Concordant Epigenetic Silencing of Transforming Growth Factor-β Signaling Pathway Genes Occurs Early in Breast Carcinogenesis

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Abstract

Human mammary epithelial cells (HMEC) grown under standard cell culture conditions enter a growth phase referred to as selection, but a subpopulation is able to escape from arrest and continue to proliferate. These cells, called post-selection or variant HMECs, may be derived from progenitor cells found in normal mammary epithelium that subsequently acquire premalignant lesions, including *p16^{INK4A}* promoter hypermethylation. Epigenetic silencing of tumor suppressor genes through DNA methylation and histone modification is an early event in tumorigenesis. A major challenge is to find genes or gene pathways that are commonly silenced to provide early epigenetic diagnostic and therapeutic cancer targets. To identify very early epigenetic events that occur in breast cancer, we used microarrays to screen for gene pathways that were suppressed in post-selection HMECs but reactivated after treatment with the demethylation agent 5-aza-2′-deoxycytidine. We found that several members of the transforming growth factor β (*TGF-β*) signaling pathway were consistently down-regulated in the post-selection HMEC populations, and this was associated with a marked decrease in Smad4 nuclear staining. Gene suppression was not associated with DNA methylation but with chromatin remodeling, involving a decrease in histone H3 lysine 27 trimethylation and an increase in histone H3 lysine 9 dimethylation and deacetylation. These results show for the first time that *TGF-β2*, its receptors *TGF-βR1* and *TGF-βR2*, and activator thrombospondin-1 are concordantly suppressed early in breast carcinogenesis by histone modifications and indicate that the *TGF-β* signaling pathway is a novel target for gene activation by epigenetic therapy. [Cancer Res 2007;67(24):11517–27]

Introduction

Major progress in controlling mortality and morbidity from cancer requires an understanding of the molecular mechanisms that are responsible for initiating the disease. Analysis of these early aberrant events is complex because by the time the tumor is detected, the cancer progenitor cells have already undergone multiple genetic and epigenetic changes that may be a consequence, rather than a cause, of malignancy. Genetic changes include DNA mutations and changes in DNA copy number due to gene deletions, rearrangements, and amplification, whereas epigenetic lesions involve alterations to both the chromatin structure and the DNA methylation pattern of the cancer genome (1). Changes in the DNA methylation pattern in cancer include global hypomethylation of CpG dinucleotides in DNA repeated regions (2) in conjunction with hypermethylation of CpG island promoter–associated genes (1, 3). CpG islands (4) are ~200 bp to several kilobases in length and typically are unmethylated in a normal cell and span the proximal promoter and first exon of most tumor suppressor genes. Repressive chromatin modifications are also associated with tumor suppressor gene silencing and hypermethylation of CpG island promoters. Common chromatin modifications leading to repression include histone H3 lysine 9 (H3K9) methylation, H3K9 deacetylation (5), and histone H3 lysine 27 (H3K27) trimethylation, all of which contribute to the inaccessibility of the promoter elements to transcription factor binding (6). Although epigenetic changes are a common feature of human cancer cells compared with their normal counterparts, it is not clear at what stage in the transformation process these epigenetic changes occur and whether they occur concurrently or independently.

