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Estrogen-Regulated Gene Networks in Human Breast Cancer Cells: Involvement of E2F1 in the Regulation of Cell Proliferation

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Estrogens generally stimulate the proliferation of estrogen receptor (ER)-containing breast cancer cells, but they also suppress proliferation of some ER-positive breast tumors. Using a genome-wide analysis of gene expression in two ER-positive human breast cancer cell lines that differ in their proliferative response to estrogen, we sought to identify genes involved in estrogen-regulated cell proliferation. To this end, we compared the transcriptional profiles of MCF-7 and MDA-MB-231ER+ cells, which have directionally opposite 17β-estradiol (E2)-dependent proliferation patterns, MCF-7 cells being stimulated and 231ER+ cells suppressed by E2. We identified a set of approximately 70 genes regulated by E2 in both cells, with most being regulated by hormone in an opposite fashion. Using a variety of bioinformatics approaches, we found the E2F binding site to be overrepresented in the potential regulatory regions of many cell cycle-related genes stimulated by estrogen in MCF-7 but inhibited by estrogen in 231ER+ cells. Biochemical analyses confirmed that E2F1 and E2F downstream target genes were increased in MCF-7 and decreased in 231ER+ cells upon estrogen treatment. Furthermore, RNA interference-mediated knockdown of E2F1 blocked estrogen regulation of E2F1 target genes and resulted in loss of estrogen regulation of proliferation. These results demonstrate that regulation by estrogen of E2F1, and subsequently its downstream target genes, is critical for hormone regulation of the proliferative program of these breast cancer cells, and that gene expression profiling combined with bioinformatic analyses of transcription factor binding site enrichment in regulated genes can identify key components associated with nuclear receptor hormonal regulation of important cellular functions. (*Molecular Endocrinology* 21: 2112–2123, 2007)

ESTROGENS STIMULATE THE growth of many breast cancers via the estrogen receptor (ER) and, therefore, the ER, a member of the nuclear hormone receptor transcription factor family, has proven to be a valuable target for endocrine-based therapies (1–5). Upon hormone binding, ER exerts many of its effects by interacting with DNA elements in target gene promoters either directly or through tethering to other transcription factors (6–10), and orchestrating the assembly of coregulator and mediator proteins (11, 12), chromatin remodeling complexes (13, 14), and the basal transcription machinery to regulate transcription (3, 13–19). In some manner, these transcriptional responses drive estrogen's regulation of cell proliferation (20) and other functional changes in target cells (21, 22). Understanding the manner in which estrogen regulates the proliferation of breast cancer cells is key to the development of novel targeted therapies for cancer prevention and treatment.

Through the use of gene expression profiling with DNA microarrays, 17β-estradiol (E2) has been found to regulate diverse gene targets and functional pathways in ER-containing cancer cells (23, 24). Although estrogen usually stimulates the proliferation of ER-containing human breast cancer cells, such as MCF-7 and ZR75 (23, 25, 26) and of ER-positive breast tumors in women, some breast cancer cells and tumors show reduced proliferation and tumor regression when treated with estrogen (27–32). The sequencing of the human genome, in addition to allowing examination of the effects of hormonal agents on a wide range of genes, provides an opportunity to extract the potential regulatory regions for all genes within a gene expression dataset. Bioinformatic analysis of these regulatory regions for transcription factor binding sites has provided information about potential coordinated reg-

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Abbreviations: E2, 17β-Estradiol; ER, estrogen receptor; ICI, the antiestrogen ICI 182,780; siRNA, small interfering RNA.

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ulatory mechanisms in yeast and *Escherichia coli* and allowed for the construction of condition-specific gene-regulatory networks (33–37). Therefore, searching the potential regulatory regions of coregulated genes under specific experimental conditions, *i.e*. ligand, time, *etc*. for overrepresented *cis*-regulatory elements should yield valuable information regarding mechanisms of common gene regulation.

Breast cancer cell lines, such as MCF-7, undergo a robust proliferative response to estrogens. However, because estrogen regulates a very large number of genes in these cells (23), it is challenging to determine which genes or gene networks underlie the proliferative response. Comparative analysis can be a powerful approach to elucidating gene function. In this regard, the fact that MDA-MB 231 breast cancer cells expressing stably introduced ER show a suppression of proliferation in the presence of estrogen (38) provides a valuable phenotypic comparator for the discovery of gene networks that are involved in the regulation of cell proliferation by estrogen.

To elucidate gene networks involved in estrogen regulation of the proliferative program of human breast cancer cells, we compared gene expression profiles of two ER-positive breast cancer cell lines, MCF-7 and MDA-MB-231 stably expressing ER (231ER+), which exhibit opposite E2-dependent growth patterns. From this comparative analysis, we established a common set of E2-regulated genes. Interestingly, although there were genes regulated in a similar or concordant fashion by E2 in both cell types, the majority of the genes commonly regulated in both cell types were regulated in an opposite or discordant fashion. Such discordantly regulated genes are potential candidates for genes involved in estrogen regulation of proliferation.

Through comparative biochemical and bioinformatic analyses in these two ER-containing breast cancer cells, described in this report, we have identified E2F1 as an early target of estrogen action, find the E2F binding site to be greatly overrepresented in the regulatory regions of many cell cycle-related genes regulated by estrogen, and show E2F1 to be a critical component in estrogen regulation of the proliferative program in these breast cancer cells.

RESULTS

Estrogen Regulation of the Proliferation of ER-Containing Breast Cancer Cells

In our studies with two ER-containing breast cancer cell lines, we observed opposite effects of E2 on their cell proliferation (Fig. 1). E2 treatment of MCF-7 cells resulted in a marked increase in cell number compared with cells treated with vehicle control (Fig. 1A). Treatment with the antiestrogen ICI 182,780 slowed cell proliferation, and administration of ICI with E2 resulted in reversal of the E2-dependent proliferation stimulation in MCF-7 cells (Fig. 1A). In contrast, exposure of MDA-MB-231 cells stably expressing ERα (231ER+ cells) to E2 decreased cell proliferation, as observed previously (27, 38) (Fig. 1B), and incubation with E2 and excess ICI reversed the E2 suppression. These results demonstrate that E2 stimulates proliferation of MCF-7 cells, while inhibiting the proliferation of 231ER+ cells.

