Associations and Interactions between Ets-1 and Ets-2 and Coregulatory Proteins, SRC-1, AIB1, and NCoR in Breast Cancer

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ABSTRACT

Purpose: Associations between p160 coactivator proteins and the development of resistance to endocrine treatment have been described. We hypothesized that nuclear receptor coregulatory proteins may interact with nonsteroid receptors. We investigated the mitogen-activated protein kinase–activated transcription factors, Ets, as possible interaction proteins for the coactivators SRC-1 and AIB1 and the corepressor NCoR in human breast cancer.

Experimental Design: Expression and coexpression of Ets and the coregulatory proteins was investigated using immunohistochemistry and immunofluorescence in a cohort of breast tumor patients (N = 134). Protein expression, protein-DNA interactions and protein-protein interactions were assessed using Western blot, electromobility shift, and coimmunoprecipitation analysis, respectively.

Results: Ets-1 and Ets-2 associated with reduced disease-free survival (P < 0.0292, P < 0.0001, respectively), whereas NCoR was a positive prognostic indicator (P < 0.00297). Up-regulation of Ets-1 protein expression in cell cultures derived from positive tumors in the presence of growth factors associated with tumor grade (P < 0.0013; n = 28). In primary breast tumor cell cultures and in the SKBR3 breast cell line, growth factors induced interaction between Ets and their DNA response element, induced recruitment of coactivators to the transcription factor-DNA complex, and up-regulated protein expression of HER2. Ets-1 and Ets-2 interacted with the coregulators under basal conditions, and growth factors up-regulated Ets-2 interaction with SRC-1 and AIB1. Coexpression of Ets-2 and SRC-1 significantly associated with the rate of recurrence and HER expression, compared with patients who expressed Ets-2 but not SRC-1 (P < 0.0001 and P < 0.0001, respectively).

Conclusions: These data describe associations and interactions between nonsteroid transcription factors and coregulatory proteins in human breast cancer.

INTRODUCTION

In breast cancer, current endocrine therapies, such as tamoxifen, are based on targeting the estrogen receptor (ER). ER interacts with steroid nuclear regulatory proteins (coactivators and corepressors) in a ligand-dependent manner to regulate gene transcription. The coactivator proteins amplified in breast cancer 1 (AIB1/pCIP/RAC3/ACTR/SRC-3) and steroid receptor coactivator 1 (SRC-1/NCoA-1) are both members of the p160 family of coactivator proteins whose expression has been shown to be elevated in human breast cancer (1–3). These coregulatory proteins interact with nuclear receptors at a conserved LXXLL motif within the receptor interacting domain of the protein to drive target gene expression (4). In contrast, corepressor proteins such as NCoR interact with antagonist-bound ER to maintain transcriptional silence (5). Although the steroid coregulatory proteins were previously thought to exclusively associate with nuclear receptors, there is now evidence to suggest that they can also complex with other transcription factors including activator protein (6), nuclear factor κB (7), and p53 (8).

Abnormalities in growth factor signaling pathways play an intrinsic role in disease progression. In human breast cancer, the growth factor receptor, HER2, is overexpressed in 20% to 30% of breast cancers and is associated with enhanced tumorigenicity and resistance to endocrine therapy (9, 10). Molecular and clinical evidence suggests that cross talk between ER and growth factor pathways contribute to endocrine resistance, at least in part through the phosphorylation of coactivator proteins (11, 12). We have previously described a positive association between expression of the p160 proteins, SRC-1 and AIB1, and HER2 in a cohort of patients with breast tumor (3).

Ets proteins are a family of mitogen-activated protein kinase (MAPK)–dependent transcription factors, which have been implicated as downstream effectors of HER2 signaling (13). They contain a conserved winged helix-turn-helix DNA-binding domain, regulating gene expression by binding to Ets-binding sequences found in promoter/enhancer regions of their target genes. The Ets proteins have been shown to be expressed in both primary human breast cancers and breast cancer cell lines and their expression has been associated with disease progression and metastasis (14, 15). Known Ets target genes include the extracellular proteases, urokinase-type plasminogen activator and matrix metalloproteinases, and the growth factor receptor HER2 (16–18). Ets transcription factors are thought to bind to coregulatory proteins to modulate their transcriptional regulatory properties. The highly homologous Ets-1 and Ets-2 and the
PEA3 family member, ER81, have been shown to recruit the transcription adapter proteins p300 and CBP (19–21). More recently, the p160 coactivator, AIB1, was identified as an interaction partner for ER81 (22). Furthermore, a consensus recognition site for the steroid nuclear interacting protein SRC/p160 binding domain, LXXLL, is conserved in loop 1 of the Ets domain in all Ets family transcription factors, with the exception of PEA3 (23). These observations raise the possibility that Ets family members could recruit steroid coregulatory proteins either directly or through adapter proteins, such as CBP/p300, to modulate their transcriptional activity.

We hypothesized that in human breast cancer steroid coregulatory protein interactions are not restricted to nuclear receptors but can complex with MAPK effectors such as the Ets transcription factors. Here we provide evidence that growth factors can induce Ets DNA interaction and initiate recruitment of the p160 coactivator proteins to the transcription factor DNA complex. Furthermore, we describe positive associations between Ets and p160 protein expression and disease recurrence in human breast cancer.

MATERIALS AND METHODS

Patient Selection. One hundred and thirty-four breast tumor specimens and six reduction mammoplasties were included in this study. All patients had stage I/II breast cancer at presentation and were assessed by abdominal ultrasound, chest X-ray, and bone scintigraphy before surgery. All patients received adjuvant tamoxifen (20 mg/d for 5 years); where patients were ER negative they received tamoxifen based on a positive progesterone receptor status. All recurrences occurred while patients were on endocrine therapy.