Human mammary epithelial cells (HMEC) isolated from disease-free breast tissue provide an excellent system to study early events in tumorigenesis. When cultured in serum-free medium, HMECs can exhibit two phases of growth (7, 8). The first growth phase, referred to as pre-selection, lasts for several passages, after which growth ceases (termed selection). However, some cells with enhanced growth capacity are able to escape this plateau and continue into a second growth phase where they proliferate for another 20 to 40 generations before entering a second growth plateau referred to as agonescence (9). Cells in the second phase of growth are termed post-selection (10) or variant HMECs (11, 12) and exhibit various preneoplastic characteristics including DNA methylation and silencing of the *p16^{INK4A}* tumor suppressor gene (9, 10, 12–15). We and others have previously shown that the silencing and DNA methylation of the p16^{INK4A} locus occurs during selection (9, 10, 12–15), and it has been suggested that epigenetic deregulation of *p16^{INK4A}* occurs commonly in premalignant breast lesions. Indeed, rare foci of morphologically normal epithelial cells with hypermethylation of the tumor suppressor gene *p16^{INK4A}* have been identified *in vivo* in disease-free breast tissue (12) and these foci have been hypothesized to be precursors to cancer (16).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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The transforming growth factor β (TGF-β) family of growth factors comprises three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) and plays a pivotal role in the regulation of many cellular processes such as cell division, differentiation, motility, adhesion, and death (17, 18). Deregulation of TGF-β has been shown to be involved in the pathogenesis of cancer, including that of the breast, but the precise role it plays in cancer biology is complex. In normal cells of epithelial, endothelial, and hematopoietic cell lineages, TGF-β functions as a potent tumor suppressor (19). At some point along the neoplastic continuum, cells become unresponsive to TGF-β growth inhibition, and as malignant disease progresses, TGF-β can actually promote tumor progression by enhancing tumor cell motility and invasiveness (17, 19, 20). Several members of the *TGF-β* pathway, including TGF-β receptor 1 (*TGF-βR1*), TGF-β receptor 2 (*TGF-βR2*), and members of the downstream Smad signaling family, are often mutated and/or functionally inactivated in human cancers (21). In addition to genetic changes, epigenetics may also play a role in regulating individual members of the TGF-β pathway, including *TGF-βR1* and *TGF-βR2* (22–26), the Smad molecules (27), and thrombospondin (*THBS1*), an activator of TGF-β (28); however, there are no reports of coordinate epigenetic suppression of these genes in breast cancer.

In this study, we used HMECs to identify genes or gene pathways that may succumb to epigenetic down-regulation in the early stages of breast cancer. Using microarrays, we identified gene suppression of transforming growth factor β2 (*TGF-β2*), its receptors *TGF-βR1* and *TGF-βR2*, and its activator *THBS1* in post-selection HMECs. Bisulfite methylation analysis and chromatin immunoprecipitation assays showed that the down-regulation was not under the control of DNA hypermethylation but was associated with methylation and deacetylation of H3K9 residues and demethylation of H3K27 residues in post-selection HMECs and in the breast cancer cell line MDAMB453. Decreased expression of TGF-β2 and its associated genes was also observed in breast tumor samples. This study shows (*a*) the epigenetic regulation of multiple members in a gene pathway; (*b*) that this can be controlled by histone methylation and deacetylation rather than DNA methylation; and (*c*) that epigenetic deregulation of the *TGF-β* gene pathway members is likely to be an early event in breast cancer formation.

Materials and Methods

Cells, cell culture, and tissue samples. Breast tissue removed at reduction mammoplasty was obtained with institutional ethics committee approval and informed donor consent. HMEC cultures were prepared from normal breast tissues as previously described (10) and further details about the HMEC samples are provided in Supplementary Methods. The breast cancer samples were obtained with institutional ethics committee approval and were previously described (29). Breast cancer cell lines (T47D, MDAMB453, MDAMB468, MDAMB231, MDAMB157, SKBR3, MCF-7, KPL1, DU4475, and MCF-10A) were obtained from American Type Culture Collection and cultured in RPMI 1640 with 10% fetal bovine serum.

Immunocytochemistry. The localization of Smad4 was detected by immunofluorescence staining with a primary Smad4 (B-8) mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.) and a secondary goat anti-mouse IgG (H+L) Alexa Fluor 488 antibody (Molecular Probes, Invitrogen). For more details, see Supplementary Methods.

5-Aza-2′-deoxycytidine and trichostatin A treatment. MDAMB453 and T47D cells were treated with 5-aza-2′-deoxycytidine (5-aza-dC) and trichostatin A (TSA) essentially as previously described (30) with the modifications described in Supplementary Methods.

Microarray experiment and analysis. The details are described in Supplementary Methods.

Gene expression analysis. Total RNA was extracted from pre- and post-selection HMEC, breast cancer cell lines, and fresh frozen normal breast tissue using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was reverse transcribed using SuperScript III RNase H$^-$ Reverse Transcriptase (Invitrogen Life Technologies) and random hexamers (Roche) according to the manufacturers' instructions. Primer sequences are described in Supplementary Table S1. Expression of *TGF-β2, TGF-βR1, TGF-βR2, THBS1*, and $p16^{INK4A}$ was quantitated with a fluorogenic real-time detection method using the ABI Prism 7900HT Sequence Detection System and normalized against endogenous control 18S rRNA mix (Applied Biosystems). For more details, see Supplementary Methods.

