Down-regulation of PROS1 Gene Expression by 17\(\beta\)-Estradiol via Estrogen Receptor \(\alpha\) (ER\(\alpha\))-Sp1 Interaction Recruiting Receptor-interacting Protein 140 and the Corepressor-HDAC3 Complex

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Pregnant women show a low level of protein S (PS) in plasma, which is known to be a risk for deep venous thrombosis. 17\(\beta\)-Estradiol (E\(_2\)), an estrogen that increases in concentration in the late stages of pregnancy, regulates the expression of various genes via the estrogen receptor (ER). Here, we investigated the molecular mechanisms behind the reduction in PS levels caused by E\(_2\) in HepG2-ER\(\alpha\) cells, which stably express ER\(\alpha\), and also the genomic ER signaling pathway, which modulates the ligandindependent repression of the PS\(\alpha\) gene (PROS1). We observed that E\(_2\) repressed the production of mRNA and antigen of PS. A luciferase reporter assay revealed that E\(_2\) down-regulated PROS1 promoter activity and that this E\(_2\)-dependent repression disappeared upon the deletion or mutation of two adjacent GC-rich motifs in the promoter. An electrophoretic mobility shift assay and DNA pulldown assay revealed that the GC-rich motifs are associated with Sp1, Sp3, and ER\(\alpha\). The major source of circulating plasma PS is the hepatocyte and liver disease (8). Furthermore, acquired PS deficiency has been reported in individuals with high levels of estrogen during pregnancy and in those taking oral contraception (9–11). The major source of circulating plasma PS is the hepatocyte (12), but PS is also produced constitutively at low levels by a variety of other cell types throughout the body (13–17). PS circulates in human plasma at a concentration of 0.35 \(\mu\)M in a free form (40%) and a C4b-binding protein-bound form (60%) (18, 19). Two copies of the PS gene located on chromosome 3, the active PS\(\alpha\) gene (PROS1) and the inactive PS\(\beta\) pseudogene (PROS2), share 96% homology in their coding sequences (20–22). The promoter and first exon are absent from the PROS2 gene, and the promoter region of PROS1 has been poorly investigated in contrast to the coding regions. It has been reported that transcription from the PROS1 promoter is directed from multiple start sites and that the PROS1 5′-flanking region lacks the characteristic “CAAT” and “TATA” boxes (23).

Estrogens are important regulators of mammalian growth and metabolism, accomplishing these functions by controlling the expression of specific genes via estrogen receptors (24). The estrogen receptor (ER) is a member of the steroid/nuclear receptor superfamily of transcription factors and is required for the mediation of 17\(\beta\)-estradiol (E\(_2\))-induced responses in multiple tissues and organs (25). The classical ER mechanism of ligand binding to the receptor leads to several downstream events, including the recruitment of coactivators, histone acetyltransferases, and cell-specific transcription factors and corepressors, such as RIP140 and the NCoR-SMRT-HDAC3 complex to the PROS1 promoter, which hypoacetylated chromatin. Taken together, this suggested that E\(_2\) might repress PROS1 transcription depending upon ER\(\alpha\)-Sp1 recruiting transcriptional repressors in HepG2-ER\(\alpha\) cells and, consequently, that high levels of E\(_2\) leading to reduced levels of plasma PS would be a risk for deep venous thrombosis in pregnant women.

Protein S (PS)\(^2\) is a vitamin K-dependent plasma protein that functions as a nonenzymatic cofactor for activated protein C in the down-regulation of the blood coagulation cascade via pro-

eolytic inactivation of coagulant factors Va and VIIIa (1). PS has been also shown to display activated protein C-independent anticoagulant activity in purified systems as well as in plasma (2, 3).

Over the past 2 decades, low levels of plasma PS have become a well established risk factor for the development of deep venous thrombosis (4–6). Hereditary PS deficiency has been shown to be an autosomal dominant trait, and many causative genetic mutations have been described in the PS\(\alpha\) gene (PROS1) (7). However, PS deficiency can also occur throughout life under acquired conditions such as oral anticoagulant use and liver disease (8). Furthermore, acquired PS deficiency has been reported in individuals with high levels of estrogen during pregnancy and in those taking oral contraception (9–11).

