

Estradiol Regulates Different Genes in Human Breast Tumor Xenografts Compared with the Identical Cells in Culture

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In breast cancers, estrogen receptor (ER) levels are highly correlated with response to endocrine therapies. We sought to define mechanisms of estrogen (E) signaling in a solid breast tumor model using gene expression profiling. ER⁺ T47D-Y human breast cancer cells were grown as xenografts in ovariectomized nude mice under four conditions: 1) 17 β -estradiol for 8 wk (E); 2) without E for 8 wk (control); 3) E for 7 wk followed by 1 wk of E withdrawal (Ewd); or 4) E for 8 wk plus tamoxifen for the last week. E-regulated genes were defined as those that differed significantly between control and E and/or between E and Ewd or control and Ewd. These protocols generated 188 *in vivo* E-regulated genes that showed two major patterns of regulation. Approximately 46% returned to

basal states after Ewd (class I genes); 53% did not (class II genes). In addition, more than 70% of class II-regulated genes also failed to reverse in response to tamoxifen. These genes may be interesting for the study of hormone-resistance issues. A subset of *in vivo* E-regulated genes appears on lists of clinical ER discriminator genes. These may be useful therapeutic targets or markers of E activity. Comparison of *in vivo* E-regulated genes with those regulated in identical cells *in vitro* after 6 and 24 h of E treatment demonstrate only 11% overlap. This indicates the extent to which gene expression profiles are uniquely dependent on hormone-treatment times and the cellular microenvironment. (*Endocrinology* 147: 700–713, 2006)

MORE THAN two thirds of human breast tumors express estrogen receptors (ERs) and are hormone responsive (1). Because of the importance of estrogens (E) and ER activities, multiple studies have sought to define E-regulated signaling pathways by assessment of individual genes or, more globally, by expression profiling (2–16). Most of these studies have used established human breast cancer cell lines to compare E signaling in ER⁺ vs. ER⁻ settings and to assess the functions of phytoestrogens, xenobiotics, or antiestrogens (2–5, 7–10, 12). These studies usually involve hormone-treatment times of 1–72 h using cells cultured *in vitro*. Generally, ER⁺ human breast cancer cells such as MCF-7 or ZR-75 are used, and these *in vitro* approaches, especially shorter treatment times of approximately 6 h, have yielded a number of novel and interesting direct E target genes (2–5, 7–9, 12).

Independently, several recent studies have identified genes differentially expressed in ER⁺ compared with ER⁻ solid tumors isolated from breast cancer patients (13–21). Collectively, the results of these studies have yielded lists of so-called ER discriminator genes. These lists, however, cannot address the question of whether the genes or transcription patterns identified are indeed controlled by, or even related to, E and ER signaling directly or whether they are

simply correlative, reflecting other aspects of cancer biology such as tumor-differentiation state or growth rate that may be associated with ER without being ER regulated (22).

The purpose of the present study was to define mechanisms of E signaling in ER⁺ solid tumors using an *in vivo* approach. To do this, we used ER⁺ T47D human breast cancer cells (T47D-Y) grown as *in vivo* E-dependent solid tumor xenografts in nude mice (23). This allowed us to define E-regulated genes as ones that are differentially expressed in static, non-E-treated control tumors, compared with the 17 β -estradiol (E2)-treated proliferating tumors (E).

Additionally, to further refine the definition of *in vivo* E-regulated genes, we devised an E withdrawal (Ewd) protocol in which E2 was withdrawn for 1 wk in mice bearing E-dependent tumors, and the genes expressed in these tumors were analyzed. The Ewd data were, in turn, compared with 1 wk of E2 plus tamoxifen (Tam) (E + Tam) treatment after 7 wk of E2. One week of Ewd or Tam was chosen because previous studies have shown that in xenografts, key markers of E action are altered in the first few days after E deprivation (24). We speculated that differences in gene-expression profiles between the E sets, and between the untreated control and Ewd sets, would rigorously define the set of direct and indirect genes targeted by E *in vivo* and that Tam would confirm the ER dependency of the regulation. Furthermore, we wished to compare and contrast genes expressed in our chronic E-treated *in vivo* models with: 1) genes regulated acutely by E *in vitro* using the identical T47D-Y cells; 2) previously *in vitro*-defined E-regulated genes; and 3) ER discriminator genes defined in the clinical breast tumor microarray studies.

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Abbreviations: E, Estrogen(s); ER, estrogen receptor; Ewd, estrogen withdrawal; E2, 17 β -estradiol; GO, gene ontology; IHC, immunohistochemistry; ovx, ovariectomized; PR, progesterone receptor; qRT-PCR, real-time quantitative RT-PCR; Tam, tamoxifen.

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We show here that using *in vivo* tumors, the majority of E-regulated genes identified are novel. Additionally, two categories of E-regulated genes were identified: one subset that returned to basal levels after Ewd and a second subset that remained E up- or down-regulated and appeared to be resistant to 1 wk of Ewd. Two-thirds of all E-regulated genes identified were regulated by Ewd and Tam in a similar manner. A comparative analysis of gene-expression profiles in tumors and cultured cells revealed significant differences between short-term E-regulated genes *in vitro* and long-term E-regulated genes *in vivo*. Interestingly, however, the percentage of genes involved in distinct functional pathways regulated by E *in vitro* and *in vivo* are similar except for genes associated with development and organogenesis, which are preferentially regulated *in vivo*. We conclude that both models identify similar E-targeted pathways but that the specific genes differ extensively in the two models.

Materials and Methods

Cell culture and E treatment

T47D-Y cells [ER⁺, progesterone receptor (PR⁻)] are clonally derived sublines of wild-type T47Dco (ER⁺, PR⁺) human breast cancer cells (25). MCF-7 and MDA-231 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely maintained in MEM supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT). For microarray studies, cells were maintained 4 d in phenol red-free MEM containing 5% twice dextran-coated, charcoal-stripped fetal bovine serum prepared as described previously (26). The medium was changed on d 4, and cells were treated with 10 nM E2 or ethanol vehicle in phenol red-free MEM for 6 or 24 h.

T47D-Y tumor xenografts

All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee. Ovariectomized (ovx) female athymic *nu/nu* mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) at 5–6 wk of age. The animals were housed under controlled temperature, humidity, and lighting conditions. At approximately 7 wk of age, animals were anesthetized and implanted with a SILASTIC brand silicon tube (Dow Corning, Midland, MI) containing 2 mg of E2 or placebo (cellulose) sc at the base of the neck, as described (27). At the same time, animals were injected sc on both the left and right flank, with 5×10^6 T47D-Y human breast cancer cells suspended in 50% Matrigel. Mice were divided into the following experimental groups: E2 for 8 wk (E); E2 for 7 wk followed by surgical removal of the E2-containing implant and continuation on no hormone for 1 wk (Ewd); E2 for 8 wk and 10 mg of Tam added for the last week (E + Tam); or placebo for 8 wk or placebo for 7 wk followed by pellet removal for 1 wk (control). Data from the latter two controls were combined. Tumor growth was monitored weekly by measuring length (l) and width (w) with a digital caliper. Tumor area was calculated by the formula $lw\pi/4$. Vaginal cytology was monitored weekly or daily during Ewd- or Tam-treatment periods (28). Animals were euthanized by CO₂ asphyxiation. Tumors, normal mammary glands, and uteri were immediately removed, and tumors and uteri were weighed. Selected tumors were placed in TRIzol (Invitrogen, Carlsbad, CA), frozen in liquid nitrogen, and stored at -70 C. E2 levels in sera were measured, as previously described (27), by chemiluminescence microparticle immunoassay (Abbott Laboratories, North Chicago, IL) at the University of Colorado Hospital.

Immunohistochemistry and immunoblotting

Select tumors were formalin and paraffin embedded. For immunohistochemistry (IHC), sections (4 μ m) were deparaffinized, antigen was retrieved, and ERs were detected with an anti-ER monoclonal antibody (6F11, Novocastra, Norwell, MA). Primary antiserum was omitted from select incubations to control for nonspecific labeling. Sections were coun-

terstained with Meyer's hematoxylin (Fisher Scientific, Pittsburgh, PA). Immunostained slides were blinded and scored by the method of Allred (29). For immunoblotting, cultured cells were lysed in TRIzol. Protein extracts were prepared according to the instructions of the manufacturer, and protein concentrations were determined by the method of Bradford (Bio-Rad Laboratories, Hercules, CA). Protein extracts (150 μ g) were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with an anti-ER monoclonal antibody (AB15, Lab Vision, Fremont, CA), and bands were visualized by enhanced chemiluminescence (Amersham Biosciences Pharmacia Biotech, Arlington Heights, IL).

RNA isolation and expression profiling

Total RNA was extracted from tumors and cells using TRIzol and further purified using Qiagen RNeasy columns (Qiagen, Valencia, CA). RNA samples were prepared from six independent tumors removed from six individual animals per experimental condition (control, E, and Ewd). Four tumors were removed from three different animals undergoing E + Tam treatment. Three independent cell-culture samples per four experimental groups (6 or 24 h, E2 or ethanol) were harvested. RNA integrity was confirmed with an RNA-nano Bioanalyzer (Agilent Technologies, Palo Alto, CA), and cDNA was prepared and used to generate biotin-labeled cRNA (Enzo Bioarray High Yield RNA Transcript Labeling Kit). This was fragmented, hybridized to HG-U133A Affymetrix chips (Santa Clara, CA) that contain more than 22,000 human oligonucleotide probe sets and scanned (Gene Chip Scanner 3000, Affymetrix).

