Published in final edited form as:
J Mol Endocrinol. 2009 January ; 42(1): 19–33. doi:10.1677/JME-08-0088.

Role of SP transcription factors in hormone-dependent modulation of genes in MCF-7 breast cancer cells:

microarray and RNA interference studies

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Abstract

17β-Estradiol (E_2) binds estrogen receptor α (ESR1) in MCF-7 cells and increases cell proliferation and survival through induction or repression of multiple genes. ESR1 interactions with DNA-bound specificity protein (SP) transcription factors is a nonclassical genomic estrogenic pathway and the role of SP transcription factors in mediating hormone-dependent activation or repression of genes in MCF-7 cells was investigated by microarrays and RNA interference. MCF-7 cells were transfected with a nonspecific oligonucleotide or a cocktail of small inhibitory RNAs (iSP), which knockdown SP1, SP3, and SP4 proteins, and treated with dimethylsulfoxide or 10 nM E_2 for 6 h. E_2 induced 62 and repressed 134 genes and the induction or repression was reversed in ~62% of the genes in cells transfected with iSP (ESR1/SP dependent), whereas hormonal activation or repression of the remaining genes was unaffected by iSP (SP independent). Analysis of the ESR1/SP-dependent and SP-independent genes showed minimal overlap with respect to the GO terms (functional processes) in genes induced or repressed, suggesting that the different genomic pathways may contribute independently to the hormone-induced phenotype in MCF-7 cells.

Introduction

The estrogen receptor (ER) and other steroid hormone receptors are members of the nuclear receptor (NR) superfamily of transcription factors that are characterized by their common modular structure and similar ligand-dependent mechanisms of action. NRs express an N-terminal A/B domain that contains a ligand-independent activation function-1, a DNA-binding domain C, a flexible hinge region (D), and a C-terminal E/F domain that contains a ligand-dependent AF-2 and a region that binds ligands (Katzenellenbogen *et al.* 1996, Hall *et al.* 2001, Nilsson & Gustafsson 2002, Evans 2005, O'Malley 2005).

ESR1 and ESR2 are the two ER subtypes that exhibit overlapping and different patterns of expression in various tissues, and there is extensive evidence from *in vitro* cell culture, gene ablation, and *in vivo* studies that these receptors primarily have different functions (Matthews & Gustafsson 2003). ESR1 was the first ER subtype identified and its role is associated with

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

many of the familiar estrogen-mediated functions including female reproductive tract development, bone growth, and overexpression in early stage mammary tumors in women and in ER-positive (ER^+) breast cancer cells such as the widely used MCF-7 cell line (Levenson & Jordan 1997). MCF-7 and other ER^+ breast cancer cell lines are highly responsive to the mitogenic activity of 17β-estradiol (E_2) and have been extensively used to understand the complex molecular biology of estrogen action. The classical mechanism of E_2-induced gene expression involves ligand-dependent formation of a nuclear ER homodimer bound to an estrogen-responsive element (ERE) that in turn recruits multiple elements of the transcriptional machinery to activate gene expression (Katzenellenbogen *et al.* 1996, Hall *et al.* 2001, Nilsson & Gustafsson 2002, Matthews & Gustafsson 2003, Evans 2005, O'Malley 2005). Subsequent studies have identified multiple pathways of hormone-dependent activation of ER and these include interactions of the receptor with ERE half-sites alone and in combination with other DNA-bound transcription factors such as GATA1, NFκB, and specificity protein-1 (SP1). In addition, ER activates genes through protein-protein interactions with other DNA-bound transcription factors such as the activator protein-1 (JUN) complex and SP proteins (Blobel & Orkin 1996, Paech *et al.* 1997, Webb *et al.* 1999, Inadera *et al.* 2000, Pelzer *et al.* 2001, Safe 2001, Pratt *et al.* 2003, Safe & Kim 2004, Chadwick *et al.* 2005, Ghisletti *et al.* 2005, Kalaitzidis & Gilmore 2005).

Several E_2-responsive genes contain E_2-responsive GC-rich promoters (Safe 2001, Safe & Kim 2004), and RNA interference studies using small inhibitory RNAs for SP1 (iSP1), SP3 (iSP3), SP4 (iSP4) or their combination (iSP) show that all three SP proteins play a role in hormonal activation of three E_2-responsive genes, namely *E2f1*, *Cad*, and *Rara* (Khan *et al.* 2007). Transfection with iSP was the most effective inhibitor of hormone activation of these genes.

E_2 and various selective ER modulators induce or repress a broad spectrum of genes in breast cancer cells (Soulez & Parker 2001, Inoue *et al.* 2002, Levenson *et al.* 2002, Lobenhofer *et al.* 2002, Coser *et al.* 2003, Cunliffe *et al.* 2003, Frasor *et al.* 2003, 2004, Scafoglio *et al.* 2006) and, in some microarray studies, E_2 decreased expression of more genes than it induced. In this study, we investigated the role of SP proteins in hormonal modulation of gene expression in MCF-7 cells by treating cells for 6 h with dimethylsulfoxide (DMSO; solvent control) or 10 nM E_2 and transfecting cells with either a nonspecific oligonucleotide (iNS) or iSP cocktail containing iSP1, iSP3, and iSP4. Using this approach, we showed that E_2 induced 67 and repressed 134 genes and 62% of these genes were SP dependent. Genes regulated by ESR1/SP could be further subdivided into three subclasses based on the effects of iSP on basal activity of these genes in which basal expression was unaffected (B^0), enhanced (B^+), or decreased (B^-). Interestingly, the B^0 and B^+ subcategories were predominant for genes induced and the B^- subcategory predominated for genes repressed by E_2-mediated activation of ESR1/SP. Our results show that the genomic ESR1/SP pathway is important for hormonal regulation of genes in MCF-7 cells.

Materials and methods

Cell culture

MCF-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM/nutrient mixture Ham's F12 (DMEM/F12; Sigma-Aldrich) supplemented with 2·2 g/l sodium bicarbonate, 5% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA), and 5 ml/liter antibiotic antimycotic solution (AAS, Sigma-Aldrich). Cells were cultured and grown in a 37 °C incubator with humidified 5% CO_2:95% air.

Reverse transcription PCR for determination of mRNA levels

MCF-7 cells were cultured in serum-free DMEM/F12 for 1 day before treatment with 20 nM E_2 or 20 nM E_2+2 μM ICI 182780. DMSO as a solvent control for 24 h or with 20 nM E_2 for 0, 2, 4, 6, 9, and 24 h. For the RNA interference studies, cells were cultured in phenol red-free DMEM/F12 supplemented with 2·5% charcoal-stripped FBS in six-well plates until 50-70% confluent. Cells were washed once with serum-free, antibiotic-free, phenol red-free DMEM/F12. The amount of siRNA to give a maximal decrease of each target protein was determined experimentally (50 nM final concentration in the well). Lipofectamine 2000 reagent (Invitrogen) was used to transfect MCF-7 cells with siRNA (iSP1, 3, 4, or iNS) according to the manufacturer's protocol and as indicated above under RNA interference and microarray assay. The next day, cells were treated with 20 nM E_2 or DMSO as a solvent control in serum-free, antibiotic-free, phenol red-free DMEM/F12. Cells were harvested 2, 4, 6, 9, or 24 h after treatment or as indicated in the different experiments. The siRNA oligonucleotides for nonspecific small inhibitory RNA (iNS), SP1, SP3, and SP4 were obtained from Dharmacon, Lafayette, CO, USA as described above. Total RNA from the studies was isolated using the RNeasy Protect Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration was measured by u.v. 260:280 nm absorption ratio; 1 μg RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The genes of interest were amplified from the generated cDNA by PCR cycles using Taq polymerase (Invitrogen). The PCR conditions were as follows: initial denaturation at 94 °C (2 min) followed by 29 cycles (E2F1 and KLK6), 30 cycles (VEGFA), 28 cycles (HMGB1), or 27 cycles (GAPDH) of denaturation for 30 s at 94 °C; annealing for 30 s at 54 °C; extension at 72 °C for 1 min; and a final extension step at 72 °C for 10 min. The mRNA levels were normalized using *Gapdh* as an internal housekeeping gene. PCR products were electrophoresed on 1·5% agarose gels containing ethidium bromide and visualized under u.v. transillumination, then quantitated using Image-J software. Primers obtained from IDT (Coralville, IA, USA) and used for amplification were E2F1 (sense, 5′-CGC ATC TAT GAC ATC ACC AAC G-3′; antisense, 5′-GAA AGT TCT CCG AAG AGT CCA CG-3′); VEGFA (sense, 5′-CCA TGA ACT TTC TGC TGT CTT-3′; antisense, 5′-ATC GCA TCA GGG GCA CAC AG-3′); HMGB1 (sense, 5′-AAC ATG GGC AAA GGA GAT CC-3′); antisense, 5′-TAC CAG GCA AGG TTA GTG GC-3′); KLK6 (sense, 5′-TAC CAA GCT GCC CTC TAC AC-3′; antisense, 5′-ACA AGG CCT CGG AGG TGG TC-3′); GAPDH (sense, 5′-AAT CCC ATC ACC ATC TTC CA-3′; antisense, 5′-GTC ATC ATA TTT GGC AGG TT-3′).

