Transcriptional Repression of ErbB2 by Histone Deacetylase Inhibitors Detected by a Genomically Integrated ErbB2 Promoter-reporting Cell Screen[1]

Gary K. Scott, Chloe Marden, Fan Xu, Liana Kirk, and Christopher C. Benz[2]

Program of Cancer and Development Therapeutics, Buck Institute for Age Research, Novato, California 94945

Abstract

The antitumor activity of histone deacetylase (HDAC) inhibitors has been linked to gene expression induced by acetylation of histone and nonhistone proteins; but the molecular basis for their antitumor selectivity remains largely unknown. With development of a genomically integrated, ErbB2 promoter-reporting breast cancer cell screen, ErbB2 promoter inhibiting activity was observed by the HDAC inhibitors trichostatin A (TSA) and sodium butyrate. Paradoxically, these agents stimulated the episomal form of this ErbB2 promoter-reporter introduced by transient transfection. Transcriptional run-off assays in ErbB2 amplified and overexpressing breast cancer cells confirmed that within 5 h, TSA exposure profoundly inhibits ErbB2 transcript synthesis from the amplified oncogene yet preserves transcription from single copy genes such as the epithelial-specific Ets family member, ESX. Northern analyses of ErbB2-overexpressing breast cancer lines (SKBR3, BT-474, and MDA-453) showed that within 24 h of submicromolar treatment by TSA, ESX transcript levels increase while ErbB2 transcript levels rapidly decline, with no TSA effect apparent on the open chromatin configuration of either gene as monitored by DNase I hypersensitivity. Actinomycin D studies confirmed that in addition to inhibiting ErbB2 transcript synthesis, TSA selectively destabilizes mature ErbB2 transcripts enhancing their decay. Whereas TSA markedly reduced ErbB2 protein levels in these overexpressing cell lines, TSA treatment of MCF/HER2–18 cells engineered to overexpress the ErbB2 receptor under control of a heterologous promoter increased their expression of ErbB2 protein. These findings suggest that further studies are warranted to determine whether ErbB2-positive human cancers represent unusually sensitive clinical targets for HDAC inhibitor therapy.

Introduction

Despite recent approval of an anti-ErbB2 therapeutic antibody (trastuzumab) to treat advanced breast cancer and the clinical promise of even newer ErbB2 receptor-targeted therapeutics (1, 2), there is increasing interest in *erb*B2 oncogene-silencing strategies because the amplified oncogene and its expressed transcripts/tumor cell are far fewer in copy number than the overexpressed ErbB2 protein product. In addition, the prevalence of obvious resistance mechanisms to ErbB2 receptor-based therapy points to the clinical need for alternative anti-ErbB2 strategies and combinatorial approaches (1).

Antisense and ribozyme strategies have proven partially successful at down-regulating ErbB2 transcript and protein expression in preclinical models but have thus far failed to enter clinical trials (3–6). Efforts to target the 2-to-10-fold amplified copies/tumor cell of the *erb*B2 oncogene have also been explored. EIA induced repression of the ErbB2 promoter has already entered clinical trials, but its CBP/p300-mediated repression mechanism is not specific to the ErbB2 promoter, and this therapeutic agent requires efficient intratumor gene delivery and expression (7). Other more specific ErbB2 promoter-targeting approaches that have shown promise *in vitro* but have not yet been evaluated *in vivo* include ErbB2 promoter-binding and triplex-forming oligonucleotides (8), polyamides with nanomolar affinity for the EBS[3] of the ErbB2 promoter (9), and EBS-targeted chimeric transcriptional repressors (10). It is expected that virtually all of these ErbB2 transcript- and promoter-targeted strategies are compromised most by their limited *in vivo* bioavailability and/or solid tumor uptake and also face significant intracellular and intranuclear delivery and trafficking challenges prior to their clinical advancement (10).

Exploring additional ErbB2 promoter-silencing strategies not encumbered by the above intratumor delivery and intranuclear distribution limitations, we developed a whole-cell high-throughput screen to identify cell-permeable small molecule inhibitors of the ErbB2 promoter. To this end, stable transfection of the ErbB2-independent breast cancer cell line MCF-7 was undertaken to produce a subline (MCF/R06pGL-9) bearing a genomically integrated and chromatinized ErbB2 proximal promoter construct driving a luciferase reporter gene for use in high-throughput screening of chemical libraries for compounds capable of inhibiting ErbB2-

Received 12/26/01; revised 2/14/02; accepted 2/22/02.
[1] This research was supported in part by Grants R01-CA36773 and P50-CA58207 from the NIH as well as grants from the Hazel P. Munroe and Janet Landfear (Mt. Zion Health Systems) memorial funds.
[2] To whom requests for reprints should be addressed, at Buck Institute for Age Research, 8001 Redwood Boulevard, Novato, CA 94945. Phone: (415) 209-2092; Fax: (415) 209-2232; E-mail: cbenz@buckinstitute.org.

[3] The abbreviations used are: EBS, Ets binding site; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HDAC, histone deacetylase; TSA, trichostatin A; Act D, actinomycin D; CMV, cytomegalovirus; HTS, high-throughput screening; ER, estrogen receptor; UTR, untranslated region; NCI/DTP, National Cancer Institute/Developmental Therapeutics Program.

driven luciferase activity without producing general cytotoxicity (as measured by MTT viability assay). Using this screening assay, the HDAC inhibitors sodium butyrate and TSA were identified as potent and relatively specific ErbB2 promoter-inhibiting agents. After validating their ErbB2 promoter- and transcript-inhibiting potential against a panel of breast cancer cell lines that endogenously overexpress ErbB2, we also observed that HDAC inhibitor treatment selectively destabilizes preexistent ErbB2 transcripts, leading to an accelerated loss of intracellular ErbB2 mRNA and protein.

Materials and Methods

Drugs, Breast Cancer Cell Lines, Probes, and Antibodies. The HDAC inhibitors, sodium butyrate and TSA, as well as the RNA polymerase inhibitor, Act D, and the ribosome translocation inhibitor, cycloheximide, were all commercially obtained (Sigma Chemical Co.). ErbB2-independent (MCF-7) and ErbB2-dependent/overexpressing (SKBR3, BT-474, and MDA-MB-453) breast cancer cell lines were originally obtained from American Type Culture Collection and were passaged in tissue culture as described recently (10). MCF7/HER2–18 were derived by stable transfection into MCF-7 cells of a human HER2/ErbB2 cDNA (coding region only) within a 4.7-kb pRK5 expression plasmid downstream and under the control of a CMV promoter/enhancer and with SV40 termination and polyadenylation signals, as has been described previously (11). ErbB2 genomic and cDNA probes used for Southern and Northern blots and transcriptional run-off slot-blots have been described (11–13); monoclonal antibody to ErbB2 protein used for Western blots was commercially obtained (Calbiochem). ESX genomic and cDNA probes used for Southern and Northern blots and transcriptional run-off slot-blots, as well as the anti-ESX polyclonal antibody used for Western blots, have all been described (14, 15). Monoclonal antibody to α-tubulin used on Western blots was commercially obtained (Calbiochem).