Data acquisition and analysis

Initial analyses from scanned image files were performed using Microarray Suite Version 5 (Affymetrix), with global scaling to correct for technical variations among chips. Thus, each chip was normalized to an arbitrary value of 500. For quality assurance, hybridization, RNA quality, and 5'- to 3'-end ratios for control genes and internal controls were examined. For subsequent analyses, data were imported from Microarrays Suite into GeneSpring version 6.1 (Silicon Genetics, Redwood City, CA), normalized, and used to identify patterns of gene expression. Genes were defined as E regulated when: 1) the gene was identified as present (P) or marginal (M) by GeneSpring in at least one sample studied (n = 6 in control, E, and E + Ewd tumors; n = 3 in cell cultures; n = 4 for E + Tam); 2) expression changes were greater than 1.5-fold between the control and E, E and Ewd, or control and Ewd sets; and 3) differences in expression were statistically significant ($P > 0.05$; see below). Functional classifications were based on gene ontology (GO) annotation obtained through the NetAffx server (Affymetrix, Santa Clara, CA) (30).

Real-time quantitative RT-PCR

To confirm the expression profiles of selected genes, real-time quantitative RT-PCR (qRT-PCR) was performed using an ABI Prism 7700 sequence detector (PerkinElmer Applied Biosystems, Inc., Foster City, CA), and RNA isolated separately from parallel tumors was used for microarray. cDNA was reverse-transcribed from 1 μ g of total RNA using 0.4 mM random hexamers, 1 \times PCR buffer (5 mM MgCl₂), 20 U of RNase Inhibitor, 4 mM deoxynucleotide triphosphates, and 125 U of Moloney Murine leukemia virus reverse transcriptase. The resulting cDNA reaction (5 μ l) was used in real-time PCR conditions. Amplification was carried out in a 50 μ l of reaction as previously described (31). Three independent cDNA samples per experimental group were used as template RNA. Primers and probes for selected genes were designed using Prism 7700 sequence detection software (Primer Express, PerkinElmer Applied Biosystems, Inc.). Primers were: ADD3 (forward, 5'-TGG AGG AGG TGT GAA TAT GGG-3'; reverse, 5'-CTC ATA AGC CCT TCA AAC TCA A-3'); AREG (forward, 5'-TGA TAC TCG GCT CAG GCC AT-3'; reverse, 5'-ATG GTT CAC GCT TCC CAG AG-3'); CXCR4 (forward, 5'-CCT GGC CTT CAT CAG TCT GG-3'; reverse, 5'-TTG GCC TCT GAC TGT TGG TG-3'); LMO4 (forward, 5'-GGA AAT AGC GGT GCT TGC A-3'; reverse, 5'-CGC CCT CAT GAC GAG TTC A-3'); RARESS (forward, 5'-CAA CAA CAG CTT GGA CCA TGA G-3'; reverse, 5'-TCC TTC GCA GAA CTG ATG ATC A-3'); and SDF-1 (forward, 5'-CCT GAG CTA CAG ATG CCC ATG-3'; reverse, 5'-TTT GAG ATG CTT GAC GTT GGC-3'). After an initial 10-min incubation at 95 C, 40

cycles of 95 C for 15 sec and 60 C for 1 min were performed. A standard curve was generated using appropriate dilutions of cDNA or plasmid controls. PCR for the target of interest was performed in parallel with reactions using glyceraldehyde-3-phosphate dehydrogenase internal reference-specific primers after the amount of both were determined from the standard curve. The target concentration was normalized to glyceraldehyde-3-phosphate dehydrogenase.

Statistics

Data represent the mean \pm SEM. Differences between means were assessed using one-way ANOVA and individual comparisons using Student's *t* test. For microarray analyses of tumors, *P* values were derived from nonparametric *t* test and ANOVA. For cultured cells, *P* values were derived from parametric *t* test and ANOVA. Both data sets were analyzed using GeneSpring statistical software. Genes that passed both the *t* test and ANOVA were scored as candidate genes. Genes identified in tumors that were at least 1.5-fold regulated are shown in the tables. Gene sets derived from cultured cells using a 1.5-fold cutoff are available as supplemental data, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>. Statistical analyses of qRT-PCR were performed on normalized target values using one-way ANOVA and *P* < 0.05 was considered significant.

Results

The Ewd model

We investigated mechanisms of E-mediated signaling in breast cancers using both *in vivo* and *in vitro* approaches. T47D-Y human breast cancer cells that were ER⁺ but lacked PR were grown *in vivo* as solid tumors in ovx nude mice implanted with: 1) E2 for 8 wk; 2) E2 for 7 wk followed by 1 wk of Ewd; or 3) cellulose for 7 or 8 wk with or without sham withdrawal (control). In the absence of E, viable but nonproliferating tumor cells are retained at the injection site, whereas 8 wk of E-dependent growth yields small to intermediate sized tumors. The 1-wk time point for Ewd was chosen to target genes that change upon E cessation under conditions of minimal tumor regression. Other *in vivo* studies have shown that E-dependent markers change significantly in this time (24). In parallel studies, T47D-Y cells were also cultured *in vitro* and treated with or without E2 for 6 and 24 h to compare these typically studied experimental conditions (2, 4, 7, 32) with the *in vivo* results.

To document *in vivo* estrogenization and Ewd, vaginal cytology was monitored weekly and daily during the last 2 wk of the study. Mice treated with E2 for 8 wk had estrogenized vaginal epithelia that converted to diestrus after 1 wk of Ewd (not shown). At the end of the study, circulating E2 levels, uterine wet weights and morphology (Fig. 1), mammary gland morphology, and ER expression (not shown) were assessed as additional indicators of estrogenization and Ewd. Circulating E2 levels in untreated control mice averaged 55 pg/ml. They rose to 129 pg/ml after 8 wk of E2 and returned to control levels within 1 wk of Ewd (Fig. 1A). The uterus is a major E target. At the end of the studies, average uterine weights were 21, 114, and 29 mg, respectively, in control, E-supplemented, and Ewd mice. The gross morphology of the uterus mirrored these changes (Fig. 1B). Lastly, E delivery was monitored by assessment of normal mammary gland ER levels. ER expression levels were high in control glands, extensively down-regulated in E-supplemented mice, and restored to control levels by Ewd (not shown). Together, these data demonstrate the physiological

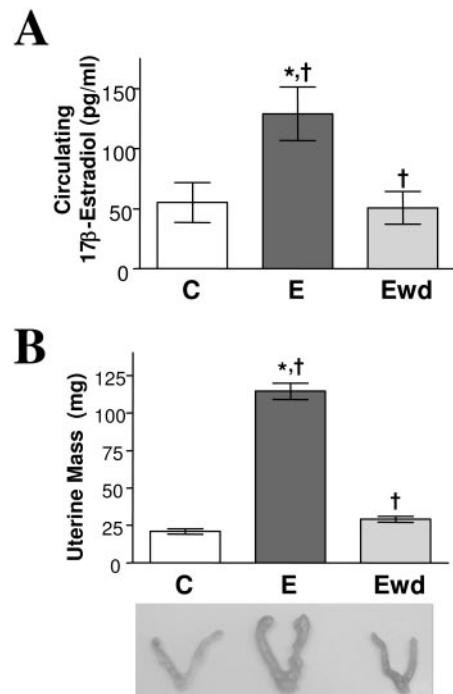


FIG. 1. E withdrawal decreases circulating E2 levels and uterine mass. Ovariectomized nude mice were treated with either placebo control (C), E2 for 8 wk (E), or E2 for 7 wk followed by 1 wk of E withdrawal (Ewd). At the end of the experiment, mice were euthanized, whole blood collected, and uteri removed and weighed. Sera were assayed for E2 by immunoassay as described in the *Materials and Methods*. Each data bar represents the mean circulating E2 levels \pm SEM (n = 5–6) (A) or the mean uterine mass \pm SEM (n = 13–16) (B). *, Statistically significant difference (*P* \leq 0.05) between untreated and treated groups; †, statistically significant difference (*P* \leq 0.05) between treated groups.

validity of the E-treatment regimen and of the 1 wk Ewd approach to alter or reverse the estrogenization state of mice. The same protocols were then used to identify E-regulated genes in tumors.

E-dependent gene regulation in solid tumors

Tumor biology. Continuous E treatment induced slow tumor growth in ovx nude mice inoculated with T47D-Y cells, whereas tumors failed to grow without E. However, non-growing tumor nodules persisted at the injection site of untreated control mice, with the average residual tumor area starting and remaining at 27–30 mm² over the course of the study (Fig. 2A). These nodules served as important E⁻ controls. In mice supplemented with E, tumor area averaged 51 mm² after 8 wk and all mice responded; 1 wk of Ewd reduced tumor area to 43 mm², and this was statistically significant (*P* < 0.05). At necropsy, tumor mass averaged 71, 152, and 119 mg in control, E, and Ewd mice, respectively; however, differences between E and Ewd were not statistically significant (Fig. 2B), demonstrating that our aim of reversing the estrogenization state of the tumors could be achieved (see above) without marked tumor regression. Overall, these data indicate that the xenografts are E dependent for growth, but E is not required for cell survival, as shown by the residual tumor nodules in control mice.

