Role of specificity protein transcription factors in estrogen-induced gene expression in MCF-7 breast cancer cells

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Abstract

Deletion analysis of several 17 β -estradiol (E₂)-responsive genes have identified GC-rich sites that are associated with hormone-induced transactivation in MCF-7 breast cancer cells. However, the role of individual specificity proteins (Sp) in mediating hormone-induced gene expression has not been unequivocally determined. In transient transfection studies using E₂-responsive GC-rich promoters from the E₂F1, carbamoylphosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD), and retinoic acid receptor α (RAR α) genes, RNA interference using small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4) decreased both basal and E₂-induced transactivation. The contributions of individual Sp proteins to basal and E₂-induced activity were promoter dependent. iSp1, iSp3, and iSp4 also significantly inhibited hormonal induction of E₂F1, CAD, and RAR α mRNA levels; however, the enhanced inhibitory effects of the latter two small inhibitory RNAs suggest that Sp3 and Sp4 play a major role in estrogen receptor α /Sp-mediated gene expression in MCF-7 cells.

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Introduction

The estrogen receptor (ER) is a member of the nuclear receptor superfamily of transcription factors and is required for mediating 17 β -estradiol (E₂)-induced responses in multiple tissues and organs (Chow *et al.* 1992, Turner *et al.* 1994, Farhat *et al.* 1996, Clark 1998). Two major ER subtypes (ER α and ER β) and multiple splice variants have been identified and these various forms exhibit both overlapping and different activities (Hall *et al.* 2001, Olefsky 2001, Nilsson & Gustafsson 2002, Matthews & Gustafsson 2003). ER α and ER β exhibit the typical domain structures of nuclear receptors, and similarities in their DNA binding (C) and ligand binding (EF) domains are correlated with comparable mechanisms of DNA binding and similar but not identical interactions with various estrogenic ligands (Hall *et al.* 2001, Olefsky 2001, Nilsson & Gustafsson 2002, Matthews & Gustafsson 2003). The N-terminal regions (AB) of human ER α and ER β exhibit only 17% amino acid homology and this variability may account for some of the differences between the ER subtypes such as those observed in the murine uterus where ER β tends to inhibit ER α -dependent uterotrophic responses (Krege *et al.* 1998, Weihua *et al.* 2000).

The classical ER mechanism of action involves ligand-induced formation of an ER homodimer which interacts with estrogen responsive elements (EREs; Klein-Hitpass *et al.* 1986, Cowley *et al.* 1997, Hyder *et al.* 1999) in target gene promoters and recruits cofactors necessary for

transactivation. There is an increasing evidence that the classical genomic ER–ERE complex formation is only one of several genomic and non-genomic pathways of estrogen action (Blobel & Orkin 1996, Delfino & Walker 1999, Kelly & Wagner 1999, Kushner *et al.* 2000, Watson *et al.* 2002, Razandi *et al.* 2004, Safe & Kim 2004, Levin 2005). Genomic ER associates with other transcription factors such as the activating protein-1 (AP-1) complex, nuclear factor kappa B (NFkB), and specificity proteins (Sp) to modulate ligand-dependent gene expression. For example, studies in this laboratory and others have demonstrated that deletion and mutational analysis of promoters derived from several E₂-responsive genes contain GC-rich motifs that bind Sp proteins (Safe & Kim 2004). Some of the genes that fall into this category are important for cell proliferation, angiogenesis, and nucleotide metabolism and include E₂F1, retinoic acid receptor α (RAR α), carbamoylphosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD), bcl-2, DNA polymerase α , vascular endothelial growth factor (VEGF), VEGFR receptor 2 (VEGFR2), progesterone receptor, creatine kinase B, thymidylate synthase, insulin-like growth factor binding protein 4, epidermal growth factor receptor, receptor for advanced glycation end products, low density lipoprotein receptor, vitamin D receptor, pS2, LRP16, metastasis associated protein 3, and SK3 (Porter *et al.* 1996, 1997, Duan *et al.* 1998, Sun *et al.* 1998, 2005, Dong *et al.* 1999, Qin *et al.* 1999, Wang *et al.* 1999, 2002, Xie *et al.* 1999, 2000, Byrne *et al.* 2000, Salvatori *et al.* 2000, Saville *et al.* 2000, Stoner *et al.* 2000, 2004,

Tanaka *et al.* 2000, Castro-Rivera *et al.* 2001, Li *et al.* 2001, Samudio *et al.* 2001, Bruning *et al.* 2003, Jacobson *et al.* 2003, Khan *et al.* 2003, Ngwenya & Safe 2003, Schultz *et al.* 2003, 2005, Fujita *et al.* 2004, Bardin *et al.* 2005, Zhao *et al.* 2005, Higgins *et al.* 2006b). In addition, RNA interference studies with a small inhibitory RNA (siRNA) for Sp1 (iSp1) show that after transfection with iSp1, there was a significant decrease in hormone-induced G₀/G₁ to S-phase progression, suggesting that ER α /Sp1 plays an important role in this process (Abdelrahim *et al.* 2002).

MCF-7 cells express multiple Sp proteins including Sp1, Sp3, and Sp4, and the role of these individual proteins in mediating E₂-induced transactivation has not been determined. Using the E₂F1 gene as a model, we now show that hormonal activation of a GC-rich E₂F1 promoter construct is inhibited in cells cotransfected siRNAs for Sp1, Sp3 (iSp3), and Sp4 (iSp4), and using this approach, it was shown that these Sp proteins are also important for hormone-induced expression of E₂F1 mRNA and protein. Similar results were observed for two other E₂-responsive genes, CAD and RAR α , demonstrating that all three Sp proteins and particularly Sp3 and Sp4 are important for ER α /Sp-mediated gene expression in MCF-7 cells.

Materials and methods

Cells, chemicals, biochemicals, and plasmids

MCF-7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DME/F12 (Sigma Chemical Co.) media supplemented with 5% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA; or Atlanta Biologicals Inc., Norcross, GA, USA), 1.5 g/l sodium bicarbonate, and 10 ml/l antibiotic-antimycotic solution (Sigma). Cells were maintained in incubators at 37 °C under humidified 5% CO₂: 95% air. Dimethyl sulfoxide (Me₂SO), PBS, and E₂ were purchased from Sigma. Reporter lysis buffer and luciferase assay reagent were purchased from Promega Corp. and/or Boehringer Mannheim. β -Galactosidase activity was measured using Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA, USA). Instant Imager and Lumicount micro-well plate reader were purchased from Packard Instrument Co. (Downers Grove, IL, USA). β -Actin antibody was obtained from Sigma and all other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human ER α expression plasmid was originally provided by Dr Ming-Jer Tsai (Baylor College of Medicine, Houston, TX, USA) and was recloned into pcDNA3 in this laboratory. Promoter luciferase construct pRAR α 2 (−79/−49) was made by inserting the oligonucleotide spanning −79 to −49 of the human RAR α 1 promoter into the

pGL2 basic luciferase reporter plasmid (Promega Corp.). CAD promoter luciferase construct pCAD2 (−90/+25) was kindly provided by Dr Peggy Farnham (University of Wisconsin, Madison, WI, USA). E₂F1 promoter luciferase construct pE₂F1h was made in this laboratory as described (Wang *et al.* 1999). Plasmid pSp1₃ containing three consensus Sp1 binding sites linked to a luciferase reporter gene was cloned into pXP2 plasmid in this laboratory.