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2 The abbreviations used are: PS, protein S; ChIP, chromatin immunoprecipitation; CS–FBS, charcoal-stripped FBS; DME, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility shift assay; E\(_2\)-estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; FBS, fetal bovine serum; HDAC, histone deacetylase; LCoR, ligand-dependent corepressor; NCoR, nuclear receptor corepressor; re-IP, re-immunoprecipitation; RIP140, receptor-interacting protein 140; RT, reverse transcription; siRNA, small interfering RNA; iNS, nonspecific siRNA; SMRT, silencing mediator of retinoid and thyroid hormone receptors; Sp, specificity protein; TSA, trichostatin A; WT, wild type.
action involves ligand-induced formation of an ER homodimer that interacts with estrogen-responsive elements (EREs) in target gene promoters and recruits cofactors necessary for transactivation (25). There is increasing evidence that the formation of a classical genomic ER-ERE complex is only one of several genomic and non-genomic pathways of estrogen actions (26–28). Genomic ER associates with other transcription factors such as the activating protein-1 (AP-1) complex, nuclear factor κB (NFκB), and specificity proteins (Sp) to modulate ligand-dependent gene expression (26, 27, 29). In this study, we investigated the molecular mechanisms of the reduction in PS production caused by E2, as well as the genomic ER signaling pathway that modulates ligand-dependent PROS1 gene repression in HepG2-ERα cells stably expressing human ERα.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human hepatoma cell line HepG2 and human breast cancer cell line MCF7 were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako, Tokyo) supplemented with 5% or 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) and 100× antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan). Human normal hepatocytes (hNhep) were purchased from Lonza (Walkersville, MD) and cultured in collagen I-coated dishes using the HCM™ BulletKit according to the manufacturer’s protocols. For estrogen assays, cells were cultured in phenol red-free DMEM (Invitrogen) supplemented with 10% charcoal-stripped FBS (CS-FBS) for 3 days. Cells were cultured in phenol red-free DMEM supplemented with 1% CS-FBS and treated with ethanol (vehicle) or 100 nM E2 for 48 h. E2 and trichostatin A (TSA) were supplemented with 1% CS-FBS and treated with ethanol (vehicle) for 48 h, and the culture medium was harvested. A rabbit IgG antibody against PS (2.4 ng/100 μl) in bicarbonate buffer (15 mm Na2CO3, 35 mm NaHCO3, and 3 mm NaNO3) was coated onto each well of a microtiter plate (Nunc, Roskilde, Denmark). After three washes with 150 μl of Tris-buffered saline (TBS; 50 mm Tris, pH 7.4, 150 mm NaCl), the wells were blocked with 1% bovine serum albumin in TBS and then incubated with the plasma standards or culture medium samples. After three more washes with TBS, the biotinylated anti-PS polyclonal antibody (0.1 μg/100 μl) was added followed by dilution (1:1000) streptavidin-horseradish peroxidase conjugate (Amersham Biosciences). After incubation, the substrate buffer (0.65 mg/ml o-phenylenediamine (Wako) and 0.06% H2O2 in 0.1 M citrate, 0.2 M sodium phosphate buffer, pH 5.0) was added to each well. After further incubation at room temperature for 20 min, the peroxidase reaction was stopped by the addition of 50 μl of 2 M H2SO4, and absorbance was measured at 490 nm.

Quantitative RT-PCR for Measurement of PS mRNA—Total RNA of the cells was extracted using an RNeasy mini kit (Qiagen, GmbH, Germany). The first-strand cDNA was prepared with 5 μg of total RNA using the SuperScript III first strand system (Invitrogen). Quantitative RT-PCR was performed with a Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and ABI PRISM 7000 sequence detection systems (Applied Biosystems) were used for measurement. Relative PS mRNA was calculated as the respective PS
mRNA/GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA as described previously (33).

**Western Blot Analysis**—Proteins were extracted from the cultured cells by harvesting in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 850 mM 2-mercaptoethanol, 5% glycerol, and 0.001% bromphenol blue). Samples were resolved by 10% SDS-PAGE and transferred to Immonilon-P membranes (Milipore, Bedford, MA). Membranes were blocked with excess protein (2% skim milk) and probed with primary antibody (1:1000) against ER protein (2% skim milk). Membranes were blocked with excess protein (2% skim milk) and probed with primary antibody (1:1000) against ERα, Sp1, Sp3 (Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (Cytoskeleton Inc., Denver, CO). After being washed with phosphate-buffered saline containing 0.05% Tween 20, membranes were probed with a horseradish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling Technology, Danvers, MA) for 1 h. Signals were visualized with a chemiluminescent substrate (ECL Plus Western blotting detection system, Amersham Biosciences).