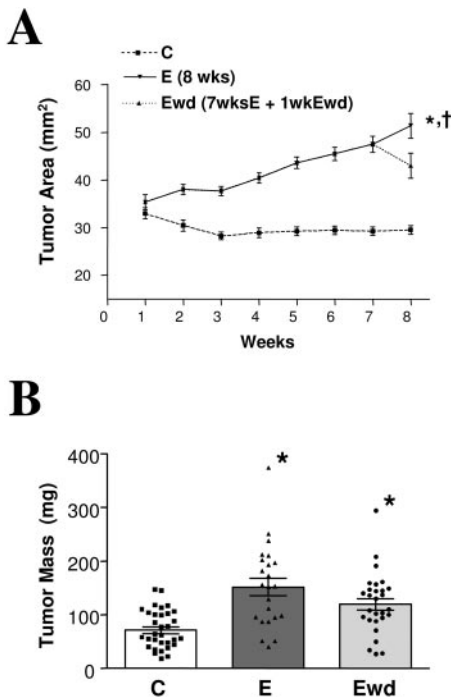


FIG. 2. Tumor growth and regression during E2 treatment without or with 1 wk of Ewd. Ovariectomized mice were injected with T47D-Y breast cancer cells and implanted with an E2-releasing pellet. Tumor growth was monitored weekly in untreated mice (C) ($n = 16$), mice treated with E2 for 8 wk (E) ($n = 13$), or treated with E2 for 7 wk followed by 1 wk of Ewd ($n = 14$). A, Each *data point* represents the mean tumor area \pm SEM over the course of the experiment. B, Each *bar* represents the mean tumor wet mass \pm SEM at the time of death. *, Statistically significant difference ($P \leq 0.05$) between untreated and treated groups; †, statistically significant difference ($P \leq 0.05$) between treated groups.

ER in tumors and cells. To additionally document E and Ewd states, ER levels were assessed in tumors and cultured cell lines. ER immunoblotting data for MCF-7 cells (ER⁺), MDA-231 (ER⁻), and untreated or 6- or 24-h E-treated T47D-Y cells are shown in Fig. 3A. Untreated MCF-7 cells express high ER levels; these are absent in MDA cells and lower in T47D-Y cells. ER were down-regulated in breast cancer cells by 6 or 24 h of E2 treatment *in vitro* as previously described (33). Tumor ER levels after chronic *in vivo* E treatment were assessed by IHC and showed a different pattern. ER, quantified by the method of Allred (29) were 3.0 in ovx, 4.0 in E, and 3.3 in Ewd; these did not differ significantly from one another. Clearly, the down-regulation pattern observed after acute E treatment *in vitro*, as assessed by immunoblotting, was not observed after chronic E treatment *in vivo*, as assessed by IHC. The differences may be due to technical differences in assay methods and antibodies or may represent real differences in ER response to transient *vs.* chronic E exposure.

E-regulated genes in tumors *in vivo*. To identify E-regulated genes *in vivo*, expression profiles were assessed in tumors of control mice, after 8 wk of E2, and after 7 wk of E2 followed by 1 wk of Ewd. RNA was isolated from six independent tumors per experimental group and expression profiling was performed with Affymetrix U133A gene chips interrogating approximately 22,000 genes. Two types of statistical analy-

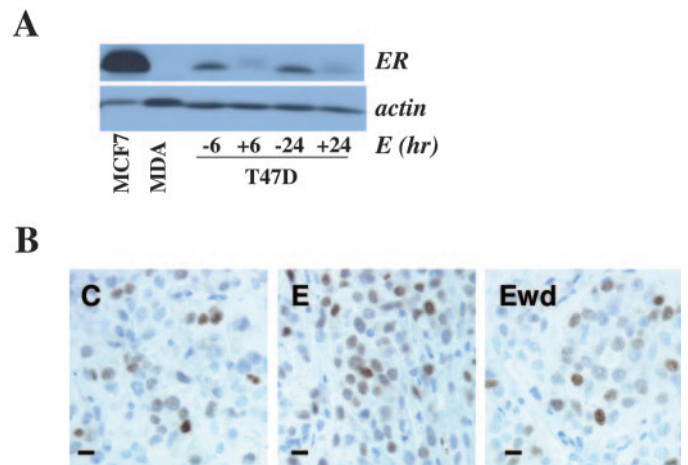


FIG. 3. ER levels in various treatment groups of cells and tumors. A, MCF-7 and MDA cells were grown in culture without (–) or with (+) E2 for 6 or 24 h. Immunoblot of protein extracts (150 μ g) from cells were probed for ER with mouse monoclonal antibody AB15. B, Paraffin sections of T47D-Y tumors [control (C), E, Ewd] were processed and stained for ER expression by IHC using mouse monoclonal antibody 6F11 ($\times 40$ magnification). *Bar*, 10 μ m.

ses, nonparametric ANOVA and Student's *t* test, were performed. E-regulated genes were defined as ones that differ between: 1) control *vs.* E; 2) E *vs.* Ewd or; 3) control *vs.* Ewd. All genes passed both statistical tests and a more than 1.5-fold change was set as cutoff. One hundred eighty-eight genes passed both statistical tests and were scored as significantly regulated by E (Tables 1 and 2). One hundred eighty-six differed significantly between control and E. Among these, the majority 114 (61%) were up-regulated; the remaining 72 were (39%) were down-regulated. Two genes were unchanged between control and E but were down-regulated by Ewd and could not be classified. We defined them as E-regulated based on their sensitivity to E deprivation.

Patterns of gene regulation in tumors in response to Ewd. Gene cluster analysis was performed using GeneSpring software and three main patterns—designated as classes I, II, and III—were observed (Fig. 4). Of the 188 E-regulated genes, 87 or approximately 46% were up- or down-regulated at least 1.5-fold by E relative to control, and then either completely or partially returned to baseline in a statistically significant manner after Ewd. These were designated as class I genes and represent the expected pattern. Table 1 lists these genes, their fold regulation, and whether they went up or down in response to E. Seventeen of these genes (italicized in Table 1) were previously reported to be E regulated.

Approximately half of the E-regulated genes (99 genes or ~53%) were unaffected in a statistically significant manner by 1 wk of Ewd, and several E up-regulated genes paradoxically rose further after Ewd. These were designated as class II genes and are listed in Table 2. Twenty-five of these were previously reported to be E-regulated and are italicized in Table 2. A minor subset (1%) of two genes, GABPB2 and an EST, were designated as class III, because they did not change in response to E but were affected by Ewd.