Immunohistochemistry. Five-micrometer-thick tissue sections were cut from paraffin-embedded breast tumor tissue blocks and mounted on Superfrost Plus slides (BDH, Poole, United Kingdom). Sections were dewaxed, rehydrated, and washed in PBS. Endogenous peroxidase was blocked using 3% hydrogen peroxide in PBS for 10 minutes. Antigen retrieval was done by immersing sections in 0.6 mol/L citrate buffer and microwaving on high power for 7 minutes. Antigens were detected using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Briefly, sections were blocked in serum for 90 minutes. Sections were incubated with primary antibodies: rabbit anti-human Ets-1 (1 μg/mL), rabbit anti-human Ets-2 (1 μg/mL), rabbit anti-human AIB1 (1 μg/mL), goat anti-human SRC-1 (1 μg/mL), rabbit anti-human NCoR (1 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-phospho-Raf (1:50, Cell Signaling, Beverly, MA) for 60 minutes at room temperature. Subsequently, sections were incubated in the corresponding fluorochrome-conjugated antibody (1 in 100) incubated with either goat anti-human AIB1, goat anti-human SRC-1, or goat anti human NCoR (all at 10 μg/mL in 10% human serum) was placed on each slide for 90 minutes. The sections were incubated with the corresponding secondary fluorochrome-conjugated antibody (1 in 100) for 60 minutes. Rabbit anti-human Ets-1 or Ets-2 (10 μg/mL in 10% human serum) was placed on each slide for 90 minutes. The slides were blocked in rabbit serum for 90 minutes. Each slide was incubated with either goat anti-human AIB1, goat anti-human SRC-1, or goat anti human NCoR (all at 10 μg/mL in 10% human serum) for 90 minutes. The slides were incubated with the corresponding fluorochrome-conjugated antibody (1 in 100) for 60 minutes. All steps were preceded by a wash with PBS. Sections were mounted using fluorescent mounting media (Dako). Slides were examined under a fluorescent microscope. Negative controls were done using matched IgG.

Cell Culture Stimulations. After ethical approval, breast tumor specimens were obtained from 28 patients undergoing surgery for removal of a histologically confirmed breast tumor. Breast tumor cell cultures were established and validated as previously described (2). In brief, primary tumor epithelial cells were extracted in HBSS without calcium or magnesium (Life Technologies, Inc., Paisley, Scotland) supplemented with 1 μmol/L EDTA and 1 μmol/L diithiothreitol for 40 minutes. Cells were cultured in RPMI containing 5 μg/mL insulin, 10 μg/mL transferrin, 30 nmol/L sodium selenate, 10 nmol/L hydrocortisone, 10 nmol/L β-estradiol, 10 mmol/L HEPES, 2 mmol/L glutamine, 10% FCS (w/v), and 5% ultrroser G on a growth factor reduced Matrigel matrix (BD Biosciences, San Jose, CA; 60 ng/cm²). Examination of primary breast cultures by staining with ethidium bromide and flow cytometric analysis using the phycoerythrin-labeled pan-leukocyte marker (CD45 RA and RO), confirmed cell viability and epithelial origin of tumor cells (2). Phenotypically distinct progenitor epithelial cell populations within the mammary epithelium were characterized by flow cytometry using a phycoerythrin-labeled mouse anti-human EpCAM (epithelial specific antigen) antibody and FITC-conjugated mouse anti-human CD227 (MUC1) monoclonal mouse antibody (BD Biosciences). Bipotent progenitors (EpCAM MUC1⁻), which can generate both luminal and myoepithelial cells, were found to represent 51.9% of the epithelial cell population, whereas the luminal restricted progenitor (EpCAM MUC1⁻) were found to represent 48.1%. The SK-BR3 breast cancer cell line (European Collection of

Assessment of HER2 Status. HER2 status was evaluated using the Dako HercepTest immunocytochemical assay. Scoring was assessed according to the manufacturer’s instructions. A score was assigned according to the intensity and pattern of cell membrane staining: 0 = no staining; +1 = weak staining; +2 = weak to moderate staining; +3 = strong staining. In tumors samples scoring +2 with the Hercept test, HER2 status was confirmed by fluorescent in situ hybridization using the PathVision kit probe to detect amplification of the HER2 gene (spectrum orange labeled HER2 and spectrum green labeled α satellite centromeric region for chromosome 17; Vysis Inc, Downers Grove, IL) according to the manufacturer’s instructions. Criteria for gene amplification were tight clusters of HER2 signals in multiple cells at least twice more HER2 signal than centromeric 17.

Immunofluorescent Microscopy. Breast cancer sections were prepared as above and incubated in goat serum for 60 minutes. Rabbit anti-human Ets-1 or Ets-2 (10 μg/mL in 10% human serum) was placed on each slide for 90 minutes. The sections were incubated with the corresponding secondary fluorochrome-conjugated antibody (1 in 100; Sigma-Aldrich, Steinheim, Germany) for 60 minutes. Subsequently, the slides were blocked in rabbit serum for 90 minutes. Each slide was incubated with either goat anti-human AIB1, goat anti-human SRC-1, or goat anti human NCoR (all at 10 μg/mL in 10% human serum) for 90 minutes. The slides were incubated with the corresponding fluorochrome-conjugated antibody (1 in 100) for 60 minutes. All steps were preceded by a wash with PBS. Sections were mounted using fluorescent mounting media (Dako). Slides were examined under a fluorescent microscope. Negative controls were done using matched IgG.
Animal Cell Cultures, Wiltshire, United Kingdom) was maintained in RPMI medium (Life Technologies) supplemented with 5% FCS, 200 μg/mL penicillin-streptomycin, and 5 μg/mL fungizone (Life Technologies).

Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Experiments were carried out when cells reached 90% confluence. Cells were serum and steroid depleted for 24 hours before stimulation and then incubated in the presence and absence of basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) for 24 hours and harvested. Total protein was extracted using lysis buffer (1% Igepal, 0.5% deoxycholic acid, 0.1% SDS, and 1× PBS) with pefabloc (5 μg/mL). Cell lysates were subsequently normalized for protein content.

**Western Blotting.** Proteins (30-100 μg) were resolved on a polyacrylamide gel (12% for Ets-1, Ets-2, AIB1, SRC-1, and HER2 and 7% for NCoR) at 110 V for 120 minutes and were transferred to a nitrocellulose membrane (250 mA for 60 minutes for Ets-1, Ets-2, AIB1, SRC-1, and HER2 and 90 minutes for NCoR). Membranes were incubated for 60 minutes in blocking buffer (5% nonfat dry milk, 0.1% Tween in PBS) at room temperature and subsequently with primary antibody, rabbit anti-human Ets-1 (1 μg/mL), rabbit anti-human Ets-2 (1 μg/mL), rabbit anti-human AIB1 (2 μg/mL), goat anti-human SRC-1 (2 μg/mL), rabbit anti-human NCoR (2 μg/mL), or mouse anti-human HER2 (1/100; Serotec, Raleigh, NC) in blocking buffer overnight at 4°C. The membranes were washed before incubation with the corresponding horseradish peroxidase secondary antibody (Santa Cruz Biotechnology; 1 in 2,000) in blocking buffer for 60 minutes at room temperature. The membranes were washed and developed with either chemiluminescence (Santa Cruz Biotechnology) for Ets-1, Ets-2, and HER2 or intensified luminescence (Pierce). Jurkat nuclear cell lysates and membranes were subsequently normalized for protein content.

**Electrophoretic Mobility Shift Assays.** Nuclear protein extracts were obtained using a Ne/Per kit according to the manufacturer's instructions (Pierce). For electrophoretic mobility shift assay, 1 μg of nuclear extract was incubated for 30 minutes in the presence of 20 mmol/L HEPES (pH 7.9), 5 mmol/L MgCl₂, 20% glycerol, 100 mmol/L KCl, 0.2 mmol/L EDTA, 8% Ficoll, 600 mmol/L KCl, 500 ng/μL poly(deoxyinosinic-deoxycytidylic acid), 50 mmol/L dithiothreitol, and [α-32P]dCTP-labeled double-stranded oligonucleotide for Ets response element. Oligonucleotides were designed to incorporate the native human HER2/ERBB2 (NM 001005852) promoter (−287 to −270) 5’-CATGCGCTAGGAATTTACTC-3’, with the consensus sequence of Ets binding elements underlined. For supershift experiments, antibodies against Ets-1, Ets-2, AIB1, SRC-1, and NCoR were added after the initial incubation, and samples were then incubated for a further 20 minutes. The samples were electrophoresed through a 5.5% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. For competition studies the reaction was done as described with 50× molar excess of unlabelled probe. Supershift negative controls were done using matched IgG control.

To determine the relative expression of coregulatory proteins at the Ets response element, electrophoretic mobility shift assay gels were transferred to a nitrocellulose membrane (250 mA for 80 minutes) and were subsequently immunoblotted with antibodies directed against AIB1, SRC-1, and NCoR.

**Immunoprecipitation.** Complex formation between Ets-1, Ets-2, and the coregulatory proteins was examined by using breast tumor cell lysates. Whole-cell lysates were prepared as described above. Fifty micrograms of the lysate was immunoprecipitated with 2 μg of either anti-AIB1, SRC-1, or NCoR (Santa Cruz Biotechnology) for 60 minutes at 4°C. The precipitates were collected for 1 hour on protein A/G-agarose (Santa Cruz Biotechnology). After washing with radioimmunoprecipitation assay buffer, precipitates were resuspended in Laemmli SDS sample buffer and resolved on 12% SDS-PAGE. After transfer to nitrocellulose membrane the proteins were probed with either anti–Ets-1 or anti–Ets-2 (both 1 μg/mL), followed by the corresponding peroxidase-conjugated secondary antibody (1 in 2,000). Labeled bands were detected by using intensified luminescence (Pierce). Jurkat nuclear cell lysates and matched IgG were used as positive and negative controls respectively.

**Clinicopathologic Parameters.** Variables analyzed included tumor grade, axillary nodal status, and ER status. A recurrence was defined as any local (chest wall) or systemic recurrence during the follow-up period.

**Statistical Analysis.** Statistical analysis was carried out using the Fisher’s exact test for categorical variables to compare two proportions. Kaplan-Meier estimates of survival functions were computed and the Wilcoxon test was used to compare survival curves. In addition, the Wilcoxon rank sum test was used to compare two medians. Two-sided P values of <0.05 were considered to be statistically significant.

**RESULTS**

**Localization of Ets-1, Ets-2, SRC-1, AIB1, and NCoR in Human Breast Cancer.** The transcription factors Ets-1 and Ets-2, the p160 coactivators SRC-1 and AIB1 and the corepressor NCoR were localized within paraffin-embedded human breast tissue using immunohistochemistry. Both Ets-1 and Ets-2 were detected within the nuclei of invasive ductal and invasive lobular tumor epithelial cells, whereas expression within the cytosol was negligible (Fig. 1A). As previously reported, both SRC-1 and AIB1 were found to be expressed in the nuclei of breast epithelial cells, predominantly in those of the duct (3). Expression of NCoR was found in the nuclei of tumor epithelial cells; however, scant staining was also detected within the cytosol of tumor cells and within the nuclei of surrounding normal breast cells. Immunofluorescence was undertaken to determine if Ets-1 and Ets-2 could be localized to the same breast tumor cell as steroid coregulators SRC-1, AIB1, and NCoR. Both Ets-1 and Ets-2 were found to colocalize with each of the coregulatory proteins in a subset of breast tumor epithelial cells (Fig. 1B).

