

# Estrogen-mediated Down-Regulation of E-cadherin in Breast Cancer Cells<sup>1</sup>

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## Abstract

E-cadherin is an important mediator of cell-cell interactions, and has been shown to play a crucial role in breast tumor suppression. Its inactivation occurs through instability at its chromosomal locus and mutations, but also through epigenetic mechanisms such as promoter hypermethylation and transcriptional silencing. We show here that the potent mitogen estrogen causes down-regulation of E-cadherin levels in both normal and tumorigenic breast epithelial cells, and that this down-regulation is reversed by antiestrogens. The reduction in E-cadherin levels is via a decrease in promoter activity and subsequent mRNA levels. Chromatin immunoprecipitation assays revealed that estrogen receptor and corepressors were bound to the E-cadherin promoter, and that overexpression of corepressors such as scaffold attachment factor B resulted in enhanced repression of E-cadherin. We propose that estrogen-mediated down-regulation of E-cadherin is a novel way of reducing E-cadherin levels in estrogen receptor-positive breast cancer.

## Introduction

E-cadherin is a glycoprotein with a large extracellular domain, a transmembrane domain, and a short intracellular domain that interacts with catenins. Recently there has been increased interest in E-cadherin as a mediator of cell-cell adhesions and as a tumor suppressor gene (reviewed in Ref. 1). E-cadherin maps to a region on chromosome 16q22.1 that shows frequent loss of heterozygosity in sporadic breast cancer. Although loss of heterozygosity-concurrent mutations have been found on the second allele in lobular breast tumors, very few mutations have been found in ductal breast carcinoma (1). This finding suggests that other epigenetic mechanisms such as hypermethylation and transcriptional silencing might play a role in E-cadherin inactivation. Indeed, methylation of the E-cadherin promoter has been shown to correlate with loss of E-cadherin expression in breast cancer cell lines and primary ductal and lobular breast cancers (2, 3). However, the decrease of E-cadherin expression is not simply attributable to hypermethylation, because treatment with 5-aza-2'-deoxycytidine fails to reactivate E-cadherin expression (4). Increased internalization and degradation via Hakai overexpression (5), as well as overexpression of transcriptional repressors known to inactivate the E-cadherin promoter such as Snail (6, 7) and SIP1/ZEB2 (8), are alternative mechanisms for its inactivation in breast tumors. Indeed, a recent study by Fujita *et al.* (9) showed that aberrant expression of

Snail in ER<sup>3</sup>  $\alpha$  (called ER throughout the manuscript)-negative breast cancer cell lines results in the loss of E-cadherin expression.

In this report, we show that, in ER-positive breast cancer cell lines, the steroid hormone E<sub>2</sub> down-regulates levels of E-cadherin protein and mRNA. This down-regulation can be reversed by antiestrogens used in the clinical management of breast cancer. We have evidence that the observed down-regulation depends not only on ER but also on the cross-talk with other pathways because it can be observed only when cells are kept in serum-containing media, not in serum-free media. The down-regulation involves direct recruitment of ER and ER corepressors at the most proximal E-cadherin promoter. This study is intriguing because (a) few estrogen-down-regulated genes have been described to date; (b) it provides evidence for a direct involvement of ER-corepressors (such as SAFB) in estrogen-mediated down-regulation of genes; and, finally (c) it presents a novel mechanism for E-cadherin inactivation in breast tumors.

## Materials and Methods

**Cells, Transfections, and CAT Assay.** Human breast cancer cells (MCF-7L, MDA-MB-231, MDA-MB-435, T47D, ZR75) were maintained in IMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), 200 IU/ml penicillin, 200  $\mu$ g/ml streptomycin, and 6 ng/ml insulin. The immortalized human breast epithelial MCF10A cells were kept in DMEM/F12 medium supplemented with 5% horse serum, 10 ng/ml insulin, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 2 mM glutamine, 500 ng/ml hydrocortisone, 200 IU/ml penicillin, and 200  $\mu$ g/ml streptomycin. To express ER in these cells,  $5 \times 10^5$  cells were plated in a 6-cm dish and were transfected with 1  $\mu$ g of HA-tagged ER (ER-HA-pcDNA3.1) for 5 h. To generate the ER-HA-pcDNA3.1 plasmid, we released the ER-HA cDNA from pcDNA3.1/V5/His-TOPO (10) with *EcoRI*, and ligated it into pcDNA3.1. After a 24-h recovery, the cells were placed in phenol red-free IMEM containing 5% CSS for an additional 24 h and then stimulated with  $10^{-8}$  M E<sub>2</sub> for another 24 h before being lysed in 5% SDS for subsequent immunoblotting (see "Western and Northern Blot Analysis" below). The experiments were performed three independent times.