TABLE 1. Class I E-regulated genes in tumors

GenBank accession no.	Gene symbol	Gene name (Ref.)	Fold	
			Control <i>vs.</i> E	Ewd <i>vs.</i> E
Up-regulated				
NM_000550.1	TYRP1	Tyrosinase-related protein 1	↑ 7.8	↓ 2.0 ^a
NM_014932.1	NLGN1	Neurologin 1	↑ 4.5	↓ 1.3
AI337584	NUMA1	Nuclear mitotic apparatus protein 1	↑ 3.2	↓ 2.4
Z54367	PLEC1	Plectin 1, intermediate filament binding protein	↑ 2.8	↓ 3.8
U16797.1	EFNB2	<i>Ephrin-B2</i> (6)	↑ 2.8	↓ 3.1 ^a
BC001012.1	FLJ20151	Hypothetical protein FLJ20151	↑ 2.3	↓ 1.8
NM_014362.1	HIBCH	3-Hydroxyisobutyryl-Coenzyme A hydrolase	↑ 2.3	↓ 1.3
NM_017949.1	C14ORF34	Chromosome 14 open reading frame 34	↑ 2.2	↓ 2.7
AL050090.1	MYRIP	Myosin VIIA and Rab interacting protein	↑ 2.2	↓ 2.6
T93562	RAD23B	RAD23 homolog B (<i>S. cerevisiae</i>)	↑ 2.2	↓ 2.0
NM_004866.1	SCAMP1	Secretory carrier membrane protein 1	↑ 2.2	↓ 1.6
NM_015626.1	WSB1	SOCS box-containing WD protein SWiP-1	↑ 2.2	↓ 1.3
NM_014739.1	BTF	Bcl-2-associated transcription factor	↑ 1.9	↓ 1.8 ^a
AI829170	GOLGIN-67	Golgin-67	↑ 1.9	↓ 1.6
D55639.1	KYNU	<i>Kynureninase (L-kynurenine hydrolase)</i> (9)	↑ 1.9	↓ 1.6 ^a
AA541758	CPNE3	Copine III	↑ 1.9	↓ 1.3
Z25430.1	STK4	Serine/threonine kinase 4	↑ 1.8	↓ 3.0
N21138	RHOBTB3	<i>Rho-related BTB domain containing 3</i> (11)	↑ 1.8	↓ 2.0
AW298092	KIAA0776	KIAA0776 protein	↑ 1.8	↓ 1.9 ^a
NM_006366.1	CAP2	CAP, adenylate cyclase-associated protein, 2 (yeast)	↑ 1.8	↓ 1.6
AI694452	FLJ10618	Hypothetical protein FLJ10618	↑ 1.8	↓ 1.5
D13989.1	ARHGDI	Rho GDP dissociation inhibitor (GDI) alpha	↑ 1.8	↓ 1.5
AW978896	TRA2A	Transformer-2 alpha	↑ 1.8	↓ 1.5
D84294.1	TTC3	<i>Tetratricopeptide repeat domain 3</i> (8)	↑ 1.8	↓ 1.4 ^a
AF200348.1	D2S448	<i>Melanoma-associated gene</i> (9)	↑ 1.8	↓ 1.3 ^b
NM_017909.1	C6ORF96	Chromosome 6 open reading frame 96	↑ 1.8	↓ 1.3
NM_003107.1	SOX4	<i>SRY (sex determining region Y)-box 4</i> (67)	↑ 1.8	↓ 1.3
AC004528	C19ORF6	Chromosome 19 open reading frame 6	↑ 1.7	↓ 1.9
AK000970.1	TEB4	Similar to <i>S. cerevisiae</i> SSM4	↑ 1.7	↓ 1.9
NM_024790.1	FLJ22490	Hypothetical protein FLJ22490	↑ 1.7	↓ 1.9
NM_003358.1	UGCG	<i>UDP-glucose ceramide glucosyltransferase</i> (3)	↑ 1.7	↓ 1.7
AL050025.1	AP1G1	<i>Adaptor-related protein complex 1, gamma 1 subunit</i> (9, 68)	↑ 1.7	↓ 1.5
BE568184	15E1.2	Hypothetical protein 15E1.2	↑ 1.6	↓ 2.0
AL136621.1	ZNF198	Zinc finger protein 198	↑ 1.6	↓ 1.7
AK025348.1	KIAA1025	KIAA1025 protein	↑ 1.6	↓ 1.7
NM_024563.1	FLJ14054	Hypothetical protein FLJ14054	↑ 1.6	↓ 1.6
BC000278.1	CA12	Carbonic anhydrase XII (9, 11)	↑ 1.6	↓ 1.6
AA128023	STARD13	START domain containing 13	↑ 1.6	↓ 1.6
U22815.1	PPFIA1	Interacting protein (liprin), alpha 1	↑ 1.6	↓ 1.5
NM_020132.1	AGPAT3	1-Acylglycerol-3-phosphate <i>O</i> -acyltransferase 3	↑ 1.6	↓ 1.5
AL121981	DLG1	Discs, large homolog 1 (<i>Drosophila</i>)	↑ 1.6	↓ 1.4 ^a
AF133207.1	H11	Protein kinase H11 (5, 10)	↑ 1.6	↓ 1.4 ^a
AL157485.1	MGC25062	Hypothetical protein MGC25062	↑ 1.6	↓ 1.2 ^b
M61906.1	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1	↑ 1.6	↓ 1.4
AI628360	NPR3	Natriuretic peptide receptor C/guanylate cyclase C	↑ 1.6	↓ 1.3
NM_006275.1	SFRS6	Splicing factor, arginine/serine-rich 6	↑ 1.5	↓ 1.9
BE732345	FLJ21272	Hypothetical protein FLJ21272	↑ 1.5	↓ 1.9
T87245	FLJ10613	Hypothetical protein FLJ10613	↑ 1.5	↓ 1.9
NM_006717.1	SPIN	Spindlin	↑ 1.5	↓ 1.6
NM_007366.1	PLA2R1	Phospholipase A2 receptor 1, 180 kDa	↑ 1.5	↓ 1.4
NM_007054.1	KIF3A	Kinesin family member 3A	↑ 1.5	↓ 1.4
AU147942	PRDX2	Peroxiredoxin 2	↑ 1.5	↓ 1.4 ^a
AB011092.1	ADCY9	Adenylate cyclase 9 (7, 9, 10)	↑ 1.4	↓ 1.7
N32859	NR1D2	Nuclear receptor subfamily 1, group D, member 2	↑ 1.3	↓ 1.7
NM_003866.1	INPP4B	<i>Inositol polyphosphate-4-phosphatase</i> (6)	↑ 1.2	↓ 1.5
Down-regulated				
NM_003007.1	SEMG1	Semenogelin I	↓ 7.8	↑ 2.4 ^a
AL050152.1	DAT1	Neuronal specific transcription factor DAT1	↓ 3.8	↑ 1.4 ^a
U20350.1	CX3CR1	Chemokine (C-X3-C motif) receptor 1	↓ 2.8	↑ 1.5 ^a
NM_012338.1	TM4SF12	Transmembrane 4 superfamily member 12	↓ 2.4	↑ 1.5
AL049977.1	CLDN8	Claudin 8	↓ 2.4	↑ 1.4 ^a
NM_000674.1	ADORA1	<i>Adenosine A1 receptor</i> (3)	↓ 2.2	↑ 1.4
NM_000072.1	CD36	CD36 antigen (collagen type I receptor, thrombospondin receptor)	↓ 2.2	↑ 1.4 ^a
NM_022352.1	CARD9	Caspase recruitment domain family, member 9	↓ 2.1	↑ 1.4 ^a
NM_003064.1	SLPI	Secretory leukocyte protease inhibitor	↓ 2.0	↑ 1.3 ^b
NM_014624.2	S100A6	S100 calcium binding protein A6 (calcyclin)	↓ 2.0	↑ 1.3

TABLE 1. Continued

GenBank accession no.	Gene symbol	Gene name (Ref.)	Fold	
			Control <i>vs.</i> E	Ewd <i>vs.</i> E
NM_000064.1	C3	Complement component 3	↓ 1.9	↑ 1.6
AF132818.1	KLF5	Kruppel-like factor 5 (intestinal)	↓ 1.9	↑ 1.5
NM_014256.1	B3GNT3	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3	↓ 1.8	↑ 1.3
AI923492	HLA-A	Major histocompatibility complex, class I, A	↓ 1.7	↑ 1.7
AF305083.1	FUT4	<i>Fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific) (70)</i>	↓ 1.7	↑ 1.4
BC000723.1	CRAT	Carnitine acetyltransferase	↓ 1.7	↑ 1.3 ^b
NM_005975.1	PTK6	PTK6 protein tyrosine kinase 6	↓ 1.6	↑ 1.6
NM_001710.1	BF	B-factor, properdin	↓ 1.6	↑ 1.5
AB023173.1	ATP11B	ATPase, Class VI, type 11B	↓ 1.6	↑ 1.4
NM_024574.1	FLJ23191	Hypothetical protein FLJ23191	↓ 1.5	↑ 1.8
NM_001647.1	APOD	<i>Apolipoprotein D (71)</i>	↓ 1.5	↑ 1.7
NM_004585.2	RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	↓ 1.5	↑ 1.5
A1823980	RBSK	Ribokinase	↓ 1.5	↑ 1.4
AF070528.1	MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1	↓ 1.5	↑ 1.4
AI554300	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B, member 1	↓ 1.5	↑ 1.4
AD000092	DNASE2	Deoxyribonuclease II, lysosomal	↓ 1.5	↑ 1.3 ^b
M19720	MYCL1	L-myc protein gene	↓ 1.4	↑ 1.5
AK000345.1	DHRS2	<i>Dehydrogenase/reductase (SDR family) member 2 (6)</i>	↓ 1.3	↑ 1.6
D86980.1	KIAA0227	KIAA0227 protein	↓ 1.3	↑ 1.5
NM_004529.1	MLLT3	Myeloid/lymphoid or mixed-lineage leukemia; translocated to, 3	↓ 1.3	↑ 1.5
U47674.1	ASAH1	<i>N</i> -acylsphingosine amidohydrolase (acid ceramidase) 1	↓ 1.2	↑ 1.6
U19179.1	NCOA1	<i>Nuclear receptor coactivator 1 (72)</i>	↓ 1.2	↑ 1.5

Genes in *bold* are also found in T47D-Y cell lines to be E regulated. Genes in *italics* have been identified previously in the literature to be estrogen regulated.

^a Genes that were changed more than 1.3-fold but not in a statistically significant manner.

^b Genes that were changing in a statistically significant manner but changed less than 1.3-fold.

Ewd compared with Tam. The antiestrogen Tam is used clinically to suppress actions of ER in E-dependent breast cancers. Effects on E-regulated genes of 1 wk of Tam treatment (E + Tam) were compared with the effects of 1 wk of Ewd. One week of Tam partially suppressed E-stimulatory effects on uterine weights demonstrating efficiency of Tam delivery. Like with Ewd, E + Tam minimally suppressed tumor growth (data not shown). Expression profiling was assessed using four E + Tam tumors using Affymetrix U133A gene chips interrogating approximately 22,000 genes. Of the 188 E-regulated genes, 124 (66%) responded in the same class I, class II (supplemental Tables 1A and 1B), or class III manner to Tam as they did to Ewd (data not shown). The remaining ~35% of genes either suppressed E effects when Ewd did not or failed to suppress E effects when Ewd did. That two-thirds of chronically E-regulated genes respond to Tam suggests that after 8 wk of E treatment, ERs remain engaged in regulating these genes. Interestingly, like the failure of class II genes to respond to Ewd, approximately 72% also failed to respond to Tam, suggesting that E removal, as accomplished by aromatase inhibitors, has extensive (but not complete) overlapping gene regulatory effects as Tam-induced ER blockade.

Confirmation by real-time RT-PCR. To confirm the expression-profiling results among the two major gene classes in tumors, selected class I and II genes were reanalyzed by qRT-PCR. (The expression level of class III genes was too low for accurate quantitation.) As shown in Fig. 5, SDF-1 (CXCL12), LMO4, RARESS, ADD3, AREG, and CXCR4 showed similar expression patterns by both array profiling and qRT-PCR. Although gene profiling data indicate that SDF-1 is not significantly up-regulated by E in tumors, it was of interest because it is the ligand for the CXCR4 receptor and is a protein previously shown to be E-regulated *in vitro* (7).