Comparison of Estrogen-Regulated Gene Expression Profiles

To identify genes that might be involved in mediating this directionally opposite effect of estrogen on the proliferation of these ER-positive breast cancer cells, we used Affymetrix GeneChip DNA microarrays to

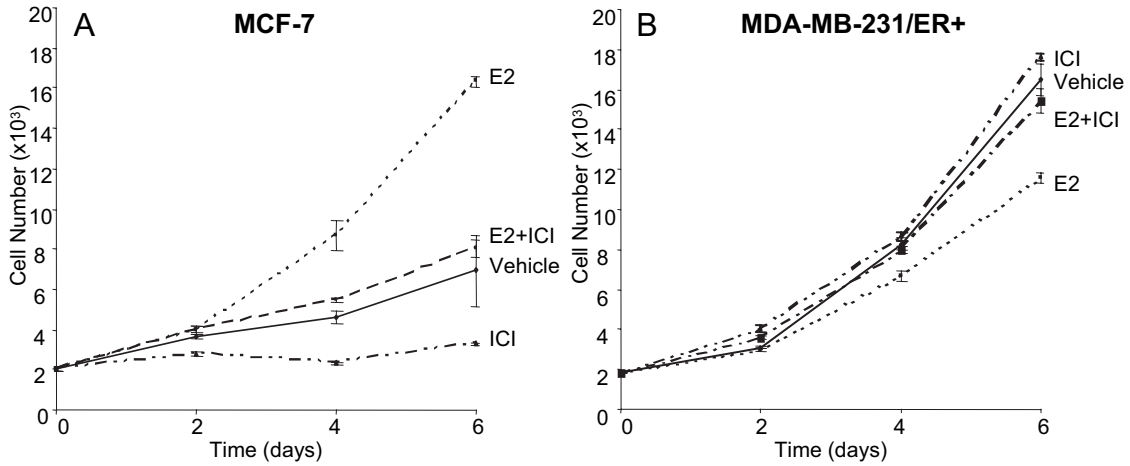


Fig. 1. Estrogen Regulates Proliferation in an Opposite Fashion in Two Different ER-Positive Breast Cancer Cell Lines

Proliferation assays for MCF-7 (panel A) and 231ER+ (panel B) cells were performed as stated in *Materials and Methods*. Cells were treated with control 0.1% ethanol vehicle, 10 nM E2, 1 μM ICI 182,780 (ICI), or E2 plus ICI, and cell numbers were monitored over time. Values are the mean ± SEM of triplicate determinations. This time course was repeated twice with very similar findings.

compare gene expression profiles. Time course experiments up to 48 h of E2 treatment were performed for both cell types (MCF-7 and 231ER+ cells), and gene expression profiles were generated using U95A Affymetrix Genechips, containing probes for approximately 12,000 genes. Analysis for significantly regulated genes, as described in *Materials and Methods*, identified 462 and 340 genes to be regulated (*i.e*. either up- or down-regulated) by estrogen in MCF-7 and 231ER+ cells, respectively (Fig. 2A). A comparison analysis of genes regulated by E2 in these two cell lines using GeneSpring software identified 71 genes that were commonly regulated. Hierarchical gene tree clustering analysis of these 71 commonly regulated genes identified 25 genes that were regulated in a similar direction by E2 in both cell types (Fig. 2B) and 46 genes that were oppositely regulated by E2 in both cell types. Cluster I encompasses 12 genes that were inhibited by E2 in both cell types and include SMAD3, SMAD6, and PMP22 (Fig. 2B). In addition, cluster II (Fig. 2B) encompasses 13 genes that were similarly stimulated by E2 in both cell types and include known target genes regulated by the ER such as pS2, WISP2, and NRIP1 (23, 39, 40).

Interestingly, the majority of genes commonly regulated in MCF-7 and 231ER+ cells were regulated by estrogen in an opposite fashion, with 22 genes in cluster III being inhibited in 231ER+ but stimulated in MCF-7 cells by E2, and 24 genes in cluster IV being stimulated in 231ER+ cells but inhibited in MCF-7 cells by E2 (Fig. 2B). Several genes associated with cluster III (Fig. 2B) are essential for cell division and include cell cycle regulators (STK6, CDC2, CDC20), enzymes involved in nucleotide biosynthesis (TK1, RRM2), and major components of the DNA replication machinery (POLA2, TOP2A, RFC5, MCM2). Cluster IV contains genes known to block cell cycle progression, *e.g*. CDKN1A and TGFB3, suggesting that the genes in these two clusters (III and IV) might underlie estrogen's differential proliferative programs in these two cells.

To confirm the microarray data, we performed quantitative real-time PCR for selected members of each regulation pattern identified by gene tree clustering. As seen in Fig. 3, expression of SMAD3, a cluster I gene, was reduced in both MCF-7 and 231ER+ cells as early as 4 h of treatment, and its level remained suppressed through 48 h. By contrast, E2 stimulated the expression of NRIP1, a cluster II gene, within 4 h of treatment, and its expression remained elevated through 48 h of E2 treatment in both MCF-7 and 231ER+ cells. Further, E2 reduced the expression of CDC6 (in gene cluster III) in 231ER+ cells at 24 and 48 h, while stimulating CDC6 in MCF-7 cells throughout the time course studied. For the cluster IV cell cycle inhibitor gene p21 (also known as CDKN1A), E2 robustly increased its expression in 231ER+ cells, whereas E2

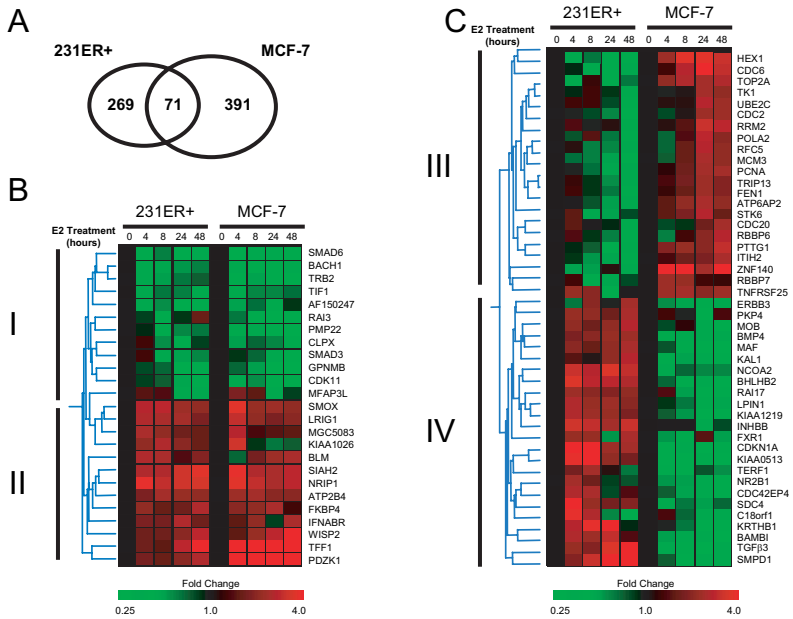


Fig. 2. Gene Tree Cluster Analysis for Genes Commonly Regulated by E2 in 231ER+ and MCF-7 Breast Cancer Cells