MCF/R06pGL-9 Construction and Drug Screening. A luciferase reporter plasmid (designated R06pGL-luc) was constructed by inserting the ~500-bp *Sma*I-*Sma*I fragment of the human ErbB2 proximal promoter (12) into the pGL3Basic plasmid driving a luciferase reporter gene, as has been described (10). R06pGL-luc and a plasmid expressing neomycin phosphotransferase were cotransfected into the MCF-7 cells (American Type Culture Collection) using Effectene (Qiagen). Cells were selected for stable expression of luciferase and neomycin (G418) resistance. Single cell clones including MCF/R06pGL-9 were isolated and maintained in DME H-16 medium supplemented with 10% fetal bovine serum, 10 μg/ml insulin, 100 μg/ml penicillin/streptomycin, and 500 μg/ml G418. MTT cell viability and luciferase expression assays on the MCF/R06pGL-9 subline were performed after plating the subline into 96-well culture dishes at a density of 10^4 cells/well in 100 μl of medium. After 24-h cell plating, TSA from stock DMSO solution was diluted into 100 μl of medium and then added into eight replicate wells at the indicated concentrations. After 24 h of drug (or 0.5% DMSO vehicle control) treatment, MTT (Sigma Chemical Co.) was added to the cells to a final concentration of 0.5 mg/ml and incubated at 37°C for 4 h. The medium was carefully aspirated. The colored formazan product was solubilized in 100 μl of 0.1 N HCl in isopropanol. The reaction was quantified by absorbance at 570 nm measured with a microplate reader (Molecular Devices), and the mean ($\pm$SD) absorbance was recorded after normalization by the vehicle-treated controls. In parallel, plated and drug-treated cells were washed once with PBS and lysed for 15 min at room temperature in lysis buffer (Promega), and luciferase activity was measured immediately from the cell extract by commercial assay kit (Promega) and using a microplate luminometer (LabSystems), with results similarly expressed as the mean ($\pm$SD) of control activity after normalization for vehicle-treated controls. The $\leq$3 h intracellular half-life of the luciferase product from the R06pGL reporter construct detected by this commercial luminescence assay allows for rapid and sensitive detection of virtually complete ($\geq$8 half-life reduction in reporter activity) inhibition of the intracellular ErbB2 promoter within 24 h of drug treatment.

DNase I Hypersensitivity and Southern Blotting, Northern Assays and Transcriptional Run-Off Slot-Blotting, and Western Immunoblotting. The conserved and singular DNase I hypersensitivity sites found in the proximal ErbB2 and ESX promoters (12, 15) were assayed as before (12). Briefly, after culture treatment of cells, nuclei isolated by mild detergent lysis in a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.4% NP40, 10% glycerol, and 1 mM DTT. Partial DNase I digestions were carried out by varying DNase I treatment times (0–15 min at 37°C) at fixed DNase I concentrations (0.5–1.0 units/10^6 nuclei). After purifying and restricting the DNA, it was electrophoresed through 0.7% agarose gels and blotted onto nylon membranes, UV-cross-linked, and hybridized with randomly ^{32}P-labeled ErbB2 and ESX promoter probes, as similarly performed for routine Southern blotting. For Northern blotting, total (treated *versus* control) cell RNA (10 μg/sample lane) was isolated using TRIzol (Invitrogen) according to the manufacturer's specifications, electrophoresed into 1% agarose-formaldehyde gels, and transferred onto membranes that were then hybridized with ^{32}P-labeled ErbB2 and ESX cDNA probes. For transcriptional run-off assays, cell nuclei were first isolated from treated and control cells as described for the DNase I studies. Elongation of initiated nascent RNA chains was performed at 37°C for 30 min using ~5 $\times$ 10^6 nuclei/reaction in a buffer containing 10 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 150 mM KCl, 1 mM DTT, 10% glycerol, 0.5 mM ATP, GTP, and CTP, and 100 μCi of [α-^{32}P]UTP (800 Ci/mmol). Nuclear RNA was purified by the addition of 100 units of RNase-free DNase I (Roche) per reaction for 2 min, followed by TRIzol processing. Radiolabeled RNA was hybridized (~4 $\times$ 10^6 cpm) at 68°C in ExpressHyb (Clontech) for 24 h to nylon filter membranes slotted previously with 0.5 μg/slot of unlabeled ErbB2 cDNA fragments (from either the transmembrane or COOH-terminal domains), ESX cDNA fragments, and empty plasmid control, as we have described previously (16). Filters were washed at 64°C in 0.2$\times$ SSC and 0.5% SDS. For Western blotting, whole-cell extracts from control *versus* treated cells were boiled in sample loading buffer (1% SDS, 20% glycerol, 100 mM DTT, and 50 mM Tris,