Transient transfection assays of small inhibitory RNA

Validated, non-targeting small inhibitory RNA (siRNA; Silencer Negative Control siRNA; iNS1) was purchased from Ambion (Austin, TX, USA) and siRNAs for Sp1, Sp3, and Sp4 were purchased from Dharmacon Research (Lafayette, CO, USA). MCF-7 cells were seeded in DME/F12 medium supplemented with 2.5% charcoal-stripped serum overnight in 12-well plates. After 16–20 h, siRNA duplexes were transfected using Lipofectamine 2000 Reagent (Invitrogen Life Technologies). After 24 h, reporter plasmids (200 ng) were cotransfected with ER α expression plasmid (150 ng) and 50 ng pCDNA3.1-His-LacZ using GeneJuice (Novagen, EMD Biosciences Inc., Madison, WI, USA) according to the manufacturer's protocol. Cells were then treated for 36 h with Me₂SO or E₂ and harvested in 100 μ l of cell lysis buffer (Promega Corp.). Luciferase activities in the various treatment groups were performed on 20 μ l of cell extract using the luciferase assay system (Promega Corp.) in a luminometer (Packard Instrument Co., Meriden, CT, USA) and results were normalized to β -galactosidase enzyme activity, which was carried out on 20 μ l of cell extract. Sequences of siRNA oligonucleotides for Sp1, Sp3, and Sp4 are summarized below.

Sp1: 5'-AUC ACU CCA UGG AUG AAA UGA dTdT-3'
 Sp3: 5'-GCG GCA GGU GGA GCC UUC ACU dTdT-3'
 Sp4: 5'-GCA GUG ACA CAU UAG UGA GC dT dT-3'

Preparation of whole cell extracts and western blot analysis

MCF-7 cells were seeded into six-well plates in DME/F12 medium supplemented with 2.5% charcoal-stripped serum. The next day, cells were transfected with siRNAs using Lipofectamine 2000 reagent according to the manufacturer's protocol. After 55–60 h, cells were dosed with Me₂SO and E₂ for 18 h and then harvested with ice-cold lysis buffer (50 mM HEPES (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl₂, and 1 mM EGTA) and supplemented with protease inhibitor cocktail (Sigma). Equal amounts of protein from each treatment group were boiled in 1 $\times$ sample buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue,

and 175 mM β -mercaptoethanol) for 5 min and separated on 7.5 or 10% gel, and then transferred to polyvinylidene difluoride membrane (Bio-Rad) overnight at 30 V. Membranes were blocked in Blotto (5% milk, Tris-buffered saline (10 mM Tris-HCl (pH 8.0) and 150 mM NaCl), and 0.05% Tween 20) for 30 min and probed with primary antibodies for 2–4 h. Membranes were washed for 30 min in 1 $\times$ TBS-Tween, probed with peroxidase-conjugated secondary antibody for 1–2 h, and then washed in 1 $\times$ TBS-Tween for 30 min. Ten millilitres of HRP substrate (Dupont-NEN, Boston, MA, USA) were added and incubated for 1 min and visualized by autoradiography. Protein band intensities were scanned on a JX-330 scanner (SHARP Corp., Mahwah, NJ, USA) using Adobe Photoshop 3.0 (Adobe Systems Inc., San Jose, CA, USA).

Immunohistochemistry

For ER α -Sp1 colocalization experiments, MCF-7 cells were seeded onto two-well glass chamber slides (Nalge Nunc International, Naperville, IL, USA) at 100 000 cells per well in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 5% charcoal stripped FBS, incubated in a 37 °C incubator with 5% CO₂ for 24 h, and treated with Me₂SO vehicle or 10 nM E₂ for 1 h. Slides were then washed with PBS, fixed with –20 °C methanol, air dried, and washed with PBS + 0.3% Tween 20 (PBS/Tween). Slides were blocked for 1 h with 5% donkey serum in antibody dilution buffer (1% BSA in PBS/Tween), washed with PBS/Tween briefly, incubated with anti-Sp1 PEP-2 (goat) antibody (Santa Cruz) at 1:100 dilution (goat serum for controls) in antibody dilution buffer at 4 °C for 12 h. Slides were washed with PBS/Tween (3 $\times$ 10 min), incubated with donkey anti-goat immunoglobulin G (IgG) fluorescein isothiocyanate (FITC) (Santa Cruz) at 1:200 dilution in antibody dilution buffer for 1 h and washed with PBS/Tween (3 $\times$ 10 min). Slides were subsequently blocked with 5% donkey serum in antibody dilution buffer for 1 h, washed with PBS/Tween briefly, incubated with anti-ER α H-184 antibody (Santa Cruz) at 1:100 dilution in antibody dilution buffer at 4 °C for 12 h (rabbit serum for controls), washed with PBS/Tween (3 $\times$ 10 min), incubated with donkey anti-rabbit IgG Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA) at 1:500 dilution in antibody dilution buffer for 1 h, and washed with PBS/Tween (3 $\times$ 10 min). Slides were finally washed in deionized water, and cover glass mounted using Prolong Gold antifade reagent with DAPI (Molecular Probes). Immunofluorescence images of Sp1 and ER α were examined using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY, USA) fitted with an Axiocam high-resolution digital camera. Digital images were captured using Axiovision 4.1 software (Carl Zeiss).

For ER α -Sp4 colocalization experiments, COS-7 cells were seeded as described above, transfected with 250 ng

of ER α and 250 ng of Sp4 expression plasmid using GeneJuice transfection reagent (Novagen), incubated at 37 °C with 5% CO₂ for 24 h, and then treated with Me₂SO vehicle or 10 nM E₂ for 1 h, and immunostaining experiments were carried out as described above using ER α D-12 (Santa Cruz) paired with donkey anti-mouse Alexa Fluor 488 (Molecular Probes) and Sp4 V-20 (Santa Cruz) paired with donkey anti-rabbit IgG Alexa Fluor 594 (Molecular Probes) antibodies.

Real-time PCR

Cells were seeded into six-well plates in DME/F12 medium supplemented with 2.5% charcoal-stripped serum overnight. The next day cells were transfected with siRNAs using Lipofectamine 2000 Reagent and, after 55–60 h, cells were treated with Me₂SO or E₂ for 6 or 12 h. RNA was extracted using Qiagen RNeasy minikit (Qiagen) following the manufacturer's protocol and was reverse transcribed for cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The cDNA reaction mixture was then used to carry out PCR using SYBR Green PCR Master Mix from PE Applied Biosystems (Warrington, UK) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The relative quantitation of samples was carried out using comparative C_T method and TATA binding protein (TBP) was used for normalization. Quantitect primer assays (Qiagen) for Sp1, Sp3, Sp4, E₂F1, and CAD were used to perform PCR, whereas primers for RAR α and TBP were purchased from Integrated DNA technologies (Coralville, IA, USA) and are as follows:

RAR α (Fwd): 5'-TCC ATG CCG CCT CTC ATC-3'
 RAR α (Rev): 5'-CGG CTG TCC GCT CAG AGT-3'
 TBP (Fwd): 5'-TGC ACA GGA GCC AAG AGT GAA-3'
 TBP (Rev): 5'-CAC ATC ACA GCT CCC CAC CA-3'

Chromatin immunoprecipitation (ChIP) assay

MCF-7 cells (1 $\times$ 10⁷) were treated with Me₂SO (time 0) or E₂ (10 nM) for different times. Cells were then fixed with 1.5% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. Cells were scraped, pelleted, and hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (~500 bp). The chromatin was precleared by addition of protein A/G-conjugated beads (Pierce, Rockford, IL, USA). The precleared chromatin supernatants were immunoprecipitated with antibodies specific to IgG, ER α , Sp1, Sp3, and Sp4 (Santa Cruz Biotechnology) at 4 °C overnight. The protein-antibody complexes were collected by addition of protein A/G-conjugated beads for 1 h, and the beads