Cells were treated with 10 nM E2 for 0, 4, 8, 24, and 48 h. A, Venn diagram showing comparison of total E2-regulated genes between 231ER+ and MCF-7 cells. B, Gene tree cluster analysis was performed on 25 genes regulated similarly by E2 in both cell lines. C, Gene tree clustering analysis was performed on 46 genes regulated in opposite fashion in both cell lines. *Color bar at the bottom* indicates magnitude of gene regulation, with *green* and *red* indicating repression and stimulation, respectively. This analysis identifies in panel B (group I) 12 genes repressed by E2 in both cell types and (group II) 13 genes stimulated by E2 in both cell types; and in Panel C (group III) 22 genes repressed in 231ER+ but stimulated in MCF-7 cells by E2 and (group IV) 24 genes stimulated in 231ER+ but repressed in MCF-7 cells by E2. *Color bar* indicates magnitude of gene regulation with *green* and *red* indicating repression and stimulation, respectively.

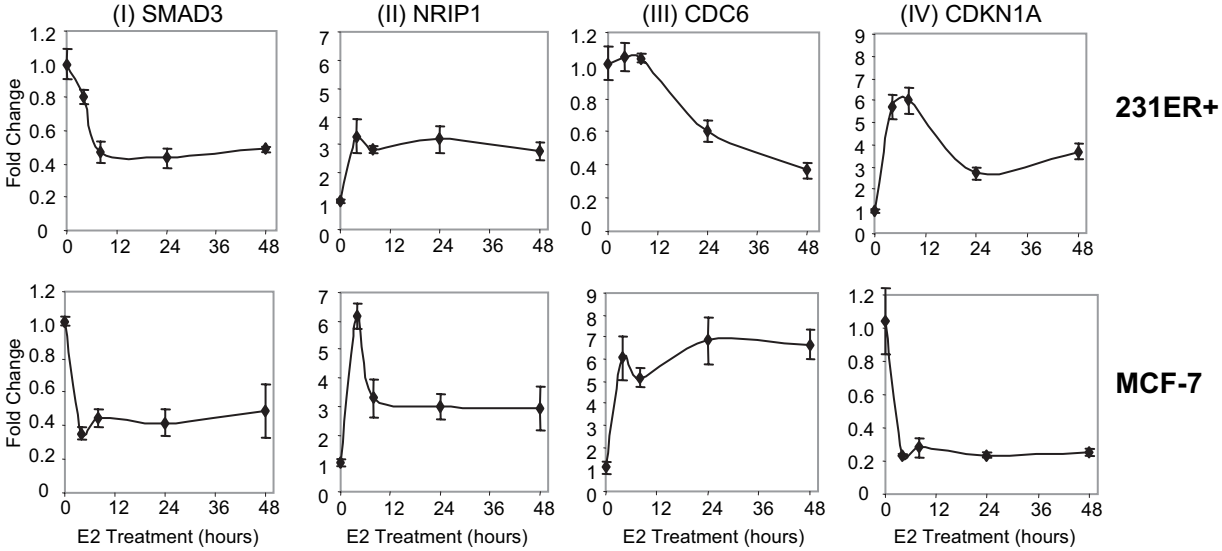


Fig. 3. Quantitative Real-Time PCR Analysis of Gene Expression Regulation Patterns in Response to E2 in 231ER+ and MCF-7 Breast Cancer Cells

Real-time PCR was carried out as described in *Materials and Methods* for SMAD3, NRIP1, CDC6, and CDKN1A mRNA from 231ER+ (*top*) or MCF-7 (*bottom*) cells treated with 10 nM E2 for 0, 4, 8, 24, and 48 h. Values are mean ± SEM of three independent experiments.

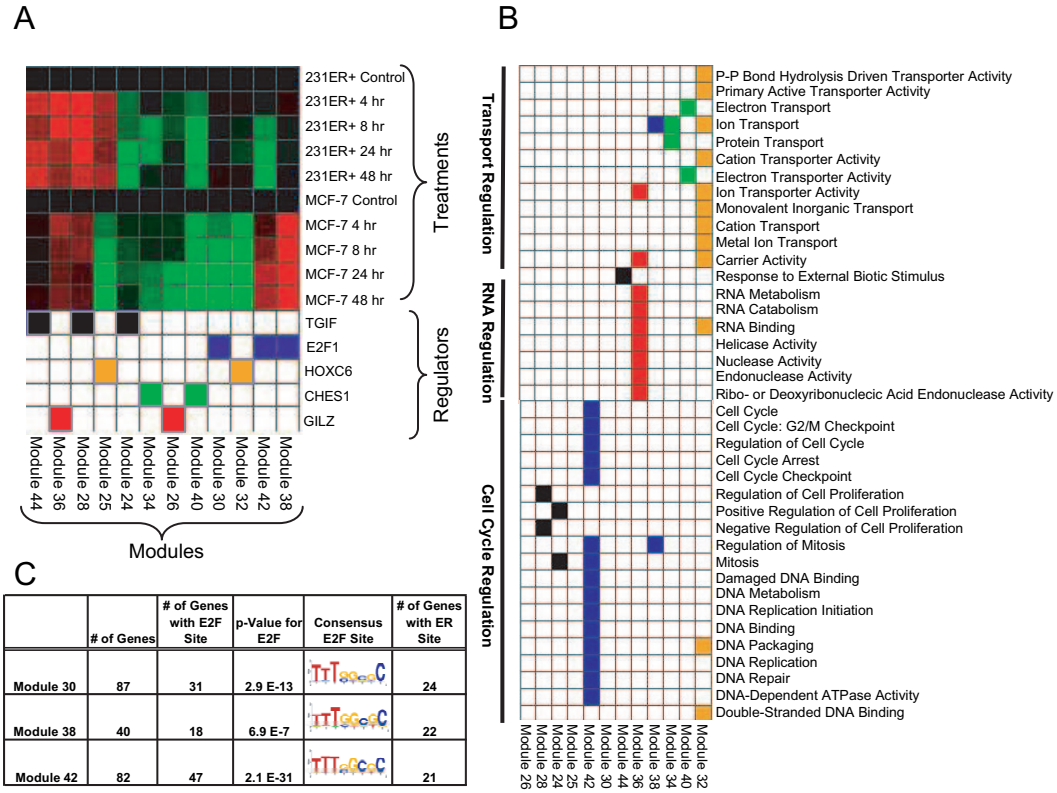
rapidly inhibited CDKN1A expression in MCF-7 cells (Fig. 3). These findings confirm the four distinct regulation patterns for genes identified by microarray analysis of the MCF-7 and 231ER+ breast cancer cells.