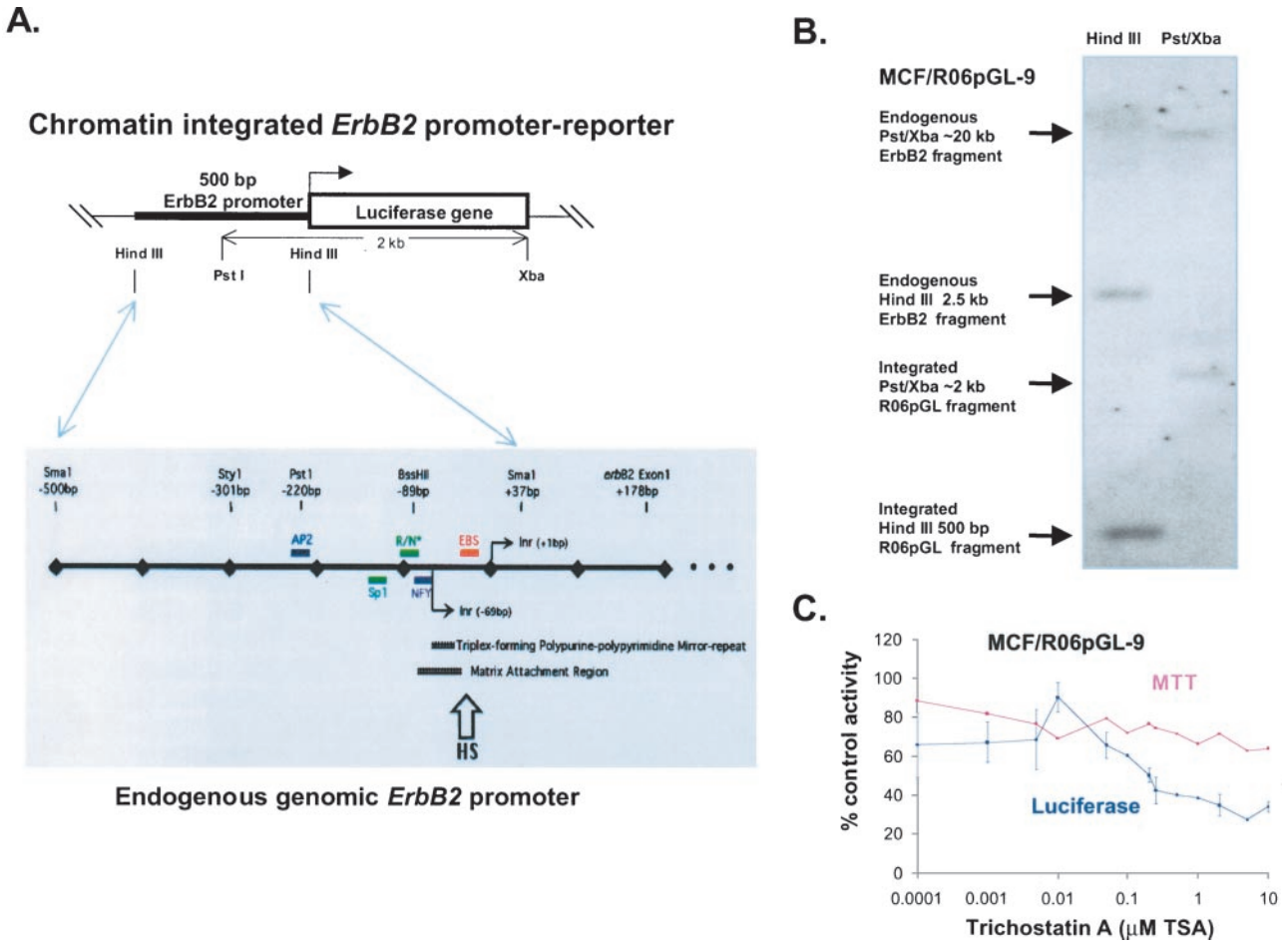


Fig. 1. Structure, genomic verification, and TSA responsiveness of an ErbB2 promoter-reporter stably integrated into the MCF-7 subline, MCF/R06pGL-9. *A,* restriction enzyme map of ErbB2-promoter-luciferase reporter construct (R06pGL) above a map of known ErbB2 promoter features (*R/N**, RPBj/Notch, AP2, Sp1, and NFY response elements) including transcription initiation sites (Inr −69 and Inr +1) and DNase I hypersensitivity (HS) site, contained within the 500-bp *Sma*I-*Sma*I promoter fragment as has been detailed previously (10). *B,* Southern analysis of the MCF/R06pGL-9 subline demonstrating restriction fragment lengths consistent with faithful genomic integration of the ErbB2 promoter-reporter. Hybridization probe was a 257-bp *Pst*I-*Sma*I ErbB2 promoter fragment. *C,* HTS assay for luciferase activity and cell viability (MTT assay) from 96-well replicate cultures of MCF/R06pGL-9 cells showing their responsiveness to a 24-h treatment with the indicated TSA doses.

pH 6.8); gel lanes were loaded for constant total protein (15 μg), electrophoresed into 9% SDS-PAGE gels, and transferred onto membranes (Immobilon-P, Millipore), and the protein-bound membranes were hybridized with a primary antibody followed by a horseradish peroxidase-conjugated secondary antibody (Sigma Chemical Co.). Specific protein bands were visualized by chemiluminescent substrate (Pierce), as described previously (10).

Results

Genomically Integrated *versus* Episomal ErbB2 Promoter Constructs. To establish an assay system capable of reflecting endogenous ErbB2 promoter activity in human breast cancer cells lines, stable integration of an ErbB2 promoter-luciferase reporter construct (R06pGL) into various breast cancer cell lines was undertaken. ErbB2 amplified and overexpressing SKBR3 and MDA-453 cells and the low ErbB2-expressing MCF-7 cells were cotransfected with both a 500-bp ErbB2 promoter-driven luciferase construct (Fig. 1) and a Neo (G418) selection plasmid. Stable integration of the ErbB2 promoter-reporter was successfully achieved only in the MCF-7 cell line, producing the clonally isolated MCF/R06pGL-9 subline with faithful integration of the 500-bp ErbB2 promoter-reporter documented by Southern blot analysis (Fig. 1). No luciferase-expressing clones could be isolated from multiple transfection attempts into SKBR3 cells; and multiple luciferase-expressing MDA-453 clones showing very slow initial growth all reverted to wild-type culture growth in association with loss of their genomically integrated 500-bp ErbB2 promoter-driven reporter constructs (data not shown).

Using the genomically integrated ErbB2 promoter-reporter subline MCF/R06pGL-9 in a 96-well HTS format to begin screening chemical libraries (*e.g.,* the NCI/DTP Diversity Set), we were surprised to observe that the HDAC inhibitor TSA resulted in significant reduction of luciferase activity with little