**Luciferase Reporter Assay**—Cells were seeded in 35-mm dishes at a concentration of 1.0 × 10^5 cells in phenol red-free DMEM supplemented with 10% CS-FBS. After 18 h, the appropriate PROS1 luciferase reporter plasmids (3 μg) and pSV-β-galactosidase plasmid (0.2 μg; Promega, Madison, WI) were transiently co-transfected using Lipofectin® reagent (Invitrogen) according to the manufacturer’s protocol.

After a 6-h transfection, the cells were washed and treated for 48 h with fresh phenol red-free DMEM supplemented with 1% CS-FBS containing 100 mM E2, 100 mM E2/1 mM ICI 182,780, 1 mM ICI 182,780 only dissolved in ethanol, or ethanol alone as a vehicle control. The cells were harvested, and subsequently luciferase activity was determined with a luciferase assay system (Promega) according to the manufacturer’s directions. Luciferase activity was normalized to the activity of co-transfected β-galactosidase as an internal control for transfection efficiency.

**Transient Transfection of siRNA**—HepG2-ERα cells were cultured in phenol red-free DMEM with 10% CS-FBS and transfected with siRNA against Sp1 or Sp3 (Ambion, Austin, TX) or nonspecific siRNA using Oligofectamine reagent (Invitrogen) according to the manufacturer’s directions.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts were prepared from HepG2-ERα cells using NE-PER® nuclear and cytoplasmic extraction reagents (Pierce) and stored in aliquots at −80 °C until further use. The protein concentration of the nuclear extracts was measured with the Bio-Rad protein assay kit (Bio-Rad). DNA probes containing the PROS1 promoter fragment (from −176 to −147) were synthesized, biotinylated, and annealed. EMSA was performed according to a method described previously (32). Briefly, nuclear extract (5 μg) and a biotin-labeled double-stranded DNA probe (600 fmol), with or without an unlabeled competitor, were treated with a LightShift™ chemiluminescent EMSA kit (Pierce) according to the manufacturer’s instructions. In supershift experiments, the nuclear extract was incubated on ice for 10 min with the biotin-labeled double-stranded DNA probe after which an anti-Sp1, anti-Sp3, or anti-ERα antibody was added, and the incubation was continued for another 20 min. Samples were loaded on a 6% nondenaturing polyacrylamide gel in 0.5× TBE buffer (0.089 M Tris borate, pH 8.0, 0.089 M boric acid, and 10 mM EDTA) and electrophoresed for 3.5 h at 100 V. Biotin-labeled DNA probes were transferred to Hybond™N+ membranes (Amersham Biosciences) and then integrated with streptavidin-horseradish peroxidase conjugate.

**DNA Pulldown Assay**—The DNA pulldown assay (DNA affinity precipitation; DNAP assay) was carried out with biotin-labeled DNA probes as described previously (34). The nuclear extracts (100 μg) were prepared from HepG2-ERα cells and incubated with biotin-labeled DNA probes (100 pmol) and 15 μg of polydI-dC in DNAP buffer (20 mM HEPES-KOH, pH 7.9, 80 mM KCl, 1 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 0.1% Triton X-100) on ice for 45 min. Subsequently, 500 μg of Dynabeads® M-280 streptavidin (Invitrogen) was added and incubated further at 4 °C for 1 h. The beads were washed three times with DNAP buffer, and the bound proteins were eluted in SDS sample buffer, separated by 10% SDS-PAGE, and characterized by Western blot analysis with the respective specific antibodies.