Figure 1 siRNAs for Sp1, Sp3, and Sp4. Effects of (A) iSp1, (B) iSp3, and (C) iSp4 on mRNA expression. MCF-7 cells were transfected with iNS1, iSp1, iSp3, or iSp4, treated with Me₂SO (D), 10 nM E₂ (E), and Sp mRNA levels were determined by real-time PCR as described in the Materials and methods. Results are expressed as means $\pm$ S.E.M. for three replicated determinations for each treatment group. Significantly ($P < 0.05$) decreased mRNA levels by iSps is indicated by an asterisk. Effects of (D) iSp1, (E) iSp3, and (F) iSp4 on protein expression. MCF-7 cells were transfected with siRNAs as described above, treated with Me₂SO (D) or 10 nM E₂ (E) and whole cell lysates were analyzed by western blot analysis as described in Materials and methods.

were extensively washed. The protein–DNA crosslinks were eluted and reversed. DNA was purified by Qiaquick Spin Columns (Qiagen) followed by PCR amplification. The pS2 primers are: 5'-CTA GAC GGA ATG GGC TTC AT-3' (forward) and 5'-ATG GGA GTC TCC TCC AAC CT-3' (reverse), which amplify a 209-bp region of the human pS2 promoter containing an estrogen response element (ERE). The CAD primers are: 5'-CTT GGG GTG GGA GGG ACT-3' (forward) and 5'-GCG GCA GCA

GCA GAG ACT-3' (reverse), which amplify a 158-bp GC-rich region of the human CAD promoter. The RAR α primers are: 5'-GCC CTT CCC GAG GTC TAT TA-3' (forward) and 5'-GAG GGT ACG GAG CAG AGG TA-3' (reverse), which amplify a 138-bp GC-rich region of the human RAR α promoter. The E₂F1 primers are: 5'-CTG gta cca tcc gga caa ag-3' (forward) and 5'-TTT TTG CCG CGA AAG AGC-3' (reverse), which amplify a 198-bp region of the human E₂F1 promoter containing GC-rich

Figure 2 Role of Sp proteins in hormonal activation of pSp1₃. MCF-7 cells were cotransfected with pSp1₃ and iNS1, iSp1, iSp3, iSp4, or their combinations, treated with Me₂SO (D) or 10 nM E₂ (E), and (A) luciferase activity or (B) fold induction of luciferase activity by E₂ was determined as described in the Materials and methods. Results are expressed as means $\pm$ s.e.m. for at least three replicate determinations for each treatment group, and significantly ($P < 0.05$) decreased basal or E₂-induced activity (compared with iNS1) is indicated by a double asterisk. Significant ($P < 0.05$) induction by E₂ compared with Me₂SO is also indicated (*).

Sp-binding sites. PCR products were resolved on a 2% agarose gel in the presence of 1:10 000 SYBR gold (Molecular Probes).

Statistical analysis

Experiments were repeated two or more times, and data are expressed as mean $\pm$ s.e.m. for at least three replicates for each treatment group. Statistical differences between treatment groups were determined using Super ANOVA

and Scheffé's test. Treatments were considered significantly different from controls if $P < 0.05$.

Results

Role of Sp proteins of hormonal activation of pSp1₃

Analysis of several gene promoters from E₂-responsive genes have identified GC-rich motifs that are required

Figure 3 Role of Sp proteins in hormonal activation of E₂F1. (A) Luciferase activity and (B) fold induction by E₂ in MCF-7 cells cotransfected with ER α expression plasmid (150 ng) and pE₂F1 (-161/+54). MCF-7 cells were transfected with pE₂F1 (-161/+54) and iNS1, iSp1, iSp3, iSp4, or their combinations, and luciferase activity determined as described in the Materials and methods. Hormonal activation of pE₂F1 requires cotransfection with ER α . Results are expressed as means $\pm$ S.E.M. for three replicate determinations for each treatment group, and significantly ($P < 0.05$) decreased basal or hormone-induced activity (compared with iNS1) is indicated (**). Role of Sp proteins in hormonal activation of (C) E₂F1 mRNA and (D) protein. Cells were transfected with iNS1, iSp1, iSp3, or iSp4, and then treated with Me₂SO (D) or 10 nM E₂ (E) for 12 h. Whole cell extracts were analyzed for E₂F1 mRNA or protein as described in the Materials and methods. E₂F1 mRNA data were determined in three separate experiments and results (normalized to TBP mRNA) are means $\pm$ S.E.M.. Significant ($P < 0.05$) inhibition of E₂-induced mRNA levels by iSp1, iSp2, and iSp3 are indicated (**). Western blot analysis of whole cell lysates was determined as described in the Materials and methods. The immunoblot shown in Fig. 3D was one of two replicates that gave similar results. Significant induction by E₂ compared with Me₂SO (D) is indicated (*).

for hormone-induced transactivation in breast cancer and other cell lines (Porter *et al.* 1996, 1997, Duan *et al.* 1998, Sun *et al.* 1998, 2005, Dong *et al.* 1999, Qin *et al.* 1999, Wang *et al.* 1999, 2002, Xie *et al.* 1999, 2000, Byrne *et al.* 2000, Salvatori *et al.* 2000, Saville *et al.* 2000, Stoner *et al.* 2000, 2004, Tanaka *et al.* 2000, Castro-Rivera *et al.* 2001, Li *et al.* 2001, Samudio *et al.* 2001, Bruning *et al.* 2003, Jacobson *et al.* 2003, Khan *et al.* 2003, Ngwenya & Safe 2003, Schultz *et al.* 2003, 2005, Fujita *et al.* 2004, Bardin *et al.* 2005, Zhao *et al.* 2005, Higgins *et al.* 2006*b*). In this study, we have also used RNA interference and small inhibitory RNAs (siRNAs) for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4) to investigate the role of individual Sp proteins in ER α /Sp-mediated transactivation in MCF-7 cells. Previous studies in cancer cell lines have demonstrated the specificity and effectiveness of iSp1, iSp3, and iSp4 in Sp protein knockdown (Abdelrahim *et al.* 2002, 2004, Higgins *et al.* 2006*a,b*), and results in Fig. 1A–C illustrate that transfected iSp1, iSp3, and iSp4 significantly decreased Sp1, Sp3, and Sp4 mRNA levels in whole cell extracts from MCF-7 cells. In parallel studies, transfected iSp1, iSp3, and iSp4 also decreased Sp1, Sp3, and Sp4 protein expression (Fig. 1D–F) in MCF-7 cells.

The pSp1₃ construct has been extensively used as a model to investigate the mechanisms of ER α /Sp-mediated transactivation (Saville *et al.* 2000, Kim *et al.* 2003, 2005). This construct contains three tandem consensus GC-rich binding sites linked to a minimal TATA-luciferase and is E₂ responsive only after cotransfection with ER α and this is due, in part, to overexpression of pSp1₃ in transfected cells and limiting levels of ER α . This system has been ideal for studying hormone activation of pSp1₃ in ER-positive or ER-negative cell lines thereby determining the contributions of cell context on the induction response (Saville *et al.* 2000, Kim *et al.* 2003, 2005). Results illustrated in Fig. 2A show that E₂ induces transactivation in MCF-7 cells transfected with pSp1₃ and non-specific RNA (iNS1) and similar results were obtained in the presence or absence of transfected iNS1 oligonucleotide (data not shown). The results show that all three siRNAs or their combinations significantly decreased basal luciferase activity, and iSp1 and iSp4 were more effective than iSp3. The fold induction of luciferase by E₂ compared with solvent (Me₂SO) control shows that the effectiveness of the siRNAs for decreasing fold induction was iSp1 > iSp4 > iSp3 (Fig. 2B) with only minimal changes in fold induction observed for iSp4 and iSp3. The apparent lack of effectiveness of iSp4 and iSp3 for inhibiting fold induction of luciferase activity when compared with iSp1 was due, in part, to their differential inhibitory effects on basal luciferase activity. In cells transfected with iSps in combination, the maximal inhibition of fold induction was observed in cells transfected with iSp1/iSp3 and iSp1/iSp4, whereas minimal changes were observed in cells transfected with iSp3/iSp4. However, these data suggest that for the pSp1₃

construct containing three consensus GC-rich sites, Sp1 plays the most important role in E₂-dependent activation of ER α /Sp.