Ets-1 and Ets-2 were found to be expressed in 52% and 54% of breast tumor patients, respectively. The coactivators SRC-1 and AIB1 were expressed in 22% and 54% of breast tumors, whereas the corepressor NCoR was observed in 45%. Coexpression of Ets-1 with SRC-1, AIB1, and NCoR was detected in 15%, 36%, and 15% of patients respectively.
likewise, coexpression of Ets-2 with SRC-1, AIB1, and NCoR was detected in 21%, 40%, and 18% of patients. There was a significant association detected between the expression of Ets-1 and each of the coactivators, SRC-1 and AIB1 ($P = 0.006$ and $P = 0.003$, respectively), and the corepressor, NCoR ($P < 0.001$).

Similarly, a significant relationship between Ets-2 and each of the coregulatory proteins SRC-1 ($P < 0.001$), AIB1 ($P = 0.015$), and NCoR ($P = 0.012$) was observed.

**Growth Factor Induction of Ets-1 and Ets-2 in Breast Cancer Cells.** The ability of growth factors bFGF and EGF to

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**Fig 1 A.** Immunohistochemical localization of Ets-1 (magnification ×200), Ets-2 (magnification ×200), SRC-1 (magnification ×200), AIB1 (magnification ×200), and NCoR (magnification ×200) counterstained with hematoxylin and matched IgG negative controls in human breast cancer tissue. **B,** immunofluorescent colocalization of Ets-1 with SRC-1 (magnification ×200), Ets-1 with AIB1 (magnification ×200), Ets-1 with NCoR (magnification ×200), Ets-2 with SRC-1 (magnification ×200), Ets-2 with AIB1 (magnification ×200), and Ets-2 with NCoR (magnification ×200).
induce Ets expression in primary breast cancer cells and ER-
negative SKBR3 breast cell lines was determined by Western
blotting. Both Ets-1 and Ets-2 were found to be expressed in
SKBR3 cells and expression of the transcription factor was
found to be increased in the presence of both bFGF and EGF
data not shown). Expression of Ets-1 and Ets-2 could be
detected in primary breast cancer cell cultures derived from
patient tumors. Of tumors found positive for the Ets
transcription factors, increased Ets-1 and Ets-2 expression was
found in a subset of tumors in response to growth factor
treatment (Fig. 2A).

To determine the ability of Ets-1 and Ets-2 to bind to the Ets
response element in the presence of growth factors, bFGF and
EGF, gel shift assays were done. Using oligonucleotide
sequences, which are specific for the HER2 promoter, the ability
of nuclear extracts from nontreated SKBR3 breast cancer cells
(Fig. 2B) and primary breast cancer cell cultures (Fig. 2C) to
bind to the DNA response element was compared with cells
treated with bFGF and EGF. Ets-1 and Ets-2 response element
binding was induced in the presence of both growth factors in
comparison with control. An immunodepletion induced by
preincubation of the nuclear extracts with anti–Ets-1 and anti–
Ets-2 established that both Ets transcription factors were present
at the protein-DNA complex.

The ability of growth factors to induce expression of the
Ets target gene, HER2, was examined. In primary breast cell
cultures derived from patient tumors, which had a positive
HER2 status and expressed both Ets-1 and Ets-2, HER2 protein
expression was increased in response to treatment with EGF
(Fig. 2D).

![Fig. 2](https://example.com/fig2.png)

**Fig. 2**  **A**, Western blot analysis of Ets-1 and Ets-2 protein levels in primary breast cultures. Illustrative blots of primary tumor response to stimulation (Stim) with EGF (10 ng/mL). Positive controls, Jurkat cells, for Ets-1 and Ets-2 (+). Relative absorbance of Ets-1 and Ets-2 immunoblots were obtained (Eagle Eye, Stratagene, La Jolla, CA), absorbance readings of control values were normalized to 1 and treated groups were expressed as a ratio. Results are expressed as mean ± SE (n = 28). Electrophoretic mobility shift analysis of nuclear extracts from the SKBR3 breast tumor cell line (β) and primary breast cancer cultures (C). Nuclear protein extracts from primary breast cancer cells in the presence and absence of bFGF and EGF was compared for increased binding to an [α-32P]dCTP-labeled Ets response element. DNA protein interactions were assayed in the presence of 50× molar excess of homologous oligonucleotide. Nuclear protein extracts were preincubated in the presence of anti–Ets-1 and anti Ets-2. **D**, Western blot analysis of HER2 protein levels in primary tumor cell cultures in response to treatment with EGF (10 ng/mL). Relative absorbance readings of control values were normalized to 1 and treated groups were expressed as a ratio. Results are expressed as mean ± SE (n = 4).
Growth Factor Induced Coregulatory Protein Expression and Recruitment to the Ets Response Element. Growth factor regulation of the coactivators SRC-1 and AIB1 and the corepressor NCoR was assessed in SKBR3 cell lines and in primary breast cell cultures. Protein expression of each of the coregulators was detected in SKBR3 cells and growth factors induced an up-regulation of SRC-1 and AIB1, whereas no regulation of NCoR was observed (data not shown). Protein expression of SRC-1, AIB1, and NCoR was detected in primary breast tumor cell cultures. Of the patients that expressed the coregulatory proteins, a subset of tumors was found to up-regulate SRC-1 and AIB1 expression in response to growth factors; however, no regulation of NCoR was detected in the presence of either bFGF or EGF (Fig. 3A).