For reporter assays, cells were transiently transfected using Fugene (Roche, Indianapolis, IN) following the manufacturer's protocol. One day before transfection, cells were plated at  $2 \times 10^5$  in 6-well plates. For E<sub>2</sub> induction experiments, the cells were treated either in SFM, which consisted of phenol red-free IMEM + 10 mM HEPES (pH 7.4) + 1  $\mu$ g/ml fibronectin (Invitrogen, Carlsbad, CA) + trace elements (Biosources, Worcester, MA) + 1  $\mu$ g/ml transferrin (Invitrogen) or in phenol red-free IMEM containing 5% CSS (Hyclone). Most E<sub>2</sub> induction experiments were performed at least twice; the experiments in MCF-7L cells were performed at least three times each. For the promoter analysis, 1  $\mu$ g of E-cadherin promoter (−178/+92 bp) CAT construct (11) was transfected, and 24 h later, the medium was replaced with IMEM + 5% CSS containing the appropriate ligand. Forty-eight h later, cells were washed twice with PBS, and CAT activity was measured using the CAT

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<sup>3</sup> The abbreviations used are: ER, estrogen receptor ( $\alpha$ ); IMEM, Iscove's MEM; CSS, charcoal-stripped serum; SFM, serum-free medium; E<sub>2</sub>, estradiol; IRS-1, insulin-receptor substrate 1; PgR, progesterone receptor; ChIP, chromatin immunoprecipitation; TAM, 4-hydroxytamoxifen; SAFB, scaffold attachment factor B; SAGE, serial analysis of gene expression; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ELISA from Roche (Indianapolis, IN). Values were corrected for protein concentrations and are presented as relative CAT activity. For transient transfections, triplicate samples were measured in each experiment. The data are presented as the average  $\pm$  SE and are representative of three independent experiments.

**SAFB1 Overexpression in MCF-7 Cells.** To transiently overexpress SAFB1, subconfluent MCF-7L cells plated in a 10-cm dish were transfected overnight with 2  $\mu$ g of SAFB1-HA-pcDNA1 (12, 13) using Fugene (Roche, Indianapolis, IN) following the manufacturer's protocol. The next morning, the medium was changed, and 24 h later, the cells were lysed in 5% SDS. The generation of MCF-7 cells expressing inducible HA-SAFB1 has recently been described elsewhere (13).

**Western and Northern Blot Analyses.** Proteins were resolved on 8% SDS-PAGE, and electrophoretically transferred to nitrocellulose. The membrane was blocked in PBS/0.1% Tween 20 (PBST) + 5% milk for 1 h at room temperature. Antibodies to E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), IRS-1 (Upstate Biotechnology Inc., Waltham, MA), HA (Covance), SAFB (Upstate Biotechnology Inc.), PgR (Santa Cruz Biotechnology), ER (Vector, Novacastra, Burlingame, CA), and  $\beta$ -actin (Sigma, St. Louis, MO) were diluted at 1:1000, 1:1000, 1:1000, 1:500, and 1:5000, respectively, in PBST + 5% milk. After washing six times for 5 min each time with PBST, the membrane was incubated with horseradish peroxidase-linked antimouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ) at 1:1000 in PBST + 5% milk and washed six times for 5 min each time, and the signal was developed using enhanced chemiluminescence according to the manufacturers instructions (Pierce, Rockford, IL).

For Northern blots, 10  $\mu$ g of total RNA were separated by electrophoresis in a 1.2% formaldehyde-agarose gel. RNA isolation (CsCl gradient) and Northern blotting was performed after standard procedures. The human E-cadherin probe for hybridization was purchased from Research Genetics (Clone ID 2286727), and fold changes in RNA levels were determined using software on the Molecular Imager FX (Bio-Rad). The presented Northern blot is representative of two independent experiments.