ER α /Sp-mediated activation of E₂F1

E₂ induces E₂F1 gene expression in MCF-7 cells (Wang *et al.* 1999, Ngwenya & Safe 2003), and promoter analysis has identified critical GC-rich sites at between –169 and –111 in the proximal region of the E₂F1 promoter. Figure 3A illustrates luciferase activity in MCF-7 cells transfected with pE₂F1 (–169/–54) and various iSps or their combinations. The results show that individual siRNAs for Sp1, Sp3, and Sp4 decreased luciferase by 70–90% with iSp3 as the most effective siRNA. These results indicate that Sp proteins play the major role in regulating basal luciferase activity in MCF-7 cells transfected with pE₂F1 (–169/–54). Moreover, the effectiveness of iSp1, iSp3, and iSp4 in decreasing activity suggests that the loss of any single Sp protein was important for transcription, indicating cooperative interactions among these transcription factors for regulating luciferase activity. The effects of individual and combined Sp protein knockdown in MCF-7 cells transfected with pE₂F1 (–169/–54) and treated with Me₂SO or E₂ were also determined, and the effects on fold induction by E₂ are illustrated in Fig. 3B. With the exception of the results observed for iSp3, the fold induction by E₂ was decreased 50–65% by iSp1, iSp4, and their combinations (including iSp3 + iSp1 and iSp3 + iSp4). iSp3 only slightly decreased the fold induction by E₂ and was less effective than either iSp1 or iSp4. These results were similar to those observed for the effects of iSps on hormone inducibility in MCF-7 cells transfected with pSp1₃ (Fig. 2A).

The effects of siRNAs for Sp proteins on induction of E₂F1 mRNA levels in MCF-7 cells were also investigated. Cells were transfected with iNS1 (non-specific RNA), iSp1, iSp3 or iSp4 for 55–60 h, then treated with Me₂SO or 10 nM E₂ for an additional 12 h and mRNA levels were determined in whole cell extracts by real-time PCR. Previous studies show that within this time period, there is a maximal decrease of Sp proteins using these siRNAs (Abdelrahim *et al.* 2002, 2004, Higgins *et al.* 2006*a,b*). Basal E₂F1 mRNA levels were significantly decreased by transfecting iSp1, iSp3, and iSp4 by 40, 86, and 77% respectively, demonstrating the role of Sp proteins in mediating E₂F1 mRNA expression. E₂ induced an approximately threefold increase in E₂F1 mRNA expression and in cells transfected with iSp1, iSp3, and iSp4, there were significant decreases in hormone-induced E₂F1 mRNA levels and cells transfected with iSp4 gave the lowest inducibility. However, the results suggest that all three Sp proteins are involved in ER α /Sp-dependent activation of E₂F1. iSp3 was more effective in decreasing induction of E₂F1 mRNA by E₂ (Fig. 3C)

Figure 4 Role of Sp proteins in hormonal activation of CAD. (A) Luciferase activity and (B) fold induction by E₂ in MCF-7 cells cotransfected with ERα expression plasmid (150 ng) and pCAD (−90/+25). MCF-7 cells were transfected with pCAD (−90/+25) and iNS1, iSp1, iSp3, iSp4, or their combinations, and luciferase activity determined as described in the Materials and methods. The requirement of ERα for hormonal activation of this construct is also indicated (B). Results are expressed as means ± S.E.M. for three replicate determinations for each treatment group, and significantly ($P < 0.05$) decreased basal or hormone-induced activity (compared with iNS1) is indicated (**). The effects of ER expression plasmid on hormone inducibility is also indicated. (C) Role of Sp proteins in hormonal activation of CAD mRNA. MCF-7 cells were transfected with iNS, iSp1, iSp3, or iSp4, treated with ME₂SO (D) or 10 nM E₂ (E) for 6 h, and mRNA levels determined as described in the Materials and methods. CAD mRNA levels (normalized to TBP mRNA) are means ± S.E.M. for at least three determinations and significantly ($P < 0.05$) decreased hormone-induced CAD mRNA is indicated (**). Significant induction by E₂ compared with ME₂SO is indicated (*).

than decreasing induction of luciferase activity by E₂ in cells transfected with pE₂F1h (Fig. 3B). This suggests that other regulatory regions of E₂F1 that are affected by Sp3 are important for induction of E₂F1 mRNA levels by E₂. We also observed in a separate experiment that induction of E₂F1 protein by E₂ and basal E₂F1 protein expression were also decreased in MCF-7 cells transfected with iSp1, iSp3, and iSp4 (Fig. 3D). These results demonstrate the hormone inducibility of E₂F1 is dependent on Sp transcription factors. Thus, although Sp3 plays a minimal role in hormone-induced activation of pE₂F1 (−169/−54), this transcription factor is important for mediating hormone-dependent induction of E₂F1 mRNA and protein.

ER α /Sp-mediated activation of CAD and RAR α

The −90 to +25 region of the CAD gene promoter is also E₂ responsive in transfection assays, and deletion and mutation analyses have identified three proximal GC-rich sites which are required for hormone responsiveness (Khan *et al.* 2003). Results in Fig. 4A show that in MCF-7 cells transfected with pCAD (−90/+25), cotransfection with iSp1, iSp3, iSp4, or their combinations significantly decreased activity (>50%); however, the magnitude of the decreased activity was significantly less than observed for pE₂F1 (−169/−54; Fig. 3A). Since the magnitude of the decreased response was similar after transfection with iSp1, iSp3, or iSp4, it is likely that all three transcription factors are required for basal activity. In MCF-7 cells transfected with pCAD (−90/+25) plus iNS1 and treated with Me₂SO or E₂, cotransfection with iSp1, iSp3, iSp4, or their combinations significantly decreased hormone-induced transactivation (Fig. 4B). Fold induction was decreased only slightly more effectively in cells transfected with iSp1 and iSp4 when compared with iSp3; however, all three Sp transcription factors were important for hormone responsiveness and combinations of iSps inhibited hormone inducibility more than individual siRNAs. Similar results were observed for induction of CAD mRNA levels by E₂ (Fig. 4C) where the induction response was significantly decreased in MCF-7 cells transfected with iSp1, iSp3, and iSp4. In contrast, iSps had only a minimal effect of CAD mRNA levels (data not shown).