The ability of growth factors to induce coregulatory protein recruitment to the Ets response element was investigated using shift analysis in the presence of antibodies directed against SRC-1, AIB1, and NCoR in SKBR3 cells (Fig. 3B) and in primary breast cell cultures (Fig. 3C). SRC-1 was found to complex at the response element in untreated cells and largely in the presence of growth factors bFGF and EGF. AIB1 was found to be recruited to the protein-DNA complex predominantly in the presence of EGF, in both the cell line and in primary cell cultures. In contrast, although NCoR was present at the DNA complex under control conditions, its recruitment was not found to be regulated in the presence of growth factors.

To confirm the relative expression of the coregulators at the transcription factor-response element complex, the DNA protein gels were transferred to a nitrocellulose membrane and immunoblotted with antibodies directed against SRC-1, AIB1, and NCoR (Fig. 3D). Bands detected were found to migrate to the same height as those detected using the radiolabeled Ets response element. In the presence of growth factors, SRC-1 and AIB1 expression was induced at the Ets-response element complex. Conversely, no alteration in NCoR expression at the protein-DNA complex was detected in growth factor–treated cells.

Interactions between Ets-1 and Ets-2 and the coregulatory proteins were investigated using communoprecipitation studies. Both Ets-1 and Ets-2 were found to interact with coactivators AIB1 and SRC-1 in SKBR3 cells (Fig. 4A) and in primary breast tumor cell cultures (Fig. 4B). Increases in protein-protein interaction between Ets-2 and SRC-1 and AIB1 occurred in the presence of bFGF and EGF in both the cell line and in primary cultures; however, no modulation of the Ets-1 coactivator complexes was observed. The corepressor NCoR interacted with both Ets-1 and Ets-2; no regulation of the interaction was detected in growth factor–treated cells compared with control (Fig. 4A and B). Immunoprecipitated SRC-1, AIB1, and NCoR were confirmed by immunoblot using antibodies corresponding to the relevant coregulatory protein (Fig. 4C).

Associations between Expression of Ets Transcription Factors/Coregulatory Proteins and Clinical Variables/Growth Factor Markers. Associations between the qualitative expression of Ets-1, Ets-2, SRC-1, AIB1, and NCoR and clinicopathologic parameters were examined. No relationship between the expression of Ets transcription factors and the coregulatory proteins was observed in relation to tumor grade and axillary node status, with the exception of an association between expression of SRC-1 and tumor grade (P < 0.0038). A significant association was found between disease recurrence and expression of both the transcription factors Ets-1 and Ets-2 (P < 0.0325 and P < 0.0001, respectively) and the coactivator proteins SRC-1 and AIB1 (P < 0.0001 and P < 0.0328, respectively). Conversely, NCoR was found to inversely associate with disease recurrence (P < 0.0325). No relationship was detected between either the transcription factors or the coregulatory proteins and ER status (Table 1).

From Kaplan-Meier estimates of survival, both Ets-1 and Ets-2 proteins were found to significantly associate with time to disease recurrence (P < 0.0292 and P < 0.0001, respectively; N = 134; Fig. 5A and B). In line with our previous findings, a significant relationship between the coactivators AIB1 and SRC-1 and time to recurrence on endocrine treatment was observed (3). Conversely, expression of the corepressor NCoR was found to associate with disease-free survival (P < 0.0297, N = 134; Fig. 5A).

Expression of the Ets target gene, HER2, in breast tumor tissue was determined (Fig. 5B). In line with previous observations, a significant association was detected between the coactivators SRC-1 and AIB1 and the growth factor receptor, HER2 (3). In this study Ets-1 and Ets-2 were found to associate with HER2 (P < 0.0137 and P < 0.0006, respectively), whereas an inverse relationship was observed between the expression of the corepressor NCoR and HER2, (P < 0.0001; Table 1). To assess associations between expression of Ets transcription factors and coregulatory proteins and an activated growth factor pathway, expression of the MAPK kinase kinase protein phospho-Raf was examined (Fig. 5B). Phospho-Raf was associated with Ets-1 and Ets-2 (P < 0.0002 and P < 0.0001, respectively) and the coactivators SRC-1 and AIB1 (P < 0.0001 and P < 0.0001) and inversely associated with the NCoR (P < 0.0024).

The ability of breast cancer cells derived from patient tumors to regulate Ets-1 and Ets-2 protein expression in the presence of growth factors was related to clinicopathologic parameters. Up-regulation of Ets-1 and Ets-2 was detected in 60% and 62% of tumors, respectively. Relative increases in Ets protein expression are given in Table 2. Growth factor induction of Ets-1 expression was found to significantly associate with tumor grade (P < 0.0013).

Coexpression of Ets Transcription Factors and Coactivator Proteins. From our molecular observations of inducible interactions between Ets-2 and the coactivator proteins, we looked for associations between disease recurrence and coexpression of Ets-2 and the coactivators SRC-1 and AIB1. Coexpression of Ets-2 and SRC-1 significantly increased the rate of recurrence, compared with patients who expressed Ets2, but not SRC-1 (P < 0.0001, n = 72); however, no significant association between coexpression of Ets-2 and AIB1 was observed (P = 0.2917, n = 72). Furthermore, coexpression of Ets-2 and SRC-1 significantly associated with expression of HER2 (P < 0.0001, n = 72), whereas no association between Ets-2 and AIB1 coexpression and HER2 status was detected (P = 0.5818, n = 72). Coexpression of the Ets transcription factor and coactivator proteins was not found to significantly associate with expression of the MAPK kinase kinase protein, phospho-Raf.
DISCUSSION

Up-regulation of expression of Ets genes has been described in many types of human tumors; expression levels correlate with invasion and metastasis and can be useful in predicting tumor progression in cancer patients. In breast cancer, the Ets transcription factors are induced by, or required for the activation of, several genes involved in angiogenesis and extracellular matrix remodeling (25, 16, 17). Ets-1 transcript