**Chromatin Immunoprecipitation (ChIP) and ChIP Reimmunoprecipitation (Re-IP) Assays**—HepG2-ERα cells were treated with E$_2$ or vehicle and fixed with 2% formaldehyde. The cross-linking reaction was stopped by the addition of 0.125 M glycine. After two washes with phosphate-buffered saline, the cells were resuspended in a swelling buffer (10 mM Tris-HCl, pH 7.6, 3 mM CaCl$_2$, 0.1% Nonidet P-40, and 1× protease inhibitor mixture (Nakalai Tesque)). After incubation on ice for 10 min, the samples were mixed by vortex, and the nuclei were collected. Isolated nuclei were resuspended in SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA, and 1× protease inhibitor mixture) and sonicated to the desired chromatin length (0.5 kb). After centrifugation, the supernatant was diluted with dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, and 1.1% Triton X-100) containing 1× protease inhibitor mixture, divided into aliquots, and precleared by the addition of protein A-agarose or protein G PLUS-agarose (Santa Cruz Biotechnology). In the ChIP assay, the precleared chromatin supernatants were immunoprecipitated with the respective antibodies specific to ERα, Sp1, Sp3, nuclear receptor corepressor (NCoR), silencing mediator of retinoid and thyroid hormone receptors (SMRT), histone deacetylase 1 (HDAC1), HDAC3, HDAC4, HDAC5, receptor-interacting protein 140 (RIP140), ligand-dependent corepressor (LCoR) (Santa Cruz Biotechnology), and ACh4 (Millipore) or nonspecific IgG at 4 °C overnight. The protein-antibody complexes were incubated with protein A-agarose or protein G PLUS-agarose at 4 °C overnight. The beads were washed extensively in the following buffers: low salt wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and 1× protease inhibitor mixture); high salt wash buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA, and 1× protease inhibitor mixture) and stored in aliquots at −80 °C until further use. The protein concentration of the nuclear extracts was measured with the Bio-Rad protein assay kit (Bio-Rad). DNA probes containing the PROS1 promoter fragment (from −176 to −147) were synthesized, biotinylated, and annealed. EMSA was performed according to a method described previously (32). Briefly, nuclear extract (5 μg) and a biotin-labeled double-stranded DNA probe (600 fmol), with or without an unlabeled competitor, were treated with a LightShift™ chemiluminescent EMSA kit (Pierce) according to the manufacturer’s instructions. In supershift experiments, the nuclear extract was incubated on ice for 10 min with the biotin-labeled double-stranded DNA probe after which an anti-Sp1, anti-Sp3, or anti-ERα antibody was added, and the incubation was continued for another 20 min. Samples were loaded on a 6% nondenaturing polyacrylamide gel in 0.5× TBE buffer (0.089 M Tris borate, pH 8.0, 0.089 M boric acid, and 10 mM EDTA) and electrophoresed for 3.5 h at 100 V. Biotin-labeled DNA probes were transferred to Hybond™N+ membranes (Amersham Biosciences) and then integrated with streptavidin-horseradish peroxidase conjugate.
Down-regulation of PROS1 by 17β-Estradiol

GCTCCGAAAAGCTTCTGGAA-3′ (−236/−217, forward) and 5′-CGCCCTCGTCTGAGCCGT-3′ (−88/−105, reverse), which amplified a 149-bp region of the PROS1 promoter containing target GC-rich motifs. The primers amplifying a 159-bp region of the PROS1 promoter that contained no GC-rich motif were 5′-GGAGAATGAGGGGCAAGA-3′ (−4033/−4016, forward) and 5′-CATTTCATCACCTTAGCCT-3′ (−3875/−3896, reverse), and those amplifying a 175-bp region of the PROS1 promoter containing a non-target GC-rich motif were 5′-AGGAGAGCGGACAGGATTAA-3′ (−3748/−3729, forward) and 5′-GGACAGAAGGCCCAATC-3′ (−3574/−3598, reverse). PCR products were resolved on a 2% agarose gel in the presence of 1 μg/ml ethidium bromide.

In the ChiP re-IP assay, the pre-cleared chromatin supernatants were immunoprecipitated with the first antibody, anti-ERα or anti-RIP140, at 4 °C for overnight. The protein-antibody complexes were incubated with protein G PLUS-agarose at 4 °C for 2–4 h, eluted by incubation with 10 mM dithiothreitol at 37 °C for 30 min, and diluted 1:50 in dilution buffer. After centrifugation, the supernatants were divided in aliquots and reimmunoprecipitated with their respective second antibodies individually. The second immunocomplexes were extracted from the beads followed by PCR amplifications of a 149-bp region of the PROS1 promoter containing target GC-rich motifs from bound DNA as described above.

Statistical Analysis—Data are presented as the mean ± S.D. and are representative of at least three independent experiments. Significant differences between experimental groups in the quantitative RT-PCR, enzyme-linked immunosorbent assay, and luciferase assay were analyzed using Student’s t test. Differences were considered to be significant when p was less than 0.05.