DNA isolation and DNA methylation analysis. Details of DNA isolation and the bisulfite protocol (31) are described in Supplementary Methods. Location of the bisulfite PCR amplicons, in relation to the CpG island and start of transcription, is summarized in Supplementary Fig. S1. The primer sequences are described in Supplementary Table S1.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation assays were carried out according to the manufacturer's instructions (Upstate Biotechnology) using Bre-40 and Bre-12 pre- and post-selection HMECs or MDAMB453 cells that were either untreated or treated with 3 μmol/L 5-aza-dC and 50 nmol/L TSA. The complexes were immunoprecipitated with antibodies specific for acetylated histone H3K9 (Upstate), dimethyl-histone H3K9 (Upstate), and trimethyl-histone H3K27 (Upstate), and DNA yield was measured by quantitative real-time PCR as previously described (32). Chromatin immunoprecipitation amplification primers are described in Supplementary Table S1, and the position of the chromatin immunoprecipitation PCR amplicons in relation to the CpG island and start of transcription is summarized in Supplementary Fig. S1. For each sample, the average C_T value was obtained for the immunoprecipitated material and for the input chromatin. The difference in C_T values (δC_T) reflects the difference in the amount of material that was immunoprecipitated relative to the amount of input chromatin or the internal control genes GAPDH and 16-Cen (33) as described in ABI PRISM 7700 Sequence Detection System User Bulletin no. 2, P/N 4303859.

Results

Down-regulation of *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* gene expression in post-selection HMECs. To identify genes or gene pathways that are epigenetically down-regulated in the early stages of breast cancer, we grew normal HMECs in culture to select for post-selection HMECs. We used microarrays to compare the gene expression profiles between pre- and post-selection HMECs from two HMEC strains (designated Bre-60 and Bre-80) and from the post-selection cells following treatment with 5-aza-dC treatment. Three hundred eighty-two of 10,242 informative genes on the Compugen array were found to be down-regulated >1.5 fold in both Bre-60 and Bre-80 post-selection HMECs compared with the equivalent pre-selection HMECs (Supplementary Fig. S2). Forty-three of these genes, including several *TGF-β* associated transcripts, were up-regulated >1.5-fold in both Bre-60 and Bre-80 post-selection HMECs after 5-aza-dC treatment, indicating that the gene expression may be controlled epigenetically. *TGF-β2* was of direct interest because disruption of the TGF-β signaling pathway due to mutation is implicated in the pathogenesis of breast cancer (19). We were therefore interested to determine if epigenetic deregulation of the *TGF-β* signaling pathway also occurred in oncogenesis. Interestingly, the *TGF-β2* receptors, *TGF-βR1* and *TGF-βR2*, and *THBS1*, an activator of *TGF-β2*, were also generally down-regulated in both Bre-60 and Bre-80 post-selection cells, unlike *TGF-β1* and *TGF-β3*, which were not suppressed (Supplementary Fig. S2).

To validate if *TGF-β2* mRNA expression was down-regulated in different post-selection HMEC strains to the ones used in the array studies, we carried out quantitative reverse transcription-PCR (RT-PCR) and found that *TGF-β2* mRNA expression was also suppressed (8.1- and 5.4-fold, respectively) in two independent post-selection HMEC strains designated Bre-40 and Bre-12 (Fig. 1*A*). This suggests that *TGF-β2* gene suppression is a common and early event in post-selection HMECs that escape senescence and continue to proliferate. We also observed gene suppression of other CpG island–associated *TGF-β* pathway genes, *TGF-β* receptors *TGF-βR1* and *TGF-βR2*, and *THBS1* in post-selection Bre-40 HMECs by quantitative RT-PCR and modest reduction in mRNA expression of *TGF-βR1* and *THBS1* in post-selection Bre-12 cells (Fig. 1*A*), suggesting that the *TGF-β* associated genes may be coordinately regulated. The $p16^{INK4A}$ CpG island promoter, which we and others have reported to be methylated in post-selection HMECs (9, 10, 12–15, 34), was also found to be completely inactivated in both post-selection Bre-40 and Bre-12 HMECs (Fig. 1*A*).