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**Fig. 3 A.** Western blot analysis of SRC-1, AIB1, and NCoR protein levels in primary breast cultures. Illustrative blots of primary tumor response to stimulation with growth factors. Relative absorbance of SRC-1, AIB1, and NCoR immunoblots and absorbance readings of control values were normalized to 1 and treated groups were expressed as mean ± SE (n = 14, n = 14, and n = 5, respectively). Electrophoretic mobility shift analysis of nuclear extracts from the SKBR3 cell line (B) and primary breast cancer cultures (C). Nuclear protein extracts from primary breast cancer cells in the presence and absence of bFGF and EGF was compared for increased binding to an [α-32P]dCTP-labeled Ets response element. DNA-protein interactions were assayed in the presence of 50× molar excess of homologous oligonucleotide. Nuclear protein extracts were preincubated in the presence of anti-SRC-1, anti-AIB1 and anti-NCoR. D, the relative expression of coregulatory proteins at the Ets response element under control conditions and after stimulation with EGF (10 ng/mL) was examined by transfer of the DNA-protein blot to a nitrocellulose membrane and subsequent immunoblotting with either anti–SRC-1, AIB1, or NCoR. Migration was compared with a protein-bound, radiolabeled Ets response element (lane 1). Relative absorbance of SRC-1, AIB1, and NCoR immunoblots and absorbance readings of control values were normalized to 1 and treated groups were expressed as a ratio. Results expressed as mean ± SE (n = 3).
Fig. 4 The ability of Ets-1 and Ets-2 to interact with SRC-1, AIB1, and NCoR in the SKBR3 cell line (A) and in primary cell cultures (B), under control conditions and after stimulation with bFGF (5 ng/mL) or EGF (10 ng/mL), was determined by coimmunoprecipitation. Cell lysates were immunoprecipitated with either anti-SRC-1, anti-AIB1, or anti-NCoR and subsequently immunoblotted with anti–Ets-1 or anti–Ets-2. Positive controls (+Control) for Ets-1 and Ets-2, Jurkat cells, and negative controls, matched IgG (IgG). Relative absorbance of SRC-1, AIB1, and NCoR primary culture immunoblots and absorbance readings of control values were normalized to 1 and treated groups were expressed as mean ± SE (n = 3). C, immunoprecipitated SRC-1, AIB1, and NCoR were confirmed by immunoblot using antibodies corresponding to the relevant coregulatory protein.
levels have been shown to be a strong predictor of poor prognosis (14). Here, we describe a significant association between protein expression of Ets-1 and Ets-2 and time to disease recurrence in a cohort of breast tumor patients and show a relationship between regulation of Ets-1 expression in response to growth factors and tumor grade in primary breast cell cultures. In this study we describe associations between Ets-1 and Ets-2 and coregulatory proteins in human breast cancer. We provide preliminary evidence that Ets proteins can interact and recruit nuclear receptor coregulatory proteins, which may have implications in the transcriptional modulation of Ets target genes.

The function of Ets-1 and Ets-2 is activated by the growth factor receptor–dependent Ras-MAPK signaling pathway (17, 26). Mutation of a threonine residue located within the amino-terminal pointed domains of Ets-1 and Ets-2 has been reported to abolish Ras-responsive enhancement of their transcriptional activities (16). However, in human dermal fibroblast cultures, growth factors such as transforming growth factor–α and epidermal growth factor up-regulated the expression of the Ets target genes and may be of relevance to distinct signaling pathways previously described for Ets-2 (29). This led us to examine coexpression of Ets-2 and coactivator proteins in relation to tumor progression. Coexpression of Ets-2 with the protein SRC/p160 binding region, LXXLL has been described in loop 1 of the Ets domain of Ets proteins, with the exception of PEA3 (23). Taken together, it is attractive to postulate that the Ets family of transcription factors may represent new targets for p160 transcriptional regulation. Here we observed coexpression of the transcription factors Ets-1 and Ets-2 within breast tumor epithelial cells, indicating that these regulatory proteins may have a potential impact on the transcriptional regulation of Ets target genes.

Recent studies have shown that ACTR (AIB1) can stimulate ER1-dependent transcription in a CV-1 cell model (22), introducing the possibility that p160 nuclear coregulatory proteins could function as coactivators for the Ets family of transcription factors. In this study, using primary breast cell cultures derived from patient tumors, we observed a growth factor–dependent recruitment of coactivators SRC-1 and AIB1 to the Ets protein-DNA complex. Growth factors were found to specifically enhance Ets-2 interaction with SRC-1 and AIB1, but not interactions between Ets-1 and the coactivator proteins. Differential coactivator interactions within this subgroup of Ets family members may be important for defining how these factors selectively regulate target genes and may be of relevance to distinct signaling pathways previously described for Ets-2 (29). This led us to examine coexpression of Ets-2 and coactivator proteins in relation to tumor progression.

### Table 1: Comparisons of Ets-1, Ets-2, SRC-1, AIB1, NCoR, and phospho-Raf expression with clinicopathologic parameters and growth factor markers using the Fisher’s exact test