RNA interference and microarray assay

MCF-7 cells (5×10^4) were cultured in phenol red-free DMEM/F12 supplemented with 2·5% charcoal-stripped FBS without AAS in 12-well plates overnight. Cells were transfected with nonspecific siRNA (iNS) or the combination of iSP1/iSP3/iSP4 (iSP) by Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. After 6 h, the transfection medium was changed with fresh DMEM/F12 and 2·5% serum without phenol red. Forty-eight hours after transfection, cells were treated with (DMSO, solvent) or 10 nM E_2 (98%, Sigma-Aldrich) for 6 h. Three replicate determinations were obtained for each treatment group. Total RNA was isolated using the RNeasy Protection Mini Kit (Qiagen) according to the manufacturer's protocol.

The siRNA duplexes used in this study are indicated as follows. Silencer Negative Control #1 siRNA purchased from Ambion (Austin, TX, USA) was used as iNS. The siRNA oligonucleotides for SP1, SP3, and SP4 were obtained from Dharmacon as follows: SP1, 5′-AUC ACU CCA UGG AUG AAA UGA dTdT-3′; SP3, 5′-GCG GCA GGU GGA GCC UUC ACU dTdT-3′; and SP4, 5′-GCA GUG ACA CAU UAG UGA GCdT dT-3′.

Microarray hybridization and data analysis

Microarray studies were carried out with the three samples obtained for each treatment group using the CodeLink Whole Genome Bioarrays (#300026) with over 50 000 probe targets per slide and, after treatment with 10 nM E_2 for 6 h, RNA was isolated for reverse transcription PCR. The microarray data for each sample were imported into GeneSpring BX7 software (Silicon Genetics, Redwood City, CA, USA) for data analysis. The data were normalized in two steps. First, for each array, the expression value of each gene was divided by the median of the expression values in that array. Second, for each gene, the expression value in each array was divided by the median expression value of that gene across all of the arrays. Genes that were flagged 'L' by the CodeLink preprocessing software, i.e. with low signal to background noise ratio, were excluded from the subsequent statistical analysis. One-way parametric ANOVA test was performed to detect significant changes of gene expression. Benjamini-Hochberg false discovery rate as multiple testing correction and Tukey *post hoc* test were applied. The false discovery rate cut-off was set to 0·05 due to the large number of genes on the chip and the small number of microarrays used in the experiment.

Western blot analysis

MCF-7 cells were seeded into six-well plates in DMEM/F12 supplemented with 2·5% charcoal-stripped FBS. The next day, cells were transfected with siRNA as described above and high-salt extracts were obtained by harvesting cells in a high-salt lysis buffer (50 mM HEPES (pH 7·5), 500 mM NaCl, 10% (vol/vol) glycerol, 1% Triton X-100, 1·5 mM $MgCl_2$, 1 mM EGTA, protease inhibitor cocktail (Sigma-Aldrich)) on ice for 45-60 min with frequent vortex and centrifugation at 20 000 *g* for 10 min at 4 °C. Protein concentrations were determined using a BioRad protein assay reagent. Protein (60 μg) was diluted with Laemmli's loading buffer, boiled, and loaded onto 7·5% SDS-PAGE. Samples were resolved using electrophoresis at 150 V for 3-4 h and transferred (transfer buffer, 48 mM Tris-HCl, 29 mM glycine, and 0·025% sodium dodecyl sulfate) to a polyvinylidene difluoride membrane (BioRad) by electrophoresis at 0·2 Å for ~12-16 h. Membranes were blocked in 5% TBS-Tween 20-Blotto (10 mmol/l Tris-HCl, 150 mmol/l NaCl (pH 8·0), 0·05% Triton X-100, 5% nonfat dry milk) with gentle shaking for 30 min and incubated in fresh 5% TBS-Tween 20-Blotto with 1:1000 (for SP1 and SP3,), 1:500 (for SP4), and 1:5000 (for β-actin) primary antibody overnight with gentle shaking at 4 °C. The primary antibodies for SP1 (PEP2), SP3 (D-20), and SP4 (V-20) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and antibody for β-actin was purchased from Sigma-Aldrich. Membranes were probed with a HRP-conjugated secondary antibody (1:5000) for 3-6 h at 4 °C. Blots were visualized using the chemiluminescence substrate (Perkin-Elmer Life Sciences Warwick, RI, USA) and exposure on Image Tek-H X-ray film (American X-ray Supply). Band quantitation was performed by ImageJ (National Institutes of Health).

Results

Previous microarray studies have investigated hormonal modulation of genes in breast cancer cells under various conditions and there is evidence that genes, with different and overlapping functions, are induced and repressed (Soulez & Parker 2001, Inoue *et al.* 2002, Levenson *et al.* 2002, Lobenhofer *et al.* 2002, Coser *et al.* 2003, Cunliffe *et al.* 2003, Frasor *et al.* 2003, 2004, Scafoglio *et al.* 2006, Jonsson *et al.* 2007). Several reports show that E_2 induces and represses genes that are activated through a nonclassical ESR1/SP mechanism in which ESR1 binds SP proteins but not promoter DNA (Safe 2001, Safe & Kim 2004); however, the contributions of this pathway to hormonal activation of genes is not well defined. In this study, MCF-7 cells were treated with DMSO (solvent) or 10 nM E_2 for 6 h and transfected with a nonspecific oligonucleotide (iNS) or a combined cocktail (iSP) of small inhibitory RNAs for SP1 (iSP1), SP3 (iSP3), and SP4 (iSP4), which has previously been used to simultaneously

knockdown all three SP proteins (Khan *et al.* 2007). Western blot analysis of whole-cell lysates from cells transfected with INS or iSP confirms efficient knockdown of all three SP proteins (Fig. 1A). Results in Fig. 1B show that in cells transfected with iNS and treated with DMSO or E_2, there were 67 and 134 genes significantly ($P<0{\cdot}01$) induced and repressed respectively, and this was consistent with other studies in MCF-7 cells showing that E_2 primarily decreases gene expression (Frasor *et al.* 2003, 2004). It is important to point out that our selection of significantly expressed genes is based on a stringent condition ($P<0{\cdot}01$) that reflects the large number of probes on the slides and the small number of samples. Selection of these stringent conditions is consistent with a report by Hu and coworkers (Hu *et al.* 2005), which uses the false discovery rate to estimate the number of required samples per condition for achieving a desired level of significance. This has to be taken into account when comparing the results of our statistical analysis with the already published similar studies. For example, Frasor and coworkers (Frasor *et al.* 2003) identified over 400 genes with 'robust level of regulation,' but the microarrays used in their experiment contained ~12 000 genes and the decision to place a gene in the reported list was based on a different scoring procedure.