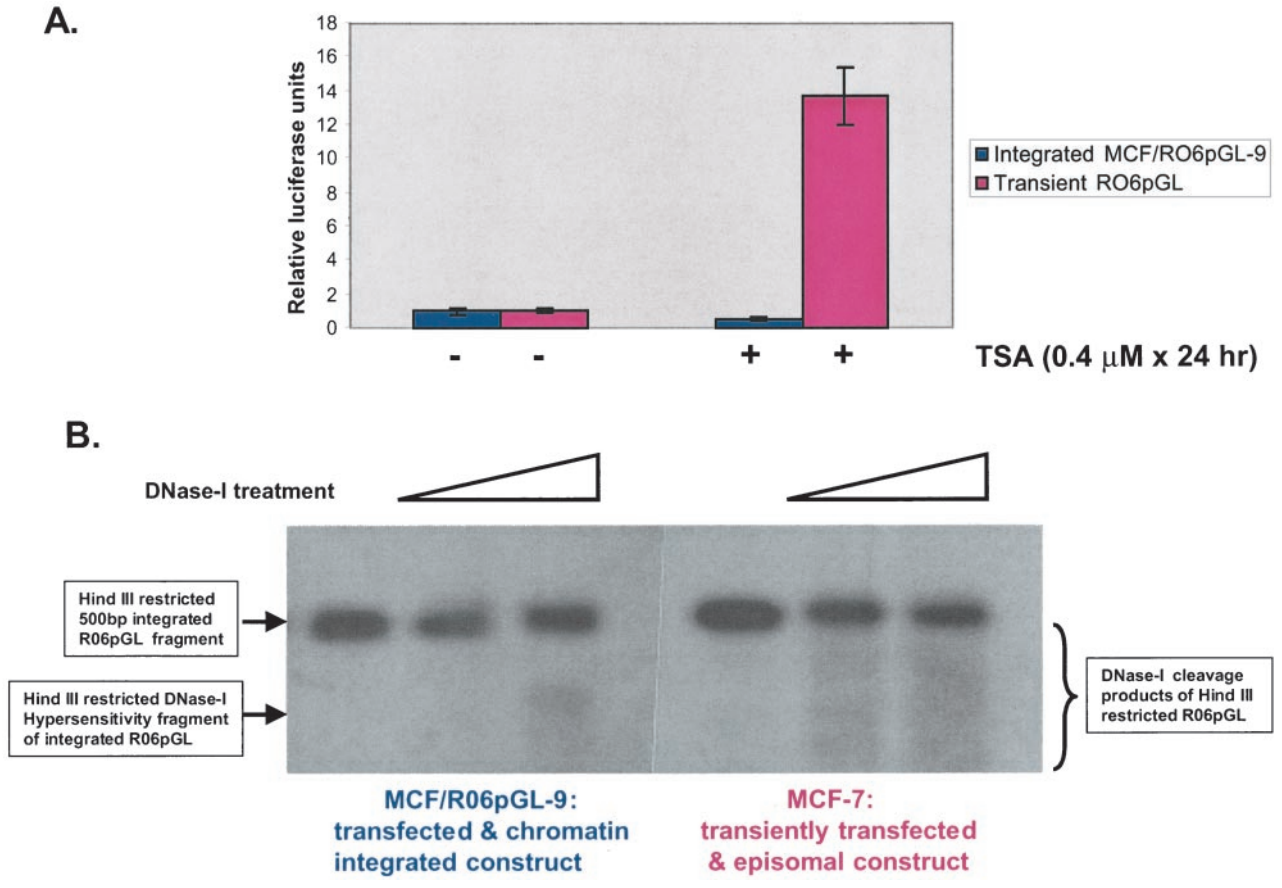
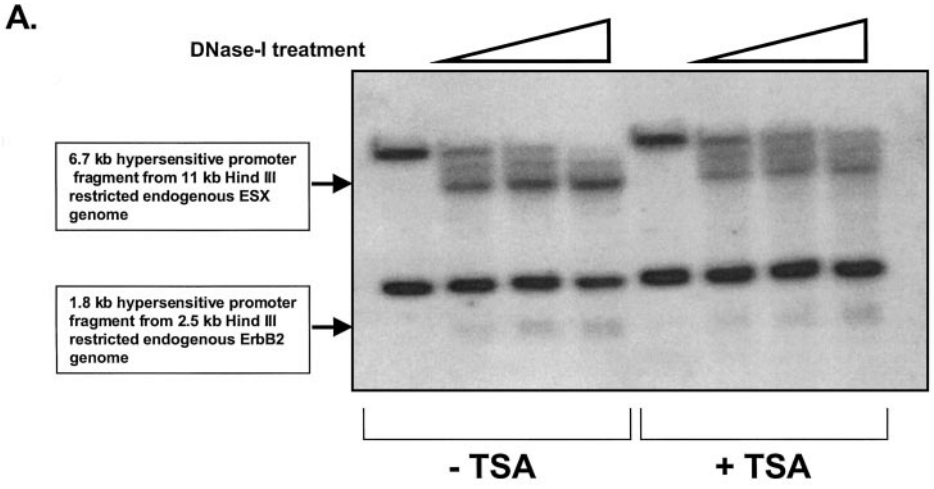


Fig. 2. TSA responsiveness and DNase I hypersensitivity comparisons between the genomically integrated *versus* episomally introduced ErbB2 promoter-reporter construct, R06pGL, in MCF-7 cells. *A,* luciferase activity (relative luminometer units) of the MCF/R06pGL-9 subline compared with the parental MCF-7 cells transiently transfected with the same ErbB2 promoter-reporter plasmid (R06pGL) and after 24-h treatment with 0.4 μM TSA, normalized against their respective nontreatment (vehicle only) control conditions. *Bars,* SD. *B,* DNase I hypersensitivity analysis (described in "Materials and Methods") of nuclei from MCF/R06pGL-9 *versus* MCF-7 cells transiently transfected with R06pGL, following similar culture treatments with TSA.

impact on cell viability (assessed by MTT assay) after 24 h of culture exposure to TSA concentrations up to 50 μM (Fig. 1). In an episomal context and after transient transfection of the R06pGL construct into MCF-7 cells, TSA produced diametrically opposite (>10-fold stimulatory) effects on this same ErbB2 promoter-driven reporter (Fig. 2). Southern blot comparisons of DNase I-treated MCF/R06pGL-9 cell nuclei and those from transfected MCF-7 cells bearing the episomally introduced R06pGL plasmid confirmed the lack of a discrete DNase I hypersensitivity site in the episomal R06pGL and the presence of such a site in the genomically integrated R06pGL, similar in location and intensity to that found within the endogenous ErbB2 promoter (Fig. 2). The strong TSA stimulatory effect on the transiently transfected ErbB2 promoter-reporter appeared independent of the amount of R06pGL plasmid introduced (1 ng to 1 μg) and was similarly observed with transient transfection and TSA treatment of ErbB2-overexpressing SKBR3 and MDA-453 cells (data not shown).

Down-Regulation of Endogenous ErbB2 Transcripts by TSA. Northern analyses were performed to determine the influence of TSA upon endogenous ErbB2 transcript levels. Gels were normalized for constant rRNA loading, and the effects of TSA treatment on long-lived 4.8-kb ErbB2 transcript levels were compared with its effects on the short-lived 2.2-kb transcripts of ESX, an epithelium-specific Ets transcription factor often coexpressed with ErbB2 in human breast cancer cell lines (14). Fig. 3 demonstrates the near total disappearance of ErbB2 transcripts in SKBR3 cells after 24 h of TSA treatment, associated with a simultaneous 5-fold increase in ESX transcript levels in these treated cells. Given these opposite TSA effects on ErbB2 and ESX transcript levels and a previous report suggesting that TSA activates transcription in association with its enhancement of DNase I hypersensitivity in the regulatory locus of the gene (17), we were also surprised to observe the lack of any TSA treatment effects on the hypersensitive loci within either the ErbB2 or ESX promoters (Fig. 3). Virtually complete elimination of ErbB2 transcript levels was also observed after similar TSA treatment of MDA-453 and BT-474 cells and could also be induced by treatment with sodium butyrate (3 mM $\times$ 24 h), another well known HDAC inhibitor (data not shown).