**ChIP assays.** MCF-7 cells ( $3 \times 10^6$ ) were plated in 15-cm dishes in phenol red-free DMEM supplemented with 10% CSS. After 36 h, the cells were transfected with 250 ng of plasmid DNA (mouse E-cadherin promoter, pCAD-Ecad-3000; Refs. 11, 14) using LipofectAMINE and following the manufacturer's protocol. The next morning, the cells were treated with vehicle only,  $10^{-8}$  M  $E_2$ , or  $10^{-6}$  M TAM for 45 min. After washing the cells with PBS (three times), they were cross-linked with 1% formaldehyde for 10 min at room temperature. Cells were rinsed three times with ice-cold PBS, were collected into 100 mM Tris-HCl (pH 9.4)-10 mM DTT, were incubated for 15 min at 30°C, and were centrifuged for 5 min at 2000  $\times$  g. Subsequently, cells were washed sequentially with 1 ml of ice-cold PBS, buffer I [0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 6.5)] and buffer II [200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 6.5)]. Cells were then resuspended in 0.3 ml of lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), protease inhibitors], sonicated three times for 10 s each time, followed by centrifugation for 10 min. Supernatants were diluted in 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1), and 250  $\mu$ g were precleared with 2  $\mu$ g of sheared salmon sperm DNA and protein G-Sepharose (40  $\mu$ l of 50% slurry) for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with specific antibodies (2  $\mu$ g protein/each). After immunoprecipitation, 50  $\mu$ l of protein G-Sepharose and 2  $\mu$ g of salmon sperm DNA were added, and the incubation was continued for 1 h. Precipitates were washed sequentially, each in TSE I [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl], TSE II [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl], and buffer III [0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)]. Precipitates were then washed three times with TE buffer [10 mM Tris (pH 8)-1 mM EDTA], extracted with 1% SDS-0.1 M NaHCO<sub>3</sub>, and heated at 65°C for at least 6 h to reverse the formaldehyde cross-linking. After DNA purification (QIAquick Spin kit), the proximal E-cadherin promoter (-234 to +62 bp) was amplified using the following primer set: forward primer, 5'-TCCTTTGTAAGTCCATGTCTCCCGT-3', and reverse primer, 5'-CGGGCAGGAGTCTAGCAGAAG-3'. The PCR of the pS2 promoter was performed as described previously (15). The antibodies for the ChIP assays were purchased from Santa Cruz Biotechnology (ER, N-CoR, rabbit IgG) and from UBI (SAFB). The experiments were performed three times.

**Animals, Treatments, and Tissues.** Animal care was in accordance with institutional guidelines. Female ovariectomized BALB/c athymic nude mice (4–6 weeks old; Harlan Sprague Dawley Inc., Madison, WI) supplemented with estrogen pellets (0.25 mg, Innovative Research, Rockville, MD) were inoculated s.c. with  $5 \times 10^6$  MCF-7 cells, as described previously (16). When tumors reached a diameter of 7–9 mm (2–4 weeks), the animals were randomly allocated to continue estrogen treatment or to discontinue estrogen treatment by removal of the estrogen pellets. In this tumor model, estrogen stimulates tumor growth and estrogen withdrawal results in tumor growth inhibition. Tumors were removed during estrogen treatment ( $E_2$  tumor group) and at 3 weeks after estrogen withdrawal treatment ( $-E_2$  tumor group) and kept at  $-70^\circ\text{C}$  for later analyses. Tumor powders were manually homogenized in a 5% SDS solution. After boiling and microcentrifugation, clear supernatants were collected, protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL), and Western blotting was performed as described above. This *in vivo* experiment was performed once.

## Results

**Estrogen Down-Regulates E-Cadherin Protein Levels in MCF-7 Cells *in Vitro* and *in Vivo*.** To analyze whether E-cadherin protein levels are regulated by  $E_2$ , we placed ER-positive MCF-7L cells in a medium containing CSS and treated them with  $E_2$  at a concentration ranging from  $10^{-12}$  to  $10^{-7}$  M for 24 h. Immunoblot analysis was performed using E-cadherin-specific antibodies, as well as  $\beta$ -actin antibodies for a loading control. As shown in Fig. 1A,  $E_2$  treatment resulted in a dose-dependent decrease of E-cadherin. Interestingly, we never observed this  $E_2$ -mediated down-regulation of E-cadherin in the absence of serum, *i.e.* when the cells were kept in SFM (Fig. 1B). This result suggests that the  $E_2$ -mediated down-regulation of E-cadherin depends on other factors present in the serum, possibly "cross-talking" with ER.

Next we asked whether the down-regulation could be reversed by antiestrogens. Therefore, we treated MCF-7 cells with  $E_2$  only, with the nonsteroidal antiestrogen TAM only, or with a combinations of both (Fig. 1C). As expected, the addition of antiestrogen to  $E_2$ -treated cells blocked E-cadherin down-regulation, reflecting the inactivation of ER activity. We observed the same effect with the pure steroidal antiestrogen ICI 182,780 (data not shown). As a control we immunoblotted for the estrogen-inducible proteins IRS-1 and PgR, levels of which were both potently increased by estrogen. Additionally, confirmation of ER function was shown by the down-regulation of ER (Fig. 1C) which is known to be degraded by  $E_2$  and stabilized by TAM (17).