Functional categories of E-regulated genes in tumors. To define molecular or biological processes among the two major classes, genes were sorted into functional categories based on detailed bioinformatics analysis using the NetAffx Gene Ontology Mining Tool. The 87 class I and 99 class II genes were compared for their expression in functional categories as defined by GO gene annotations (data not shown). GO terms that predominated among genes in both classes were metabolism, cell death, development, and transport. Immune response genes were the main down-regulated class I and class II genes. Interestingly, cell proliferation, tumor aggressiveness, and cell-cycle genes were 2–7-fold more prevalent among the class II genes that fail to respond to Ewd. These include genes involved in cell cycle regulation (cyclin G2), differentiation (SKIL), development (SOX9), vascularization (VEGF), metastasis (CXCR4), oncogenesis (LMO4), and ER signaling (ESR-1). We speculate that long-term E treatment alters the hormone sensitivity of these genes, with implications for tumor aggressiveness and resistance. Note that ER (ESR-1) transcripts fall into this Ewd-resistant category, which supports the failure of ER protein to be down-regulated by E as measured by IHC (Fig. 3B).

In vitro gene regulation by E. Because previous studies addressing E-regulated gene expression by global profiling used cultured cells exposed to short-term hormone treatments, we analyzed *in vitro* effects of 6 and 24 h E2 using the same ER⁺, T47D-Y cells from which the tumors were generated. These time points fall into the mid-range for *in vitro* studies, commonly between 1–72 h (2–5, 7–10, 12). A total of 266 genes were defined as E-regulated *in vitro* at one or both time points using a 1.5-fold cutoff; 74% up-regulated, and the rest down-regulated. A complete list is available online as Supplemental Tables 2A and 2B. Among *in vitro* up-regulated genes were: SDF-1 (CXCL12) (21.3-fold),

TABLE 2. Class II E-regulated genes in tumors

GenBank accession no.	Gene symbol	Gene name (Ref.)	Fold	
			Control <i>vs.</i> E	Ewd <i>vs.</i> E
Up-regulated				
NM_000125.1	<i>ESR1</i>	<i>Estrogen receptor 1</i> (4, 73)	↑ 2.7	NC
NM_005414.1	<i>SKIL</i>	<i>SKI-like</i> (11)	↑ 2.6	NC
AF162704.1	<i>AR</i>	<i>Androgen receptor</i> (74)	↑ 2.5	NC
AI424243	SCUBE2	Signal peptide, CUB domain, EGF-like 2	↑ 2.5	NC
AW299958	PAPSS2	3'-Phosphoadenosine 5'-phosphosulfate synthase 2	↑ 2.5	NC
NM_024826.1	FLJ21159	Hypothetical protein FLJ21159	↑ 2.4	NC
M90657.1	<i>TM4SF1</i>	<i>Transmembrane 4 superfamily member 1</i> (9)	↑ 2.4	NC
BC003600.1	LMO4	LIM domain only 4	↑ 2.3	NC
NM_017797.1	BTBD2	BTB (POZ) domain containing 2	↑ 2.1	NC
AF022375.1	<i>VEGF</i>	<i>Vascular endothelial growth factor</i> (75)	↑ 2.1	NC
BF001665	OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	↑ 2.0	NC
NM_000933.1	PLCB4	Phospholipase C, beta 4	↑ 2.0	NC
BF110421	EGFL5	EGF-like-domain, multiple 5	↑ 2.0	NC
NM_024573.1	C6ORF211	Chromosome 6 open reading frame 211	↑ 1.9	NC
NM_000637.1	GSR	Glutathione reductase	↑ 1.9	NC
NM_019058.1	<i>RTP801</i>	<i>HIF-1 responsive RTP801</i> (9)	↑ 1.9	NC
NM_004566.1	PFKFB3	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	↑ 1.8	NC
NM_007016.1	PP35	Protein similar to <i>E. coli</i> yhdg and <i>R. capsulatus</i> nifR3	↑ 1.8	NC
NM_017826.1	FLJ20449	Hypothetical protein FLJ20449	↑ 1.7	NC
NM_001873.1	<i>CPE</i>	<i>Carboxypeptidase E</i> (76)	↑ 1.7	NC
NM_002731.1	PRKACB	Protein kinase, cAMP-dependent, catalytic, beta	↑ 1.7	NC
NM_014583.1	LMCD1	LIM and cysteine-rich domains 1	↑ 1.7	NC
AF088867.1	AGR2	Anterior gradient 2 homolog (<i>Xenopus laevis</i>)	↑ 1.7	NC
AL583520	CALD1	Caldesmon 1	↑ 1.6	NC
NM_014782.1	ALEX2	Armadillo repeat protein ALEX2	↑ 1.6	NC
U70862.1	NFIB	Nuclear factor I/B	↑ 1.6	NC
BE973687	<i>HES1</i>	<i>Hairy and enhancer of split 1, (Drosophila)</i> (6)	↑ 1.6	NC
X79067.1	<i>ZFP36L1</i>	<i>Zinc finger protein 36, C3H type-like 1</i> (76)	↑ 1.6	NC
AJ002572.1	C21ORF107	Chromosome 21 open reading frame 107	↑ 1.6	NC
NM_003831.1	RIOK3	RIO kinase 3 (yeast)	↑ 1.6	NC
AL096842.1	MTSG1	Mitochondrial tumor suppressor gene 1	↑ 1.6	NC
AF118887.1	VAV3	vav 3 oncogene	↑ 1.6	NC
AF119873.1	<i>SERPINA1</i>	<i>Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1</i> (3)	↑ 1.6	NC
NM_004052.2	<i>BNIP3</i>	<i>BCL2/adenovirus E1B interacting protein 3</i> (77)	↑ 1.6	NC
NM_014380.1	NGFRAP1	Nerve growth factor receptor (TNFRSF16) associated protein 1	↑ 1.6	NC
BC002449.1	EFHD1	EF hand domain containing 1 (FLJ13612)	↑ 1.6	NC
AJ224869	CXCR4	Chemokine (C-X-C motif) receptor 4	↑ 1.5	↑ 1.4
K01144.1	CD74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	↑ 1.5	↑ 1.2
AA524505	JMJD1	Jumonji domain containing 1	↑ 1.5	↑ 1.2
NM_000346.1	SOX9	SRY (sex determining region Y)-box 9 (7)	↑ 1.5	↑ 1.2
NM_004445.1	EPHB6	EphB6	↑ 1.5	NC
U10489.1	<i>PTPRO</i>	<i>Protein tyrosine phosphatase, receptor type, O</i> (6)	↑ 1.5	NC
BC003068.1	SLC19A1	Solute carrier family 19 (folate transporter), member 1	↑ 1.5	NC
L27560.1	<i>IGFBP5</i>	<i>Insulin-like growth factor binding protein 5</i> (8)	↑ 1.5	NC
NM_001483.1	GBAS	Glioblastoma amplified sequence	↑ 1.5	NC
NM_000935.1	<i>PLOD2</i>	<i>Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2</i> (8)	↑ 1.5	NC
NM_016283.1	<i>TAF9</i>	<i>TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32 kDa</i>	↑ 1.5	NC
AA479488	AHCYL1	S-adenosylhomocysteine hydrolase-like 1	↑ 1.5	NC
W80642	NUDT13	Nudix (nucleoside diphosphate linked moiety X)-type motif 13	↑ 1.5	NC
AI761561	HK2	Hexokinase 2	↑ 1.4	NC
AL136139	NEDD9	Neural precursor cell expressed, developmentally down-regulated 9	↑ 1.4	↑ 1.5
AL523320	FLJ10097	Hypothetical protein FLJ10097	↑ 1.4	↑ 1.2
AL132665.1	BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	↑ 1.4	↑ 1.2
NM_030796.1	DKFZP564K0822	Hypothetical protein DKFZp564K0822	↑ 1.3	↑ 1.2
NM_005368.1	<i>MB</i>	<i>Myoglobin</i> (9)	↑ 1.3	↑ 1.3
NM_001975.1	<i>ENO2</i>	<i>Enolase 2, (gamma, neuronal)</i> (4)	↑ 1.3	↑ 1.2
NM_004354.1	<i>CCNG2</i>	<i>Cyclin G2</i> (7, 8, 10)	↑ 1.3	NC
NM_003116.1	SPAG4	Sperm associated antigen 4	↑ 1.2	↑ 1.4
AF109161.1	CITED2	Chp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (8)	↑ 1.2	↑ 1.3
Down-regulated				
NM_003986.1	BBOX1	Butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1	↓ 2.6	NC

