Ral displayed only partial or no agonist activity, as was the case with phospholipase C-like 2 and Rab31 (Fig. 4). These findings suggest

Table 6 *Genes up-regulated or down-regulated by one or more of the compounds [ICI, Ral or trans-hydroxytamoxifen (TOT)-1 μM] but not by E2 (10 nM)*
Numbers are fold change *versus* vehicle treated control cells.

			E2		ICI		Ral		TOT	
	Symbol	GenBank	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h
Gene name [up-regulated by selective estrogen receptor modulators (SERMs) or ICI][a,b]										
A kinase (PRKA) anchor protein 3	AKAP3	AF087003	0.90	1.24	1.92	1.53	1.48	1.53	1.66	**2.00**
ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A, isoform 1	ATP6V1A	AF113129	1.28	1.24	1.74	1.93	1.78	1.63	**2.11**	1.88
cDNA DKFZp586L081		AL080234	1.27	1.02	1.41	1.68	1.78	1.39	**2.01**	1.49
Chromosome 18 open reading frame 1	C18orf1	AF009426	0.94	0.61	1.65	**3.31**	1.69	1.77	**2.17**	1.49
C-type lectin BIMLEC precursor	DCL-1	D14664	1.24	1.21	**2.19**	**2.24**	1.81	**2.03**	**2.16**	1.85
DiGeorge syndrome critical region gene 9	DGCR9	L77571	1.20	0.87	1.87	1.05	**2.21**	1.01	1.94	1.71
Distal-less homeo box 2	DLX2	L07919	0.91	0.61	1.25	1.56	1.54	1.24	**2.06**	1.11
Dmx-like 1	DMXL1	AJ005821	0.69	0.60	1.29	**2.20**	**2.00**	1.40	1.53	0.99
Dual specificity phosphatase 10	DUSP10	AB026436	0.63	0.97	1.90	**2.22**	1.80	1.56	1.73	1.18
KIAA0776 protein	KIAA0776	AB018319	1.18	1.28	1.72	**2.31**	1.95	1.77	**2.07**	1.76
KIAA0828 protein	KIAA0828	AB020635	1.00	1.02	1.32	**2.21**	1.05	1.18	1.87	1.34
KIAA1219 protein	KIAA1219	AL035419	1.29	1.09	**2.09**	1.07	1.57	0.78	1.39	**2.05**
Neural cell adhesion molecule 2	NCAM2	U75330	1.20	1.06	1.27	1.75	1.43	**2.00**	1.55	1.30
Nuclear receptor subfamily 2, group C, member 1	NR2C1	M29960	1.29	0.85	1.99	1.64	1.64	1.55	**2.35**	1.86
Plexin C1	PLXNC1	AF030339	0.73	0.47	1.47	**2.39**	1.01	1.19	1.12	0.61
RAB30, member RAS oncogene family	RAB30	U57092	1.21	0.56	0.57	0.66	0.89	0.83	1.67	**2.22**
RB1-inducible coiled-coil 1	RB1CC1	D86958	0.98	1.27	**2.22**	**2.26**	**3.32**	**2.39**	1.49	**2.07**
Reticulocalbin 2, EF-hand calcium binding domain	RCN2	X78669	1.19	1.28	1.84	**2.01**	**2.18**	1.48	**2.15**	1.67
RNA helicase family	RNAH	AJ223948	0.52	0.74	1.72	**2.12**	1.95	**2.33**	**2.13**	**2.01**
Schwannomin interacting protein 1	SCHIP1	AF070614	0.96	0.58	1.35	**2.20**	1.25	1.23	1.87	1.26
Steroid-5-α-reductase, alpha polypeptide 1	SRD5A1	M32313	1.26	1.20	1.32	1.27	1.32	1.36	1.62	**2.34**
Syntaxin binding protein 3	STXBP3	D63506	1.04	1.29	1.94	1.75	1.89	1.42	**2.05**	**2.04**
Tropomyosin 1 (α)	TPM1	Z24727	1.08	0.60	0.89	0.85	0.91	0.84	1.18	**3.37**
Unknown RNA, EST oq28g02.s1		AA975427	1.17	1.12	1.11	**2.02**	1.38	1.98	1.95	1.33
Gene name (down-regulated by SERMs or ICI)[a,c]										
Bloom syndrome	BLM	U39817	1.35	1.57	0.98	**0.36**	0.89	0.99	0.98	1.32
BRCA1-associated RING domain 1	BARD1	U76638	1.61	1.74	0.94	**0.50**	0.95	0.99	0.95	1.17
Bromodomain and PHD finger containing, 1	BRPF1	M91585	0.85	0.79	0.67	0.64	0.60	0.71	0.71	**0.44**
