0888-8809/07/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 21(11):2615–2626

doi: 10.1210/me.2007-0110

Progressive Loss of Estrogen Receptor α Cofactor Recruitment in Endocrine Resistance

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Differential expression of estrogen receptor-α (ERα) cofactors has been implicated in endocrine resistance in breast cancer. Using a three-stage MCF-7 cell-based model that emulates the clinical manifestation of acquired endocrine resistant breast cancer we now show, using a combination of chromatin immunoprecipitation and RNA interference, that there is a progressive loss of ERα cofactor recruitment to the estrogen-dependent pS2 gene and reduced requirement for cofactor expression. Maximal estrogen induced pS2 induction requires ERα and cofactor recruitment in MCF-7 cells, but in the progression to endocrine resistance these requirements are altered and expression has become less dependent on cofactors. Additionally, in estrogen-resistant MCF-7 cells there is a global loss of requirement of individual cofactors for proliferative cell growth indicating that other genes have lost the need for transcriptional cofactors. This loss of the requirement for cofactors may represent an important mechanism for gene misregulation in cancer. (*Molecular Endocrinology* 21: 2615–2626, 2007)

ENDOCRINE RESISTANCE IS a major problem in the treatment of breast cancer patients (1). One proposed mechanism of resistance involves differential expression of estrogen receptor-α (ERα) cofactors (2). These cofactors are either coactivators or corepressors and function to regulate ERα-mediated gene expression. Estrogen binding to ERα promotes receptor dimerization and induces occupancy of estrogen target gene promoters [typically at the site of conserved estrogen response elements] by ERα (1). Estrogen binding induces a conformational change in ERα that creates a protein interaction site on the surface of the ligand binding domain that is recognized by transcriptional coactivators (3–5). Coactivators function to recruit ATP-dependent chromatin remodelling complexes, histone acetyltransferases (HATs) and methyltransferases to specific enhancer/promoter regions (6–8). This activity serves to alleviate the repressive effect of chromatin on transcription, thus facilitating recruitment of the general transcription machinery (RNA polymerase II) and initiation of target gene transcription (6, 7). The ERα transcription complex formed is a dynamic structure with components recruited in a sequential order and has been shown to cycle repeatedly on and off target promoters in the presence of estrogen (9, 10). In contrast, ERα antagonists such as tamoxifen induce an alternate conformation in ERα that occludes the co-activator binding site and recruits co-repressors, which via recruitment of histone deacetylases (HDACs), silence gene transcription (5).

The p160 family of steroid receptor coactivators comprises three homologous members: SRC-1 (steroid receptor coactivator-1/NCOA-1), SRC-2 (TIF-2/GRIP-1/NCOA-2), and AIB1 (SRC-3/ACTR/pCIP/RAC-3/TRAM-1/NCOA-3), all of which have been implicated in ERα-mediated transcription and two of which (SRC-1 and AIB1) contain HAT activities (8). Specifically, overexpression and knockout studies have defined a role for p160s in the enhancement of ERα ligand-dependent transactivation (5, 11). More recently alterations in expression of one of these coactivators, AIB1, has been implicated in breast cancer initiation and, furthermore, in endocrine resistance. Overexpression of AIB1 in transgenic mice leads to the development of mammary carcinomas (12), and AIB1 gene amplification is detected in 5–10% of breast cancers (13), whereas a further study reported AIB1 overexpression in 64% of breast tumors (14). With respect to endocrine resistance, a recent study of tamoxifen-treated patients has shown that high AIB1 expression associates with a poorer disease-free survival in these patients, suggesting that high expression of ERα coactivators can reduce the antagonist activity of tamoxifen (15).

ERα-associated corepressors include the nuclear receptor corepressor (NCoR) and silencing mediator for

First Published Online July 31, 2007

Abbreviations: ChIP, Chromatin immunoprecipitation; DCC, double charcoal stripped fetal calf serum; E_2, estradiol; ERα, estrogen receptor-α; HAT, histone acetyltransferase; HDAC, histone deacetylase; LCC, Lombardi Cancer Centre; NCoR, nuclear receptor corepressor; RNAi, RNA interference; siRNA, short interfering RNA; SDS, sodium dodecyl sulfate; SMRT, silencing mediator for retinoic and thyroid hormone receptor.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

retinoic and thyroid hormone receptors (SMRT) (16, 17). In the absence of ligand, these corepressors are associated with ERα to mediate transcriptional repression (18). In contrast to coactivators, a reduction in corepressor levels during tamoxifen treatment may be associated with resistance (19). Our understanding of how these coactivators and corepressors function at the ERα transcription complex comes from studies examining the promoter of estrogen-regulated genes, *e.g.* pS2 and Cathepsin D (9, 10, 20). pS2 [trefoil factor 1 (TFF1)] is the most frequently studied estrogen-responsive gene, with mRNA levels induced massively (up to 100-fold) by estrogen and only weakly by tamoxifen (21).

In this study we have investigated cofactor involvement in ERα-regulated transcription and growth using an *in vitro* model of endocrine resistance. We used the parental estrogen-dependent ERα+ MCF-7 breast cancer cell line and acquired resistance was examined using the MCF-7-derived Lombardi Cancer Centre (LCC) sublines MCF-7/LCC1 and MCF-7/LCC9. MCF-7/LCC1 (LCC1) cells were derived *in vivo* through selection under low estrogen conditions. These cells have become estrogen independent but remain responsive to antiestrogens (22). MCF7/LCC9 (LCC9) cells were established through stepwise *in vitro* selection of LCC1 cells against ICI 182, 780 and, in addition to acquiring ICI 182, 780 resistance, are cross resistant to tamoxifen (23). LCC1 and LCC9 cells retain functional ERα and remain dependent on ERα for maximal growth (24). However, our data suggest that in the acquisition of endocrine resistance in the low estrogen environment, growth becomes estrogen independent, involving increased ERα expression and estrogen independent gene transcription (22, 24). Using chromatin immunoprecipitation (ChIP) and RNA interference (RNAi) methodologies, we now show that there is a progressive loss of cofactor requirement in endocrine resistance for the expression of pS2, an estrogen-dependent gene. In addition, in endocrine resistant cells, there is a global loss of requirement of individual cofactors for proliferative cell growth indicating that many other proliferative genes have also lost their requirement for transcriptional cofactors.

RESULTS

ERα-Dependent Gene Expression Is Altered in Endocrine Resistance

To investigate the molecular effects of estrogen resistance in breast cancer we have used the MCF-7 breast cancer cell line and estrogen-resistant derivative MCF-7 cell lines (22, 23). The pS2 gene is estrogen dependent and shows cyclical recruitment of factors for its expression (10). In MCF-7 cells pS2 gene expression is estradiol (E_2) dependent with minimal expression in the absence of E_2 (Fig. 1). In contrast, pS2 gene expression has become E_2 independent in LCC1 and LCC9 cells, with significant levels of expression observed in the absence of E_2 treatment (MCF-7 pS2 expression at 0 min *vs.* LCC1 0 min $P = 0.016$ and LCC9 0 min $P = 0.0002$; Student's *t* test) making this gene a good model for investigating the molecular changes associated with endocrine resistance. This result supports similar previously published data from our group (24). However there is a significant induction of pS2 gene expression after 24-h E_2 treatment, showing the pS2 promoter is E_2 responsive in all cell lines (pS2 expression at 0 min *vs.* 24-h E_2 treatment: MCF-7 and LCC9 $P < 0.001$, LCC1 $P < 0.01$; Student's *t* test).