TABLE 2. Continued

GenBank accession no.	Gene symbol	Gene name (Ref.)	Fold	
			Control <i>vs.</i> E	Ewd <i>vs.</i> E
NM_005420.1	STE	Sulfotransferase, estrogen-preferring	↓ 2.5	NC
AF208043.1	IFI16	Interferon, gamma-inducible protein 16	↓ 2.4	NC
NM_024616.1	FLJ23186	Hypothetical protein FLJ23186	↓ 2.4	NC
J04162.1	FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)	↓ 2.2	NC
<i>NM_001657.1</i>	<i>AREG</i>	<i>Amphiregulin (schwannoma-derived growth factor)</i> (3,7–9, 69)	↓ 2.2	NC
U11058.2	KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, α member 1	↓ 2.1	NC
NM_018643.1	TREM1	Triggering receptor expressed on myeloid cells 1	↓ 2.1	NC
<i>A1982754</i>	<i>CLU</i>	<i>Clusterin</i> (9)	↓ 2.0	NC
NM_003570.1	CMAH	Cytidine monophosphate-N-acetylneuraminic acid hydroxylase	↓ 1.8	NC
AA631242	RAB15	RAB15, member RAS oncogene family	↓ 1.8	NC
AJ236915.1	PAK6	p21(CDKN1A)-activated kinase 6	↓ 1.8	NC
X13230.1	MCF2	MCF2 cell line derived transforming sequence	↓ 1.8	NC
NM_012294.1	GFR	Guanine nucleotide exchange factor for Rap1	↓ 1.9	NC
NM_004525.1	LRP2	Low-density lipoprotein-related protein 2	↓ 1.8	NC
<i>BC003143.1</i>	<i>DUSP6</i>	<i>Dual specificity phosphatase 6</i> (6)	↓ 1.7	NC
AL534702	ABHD3	Abhydrolase domain containing 3	↓ 1.7	NC
NM_016240.1	SCARA3	Scavenger receptor class A, member 3	↓ 1.7	NC
AL050391.1	CASP4	Caspase 4, apoptosis-related cysteine protease	↓ 1.7	NC
NM_002250.1	KCNN4	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	↓ 1.7	NC
AA995910	ALOX5	Arachidonate 5-lipoxygenase	↓ 1.6	NC
X56841.1	HLA-E	Major histocompatibility complex, class I, E	↓ 1.6	NC
AF114012.1	TNFSF13	Tumor necrosis factor (ligand) superfamily, member 13	↓ 1.6	NC
<i>BC005830.1</i>	<i>ANXA9</i>	<i>Annexin A9</i> (8)	↓ 1.6	NC
D28124	NBL1	Neuroblastoma, suppression of tumorigenicity 1	↓ 1.6	NC
NM_002863.1	PYGL	Phosphorylase, glycogen; liver	↓ 1.6	NC
AL576654	PPAP2B	Phosphatidic acid phosphatase type 2B	↓ 1.6	NC
NM_024307.1	MGC4171	Hypothetical protein MGC4171	↓ 1.6	NC
NM_000104.2	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	↓ 1.5	NC
NM_019903.1	ADD3	Adducin 3 (gamma)	↓ 1.5	NC
U84246.1	NEU1	Sialidase 1 (lysosomal sialidase)	↓ 1.5	NC
NM_006867.1	RBPMS	RNA binding protein with multiple splicing	↓ 1.5	NC
NM_018835.1	MNAB	Membrane-associated nucleic acid binding protein	↓ 1.5	NC
U13699.1	CASP1	Caspase 1, apoptosis-related cysteine protease	↓ 1.6	NC
NM_000170.1	GLDC	Glycine dehydrogenase (decarboxylating; glycine decarboxylase, glycine cleavage system protein P)	↓ 1.6	↑ 1.2
NM_001432.1	EREG	Epiregulin	↓ 1.6	NC
NM_015254.1	KIF13B	Kinesin family member 13B	↓ 1.5	NC
AU145127	FBXL7	F-box and leucine-rich repeat protein 7	↓ 1.5	NC
NM_004528.1	MGST3	Microsomal glutathione S-transferase 3	↓ 1.5	NC
<i>NM_005544.1</i>	<i>IRS1</i>	<i>Insulin receptor substrate 1</i> (6)	↓ 1.2	↓ 1.4

Genes in *bold* are also found in T47D-Y cell lines to be E regulated. Genes in *italics* were previously identified in the literature to be E regulated. NC, Genes that did not change in a statistically significant manner and changed less than 1.3 fold.

CYP2B6 (16.6-fold), and MYC (11-fold). Down-regulated genes include: RNF32 (4.2-fold), P53AIP1 (4.1-fold), and SERHL (3.2-fold). Forty-one of these genes (*italicized* and referenced in the Supplemental Tables 2A and 2B) were previously reported to be E regulated. In general, fold changes observed with short-term E treatment *in vitro* tended to greatly exceed the fold changes measured *in vivo*.

Comparison of genes regulated by acute E in vitro vs. chronic E in vivo. To determine the extent to which gene expression was modulated similarly by short-term E *in vitro* vs. long-term E *in vivo*, expression patterns between the two experimental conditions were compared. Of 266 *in vitro* E-regulated genes and 188 *in vivo* E-regulated genes, only 20 are common to both conditions: approximately 11% of the total number of genes defined as E-regulated *in vivo* are also E-regulated *in vitro* (Fig. 6 and Supplemental Tables IIA and IIB). Differences in expression profile patterns between *in vitro* and *in vivo* states are shown for four genes (Fig. 7). The CA12 (car-

bonic anhydrase XII) transcript is up-regulated by E2 at 6 and 24 h *in vitro* and is a class I gene in tumors. This is an example of a gene that is similarly regulated in both states. PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3) is up-regulated by E2 at 6 and 24 h *in vitro* but is a class II gene *in vivo*. Some genes were regulated in opposite directions *in vitro* vs. *in vivo*. For example, CITED 2 (Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2) and SOX9 (sex-determining region Y-box 9) are E down-regulated *in vitro* but up-regulated *in vivo*.

Development and organogenesis genes are overrepresented in tumors. To understand the differences between *in vitro* and *in vivo* gene profiles, E-regulated genes identified in both models were organized into functional categories. Of 14 functional GO terms searched, most were represented to a similar extent in cultured cells and tumors. This suggests that, whereas individual genes in a functional pathway may differ *in vitro* vs. *in vivo*, in general, similar functions are maintained

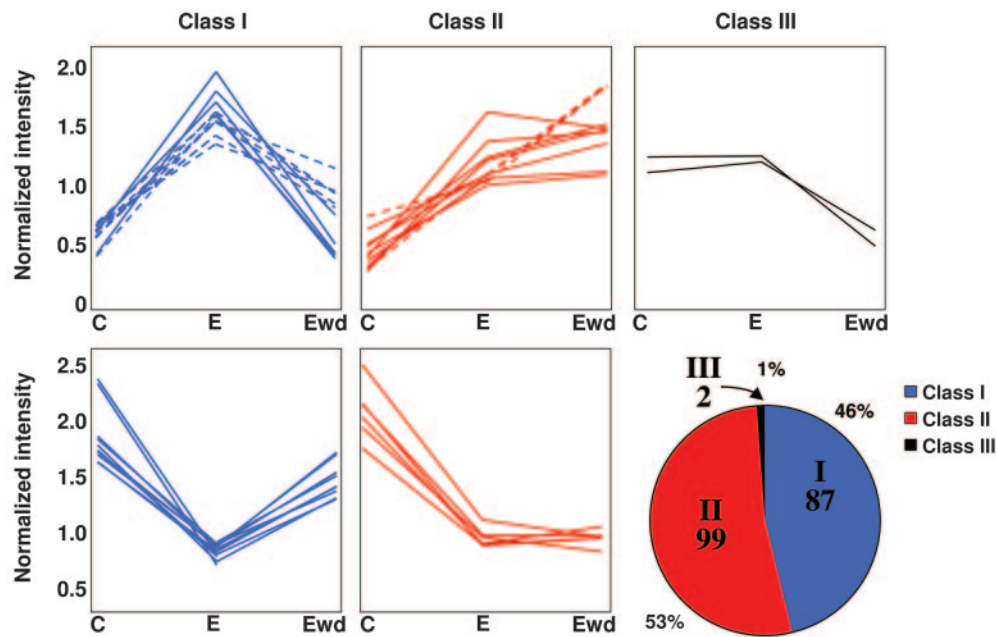


FIG. 4. E-regulated genes in tumors fall into three classes depending on their response to Ewd. Panels, Total RNA was collected from 6 sets each of T47D-Y tumors growing in nude mice under control (C), E (8 wk E2), and Ewd (7 wk E2, 1 wk Ewd) conditions. cDNA was synthesized and used to generate biotin-labeled cRNA to hybridize with microarrays (Affymetrix U133A) interrogating ~22,000 human genes. Genes were defined as E regulated in a statistically significant manner, as assessed by nonparametric ANOVA and Student's *t* test using GeneSpring software. Clustering of these genes resulted in three main patterns. Examples of these patterns are illustrated. Each solid or dashed line represents a specific gene. Class I are E-regulated genes that partially or completely returned to basal after Ewd; class II are E-regulated genes that did not respond to Ewd; and class III are non-E-regulated genes that changed only upon Ewd. Class I and II genes were either up-regulated (top panel) or down-regulated (bottom panel) by E. Pie chart, The percentage of genes in a class represents the number of genes assigned to that class divided by the total number of E-regulated genes. The number of genes in each class is also shown.

under both conditions. However, several functional sets were more common (>2.5-fold) to tumors: ones involved in development/morphogenesis, differentiation, cell death, and immune response (Fig. 8). Together, the data indicate that, whereas E regulates unique genes *in vitro vs. in vivo*, functional pathways tend to remain similar in both states, with the exception of genes involved in processes that are unique to a three-dimensional, *in vivo* environment.

ER discriminator genes. Recent studies (4, 13–21) of breast cancer patients have assessed the gene expression profiles of ER⁺ tumors. They have generated poorly overlapping lists of ER discriminator genes: genes that are coexpressed with ER, but that may or may not be under control of E signaling pathways. Of the 188 genes we define as E regulated in our experimental solid tumor models, 25 genes (Table 3), including ER (ESR1), appear at least once on one of four lists (13–16). They may be particularly interesting targets for future clinical studies focused on E signaling.