c-ABL	ABL1	U07563	1.02	1.13	0.65	**0.49**	0.87	0.85	1.00	0.95
CAP, adenylate cyclase-associated protein, 2	CAP2	BC008481	1.19	1.23	0.71	**0.35**	0.80	**0.44**	0.59	0.85
CDC20 cell division cycle 20 homologue (*S. cerevisiae*)	CDC20	U05340	1.11	1.86	0.96	**0.20**	0.88	0.88	0.75	0.88
CDC28 protein kinase regulatory subunit 1B	CKS1B	BC007751	1.20	1.56	1.16	**0.42**	1.08	1.05	1.18	1.27
Cell division cycle 2, G_1–S and G_2–M	CDC2	X05360	1.87	1.87	1.81	**0.42**	1.29	1.24	1.22	1.92
Cell division cycle 25B	CDC25B	S78187	0.85	1.65	1.11	**0.39**	0.85	0.93	1.04	1.02
Centromere protein F, 350/400ka (mitosin)	CENPF	U30872	0.88	1.51	1.13	**0.43**	1.09	1.02	1.10	1.31
Chromatin assembly factor 1, subunit A (p150)	CHAF1A	U20979	1.33	1.57	1.03	**0.38**	0.98	1.02	1.10	1.42
c-myb	MYB	U22376	1.37	1.20	**0.42**	**0.44**	0.77	0.68	0.89	0.87
Cyclin A2	CCNA2	X51688	1.03	1.52	1.00	**0.21**	0.89	1.02	0.91	1.28
Cyclin-dependent kinase 8	CDK8	X85753	0.79	0.95	**0.49**	**0.42**	0.65	0.60	0.58	0.64
Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	CDKN2C	AF041248	1.01	1.69	0.97	**0.45**	1.15	1.42	0.94	1.53
E2F transcription factor 1	E2F1	M96577	0.91	1.15	0.90	**0.38**	1.13	1.24	0.97	1.04
Eukaryotic translation elongation factor 1 α 1	EEF1A	W28170	1.12	0.91	1.14	1.48	0.74	**0.41**	0.78	1.05
Fk506 binding protein 1A	FKBP1A	AL136531	0.87	1.16	0.84	**0.44**	0.80	0.65	0.82	0.78
Flap structure-specific endonuclease 1	FEN1	AC004770	1.71	1.67	1.14	**0.44**	1.25	1.22	1.30	1.52
Forkhead box M1	FOXM1	U74612	0.84	1.74	1.04	**0.32**	0.87	1.00	0.88	1.15
Galanin	GAL	M77140	1.17	1.45	0.79	**0.48**	0.92	0.89	1.05	0.93
H2A histone family, member X	H2AFX	X14850	0.93	1.10	0.71	**0.36**	0.68	0.82	0.74	0.90
Homeobox A11	HOXA11	H94842	2.11	1.37	**0.46**	0.71	0.99	1.28	1.11	0.88
Hypothetical protein FLJ20552		AI627877	0.80	0.84	0.66	**0.44**	0.63	0.73	0.64	0.73
KIAA0173 gene product	KIAA0173	D79995	0.79	1.02	**0.49**	0.65	1.08	0.84	0.81	1.07
Kinesin-like 1	KIF11	U37426	1.14	1.63	1.22	**0.35**	1.19	1.49	1.35	1.70
Kinesin-like 6 (mitotic centromere-associated kinesin)	KIF2C	U63743	1.26	1.91	1.12	**0.26**	1.05	1.08	1.24	1.40
MCM2 minichromosome maintenance deficient 2, (*S. cerevisiae*)	MCM2	D21063	1.23	1.50	0.81	**0.33**	0.93	0.98	0.98	1.22
MCM3 minichromosome maintenance deficient 3 (*S. cerevisiae*)	MCM3	D38073	1.12	1.13	0.81	**0.34**	0.98	1.00	0.91	1.10
MCM5 minichromosome maintenance deficient 5, (*S. cerevisiae*)	MCM5	X74795	1.00	1.18	0.69	**0.26**	0.60	0.73	0.67	1.08
MCM6 minichromosome maintenance deficient 6 (*S. cerevisiae*)	MCM6	D84557	1.47	1.19	0.86	**0.39**	0.84	0.88	0.92	1.17
MCM7 minichromosome maintenance deficient 7 (*S. cerevisiae*)	MCM7	D55716	1.41	1.38	0.98	**0.48**	0.89	0.97	0.90	1.06
Methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	MTHFD1	J04031	1.03	1.13	0.92	**0.40**	0.86	0.79	0.95	0.98
MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	D85131	0.80	1.66	0.88	**0.49**	0.70	0.72	0.95	0.92
Nuclear factor related to κB binding protein	NFRKB	X80878	0.81	0.84	0.85	0.86	0.76	0.68	0.86	**0.50**