Nuclear run-off experiments were performed on ErbB2-overexpressing breast cancer cells to confirm the conclusion



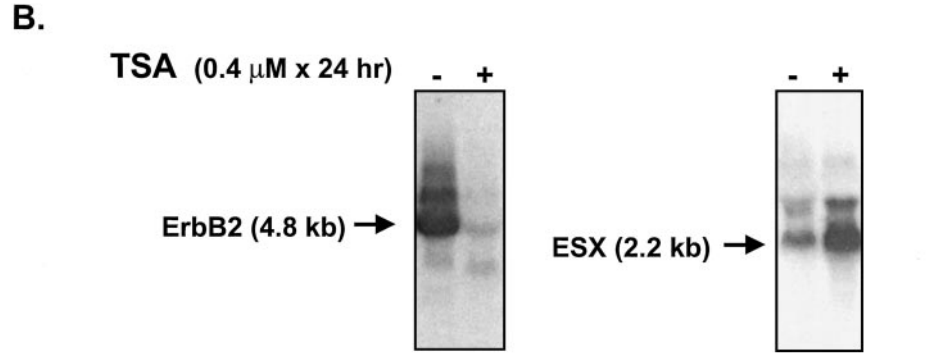


Fig. 3. Comparison of TSA effects on the transcript expression and DNase I hypersensitivity of ErbB2 and ESX genes. *A,* DNase I hypersensitivity analysis of nuclei from SKBR3 cells after treatment with (+) or without (−) 24-h exposure to 0.4 μM TSA. Southern blot prepared from *Hin*dIII-digested DNA was probed first with an ErbB2 promoter probe, followed by an ESX cDNA probe for correct hypersensitive fragment band assignments. Lanes without DNase I treatment define clearly the endogenous 2.5-kb ErbB2 *Hin*dIII fragment and 11-kb ESX *Hin*dIII fragment. *B,* Northern blot of total RNA isolated from SKBR3 cells after 24-h culture treatment with (+) or without (−) 0.4 μM TSA, probed first with ErbB2 cDNA (*left panel*) and then reprobed with ESX cDNA (*right panel*) to reveal their respective (4.8- and 2.2-kb) transcript bands.

drawn from TSA induced down-regulation of luciferase expression in MCF/R06pGL-9 cells that HDAC inhibitors can suppress ErbB2 promoter activity and presumably also repress ErbB2 transcription. Nuclei from SKBR3 cells treated for 5 h with TSA were isolated, and their nascent nuclear transcripts were elongated in the presence of radiolabeled nucleotides; the labeled RNA was isolated and hybridized to membranes slotted with ESX cDNA and ErbB2 cDNA fragments, including those from either the COOH terminus or transmembrane domain to assure good transcript representation from this ~30-kb oncogene. Slot-blot hybridization stringency was adjusted so that no signal could be detected from those control slots containing the empty plasmid. As shown in Fig. 4, TSA culture treatment for only 5 h profoundly suppressed synthesis of new ErbB2 transcripts from the amplified oncogene yet preserved ESX mRNA synthesis from this early response gene.

The rate of ErbB2 transcript loss observed in TSA-treated ErbB2-positive breast cancer cells (*e.g.,* Fig. 3) suggested a TSA-induced accelerated decay of the normally long-lived intracellular ErbB2 mRNA, because an 8-h (approximate half-life of ErbB2 mRNA) TSA treatment also resulted in <20% of control ErbB2 transcript levels as detected by Northern assays (data not shown). To test this possibility, Northern assays were performed on RNA isolated from SKBR3 cells after 5 h of treatment with either Act D or TSA (0.05 or 0.40 μM). As shown in Fig. 4, TSA greatly enhanced the rate of ErbB2

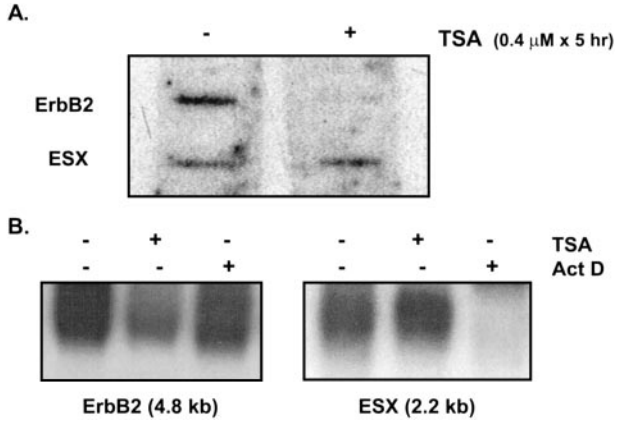


Fig. 4. Influence of TSA on ErbB2 and ESX transcript synthesis and stability. *A,* nuclear run-offs showing radiolabeled and newly synthesized RNA from nuclei of SKBR3 cells pretreated (+) or not pretreated (−) for 5 h with 0.4 μM TSA, hybridized to membranes slotted with ErbB2 COOH-terminus and ESX cDNA fragments. *B,* Northern blot of total RNA from SKBR3 cells similarly treated for 5 h ± 0.4 μM TSA or ± 10 μg/ml of the RNA polymerase inhibitor, Act D, probed first with ErbB2 cDNA (*left panel*) and then with ESX cDNA (*right panel*) to reveal the treatment effects on their total intracellular transcript (4.8- and 2.2-kb) levels.

mRNA decay relative to Act D-treated cells (which show only a slight reduction in ErbB2 mRNA level), yet this exposure to Act D was sufficient to fully inhibit transcription and result in the complete loss of the short-lived ESX mRNA. Of interest,

a 5-h SKBR3 treatment with either 0.05 or 0.40 μM TSA dose produced comparable reductions in Northern blot ErbB2 transcript levels (data not shown); this apparent plateau in the 5-h TSA dose-response suggests that any observed decline in total ErbB2 transcript levels likely reflects at least two independent CxT responses to TSA, one for its inhibition of transcript synthesis and another for its acceleration of ErbB2 transcript decay. TSA-treated BT-474 and MDA-453 cells showed a similarly accelerated loss of ErbB2 transcript levels as compared with their treatment with Act D (data not shown). In additional experiments to explore the nature of this TSA induced posttranscriptional decay of ErbB2 transcripts, SKBR3 cells were treated with a dose of cycloheximide sufficient to block mRNA ribosomal translocation (50 μg/ml), and although this was observed to have no effect on ErbB2 transcript levels in control cells, it completely inhibited the accelerated ErbB2 mRNA degradation induced by TSA. However, a similar cyclohexamide treatment had no ability to arrest TSA-induced ErbB2 transcript decay in BT-474 or MDA-453 cells (data not shown). These observations indicate that the unknown mechanism(s) underlying TSA-induced ErbB2 mRNA decay may be distinct in different cell lines and likely independent from those underlying the inhibition of ErbB2 transcript synthesis.