ERα-Independent pS2 Gene Expression in Endocrine Resistance

In a parallel study we have shown that pS2 gene expression has become ERα independent in the endocrine resistant LCC9 cell line (24). These experiments exploited Fulvestrant as an ERα down-regulator and demonstrated reduction of ERα expression in all three cell lines (MCF-7, LCC1, and LCC9). However, unlike MCF-7 and LCC1 cell lines, loss of ERα did not affect pS2 mRNA expression in LCC9 cells.

To examine how the mechanisms of pS2 gene transcription have changed in endocrine resistance, we used ChIP to investigate chromatin marks and cellular factors on the pS2 promoter. To determine whether the enhanced basal pS2 expression of LCC1 and LCC9 cells was regulated by ERα binding, ChIP analysis was used to examine ERα-ERE association at the pS2 promoter in each cell line. In MCF-7 cells, ERα binding to the pS2 promoter is minimal under basal conditions (Fig. 2A), but even in the absence of estrogen it was considerably higher in LCC1 cells (~8-fold). Importantly, in LCC9 cells there was little ERα, but a high level of pS2 expression, suggesting that in these very endocrine-resistant cells, there is no requirement of ERα for cofactor binding. These data are in agreement with our previously published observation, but novel associations with histone acetylation, cofactor binding, and gene expression are reported below (24).

E_2-Independent Gene Expression Associated with Altered Cofactor Binding to the pS2 Promoter

Histone acetylation is correlated to gene expression, and in MCF-7 cells histone H4 acetylation levels are low (Fig. 2A). Uninduced LCC1 cells have an increased level of histone acetylation (~2-fold) that is associated with maximal E_2-induced pS2 gene expression observed in MCF-7 cells. Uninduced LCC9 cells display markedly enhanced H4 acetylation (>6-fold above MCF-7 levels) reflecting high basal expression within these cells. The considerable differences in histone acetylation on the pS2 promoter between the endocrine-resistant MCF-7 cells is probably due to a further misregulation of HATs or HDACs in LCC9 cells compared with LCC1 cells.

ERα cofactors are recruited to the ERα-transcription complex after ligand binding (9, 10). Many ERα cofactors have HAT or HDAC activity (5, 8). By Western analysis we examined the global expression levels of

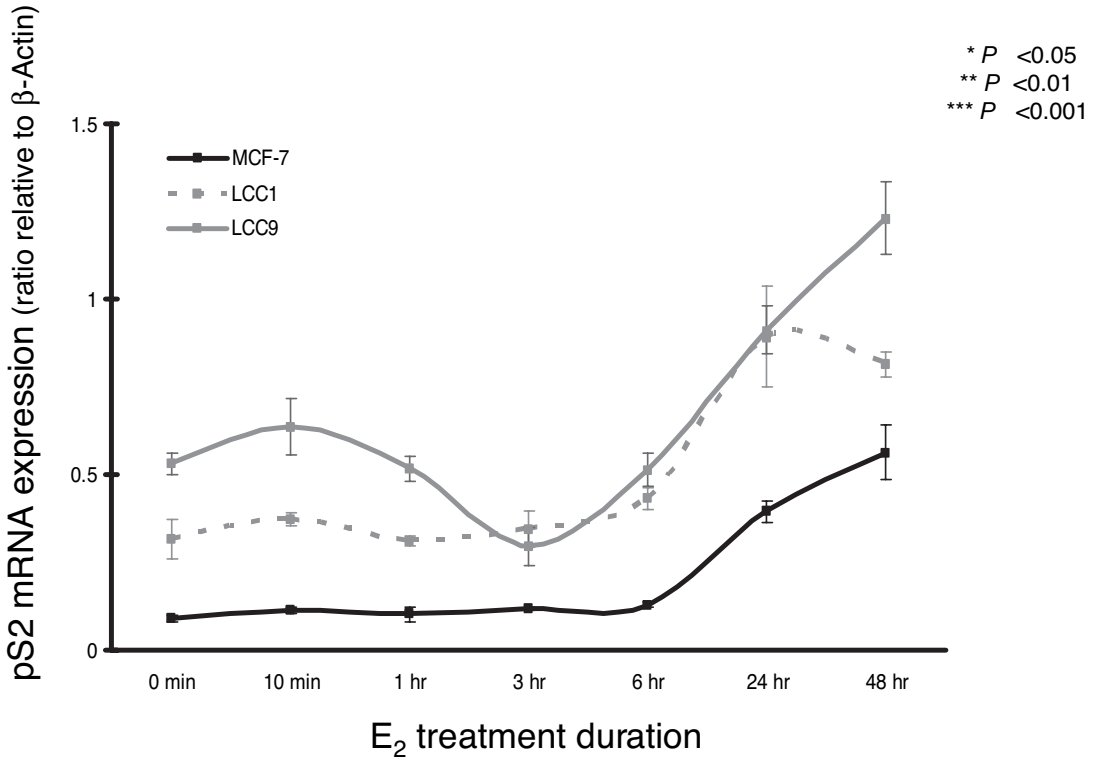

Fig. 1. Effects of Estrogen on pS2 mRNA Expression

Relative pS2 mRNA expression levels within each cell line were determined by RT-PCR after E_2 (10^{-9} M) treatment over a 48-h time course. Data are presented as means ± SE of actin-corrected values from quadruplicate samples.

the p160 coactivators SRC-1, SRC-2, and AIB1 and the corepressors NCoR and SMRT in the MCF-7 cell lines (Fig. 2B). We observed that progressive endocrine resistance is accompanied by alterations in cofactor levels such that expression of all cofactors examined decreased in the LCC9 cells.

Using ChIP we assessed whether the enhanced basal gene expression and increased histone acetylation in LCC1 and LCC9 cells is due to alterations in cofactor binding to the pS2 promoter. We found that E_2-independent pS2 gene activation in LCC1 cells might be due to increased HAT activity via enhanced AIB1 binding, whereas in LCC9 cells the increased pS2 gene activation is unlikely to be caused by a high level of HAT activity because both SRC-1 and AIB1 levels at the pS2 promoter are no higher than those observed in MCF-7 cells (Fig. 2C). However, ChIP analysis of H4 acetylation on the pS2 promoter after AIB1 short interfering RNA (siRNA) disproved this hypothesis (data not shown). Similarly enhanced pS2 gene activation could also be due to a loss of HDAC activity on the promoter as the level of SMRT associated with the pS2 promoter appears reduced in both LCC1 and LCC9 cells (Fig. 2D). In an effort to determine the importance of the individual cofactors for basal pS2 expression, an RNAi approach was used (Fig. 3). These data confirm that basal pS2 expression is enhanced after SMRT depletion (Fig. 3D).

Endocrine Resistance Is Not Associated with Altered ERα Recruitment

We have previously reported enhanced ERα binding at the pS2 promoter may be associated with increased basal ERα protein expression (Fig. 2A) (24). To further explore ERα involvement in endocrine resistance, ChIP analysis was used to explore E_2-induced ERα recruitment in all cell lines over a 90-min period. E_2 treatment strongly induced cyclical ERα recruitment to the pS2 promoter in MCF-7 cells (Fig. 4A). A similar response was observed for LCC1 and LCC9 cells. As a control we confirmed that ERα recruitment was not observed at a distal pS2 region (Fig. 4B).

Significant levels of E_2-induced ERα recruitment to the pS2 promoter were observed by 15 min in all cell lines, with maximal levels observed at 50 min. ERα recruitment was significantly greater in MCF-7 cells than in their endocrine-resistant derivatives, suggesting that although the ERα protein is intact and that mechanisms of ERα recruitment are not altered in hormone resistance, dependency on ERα as a cofactor for pS2 expression is reduced. Although E_2-induced ERα recruitment is associated with enhanced H4 acetylation in both MCF-7 and LCC1 cells, this requirement has been lost in LCC9 cells where H4 acetylation is consistently high (Fig. 4C).