Table 6 *Continued*

	Symbol	GenBank	E2		ICI		Ral		TOT	
			8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h
Nudix (nucleoside diphosphate linked moiety X)-type motif 1	NUDT1	D16581	1.00	1.57	0.89	**0.45**	0.85	0.91	0.85	1.07
Polymerase (DNA directed), [ϵ]	POLE	AL080203	0.85	1.17	0.79	**0.46**	0.79	0.74	0.70	0.92
Polymerase (DNA-directed), α (70kD)	POLA2	L24559	0.94	1.27	0.70	**0.24**	0.82	0.80	0.74	0.90
Polymerase (RNA) mitochondrial (DNA directed)	POLRMT	U75370	0.86	1.02	0.52	**0.45**	0.54	0.59	0.79	**0.49**
Primase, polypeptide 1, 49kDa	PRIM1	X74330	1.76	1.82	1.27	**0.28**	1.07	1.18	1.11	1.27
Replication factor C (activator 1) 2, 40kDa	RFC2	M87338	0.92	0.83	0.81	**0.32**	0.75	0.76	0.98	1.13
Replication factor C (activator 1) 5, 36.5kDa	RFC5	L07540	1.23	1.24	0.90	**0.36**	0.78	0.93	0.85	1.18
Ribonuclease H2, large subunit	RNASEH2A	Z97029	1.07	1.03	0.75	**0.49**	0.59	0.70	0.76	0.79
Ribonucleotide reductase M1 polypeptide	RRM1	X59543	1.11	1.46	1.05	**0.47**	1.16	1.10	1.06	1.35
Serine/threonine kinase 6	STK6	AF011468	1.06	2.43	1.22	**0.33**	1.14	1.22	1.31	1.19
Stathmin 1/oncoprotein 18	STMN1	M31303	1.04	1.50	1.20	**0.43**	1.15	1.33	1.10	1.40
Thymidine kinase 1, soluble	TK1	K02581	0.89	1.02	0.88	**0.25**	0.78	0.95	0.81	1.07
Timeless homologue (Drosophila)	TIMELESS	AF098162	0.80	1.27	0.94	**0.38**	0.95	0.80	0.97	1.21
Ubiquitin-conjugating enzyme E2C	UBE2C	U73379	1.09	1.83	1.10	**0.18**	0.94	1.05	0.97	1.24
v-myc myelocytomatosis viral oncogene homologue (avian)	MYC	V00568	1.47	0.95	**0.48**	0.53	0.75	0.86	0.78	0.80
WEE1 homologue (*S. pombe*)	WEE1	U10564	0.89	0.90	0.79	**0.45**	0.66	0.68	0.74	0.84