Interestingly, treatment with TAM alone increased levels of E-cadherin protein over baseline, reflecting the inhibitory effects of the residual  $E_2$  in the CSS. Indeed, CSS can contain up to  $10^{-11}$  M  $E_2$  (data sheet from manufacturer). Further supporting this is our finding that increasing amounts of CSS resulted in a dose-dependent decrease of E-cadherin levels (Fig. 1D). We think that this is attributable to residual  $E_2$  because (a) it can be reversed by TAM (Fig. 1C and data not shown); and (b) treatment with a range of growth factors such as epidermal growth factor, insulin-like growth factor-I (IGF-I), or heregulin in SFM did not lower E-cadherin levels (data not shown).

Next we asked whether this estrogen-mediated down-regulation of E-cadherin in MCF-7 cells in tissue culture could also be observed when MCF-7 cells were grown as xenografts in athymic mice. Therefore, athymic ovariectomized mice were given injections of MCF-7 cells, and tumors were allowed to grow in the presence of  $E_2$  ( $+E_2$ ). When the tumor reached 7–9 mm (2–4 weeks), the pellet was removed ( $-E_2$ ), and the tumors stopped growing, as reported previously (16). We analyzed E-cadherin expression in estrogen-stimulated ( $n = 4$ ) and estrogen-deprived tumors ( $n = 4$ ). As shown in Fig. 1E, the E-cadherin levels were significantly lower in the  $+E_2$  group as compared with the  $-E_2$  group (Fig. 1E). Thus, estrogen treatment