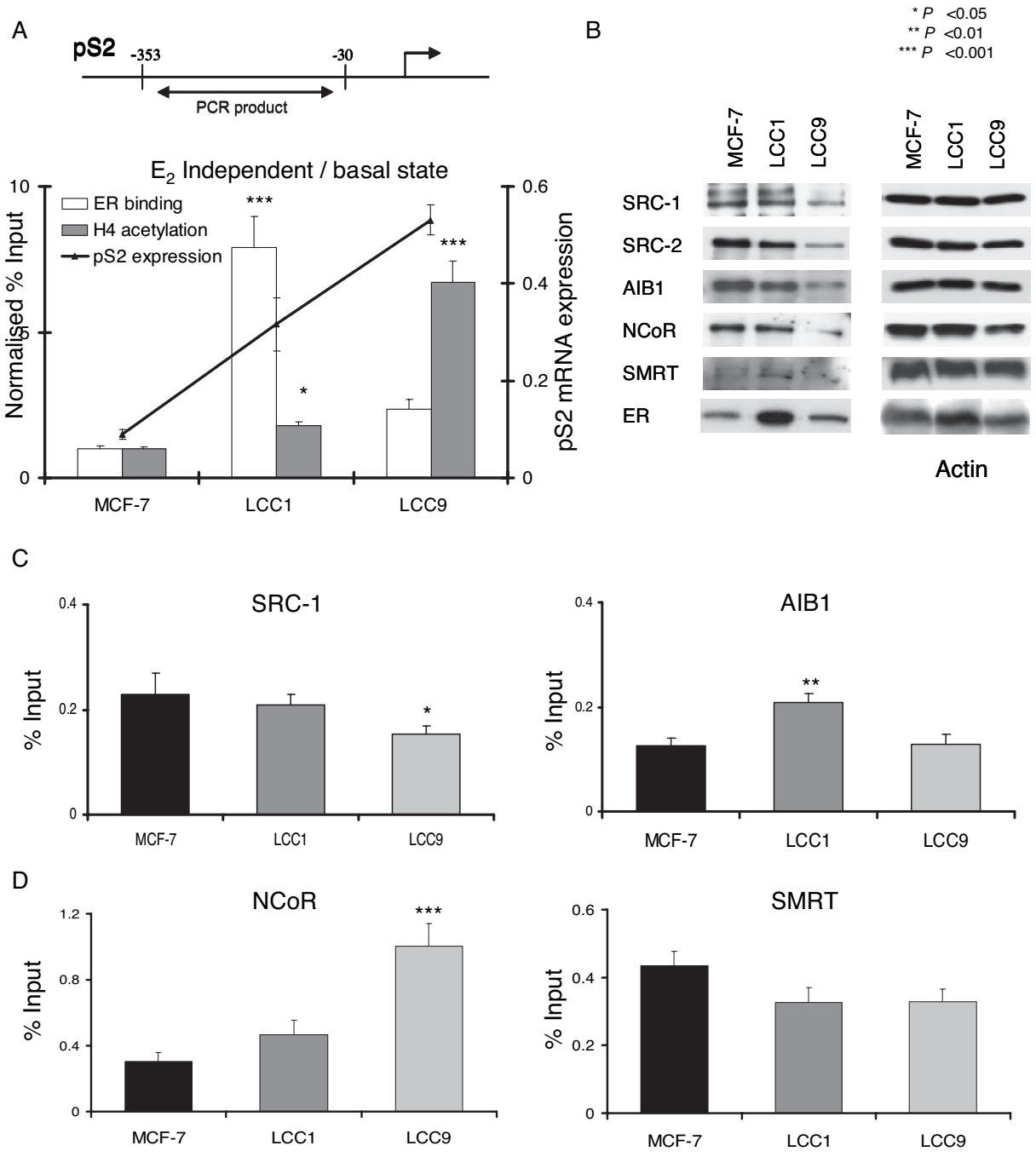

Fig. 2. pS2 Promoter Acetylation and Occupancy by Cofactors

A, Basal H4 acetylation and ERα binding to the pS2 promoter were determined by ChIP analysis on untreated cells. Immunoprecipitated pS2 promoter was quantified by real-time PCR using primers that cover the region indicated (−353 to −30). H4 acetylation/ERα levels are normalized to the inputs and each is the mean ± SE (of 10 samples). Basal pS2 expression data from Fig. 1 is shown. B, Cofactor and ERα protein levels were determined by Western blotting whole cell extracts. Actin is shown as a loading control. C and D, ChIP for basal pS2 promoter occupancy by coactivators SRC-1, AIB1 (C) and corepressors NCoR, SMRT (D). Data are presented as the mean ± SE for three independent experiments. In all ChIP experiments negative control IgG samples gave expression values < 0.1.

ERα Cofactor Recruitment Is Progressively Lost in Endocrine Resistance

High quality, cyclical gene transcription involves sequential recruitment of coactivators to the pS2 promoter (10). Because 15-min E_2 exposure is sufficient to produce significant ERα recruitment in all three cell lines (Fig. 4A), we examined cofactor levels on the pS2 promoter at this time point (Fig. 5A–D). MCF-7 cells show E_2-induced recruitment of SRC-1 and AIB1 to