Down-Regulation of Endogenous ErbB2 Protein by TSA. That a genomically integrated but not episomal ErbB2 promoter-reporter construct correctly recapitulated the endogenous ErbB2 promoter's response to TSA underscores the need to develop ErbB2 models that better resemble the endogenously overexpressed oncogene. In this regard, TSA also failed to repress (and actually stimulated) ErbB2 overexpression in another MCF-7 subline, MCF/HER2–18, engineered to transcriptionally overexpress ErbB2 and its functional M_r 185,000 surface receptor, and now used commonly to evaluate novel ErbB2 receptor-targeted therapeutics (11, 18, 19). The ectopically introduced and genomically integrated ErbB2 expression construct in this subline was placed under the control of a CMV promoter and contains only the ErbB2 protein coding sequences fused to an SV-40 polyadenylation signal (11). As shown by the Western analyses in Fig. 5, 24-h TSA treatment of SKBR3, MDA-453, and BT-474 cells produced the expected marked decline in their endogenous M_r 185,000 ErbB2 protein levels (normalized to α-tubulin levels). In contrast, the level of ectopic M_r 185,000 ErbB2 protein overexpressed in MCF/HER2–18 cells is actually further stimulated under these same TSA treatment conditions.

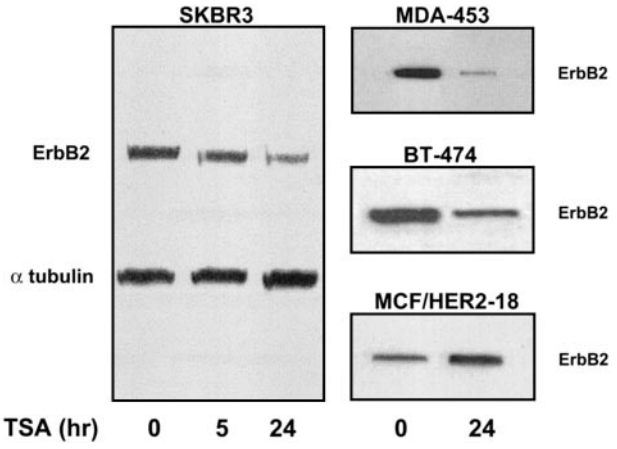


Fig. 5. ErbB2 protein levels after TSA treatment of various ErbB2-overexpressing breast cancer cell lines. Western blots of whole-cell extracts from four different cell lines (SKBR3, MDA-453, BT-474, and MCF/HER2–18) after treatment with 0.4 μM TSA for the indicated times (hours) are shown. Membranes were probed with antibodies to the ErbB2 and α-tubulin proteins, with the resulting band intensities as indicated. SKBR3, MDA-453, and BT-474 cells overexpress M_r 185,000 ErbB2 protein from their endogenously amplified oncogenes, whereas MCF/HER2–18 cells overexpress M_r 185,000 ErbB2 protein from a genomically integrated but ectopically introduced ErbB2 expression vector lacking native ErbB2 promoter and noncoding cDNA sequences.

Discussion

We developed a whole-cell HTS for cell-permeable agents capable of selectively inhibiting the ErbB2 promoter without producing generalized cytotoxicity. After verifying by Southern blot and DNase I hypersensitivity assays, the integrity of the chromatinized ErbB2 promoter-reporter construct integrated within the genome of the MCF/R06pGL-9 subline, the HDAC-inhibiting agents TSA, and sodium butyrate tested in this cell screen were identified as potent ErbB2 promoter-repressing candidates. Consistent with earlier reports questioning conclusions drawn from cell studies using nonintegrated (*e.g.,* episomal or transiently transfected) promoter-reporter constructs in favor of genomically integrated and chromatinized promoter-reporters (20), we observed significant suppression by TSA of the chromatinized ErbB2 promoter-reporter in the MCF/R06pGL-9 subline in contrast with a paradoxical >10-fold stimulating effect of TSA on parental MCF-7 cells transiently transfected with the same ErbB2 promoter-reporter construct (R06pGl). This HTS format using a genomically integrated promoter driving a short half-life (≤3 h) luciferase reporter appears particularly useful for rapid and sensitive monitoring of drug-induced effects targeted to the regulatory region of genes such as *erb*B2, the endogenous transcript and protein products of which can be so long-lived as to require days of continuous promoter inhibition to measure significant declines in intracellular levels of these gene products.

Our HTS identification of an HDAC inhibiting effect on the chromatinized ErbB2 promoter was validated by showing that comparable submicromolar exposure to TSA significantly reduces intracellular ErbB2 mRNA and protein levels in a panel of culture-treated breast cancer cell lines (SKBR3, MDA-453, and BT-474) known to contain the endogenously amplified and overexpressing *erb*B2 oncogene. This TSA-induced repression of endogenously ErbB2 expression was not attributable to a generalized repression of intracellular gene expression because reprobing the Northern and Western blots containing the same RNA and protein from these TSA-treated cell lines revealed a strong TSA-stimulating effect on the expression of other endogenous genes such as the Ets family transcription factor, ESX. Given the recent report describing TSA activation of ER (α isoform) transcription in similarly treated breast cancer cells associated with TSA-enhanced ER gene locus sensitivity to DNase I (17), we

evaluated TSA treatment effects on the singular DNase I hypersensitivity sites associated with endogenous *erb*B2 and ESX promoter loci. Unlike the reported induction of ER genomic sensitivity to DNase I potentially explaining TSA-stimulated ER gene expression (17), TSA produced no significant change in the prominent DNase I hypersensitivity sites of either *erb*B2 or ESX, suggesting that alteration of the already open chromatin configuration of these two actively transcribing genes cannot adequately explain the opposing effects of TSA on their intracellular transcript levels.

Nuclear run-off assays performed on ErbB2-overexpressing and ESX-expressing SKBR3 cells confirmed that the ErbB2 transcriptional silencing effect of this HDAC inhibitor is profoundly evident within 5 h of TSA treatment and concurrent with preserved ESX transcription. Given the well-established long half-life of intracellular ErbB2 transcripts in contrast with the short (<2 h) half-life of ESX transcripts, the observed decline in total SKBR3 ErbB2 transcript levels within 5 h of TSA treatment suggested that HDAC inhibition might also affect the stability of mature ErbB2 transcripts in addition to inhibiting synthesis of new ErbB2 mRNA. Treating SKBR3 cells for 5 h with a RNA polymerase-inhibiting dose of Act D sufficient to completely deplete endogenous ESX transcript levels produced little detectable change in total ErbB2 transcript levels, in keeping with the long half-life of these transcripts and in contrast with the marked decline in ErbB2 transcript levels caused by TSA treatment of similar duration. Comparable results were observed in TSA and sodium butyrate-treated BT-474 and MDA-453 cells, indicating that HDAC inhibitors not only repress the synthesis of endogenous ErbB2 transcripts but also accelerate the decay of preexistent mature ErbB2 transcripts. Further studies are needed to explore the likelihood that HDAC inhibitors produce reductions in total ErbB2 transcript levels based on two probably independent subcellular mechanisms, each with different dose responses to this class of drugs: one resulting in inhibition of ErbB2 transcript synthesis, and another resulting in enhanced ErbB2 transcript decay.