Discussion

Models

These studies sought to define E-regulated gene expression profiles in an ER⁺, E-dependent xenograft model of human breast cancer grown in ovx nude mice. To our knowledge, no studies have analyzed E signaling in solid breast tumors or after long-term hormone treatments under conditions that resemble physiological and clinical states. *In vivo* E-regulated genes were defined by assessing differences in expression profiles in residual tumor nod-

ules of ovx, E-untreated mice compared with E-treated mice, and/or by assessing differences in expression profiles in 8-wk E-treated tumors compared with ones in which E had been withdrawn for 1 wk or in which E-stimulated tumors were treated with Tam for 1 wk. We speculated that the genes expressed in the control and Ewd states would be similar, adding a second layer of control and minimizing the possibility that differences between control and E were due to generic growth signaling pathways rather than to pathways under E control. Tam treatment also indicates ER-dependent pathways.

We additionally assessed E-dependent gene expression profiles in cultured cells treated acutely with E. This allowed us to perform a detailed comparison of data generated in the same cells, under solid tumor conditions, against: 1) *in vitro* conditions commonly used to profile gene regulation and 2) clinical ER discriminator genes. We found that despite the fact that specific genes regulated acutely *in vitro* were substantially different from ones regulated chronically *in vivo*, functional pathways regulated by E *in vitro* and *in vivo* were similar. These data demonstrate the extent to which specific gene expression in human breast cancer cells is modulated by the microenvironment in which they reside.

Two classes of E-regulated genes *in vivo*

We identified a total of 188 genes regulated by E *in vivo*. Eighty-seven (46%) of these returned to pre-E levels after Ewd (class I). The majority of these class I genes (53, or 61%) also responded to Tam, suggesting that they are direct or indirect ER target genes. Because expression changes in class

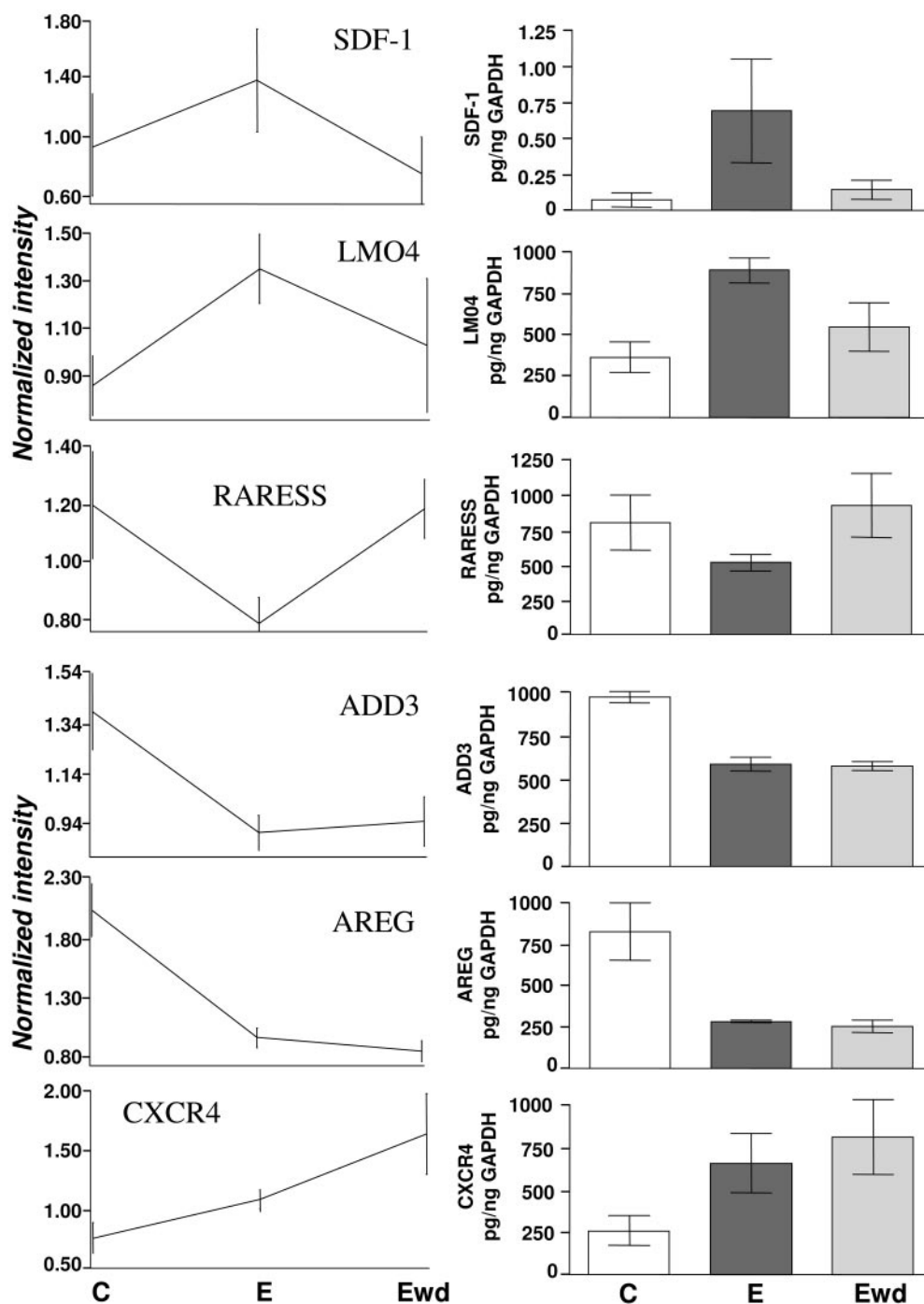


FIG. 5. qRT-PCR analysis of selected class I and II E-regulated genes. T47D-Y tumors were grown in ovx nude mice without hormone (C), with 8 wk of E2 treatment (E), or with 8 wk of E2 followed by 1 wk of Ewd. *Left panels*, Expression array profiling data for the six transcripts indicated. *Right panels*, qRT-PCR data for the same transcripts and conditions using specific primers as described in *Materials and Methods*.

I genes correlated with initiation of tumor regression they may be involved in E-dependent tumor growth. Interestingly, approximately half (53%) of E-regulated genes did not return to basal levels after Ewd. These genes, which we call class II (Table 2), include transcripts for proteins thought to be involved in E-mediated growth or tumor progression, including ER (ESR1) (34); CXCR4 (chemokine C-X-C motif receptor 4) (35); CXCL12, the receptor for SDF-1; VEGF (vas-

cular endothelial growth factor) (36, 37); and LMO4 (LIM domain only 4) (38). We speculate that this phenomenon plays a role in the resistance that invariably accompanies long-term hormone treatments of tumors. Importantly, many (>70%) of these class II genes were also Tam resistant. If the clinical objective of Ewd using AI, or ER blockade using antiestrogens, is to shut down E signaling, then our data would indicate that after chronic E treatment, only half of the

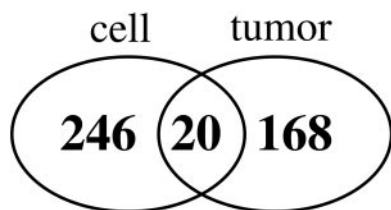


FIG. 6. Venn diagram comparing the number of genes regulated by E2 *in vitro* and in tumors *in vivo* and the overlap between them. T47D-Y cells were grown in culture and treated without or with E2 for 6 or 24 h. T47D-Y tumors were grown in ovx nude mice treated without or with E2 for 8 wk or Ewd for 1 wk. E-regulated genes in cells and tumors were assessed by expression profiling and defined as described in *Materials and Methods*. Shown are total gene numbers that were statistically significant by both *t* test and ANOVA and regulated at least 1.5-fold in cells and/or tumors. In the center are the number of overlapping genes regulated by E in both cells and tumors. Specific genes are listed in Tables 1 and 2 and Supplemental Tables 1A and 1B.

signaling activity is effectively blocked; the rest remains activated and insensitive to suppression.

With regard to the class II genes, establishment of a permanently estrogenized phenotype could be due to epigenetic changes (39) in specific cell lineages, analogous to developmental imprinting (40, 41). Such changes can be hormonally driven (40, 42). Alternatively, it is possible that the class II gene subset is controlled not only by E but also by other pathways, including translational factors (43), growth factors (44–50), *etc.* This suggests mechanisms for resistance in which control over key genes, initially mediated by E, is seized by other factors over time (51). Under these conditions, E suppression or anti-ER therapies would fail to work even in ER⁺ settings. Class II transcripts may be excellent targets for studies aimed at understanding how tumors acquire resistance to long-term, ER-targeted treatments. Long-term Ewd studies of several weeks are necessary to further define the class II genes that do not return to baseline. This would distinguish more sensitive genes that can be regulated after longer periods of Ewd, from those whose expression is indeed permanently altered. However, the extensive overlap in class II genes exhibiting resistance to both Ewd and Tam suggests that the 1 wk test is valid.

E-regulated genes in tumors and cell lines

The majority of E-regulated genes defined to date—whether as single genes or by global profiling—are based on *in vitro* studies, mostly using ER⁺ MCF-7 cells. Short-term hormone treatments, in the range of hours to several days, are used in an effort to ensure that targets are direct. Gene expression profiles in these cells have sought to identify novel downstream E effectors in response to E, Tam, other selective ER modulators or xenoestrogens (2, 4, 7, 9, 32, 52–61). Most of the studies conclude that the gene profiles described provide new therapeutic targets for breast cancer prevention or treatment.