We also investigated the role of Sp proteins in hormonal activation of RAR α since previous studies have identified proximal GC-rich motifs at −68 to −62 and −59 to −52 required for hormonal activation of promoter constructs (Sun *et al.* 1998). In contrast to the pCAD (−90/+25) and pE₂F1 (−169/−54) constructs, basal luciferase activity was not decreased in MCF-7 cells transfected with pRAR α (−79/−49) and iSp1 or iSp4 but was significantly decreased only after cotransfection with iSp3 (Fig. 5A). Cotransfection with iSp combinations (except iSp1+iSp3) decreased activity; however, it was

evident that despite the presence of GC-rich motifs in pRAR α (−79/−49), Sp proteins were not the predominant transcription factors required for basal activity. In contrast, fold inducibility of luciferase activity in MCF-7 cells treated with Me₂SO or 10 nM E₂ and transfected with pRAR α (−79/−49) was primarily decreased in cells transfected with iSp1 and iSp4 but not iSp3 (Fig. 5B). Since both iSp1 and iSp4 significantly decreased hormone responsiveness, any combination of these siRNAs inhibited E₂-induced transactivation. Thus, hormone-dependent activation of pRAR α (−79/−49) was primarily dependent on ER α /Sp1 and ER α /Sp4, and this was similar to results obtained with pSp1₃ (Fig. 2B) and pE₂F1 (−169/−54; Fig. 3B). Basal expression of RAR α mRNA was only minimally affected by iSps (data not shown); however, induction of RAR α mRNA levels by E₂ was decreased after cotransfection with iSp1, iSp3, and iSp4 with iSp3 being the most effective siRNA (Fig. 5C). iSp3 completely abrogated induction of RAR α by E₂, whereas in transfection studies with pRAR α 2, which contains a −79 to −49 RAR α promoter insert, iSp3 did not significantly decrease hormone responsiveness (Fig. 5B). These results suggest that GC-rich sites in addition to those at −79 to −49 may be important for ER α /Sp-mediated induction of RAR α mRNA by E₂.

Colocalization of ER α with Sp1 and Sp4

Previous studies showed that ER α interacts with Sp1 and Sp3 (Porter *et al.* 1996, 1997, Saville *et al.* 2000, Stoner *et al.* 2000); however, the results of RNA interference assays now demonstrate that, in MCF-7 cells, Sp4 plays an important role in E₂-dependent activation of ER α /Sp. Therefore, we have investigated colocalization of ER α and Sp1 in MCF-7 cells, which exhibit endogenous expression of both proteins (Fig. 6A). ER α and Sp1 proteins alone exhibit nuclear staining and the colocalized proteins exhibit a punctate staining pattern, and these results complement previous studies showing direct interactions of both proteins (Porter *et al.* 1997). Colocalization experiments with ER α and Sp4 were examined in COS-7 cells transfected with ER α and Sp4 expression plasmids (Fig. 6B). Immunostaining of endogenous Sp4 in MCF-7 cells was weak due to the relative insensitivity of the commercially available antibodies; however, in transfected COS-7 cells, Sp4 staining is observed and is more intense in the central part of the nucleus. ER α alone is uniformly observed in the nucleus and, after treatment with E₂, a more punctate staining is observed and the colocalized ER α /Sp4 also exhibited punctate staining. Several attempts to investigate colocalization of ER α and Sp3 were unsuccessful, due to the failure of available Sp3 antibodies to detect Sp3 by this method. However, these results demonstrate that ER α colocalizes with Sp1 and Sp4 and these transcription factors play an important role in ER α /Sp-mediated expression of E₂F1, CAD, and RAR α .

Figure 5 Role of Sp proteins in hormonal activation of RAR α . (A) Luciferase activity and (B) fold induction by E₂ in MCF-7 cells cotransfected with ER α expression plasmid (150 ng) and pRAR α (–79/–49). MCF-7 cells were transfected with pRAR α (–79/–49) and iNS1, iSp1, iSp3, iSp4, or their combinations, and luciferase activity determined as described in the Materials and methods. Hormonal activation of pRAR α requires cotransfection with ER α . Results are expressed as means $\pm$ S.E.M. for three replicate determinations for each treatment group, and significantly ($P < 0.05$) decreased basal or hormone-induced activity (compared with iNS1) is indicated (**). (C) Role of Sp proteins in hormonal activation of RAR α mRNA. MCF-7 cells were transfected with iNS, iSp1, iSp3, or iSp4, treated with Me₂SO (D) or 10 nM E₂ (E) for 12 h, and mRNA levels determined as described in the Materials and methods. RAR α mRNA levels (normalized to TBP mRNA) are expressed as means $\pm$ S.E.M. for at least three determinations and significantly ($P < 0.05$) decreased induction (fold) of RAR α mRNA by E₂ is indicated (**). Significant induction by E₂ compared with ME₂SO is indicated (*).

Figure 6 Colocalization of ER α with Sp1 and Sp4 in (A) MCF-7 and (B) COS-7 cells. MCF-7 and COS-7 cells were seeded in two-well chamber slides. The latter cells were transfected with ER α and Sp4 expression plasmids. Both cell lines were treated with Me₂SO or 10 nM E₂ for 1 h, and cells were immunostained as described in the Materials and methods.

in MCF-7 cells. However, the effects of E₂ on colocalization are minimal, suggesting interactions of ER α with Sp proteins in the presence or absence of hormone, and this is consistent with previous ChIP assays (Higgins *et al.* 2006a) and the reported coimmunoprecipitation of ER α and Sp1 ($\pm$ hormone; Saville *et al.* 2000).

Interactions of ER α and Sp proteins with the pS2, E₂F1, CAD, and RAR α gene promoters

Previous studies show that the E₂-responsive region of the pS2 gene promoter contains GC-rich motifs and ChIP analysis of the pS2 promoter (Fig. 7A) shows constitutive binding of Sp1, Sp3, and Sp4. The band intensities exhibit minimal changes after treatment with 10 nM E₂ for up to

2 h. However, as previously reported (Shao *et al.* 2002, Metivier *et al.* 2003, Acevedo *et al.* 2004, Krieg *et al.* 2004, Sun *et al.* 2005), treatment of MCF-7 cells recruits ER α to the pS2 promoter, and this is consistent with interactions with the ERE motif at -405 to -393. Results in Fig. 7B–D summarize ChIP assays, which examine basal and hormone-induced interactions of ER α , Sp1, Sp3, and Sp4 with the GC-rich regions of the RAR α , E₂F1, and CAD gene promoters respectively. Sp1, Sp3, and Sp4 are constitutively bound to all three promoters and treatment with E₂ does not increase or decrease Sp interactions with these promoters. Moreover, similar results were also observed for ER α which was bound to the GC-rich RAR α , E₂F1, and CAD gene promoters in the presence or absence of hormone treatment. Since Sp1, Sp3, and Sp4

Figure 7 ChIP assay of ER α and Sp protein interactions with E₂-responsive gene promoters. MCF-7 cells were treated with Me₂SO (D) or 10 nM E₂ for different time points, and the interaction of ER α and Sp proteins to the (A) pS2, (B) RAR α , (C) E₂F1, and (D) CAD gene promoters was determined in a ChIP assay as described in the Materials and methods. Similar results were obtained in duplicate experiments. We also observed specific binding of transcription factor IIB to the glyceraldehyde 3-phosphate dehydrogenase promoter and not to exon 1 of the CNAP1 gene (data not shown). This serves as an additional positive control for the ChIP assay (Higgins *et al.* 2006b).

are important for basal expression of the genes examined in this study, it is not surprising that these proteins are associated with these GC-rich promoters. These results are consistent with the known ligand-independent interactions of ER α with Sp proteins (Porter *et al.* 1997, Saville *et al.* 2000, Stoner *et al.* 2000), and the colocalization of ER α with Sp1 and Sp4 in Me₂SO (and

E₂)-treated cells (Fig. 6). Moreover, we have recently reported similar ER α and Sp interactions with the VEGFR2 promoter in ZR-75 cells using a ChIP assay (Higgins *et al.* 2006b). Our results demonstrate for the first time that hormonal activation of genes containing E₂-responsive GC-rich promoters in MCF-7 cells requires ER α interactions with Sp1, Sp3, and Sp4.