Transcript stability is thought to be regulated by *trans*-acting factors that bind to *cis*-acting elements within UTRs in the 5′ and/or 3′ termini of mature mRNA molecules, mediating mRNA decay through poorly defined mechanisms (21). To test the dependence of ErbB2 transcriptional repression by HDAC inhibitors on regulatory elements contained within both the native ErbB2 promoter and UTRs of its endogenous transcript, we turned to another MCF-7 subline, MCF/HER2–18, engineered to overexpress ErbB2 (45-fold over parental line) and commonly used to assess the activity of ErbB2 receptor-targeted therapeutics (11, 18, 19). Overexpression of ErbB2 protein in MCF/HER2–18 cells results from a stably transfected and genomically integrated ErbB2 expression vector lacking the native ErbB2 promoter (replaced by a CMV promoter) and all noncoding ErbB2 cDNA sequence (*i.e.,* 5′ and 3′ UTRs). Interestingly, TSA treatment of MCF/HER2–18 cells does not repress transcription from this engineered and genomically integrated ErbB2 construct but rather stimulates additional ectopic gene expression. This observation not only points to the limited use of such artificial cell lines to evaluate novel anti-ErbB2 therapeutics but also suggests that the full ErbB2 promoter-repressing and transcript-destabilizing activity of TSA requires both native ErbB2 promoter sequence as well as some (as yet undefined) 5′ or 3′ UTR regulatory element within the mature ErbB2 transcript.

HDAC inhibitors such as sodium butyrate and the hydroxamic acid derivatives TSA and suberoylanilide hydroxamic acid, as well as other structurally unrelated HDAC inhibitors, are known to produce *in vitro* and *in vivo* antiproliferative and differentiation- and apoptosis-inducing effects against breast and other epithelial cancers, and some of these are showing promise in early-phase clinical trials (22–28). The antitumor effects of HDAC inhibitors are thought to arise from acetylation of both histones (H3 and H4) and nonhistone transcription-related factors (29, 30), leading to enhanced expression of such cell cycle-regulating genes as *p21Waf1* (22, 23). However, the molecular basis for the *in vitro* or *in vivo* tumor selectivity of HDAC inhibitors remains largely unknown. Microarray studies indicate that of the <10% of actively transcribing genes whose cellular expression are significantly affected by HDAC inhibitors such as sodium butyrate and TSA, the vast majority are up-regulated, with very few genes of known function identified as being down-regulated within 48 h of treatment (31, 32). Moreover, preliminary assessment of the NCI/DTP Diversity Set of nearly 2000 chemical compounds against our genomically integrated ErbB2 promoter-reporting MCF/R06pGL-9 cell screen indicates that <0.3% of potential anticancer compounds have ErbB2 promoter-inhibiting specificity and potency approaching that of HDAC inhibitors (33).

The critical growth and developmental role of normal ErbB2 as well as its amplification and overexpression during human epithelial tumorigenesis highlight the potential mechanistic and clinical significance of our observation showing selective down-regulation of ErbB2 within hours after cellular exposure to an HDAC inhibitor such as TSA. Recent studies reporting on HDAC inhibitor activity against human breast cancer cells did not evaluate a sufficient number of ErbB2-positive *versus* ErbB2-negative cell lines to determine whether ErbB2 overexpression represents a predictive tumor marker for HDAC inhibitor antitumor activity (27, 28). Furthermore, the NCI/DTP's screen of TSA (NSC-709238) determined a median cytotoxic concentration (LC_{50}) for TSA of >10 μM against their 60 human cancer cell line panel (data courtesy of J. Johnson, NCI/DTP), much higher than the submicromolar concentrations we observed to markedly repress ErbB2 transcript levels and others have shown to significantly inhibit growth of the ErbB2-positive SKBR3, BT-474, and MDA-453 breast cancer cell lines (27). In this regard, it must be noted that the NCI/DTP 60 cell line panel, which continues to be used to assess and compare the potential anticancer activity of thousands of synthetic and natural compounds, contains eight breast cancer cell lines but none of the well-characterized ErbB2-positive breast cancer models we have studied.[4] Our findings, therefore, suggest that additional *in vitro* and *in vivo* studies are warranted to determine whether human breast cancers with

[4] Internet address: http://dtp.nci.nih.gov/webdata.html.

ErbB2 amplification and overexpression represent unusually sensitive clinical targets for HDAC inhibitor therapy.

Acknowledgments

We sincerely appreciate the contribution of J. Johnson (NCI/DTP) in providing NCI/DTP *in vitro* activity data (G150, TGI, and LC_{50}) for NSC-709238 (TSA) treatment of the NCI's panel of 60 different human cancer cell lines.

References

1. Benz, C. C., and Tripathy, D. ErbB2 overexpression in breast cancer: biology and clinical translation. J. Women's Cancer, *2:* 33–40, 2000.

2. Krauss, W. C., Park, J. W., Kirpotin, D. B., Hong, K., and Benz, C. C. Emerging antibody-based HER2(ErbB2/*neu*) therapeutics. Breast Disease, *11:* 113–124, 2000.

3. Monia, B. P., Holmlund, J., and Dorr, F. A. Antisense approaches for the treatment of cancer. Cancer Investig., *18:* 635–650, 2000.

4. Roh, H., Pippin, J., and Drebin, J. A. Down-regulation of HER2/*neu* expression induces apoptosis in human cancer cells that overexpress HER2/*neu*. Cancer Res., *60:* 560–565, 2000.

5. Suzuki, T., Anderegg, B., Ohkawa, T., Engebraaten, O., Halks-Miller, M., Holm, P. S., Curiel, D. T., Kashani-Sabet, M., and Scanlon, K. J. Adenovirus-mediated ribozyme targeting of HER2/*neu* inhibits *in vivo* growth of breast cancer cells. Gene Ther., *7:* 241–248, 2000.

6. Wright, L., and Kearney, P. Current status of ribozymes as gene therapy agents for cancer. Cancer Investig., *19:* 495–509, 2001.

7. Hortobagyi, G. N., Ueno, N. T., Xia, W., Zhang, S., Wolf, J. K., Putnam, J. B., Weiden, P. L., Willey, J. S., Carey, M., Branham, D. L., Payne, J. Y., Tucker, S. D., Bartholomeusz, C., Kilbourn, R. G., DeJager, J. L., Sneige, N., Katz, R. L., Anklesaria, P., Ibrahim, N. K., Murray, J. L., Theriault, R. L., Valero, V., Gershenson, D. M., Bevers, M. W., Huang, L., Lopez-Berestein, G., and Hung, M-H. Cationic liposome-mediated *EIA* gene transfer to human breast and ovarian cancer cells and its biologic effects: a Phase I clinical trial. J. Clin. Oncol., *19:* 3422–3433, 2001.