<table>
<thead>
<tr>
<th>Grade</th>
<th>Total (N = 134)</th>
<th>Ets-1 (n = 70), %</th>
<th>P</th>
<th>Ets-2 (n = 72), %</th>
<th>P</th>
<th>AIB-1 (n = 76), %</th>
<th>P</th>
<th>SRC-1 (n = 32), %</th>
<th>P</th>
<th>NCoR (n = 64), %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade III</td>
<td>57</td>
<td>54</td>
<td>0.7279</td>
<td>0.076</td>
<td>60</td>
<td>0.5996</td>
<td>0.0038</td>
<td>39</td>
<td>0.0813</td>
<td></td>
<td></td>
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<tr>
<td>Non grade III Axilla</td>
<td>77</td>
<td>51</td>
<td>0.0100</td>
<td>0.55</td>
<td>55</td>
<td>14</td>
<td>55</td>
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<td></td>
<td></td>
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<tr>
<td>Positive</td>
<td>82</td>
<td>52</td>
<td>0.0001</td>
<td>0.0095</td>
<td>56</td>
<td>0.8347</td>
<td>0.4659</td>
<td>46</td>
<td>0.5377</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>30</td>
<td>50</td>
<td>0.0137</td>
<td>0.0006</td>
<td>77</td>
<td>0.0037</td>
<td>62</td>
<td>&lt;0.0001</td>
<td>21</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>ER status</td>
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<td>53</td>
<td>0.8373</td>
<td>58</td>
<td>0.0995</td>
<td>56</td>
<td>0.8347</td>
<td>0.4659</td>
<td>46</td>
<td>0.5377</td>
<td></td>
</tr>
<tr>
<td>HER2</td>
<td>39</td>
<td>69</td>
<td>0.0137</td>
<td>0.0006</td>
<td>77</td>
<td>0.0037</td>
<td>62</td>
<td>&lt;0.0001</td>
<td>21</td>
<td>&lt;0.0001</td>
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<tr>
<td>Positive</td>
<td>106</td>
<td>47</td>
<td>0.8373</td>
<td>58</td>
<td>0.0995</td>
<td>56</td>
<td>0.8347</td>
<td>0.4659</td>
<td>46</td>
<td>0.5377</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>28</td>
<td>71</td>
<td>0.0325</td>
<td>89</td>
<td>&lt;0.0001</td>
<td>75</td>
<td>0.0328</td>
<td>89</td>
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<td>29</td>
<td>0.0325</td>
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<td>Recurrence</td>
<td>39</td>
<td>69</td>
<td>0.0137</td>
<td>0.0006</td>
<td>77</td>
<td>0.0037</td>
<td>62</td>
<td>&lt;0.0001</td>
<td>21</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>P &lt;0.0001</td>
<td>29</td>
<td>0.0325</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-Raf</td>
<td>52</td>
<td>71</td>
<td>0.0002</td>
<td>86</td>
<td>&lt;0.0001</td>
<td>81</td>
<td>&lt;0.0001</td>
<td>40</td>
<td>0.0001</td>
<td>26</td>
<td>0.0024</td>
</tr>
<tr>
<td>Negative</td>
<td>82</td>
<td>37</td>
<td>0.8373</td>
<td>58</td>
<td>0.0995</td>
<td>56</td>
<td>0.8347</td>
<td>0.4659</td>
<td>46</td>
<td>0.5377</td>
<td></td>
</tr>
</tbody>
</table>
coactivator protein SRC-1 associated with expression of the Ets target gene HER2 and reduced disease-free survival, compared with patients who expressed Ets-2 alone. Coexpression of AIB1, however, had no effect on Ets-2–related disease progression. In line with these observations implicating a functional consequence of Ets-2 and SRC-1 coexpression, studies in mice have shown that expression of only one wild-type Ets-2 gene results in reduced breast tumor size, and loss of SRC-1 function is associated with resistance to endocrine hormones (30, 31).

Classically, both Ets-1 and Ets-2 were thought to function exclusively as transcriptional coactivators; however, recent studies suggest that both of these Ets subfamily members can also act as repressors of gene expression (27, 32). Moreover, Ets-2 interactions with the chromatin remodeling complex SW1/SNF has been shown to be central to the silencing of the tumor repressor gene BRCA1 (32). Although few studies have addressed the role of corepressors in breast tumor progression, Kurebayashi et al. have showed that NCoR and its close family

![Figure 5 A](https://example.com/fig5a.png)

**Fig. 5 A.** Kaplan-Meier estimates of disease free survival (N = 134). Disease-free survival according to Ets-1, Ets-2, and NCoR expression. B, immunohistochemical localization of HER2 protein (magnification ×200), fluorescent in situ hybridization of HER2 gene amplification (magnification ×200), and immunohistochemical localization of phospho-Raf in primary breast tumor tissue (magnification ×200). C, disease-free survival according to SRC-1 and AIB1 expression in Ets-2–positive breast tumor patients (n = 72).

| coactivator protein SRC-1 associated with expression of the Ets target gene HER2 and reduced disease-free survival, compared with patients who expressed Ets-2 alone. Coexpression of AIB1, however, had no effect on Ets-2–related disease progression. In line with these observations implicating a functional consequence of Ets-2 and SRC-1 coexpression, studies in mice have shown that expression of only one wild-type Ets-2 gene results in reduced breast tumor size, and loss of SRC-1 function is associated with resistance to endocrine hormones (30, 31). Classically, both Ets-1 and Ets-2 were thought to function exclusively as transcriptional coactivators; however, recent studies suggest that both of these Ets subfamily members can also act as repressors of gene expression (27, 32). Moreover, Ets-2 interactions with the chromatin remodeling complex SW1/SNF has been shown to be central to the silencing of the tumor repressor gene BRCA1 (32). Although few studies have addressed the role of corepressors in breast tumor progression, Kurebayashi et al. have showed that NCoR and its close family

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Relative levels of protein expression of Ets-1 and Ets-2 in primary breast tumor cell cultures in the presence of growth factors (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. positive patients</td>
<td>Ets-1stimulated−Ets-1control</td>
</tr>
<tr>
<td>Grade 3</td>
<td>474 (314-540)</td>
</tr>
<tr>
<td>Non-grade 3</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>Tumour size, median (range)</td>
<td>338 (270-490)</td>
</tr>
<tr>
<td>&gt;2.5 mm</td>
<td>183 (0-480)</td>
</tr>
<tr>
<td>&lt;2.5 mm</td>
<td></td>
</tr>
</tbody>
</table>

Note: Comparisons analyzed using Wilcoxon rank sum test.