Discussion

The mechanism of ligand-dependent activation of nuclear receptors involves a simple concept in which receptor ligands induce formation of a transcriptionally active receptor complex bound to a cognate response element in the target gene promoter. However, transactivation mediated by nuclear receptors such as steroid hormone receptors is highly complicated and is dependent on ligand structure, hormone receptor subtype, tissue-specific expression of coactivators, other nuclear cofactors, and promoter context (Katzenellenbogen *et al.* 1996, Lemon & Freedman 1999, Klinge 2000, Rosenfeld & Glass 2001, Smith & O'Malley 2004). For example, although E₂ activates hormone-responsive gene expression in multiple tissues, other ER agonists such as the antiestrogen tamoxifen exhibit tissue-specific ER agonist and antagonist activities (Jordan 2003a,b). Differences between the activities of E₂ and tamoxifen and other antiestrogens are due, in part, to their ligand-induced conformational changes in the ER and their subsequent differences in binding affinities for specific nuclear coactivators (Schwabe *et al.* 1993, Brzozowski *et al.* 1997, Shiau *et al.* 1998, 2002, Pike *et al.* 1999, Jordan 2003a).

ER/Sp1-dependent transactivation is also complex and dependent on multiple variables. For example, E₂ activates ER α /Sp but not ER β /Sp in several different cell lines and in HeLa cells, E₂ decreases ER β /Sp1-dependent transactivation and this may be a pathway for decreased gene expression by hormones (Saville *et al.* 2000). In addition, there is also evidence that activation of ER α /Sp1 involves multiple domains of ER α and domain requirements are also dependent on the structure of the ER agonist (Saville *et al.* 2000, Kim *et al.* 2003). Previous studies have identified multiple gene promoters activated by ER α /Sp and Sp1 knockdown by RNA interference inhibits hormone-induced G₀/G₁ to S-phase progression in MCF-7 cells (Abdelrahim *et al.* 2002). However, the role of individual Sp1, Sp3, and Sp4 proteins in mediating ER α /Sp-induced gene expression in MCF-7 cells has not been determined. In this study, we have used RNA interference to investigate the effects of Sp protein knockdown (individual and combined) on induction of E₂F1, RAR α , and CAD gene expression by E₂ since all three genes contain GC-rich promoters that are E₂-responsive in transfection studies (Wang *et al.* 1999, Khan *et al.* 2003, Ngwenya & Safe 2003).

The siRNAs for Sp proteins are highly specific and have been used in several studies (Abdelrahim *et al.* 2002, 2004, Higgins *et al.* 2006a,b), and they efficiently decrease expression of both Sp1, Sp3, and Sp4 mRNA and protein (Fig. 1). The effects of iSpS on basal luciferase activity in MCF-7 cells transfected with pSp1₃, pE₂F1 (−169/−54), pCAD (−90/+25), and pRAR α

(−79/−49) demonstrated that despite common GC-rich sites in all three promoters, decreased Sp protein expression differentially affected activity. Luciferase activity in cells transfected with pE₂F1 (−169/−54) was decreased ~70–90% after cotransfection with iSp1, iSp3, and iSp4, whereas in cells transfected with pRAR α (−79/−49), only iSp3 decreased activity and iSp1 actually increased activity. Although basal activity of the four promoters were differentially affected by Sp protein knockdown, E₂-induced transactivation in cells transfected with these constructs, and fold induction was decreased in cells transfected with iSp1 or Sp4 (and their combinations), whereas iSp3 was the least effective or ineffective at decreasing fold induction. However, this may be, in part, due to the effects of iSp3 on decreasing basal activity in cells transfected with iSp1₃, pRAR α (−79/−49), and pE₂F1 (−169/−54). All three iSpS induced a comparable decrease in basal activity in MCF-7 cells transfected with pCAD (−90/+25) and iSp3 also decreased hormone-induced activity with this construct. Transfected iSpS significantly decreased induction of E₂F1, RAR α , and CAD mRNA levels by E₂ in MCF-7 cells (Figs 3–5), and the order of effectiveness was iSp4 ≥ iSp3 > iSp1. Knockdown of Sp3 resulted in a complete loss of hormone-induced RAR α mRNA, whereas in cells transfected with pRAR α (−79/−49), iSp3 did not affect E₂-induced transactivation. This suggests that other GC-rich sites in the RAR α promoter are required for ER α /Sp-mediated expression of this gene. Previous studies demonstrate that both Sp1 and Sp3 interact with ER α (Porter *et al.* 1996, 1997, Saville *et al.* 2000, Stoner *et al.* 2000) and we now show that both Sp1 and Sp4 colocalize with ER α in MCF-7 cells (Fig. 6). Thus, at least for the RAR α , CAD, and E₂F1 genes, all three Sp proteins play a role in ER α /Sp-induced transactivation and, for E₂F1, we also showed decreased protein expression (Fig. 3).

The ChIP assay has been used extensively to investigate the ligand-induced assembly of ER α and other coactivators and nuclear cofactors on hormone-responsive gene promoters, and pS2 has been used extensively as a model for these studies (Shao *et al.* 2002, Metivier *et al.* 2003, Acevedo *et al.* 2004, Krieg *et al.* 2004, Sun *et al.* 2005). This gene has a well-characterized E₂-responsive non-consensus ERE motif, and several studies have reported that treatment of MCF-7 cells with E₂ recruits ER α to the pS2 promoter. Similar results were observed in this study (Fig. 7A). Moreover, Sp1, Sp3, and Sp4 were also bound to the pS2 promoter, and PCR bands associated with the Sp protein-promoter interactions were not appreciably changed after treatment with E₂. Sun *et al.* (2005) also reported interactions of Sp1 and Sp3 with the pS2 promoter. Band intensities associated with Sp3-promoter interactions were unchanged after treatment with E₂, whereas Sp1-promoter interactions were decreased; however, these changes were not observed in this study.

We further investigated Sp protein interactions with the E₂-responsive GC-rich regions of the E₂F1, CAD, and RAR α promoters in a ChIP assay and observed interactions of Sp1, Sp3, and Sp4 with these promoters in untreated MCF-7 cells (Fig. 7B and D). After treatment of MCF-7 cells with E₂, Sp protein-promoter interactions did not appreciably change, and this was similar to that observed for interactions of Sp proteins with the pS2 promoter (Fig. 7A). Binding of Sp1, Sp3, and Sp4 to the CAD, E₂F1, and RAR α promoters was similar in solvent and E₂-treated cells, and this is consistent with the role of all three proteins in mediating basal expression and hormonal activation of ER α /Sp-dependent CAD, E₂F1, and RAR α mRNAs (Figs 3C, 4C, and 5C).

ER α was also constitutively bound to the GC-rich promoters and, in contrast to observations with the pS2 promoter (Fig. 7A; Shao *et al.* 2002, Metivier *et al.* 2003, Acevedo *et al.* 2004, Krieg *et al.* 2004, Sun *et al.* 2005), E₂ did not enhance ER α interactions with the CAD, E₂F1, and RAR α promoters in MCF-7 cells. Similar results were reported for the VEGFR2 promoter in ZR-75 cells (Higgins *et al.* 2006a) suggesting that there are fundamental differences in hormone-dependent interactions of ER α with E₂-responsive promoters containing GC-rich or ERE motifs. We have carried out ChIP assays on several GC-rich promoters under a variety of conditions but invariably find that ER α , Sp1, Sp3, and Sp4 are associated with these promoters in the presence or absence of E₂. These results suggest that there may also be differences in the recruitment of other nuclear cofactors to these gene promoters, and promoter-dependent differences in coactivator recruitment are currently being investigated.