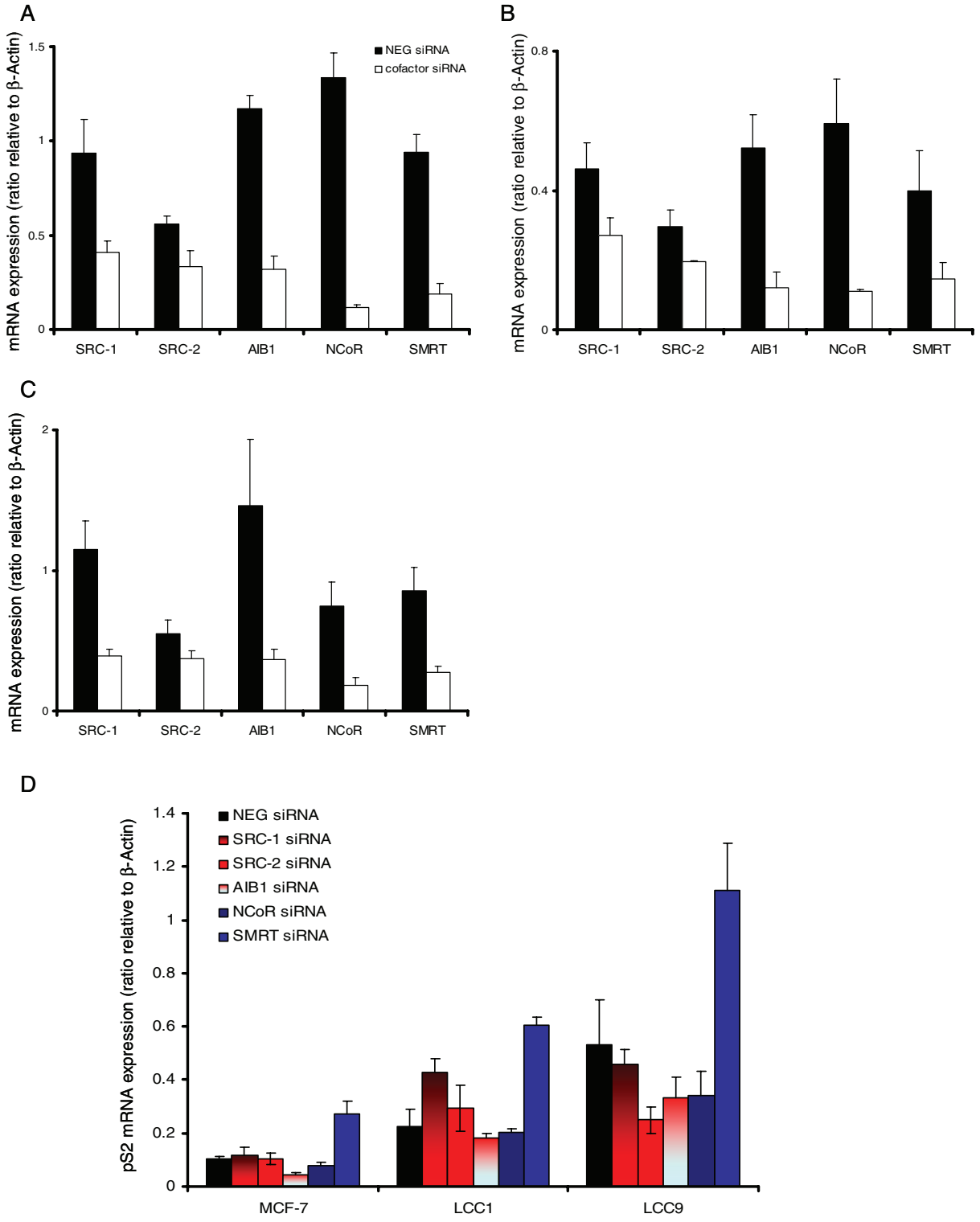

Fig. 3. Effects of siRNA Cofactor Depletion on Basal pS2 mRNA Expression

Transcriptional cofactors were depleted using an RNAi-based approach (see *Materials and Methods*). mRNA analysis confirming gene expression knockdown in (A) MCF-7 cells (B) LCC1 cells, and (C) LCC9 cells 48 h after siRNA treatment. D, pS2 mRNA expression was measured by quantitative RT-PCR on mRNA samples after 48-h siRNA treatment. Data are presented as means ± SD of actin-corrected values from triplicate samples.

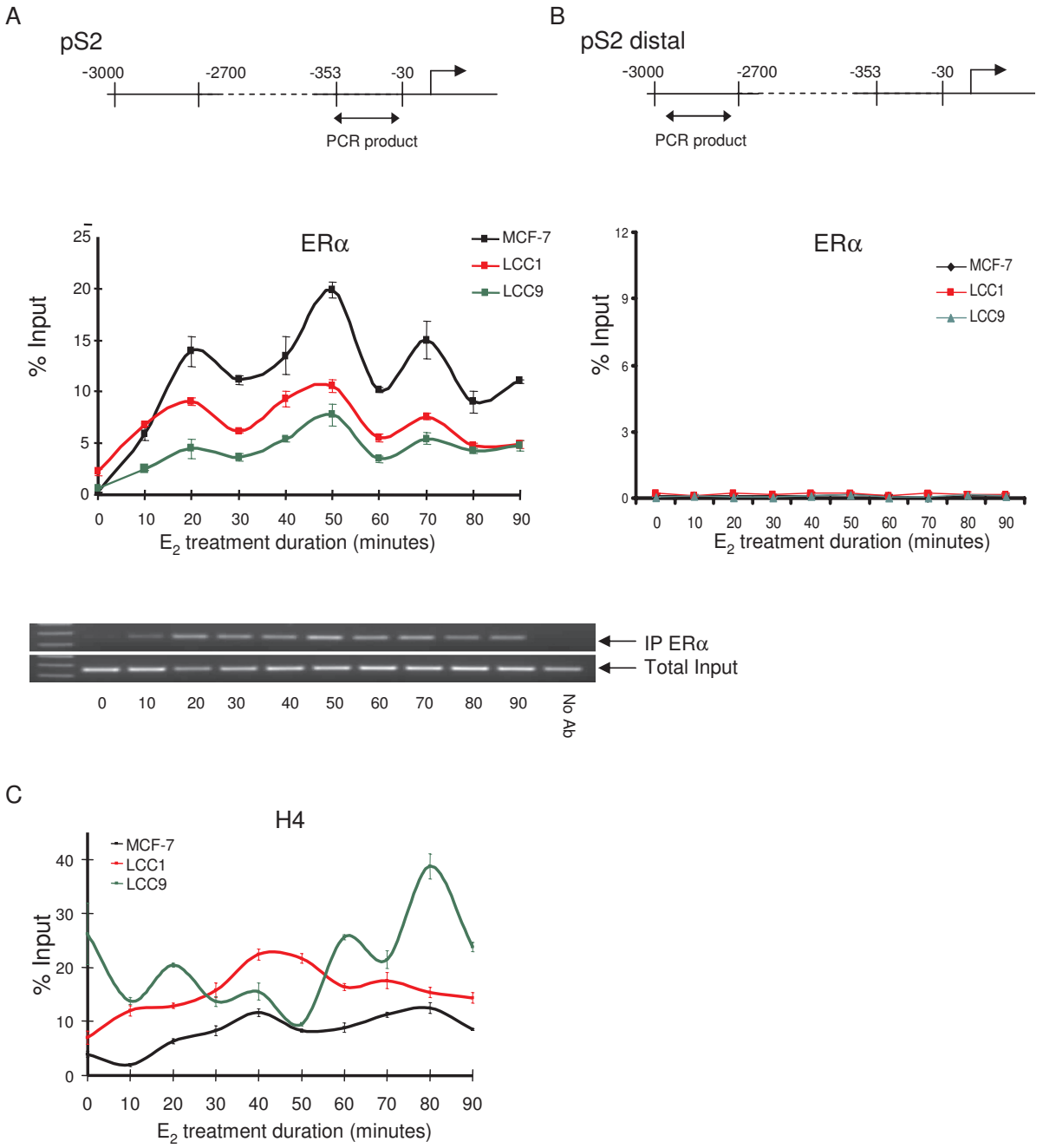

Fig. 4. E_2-Induced ERα Recruitment to the pS2 Promoter

E_2-induced ERα recruitment to the pS2 promoter (A) and distal region (B) were examined in MCF-7, LCC1, and LCC9 cells over a 90-min time course. At least three independent experiments were done in triplicate. Data are presented as the mean ± SE for a representative experiment. In addition we show agarose gel electrophoresis of the MCF-7 PCR products from the 0- to 90-min samples, inputs, and negative IgG control. C, E_2-induced H4 acetylation at the pS2 promoter was examined over a 90-min time course. Data are presented as the mean ± SE for three independent experiments.

the pS2 promoter, whereas the corepressors NCoR and SMRT remain associated. LCC1 cells only show recruitment of AIB1 and have reduced association of NCoR and SMRT. In the fully estrogen-independent and tamoxifen-resistant LCC9 cells, AIB1 recruitment is reduced as compared with MCF-7 and LCC1 cells. Additionally, SRC-1 recruitment was not induced, and NCoR and SMRT levels were significantly decreased, thus showing the progressive loss of cofactor recruitment in hormone resistance.