After transfection of cells with iSP, hormone-regulated genes in MCF-7 cells can be subdivided into four major categories (Fig. 1C). Categories A and B represent SP-dependent genes induced or repressed by E_2 (Tables 1 and 2) and categories C and D represent SP-independent genes induced or repressed by E_2 respectively (Tables 3 and 4). A fifth category, F, represents a substantial number of genes (85) that are not affected by treatment with E_2; however, after SP protein knockdown (Fig. 1A), category F genes are either induced or repressed by E_2 (Table 5). SP proteins are overexpressed in breast tumor compared with nontumor cells (Mertens-Talcott *et al.* 2007), and it is possible that as SP proteins increase during mammary carcinogenesis, category F genes become hormone insensitive. The decision to assign a gene to one of the five categories was based on two cut-off points; genes with fold change of 0·8 or less were considered repressed while the genes with fold change of 1·3 or greater were considered induced. The selection of these thresholds was based on the observation that varying them by as much as 0·02 produced small changes in the categories; only one to five genes would change their category membership.

Category A genes were induced by E_2 and induction of these genes was inhibited after cotransfection with small inhibitory RNAs for SP1, SP3, and SP4 (combined; iSP). Table 1 summarizes one subset of genes induced by E_2 (1·3- to 2·4-fold) in which transfection with iSP significantly decreased fold induction, whereas basal activity was minimally affected by iSP since the ratio of the DMSO values in the iSP and iNS treated groups (iSPD/iNSD) varied only from 0·81 to 1·77 and were considered to be unchanged. These were classified as $^{+}$ESR1/SP (B^0)-inducible genes. A second subset of genes was also induced by E_2 (1·30- to 4·38-fold), but the iSPD/iNSD ratios varied from 1·35 to 3·9, suggesting that basal activities of these hormone-inducible genes were repressed by SP proteins. These genes were classified as $^{+}$ESR1/SP (B^+). The third subset of $^{+}$ESR1/SP-regulated genes were induced 1·32- to 1·84-fold by E_2 and after cotransfection with iSP, the fold induction responses were significantly decreased to 0·83- to 1·17-fold. This was accompanied by iSPD/iNSD ratios of 0·54-0·79. This subset of genes induced by E_2 (category A) was designated at $^{+}$ESR1/SP (B^-), signifying that their basal activity was also SP dependent. There were 42 genes induced by E_2 that were classified as $^{+}$ESR1/SP (B^0), $^{+}$ESR1/SP (B^+), or $^{+}$ESR1/SP (B^-).

The classification of genes downregulated by E_2 and reversed after cotransfection with iSP (category B) was also subdivided into $^{-}$ESR1/SP (B^0), $^{-}$ESR1/SP (B^+), and $^{-}$ESR1/SP (B^-) subsets using the same criteria as indicated for genes induced by E_2 (category A). Table 2 summarizes the 21 genes decreased (0·45 to 0·80) after treatment with E_2 (+ iNS) and this response was reversed after transfection with iSP (category B). The iSPD/iNSD ratios (0·80-1·10) were relatively unchanged and these genes were designated as $^{-}$ESR1/SP (B^0). We

detected only a single gene classified as $^{-}$ESR1/SP (B^{+}) where loss of SP protein increased basal activity. Sixty genes were classified as ESR1/SP (B^{-}); E_2 decreased expression (0·80-0·52) of these genes and transfection with iSP reversed this response (0·80-1·20) for 54 of these genes. Noticeably, the remaining six genes showed similar yet much more pronounced fold induction (1·35-4·55) after transfection with iSP. Thus, E_2 decreased the expression of 75 genes that were SP dependent (category B) compared with the induction of 42 SP-dependent genes (category A).

Categories C and D contain 25 and 51 genes induced (≥1·3-fold) or repressed (≤ 0·8) by E_2 respectively, and after transfection with iSP, the fold induction or repression was either unaffected or only partially modulated by SP protein knockdown. Despite the fact that E_2-mediated modulation of these genes was SP independent, the categories C ($^{+}$ER) and D ($^{-}$ER) genes (Tables 3 and 4 respectively) were subdivided into B^{0}, B^{+}, and B^{-} subcategories where iSPD/iNSD ratios were unchanged (B^{0}), increased (B^{+}), or decreased (B^{-}). There were 5, 8, and 12 genes in the $^{+}$ER (B^{0}), $^{+}$ER (B^{+}), and $^{+}$ER (B^{-}) subcategories respectively, and 14, 4, and 33 genes in the $^{-}$ER (B^{0}), $^{-}$ER (B^{+}), and $^{-}$ER (B^{-}) subcategories respectively, indicating that the major subcategory for each set involved hormone-responsive genes whose basal expression was, in part, SP dependent (B^{-}). Statistical analysis of the data was also carried out with relaxed conditions, which included a new cut-off *P*-value of 0·05, adjustment of the thresholds for inducibility (1·1 instead of 1·3) and repression (0·9 instead of 0·8), and no cross-array normalization. Our new analysis used the software package GeneSifter and the parameters used were selected as close as possible to those in the original analysis. The number of genes in the four different categories were 57 (A), 244 (B), 93 (C), and 350 (D) compared with 43 (A), 83 (B), 25 (C), and 51 (D) genes reported in Tables 1-4 respectively. In the less stringent analysis, only 40% of the E_2-modulated genes were SP dependent and this change was primarily due to the large increase in the number of E_2-repressed genes in category D.

GeneSpring BX7 software was used to investigate the statistical significance of genes induced or repressed by E_2, and the adjusted *P*-values were obtained using Bonferroni correction. During the processing, GO terms on biological processes were assigned to each one of the significantly expressed genes in categories A-D. Tables 1-4 summarize the individual genes induced or repressed by E_2 and also includes the GO terms associated with these genes. In categories A, B, C, and D, the hormone-induced or -repressed genes were associated with 37, 68, 19, and 78 different biological processes, and most processes contained only one gene with a maximum number of four genes per individual process. However, the largest number of genes in categories A, B, C, and D were genes that were not assigned to any specific biological process and these included 30, 56, 20, and 24 genes respectively. A comparison of the biological processes induced by E_2 by ESR1/SP (category A) and SP-independent (category C) pathways exhibited minimal overlap with only ‘synaptic transmission’, ‘cell-cell signaling’, and ‘central nervous system’ genes activated in common. A comparison of the 68 and 74 biological processes downregulated by E_2 in categories B (SP dependent) and D (SP independent) indicated that only nine processes were in common. These results suggest that the SP-dependent (A, B) and SP-independent (C and D) pathways for hormone-mediated induction or inhibition of gene expression primarily target different biological processes (GO terms). At the same time, it is important to emphasize that this minimal functional overlap in the GO categories could be attributed to incomplete or inaccurate annotation in the existing databases and could change if one accepts a less conservative gene selection criteria in terms of *P*-values or FDR or if multiple cell lines with multiple time points for data collection were used. Thus, further analysis is warranted.

Distribution of genes in categories A-D and their subcategories B^{0}, B^{+}, and B^{-} is illustrated in Fig. 2 along with the number of biological processes associated with the genes in each category. Not surprisingly, the results are complex with minimal overlap between categories.

The role of SP proteins in mediating hormonal regulation of select genes was also confirmed by real-time PCR, and the four genes selected include *E2f1, Vegfa, Hmg*, and *Klk6*. Although VEGFA was not detected in the microarray studies due to the stringent conditions, we included VEGFA since ongoing studies show consistent downregulation of this gene by E_2 in MCF-7 cells (data not shown). Figure 3 illustrates the time course induction or repression of these genes after treatment with 20 nM E_2 for 2, 4, 6, 9, and 24 h. Significant effects were observed within 2 h and persisted for up to 24 h. We used a higher concentration of E_2 (20 nM) for the RT-PCR experiments and this was based on preliminary studies showing a maximal response observed using 20 nM E_2. The role of iSP in mediating hormone-dependent activation of these genes was determined in MCF-7 cells (Fig. 4) and the results were similar to those observed in the microarray experiments. Moreover, we also observed that the antiestrogen ICI 182780 (fulvestrant) also significantly inhibited the E_2-induced or -repressed genes. These results demonstrate that E2 induces and represses a diverse spectrum of genes in MCF-7 cells as previously reported (Soulez & Parker 2001,Inoue *et al.* 2002,Levenson *et al.* 2002,Lobenhofer *et al.* 2002,Coser *et al.* 2003,Cunliffe *et al.* 2003,Frasor *et al.* 2003,2004,Scafoglio *et al.* 2006,Jonsson *et al.* 2007), and this study shows that >60% of these genes are dependent on SP proteins.