In summary, results of this study demonstrate that Sp1, Sp3, and Sp4 transcription factors are important for hormone-induced E₂F1, CAD, and RAR α mRNA expression. This is the first report showing the different contributions of ER α /Sp1, ER α /Sp3, and ER α /Sp4 on hormone-induced transactivation in MCF-7 cells. The relative effects of these individual proteins on ER α /Sp-mediated transactivation were dependent on the specific gene and gene promoter. Basal activity of the GC-rich proximal promoter regions of the E₂F1, CAD, and RAR α were differentially effected by Sp protein knockdown (Figs 3A, 4A, and 5A) and activity in cells transfected with pRAR α -2 was only minimally decreased by iSps. Despite these differences, results of ChIP assays show that Sp1, Sp3, Sp4, and ER α were associated with all three promoters in the absence or presence of E₂ (Fig. 7). In ongoing studies, we are further analyzing interactions of ER α and Sp proteins with the E₂F1, RAR α , CAD, and other gene promoters and comparing their binding to E₂-responsive and non-responsive promoter sequences. We are also investigating the effects of ligand structure, coactivators, and cell context on this important genomic

pathway of estrogen action using both cell culture and *in vivo* models.

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Disclosure statement

The authors have nothing to disclose.

References

- Abdelrahim M, Samudio I, Smith R, Burghardt R & Safe S 2002 Small inhibitory RNA duplexes for Sp1 mRNA block basal and estrogen-induced gene expression and cell cycle progression in MCF-7 breast cancer cells. *Journal of Biological Chemistry* **277** 28815–28822.
- Abdelrahim M, Smith R, III, Burghardt R & Safe S 2004 Role of Sp proteins in regulation of vascular endothelial growth factor expression and proliferation of pancreatic cancer cells. *Cancer Research* **64** 6740–6749.
- Acevedo ML, Lee KC, Stender JD, Katzenellenbogen BS & Kraus WL 2004 Selective recognition of distinct classes of coactivators by a ligand-inducible activation domain. *Molecular Cell* **13** 725–738.
- Bardin A, Moll F, Margueron R, Delfour C, Chu ML, Maudelonde T, Cavailles V & Pujol P 2005 Transcriptional and posttranscriptional regulation of fibulin-1 by estrogens leads to differential induction of messenger ribonucleic acid variants in ovarian and breast cancer cells. *Endocrinology* **146** 760–768.
- Blobel GA & Orkin SH 1996 Estrogen-induced apoptosis by inhibition of the erythroid transcription factor GATA-1. *Molecular and Cellular Biology* **16** 1687–1694.
- Bruning JC, Lingohr P, Gillette J, Hanstein B, Avci H, Krone W, Muller-Wieland D & Kotzka J 2003 Estrogen receptor- α and Sp1 interact in the induction of the low density lipoprotein-receptor. *Journal of Steroid Biochemistry and Molecular Biology* **86** 113–121.
- Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA & Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389** 753–758.
- Byrne IM, Flanagan L, Tenniswood MP & Welsh J 2000 Identification of a hormone-responsive promoter immediately upstream of exon 1c in the human vitamin D receptor gene. *Endocrinology* **141** 2829–2836.
- Castro-Rivera E, Samudio I & Safe S 2001 Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. *Journal of Biological Chemistry* **276** 30853–30861.
- Chow J, Tobias JH & Colston KW 1992 Estrogen maintains trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. *Journal of Clinical Investigation* **89** 74–78.
- Clark JH 1998 Female reproduction and toxicology of estrogens. In *Reproductive and Developmental Toxicology*, pp 259–275. Ed KS Korach. New York: Marcel Dekker.

- Cowley SM, Hoare S, Mosselman S & Parker MG 1997 Estrogen receptors α and β form heterodimers on DNA. *Journal of Biological Chemistry* **272** 19858–19862.
- Delfino F & Walker WH 1999 Hormonal regulation of the NF- κ B signaling pathway. *Molecular and Cellular Endocrinology* **157** 1–9.
- Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, Kladde M, Vyhldal C & Safe S 1999 Mechanisms of transcriptional activation of *bcl-2* gene expression by 17 β -estradiol in breast cancer cells. *Journal of Biological Chemistry* **274** 32099–32107.
- Duan R, Porter W & Safe S 1998 Estrogen-induced *c-fos* protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology* **139** 1981–1990.
- Farhat MY, Lavigne MC & Ramwell PW 1996 The vascular protective effects of estrogen. *FASEB Journal* **10** 615–624.
- Fujita N, Kajita M, Taysavang P & Wade PA 2004 Hormonal regulation of metastasis-associated protein 3 transcription in breast cancer cells. *Molecular Endocrinology* **18** 2937–2949.
- Hall JM, Couse JF & Korach KS 2001 The multifaceted mechanisms of estradiol and estrogen receptor signaling. *Journal of Biological Chemistry* **276** 36869–36872.
- Higgins KJ, Abdelrahim M, Liu S, Yoon K & Safe S 2006a Regulation of vascular endothelial growth factor receptor-2 expression in pancreatic cancer cells by Sp proteins. *Biochemical and Biophysical Research Communications* **345** 292–301.
- Higgins KJ, Liu S, Abdelrahim M, Yoon K, Vanderlaag K, Porter W, Metz RP & Safe S 2006b Vascular endothelial growth factor receptor-2 expression is induced by 17 β -estradiol in ZR-75 breast cancer cells by estrogen receptor α /Sp proteins. *Endocrinology* **147** 3285–3295.
- Hyder SM, Chiappetta C & Stancel GM 1999 Interaction of human estrogen receptors alpha and beta with the same naturally occurring estrogen response elements. *Biochemical Pharmacology* **57** 597–601.
- Jacobson D, Pribnow D, Herson PS, Maylie J & Adelman JP 2003 Determinants contributing to estrogen-regulated expression of SK3. *Biochemical and Biophysical Research Communications* **303** 660–668.
- Jordan VC 2003a Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. *Journal of Medicinal Chemistry* **46** 883–908.
- Jordan VC 2003b Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *Journal of Medicinal Chemistry* **46** 1081–1111.
- Katzenellenbogen JA, O'Malley BW & Katzenellenbogen BS 1996 Tripartite steroid hormone receptor pharmacology—interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. *Molecular Endocrinology* **10** 119–131.
- Kelly MJ & Wagner EJ 1999 Estrogen modulation of G-protein-coupled receptors. *Trends in Endocrinology and Metabolism* **10** 369–374.
- Khan S, Abdelrahim M, Samudio I & Safe S 2003 Estrogen receptor/Sp1 complexes are required for induction of *cad* gene expression by 17 β -estradiol in breast cancer cells. *Endocrinology* **144** 2325–2335.
- Kim K, Thu N, Saville B & Safe S 2003 Domains of estrogen receptor α (ER α) required for ER α /Sp1-mediated activation of GC-rich promoters by estrogens and antiestrogens in breast cancer cells. *Molecular Endocrinology* **17** 804–817.
- Kim K, Barhoumi R, Burghardt R & Safe S 2005 Analysis of estrogen receptor α -Sp1 interactions in breast cancer cells by fluorescence resonance energy transfer. *Molecular Endocrinology* **19** 843–854.
- Klein-Hitpass L, Schorpp M, Wagner U & Ryffel GU 1986 An estrogen-responsive element derived from the 5'-flanking region of the xenopus vitellogenin A2 gene functions in transfected human cells. *Cell* **46** 1053–1061.
- Klinge CM 2000 Estrogen receptor interactions with co-activators and corepressors. *Steroids* **65** 227–251.
- Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA & Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor β . *PNAS* **95** 15677–15682.
- Krieg AJ, Krieg SA, Ahn BS & Shapiro DJ 2004 Interplay between estrogen response element sequence and ligands controls *in vivo* binding of estrogen receptor to regulated genes. *Journal of Biological Chemistry* **279** 5025–5034.
- Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM & Webb P 2000 Estrogen receptor pathways to AP-1. *Journal of Steroid Biochemistry and Molecular Biology* **74** 311–317.
- Lemon BD & Freedman LP 1999 Nuclear receptor cofactors as chromatin remodelers. *Current Opinion in Genetics and Development* **9** 499–504.
- Levin ER 2005 Integration of the extranuclear and nuclear actions of estrogen. *Molecular Endocrinology* **19** 1951–1959.
- Li C, Briggs MR, Ahlborn TE, Kraemer FB & Liu J 2001 Requirement of Sp1 and estrogen receptor α interaction in 17 β -estradiol-mediated transcriptional activation of the low density lipoprotein receptor gene expression. *Endocrinology* **142** 1546–1553.
- Matthews J & Gustafsson JA 2003 Estrogen signaling: a subtle balance between ER α and ER β . *Molecular Interventions* **3** 281–292.
- Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M & Gannon F 2003 Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* **115** 751–763.
- Ngwenya S & Safe S 2003 Cell context-dependent differences in the induction of E2F-1 gene expression by 17 β -estradiol in MCF-7 and ZR-75 cells. *Endocrinology* **144** 1675–1685.
- Nilsson S & Gustafsson JA 2002 Biological role of estrogen and estrogen receptors. *Critical Reviews in Biochemistry and Molecular Biology* **37** 1–28.
- Olefsky JM 2001 Nuclear receptor minireview series. *Journal of Biological Chemistry* **276** 36863–36864.
- Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA & Carlquist M 1999 Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO Journal* **18** 4608–4618.
- Porter W, Wang F, Wang W, Duan R & Safe S 1996 Role of estrogen receptor/Sp1 complexes in estrogen-induced heat shock protein 27 gene expression. *Molecular Endocrinology* **10** 1371–1378.
- Porter W, Saville B, Hoivik D & Safe S 1997 Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Molecular Endocrinology* **11** 1569–1580.
- Qin C, Singh P & Safe S 1999 Transcriptional activation of insulin-like growth factor binding protein 4 by 17 β -estradiol in MCF-7 cells: role of estrogen receptor-Sp1 complexes. *Endocrinology* **140** 2501–2508.
- Razandi M, Pedram A, Merchanthaler I, Greene GL & Levin ER 2004 Plasma membrane estrogen receptors exist and functions as dimers. *Molecular Endocrinology* **18** 2854–2865.
- Rosenfeld MG & Glass CK 2001 Coregulator codes of transcriptional regulation by nuclear receptors. *Journal of Biological Chemistry* **276** 36865–36868.
- Safe S & Kim K 2004 Nuclear receptor-mediated transactivation through interaction with Sp proteins. *Progress in Nucleic Acid Research and Molecular Biology* **77** 1–36.
- Salvatori L, Ravenna L, Felli MP, Cardillo MR, Russo MA, Frati L, Gulino A & Petrangeli E 2000 Identification of an estrogen-mediated deoxyribonucleic acid-binding independent transactivation pathway on the epidermal growth factor receptor gene promoter. *Endocrinology* **141** 2266–2274.
- Samudio I, Vyhldal C, Wang F, Stoner M, Chen I, Kladde M, Barhoumi R, Burghardt R & Safe S 2001 Transcriptional activation of DNA polymerase α gene expression in MCF-7 cells by 17 β -estradiol. *Endocrinology* **142** 1000–1008.
- Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA & Safe S 2000 Ligand-, cell- and estrogen receptor subtype (α / β)-dependent activation at GC-rich (Sp1) promoter elements. *Journal of Biological Chemistry* **275** 5379–5387.
- Schultz JR, Petz LN & Nardulli AM 2003 Estrogen receptor α and Sp1 regulate progesterone receptor gene expression. *Molecular and Cellular Endocrinology* **201** 165–175.