To investigate if gene suppression of *TGF-β2* and associated genes resulted in impaired TGF-β signaling, we examined the nuclear localization of Smad4 fluorescence in the pre- and post-selection HMECs because Smad4 is only translocated to the nucleus if the TGF-β receptor is activated. As previously reported, the cell morphology is different and heterogeneous in pre- and post-selection HMECs (10, 35). We found a marked and significant ($P < 0.0001$, χ^2 test) difference in the number of cells with intense Smad4 nuclear staining in the post-selection HMECs in

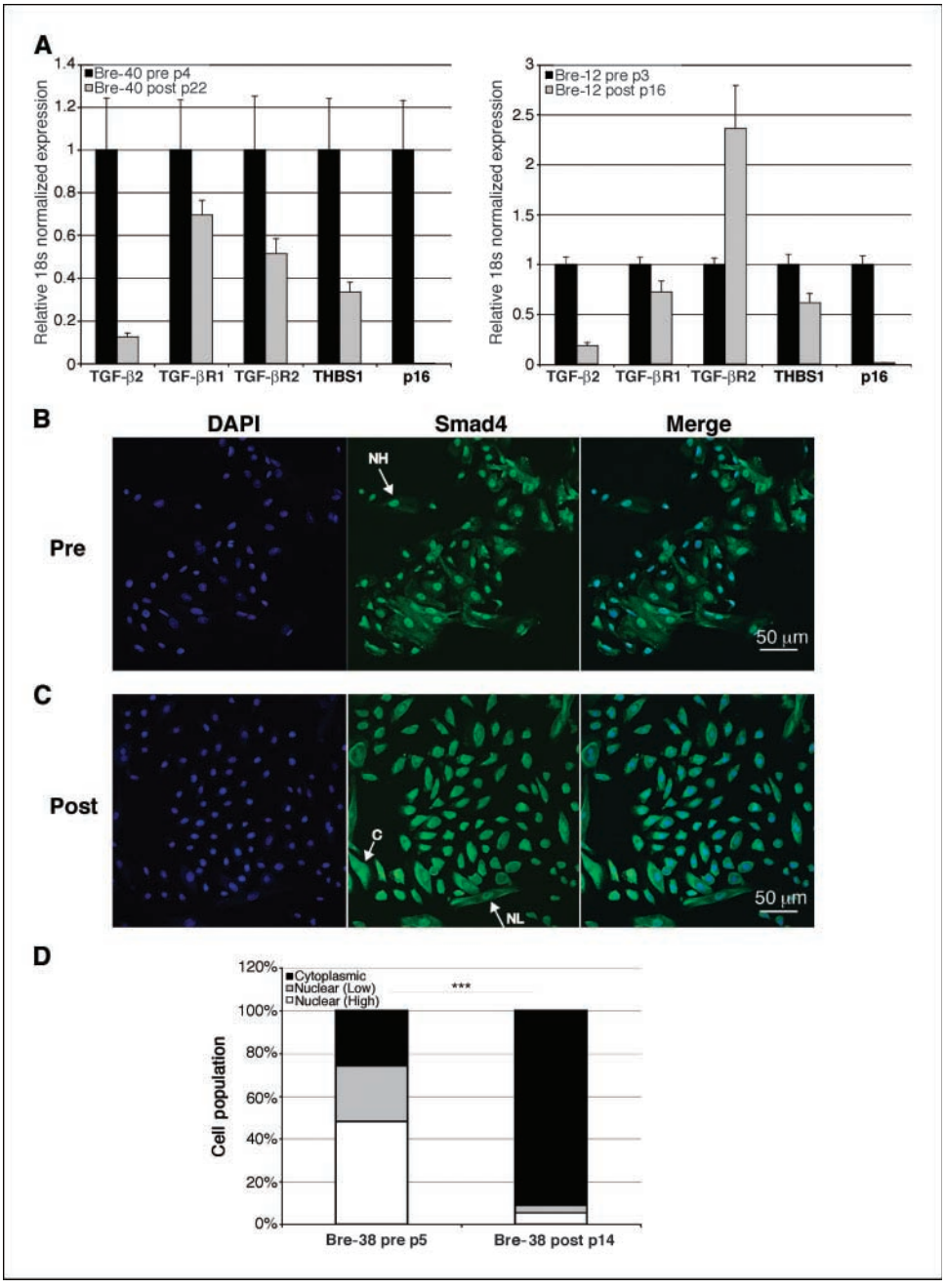


Figure 1. Suppression of TGF-β gene expression and TGF-β signaling in pre- and post-selection HMECs. *A,* mRNA levels of *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* in Bre-40 and Bre-12 pre- and post-selection HMECs were determined by quantitative RT-PCR. After normalizing expression to 18S rRNA, the fold change in expression levels was made relative to pre-selection HMECs. $p16^{INK4A}$ is shown as a positive control for gene suppression in post-selection HMECs. *B,* Smad4 immunostaining in Bre-38 pre-selection cells showing predominant Smad4 nuclear localization. *C,* Smad4 immunostaining of Bre-38 post-selection cells showing predominantly cytoplasmic localization. Blue signal corresponds to 4′,6-diamidino-2-phenylindole (*DAPI*) and the green signal represents Smad4 expression. *Arrows,* examples of nuclear high staining (*NH*), nuclear low staining (*NL*), and cytoplasmic staining (*C*). *D,* quantification of Smad4 immunostaining pattern shown in *B* and *C*. The percentages of cells in which Smad4 staining was high or low in the nucleus or evenly distributed throughout the cytoplasm were determined by randomly counting 260 and 226 individual pre- and post-selection Bre-38 HMECs in >40 independent fields, respectively. ***, $P < 0.0001$, Smad4 localization found to be significantly different between pre- and post-selection HMECs.