Discussion

Gene expression profiling has been extensively used to understand global changes in gene expression in various endogenous biochemical processes and during development of various diseases including breast cancer (Sorlie *et al.* 2003, Andersson *et al.* 2005). The patterns of gene expression in different tumor types have been extensively investigated and are used for disease prognosis and for predicting responses to various chemotherapies. For example, gene expression profiling has been used for diagnosis, prognosis, and treatment of breast cancer metastasis and for recommending adjuvant chemotherapy for breast cancer (Driouch *et al.* 2007, Henry & Hayes 2007). Gene expression profiles in breast cancer cells have been extensively investigated in several different ER^+ and ER^- breast cancer cell lines, and the effects of time, concentration, and structure of ER agonists and antagonists and other growth regulatory factors on modulation of gene expression have been investigated (Soulez & Parker 2001, Inoue *et al.* 2002, Levenson *et al.* 2002, Lobenhofer *et al.* 2002, Coser *et al.* 2003, Cunliffe *et al.* 2003, Frasor *et al.* 2003, 2004, Scafoglio *et al.* 2006, Jonsson *et al.* 2007). Results of these studies demonstrate that hormone-activated genes and gene networks are highly complex and variable and demonstrate the broad effects of E_2 on gene expression. For example, Frasor *et al.* (2003) showed that E_2 regulated over 400 genes in 12 different functional categories in MCF-7 cells and the majority (76%) of these genes were downregulated by E_2. Similar results were observed in this study (Fig. 1B and C). Frasor *et al.* also demonstrated distinct temporal differences in the expression of some E_2-induced and repressed genes (Frasor *et al.* 2003, 2004).

Several studies show that SP proteins play a pivotal role in hormone-dependent induction of genes (Safe 2001, Safe & Kim 2004), and recent reports have shown that these transcription factors are also involved in gene repression (Higgins *et al.* 2006, Stossi *et al.* 2006). Recent studies on genome-wide interactions of ESR1 with DNA demonstrate that many ESR1-binding sites for specific genes are distal from their corresponding transcription start sites (Carroll & Brown 2006, Carroll *et al.* 2006, Lin *et al.* 2007), whereas most promoter studies have focused on proximal E_2-responsive promoter sites. Thus, the E_2-responsive proximal GC-sites identified in previous studies (Safe & Kim 2005) may also contain functional E_2-responsive distal sites and further analysis of the potentially ESR1/SP-dependent genes listed in Tables 1 and 2 will have to examine both proximal and distal *cis*-elements. For example, based on proximal promoter analysis, we previously identified retinoic acid receptor α1 (RARA) as an E_2-responsive gene regulated by ESR1/SP (Sun *et al.* 1998); however, Laganiere *et al.*

(2005) used a functional genomics approach to identify a functional ERE 3·7 kb downstream from the RARA transcription start site. Another study demonstrated the potential involvement of GC-rich binding sites in the global regulation of some ESR1-dependent genes using a whole-genome cartography approach for identifying ESR1-binding sites (Lin *et al.* 2007).

Most of the previous research have focused on SP1 protein; however, studies in this laboratory have now shown that SP1, SP3, and SP4 proteins are overexpressed in breast cancer cells (Mertens-Talcott *et al.* 2007) and simultaneous knockdown of one or all three SP proteins block E_2-dependent expression of the *Rara*, *E2f*, and *Cad* genes (Khan *et al.* 2007). These genes are primarily activated through ESR1/SP binding to GC-rich promoter sites (ER:DNA independent). In this study, we have selected a single concentration (10 nM) of E_2 and one time point (6 hr) to investigate the role of SP proteins in mediating hormonal activation of genes in MCF-7 cells. This approach was not designed to identify all hormonally regulated genes but to investigate the importance of SP proteins in this induction response. Results of this study demonstrate that in MCF-7 cells treated with E_2, 67 genes are induced and 134 genes are repressed (Fig. 1B), and this ratio of induced/repressed hormone-responsive genes is similar to the results of previous studies (Frasor *et al.* 2003). However, the use of RNA interference shows that out of these 201 genes, over 60% of the genes affected by E_2 are also dependent on SP proteins (categories A and B). We also observed that within the four categories of genes (Tables 1-4 and Fig. 2), SP protein also markedly affected basal gene expression. By grouping genes into subcategories of B^0, B^+, and B^-, we observed that among the 201 genes induced or repressed by E_2, the basal activity of 57 genes was unaffected after transfection with iSP, whereas the basal activity of 144 genes was either decreased (110 genes; B^-) or increased (34 genes; B^+) (Fig. 3). Since SP proteins are important for constitutive expression of multiple mammalian and viral genes, it is not surprising that loss of SP1, SP3, and SP4 resulted in decreased basal expression of 110 out of 144 of those genes affected by iSP. However, SP protein knockdown also enhanced basal activity of 34 genes and this may be due to the SP3 protein that can act as both an enhancer and a suppressor of gene expression (Black *et al.* 2001, Suske *et al.* 2005). It should also be noted that knockdown of SP1, SP3, and SP4 proteins will not only affect ESR1/SP-regulated genes but may also result in altered expression of ESR1, coactivators, and other nuclear cofactors required for gene induction or expression. We are currently investigating the molecular mechanisms associated with the E_2-dependent induction and repression of individual SP-dependent genes listed in Tables 1 and 2. These studies will determine the role of SP proteins and identify both proximal and distal GC-rich sites that are functional *cis*-elements in ESR1/SP action.

Analysis of the effects of SP knockdown on hormone-responsive gene expression revealed a fifth category (F) of genes (Fig. 1C) that were not induced or repressed by E_2 in MCF-7 cells transfected with iNS. There were 85 E_2-nonresponsive genes in category F; however, after transfection of iSP, treatment with E_2 induced or repressed expression of these genes that were further subdivided into B^0, B^+, and B^- subcategories. Category F genes were further subdivided into multiple (112) biological processes and only 30 of these were in common with biological processes associated with genes in categories A-D. It has been reported that SP proteins are overexpressed in multiple tumor types (Zannetti *et al.* 2000,Shi *et al.* 2001,Chiefari *et al.* 2002,Wang *et al.* 2003,Hosoi *et al.* 2004,Yao *et al.* 2004,Mertens-Talcott *et al.* 2007), and our recent studies have shown that SP proteins are highly expressed in breast cancer cells but barely detectable in non-transformed mammary cells (Mertens-Talcott *et al.* 2007). Lou and coworkers also showed that malignant transformation of human fibroblast cells results in an 8- to 18-fold increase in the expression of SP1 in the tumor cells compared with the parental cells (Lou *et al.* 2005). These studies suggest that over-expression of SP proteins contributes to tumor formation; thus, genes in category F, which regain hormone responsiveness in MCF-7 cells transfected with iSP, may play an endogenous role in normal or precancerous mammary cells where SP protein levels are relatively low. Their hormone responsiveness is subsequently

silenced with increased expression of SP proteins found in MCF-7 and other breast cancer cell lines. Current studies are examining a variety of tumorigenic and non-tumorigenic mammary cells to further investigate the functions and changes in expression of category F genes and the role of SP proteins in mediating gene silencing.