Identification of Transcription Factor-Controlled Gene Networks

Because our microarray gene expression data clustered into two general gene regulation patterns, where genes were affected in a concordant or a discordant manner by estrogen in MCF-7 and 231ER+ cells, we hypothesized that estrogen regulation of transcription factors, and subsequently their downstream targets, might account for the distinct regulatory patterns identified by gene tree clustering (Fig. 2). Therefore, we used Genomica software, a module-level analysis program that identifies potential transcriptional regulators for distinct patterns of gene regulation. This analysis, developed by Segal *et al.* (37), takes as inputs gene expression data in addition to a potential regulator list. The analysis partitions the gene expression data into modules of genes based on gene expression data and predicts a candidate regulator that is the most highly correlated to each module.

To gain a global understanding of estrogen action in these breast cancer cells, we used as our input gene expression data for Genomica, genes that were identified to be significantly stimulated or repressed by E2 in our time course experiments in either MCF-7 or 231ER+ cells, or both. These criteria identified 731 E2-regulated genes, this number being much larger than those commonly regulated genes previously identified (Fig. 2A, intersection area), because it also includes genes that are regulated by E2 in only one cell type (Fig. 2A, nonoverlapping areas). Of the potential regulator list of 659 genes recognized as transcription factors by molecular function in Gene Ontology, 68 transcription factors were included in the 731 E2-regulated genes; only these were used in identifying regulatory modules using the Genomica program.

The Genomica analysis identified 12 modules (Fig. 4A) that had similar expression profiles as identified in our initial gene tree clustering (Fig. 2). Interestingly, five regulators, TGIF, E2F1, HOXC6, CHES1, and GILZ, were identified to potentially regulate these modules. CHES1, which was itself down-regulated by E2 in both cell lines, was predicted to regulate modules 34 and 40,which are repressed by E2 in both cell types. GILZ, which was up-regulated 2- to 3-fold by E2 in both MCF-7 and 231ER+ cells, and TGIF, which was down-regulated 2- to 3-fold by E2 in both cells lines, were predicted to regulate modules stimulated by E2 in both MCF-7 and 231ER+ cells (GILZ, module 36; TGIF, module 28). Meanwhile, HOXC6, which was up-regulated 2-fold by E2 in MCF-7 cells as reported previously (23), and found to be mildly down-regulated by E2 in 231ER+ cells, was predicted to regulate a module repressed only in MCF-7 cells (module 32) and a module (module 25) repressed in MCF-7 but stimulated in 231ER+ cells by E2. Additionally, E2F1 was predicted to regulate three modules (modules 30, 42, and 38) that demonstrated discordant E2 regulation in MCF-7 and 231ER+ cells. These data suggest that E2 modulation of CHES 1, GILZ, and TGIF may involve a conserved gene regulation mechanism in the two breast cancer cells, whereas E2 regulation of E2F1



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	# of Genes	# of Genes with E2F Site	p-Value for E2F	Consensus E2F Site	# of Genes with ER Site
Module 30	87	31	2.9 E-13		24
Module 38	40	18	6.9 E-7		22
Module 42	82	47	2.1 E-31		21

Fig. 4. Identification of E2-Regulated Module Networks in Breast Cancer Cells

A, Estrogen-regulated module networks identified by Genomica. Genes are partitioned into 12 modules based on gene expression data, with *red* and *green* indicating stimulation or repression, respectively (*top*). Potential transcriptional regulators are identified for each identified module (*bottom*). B, Modules are enriched for Gene Ontology terms associated with ion transport regulation, RNA regulation, and cell cycle regulation. Squares are colored based on enrichment of a module for the corresponding Gene Ontology term, and colors are assigned to each predicted transcription factor: E2F1 (*blue*), TGIF (*black*), HOXC6 (*orange*), CHES (*green*), and GILZ (*red*). C, Enrichment for the E2F response element and for ER binding sites in E2F1 controlled modules 30, 38, and 42. Identified E2F1 response elements are visually represented by WebLogo (http://weblogo.berkeley.edu).

and HOXC6 results in directionally opposite gene regulation in the two breast cancer cell lines.

To identify the cellular functions of these transcription factors and their associated modules, we determined gene ontology annotations that were enriched for each identified module. Several enriched gene ontology annotations clustered into functional processes including cellular transport regulation, RNA regulation, and cell cycle regulation (Fig. 4B). HOXC6 is predicted to control module 32, which was significantly enriched for annotations associated with cellular transport regulation and include ion transporter activity ($P < 6.74E-4$), primary active transporter activity ($P < 3.06E-3$), and cation transport ($P < 2.33E-3$), suggesting a role for E2 regulation of ion transport through regulation of the HOXC6 transcription factor (Fig. 4B). The transcription factor GILZ is predicted to regulate genes enriched for RNA regulation, and include RNA metabolism ($P < 1.75E-3$), RNA catabolism ($P < 1.96E-4$), and RNA binding ($P < 5.27E-4$) (Fig. 4). In addition, the E2F1-controlled module 42 was significantly enriched in annotations associated with cell cycle regulation and included DNA replication initiation ($P < 2.81E-51$), DNA replication ($P < 0.97E-9$), regulation of mitosis ($P < 0.03$), and regulation of the cell cycle ($P < 1.83E-7$) (Fig. 4B).

Due to its association with cell cycle regulation and the prediction that it controls modules that are oppositely regulated by E2 between MCF-7 and 231ER+ cells, we chose to focus on E2F1-controlled modules. To determine the likelihood of E2F1 involvement in regulating members of the predicted modules, we scanned 2000 bp directly upstream of the transcription start sites for genes in modules 30, 38, and 42 for the E2F response element. As shown in Fig. 4C, we found the E2F response element to be greatly enriched in the upstream regions of genes from module 30 (31 of 87 genes, $P < 2.91E-13$), module 38 (18 of 40 genes, $P < 6.92E-7$), and module 42 (47 of 82 genes, $P < 2.13E-31$), strongly implicating E2F1 in their regulation (Fig. 4C). In addition, we have searched a publicly available genome-wide ER binding site database for ER binding sites within 100 kb of the predicted E2F1-regulated genes (41). The findings are presented in Fig. 4C, and a full list of the genes comprising the predicted E2F1-regulated modules, including the lo-

cations of ER and E2F binding sites, is given in supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. As shown in Fig. 4C and supplemental Table 1, many of the genes in these three modules contain an E2F response element and no ER binding sites, suggesting that they are direct E2F targets and are secondary targets of ER that rely on E2F1 activation. The genes that contain E2F as well as ER binding sites might be potential targets of both E2F1 and ERα.