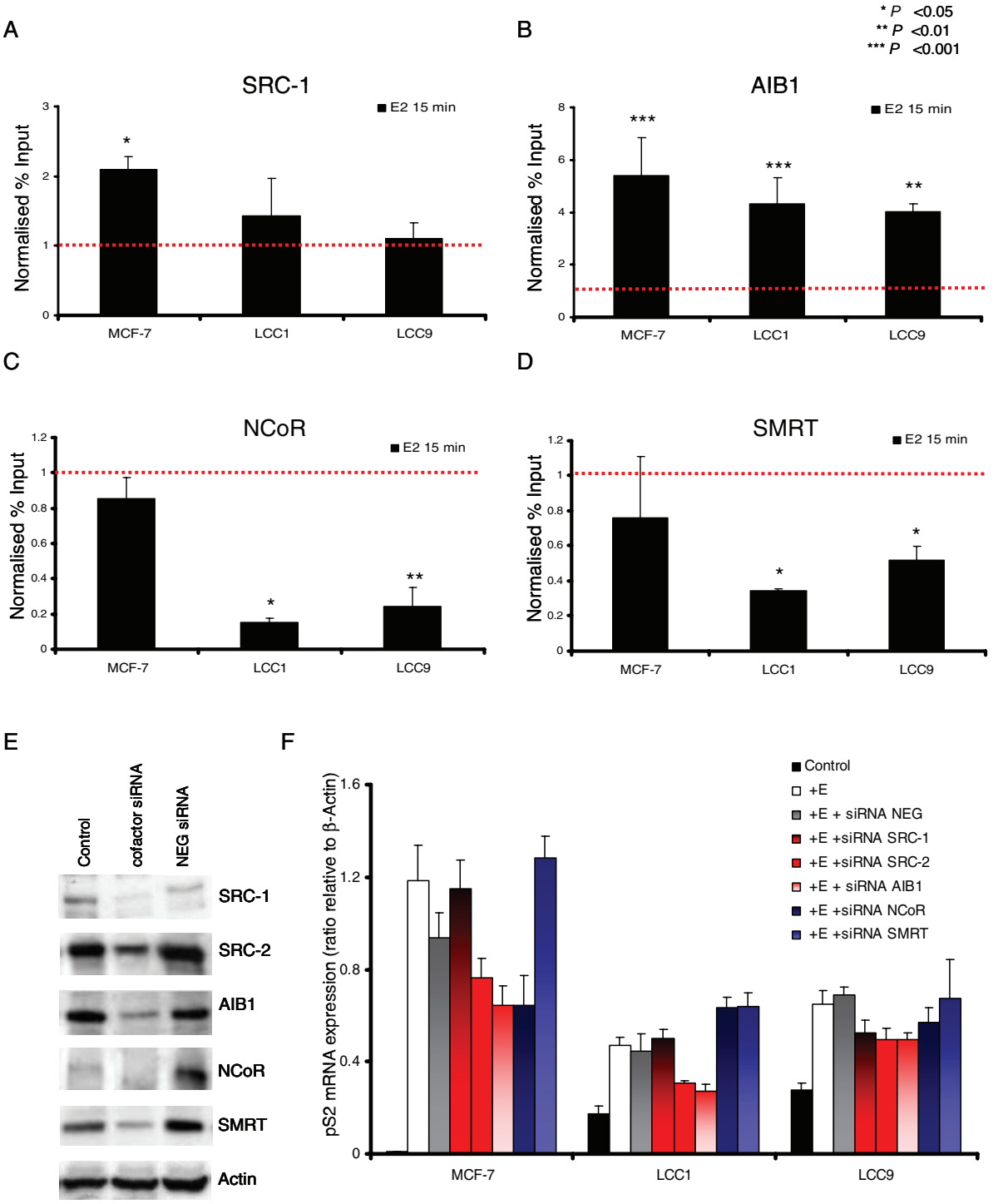

Fig. 5. E_2-Induced Cofactor Recruitment to the pS2 Promoter

E_2-induced SRC-1 (A), AIB1 (B), NCoR (C), and SMRT (D) recruitment to the pS2 promoter were determined by ChIP after 15-min E_2 (10^{-9} M) treatment in MCF-7, LCC1, and LCC9 cells. Data are normalized to basal levels from Fig. 2 and presented as the mean ± SE for three independent experiments. The *dashed red line* represents basal levels normalized to 1. Effects of siRNA cofactor ablation on E_2-induced pS2 gene expression. Transcriptional cofactors were ablated using an RNAi-based approach (see *Materials and Methods*). E, Western analysis confirming protein knockdown in MCF-7 cells 48 h after both siRNA and E_2 (10^{-9} M) treatment. Similar reductions in protein expression were observed for LCC1 and LCC9 cells (data not shown). F, pS2 mRNA expression was measured by quantitative RT-PCR on mRNA samples after 48-h siRNA and E_2 (10^{-9} M) treatment. Data are presented as means ± SD of actin-corrected values from triplicate samples.

E_2-Induced pS2 Gene Expression Is Progressively Less Dependent on p160 Coactivators in Endocrine Resistance

We have shown that basal pS2 gene expression has become ERα independent in endocrine resistance (Fig. 2A) and (24). Additionally we have shown that although this expression is dependent on AIB1 in MCF-7 cells, expression has become AIB1 independent in LCC1 and LCC9 cells (Fig. 3D). To further investigate pS2 gene expression mechanisms in endocrine resistance, we used RNAi to investigate the role of individual cofactors on E_2 induction of pS2 gene expression. RNAi was used to ablate either SRC-1, SRC-2, AIB1, NCoR, or SMRT expression (Fig. 5E). In MCF-7 cells there was a clear reduction (between 19 and 32%) in the E_2 induction of pS2 gene expression after SRC-2, AIB1, or NCoR removal (Fig. 5F). In LCC1 cells E_2-induced pS2 gene expression was only dependent on SRC-2 or AIB1 with expression reduced by between 30 and 40% after siRNA treatment. However, in the LCC9 cells E_2-induced pS2 gene expression is less coactivator dependent with minor changes in expression after cofactor removal.

Estrogen-Insensitive LCC9 Cell Growth Is Cofactor Independent

We have shown that there is a progressive loss of cofactor requirement for expression of the estrogen-dependent gene pS2. To study the requirement of ERα cofactors in the E_2 independent growth of each cell line, we ablated individual cofactors using RNAi. The efficiency and specificity of siRNA knockdown of target genes over 6 d was confirmed by Western analysis (Fig. 6A). Basal MCF-7 and LCC1 cell growth is dependent on each cofactor examined (Fig. 6B). In marked contrast, the E_2-independent cell growth demonstrated by LCC9 cells is independent of all cofactors examined.

DISCUSSION

Cofactor Dynamics Regulate ERα-Dependent Gene Expression

Much work has focused on ERα cofactor functions in ERα-mediated transcription and cell proliferation. These studies have identified p160 coactivators and the corepressors NCoR and SMRT as components of the ERα transcription complex assembled after ligand activation of ERα (9, 10, 25, 26). Indeed it has been reported that the recruitment by ERα of a p160 coactivator is sufficient for gene activation and to mediate estrogen-induced cell proliferation in breast cancer (9). The current view suggests that ligand-bound receptor exists in a dynamic equilibrium with coactivator and corepressor proteins to afford a regular and tightly controlled induction curve for nuclear receptor-mediated gene expression. However, the ultimate direction of the transcriptional response in a given cell and promoter context will be determined by the relative binding affinities and cellular distributions of these cofactors (27–29).

In this study we have identified altered cofactor binding and a progressive loss of cofactor recruitment in endocrine resistance in breast cancer. We have exploited a three-stage MCF-7-based cell line model wherein cofactor functions in parental MCF-7 cells were compared firstly to their E_2-independent derivative LCC1 cells and secondly to the further derived fully endocrine-resistant LCC9 cells. We show that in the acquisition of endocrine resistance, both ERα-dependent pS2 gene expression and cell proliferation have become E_2 independent. These changes are associated with altered cofactor binding to the promoter in the absence of ligand (Fig. 2) suggesting that cofactors are a significant feature in the development of endocrine-resistant breast cancer. In support of this, data from several studies have shown that high levels of coactivator expression may enhance the agonist activity of tamoxifen, thus contributing to endocrine resistance (12–15). Additionally, expression of dominant-negative NCoR in MCF-7 cells was found to both enhance the transcriptional activity of tamoxifen bound ERα and induce cell growth (28). In further studies MCF-7 cells were implanted into nude mice, which were then treated with tamoxifen. A decrease in tumor NCoR levels was associated with acquired resistance of these tumors to the antiproliferative effects of tamoxifen (19). These studies suggest the level of coactivator/corepressor ratio plays a key role in preventing breast tumor proliferation by tamoxifen and support this as a mechanism associated with endocrine resistance (15, 19, 28).