In summary, results of these microarray studies confirm that in MCF-7 cells, E_2 predominantly inhibits expression of genes. By combining the microarray data with simultaneous knockdown of SP1, SP3, and SP4 proteins, we have demonstrated that these transcription factors are critical regulators of hormone-dependent gene induction and repression. Moreover, the latter response has recently been confirmed in two studies on SP-dependent downregulation of KDR and cyclin G2 in MCF-7 cells treated with E_2 (Stossi *et al.* 2006, Higgins *et al.* 2008). Previous studies show that VEGFA and KDR are induced by E_2 in ZR-75 cells (Stoner *et al.* 2004, Higgins *et al.* 2006), whereas in MCF-7 cells, E_2 decreases expression of both genes (Higgins *et al.* 2008; Figs 3 and 4). This unusual cell context-dependent difference in hormonal regulation of VEGFA/KDR in two ER-positive breast cancer cells lines may be due to altered expression of critical cofactors and this is currently being investigated. It should also be noted that ESR1 is constitutively associated with proximal E_2-responsive GC-rich sequences and these interactions are not enhanced by E_2 in a ChIP assay (Higgins *et al.* 2006, 2008, Khan *et al.* 2007). We are now examining coactivators as potential markers of ESR1/SP-mediated transactivation. Thus, in MCF-7 cells treated with E_2 for 6 h, most hormonally regulated genes were SP dependent and, for the 76 genes in categories C and D where iSP did not affect hormone inducibility, loss of SP affected basal expression of 53 out of these 76 genes. Thus, among the 201 genes in categories A-D, loss of SP proteins affected hormone-induced or basal expression of over 88% (178/201) of all genes. It was also apparent that hormone-mediated responses such as increased cell proliferation involves mobilization genes that regulate a large number of functional processes (Tables 1-4), and current studies are focused on interconnections between pathways that lead to E_2-induced proliferation and survival pathways in MCF-7 cells.

Acknowledgements

Funding

This work was supported by the National Institutes of Health (ES04917, CA104116, and ES09106) and the Texas Agricultural Experiment Station.

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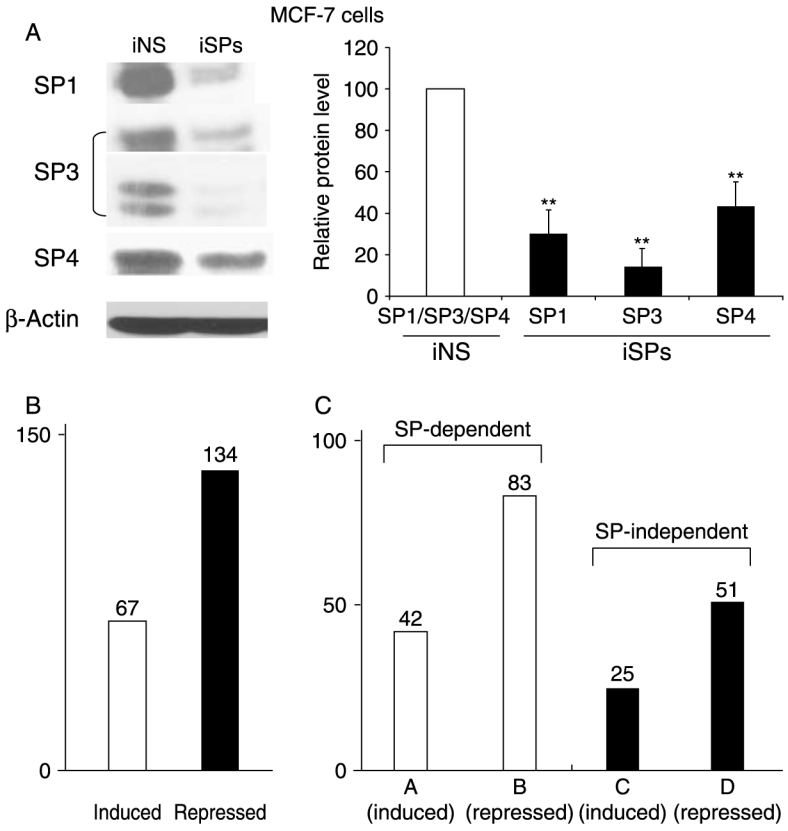

Figure 1.

Role of SP transcription factors in E_2-dependent gene expression. (A) Effect of iSP on SP1, SP3, and SP4. MCF-7 cells were transfected with the iSP cocktail or a nonspecific oligonucleotide (iNS), and SP proteins were analyzed in triplicate by western blotting as described in the Materials and methods. Significantly ($P<0{\cdot}05$) decreased expression is indicated (**). (B) Number of genes up- or downregulated by 10 nM E_2. (C) SP-dependent and -independent gene categories. Categories A and B represent SP-dependent (ESR1/SP) genes induced or repressed by E_2. Categories C and D represent SP-independent (ESR1) genes induced or repressed by E_2. Category F genes are induced or repressed by E_2 only after SP knockdown.

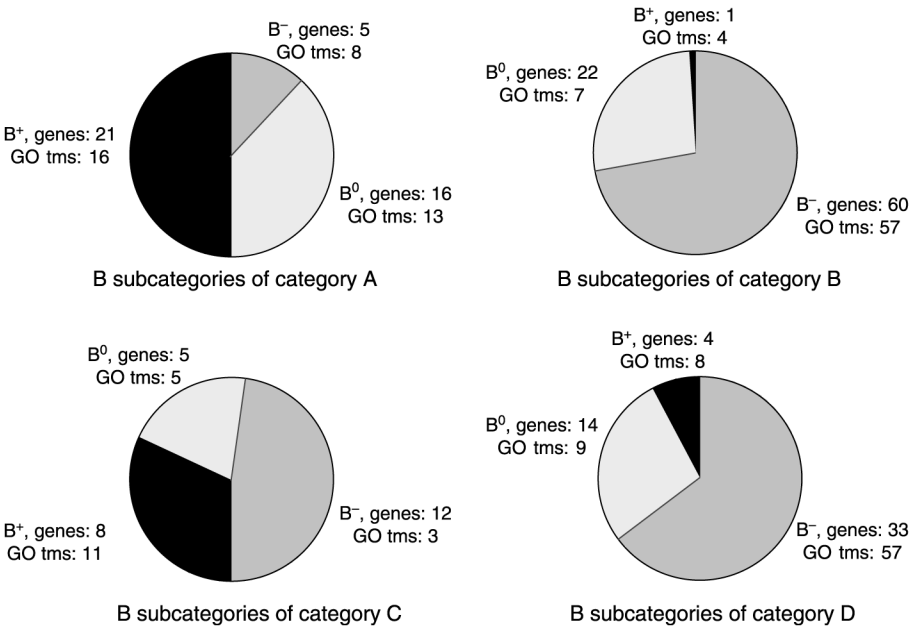

Figure 2.

Subclassification of genes in categories A-D. The effects of iSP on basal activities (B^0, B^+, and B^-) and the number of GO terms within each subclassification of categories A-D genes that are induced or repressed by E_2 in MCF-7 cells are indicated (also see Tables 1-4).

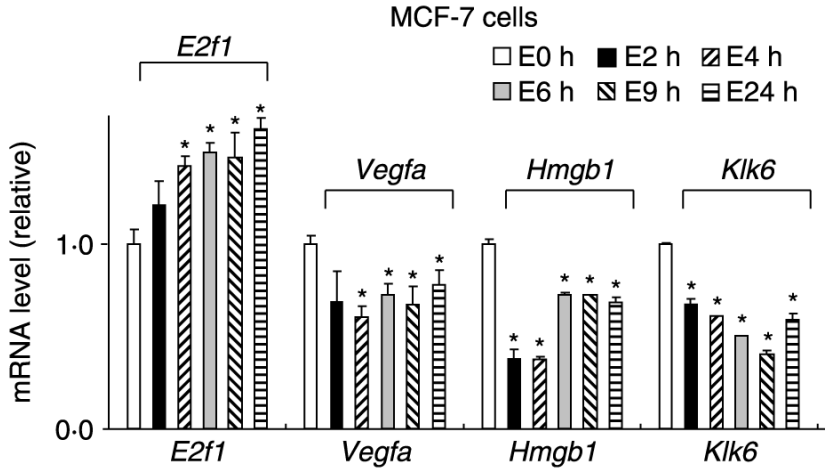

Figure 3.

Time course effects of E_2. MCF-7 cells were treated with 20 nM E_2 for various times and expression of *E2f1*, *Vegfa*, *Hmgb1*, and *Klk6* was determined (in triplicate) by real-time PCR (normalized to TBP) as outlined in the Materials and methods. Significant ($P<0{\cdot}05$) induction or inhibition is indicated by *.