Estrogen Regulation of the E2F Family of Transcription Factors

Because the E2F1 transcription factor was found to be very significantly associated with genes regulated in an opposite fashion by E2 in MCF-7 and 231ER+ cells, and the E2F1 motif was highly enriched in several genes associated with regulation of the cell cycle, it suggested that differential regulation of this factor by E2 might underlie the opposite effects of this hormone on proliferation of these two ER-positive breast cancer cells. The E2F family comprises six members, with E2F1–3 considered to usually represent transcriptional activators and E2F4–6 representing repressors (42–44). We therefore examined whether there was regulation by estrogen of E2F family members by quantitative real-time PCR in MCF-7 and 231ER+ cells.

In 231ER+ cells, estrogen inhibited E2F1 mRNA expression (Fig. 5A). E2 also transiently increased E2F2 mRNA and mildly stimulated E2F6 mRNA across the time course (Fig. 5A). Little or no regulation by E2 was seen for E2F3–5 (Fig. 6A and data not shown). In MCF-7 cells, estrogen robustly stimulated E2F1 mRNA as seen previously (43, 45), while only weakly stimulating E2F2 mRNA and E2F6 mRNA (Fig. 5B). No regulation by E2 was seen for E2F3–5 in MCF-7 cells (Fig. 6A and data not shown).

Western blots showed the E2F1 protein to exhibit opposite regulation by E2 in these breast cancer cells (Fig. 5, C and D). In 231ER+ cells, E2F1 protein remained relatively unchanged over the first 12 h of E2 exposure, but then was decreased at 16 and 24 h (Fig. 5C). Of particular note, the basal level of E2F1 protein was much higher in 231ER+ cells, as compared with MCF-7 cells, perhaps reflecting the rapid estrogen-independent growth of control 231ER+ cells (Fig. 1B). In MCF-7 cells, the E2F1 protein level was very low but was robustly increased by 8–12 h and remained elevated through the 24 h of E2 treatment (Fig. 5D). The findings in Fig. 5 demonstrate that E2 treatment regulates E2F1 mRNA and protein levels in opposite directions in 231ER+ and MCF-7 cells and because E2F1 was the E2F family member found to exhibit the most marked and directionally opposite regulation by E2 in these cells, we therefore chose to focus on E2F1 for the remaining studies.

E2F1 Knockdown Prevents Estrogen Regulation of E2F Target Genes

Because we identified enrichment of the consensus E2F binding site in several E2-regulated genes, and

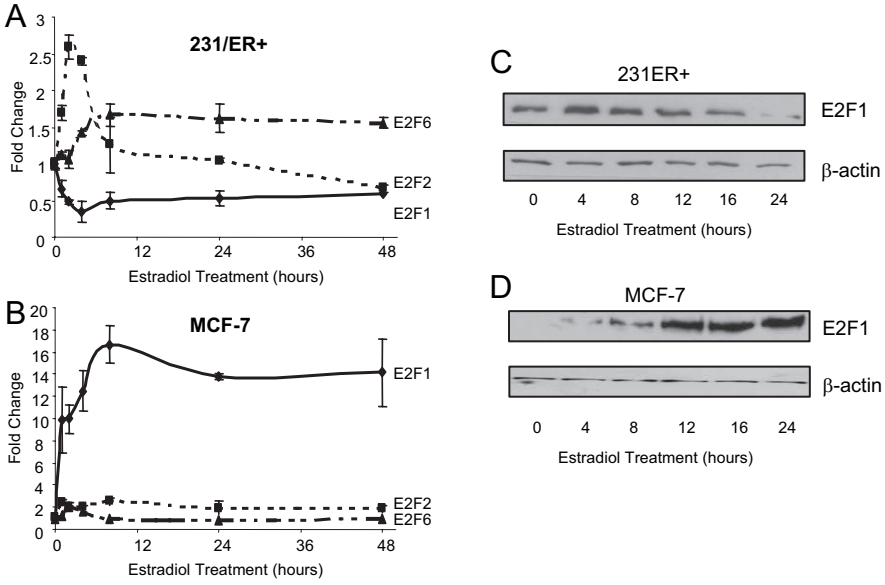

Fig. 5. Estrogen Regulation of E2F Family Members in Breast Cancer Cells

Quantitative real-time PCR data for E2F1, E2F2, and E2F6 mRNA in 231ER+ cells (panel A) and MCF-7 cells (panel B) treated with 10 nM E2 for 0, 1, 2, 4, 8, 24, and 48 h. No change was observed in E2F3, E2F4, or E2F5 mRNA with E2 treatment, and therefore these data are not shown. Values are represented as fold change ± SEM from vehicle treatments. Western blot analysis performed for E2F1, and β-actin as a loading control, in 231ER+ cells (panel C), and MCF-7 cells (panel D) treated with 10 nM E2.