RESULTS

Down-regulation of PROS1 Expression by E2 in HepG2-ERα Cells and Human Normal Hepatocytes—Because ERα was undetectable in the original HepG2 cells by Western blotting (Fig. 1A), we established a HepG2-derived cell line stably expressing human ERα (HepG2-ERα) as described under “Experimental Procedures.” We confirmed that the HepG2-ERα cells expressed large amounts of ERα protein equivalent to breast cancer-derived MCF7 cells as determined by Western blot analysis (Fig. 1A). The HepG2-ERα cells treated with E2 showed significantly decreased levels of PS mRNA, but the original HepG2 cells and HepG2-Mock cells did not (Fig. 1B). Also, E2 treatment down-regulated PS antigen significantly in the HepG2-ERα cells (Fig. 1C). In addition, we also demonstrated that E2 treatment down-regulated PS mRNA by 60% in hNHep, which expressed a high level of ERα protein (Fig. 1, D and E).

Luciferase Reporter Assay—We next examined PROS1 promoter activity by conducting a luciferase reporter assay and
observed that E₂ decreased the luciferase activity of pPROS1/−4229 in HepG2-ERα cells (Fig. 2A). In a series of 5′-truncated constructs obtained by restriction enzyme digestion and self-ligation of pPROS1/−4229, we also observed E₂-dependent repression of the luciferase activity, although the basal activity was gradually reduced (Fig. 2A). Meanwhile, a computer search for putative nuclear factor binding sites between −338 and −236 revealed AP-1 and GC-rich sites at −281 and −244 that might play a role in the basal expression of PROS1 in HepG2-ERα cells, respectively.

In a luciferase assay of further truncated forms, pPROS1/−175 showed E₂-dependent repression, but pPROS1/−137 did not, indicating that the −175 to −137 region of the PROS1 promoter, containing two adjacent GC-rich motifs at −172 to −163 and −162 to −153, was critical for E₂-induced down-regulation (Fig. 2B). To clarify the importance of those GC-rich motifs, we transfected a series of constructs containing mutations of a single GC-rich site (pPROS1/−175Mut1 or pPROS1/−175Mut2) or of both sites (pPROS1/−175Mut3). We observed that the luciferase reporter activity of the HepG2-ERα cells transfected with pPROS1/−175Mut2 was reduced by E₂ treatment, but that of the cells transfected with pPROS1/−175Mut1 or pPROS1/−175Mut3 was not (Fig. 2C).

To further investigate the importance of the two GC-rich motifs in a full-length promoter, we prepared a luciferase reporter vector with two mutated GC-rich motifs (pPROS1/−4229Mut) derived from pPROS1/−4229. In comparison with pPROS1/−4229, pPROS1/−4229Mut showed a reduced luciferase activity and loss of its E₂-dependent repression in HepG2-ERα cells (Fig. 2D). In the original HepG2 cells, which lack ERα expression, pPROS1/−4229 also did not show apparent E₂-dependent repression of the luciferase activity.

Requirement of ERα for E₂-dependent Down-regulation of PROS1 in HepG2 Cells—We also examined the ERα requirement for E₂-induced inhibition of PROS1 promoter activity in HepG2 cells transfected with pPROS1/−175. The HepG2-ERα cells stably expressing human ERα showed E₂-induced repression of luciferase activity, whereas the HepG2 mock-transfected cells with no ERα expression did not (Fig. 3A). Consistent with these observations, ICI 182,780 (a 

![FIGURE 2. Transient expression of PROS1 promoter-reporter gene constructs in HepG2-ERα cells with or without E₂ treatment.](image-url)
pure antagonist of ER) reversed the effects of E₂ on luciferase activity in HepG2-ERα cells transfected with pPROS1/-175, whereas ICI 182,780 alone had no effect (Fig. 3B).

Sp1 and Sp3 Bind to GC-rich Motifs of the PROS1 Promoter in Vitro—To identify the transcription factors binding to the GC-rich motifs at −173 and −162 of the PROS1 promoter in HepG2-ERα cells treated with E₂, EMSAs and DNA pulldown assays were carried out. The oligonucleotides used in the EMSAs and DNA pulldown assays are shown in Fig. 4A. In the EMSAs, three shifted bands (a–c) were detected using wild-type probes (GC-WT), two (a and b) of which were not detected using mutated oligonucleotides (GC-Mut) that destroyed both GC-rich motifs (Fig. 4B). Furthermore, co-incubation with a 100-fold excess of nonlabeled GC-WT oligonucleotides reduced the intensity of each of these two bands, but the third shifted band (c) was still present. These results indicate that the first two bands (a and b) were specific shifted bands, and the third (c) was nonspecific. In the supershift experiment, co-incubation with antibody against either Sp1 (a) or Sp3 (b) gave reduced shifted bands and additional supershifted bands (SS). Antibodies against ERα and nonspecific IgG, however, did not affect the intensity of the shifted bands. DNA pulldown assays gave results consistent with the EMSAs, indicating that Sp1 and Sp3 were apparently co-purified with the wild-type oligonucleotides but not with the mutated oligonucleotides (Fig. 4C). The Sp proteins were detected in the nuclear extracts regardless of E₂ treatment. In contrast, we detected more ERα signals in the nuclear extracts from E₂-treated cells than in those from untreated cells, probably because of the increase in ERα proteins in the nucleus due to E₂ stimulation.