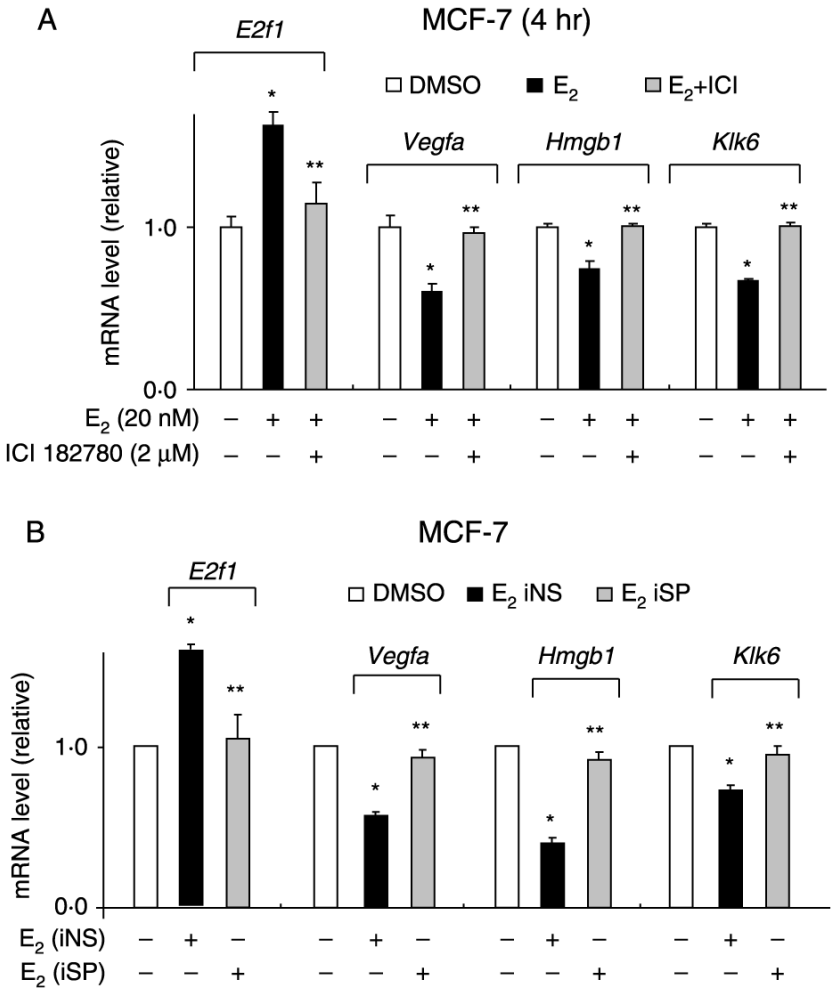

Figure 4.

Effects of (A) RNA interference or (B) ICI 182780 on E_2-dependent induction or repression of genes. MCF-7 cells were transfected with iNS or iSP and treated with DMSO or 20 nM E_2 or treated with DMSO, E_2 alone, or E_2 plus 2 μM ICI 182780, and gene expression was determined by real-time PCR as described in the Materials and methods. Results are means ±S.E.M. for three replicate determinations and significant (P<0·05) induction or repression by E_2 (*) and reversal of these effects by iSP or ICI 182780 (**) is indicated.

Table 1

Category A genes that are induced by E_2 in MCF-7 cells and reversed by SP protein knockdown (SP dependent)

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
Gene accession numbers			
Category A (B^0)			
BM459591		1·366667	No info about GO biol process
NM_178501		2·447368	No info about GO biol process
NM_005225	*E2f1*	2·338661	Apoptosis; + multiple GO terms
BF056555		2·294118	No info about GO biol process
AW002058		2·189189	No info about GO biol process
AI654853		2·179487	No info about GO biol process
H23439		2·095238	No info about GO biol process
H93315		2·022222	No info about GO biol process
AW977008		1·97561	No info about GO biol process
NM_003125	*Sprr1b*	1·826185	Epidermis development
NM_130830	*Lrrc15*	1·626459	No info about GO biol process
BQ931737		1·549451	No info about GO biol process
NM_012429	*Sec14l2*	1·477273	Transport; + multiple GO terms
NM_005551	*Klk2*	1·423529	Proteolysis and peptidolysis
NM_004973	*Jarid2*	1·32288	Central nervous system development
BG029979		1·465116	No info about GO biol process
Category A (B^+)			
BF512856		1·84127	No info about GO biol process
NM_001051	*Sstr3*	1·65625	Cell-cell signaling; + multiple GO terms
NM_003647	*Dgke*	1·326923	Intracellular signaling cascade; + multiple GO terms
BM714396		1·6	No info about GO biol process
CF147798	*P2ry10*	4·5	No info about GO biol process
AW028308		4·333333	No info about GO biol process
U91328		4·384615	No info about GO biol process
BC038785		2·914286	No info about GO biol process
AI444574		2·83871	No info about GO biol process
NM_000171	*Glra1*	2·433333	Ion transport; + multiple GO terms

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
BG541228		2·382979	No info about GO biol process
BC012108	*Ca14*	1·806452	No info about GO biol process
AI202342		1·745763	No info about GO biol process
AF130075		1·597015	No info about GO biol process
BC033385		1·604938	No info about GO biol process
NM_144715	*Efhb*	1·475728	No info about GO biol process
NM_016594	*Fkbp11*	1·347765	Protein folding
NM_033315	*Rasl10b*	1·309091	Small GTPase-mediated signal transduction
AW274516	*Itpkb*	3·607143	GO:0007165
BG498282		3·071429	No info about GO biol process
BU616743		2·761905	No info about GO biol process
Category A (B^-)			
BG211832		1·842105	No info about GO biol process
BE927766		1·755556	No info about GO biol process
NM_001165	*Birc3*	1·620818	Anti-apoptosis; + multiple GO terms
NM_005416	*Sprr3*	1·391586	GO:8544 (epidermis development);+ multiple GO terms
BX095305		1·319797	No info about GO biol process

[a] MCF-7 cells transfected with iNS (nonspecific oligonucleotide) and treated with either 10 nM E_2 (iNSE) or DMSO (iNSD).

Table 2

Category B genes that are repressed by E_2 in MCF-7 cells and reversed by SP protein knockdown (SP dependent)

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
Gene accession numbers			
Category B (B^0)			
BI520070		0·75475	No info about GO biol process
NM_005919	*Mef2b*	0·755932	Muscle development; + multiple GO terms
BG194714		0·751412	No info about GO biol process
AI748967		0·694444	No info about GO biol process
BI791886		0·685832	No info about GO biol process
BI559738		0·664634	No info about GO biol process
NM_020909	*Epb41l5*	0·661818	No info about GO biol process
NM_152429	*Fgfbp3*	0·657258	No info about GO biol process
AI459284		0·609756	No info about GO biol process
NM_018374	*Tmem106b*	0·582677	No info about GO biol process
BX093288		0·555556	No info about GO biol process
AK091299		0·556604	No info about GO biol process
XM_061614		0·527273	No info about GO biol process
AI348944		0·451923	No info about GO biol process
NM_173580		0·736842	No info about GO biol process
AI632459		0·573964	No info about GO biol process
AK023938	*Lrrfip1*	0·724638	GO:0016481;+ multiple GO terms
U66581	*Gpr22*	0·75	G-protein coupled receptor protein signaling pathway
NM_020340	*Kiaa1244*	0·795064	GO:32012 (regulation of ARF protein signal transduction)
BX103217		0·731898	No info about GO biol process
W69651		0·56	No info about GO biol process
NM_173580		0·736842	No info about GO biol process
Category B (B^+)			
NM_004799	*Zfyve9*	0·765625	Endocytosis; + multiple GO terms
Category B(B^-)			
AA323614		0·799242	No info about GO biol process
NM_024056	*Tmem106c*	0·796373	No info about GO biol process