[a] Fold change values represent the average of two independent microarray experiments.
[b] Genes listed are up-regulated by one or more of the compounds (ICI, Ral, or TOT) by ≥2.0-fold at either 8 h, 48 h, or both time points, whereas E2 stimulated <1.3-fold at both time points. The stimulations by the compounds ≥2.0-fold are indicated in bold.
[c] Genes listed are down-regulated at least 2.0-fold (*i.e.*, ≤0.5 of control level) by one or more of the compounds at either 8 h, 48 h, or both time points but are >0.78 of control for E2 at both time points. The down-regulations by the compounds to values ≤0.5 are indicated in bold.

that these ligands have different activities at different target gene sites because of their abilities to induce different conformations in the receptor (12–15, 23). The functional significance of these differences is not currently known but is under investigation.

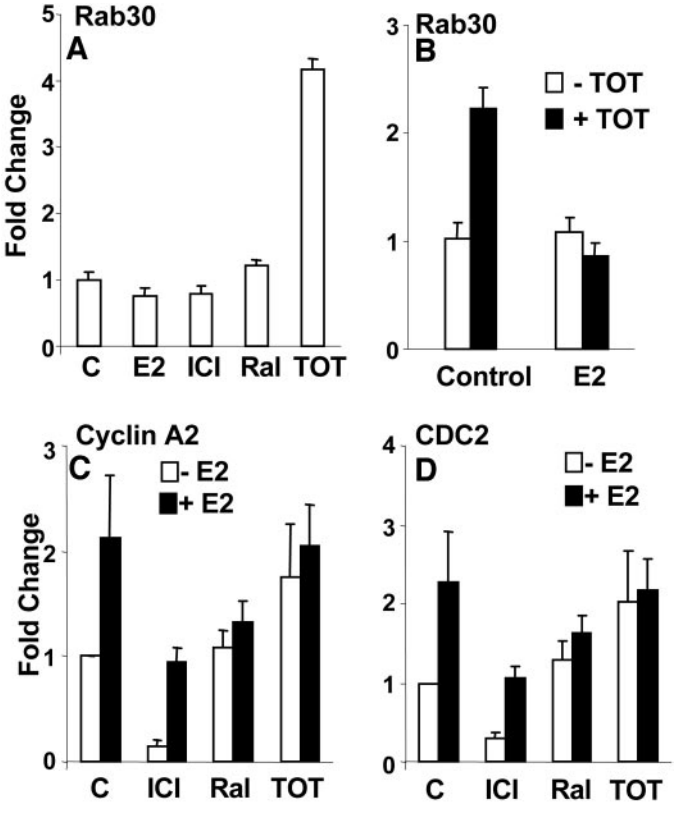

Fig. 7. Real-time PCR analysis of genes regulated by *trans*-hydroxytamoxifen (TOT) or ICI 182,780 (ICI). Real-time PCR was carried out for Rab30, which is specifically up-regulated by TOT (1 μM) and not by estradiol (E2; 10 nM) or raloxifene (Ral) or ICI (1 μM; *A*). This up-regulation by TOT (at 10 nM) was reversed by 100-fold excess of E2, indicating that TOT action on this gene is mediated by the estrogen receptor (*B*). PCR was also carried out for cyclin A2 (*C*) and CDC2 (*D*), which are two examples of cell cycle control genes specifically down-regulated by ICI, with this down-regulation being reversed by E2.

Unique Patterns of Gene Regulation by the SERMs. In addition to the agonist/antagonist activities of the SERMs and ICI on E2-regulated genes, several genes were identified that were specifically regulated by the SERMs or ICI but were not significantly regulated by E2. Very few genes showing this unique "reverse pharmacology" have previously been identified. However, quinone reductase, an important suppressor of DNA damage in breast cancer cells, was previously identified by this laboratory as an antiestrogen induced gene (38, 64). In the current study, one potentially important gene displaying a similar reverse pharmacology is retinoblastoma 1 coiled coil protein, which was significantly up-regulated by all of the SERMs but not by E2 (Table 6). This gene has been proposed to act as a tumor suppressor by its ability to up-regulate retinoblastoma 1 levels, and it could provide an additional beneficial effect of SERMs on breast cancer cells (65, 66). Although the mechanism of SERM-regulated expression of retinoblastoma 1 coiled coil protein is not known, recent evidence suggests that one mechanism by which SERMs can regulate gene expression independently from E2 action could be through a squelching mechanism, whereby SERM interaction with ER leads to the recruitment of corepressors to the ER and away from genes that are under some basal level repression (67).