![Table 2](https://example.com/table2.png)
member SMRT are up-regulated in intraductal carcinomas compared with normal mammary glands (33). It has been suggested that loss of corepressor protein may be relevant to the development of a more aggressive, hormone-unresponsive cancer (34). In this study, expression of the corepressor NCoR was found to significantly associate with disease-free survival. Furthermore, an inverse relationship was observed between NCoR and the growth factor receptor HER2. We found that NCoR could colocalize with both Ets-1 and Ets-2 in our cohort of breast tumor patients and looked for a role for NCoR in Ets-mediated transcription in primary breast tumor cell cultures. We established that NCoR can be recruited to the Ets transcription factor-DNA complex and that NCoR could interact with both Ets-1 and Ets-2 under basal conditions. Unsurprisingly, no increase in DNA recruitment or transcription factor corepressor interactions were seen in the presence of the growth factors bFGF and EGF. In line with these findings NCoR protein expression was unaltered in the presence of either bFGF or EGF.

The role of coregulatory proteins in breast tumor development has gained much attention over recent years, particularly in relation to resistance to endocrine treatment. We have previously described associations between the coactivators SRC-1 and AIB1 and disease recurrence in breast tumor patients. Here, we observed a positive relationship between the corepressor NCoR and disease-free survival. We suggest that these coregulatory proteins may play a central role in the evolution of steroid-independent tumors. Specifically, we propose that nuclear receptor coregulatory proteins may interact with nonsteroid receptor transcription factors to mediate endocrine-independent growth. As such, the MAPK effector transcription factors Ets-1 and Ets-2 are attractive targets for coregulatory protein interactions. Associations between Ets and coregulatory protein expression and reduced disease-free survival, along with preliminary evidence of Ets transcription factor coregulatory protein interactions described in this study, are suggestive of a role for these proteins in breast tumor progression.

REFERENCES


Correction: Associations and Interactions between Ets-1 and Ets-2 and Coregulatory Proteins, SRC-1, AIB1, and NCoR in Breast Cancer

In this article (Clin Cancer Res 2005;11:2111–22), which was published in the March 15, 2005, issue of Clinical Cancer Research (1), several images in Figs. 2A, 3A, and 4A were inadvertently duplicated by the authors. The authors repeated the experiments represented in the abovementioned figures. Western blot studies in endocrine sensitive and insensitive breast cancer cells confirmed an upregulation of the transcription factors Ets-1 and Ets-2 and the coactivator SRC-1 in response to treatment with EGF. In addition, immunoprecipitation studies confirmed interactions between Ets-1 and SRC-1, as well as N-CoR and interactions between Ets-2 and N-CoR. The authors claim that these experiments confirm the results presented in the original article. The corrected versions of Figs. 2A, 3A, and 4A are below. The authors regret this error.

**Figure 2.**
Western blot analysis of Ets-1 and Ets-2 protein levels in breast cancer cells. Illustrative blots of breast cancer cells. Responder (endocrine sensitive MCF7) and Non Responder (endocrine insensitive SKBR3 cells) response to stimulation (Stim) with EGF (10 ng/mL) for 24 hours. Immunoblot carried out using rabbit anti-human Ets-1 (1 mg/mL) or rabbit anti-human Ets-2 (1 mg/mL) followed by corresponding horseradish peroxidase (1:2,000).

**Note:** The original work used primary breast cancer cultures and was carried out in 2002–2004, when surgical practices were different and wide local excision of primary tumors was common, which enabled collection of sufficient material to conduct functional molecular studies. Patients now undergo routine screening and present with smaller tumors. This, in combination with breast conserving surgery, means that primary tumor material is no longer available to undertake these studies. The cell lines used here provide a similar model of patients who respond (MCF7, endocrine sensitive) or not (SKBR3, endocrine insensitive) to therapy. These studies faithfully represent the original findings in the patient primary cells.

**Figure 3.**
Western blot analysis of SRC-1 protein levels in breast cancer cells. Illustrative blots of breast cancer cells Responder (endocrine sensitive MCF7) and Non Responder (endocrine insensitive SKBR3 cells) response to stimulation (Stim) with EGF (10 ng/mL) for 24 hours. Immunoblot carried out using rabbit anti-human SRC-1 (2 mg/mL) followed by corresponding horseradish peroxidase (1:2,000).

**Note:** In the original manuscript a small increase was detected between control and stimulated in the non-responder cells. In this repeated experiment, higher levels of the protein were detected. However, greater differences were seen between control and stimulated in the sensitive cells in line with the original findings. Additionally, the original work used primary breast cancer cultures and was carried out in 2002–2004, when surgical practices were different and wide local excision of primary tumors was common, which enabled collection of sufficient material to conduct functional molecular studies. Patients now undergo routine screening and present with smaller tumors. This, in combination with breast conserving surgery, means that primary tumor material is no longer available to undertake these studies. The cell lines used here provide a similar model of patients who respond (MCF7, endocrine sensitive) or not (SKBR3, endocrine insensitive) to therapy. These studies faithfully represent the original findings in the patient primary cells.
Reference


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Figure 4.
Ets-1 and Ets-2 interaction with SRC-1 and N-CoR in the SKBR3 cell line under control conditions and after stimulation with bFGF (5 ng/mL) or EGF (10 ng/L) was determined by coimmunoprecipitation. Cell pellets were immunoprecipitated with either anti-SRC-1 or anti-N-CoR and subsequently immunoblotted with anti-Ets-1 or anti-Ets-2.
Associations and Interactions between Ets-1 and Ets-2 and Coregulatory Proteins, SRC-1, AIB1, and NCoR in Breast Cancer


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