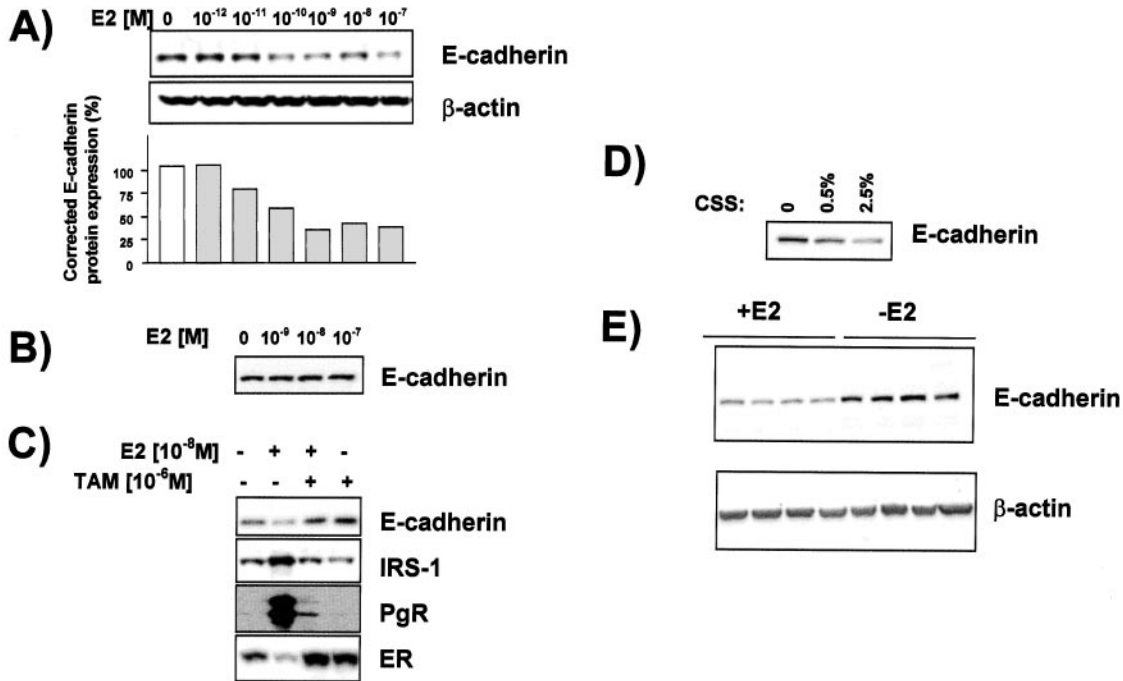


Fig. 1. Effect of  $E_2$  and antiestrogen on E-cadherin protein levels in MCF-7 breast cancer cells grown in tissue culture and *in vivo* (xenograft). Proteins were extracted from MCF-7 cells and immunoblotting was performed using specific antibodies to E-cadherin (A through E),  $\beta$ -actin (A and E), IRS-1 (C), PgR (C), and ER (C). After enhanced chemiluminescence (ECL), images were captured using a CCD video camera (Fluorimager 8000; Alpha Innotech), and pixel intensity values were obtained with this machine. Values for E-cadherin were corrected for loading by dividing the E-cadherin pixel intensity by the  $\beta$ -actin pixel intensity. A, cells were grown for 48 h in 5% CSS and then were treated with increasing concentrations of  $E_2$  for 24 h. B, cells were grown in SFM and then were treated with  $E_2$  for 24 h. C, cells were grown for 48 h in 5% CSS and then were treated with  $E_2$  and/or TAM for 24 h. D, cells were grown for 24 h in medium supplemented with increasing amounts of CSS. E, flash-frozen MCF-7 xenografts, grown in the presence and absence of  $E_2$ , were pulverized, and SDS extracts were analyzed by immunoblotting.

results in down-regulation of E-cadherin protein not only in MCF-7 cells grown in tissue culture but also *in vivo*. Although numerous (direct and indirect) factors can influence gene expression in an *in vivo* situation, we think that these data, together with our *in vitro* experiments, strongly support an estrogen-mediated down-regulation of E-cadherin.

**Estrogen Down-Regulates E-cadherin Levels in Both Normal and Transformed Breast Epithelial Cell Lines.** To exclude the possibility that the effect seen in MCF-7 was cell line-specific, we measured E-cadherin levels in two ER-negative breast cancer cell lines (MDA-MB231 and MDA-MB-435) and two other ER-positive breast cancer cell lines (T47D and ZR75). There was no expression in the ER-negative cell lines (data not shown), a finding that was recently described and analyzed by Fujita *et al.* (9). However, in the ER-positive cell lines, we detected estrogen-mediated down-regulation of E-cadherin, which was reversed by antiestrogen treatment (Fig. 2A).

We next asked whether this repression is specific to transformed cells, or whether it could also be found in cell lines with less severe genetic abnormalities. Because normal or immortalized breast epithelial cell lines do not express ER, we transiently transfected immortal but nontransformed MCF10A cells with ER to study E-cadherin regulation (Fig. 2B). Transient transfection of these cells with a green fluorescent protein-tagged ER construct revealed transfection of up to 10% of cells (data not shown). No ER was detected in cells transfected alone. Stimulation of ER-transfected cells with  $E_2$  resulted in an increase in expression of the estrogen-regulated gene *IRS-1*, and a minor decrease in ER levels. This confirmed that the ER was active in these cells. Furthermore, these cells also showed a decrease in E-cadherin levels. In other experiments, the transient expression of ER alone (*i.e.*, not simulated with  $E_2$ ) also caused a down-regulation, which is presumably caused by residual  $E_2$  action; however, decreased E-cadherin levels were always noted after  $E_2$  stimulation. Therefore, estrogen-medi-

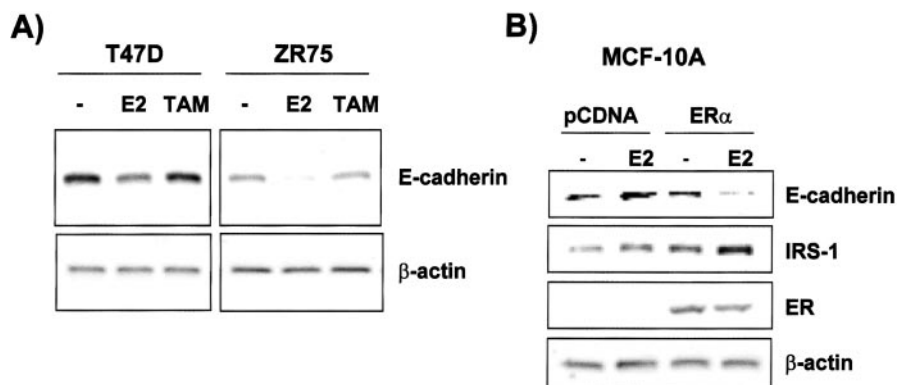


Fig. 2. Estrogen regulation of E-cadherin in immortalized and breast cancer cells. A, ER-positive breast cancer cell lines T47D and ZR75 were incubated in 5% CSS for 48 h, and, subsequently, were treated for 24 h with vehicle only,  $E_2$  ( $10^{-8}$  M), or  $E_2$  ( $10^{-8}$  M) and tamoxifen ( $10^{-6}$  M) in 5% CSS.  $\beta$ -actin was used as a loading control. B, immortalized MCF10A cells were transfected with ER-HA-pcDNA1 and were treated with  $E_2$  ( $10^{-8}$  M; + $E_2$ ) for 24 h. Cells transfected with empty vector only (pcDNA) served as negative control. SDS extracts were prepared and immunoblotted with antibodies as indicated.