ERα Cofactor Requirement Is Progressively Lost in Endocrine Resistance

High quality, cyclical gene transcription involves sequential recruitment of cofactors to the pS2 promoter (9, 10). LCC9 cells, in the acquisition of endocrine resistance, have a global reduction in cofactor protein levels (Fig. 2B). Although a reduction in corepressor proteins can be associated with endocrine resistance, coactivator expression is reported to increase (13, 14, 15, 19). Here we show that E_2-induced pS2 transcription is associated with both progressive loss of cofactor recruitment and reduced dependency on individual cofactor expression in endocrine resistance. This change in cofactor regulation at the pS2 promoter may also describe why pS2 gene activation (as determined by H4 acetylation) is altered in LCC9 cells as compared with MCF-7 or LCC1 cells (Fig. 4C). Additionally we show a global loss of individual cofactor requirements for proliferative cell growth in endocrine resistance. This suggests that in the acquisition of endocrine resistance, many other proliferative genes have also lost the requirement for transcriptional cofactors.

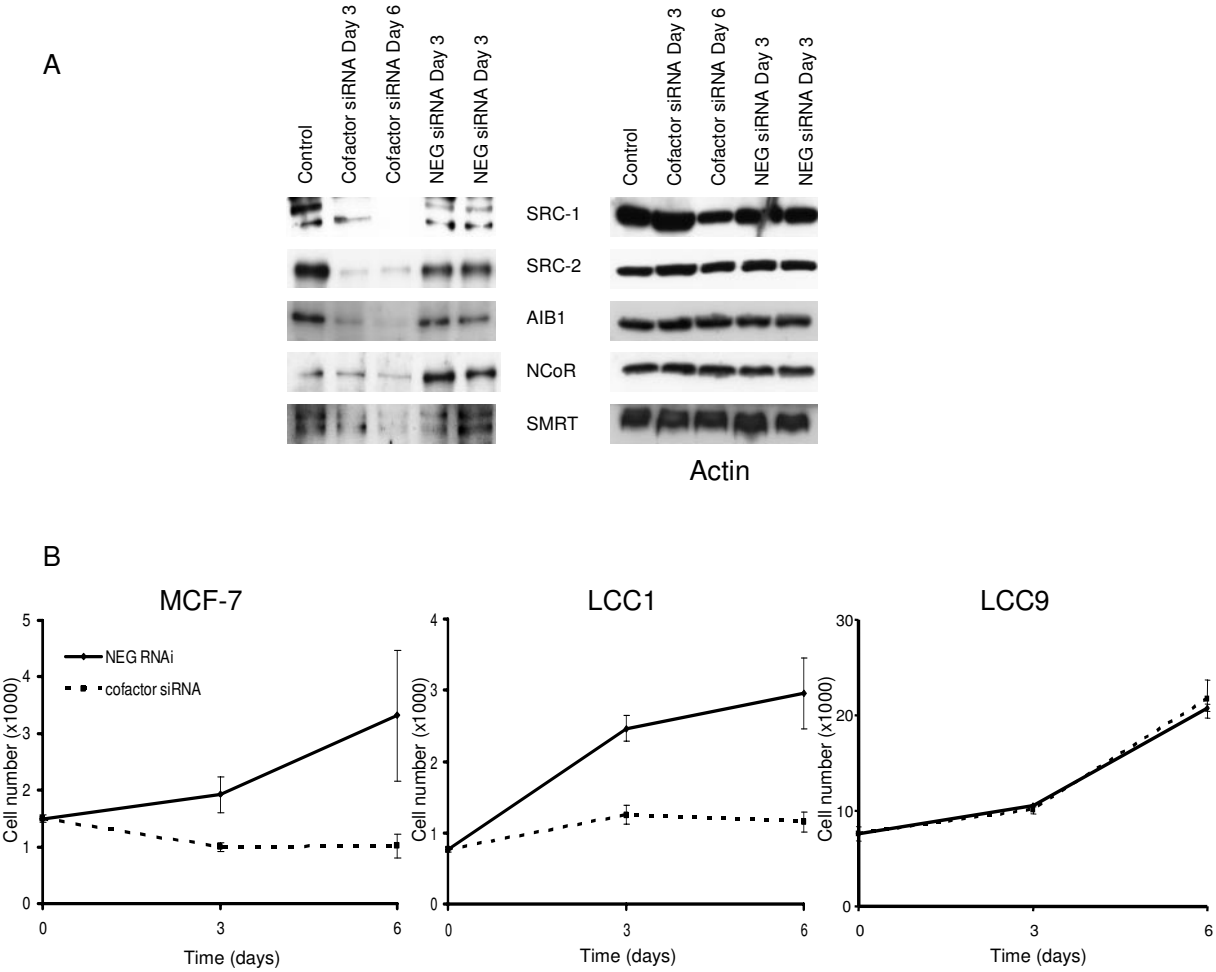

Fig. 6. Effects of Specific Cofactor siRNAs on Cell Growth

A, Western analysis confirmed cofactor protein knockdown in MCF-7 cells was maintained over a 6-d period after siRNA treatment. Similar reductions in protein expression were observed for LCC1 and LCC9 cells (data not shown). B, Effects of cofactor-specific siRNA on basal cell growth were determined by cell proliferation assays, taking cell counts before siRNA treatment (d 0) and 3 d and 6 d after treatment. Negative control data are presented as means ± SE from quadruplicate samples. As each individual cofactor siRNA produced a similar effect on cell proliferation, these data are presented as the mean ± SE of all cofactors tested (SRC-1, SRC-2, AIB1, NCoR, and SMRT).

Thus the reduction in global cofactor expression is perhaps unsurprising and could be explained by decreased requirement for cofactors producing a negative feedback on cofactor transcription. This reinforces the view that interplay between coactivators and corepressors is key to controlling gene regulation and that changes in this process are a conceivable mechanism for contributing to endocrine resistance in breast cancer.

Cofactor Regulation and Posttranslational Modifications

One mechanism that could account for this loss of cofactor recruitment to the pS2 promoter is reduced ERα activation through Ser^{118} phosphorylation (30). Indeed we have previously identified reduced basal Ser^{118} phosphorylation in both LCC1 and LCC9 cells, and estrogen-induced Ser^{118} phosphorylation was markedly reduced upon acquisition of endocrine resistance (24). In addition, recent studies have identified ERα cofactors as targets for posttranslational modification by diverse cellular signaling pathways (ERK, MAPK, p38) (25, 31–33). Phosphopeptide mapping experiments have revealed multiple phosphorylation sites within the p160 coactivator proteins (31–33). The fact that very few of these are conserved between p160s argues that this may represent an important determinant of functional specificity. Significantly, AIB1 phosphorylation has been shown to enhance its nuclear sublocalisation, ERα interaction, and have essential functions for ERα transactivation (33, 34, 35). In terms of endocrine resistance phospho AIB1 has been shown to be necessary for AIB1-induced tamoxifen agonist activity in breast cancer cells

(36). Similarly, SMRT phosphorylation has been shown to inhibit the ability of this corepressor to mediate repression (25). Increased activity in MAPK signaling pathways has been implicated in endocrine-resistant breast cancer, thus loss of cofactor functions could be accounted for via altered MAPK signaling (37, 38).