Knockdown Experiments with siRNA for Sp Proteins—To verify the function of Sp1 and Sp3 in the

FIGURE 3. E₂-dependent PROS1 down-regulation mediated by ERα. A, luciferase (LUC) activity levels of cells transfected with pPROS1/-175, with or without E₂ treatment, expressed relative to that of the pGL3 Basic-derived empty vector without E₂ treatment. B, HepG2-ERα cells transfected with pPROS1/-175 and treated with 100 nM E₂, 10 µM ICI 182,780, or both. After 48 h, luciferase activities were measured, and results were expressed as relative values to that of the vehicle-treated cells. Values are the mean ± S.D. for at least three independent experiments. *, p < 0.05 versus vehicle control.

FIGURE 4. Interaction of Sp and ERα proteins with the PROS1 promoter in vitro and importance of the GC-rich motifs. A, oligonucleotides used in EMSA and DNA pulldown analyses. GC-Mut contains mutations in both the distal and proximal GC-rich motifs. B, EMSA was performed with nuclear extracts from HepG2-ERα cells in the presence of the biotin-labeled DNA oligonucleotides GC-WT or GC-Mut. The biotin-labeled oligonucleotides were incubated alone or in combination with 5 μg of nuclear extract (N.E.) from E₂-treated HepG2-ERα cells in the presence of a 100-fold molar excess of specific unlabeled oligonucleotide (100X) or antibodies. Arrows indicate retarded (a, b, and c) and supershifted (SS) complexes. FP, free probe. C, DNA pulldown assays carried out by incubating biotin-labeled oligonucleotides containing WT or mutated GC-rich motifs with nuclear extract from HepG2-ERα cells with or without E₂ treatment. Specifically bound proteins were eluted and subjected to Western blotting using specific antibodies against Sp1, Sp3, and ERα, respectively. Similar results were obtained in multiple independent experiments. Veh, vehicle.
repression of PROS1 by E2, we carried out RNA interference experiments. A Western blot analysis of whole lysate from HepG2-ERα cells transfected with siNS, iSp1, or iSp3, and whole cell lysate was analyzed by Western blotting as described under “Experimental Procedures.” The experiments were repeated at least three times, and similar results were obtained. β-Actin was used as a loading control (Cont.). B, RNA interference-luciferase reporter analysis of HepG2-ERα cells. siRNAs (50 nM) were transfected, and the next day pPROS1/H11002 was transfected with or without E2 treatment. Luciferase activity was expressed relative to that of the cells without E2 treatment. C, quantitative RT-PCR analysis after siRNA transfection in HepG2-ERα cells. Cells were transfected with respective siRNAs for 4 h and treated with E2 for 48 h. Values are the mean ± S.D. for at least three independent experiments. *, p < 0.05 versus vehicle control.

**FIGURE 5. Knockdown of Sp1 or Sp3 by RNA interference and its effects on E2-dependent PROS1 repression.** A, knockdown of Sp was determined by Western blotting. HepG2-ERα cells were transfected with iNS, iSp1, or iSp3, and whole cell lysate was analyzed by Western blotting as described under “Experimental Procedures.” The experiments were repeated at least three times, and similar results were obtained. β-Actin was used as a loading control (Cont.). B, RNA interference-luciferase reporter analysis of HepG2-ERα cells. siRNAs (50 nM) were transfected, and the next day pPROS1/H11002 was transfected with or without E2 treatment. Luciferase activity was expressed relative to that of the cells without E2 treatment. C, quantitative RT-PCR analysis after siRNA transfection in HepG2-ERα cells. Cells were transfected with respective siRNAs for 4 h and treated with E2 for 48 h. Values are the mean ± S.D. for at least three independent experiments. *, p < 0.05 versus vehicle control.