Of the total number of genes we define as regulated by long-term E treatment in T47D human breast cancer cells *in vivo*, only 11% overlap with acutely E-regulated genes in the identical cells grown *in vitro* (Fig. 6 and Supplemental Tables IIA and IIB). This is likely the result of the disparity in the time points examined, as well as differences in the microenvironment of solid tumors compared with cells in culture. Occasionally, we

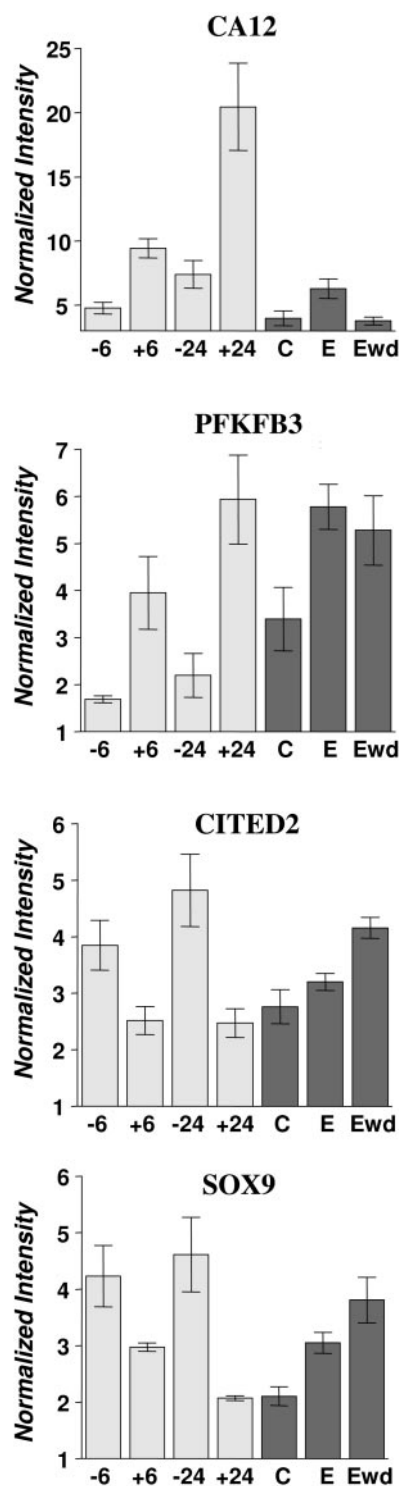


FIG. 7. Differences in expression profiles between *in vitro* and *in vivo* states of four E-regulated genes. Expression profiling was assessed by microarray analysis. Total RNA was isolated from T47D-Y cells and tumors. Labeled cRNA was generated and hybridized to Affymetrix U133A gene chips as described in *Materials and Methods*. Gene expression was assessed on three independent -6, +6, -24, and +24 h sets of cells and six independent control (C), E, and Ewd sets of tumors. CA12, PFKFB3, CITED2, and SOX9 were defined as E regulated in a statistically significant manner [assessed by parametric (cell data) and nonparametric (tumor data) ANOVA and Student's *t* test] using GeneSpring software.

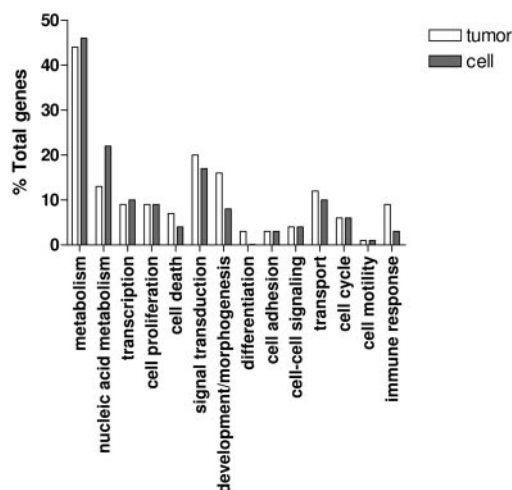


FIG. 8. E-regulated GO categories in cells vs. tumors. E-regulated genes identified in cells and tumors that met both a 1.5-fold change cutoff and the statistical criteria were organized into 1 of 14 functional categories using the NetAffx Gene Ontology Mining Tool. The percentage of genes in a category was calculated based on the number of genes assigned to that category divided by the total number of E-regulated genes identified in cells or tumors. Genes grouped into similar functional categories in cells and tumors except ones involved in development/morphogenesis, differentiation, cell death, and immune response that are enriched in tumors.

find that the same gene is regulated in opposite directions *in vitro* compared with *in vivo*. Despite these differences in specific genes regulated under the two conditions, we also show that both gene sets tend to fall into the same broad functional categories, with the exception that in tumors, E is more likely to

regulate genes involved in nucleic acid metabolism, morphogenesis/development, differentiation, and immune responses. It has been suggested that different sets of genes can specify a single pathway (e.g. growth), so that it is possible to combine genes in many ways to achieve the same end (62). This suggests that cancer cell lines grown *in vitro* accurately retain information about functional pathways (e.g. E regulation of growth) while, compared with the same cells grown *in vivo*, perhaps inaccurately specifying any one gene in that pathway (e.g. gene X as an obligate mediator of E-dependent growth). Here we show that pathways are correctly identified by both *in vitro* and *in vivo* profiling but that it may be difficult to determine specific genes to target therapeutically using only *in vitro* models.

E-regulated genes in our tumor model vs. clinical ER discriminator genes

That human breast cancer cell lines are excellent models to study the human breast tumor biology has been demonstrated by recent gene expression studies that compare normal vs. malignant breast tissues against immortalized breast cancer cell lines. They find, remarkably, that the established cell lines faithfully reflect the unique properties of tumors, rather than of the cognate normal tissue of origin (63). Because it is difficult to study E signaling in clinical samples directly, alternate approaches to assessing E-regulated pathways focus on genes that cocluster with ER α in microarray analyses of breast tumor biopsies. A number of studies have attempted to define ER discriminator gene clusters in human breast tumors (14–16, 18–20, 64–66). However, the linkage between ER discriminator genes and genes that are E regu-

TABLE 3. *In vivo* E-regulated genes that cocluster with ER α in clinical samples

GenBank accession no.	Gene symbol	Gene name (reference)	Fold	
			Control vs. E	Ewd vs. E
Up-regulated				
NM_000125.1	ESR1	Estrogen receptor 1 (13–16)	↑ 2.7	NC
AF162704.1	AR	Androgen receptor (14)	↑ 2.5	NC
M90657.1	TM4SF1	Transmembrane 4 superfamily member 1 (13)	↑ 2.4	NC
BC001012.1	FLJ20151	Hypothetical protein FLJ20151 (16)	↑ 2.3	↓ 1.8
BC003600.1	LMO4	LIM domain only 4 (13)	↑ 2.3	NC
NM_004866.1	SCAMP1	Secretory carrier membrane protein 1 (13)	↑ 2.2	↓ 1.6
NM_003358.1	UGCG	UDP-glucose ceramide glucosyltransferase (13)	↑ 1.7	↓ 1.7
AL050025.1	AP1G1	Adaptor-related protein complex 1, gamma 1 (16)	↑ 1.7	↓ 1.5
AK025348.1	KIAA1025	KIAA1025 protein (14)	↑ 1.6	↓ 1.7
BC000278.1	CA12	Carbonic anhydrase XII (13)	↑ 1.6	↓ 1.6
AF118887.1	VAV3	vav 3 oncogene (13, 16)	↑ 1.6	NC
NM_004052.2	BNIP3	BCL2/adenovirus E1B interacting protein 3 (13)	↑ 1.6	NC
AJ224869	CXCR4	Chemokine (C-X-C motif) receptor 4 (13)	↑ 1.5	↑ 1.4
AA524505	JMJD1	Jumonji domain containing 1 (16)	↑ 1.5	↑ 1.2
AB011092.1	ADCY9	Adenylate cyclase 9 (16)	↑ 1.4	↓ 1.7
NM_004354.1	CCNG2	Cyclin G2 (13)	↑ 1.3	NC
NM_003866.1	INPP4B	Inositol polyphosphate-4-phosphatase (13, 14)	↑ 1.2	↓ 1.5
Down-regulated				
NM_002250.1	KCNN4	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 (13)	↓ 1.7	NC
NM_000104.2	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1 (14)	↓ 1.5	NC
NM_005544.1	IRS1	Insulin receptor substrate 1 (13)	↓ 1.2	↓ 1.4
U47674.1	ASAH1	<i>N</i> -acylsphingosine amidohydrolase (15)	↓ 1.2	↑ 1.6
NM_003064.1	SLPI	Secretory leukocyte protease inhibitor (16)	↓ 2.0	↑ 1.3
AF305083.1	FUT4	Fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific) (13)	↓ 1.7	↑ 1.4
BC005830.1	ANXA9	Annexin A9 (16)	↓ 1.6	↑ 1.2 ^a
NM_015254.1	KIF13B	Kinesin family member 13B (13)	↓ 1.5	↑ 1.2

NC, Genes that did not change in a statistically significant manner and changed less than 1.3-fold.

^a Gene that was changing in a statistically significant manner but changed less than 1.3-fold.

lated is unclear. One possibility is that ER discriminator genes correlate with enhanced differentiation, lack of aggressiveness, slower growth, and other indices of tumor biology that are only partly related to estrogenization state. Alternatively, coclustering of these genes with ER may indeed be due to their regulation by E. We have identified 25 genes (Table 3) that are E regulated in our solid tumor models and that have also been previously reported to be ER discriminator genes. These genes may represent one set to target for E-suppression therapies or to use as markers to monitor the effectiveness of such therapies.

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