8. Basye, J., Trent, J. O., Gao, D., and Ebbinghaus, S. W. Triplex formation by morpholino oligodeoxyribonucleotides in the HER2/*neu* promoter requires the pyrimidine motif. Nucleic Acids Res., *29:* 4873–4880, 2001.

9. Chiang, S-Y., Bürli, R. W., Benz, C. C., Gawron, L., Scott, G. K., Dervan, P. B., and Beerman, T. A. Targeting the Ets binding site of the HER2/*neu* promoter with pyrrole-imidazole polyamides. J. Biol. Chem., *275:* 24246–24254, 2000.

10. Scott, G. K., Chang, C. H., Erny, K. M., Xu, F., Fredericks, W. J., Rauscher, F. J., III, Thor, A. D., and Benz, C. C. Ets regulation of the *erb*B2 promoter. Oncogene, *19:* 6490–6502, 2000.

11. Benz, C. C., Scott, G. K., Sarup, J. C., Johnson, R. M., Tripathy, D., Coronado, E., Shepard, H. M., and Osborne, C. K. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/*neu*. Breast Cancer Res. Treat., *24:* 85–95, 1992.

12. Scott, G. K., Daniel, J. C., Xiong, X., Maki, R. A., Kabat, D., and Benz, C. C. Binding of an ETS-related protein within the DNase I hypersensitive site of the HER2/*neu* promoter in human breast cancer cells. J. Biol. Chem., *269:* 19848–19858, 1994.

13. Benz, C. C., Scott, G. K., Santos, G. F., and Smith, H. S. Expression of c-*myc,* c-Ha-*ras1,* and c-*Erb*B2 proto-oncogenes in normal and malignant human breast epithelial cells. J. Natl. Cancer Inst., *81:* 1704–1709, 1989.

14. Chang, C-H., Scott, G. K., Kuo, W-L., Xiong, X., Suzdaltseva, Y., Park, J. W., Sayre, P., Erny, K., Collins, C., Gray, J. W., and Benz, C. C. *ESX*: a structurally unique *Ets* overexpressed early during human breast tumorigenesis. Oncogene, *14:* 1617–1622, 1997.

15. Chang, C-H., Scott, G. K., Baldwin, M. A., and Benz, C. C. Exon 4-encoded acidic domain in the epithelium-restricted Ets factor ESX confers potent transactivating capacity and binds to TATA-binding protein (TBP). Oncogene, *24:* 3682–3695, 1999.

16. Santos, G. F., Scott, G. K., Lee, W. M. F., Liu, E., and Benz, C. Estrogen-induced post-transcriptional modulation of c-*myc* proto-oncogene expression in human breast cancer cells. J. Biol. Chem., *263:* 9565–9568, 1988.

17. Yang, X., Ferguson, A. T., Nass, S. J., Phillips, D. L., Butash, K. A., Wang, S. M., Herman, J. G., and Davidson, N. E. Transcriptional activation of estrogen receptor α in human breast cancer cells by histone deacetylase inhibition. Cancer Res., *60:* 6890–6894, 2000.

18. Kurokawa, H., Lenferink, A. E. G., Simpson, J. F., Pisacane, P. I., Sliwkowski, M. X., Forbes, J. T., and Arteaga, C. L. Inhibition of HER2/*neu* (*erb*B2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. Cancer Res., *60:* 5887–5894, 2000.

19. Park, J. W., Kirpotin, D. B., Hong, K., Shalaby, R., Shao, Y., Nielsen, U. B., Marks, J. D., Papahadjopoulos, D., and Benz, C. C. Tumor targeting using anti-HER2 immunoliposomes. J. Controlled Release, *74:* 95–113, 2001.

20. Smith, C. L., and Hager, G. L. Transcriptional regulation of mammalian genes *in vivo*: a tale of two templates. J. Biol. Chem., *272:* 27493–27496, 1997.

21. Wilusz, C. J., Wormington, M., and Peltz, S. W. The cap-to-tail guide to mRNA turnover. Nat. Rev. Mol. Cell Biol., *2:* 237–246, 2001.

22. Marks, P. A., Richon, V. M., and Rifkind, R. A. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J. Natl. Cancer Inst., *92:* 1210–1216, 2000.

23. Weidle, U. H., and Grossmann, A. Inhibition of histone deacetylases: a new strategy to target epigenetic modifications for anticancer treatment. Anticancer Res., *20:* 1471–1485, 2000.

24. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J. Biol. Chem., *265:* 17171–17179, 1990.

25. Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., and Pavletich, N. P. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature (Lond.), *401:* 188–193, 1999.

26. Saito, A., Yamashita, T., Mariko, Y., Nosaka, Y., Tsuchiya, K., Ando, T., Suzuki, T., Tsuruo, T., and Nakanishi, O. A synthetic inhibitor of histone deacetylase, MS-27–275, with marked *in vivo* antitumor activity against human tumors. Proc. Natl. Acad. Sci. USA, *96:* 4592–4597, 1999.

27. Vigushin, D. M., Ali, S., Pace, P. E., Mirsaidi, N., Ito, K., Adcock, I., and Coombes, R. C. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer *in vivo*. Clin. Cancer Res., *7:* 971–976, 2001.

28. Munster, P. N., Troso-Sandoval, T., Rosen, N., Rifkind, R., Marks, P. A., and Richon, V. M. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res., *61:* 8492–8497, 2001.

29. Sterner, D. E., and Berger, S. L. Acetylation of histones and transcription-related factors. Microbiol. Mol. Biol. Rev., *64:* 435–459, 2000.

30. Yao, Y-L., Yang, W-M., and Seto, E. Regulation of transcription factor YYI by acetylation and deacetylation. Mol. Cell. Biol., *21:* 5979–5991, 2001.

31. Van Lint, C., Emiliani, S., and Verdin, E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr., *5:* 245–253, 1996.

32. Mariadason, J. M., Corner, G. A., and Augenlicht, L. H. Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colon cancer. Cancer Res., *60:* 4561–4572, 2001.

33. Scott, G. K., Marden, C., Andrews, D., Kirk, L., Neve, R. M., and Benz, C. C. A genomically integrated ErbB2 promoter-reporting cell system for high-throughput screening and detection of compounds with potential ErbB2 promoter silencing activity. Proceedings of the 2001 AACR-NCI-EORTC International Conference. Clin. Cancer Res., *7* (Suppl.)*:* 150, 2001.