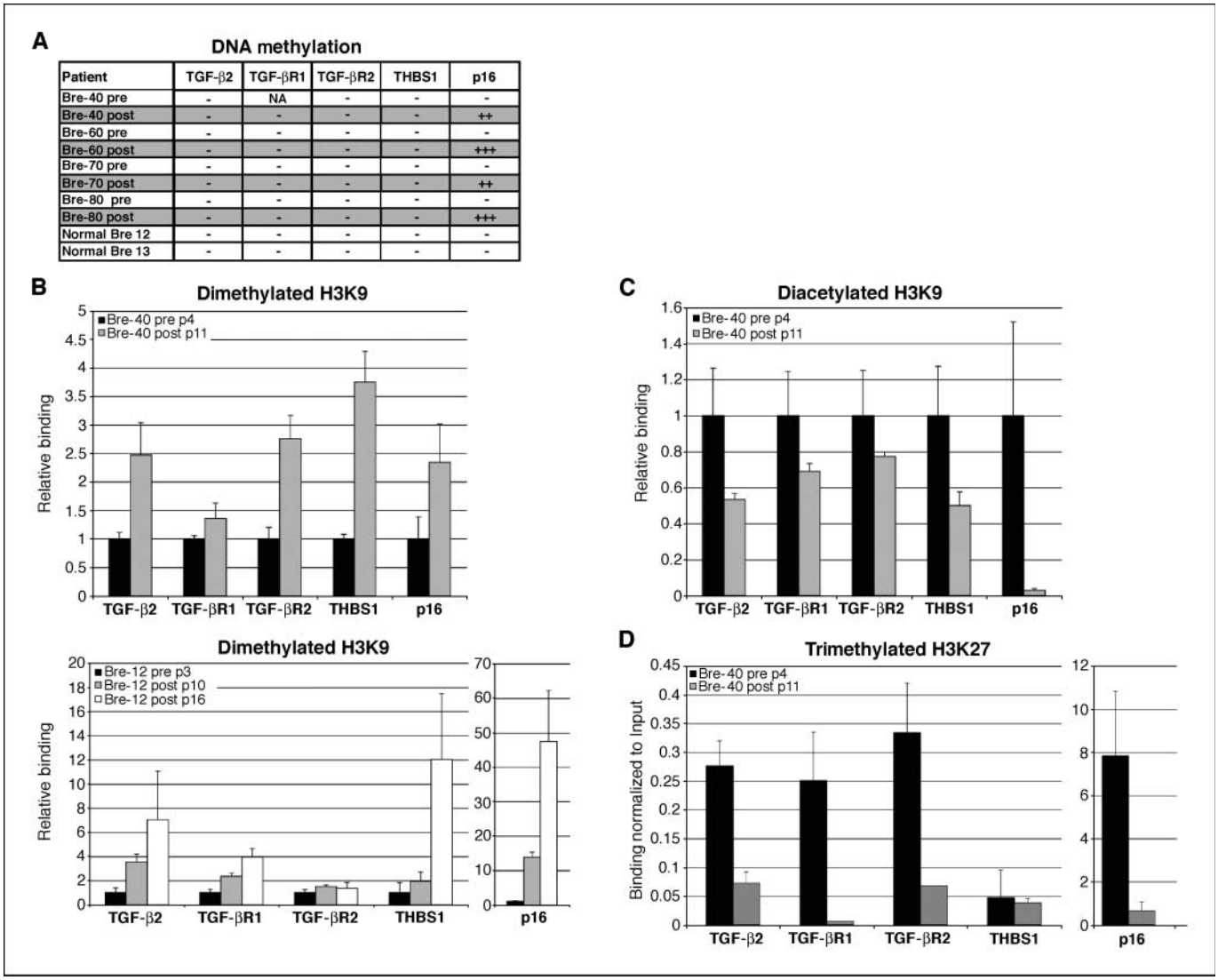


Figure 2. DNA methylation and chromatin immunoprecipitation analysis in HMECs. *A,* summary of DNA methylation status of *TGF-β2, TGF-βR1, TGF-βR2, THBS1*, and $p16^{INK4A}$ in Bre-40, Bre-60, Bre-70, and Bre-80 pre- and post-selection (*shaded*) HMECs and normal Bre-12 and Bre-13 breast tissue samples as determined by bisulfite methylation analysis (see Supplementary Fig. S3). −, unmethylated; +, methylated; *NA,* not amplified. Chromatin from HMECs was immunoprecipitated with dimethylated H3K9 (*B*), diacetylated H3K9 (*C*), and trimethylated H3K27 (*D*) antibodies. The amount of immmunoprecipitated DNA (relative binding) was quantified by real-time PCR and was calculated as a ratio of immunoprecipitated DNA to the total amount of input DNA or as a ratio relative to the amount of immunoprecipitated *16-Cen* (B:Bre-40) or *GAPDH* (C:Bre-40). *B* and *C,* data are relative to pre-selection HMECs.

comparison with the pre-selection HMECs (Fig. 1*B–D*). In the post-selection HMECs, Smad4 fluorescence was evenly distributed and predominantly cytoplasmic, supporting the gene expression results indicating that the TGF-β signaling pathway is indeed disrupted in post-selection cells.

Concordant epigenetic suppression of *TGF-β2* and associated *TGF-β* genes in post-selection HMECs. Because *TGF-β2* was identified on the microarray as a gene that was up-regulated after treatment with the demethylation agent 5-aza-dC, we asked whether suppression of *TGF-β2* and