DISCUSSION

The action of estrogen in target cells is regulated via estrogen receptors that modulate gene expression either positively or negatively. Recent studies have clarified that E2-ERα functions as a gene modulator, although its negative effects on gene expression are less well understood than its positive effects. We investigated here the molecular mechanisms by which E2-ERα negatively regulates the gene expression of the anticoagulant PS.

First, we established a cell line (HepG2-ERα) stably expressing human ERα, because ERα was undetectable in HepG2 cells, and found that E2 treatment of HepG2-ERα cells significantly down-regulated PS expression. The luciferase assays suggested that the GC-rich motif at −172 of the PROS1 promoter containing target GC-rich motifs following E2 treatment (Fig. 6B, right). Furthermore, to confirm the E2-dependent complex formation of those repressive proteins on the PROS1 promoter, we tried ChIP re-IP assays. We used anti-ERα or anti-RIP140 for primary immunoprecipitation and antibodies against the respective nuclear factors for secondary immunoprecipitation. As expected, we observed similar results in both of the ChIP re-IP assays using anti-ERα and anti-RIP140 as the primary antibodies, respectively, which showed that ERα, RIP140, Sp1, Sp3, NCoR-SMRT, and HDAC3 were present on the same region of the PROS1 promoter containing target GC-rich motifs (Fig. 6C). Additionally, we observed that the blocking of deacetylation by an HDAC inhibitor, TSA, cancelled the E2-dependent PROS1 gene repression (Fig. 6D).

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**FIGURE 5. Knockdown of Sp1 or Sp3 by RNA interference and its effects on E2-dependent PROS1 repression.** A, knockdown of Sp was determined by Western blotting. HepG2-ERα cells were transfected with iNS, iSp1, or iSp3, and whole cell lysate was analyzed by Western blotting as described under “Experimental Procedures.” The experiments were repeated at least three times, and similar results were obtained. β-Actin was used as a loading control (Cont.). B, RNA interference-luciferase reporter analysis of HepG2-ERα cells. siRNAs (50 nM) were transfected, and the next day pPROS1/H11002 was transfected with or without E2 treatment. Luciferase activity was expressed relative to that of the cells without E2 treatment. C, quantitative RT-PCR analysis after siRNA transfection in HepG2-ERα cells. Cells were transfected with respective siRNAs for 4 h and treated with E2 for 48 h. Values are the mean ± S.D. for at least three independent experiments. *, p < 0.05 versus vehicle control.

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report that a ternary ERα–Sp-DNA complex was not detected in gel mobility shift assays and that ERα enhances the formation of the Sp1-DNA complex and increases its stability (39). We hypothesized that ERα would bind to Sp-DNA complex in vivo feebly and that it could be too fragile to be detected as a supershifted band in EMSAs or in an ERα–Sp complex in co-immunoprecipitation analyses.

Through the RNA interference analysis, we found dual roles for Sp1 at the PROS1 promoter in HepG2-ERα cells. Thus, knockdown of Sp1 or Sp3 resulted in substantially decreased transcriptional activity, indicating that both Sp1 and Sp3 take part in basal PROS1 transcription as reported previously (38, 40). Intriguingly, we observed a loss of E2-dependent PROS1 repression in the cells transfected with iSp1, suggesting that Sp1 has a crucial role in the down-regulation of PROS1 transcription by E2. This highlights the potential dual function of Sp1 for basal activation and E2-dependent repression of PROS1 transcription.

Our study also showed that ERα might interact with Sp1 and repress PROS1 expression via GC-rich motifs in its promoter region. GC-rich regions are known to be involved in ERα-mediated repression at the p21/WAF1 and cyclin G2 gene promoters, where interplay with Sp proteins seems to occur (41, 42). Moreover, direct ER-Sp binding has been well documented in estrogen-stimulated genes (43). Actually, through chromatin immunoprecipitation assays, we showed that ERα and Sp1 bound to the responsive regions of the PROS1 promoter simultaneously. Safe and

EMSAs and DNA pulldown analyses, we observed that Sp1 and Sp3 bound to the GC-rich motif of PROS1, which was consistent with the findings of de Wolf et al. (38).