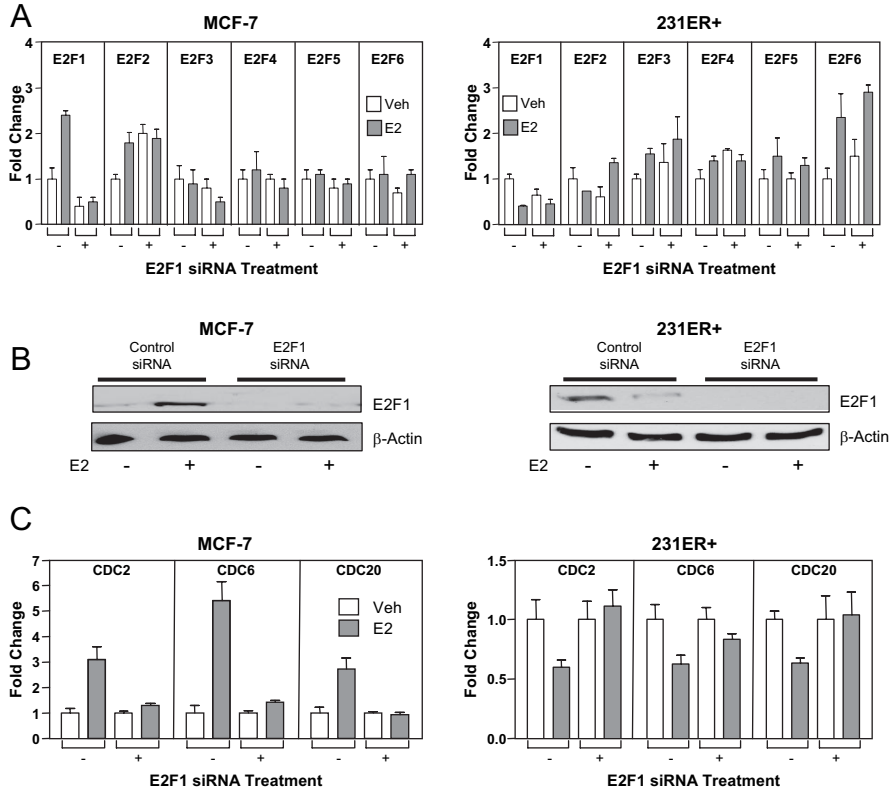

Fig. 6. Knockdown of E2F1 Prevents E2 Regulation of E2F Target Genes

siRNA for E2F1 (+) or control GL3 luciferase (−) was transfected into MCF-7 or 231ER+ cells for 24 h after which the cells were treated with either vehicle (*open bars*) or 10 nM E2 (*solid bars*) for 24 h. A, Quantitative real-time PCR for E2F1-E2F6 in MCF-7 cells (*left*) and 231ER+ cells (*right*) treated with control siRNA or E2F1 siRNA. B, Western blot analysis for E2F1 protein in siRNA-treated MCF-7 (*left*) and 231ER+ (*right*) cells. C, Quantitative real-time PCR for the E2F1 target genes CDC2, CDC6, and CDC20 in MCF-7 (*left*) and 231ER+ (*right*) cells after exposure to control siRNA or E2F1 siRNA. Veh, Vehicle.

also observed robust hormonal regulation of E2F1, we investigated the possible direct link between E2 regulation of E2F1 and the regulation of known E2F1 target genes. We therefore treated MCF-7 and 231ER+ cells with small interfering RNA (siRNA) directed toward either E2F1 or control (GL3) luciferase. To determine the effectiveness of the siRNA knockdown, we performed quantitative real-time PCR and Western blots to measure the levels of E2F1 mRNA and protein after siRNA treatments in both MCF-7 and 231ER+ cells. As seen in Fig. 6A, *left-most panel*, E2F1 knockdown was approximately 60% at the RNA level in MCF-7 cells, and whereas after 24 h of control siRNA treatment followed by 24 h vehicle or E2 exposure, E2 stimulated a 2.4-fold increase in E2F1 mRNA in the control GL3 luciferase siRNA-treated MCF-7 cells, no change in E2F1 RNA was observed with estrogen treatment in the E2F1 siRNA-treated cells. In 231ER+ cells treated with control siRNA, E2 repressed E2F1 RNA as expected, and treatment of 231ER+ cells with E2F1 siRNA reduced the basal E2F1 mRNA level, and no repression by estrogen was seen (Fig. 6A). As expected, in both MCF-7 and 231ER+ cells treated with control (GL3) siRNA, E2F1 protein levels (Fig. 6B) showed the same trend of up- or down-regulation, respectively, in response to E2 as seen previously in MCF-7 and 231ER+ cells (Fig. 5, C and D). And, in both MCF-7 and 231ER+ cells treated with E2F1 siRNA, E2F1 protein was almost undetectable, and little to no change was seen with 24 h of E2 exposure (Fig. 6B).

Furthermore, whereas estrogen stimulated in MCF-7 cells or suppressed in 231ER+ cells the known E2F1 target genes, CDC2, CDC6, CDC20, after control siRNA treatment, this estrogenic effect was completely abrogated after E2F1 siRNA treatments (Fig. 6C), suggesting that the regulation of E2F1 by estrogen is essential for the regulation of these cell cycle-associated genes in response to E2. The siRNA oligo for E2F1 used in these studies appeared to be specific for E2F1 because the mRNA levels of the other E2F family members (E2F3, E2F4, E2F5, and E2F6) remained unchanged, with the exception of E2F2 (Fig. 6A). Basal mRNA for the known E2F1 target gene, E2F2, increased in MCF-7 cells and decreased in 231ER+ cells treated with E2F1 siRNA, suggesting that E2F2 is under basal repression by E2F1 in MCF-7 cells and is basally activated by E2F1 in 231ER+. Similar results for E2F1–E2F6 were obtained with a

second E2F1 siRNA oligo and also with a SMART pool of siRNA specifically designed to target E2F1.

E2F1 Is Necessary for Estrogen Regulation of Breast Cancer Cell Proliferation

Because we demonstrated that estrogen regulation of E2F1 is important for the regulation of several cell cycle-associated genes, we investigated whether siRNA-mediated knockdown of E2F1 would impact the regulation of cell proliferation by estrogen. As seen in Fig. 7, E2 enhanced the proliferation of control siRNA-treated MCF-7 cells, and RNA interference-mediated E2F1 knockdown completely blocked E2 stimulation of proliferation. In 231ER+ cells, E2 treatment reduced the proliferation of control siRNA treated cells (Fig. 7) as observed previously (Fig. 1B), and knockdown of E2F1 eliminated the suppression of proliferation by E2. The findings highlight that E2F1 is critical for hormone-regulated proliferation in these two breast cancer cell lines.

DISCUSSION

Our findings highlight the importance of E2F1 in hormonal regulation of the proliferative response of breast cancer cells to estrogen. They also illustrate the value of combining microarray gene expression profiling with bioinformatic analyses, utilizing ER-positive cells with different phenotypic properties, to elucidate gene networks important in the regulation of proliferation.

There is evidence that ER-positive breast cancers can progress from an estrogen-responsive state, where estrogens stimulate proliferation, to a state in which estrogen instead now suppresses proliferation (1, 28, 30–32). Our comparative gene transcriptional profiling studies were conducted in two breast cancer cell lines in which estrogen has a discordant effect on proliferation to gain insight into the gene networks and pathways where an alteration in regulation might underlie the opposite response to estrogen.

Interestingly, our microarray analysis identified a distinct group of genes associated with cell cycle control that were regulated in opposite fashion by E2 in the two cell lines. These estrogen-regulated genes included those encoding proteins important in cell proliferation such as CDC2, CDC6, thymidine kinase 1, topoisomerase 2A, and inhibitory factors of cell cycle progression, such as TGFβ3 and CDKN1A. Several of these were shown previously to be under E2 control in MCF-7 cells (23), but this is the first report on their regulation by E2 in 231ER+ cells. Our microarray analysis also identified several known ER targets including WISP2, TFF1, NRIP1, and SIAH2, suggesting that some estrogen-regulated transcription networks are conserved in different ER-containing breast cancer cells. E2 also repressed the expression of SMAD3 and SMAD6 in both MCF-7 and 231/ER+ cells. Because the SMAD proteins are transcription factors that mediate TGFβ signaling, these results suggest potential cross-talk between E2 and TGFβ signaling pathways, as indicated by other previous work from our laboratory (46–48). In addition, E2 repressed BACH1, a known repressor of MAF-mediated transcription, further implicating E2 signaling cross-talk in the regulation of transcription networks.