associated *TGF-β* genes in post-selection HMECs was due to DNA hypermethylation. We carried out bisulfite methylation assays on DNA from four independent pre- and post-selection HMEC strains (Bre-40, Bre-60, Bre-70, and Bre-80) and DNA from two normal breast tissue samples (Bre-12 and Bre-13; Fig. 2*A* and Supplementary Fig. S3). After bisulfite treatment, PCR amplification within the CpG island promoters was done in triplicate and methylation of the pooled PCR products was determined by PCR heat dissociation curve analysis (Supplementary Fig. S3*A*), direct bisulfite sequencing (Supplementary Fig. S3*B*), and/or bisulfite clonal sequencing (Supplementary Fig. S3*C*). Figure 2*A* summarizes the methylation studies and shows that *TGF-β2, TGF-βR1, TGF-βR2,* and *THBS1* were unmethylated in all the pre- and post-selection HMEC strains. In contrast, $p16^{INK4A}$ was unmethylated in the normal uncultured breast tissue samples (Bre-12 and Bre-13) and in the pre-selection cells (Bre-40, Bre-60, Bre-70, and Bre-80) but was extensively methylated in all four post-selection HMEC strains studied, as determined previously (10). We concluded that unlike $p16^{INK4A}$, DNA methylation of the CpG island promoter is not responsible for the suppression of *TGF-β2, TGF-βR1, TGF-βR2*, or *THBS1* gene expression in post-selection HMEC cultures.

To determine if the down-regulation of *TGF-β2* and associated genes was due to chromatin remodeling and repression, we carried out chromatin immunoprecipitation assays on pre- and