In conclusion, we have demonstrated progressive loss of cofactor function (including regulation of growth) in progressively more estrogen-independent breast cancer cell lines. This loss of cofactor requirement may represent an important mechanism for gene misregulation in cancer and afford a suitable target to overcome endocrine resistance in the treatment of breast cancer.

MATERIALS AND METHODS

Cell Culture and Reagents

MCF-7 cells were routinely grown in phenol red containing DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). MCF-7-derived MCF-7/LCC1 and MCF-7/LCC9 cells (22, 23) (source: Dr. Robert Clarke, V. T. Lombardi Cancer Research Centre, Georgetown University Medical School, Washington, D.C.) were routinely cultured in phenol-red-free DMEM supplemented with 5% dextran activated double charcoal stripped fetal calf serum (DCC), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM Glutamine. Cells were maintained in a humidified atmosphere at 37 C and 5% CO_2. 17β-E_2 and tamoxifen were purchased from Sigma (St. Louis, MO).

Cell Proliferation Assays

MCF-7 cells were seeded at a density of 2.5×10^4 cells per well in six-well plates, and 24 h later the cells were washed twice with PBS and the media changed to phenol-red-free DMEM with 5% DCC for 48 h. The cells were then treated with media containing 10^{-9} M E_2 T and incubated for 6 d. LCC1 and LCC9 cells were seeded at a density of 2.5×10^4 cells per well in six-well plates in phenol-red-free containing DMEM with 5% DCC and after 24 h were treated with media supplemented with E_2. Cells were incubated with the drugs for 6 d, and this medium was changed and replaced with fresh medium and drugs on d 3. Cell counts on d 0, 3, and 6 were estimated using a Coulter Counter (Beckman Coulter, Inc., Fullerton, CA).

RNA Preparation and Real-Time RT-PCR Analysis

RNA from MCF-7 and LCC cells was prepared using either Stratagene's Absolutely RNA miniprep kit (Stratagene, La Jolla, CA) or Tri-Reagent (Sigma) following manufacturers' instructions. Quantification of the RNA was performed by spectrophotometry at 260 nm. RNA was analyzed by real-time RT-PCR using Rotorgene (Corbett Research, San Francisco, CA) and the QuantiTect SYBR Green system (QIAGEN, Chatsworth, CA) according to the manufacturers' instructions. The thermal cycling conditions were RT: 50 C for 30 min; PCR: initial denaturation 95 C for 15 min; followed by 40 cycles of denaturation 94 C for 15 sec, annealing 57 C for 30 sec, extension 72 C for 30 sec; and a final extension of 72 C for 60 sec. The following primers were used: pS2: fwd TTGTGGTTTTCCTGGTGTCA and rev CCGAGCTCTGGGACTAATCA; ERα: fwd CCACCA ACCAGTGCACCATT, rev GTCTTTCCGTATCCCACCTTTC; AIB1: fwd CCCTTTTATCTACTCTGTCATC, rev CCAGATGTAGAGGAGGAGAC; SRC-2: fwd AGCCTGTGAGAGGGCTGTTA, rev AATGAGAGAGGGGAAGGGAA; SRC-1: fwd CATGCTTATGAGGCAGCAAA, rev ATTCCAGTGCCAAACTGTCC; NCoR: fwd AAAGTGTGGAGACCCAGGTG, rev ACCCTCACTTCAACGTCCAC; SMRT: fwd AAGTCCATCCTCACGTCCAC, rev AAGCACACTGGGTCTCTGCT; β-actin: fwd CTACGTCGCCCTGGACTTCGAGC, rev GATGGAGCCGCCGATCCACACGG.

siRNA Transfections

MCF-7 cells were seeded at 0.5×10^6 cells per 75-cm^2 flask in DMEM as above. Twenty-four hours later after two PBS washes, the medium was changed to phenol-red-free DMEM with 5% DCC for 48 h. LCC1 and LCC9 cells were seeded directly into phenol-red-free containing DMEM with 5% DCC for 24 h before transfection. Cells were transfected with siRNA for 4 h using Oligofectamine reagent (Invitrogen, Paisley, UK) after which time 10^{-9} M E_2 was added if required for a further 48 h before RNA and protein extraction. siRNA sequences were: Negative siRNA: Upstate M-003401; AIB1: Qiagen 1024591; SRC-1: Qiagen 1024927; SRC-2: 5′AAGTCAGATGTATCCTCTACA; NCoR: 5′AAUGCUACUUCUCGAGGAAACA; SMRT: 5′AAGGGUAUCAUCACCGCUGUG. All siRNA were used at 100 nM.

Western Blot Analysis

Cells were washed twice with PBS and lysed in ice cold lysis buffer [50 mM Tris (pH 7.5), 5 mM EDTA (pH 8.5), 150 mM NaCl, 1% Triton X-100, aprotinin 10 μg/ml, and 1× protease inhibitor cocktail (Roche, Basel, Switzerland)] for 10 min, and the debris was cleared by centrifugation at 13,000 rpm for 6 min at 4 C. Protein lysates (25–100 μg) were resolved by 7–12% SDS-PAGE and electrophoretically transferred to Immobilon-P membranes. After transfer, membranes were blocked and probed with primary antibody overnight at 4 C. All blots were visualized with either BM chemiluminescence (Roche) or SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL) and Hyperfilm (Amersham Biosciences, Piscataway, NJ). Antibodies used were: ERα (F-10) (Santa Cruz Biotech SC-8002; Santa Cruz, CA), AIB1 (Affinity BioReagents, Inc., Golden, CO) (MA1-845), SRC-2 (BD Biosciences, Palo Alto, CA) (610984), SRC-1 (Upstate Biotechnology, Inc., Lake Placid, NY) (05-522) (this antibody recognizes both SRC-1 isoforms), NCoR (Upstate) (06-892), SMRT (Upstate) (06-891), and Actin (Calbiochem, La Jolla, CA) (CP01).

ChIP Assays

ChIP assays were performed as described (9). Cells were grown to 90% confluence in phenol-red-free DMEM with 5% DCC for at least 48 h. After treatment with 10^{-9} M E_2 over a 90-min time course, cells were cross-linked with 1% formaldehyde at 37 C for 10 min. Unreacted formaldehyde was quenched by gentle agitation at room temperature for 10 min with 0.125 M glycine. Cells were then washed twice with ice-cold PBS, collected into PBS containing protease inhibitors and centrifuged for 4 min at 2000 rpm at 4 C. The pellets were resuspended in 100 μl of lysis buffer per 10^6 cells [1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), and 1× protease inhibitor cocktail], incubated on ice for 10 min, and sonicated for 12 × 20 sec at 2 amplitude microns (Soniprep 150; MSE, Sanyo Gallenkamp PLC, Loughborough, UK). After centrifugation for 15 min at 13,000 rpm and 4 C, supernatants were collected and resuspended in dilution buffer (0.01% SDS;1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris HCl, pH 8.1; 167 mM NaCl; and 1× protease inhibitor cocktail). Chromatin was precleared with 1 μg anti-rabbit or antimouse IgG; 2 μg sheared salmon sperm DNA,

and protein-G-Agarose (50 μl of 50% slurry in dilution buffer) for 3 h at 4 C. Immunoprecipitation was performed overnight at 4 C with 2 μg sheared salmon sperm DNA, 50 μl protein-G-Agarose, and specific antibodies. Precipitates were washed sequentially for 5 min each at 4 C with TSE I (20 mM Tris, pH 8.1; 2 mM EDTA; 150 mM NaCl; 1% Triton X-100; and 0.1% SDS), TSE II (20 mM Tris, pH 8.1; 2 mM EDTA; 500 mM NaCl; 1% Triton X-100; and 0.1% SDS), and buffer III (10 mM Tris, pH 8.1; 0.25 M LiCl; 1 mM EDTA; 1% NP40; and 1% deoxycholate). Precipitates were then washed twice with TE buffer and the protein/DNA complexes were eluted twice with 0.1 M $NaHCO_3$ in 1% SDS. Crosslinks were reversed by incubation at 65 C overnight. DNA fragments were purified using QIAquick Spin Kit columns (Qiagen) and amplified using the QuantiTect SYBR Green system (Qiagen). Thermal cycling conditions were: 95 C for 15 min followed by 45 cycles of 94 C for 15 sec, 55 C for 30 sec, 72 C for 30 sec, and a final extension of 72 C for 5 min. PCR primer sequences for the pS2 promoter were: fwd GACGGAATGGGCTTCATGAGC and rev CTGAGACAATAATCTCCACTG. PCR primer sequences for distal pS2 (~3 kb upstream of pS2 promoter) were: fwd CTTGCCTCTGCATTCTCTCC and rev GAGTTTGGCCTCCCACATTA.