Several of the genes repressed in MCF-7 cells but stimulated in 231/ER+ cells encode proteins with roles in transcription and include the p160 coactivator NCoA1, the transcription factor MAF, and the nuclear hormone receptor NR2B1. The discordant/opposite regulation of these genes by E2 might be attributed to differences in the cellular profiles of transcription factors or to differences in the activities or levels of various coregulator proteins in the two cell types. Indeed, AP1 has been demonstrated to be stimulated by E2 in MCF-7 cells, but to be repressed in 231ER+ cells, and

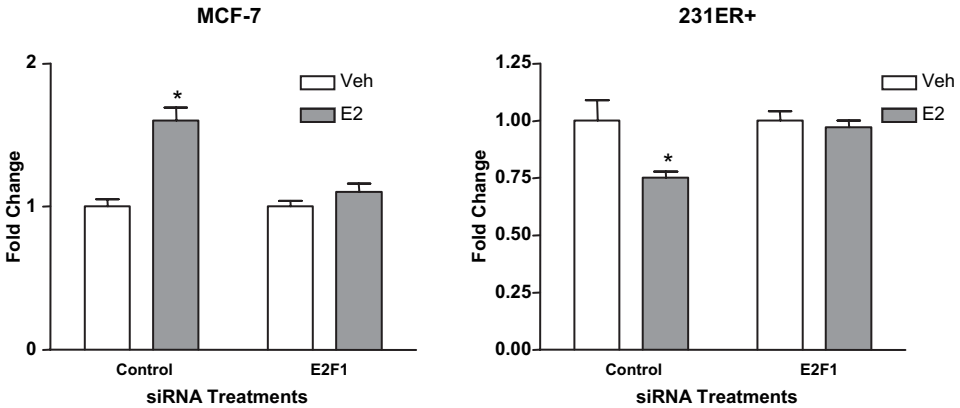

Fig. 7. Knockdown of E2F1 Prevents E2 Regulation of Breast Cancer Cell Proliferation

MCF-7 (*left*) and 231ER+ (*right*) cells were treated with control siRNA or E2F1 siRNA for 24 h after which the cells were treated with either vehicle or 10 nM E2 for 72 h and cell numbers were monitored. Values are the mean ± SEM. *, $P < 0.001$ by Student's *t* test. Veh, Vehicle.

this has been attributed to the high expression of the Fos family protein, Fra-1, in 231ER+ cells (49). Therefore, the varying expression of different transcription factors could have major implications for regulation of certain subsets of E2-regulated genes. It is also possible that the regulatory elements necessary for E2 regulation of subsets of genes might demonstrate cell type specificity or be silenced due to epigenetic modifications in one cell type *vs.* another. In this regard, it has recently been shown that the cyclin D1 gene has a cell type-specific enhancer that is used by the ER in MCF-7 cells, but not 231ER+ cells (50). In addition, although the ER may be recruited to these regulatory sites, the necessary coregulator complexes or transcriptional machinery might not be present to trigger transcriptional activation, as observed by us previously (51). Thus, the ability of ER to stimulate gene expression in one cell line but repress gene expression in another is a complex issue dependent upon many aspects: different expression levels and activity states of critical transcription factors and coregulatory proteins, chromatin structure, and epigenetic modifications.

We tested the hypothesis that these opposite regulation patterns in the two breast cancer cell lines might be attributable to a common transcription factor the level of which is being regulated in an opposite fashion by estrogen. This aspect was evaluated by applying a bioinformatic analysis of the promoters of coordinately regulated genes to identify overrepresented transcription factor binding sites. This analysis assumes that coregulated genes are regulated through a common pathway or transcription factor and also that genes regulated early by estrogen influence the expression of genes regulated at later times. Estrogen regulates the expression of many genes (23), only a portion of which are direct, primary response genes. Bioinformatic analyses enable one to trace the pathways impacted by estrogen, by identifying primary transcription factor response genes, through which estrogen can affect multiple downstream genes that are targets of these estrogen-regulated transcription factors.

These approaches, performed previously on yeast gene expression datasets, have proven quite useful in identifying subsets of genes regulated by a common transcription factor under specific experimental conditions (37, 52). The bioinformatic analysis of estrogen-regulated gene promoters in our datasets from MCF-7 and 231-ER+ cells identified overrepresented transcription factor binding motifs that are associated with distinct regulatory patterns. The E2F response element was found to be greatly overrepresented in genes stimulated in MCF-7 but repressed in 231ER+ cells, suggesting the involvement of this transcription factor in the opposite proliferative effect of estrogen in these two cells. In addition, mapping of the predicted E2F1-regulated genes to a published genome-wide ER binding site dataset enabled us to observe that many of these genes have no ER binding site within 100 kb of the gene, implicating E2F1, but not ER, in their primary regulation and suggesting that estrogen regulation of these genes is likely a secondary response mediated via the estrogen regulation of E2F1. Because many of these oppositely regulated genes are known to contribute to progression of the cell cycle and include genes known to be regulated by E2F1 (53), we investigated whether estrogen regulation of the E2F family was responsible for the opposite proliferation phenotypes observed in the two breast cancer cell lines.

The E2F family of transcription factors contains six members (E2F1–6) that can be divided into two main categories: those that usually function as activators (E2F1, E2F2, and E2F3) and are repressed by binding to pRB; and those that usually work as repressors (E2F4, E2F5, and E2F6) and bind to pRB, p107, p130, and/or polycomb proteins (42). The important role of E2F1 in E2 regulation of proliferation in breast cancer cells is supported by a number of our findings: 1) enrichment of the consensus E2F response element in the promoters of many genes that were regulated in opposite fashion by E2 in MCF-7 and MDA-MB-231-ER+ breast cancer cells; 2) our observation of the opposite regulation of E2F1, E2F2, and E2F6 by E2 in the two cell lines, with E2F1 being the most robustly regulated, corroborating prior findings (43, 45, 54) that E2F1 gene expression is highly regulated by E2 in breast cancer cells; 3) our finding that knockdown of E2F1 prevented E2 regulation of genes containing consensus E2F response elements that are associated with cell cycle progression; and 4) that E2F1 knockdown eliminated E2 regulation of cell proliferation.