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
NM_018186	*C1orf112*	0·790563	No info about GO biol process
NM_014793	*Lcmt2*	0·786538	No info about GO biol process
NM_014920	*Ick*	0·768975	Protein amino acid phosphorylation
NM_001310	*Crebl2*	0·767041	Signal transduction; transcription
NM_022746	*Mosc1*	0·765499	No info about GO biol process
BM723321		0·762322	No info about GO biol process
NM_152524	*Sgol2*	0·758025	GO:7049 (cell cycle);+ multiple GO terms
NM_025004	*Ccdc15*	0·754331	No info about GO biol process
NM_002774	*Klk6*	0·752992	Regulation of cell differentiation; + multiple GO terms
NM_016076	*Fam152a*	0·741884	No info about GO biol process
BG740877		0·741463	No info about GO biol process
NM_024430	*Pstpip2*	0·736111	No info about GO biol process
AA046178		0·731949	No info about GO biol process
AA809349		0·732218	No info about GO biol process
AK026856		0·729258	No info about GO biol process
CB962212		0·728643	No info about GO biol process
NM_003400	*Xpo1*	0·720289	GO:6406 (mRNA export from nucleus);+ multiple GO terms
NM_014240	*Limd1*	0·719298	Development; + multiple GO terms
NM_016551	*Tm7sf3*	0·718053	No info about GO biol process
NM_006479	*Rad51ap1*	0·71855	GO:6281 (DNA repair);+ multiple GO terms
NM_022374	*Atl2*	0·712202	Immune response
NM_012287	*Acap2*	0·703775	GO:43087 (regulation of GTPase activity)
NM_005226	*S1pr3*	0·703278	Morphogenesis; + multiple GO terms
AI979035		0·698925	No info about GO biol process
NM_016203	*Prkag2*	0·696429	Cholesterol biosynthesis; + multiple GO terms
AA261924		0·695868	No info about GO biol process
NM_001278	*Chuk*	0·687229	Immune response; + multiple GO terms
AA653936		0·686189	No info about GO biol process
NM_020132	*Agpat3*	0·682759	Metabolism; + multiple GO terms
NM_007027	*Topbp1*	0·677027	DNA metabolism; + multiple GO terms

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
BQ013447		0·675	No info about GO biol process
BI754506		0·670455	No info about GO biol process
NM_032876		0·668553	No info about GO biol process
NM_153826	*Capg*	0·6639	Complement activation, classical pathway
NM_016824	*Add3*	0·664298	Apoptosis; + multiple GO terms
NM_016359	*Nusap1*	0·663537	G0:0000281;+ multiple GO terms
BG285682		0·65847	No info about GO biol process
NM_172240	*Wdr51b*	0·652372	No info about GO biol process
N40495		0·647321	No info about GO biol process
NM_003415	*Znf268*	0·646	Regulation of transcription, DNA dependent
NM_139279	*Mcfd2; Lman1; Mcfd2*	0·637809	GO:6888 (ER to Golgi vesicle-mediated transport);+ multiple GO terms
NM_032525	*Tubb6*	0·633021	Microtubule-based movement
AK091460	*Znf813*	0·626354	No info about GO biol process
NM_017709	*Fam46c*	0·624658	No info about GO biol process
NM_138635	*H2afv*	0·621495	GO:6334 (nucleosome assembly);+ multiple GO terms
AK056236		0·621074	No info about GO biol process
NM_173556	*Ccdc83*	0·605682	No info about GO biol process
NM_002128	*Hmgb1*	0·59	DNA unwinding; + multiple GO terms
NM_020927	*Vat1l*	0·582734	No info about GO biol process
NM_016516	*Vps54*	0·564955	GO:15031 (protein transport);+ multiple GO terms
NM_018836	*Ajap1*	0·533835	No info about GO biol process
NM_019087	*Arl15*	0·515924	No info about GO biol process
AI218135		0·647059	No info about GO biol process
AI742318		0·314286	No info about GO biol process
BU069371		0·224138	No info about GO biol process
NM_001701	*Baat*	0·772727	Bile acid metabolism; + multiple GO terms
NM_007023	*Rapgef4*	0·675676	Exocytosis; + multiple GO terms
AI480013		0·52381	No info about GO biol process

[a] MCF-7 cells transfected with iNS (nonspecific oligonucleotide) and treated with either 10 nM E_2 (iNSE) or DMSO (iNSD).

Table 3

Category C genes that are induced by E_2 in MCF-7 cells and are unaffected by SP protein knockdown (SP independent)

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
Gene accession numbers			
Category C (B^0)			
AK098294		1·7	No info about GO biol process
BX107797		1·395349	No info about GO biol process
BC041884		3·5	No info about GO biol process
AW513673		1·895833	No info about GO biol process
NM_001321	*Csrp2*	1·529201	Cell growth; + multiple GO terms
Category C (B^+)			
AA234276		3·173387	No info about GO biol process
CA310410		2·073394	No info about GO biol process
NM_022467	*Chst8*	1·350649	Hormone biosynthesis; + multiple GO terms
NM_173505	*Ankrd29*	2·151515	No info about GO biol process
NM_000817	*Gad1*	1·39375	Amino acid metabolism; + multiple GO terms
NM_000360	*Th*	1·447205	Morphogenesis; + multiple GO terms
NM_024522	*Nkain1*	1·460317	No info about GO biol process
NM_030781		4·384615	No info about GO biol process
Category C (B^-)			
BU677122		2·575758	No info about GO biol process
BF594491		1·455682	No info about GO biol process
BM995921		1·756098	No info about GO biol process
AA470089		1·41206	No info about GO biol process
BG188765		2·434783	No info about GO biol process
AK126800		2·34375	No info about GO biol process
BX091274		2·189189	No info about GO biol process
BQ182928		2·095238	No info about GO biol process
AW291057		1·967742	No info about GO biol process
AI356266		1·630137	No info about GO biol process
NM_001657	*Areg*	1·587224	Cell proliferation; cell-cell signaling
BG025371		1·465385	No info about GO biol process

Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process

[a] MCF-7 cells transfected with iNS (nonspecific oligonucleotide) and treated with either 10 nM E_2 (iNSE) or DMSO (iNSD).

Table 4

Category D genes that are repressed by E_2 in MCF-7 cells and are unaffected by SP protein knockdown (SP independent)

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
Gene accession numbers			
Category D (B^0)			
NM_004982	*Kcnj8*	0·54631	Ion transport; + multiple GO terms
AK022053		0·430769	No info about GO biol process
CB321977		0·56338	No info about GO biol process
NM_012106	*Arl2bp*	0·600355	Signal transduction
NM_001056	*Sult1c2*	0·492958	Amine metabolism
AI492256		0·560606	No info about GO biol process
NM_015033	*Fnbp1*	0·718391	GO:6412 (protein biosynthesis)
BX114748		0·554545	No info about GO biol process
BQ024005		0·630137	No info about GO biol process
BM990273		0·730769	No info about GO biol process
AB067496	*Plekhg4b*	0·653333	GO:35023 (regulation of Rho protein signal transduction)
BC042089		0·772727	No info about GO biol process
NM_004260	*Recql4*	0·730203	DNA repair; + multiple GO terms
AW298780		0·5	No info about GO biol process
Category D (B^+)			
NM_000304	*Pmp22*	0·737374	Mechanosensory behavior; + multiple GO terms
NM_004089	*Tsc22d3*	0·794749	Regulation of transcription, DNA dependent
AV652591		0·788945	No info about GO biol process
AK126952		0·674419	No info about GO biol process
Category D (B^-)			
NM_018393	*Tcp11l1*	0·756677	No info about GO biol process
NM_003025	*Sh3gl1*	0·74812	Signal transduction; + multiple GO terms
NM_018492	*Pbk*	0·771031	Protein amino acid phosphorylation
NM_133638	*Adamts19*	0·759615	Proteolysis and peptidolysis
BI602086	*Abat*	0·756691	Aminobutyrate metabolism; + multiple GO terms
NM_017634	*Kctd9*	0·743668	GO:6813 (potassium ion transport)
NM_001668	*Arnt*	0·741218	Signal transduction; + multiple GO terms