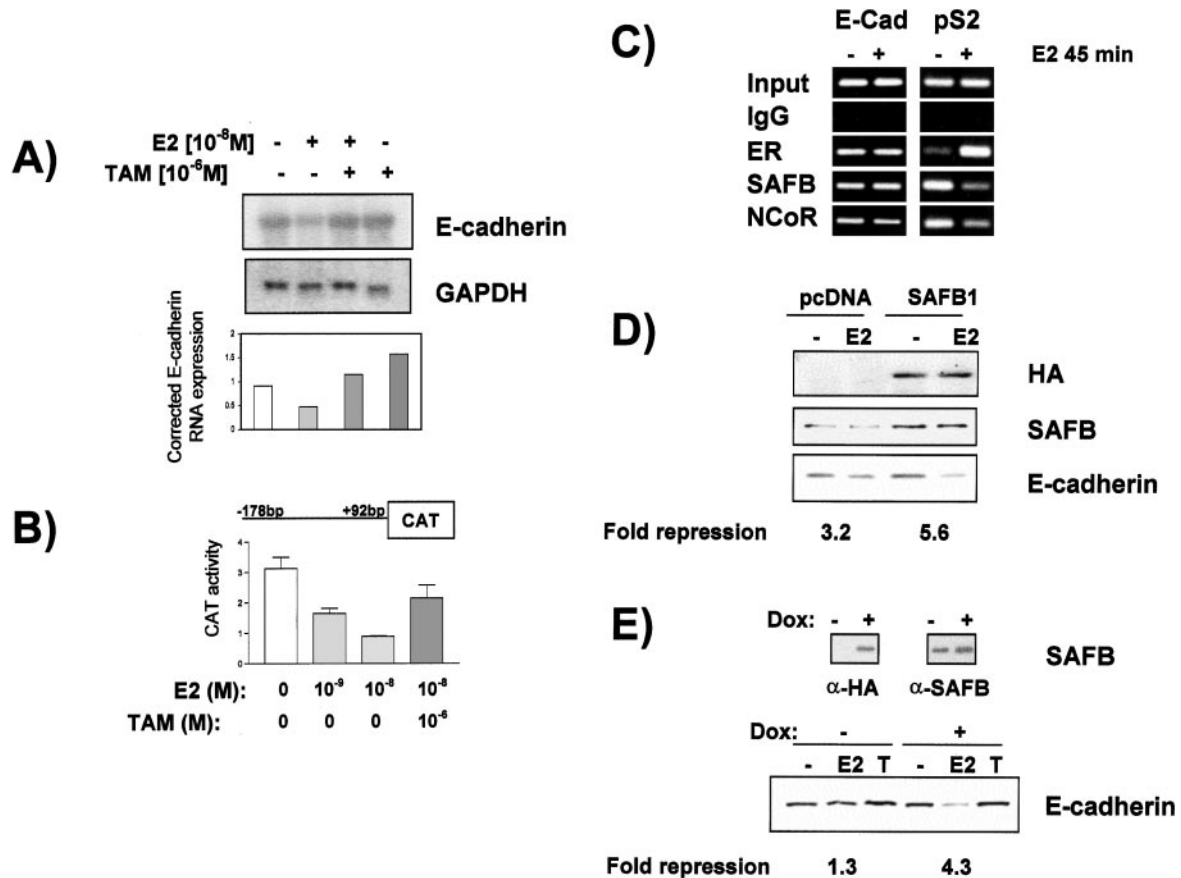


Fig. 3. Effect of E<sub>2</sub> on transcriptional regulation of E-cadherin. *A*, MCF-7L cells were treated with E<sub>2</sub> and tamoxifen for 10 h. After Northern blot analysis, data were quantified using a phosphorimager and were corrected for GAPDH. *B*, MCF-7L cells were transiently transfected with the E-cadherin promoter construct (-178/+92) and were treated for 24 h and CAT activity was measured. *C*, for the ChIP analysis, MCF-7 cells were transfected with the estrogen-responsive E-cadherin promoter. The next day, cells were treated for 45 min with vehicle only or with 10<sup>-8</sup> M E<sub>2</sub>, were cross-linked, and were subjected to immunoprecipitation and PCR as described in "Materials and Methods." *D*, MCF-7L cells were transiently transfected with vector only, or with SAFB1pcDNA1, and were treated with E<sub>2</sub> for 24 h. SDS extracts were immunoblotted, as indicated. E-cadherin levels were quantified as described above. MCF-7 cells that express tet-inducible SAFB1 (13) were treated with doxycycline for 48 h, and overexpression was confirmed by immunoblotting with HA and SAFB antibodies. Subsequently, cells were pretreated with doxycycline for 48 h, followed by treatment with vehicle only, 10<sup>-8</sup> M E<sub>2</sub>, or 10<sup>-8</sup> M E<sub>2</sub> and 10<sup>-8</sup> M tamoxifen. E-cadherin levels were quantified as described in Fig. legend 1.

ated repression of E-cadherin levels can be detected in both immortalized breast epithelial and cancer cell lines.

**Estrogen Treatment Results in Decreased E-Cadherin RNA Levels and Promoter Activity.** To determine whether estrogen decreased E-cadherin at the mRNA level, we treated MCF-7L cells with E<sub>2</sub> or a combination of E<sub>2</sub> and antiestrogen for 6 h and then isolated total RNA. Northern blot analysis was performed using an E-cadherin probe, with GAPDH as a loading control. As shown in Fig. 3A, E-cadherin RNA levels were decreased 2-fold in the presence of E<sub>2</sub>. Substantiating the estrogen regulation at the RNA level is the finding that TAM treatment blocked the E<sub>2</sub>-mediated down-regulation and, when given alone, caused an increase in the E-cadherin levels.