- Schultz JR, Petz LN & Nardulli AM 2005 Cell- and ligand-specific regulation of promoters containing activator protein-1 and Sp1 sites by estrogen receptors α and β . *Journal of Biological Chemistry* **280** 347–354.
- Schwabe JW, Chapman L, Finch JT & Rhodes D 1993 The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. *Cell* **75** 567–578.
- Shao W, Halachmi S & Brown M 2002 ERAP140, a conserved tissue-specific nuclear receptor coactivator. *Molecular and Cellular Biology* **22** 3358–3372.
- Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA & Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95** 927–937.
- Shiau AK, Barstad D, Radek JT, Meyers MJ, Nettles KW, Katzenellenbogen BS, Katzenellenbogen JA, Agard DA & Greene GL 2002 Structural characterization of a subtype-selective ligand reveals a novel mode of estrogen receptor antagonism. *Nature Structural Biology* **9** 359–364.
- Smith CL & O'Malley BW 2004 Coregulator function: a key to understanding tissue specificity of selected receptor modulators. *Endocrine Reviews* **25** 45–71.
- Stoner M, Wang F, Wormke M, Nguyen T, Samudio I, Vyhldal C, Marme D, Finkenzeller G & Safe S 2000 Inhibition of vascular endothelial growth factor expression in HEC1A endometrial cancer cells through interactions of estrogen receptor α and Sp3 proteins. *Journal of Biological Chemistry* **275** 22769–22779.
- Stoner M, Wormke M, Saville B, Samudio I, Qin C, Abdelrahim M & Safe S 2004 Estrogen regulation of vascular endothelial growth factor gene expression in ZR-75 breast cancer cells through interaction of estrogen receptor α and Sp proteins. *Oncogene* **23** 1052–1063.
- Sun G, Porter W & Safe S 1998 Estrogen-induced retinoic acid receptor α 1 gene expression: role of estrogen receptor-Sp1 complex. *Molecular Endocrinology* **12** 882–890.
- Sun JM, Spencer VA, Li L, Yu CH, Yu J & Davie JR 2005 Estrogen regulation of trefoil factor 1 expression by estrogen receptor α and Sp proteins. *Experimental Cell Research* **302** 96–107.
- Tanaka N, Yonekura H, Yamagishi S, Fujimori H, Yamamoto Y & Yamamoto H 2000 The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor- α through nuclear factor- κ B, and by 17 β -estradiol through Sp-1 in human vascular endothelial cells. *Journal of Biological Chemistry* **275** 25781–25790.
- Turner RT, Riggs BL & Spelsberg TC 1994 Skeletal effects of estrogen. *Endocrine Reviews* **15** 275–300.
- Wang W, Dong L, Saville B & Safe S 1999 Transcriptional activation of E₂F1 gene expression by 17 β -estradiol in MCF-7 cells is regulated by NF-Y–Sp1/estrogen receptor interactions. *Molecular Endocrinology* **13** 1373–1387.
- Wang F, Samudio I & Safe S 2002 Transcriptional activation of rat creatine kinase B by 17 β -estradiol in MCF-7 cells involves an estrogen responsive element and GC-rich sites. *Journal of Cellular Biochemistry* **84** 156–172.
- Watson CS, Campbell CH & Gametchu B 2002 The dynamic and elusive membrane estrogen receptor- α . *Steroids* **67** 429–437.
- Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M & Gustafsson JA 2000 Estrogen receptor (ER) β , a modulator of ER α in the uterus. *PNAS* **97** 5936–5941.
- Xie W, Duan R & Safe S 1999 Estrogen induces adenosine deaminase gene expression in MCF-7 human breast cancer cells: role of estrogen receptor–Sp1 interactions. *Endocrinology* **140** 219–227.
- Xie W, Duan R, Chen I, Samudio I & Safe S 2000 Transcriptional activation of thymidylate synthase by 17 β -estradiol in MCF-7 human breast cancer cells. *Endocrinology* **141** 2439–2449.
- Zhao YL, Han WD, Li Q, Mu YM, Lu XC, Yu L, Song HJ, Li X, Lu JM & Pan CY 2005 Mechanism of transcriptional regulation of *LRP16* gene expression by 17- β estradiol in MCF-7 human breast cancer cells. *Journal of Molecular Endocrinology* **34** 77–89.

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