post-selection Bre-40 and Bre-12 HMECs with antibodies to dimethylated and diacetylated H3K9. First, we found that the *TGF-β2* CpG island promoter selectively bound methylated H3K9 antibody in the post-selection versus pre-selection Bre-40 and Bre-12 HMECs (Fig. 2*B*). *TGF-β2* receptors (*TGF-βR1* and *TGF-βR2*) also selectively bound more methylated H3K9 antibody in the post-selection versus pre-selection Bre-40 HMECs, as did *THBS1* and $p16^{INK4A}$ (Fig. 2*B*), indicating that suppression of *TGF-β2* and associated genes in post-selection cells correlates with repressed chromatin, regardless of DNA hypermethylation. Indeed, the level of promoter-associated H3K9 methylation for Bre-12 associated with the $p16^{INK4A}$, *TGF-β2, TGF-βR1*, and *THBS1* CpG island promoters increased with successive passages (passages 3–16; Fig. 2*B*), indicating an enrichment of chromatin modification with each cell division in the post-selection HMECs. Interestingly in Bre-12 HMECs, there was no change in the *TGF-βR2* CpG island H3K9 methylation, and this also correlated with a lack of *TGF-βR2* suppression (Fig. 1*A*). Second, we found that the *TGF-β2* CpG island promoter selectively bound diacetylated H3K9 antibody in the pre-selection Bre-40 HMECs, indicating deacetylation of the H3K9 associated histones in the post-selection cells (Fig. 2*C*). Similarly, H3K9 deacetylation of $p16^{INK4A}$, receptors *TGF-βR1* and *TGF-βR2*, and *THBS1* was also observed during post-selection growth, correlating again with gene suppression. These results show that *TGF-β2* and associated pathway members *TGF-βR1, TGF-βR2,* and *THBS1* are concordantly down-regulated in post-selection HMECs by repressive chromatin remodeling associated with H3K9 deacetylation and methylation rather than DNA hypermethylation.

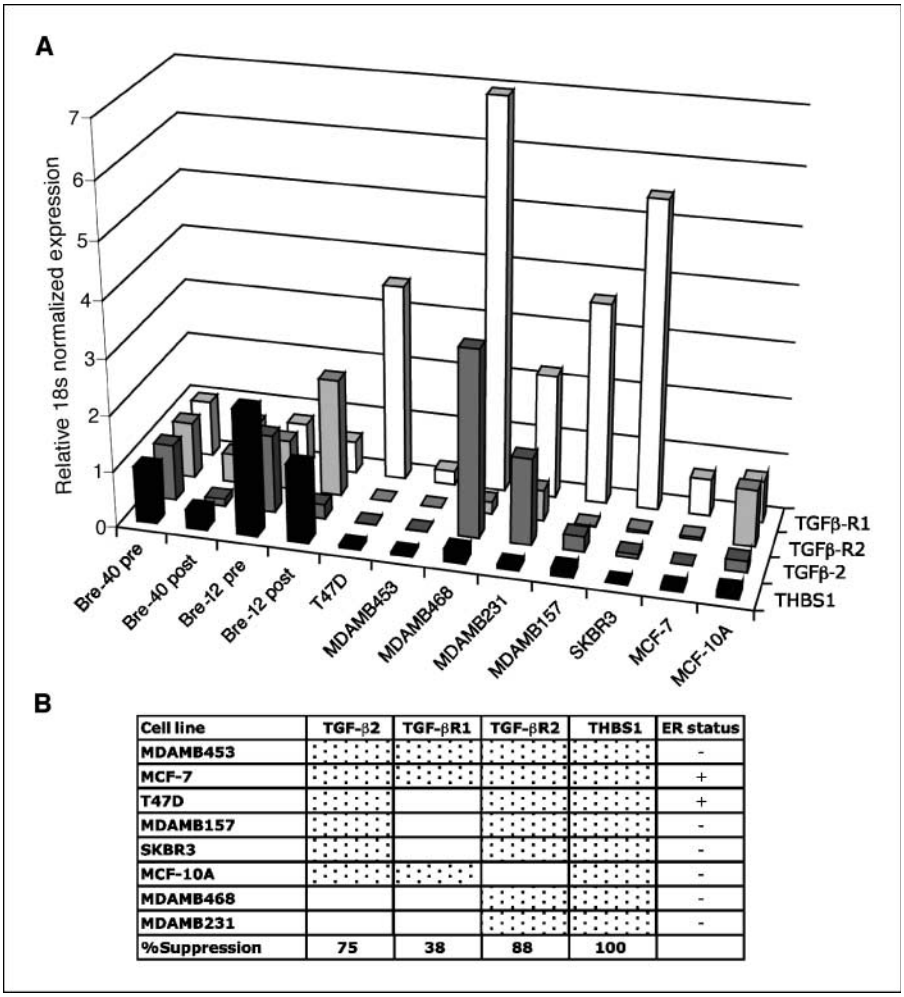
Polycomb-mediated epigenetic suppression of *TGF-β2* and associated *TGF-β* genes in post-selection HMECs. It has been proposed that CpG islands that undergo *de novo* methylation in cancer cells may be marked by polycomb-mediated H3K27 trimethylation in early development, which is then maintained in differentiated cell types by the presence of an EZH2-containing polycomb complex (36, 37). Therefore, to investigate if the $p16^{INK4A}$ gene, which is hypermethylated in post-selection cells, is associated with polycomb-mediated repression, we carried out chromatin immunoprecipitation on pre- and post-selection Bre-40 HMECs with antibodies to trimethylated H3K27. We found that the $p16^{INK4A}$ CpG island unmethylated promoter was enriched for trimethylated H3K27 in Bre-40 pre-selection cells; however, this enrichment was substantially reduced in the post-selection cells when $p16^{INK4A}$ was methylated (Fig. 2*D*). Interestingly, the unmethylated CpG island promoters associated with *TGF-β2, TGF-β2R1*, and *TGF-β2R2* also showed a reduction in H3K27 trimethylation in the Bre-40 post-selection cells despite the fact these genes did not become DNA methylated following selection. Notably, the level of H3K27 trimethylation in the pre-selection cells was greater in $p16^{INK4A}$ than the level found in the *TGF-β* associated genes, suggesting that the density of modification may be an important factor in promoting DNA methylation versus histone methylation.