This finding encouraged us to analyze whether the E-cadherin promoter might be E<sub>2</sub>-regulated in transient reporter assays. A number of studies have previously been conducted using a series of promoter constructs. We decided to use the most proximal E-cadherin mouse promoter construct (-178/+92 bp) which was previously shown to have strong activity in epithelial cells (11); however, it does not contain any classical estrogen response elements. This construct was transfected into MCF-7 cells, cells were treated with E<sub>2</sub> (10<sup>-9</sup> M and 10<sup>-8</sup> M), or with E<sub>2</sub> (10<sup>-8</sup> M) and TAM (10<sup>-6</sup> M) for 24 h, and CAT activity was measured. As shown in Fig. 3B, promoter activity was decreased in the presence of E<sub>2</sub>, and, again, this repression was relieved by the addition of TAM. Thus, *E-cadherin* is an estrogen-

down-regulated gene, and the down-regulation is mediated through the proximal promoter region.

**ER Corepressor Proteins Are Recruited to the E-Cadherin Promoter, and Corepressor Overexpression Results in Enhanced E-Cadherin Repression.** To analyze whether the decreased promoter activity was the direct result of recruitment of ER and corepressors, we performed ChIP assays. Therefore, we transfected MCF-7 cells with a plasmid containing the E-cadherin fragment known to be repressed on estrogen treatment. The cells were then treated with E<sub>2</sub> for 45 min, and the recruitment of ER and corepressors (N-CoR and SAFB1) was analyzed by ChIP (Fig. 3C) as described in "Materials and Methods." We also examined the recruitment of ER and corepressors at the pS2 promoter. As described previously (15), estrogen treatment resulted in strong recruitment of ER to the pS2 promoter. We repeatedly detected a low level of ER binding in the absence of E<sub>2</sub>, possibly reflecting low levels of E<sub>2</sub> in the stripped serum. Corepressors (N-CoR and SAFB1) showed constitutive binding to the pS2 promoter, which was released on E<sub>2</sub> treatment. In stark contrast to results obtained with the pS2 promoter, the E-cadherin promoter showed strong constitutive binding of ER in the absence of estrogen. E<sub>2</sub> treatment of cells with estrogen for 45 min did not result in release of corepressors, as observed for the pS2 promoter (Fig. 3B).

To directly test whether the corepressors might modulate E-cadherin levels, we transiently overexpressed the ER corepressor

SAFB1, and also generated stable tetracycline-inducible SAFB1 overexpressing breast cancer cell lines (Fig. 3D). Transient overexpression of SAFB1 in MCF-7L cells resulted in stronger estrogen-mediated repression of E-cadherin, as shown in Fig. 3D. We confirmed this data in MCF-7 RTA (“tet on”) cells which consistently showed 3-fold overexpression of SAFB1 on doxycycline treatment (Fig. 3E). Thus, ER corepressor levels are a major determinants in the regulation of *E-cadherin* expression.