The regulation of E2F1 by E2 may be of clinical relevance due to evidence that E2F1 expression is increased in breast tumors as compared with normal mammary tissue, and that patients with high E2F1-expressing tumors have significantly reduced disease-free and overall survival compared with patients with low-expressing E2F1 tumors (44, 55). In addition, E2F1 expression was found to increase with increased malignancy stage of breast tumors, with highest expression in invasive ductal carcinomas (56). Because many cell cycle-associated genes are regulated by E2F1, estrogen regulation of the E2F1 gene could have a broad effect on the activity of cell cycle gene targets of E2F1.

Our findings highlight the value of combined gene expression analyses and bioinformatics in elucidating gene networks and key proteins, such as those involving E2F1. Although the presence of ER in cancer cells is required for the estrogen-regulated proliferative response and for the effectiveness of endocrine therapies (*e.g*. treatment with selective ER modulators such as tamoxifen, or treatment with aromatase inhibitors), our findings suggest that certain crucial estrogen-regulated targets, such as E2F1, might also be considered as targets for therapy. Therapies directed at such targets might effectively augment endocrine ap-

proaches or be of benefit in some ER-containing yet hormone-refractory breast cancers.

MATERIALS AND METHODS

Cell Culture and Cell Proliferation Assays

MCF-7 cells, and MDA-MB-231 cells stably expressing ERα, were grown as previously described (23, 38, 57). Four days before treatment, cells were switched to phenol red-free tissue culture medium containing 5% charcoal-dextran-treated calf serum. Medium was changed on d 2 and d 4 of culture, and then cells were treated with control 0.1% ethanol vehicle, 10 nM E2, or 1 μM antiestrogen ICI 182,780 alone or with 10 nM E2 for the various times indicated.

Cell proliferation assays were performed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay as recommended by the manufacturer (Promega Corp., Madison, WI). Briefly, cells were plated at 1000 cells per well in phenol-free tissue culture media containing 5% charcoal-dextran-treated calf serum for 24 h. Cells were then treated with control 0.1% ethanol vehicle, 10 nM E2, 1 μM ICI, or the combination of E2 plus ICI for the days indicated with media changed every 2 d. For siRNA studies, cells were transfected with siRNA for 24 h (as described below), followed by hormone treatment for the times indicated. Cell number was assessed by measuring the reduction of the compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium at absorbance 490 nm.

RNA Extraction and Microarray Analysis

Preparation of RNA from cells and analysis of Affymetrix gene chip microarray data were as previously described (23). Total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was further purified using RNeasy columns (QIAGEN, Chatsworth, CA) and treatment with RNase-free DNase I (QIAGEN). Total RNA was used to generate cRNA, which was labeled with biotin as recommended by Affymetrix. cRNA was then hybridized to Affymetrix Hu95A GeneChips, which contain approximately 12,000 human oligonucleotide probe sets. After washing, the chips were scanned and analyzed using MicroArray Suite 5.0 (Affymetrix). Average intensities for each Gene Chip were globally scaled to a target intensity of 150. Further analysis was performed using Gene Spring software version 5.0.1 to obtain expression level information, fold change, and *P* values for each gene at each time point relative to untreated control as described (23).

Quantitative Real-Time PCR

Total RNA (1 μg) was reverse transcribed in a total volume of 20 μl using 200 U reverse transcriptase, 50 pmol random hexamer, and 1 mM deoxynucleotide triphosphate, and real-time PCR was performed to verify regulation of gene expression by E2. The resulting cDNA was then diluted to a total volume of 100 μl with sterile water. Each real-time PCR reaction consisted of 1 μl of diluted reverse transcriptase product, 1× SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and 50 nM forward and reverse primers. Reactions were carried out in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) for 40 cycles (95 C for 15 sec, 60 C for 1 min) after an initial 10-min incubation at 95 C. The fold change in expression for each gene was calculated as described previously, with the ribosomal protein 36B4 mRNA as internal control (25). Primer sequences will be provided upon request.

Module Discovery Analysis

We used Genomica software (37) to predict transcription factors associated with our gene regulation patterns. We imported gene expression data for genes with present cells that were regulated at least 2.0-fold by E2 in either MCF-7 or 231ER+ cells with a P value < 0.05. In addition, we generated and imported a list of 661 genes identified by the Gene Ontology Consortium to encode transcription factors. We then created a network composed of 12 modules requiring that the predicted transcription factor be correlated to its module in five of eight experimental conditions. To determine biological relevance of the identified modules, Genomica was used to determine Gene Ontology terms enriched for each module requiring three or more genes within a module to be associated with a term and P value < 0.05. All gene clustering, statistical evaluations and visualizations were performed using Genomica software.

Promoter Analysis and ER Binding Site Identification

We used GeneSpring version 5.0.1 software (Silicon Genetics, San Carlos, CA) to evaluate individual modules for the enrichment of the predicted transcription factor DNA binding motifs. We searched 2000 bp upstream of the transcriptional start site for each gene within a given module. Single *P* values were calculated by GeneSpring software and represent significance for a given motif relative to the human genome (9999 bp upstream of approximately 12,000 transcription start sites). Identified DNA motifs are visually represented by WebLogo analysis (http://weblogo.berkeley.edu).

We mapped the Affymetrix probes for E2-regulated genes to the UCSC Genome Browser. The mapped probes were intersected with a published genome-wide ER binding site dataset (41), and binding sites within 100 kb were considered to be potentially associated with the E2-regulated gene.

Western Blot Analysis

Cell protein lysates were separated in sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. Blots were incubated in blocking buffer (5% milk in Tris-buffered saline with 0.5% Tween) and then with specific antibodies for E2F1 (MS-880, NeoMarkers, Fremont, CA) and β-actin (AC-15; Sigma Chemical Co., St. Louis, MO), followed by detection using horseradish peroxidase-conjugated secondary antibodies with Supersignal West Femto Detection Kit (Pierce Chemical Co., Rockford, IL), as described by the manufacturer.

siRNA Studies

Cells were plated in phenol red-free medium containing 5% charcoal-dextran-treated calf serum at 250,000 cells per well in six-well plates, and transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions as described previously (58). Synthetic RNA oligonucleotides targeting E2F1 and control (GL3) luciferase were obtained from Dharmacon (Lafayette, CO). The siRNA sequences used for E2F1 were: *siE2F1* sense, 5′-UGGACCACCUGAUGAAUAUdTdT-3′; and antisense, 5′-AUAUUCAUCAGGUGGUCCAdTdT-3′. GL3 siRNA sequences were Dharmacon catalog number D-001400–01, as used previously (58). siRNA was used at 100 nM, as recommended by the manufacturer, and cells were exposed to the siRNA and then treated with control vehicle or hormone for the times indicated.

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