We did not observe the binding of ERα or interaction of ERα–Sp1 in the EMSAs; however, DNA pulldown assays showed that ERα bound to the PROS1 promoter (−176/−147) in an E2-dependent manner. This weak ERα binding seemed to depend on transfer from the cytoplasm to the nucleus by E2 treatment. We also tried co-immunoprecipitation analyses but could not detect either the ERα–Sp1 or the ERα–Sp3 complex (data not shown). These observations were consistent with the
Down-regulation of PROS1 by 17β-Estradiol

A: E2 (-)

![Diagram](image)

B: E2 (+)

![Diagram](image)

FIGURE 7. A proposed model for down-regulation of PROS1 promoter by 17β-estradiol in HepG2-ERα cells. A, in HepG2-ERα cells without E2 treatment, basal PROS1 expression was regulated by Sp1 and Sp3, probably together with other transcriptional coactivators. B, in HepG2-ERα cells with E2 treatment, PROS1 expression was repressed by ERα-Sp1 interaction recruiting RIP140 and the NCoR-SMRT-HDAC3 corepressor complex sequentially, which consequently induced histone deacetylation.

non-classical pathway, ligand-bound ERs do not bind to ERE directly; instead, they interact with other transcription factors such as Sp and AP-1 (39). ER-Sp or ER-AP-1 interaction mediates transcriptional gene regulation, recruiting cofactors or chromatin remodeling complexes to GC-rich motifs or to the AP-1 site in the target gene promoter. In this study, we have demonstrated that ERα-Sp1-RIP140 interaction regulates PROS1 expression by recruiting the NCoR-SMRT corepressor complex and also HDAC3, which induces histone hypoacetylation and less permissive transcription of the PROS1 gene.

An in vivo chromatin immunoprecipitation analysis of the E2-responsive region in the PROS1 promoter further revealed the recruitment of NCoR and SMRT to the PROS1 modulator complex. NCoR and SMRT are now documented corepressors for nuclear receptors such as antagonist-bound estrogen receptors and progesterone receptors (44). It was reported that NCoR forms several different complexes with other transcription factors such as SMRT, Sin3a, and HDACs (44). In this study, we observed the recruitment of NCoR and SMRT corepressors to the PROS1 promoter after E2 treatment.

Liganded ERα, however, is not known to recruit NCoR-SMRT corepressors directly. Therefore, we tried ChIP assays for RIP140, which is known to interact with liganded ER and mediate ER-mediated transcription (35), and for LCoR, which is an NR-box-containing factor the same as RIP140 (36). We demonstrated that RIP140 was recruited to the target GC-rich region of the PROS1 promoter after E2 stimulation, but LCoR was not. The recruitment of RIP140 was theorized to correlate with the interaction between ERα and Sp1 because the effect of E2 was destroyed by the knockdown of Sp1 in RNA interference analysis. We also found that the recruitment of NCoR-SMRT corepressors depended on ERα-Sp1 interaction and that the complex contained HDAC3. The NCoR-SMRT-HDAC3 inter-

action has been reported by several groups (45–48), consistent with our analysis of the PROS1 promoter. The ChIP re-IP assays demonstrated that ERα-Sp1, RIP140, and NCoR-SMRT-HDAC3 were present in the same region of the PROS1 promoter. Taken together with these data, this suggests that NCoR and SMRT were indirectly recruited as components of the HDAC3 complex by RIP140 through ERα-Sp1 interaction following E2 stimulation.

Our proposed model for the down-regulation of PROS1 expression by E2 is depicted in Fig. 7. The repression of PROS1 resembles the situation described for p21/WAF1 or cyclin G2 (39, 49). In the absence of E2, basal transcriptional regulation of PROS1 seems to be mediated via multiple GC-rich regions including one at −172 to −153, as shown in this study and previously (38). In basal PROS1 transcription, Sp1 and
than the proximal GC-rich motif. A patient carrying the g.-1668c>t mutation showed decreased levels of plasma PS but supposedly did not show E2-dependent PROS1 repression (53).

In conclusion, we have demonstrated that PROS1 expression is down-regulated by 17β-estradiol via ERα. ERα interacts with Sp1 and recruits RIP140. RIP140 associates directly with the HDAC3 complex containing NCoR-SMRT corepressors and induces the deacetylation of histones in the PROS1 promoter. We have also revealed the dual roles of Sp1, which regulates PROS1 expression both positively and negatively. E2-dependent repression of PROS1 results in reduced plasma PS levels, leading to the risk of deep venous thrombosis during pregnancy and oral contraceptive use. Further study will be required to fully characterize the mechanisms of reduction in PS and other possible mechanisms of regulation, such as using other hormones during pregnancy.

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