## Discussion

In this study, we have shown that *E-cadherin* is an estrogen-down-regulated gene in human breast cancer cells. A number of studies in various tissues and cell lines have previously described connections between steroid receptor pathways and E-cadherin. Prinsac *et al.* (18) and Habermann *et al.* (19) have shown that developmental exposure to estrogen was associated with changes in epithelial cell adhesion and decreased E-cadherin levels in the adult rat prostate. E<sub>2</sub> treatment resulted in a decrease of N-cadherin (20) and increase of E-cadherin (21) levels in the mouse ovary. In breast cancer cells, estrogen treatment was reported to induce cytoskeletal rearrangements (22) including delocalization of E-cadherin (23). Interestingly, tamoxifen restored the function of E-cadherin in an MCF-7 subline with a functionally inactive cell surface E-cadherin (MCF-7/6); however, this was an extremely rapid event (30 min) and did not require protein synthesis (24). Thus, although a number of reports have addressed a potential effect of estrogen on E-cadherin, our study represents the first attempt to investigate estrogen-mediated down-regulation of E-cadherin as a novel mechanism of its inactivation in human breast cancer.

In contrast to the well-characterized estrogen induction of a number of genes, estrogen-mediated down-regulation of genes has only recently gained more attention. In a SAGE study using estrogen-treated MCF-7 cells, an equal number of induced and repressed genes were identified (25). A recent study has shown that transcription of the nuclear coactivator src-3/AIB1 (amplified in breast cancer) is repressed by E<sub>2</sub> (26). We think that estrogen-mediated repression of genes is a critical regulatory pathway in ER-positive cells, and that deregulation of this repression in breast cancer may have dramatic effects such as the promotion of transformation and metastasis. The observation that a number of genes, including *E-cadherin*, have been described as both induced and repressed might be explained by our finding that the repression can only be seen in the presence of serum but not in SFM, suggesting that cross-talk with other pathways is necessary. For instance, kinases can regulate ER and coregulators (for a recent review, see Ref. 27), and the absence or presence of a specific kinase might determine whether the gene becomes induced or repressed on estrogen treatment. We hypothesize that there are three distinct sets of genes: one that can only be induced by estrogen, one that can only be repressed by estrogen, and one that can be induced or repressed depending on cellular context. Experiments are ongoing to test this hypothesis.

As for the mechanism of estrogen down-regulation of gene expression, our ChIP analyses suggest an involvement of ER corepressors and ER in a complex at the E-cadherin promoter. Several previous studies have suggested that a balance of coactivators and corepressors may modulate ER action, and may be deregulated in breast cancer and, in particular, in endocrine resistance. Our data support this hypothesis by showing that an excess of SAFB1 enhances the ability of ER to down-regulate E-cadherin levels. Thus, our data imply that a critical balance between ER and ER cofactors is a determinant in the regulation of E-cadherin levels in breast cancer. However, a potential caveat of our experiments is the use of a transiently transfected

promoter. Studies analyzing the recruitment of both coactivators and corepressors to the endogenous E-cadherin promoter in mouse and human cell lines are ongoing.

The connection between ER and E-cadherin is obviously very complex. ER-negative cell lines are often (but not always, as shown in Fig. 2B in MCF10A cells) E-cadherin-negative, and this has recently been analyzed in more detail. The repressor *MTA3* is an estrogen-regulated gene that regulates Snail expression, which in turn represses E-cadherin (9). Thus, in the absence of ER (and *MTA3*), aberrant expression of Snail results in a loss of expression of E-cadherin. Additionally, as shown here, in ER-positive tumors, estrogen can result in down-regulation of E-cadherin expression. How do these findings relate to known clinical data? Not surprisingly, there is little consensus between numerous studies addressing the relationship between hormone receptor status and E-cadherin expression. There have been studies showing a positive (28), a negative (29), or no correlation (30) between E-cadherin and ER levels. This might, at least in part, be explained by the analysis of “mixed samples,” *i.e.*, ER-positive as well as ER-negative samples. As clearly shown by Fujita *et al.* (Fig. 7 in Ref. 9), ER-positive and ER-negative tumors display very different and even opposite correlations between ER, E-cadherin, Snail, and *MTA3*. We would like to propose that this, at least in part, results from the estrogen-mediated down-regulation of E-cadherin in ER-positive samples. More clinical studies analyzing either only ER-positive or only ER-negative cases are needed to support these models. Potentially, our findings could have clinical impact, because restoration of E-cadherin expression might be an important result of antiestrogen therapy, and, thus, selective estrogen receptor modulators (SERMs) should be tested regarding their effects on E-cadherin expression.

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