ChIP Antibodies

H4 (Upstate, 06-866), ERα HC-20 (Santa Cruz, sc543), AIB-1 C-20 (Santa Cruz, sc7216), SRC-1 (Upstate, 05-522), NCoR (Santa Cruz, sc1609), and SMRT (Santa Cruz sc20778).

Acknowledgments

This work was supported by Cancer Research UK. We thank Dr. Nick Gilbert for helpful discussions and assistance in the preparation of the manuscript.

Received February 27, 2007. Accepted July 25, 2007.

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This work was supported by Cancer Research UK.

Disclosure Statement: The authors have nothing to disclose.

REFERENCES

1. Ali S, Coombes RC 2002 Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2:101–112
2. Normanno N, Di Maio M, De Maio E, De Luca A, de Matteis A, Giordano A, Perrone F 2005 Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. Endocr Relat Cancer 12:721–747
3. Danielian PS, White R, Lees JA, Parker MG 1992 Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J 11:1025–1033
4. Parker MG 1995 Structure and function of estrogen receptors. Vitam Horm 51:267–87
5. 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
6. Brownell JE, Allis CD 1996 Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. Curr Opin Genet Dev 6:176–184
7. Belandia B, Parker MG 2003 Nuclear receptors: a rendez-vous for chromatin remodeling factors. Cell 114: 277–280
8. Xu J, Li Q 2003 Review of the *in vivo* functions of the p160 steroid receptor coactivator family. Mol Endocrinol 17: 168116–92
9. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M 2000 Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103:843–852
10. Métivier R, Penot G, Hübne MR, Reid G, Brand H, Koš M, Gannon F 2003 Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115:751–763
11. Cavarretta ITR, Mukopadhyay R, Lonard DM, Cowsert LM, Bennett CF, O'Malley BW, Smith CL 2002 Reduction of coactivator expression by antisense oligodeoxynucleotides inhibits ERα transcriptional activity and MCF-7 proliferation. Mol Endocrinol 16:253–270
12. Torres-Arzayus MI, Font de Mora J, Yuan J, Vazquez F, Bronson R, Rue M, Sellers WR, Brown M 2004 High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. Cancer Cell 6:263–274
13. Bautista S, Valles H, Walker RL, Anzick S, Zeillinger R, Meltzer P, Theillet C 1998 In breast cancer, amplification of the steroid receptor coactivator gene AIB1 is correlated with estrogen and progesterone receptor positivity. Clin Can Res 4:2925–2929
14. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan X, Sauter G, Kallioniemi O, Trent JM, Meltzer PS 1997 AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277:965–968
15. Osborne CK, Bardou B, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SAW, Wong J, Allred DC, Clark GM, Schiff R 2003 Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 95:353–361
16. Heinzel T, Lavinsky RM, Mullen TM, Soderstrom M, Laherty CD, Torchia J, Yang WM, Brard G, Ngo SD, Davie JR, Seto E, Eisenman RN, Rose DW, Glass CK 1997 A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature 38: 43–48
17. Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL, Evans RM 1997 Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell 89:373–380
18. Chen JD, Evans RM 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
19. Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen T, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J, Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG, Rose DW 1998 Diverse signalling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc Nat Acad Sci 95:2920–2925
20. Liu X, Bagchi MK 2004 Recruitment of distinct chromatin-modifying complexes by tamoxifen-complexed estrogen receptor at natural target gene promoters in vivo. J Biol Chem 279:15050–15058
21. Prest SJ, May FEB, Westley BR 2002 The estrogen-regulated protein, TFF1, stimulates migration of human breast cancer cells. FASEB J 16:592–594
22. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R 1993 Acquisition of hormone-independent

growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53:283–290
23. Brunner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua AW, Clarke R 1997 MCF7/LCC9: an antiestrogen-resistant variant in which acquired resistance to the steroidal antiestrogen ICI 182780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res 57:3486–3493
24. Kuske B, Naughton C, Moore K, MacLeod KG, Miller WR, Clarke R, Langdon SP, Cameron DA 2006 Endocrine therapy resistance can be associated with high estrogen receptor α (ERα) espression and reduced ERα phosphorylation in breast cancer models. Endocrine Related Cancer 13:1121–1133
25. Hong, S, Privalsky ML 2000 The SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export. Mol Cell Biol 20:6612–6625
26. Smith CL, Nawaz Z, O'Malley BW 1997 Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 11:657–666
27. Zhang X, Jeyakumar M, Petukhov S, Bagchi MK 1998 A nuclear receptor corepressor modulates transcriptional activity of antagonist-occupied steroid hormone receptor. Mol Endocrinol 12:513–524
28. Fujita T, Kobayashi Y, Wada O, Tateishi Y, Kitada L, Yamamoto Y, Takashima H, Murayama A, Yano T, Baba T, Kato S, Kawabe Y, Yanagisawa J 2003 Full activation of estrogen receptor α activation function-1 induces proliferation of breast cancer cells. J Biol Chem 278: 26704–26714
29. McKenna NJ, O'Malley BW 2002 Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108:465–474
30. Dutertre M, Smith CL 2003 Ligand-independent interactions of p160/steroid receptor coactivators and CREB-binding protein (CBP) with estrogen receptor-α: regulation by phosphorylation sites in the A/B region depends on other receptor domains. Mol Endocrinol 17: 1296–1314
31. Lopez GN, Turck CW, Schaufele F, Stallcup MR, Kushner PJ 2001 Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. J Biol Chem 276: 22177–22182
32. Rowan B, Weigel N, O'Malley BW 2000 Phosphorylation of steroid receptor coactivator-1. J Biol Chem 275: 4475–4483
33. Wu R, Qin J, Yi P, Wong J, Tsai SY, Tsai M, O'Malley BW 2004 Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic reponses to multiple cellular signaling pathways. Mol Cell 15:937–949
34. Wu R, Qin J, Hashimoto Y, Wong J, Xu J, Tsai YS, Tsai M, O'Malley BW 2002 Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) coactivator activity by IκB kinase. Mol Cell Biol 22:3549–3561
35. Wu R, Smith CL, O'Malley BW 2005 Transcriptional regulation by steroid receptor coactivator phosphorylation. Endocr Rev 26:393–399
36. Shao W, Krasnickas Keeton E, McDonnell DP, Brown M 2004 Coactivator AIB1 links estrogen receptor transcriptional activity and stability. Proc Natl Acad Sci 101: 11599–11604
37. Ciocca DR, Elledge R 2000 Molecular markers for predicting response to tamoxifen in breast cancer patients. Endocrine 13:1–10
38. Mass R 2000 The role of HER-2 expression in predicting response to therapy in breast cancer. Semin Oncol 27:46–52

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