In addition to genes up-regulated by all of the SERMs, several genes were identified in this study as being specifically up-regulated by TOT but not E2, Ral, or ICI. This included Rab30, a small GTPase, the enzyme 5α-reductase type I, which converts testosterone to dihydrotestosterone, tropomyosin 1, which is a cytoskeletal protein (Table 6), and IER3/IEX-1 (Table 2). All of these genes appear to be regulated through the ER because excess E2 or ICI can block up-regulation of these genes (Fig. 7 and data not shown). This finding additionally supports the idea that TOT is capable of inducing a different and unique receptor activity, most likely through a different receptor conformation, from that of the other ligands (23).

It is of interest that several of these genes have potential tumor suppressor or antiproliferative activities in breast cancer cells and could contribute to the beneficial effects of TOT in breast cancer. For example, the up-regulation of 5α-reductase could potentially reduce local E2 levels *in vivo* through the conversion of androgens to more potent, nonaromatizable androgens rather than to estrogens. Further-

more, 5α-reductase expression has been detected in breast cancer cells and is inversely correlated with proliferation markers such as Ki67 (68). There is also evidence that dihydrotestosterone decreases breast cancer cell proliferation (69, 70). Both tropomyosin and IEX-1, which are up-regulated by TOT, have been shown to inhibit proliferation of breast cancer cells, although the mechanisms for these antiproliferative effects are not known (63, 71). These findings suggest that TOT, in addition to antagonizing estrogen action through the ER at certain genes, may have additional beneficial effects through its ability to up-regulate other specific target genes.

Unique and specific gene regulation was also seen with ICI, but in contrast to TOT, these genes were specifically down-regulated by ICI but not Ral or TOT, and this down-regulation was reversed by E2. The majority of these genes appear to be regulators of the cell cycle, cell proliferation, and DNA synthesis. This is supportive of observations that ICI very effectively arrests the proliferation of breast cancer cells in the G_0 phase of the cell cycle (72). Therefore, by down-regulating the expression of these genes, ICI may have an additional beneficial effect over the other SERMs. Several of these genes are known to actually be up-regulated by E2; however, as we have shown previously, E2 stimulation of these genes occurs at late time points only and may be secondary responses to E2 (59). Two potential upstream transcriptional regulators of these genes are c-Myc and E2F1, which are also down-regulated by ICI (Table 6). One mechanism that might explain the down-regulation of these genes by ICI is that ICI can increase turnover and decrease ER protein levels in breast cancer cells, thereby suppressing any potential ligand-independent activity of the receptor in these cells (73). Thus, ICI may be suppressing growth factor activity through the ER, which has been previously demonstrated for epidermal growth factor and insulin-like growth factor I actions in both breast cancer and uterine cells (74–77).

Here, we demonstrate that the major actions of the SERMs tamoxifen and Ral and of the antiestrogen ICI are largely antagonistic of E2 action. Because E2 regulates a large number of genes in several different pathways that promote cell proliferation, decrease apoptosis, and regulate other activities in these breast cancer cells (59), these antagonistic, tumor-suppressive actions of SERMs should be very desirable. Despite these common antagonistic actions, clearly distinct patterns of gene regulation were observed by microarray profiling for each of these three ligands, indicating that conformational differences in these ER-ligand complexes translate into different pharmacological phenotypes. Some of the genes that are regulated uniquely by the SERMs or ICI might also be contributing to the beneficial and somewhat different effects of these compounds when they are used as endocrine therapies for breast cancer.

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