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
W31852		0·73236	No info about GO biol process
NM_001798	*Cdk2*	0·726695	Cytokinesis; + multiple GO terms
NM_006751	*Ssfa2*	0·720841	No info about GO biol process
NM_015368	*Panx1*	0·707317	GO:0006812;+ multiple GO terms
NM_014900	*Cobll1*	0·701372	No info about GO biol process
NM_004523	*Kif11*	0·698872	Mitotic spindle assembly
NM_001067	*Top2a*	0·697188	GO:6266 (DNA ligation);+ multiple GO terms
NM_021211		0·693424	No info about GO biol process
BQ350534		0·671694	No info about GO biol process
NM_012381	*Orc3l*	0·667582	DNA replication
NM_005573	*Lmnb1*	0·66129	No info about GO biol process
NM_022748	*Tns3*	0·655586	Intracellular signaling cascade; + multiple GO terms
NM_004298	*Nup155*	0·649412	Nucleocytoplasmic transport
NM_016121	*Kctd3*	0·649156	GO:6813 (potassium ion transport)
NM_022918	*Tmem135*	0·637079	No info about GO biol process
NM_004856	*Kif23*	0·637931	GO:7049 (cell cycle);+ multiple GO terms
NM_144717	*Il20rb*	0·628205	No info about GO biol process
NM_005139	*Anxa3*	0·624911	GO:7165 (signal transduction)
NM_006219	*Pik3cb*	0·623397	Activation of MAPK; + multiple GO terms
W38778		0·591923	No info about GO biol process
NM_007145	*Znf146*	0·587372	Regulation of transcription, DNA dependent
NM_004087	*Dlg1*	0·58216	GO:31575 (G1/S transition checkpoint);+ multiple GO terms
NM_005180	*Commd3*	0·576584	Cell growth and/or maintenance; + multiple GO terms
BM145609		0·56044	No info about GO biol process
AW573130		0·506667	No info about GO biol process
AW237319	*Kiaa1614*	0·477273	No info about GO biol process

[a]MCF-7 cells transfected with iNS (nonspecific oligonucleotide) and treated with either 10 nM E_2 (iNSE) or DMSO (iNSD).

Table 5

Category F genes that are only induced or repressed by E_2 after SP protein knockdown

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
Gene accession numbers			
Category F (B^0)			
AI693142		0·880041365	No info about GO biol process
NM_013314	*Blnk*	0·937625755	B-cell differentiation; + multiple GO terms
BM724201		0·90070922	No info about GO biol process
NM_006849	*Pdia2*	1·201550388	Electron transport; + multiple GO terms
NM_001343	*Dab2*	0·82967033	Cell proliferation
AL080280		1·157894737	No info about GO biol process
AW294582		1·278106509	No info about GO biol process
NM_004998	*Myo1e*	1·052631579	Actin filament-based movement
NM_014930	*Znf510*	0·919254658	No info about GO biol process
NM_153020	*Rbm24*	0·883024251	No info about GO biol process
BE350156		0·843283582	No info about GO biol process
NM_006731	*Fktn*	1·070850202	Muscle development; + multiple GO terms
NM_004948	*Dsc3*	0·905405405	Cell adhesion; + multiple GO terms
NM_144726	*Rnf145*	0·898989899	GO:6512 (ubiquitin cycle)
NM_145646		0·954356846	No info about GO biol process
NM_001408	*Celsr2*	0·935185185	Development; + multiple GO terms
NM_016424	LUC7A	0·936090226	RNA splicing
NM_033083	*Eaf1*	0·844650206	GO:6350 (transcription);+ multiple GO terms
AW869316		0·956521739	No info about GO biol process
NM_016014	*Fam108b1*	1·03	No info about GO biol process
NM_014650	*Znf432*	0·861751152	GO:6350 (transcription);+ multiple GO terms
NM_173683	*Xkr6*	0·851851852	No info about GO biol process
AW007727		0·80952381	No info about GO biol process
N52436		0·827922078	No info about GO biol process
CD104312		0·830508475	No info about GO biol process
NM_012193	*Fzd4*	0·830065359	Development; + multiple GO terms
NM_003839	*Tnfrsf11a*	0·993197279	Cell-cell signaling; + multiple GO terms

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
BF963928		0·903100775	No info about GO biol process
NM_020246	*Trip6*	1·103030303	Amino acid transport
NM_006651	*Cplx1*	0·873563218	Exocytosis; + multiple GO terms
NM_002750	*Mapk8*	0·91503268	JNK cascade; + multiple GO terms
Category F (B^+)			
NM_013301	*Ccdc106*	0·923547401	No info about GO biol process
NM_012324	*Mapk8ip2*	1·079207921	Positive regulation of anti-apoptosis; + multiple GO terms
NM_032431	SVN1	0·857777778	GO:0030433
NM_020820	*Prex1*	0·897926635	Actin filament polymerization; + multiple GO terms
NM_052880	*Pik3ip1*	0·934362934	No info about GO biol process
CA337205		0·947174447	No info about GO biol process
BX101252		1·01509434	No info about GO biol process
BX089473		1·120930233	No info about GO biol process
NM_031464	*Rps6kl1*	1·095	GO:6468 (protein amino acid phosphorylation)
AB065764		0·952380952	No info about GO biol process
BX470892		0·873015873	No info about GO biol process
NM_181535		1·056603774	No info about GO biol process
AL049274		0·945273632	No info about GO biol process
NM_022818	*Map1lc3b*	0·948138298	GO:6914 (autophagy);+ multiple GO terms
AK097239		0·892592593	No info about GO biol process
BQ276710		0·949275362	No info about GO biol process
BM712945		1·021276596	No info about GO biol process
AK092442	PP14571	0·854166667	No info about GO biol process
NM_014751	*Mtss1*	0·9360519	Cell adhesion; + multiple GO terms
AK097032	MGC17403	0·950657895	Transcription
NM_032412	*C5orf32*	0·988957055	Visual perception; + multiple GO terms
BC042976	*Jmjd2c*	1·114035088	GO:0008150
AA324557		1·056818182	No info about GO biol process
NM_014428	*Tjp3*	0·847682119	No info about GO biol process
BG009563		0·873846154	No info about GO biol process

	Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process
NM_000076	*Cdkn1c*	1·008213552	Cell cycle arrest; + multiple GO terms
AW299364		1	No info about GO biol process
BG032839	PCOTH	1·118715084	No info about GO biol process
BM743413		1·162576687	No info about GO biol process
NM_022912	*Reep1*	0·972665148	GO:0051205
NM_014509	*Serhl2*	0·991071429	No info about GO biol process
NM_033467	*Mmel1*	1·016348774	Proteolysis and peptidolysis
BM991890		0·819787986	No info about GO biol process
AK130231		1·009433962	No info about GO biol process
NM_016162	*Ing4*	0·878472222	Regulation of transcription, DNA dependent
BF062651		0·875	No info about GO biol process
Category F (B^-)			
NM_006461	*Spag5*	0·835327234	GO:7049 (cell cycle);+ multiple GO terms
NM_199050	*C2cd2*	0·826548673	No info about GO biol process
NM_015341	*Ncaph*	0·814073227	Cell cycle; + multiple GO terms
NM_181353	*Id1*	0·807936508	Development; + multiple GO terms
NM_005547	*Ivl*	0·885714286	Keratinocyte differentiation
NM_153750		0·913043478	No info about GO biol process
BX451454		1·083333333	No info about GO biol process
CB052574		1·153153153	No info about GO biol process
NM_138573	*Nrg4*	1·015384615	No info about GO biol process
AI349515		0·824561404	No info about GO biol process
NM_016823	*Crk*	0·869067103	Cell motility; + multiple GO terms
NM_002766	*Prpsap1*	0·814229249	Nucleoside metabolism; + multiple GO terms
BG164253		1·119565217	No info about GO biol process
NM_004494	*Hdgf*	0·875396825	Cell proliferation; + multiple GO terms
NM_002755	*Map2k1*	0·806122449	GO:6928 (cell motility);+ multiple GO terms
NM_014553	*Tfcp2l1*	0·877777778	Steroid biosynthesis; + multiple GO terms
NM_001262	*Cdkn2c*	0·826974268	Cell cycle; + multiple GO terms
NM_024590	*Arsj*	0·87037037	GO:8152 (metabolism)

Gene name	Fold induction[a] (iNSE/iNSD)	GO biol process

[a] MCF-7 cells transfected with iNS (nonspecific oligonucleotide) and treated with either 10 nM E_2 (iNSE) or DMSO (iNSD).