Epigenetic deregulation of *TGF-β2, TGF-β* receptors, and *THBS1* in breast cancer cell lines. Because post-selection HMECs may mimic the early epigenetic lesions that occur in breast cancer initiation, we next asked if the coordinate epigenetic gene suppression of *TGF-β2, TGF-βR1* and *TGF-βR2* receptors, and *THBS1* that was observed in post-selection HMECs was also observed in breast cancer cell lines. A summary of the expression data for eight breast cancer cell lines (T47D, MDAMB453, MDAMB468, MDAMB231, MDAMB157, SKBR3, MCF-7, and MCF-10A) is shown in Fig. 3*A* and Supplementary Fig. S4. By comparison with pre-selection HMECs, reduced gene expression was observed for *TGF-β2* in 6 of 8 (75%) breast cancer cell lines, *TGF-βR1* in 3 of 8 (38%), *TGF-βR2* in 7 of 8 (88%), and *THBS1* in 8 of 8 (100%; Fig. 3*B*). The level of concordant gene suppression varied between the breast cancer lines. In MDAMB453 and MCF7, all four genes (*TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1*) were suppressed relative to expression in pre-selection Bre-40 cells; expression of three genes (*TGF-β2, TGF-βR1* or *TGF-βR2*, and *THBS1*) was reduced in T47D, MDAMB157, SKBR3, and MCF10-A; and expression of two genes (*TGF-βR2* and *THBS1*) was down-regulated in MDAMB468 and MDAMB231. There was no apparent correlation with estrogen receptor status.

To determine if the reduced expression of *TGF-β2* and associated genes in the breast cancer cell lines was associated with epigenetic suppression, we treated MDAMB453 and T47D cells with 5-aza-dC, TSA, or a combination of both. In MDAMB453 cells, expression of *TGF-β2* mRNA increased 30-fold following treatment with 5-aza-dC, but showed limited activation after TSA alone (Fig. 4*A*). In T47D cells, expression of *TGF-β2* mRNA increased ~3-fold following treatment with 5-aza-dC and showed ~6-fold activation after TSA alone (Fig. 4*B*). In both MDAMB453 and T47D cells, *TGF-βR1, TGF-βR2, THBS1*, and $p16^{INK4A}$ all showed minimal gene activation after 5-aza-dC treatment (1.2- to 3-fold) or TSA treatment (Fig. 4*A* and *B*). However, when the two breast cancer cell lines were treated with 5-aza-dC in conjunction with TSA, all genes commonly exhibited an enhanced induction ranging from 1.5- to 167-fold, most notably in MDAMB453 cells.

To ascertain whether the combined 5-aza-dC and TSA treatment was inducing expression via DNA demethylation, *TGF-β2, TGF-βR1, TGF-βR2, THBS1*, and $p16^{INK4A}$ were assayed for DNA methylation in MDAMB453 cells and nine other breast cancer cell lines (T47D, MDAMB468, SKBR3, KPL1, MDAMB231, DU4475, MCF-7, MDAMB157, and MCF-10A). A summary of the methylation status in the 10 breast cancer cell lines is shown in Fig. 4*C*. Interestingly, the CpG islands spanning *TGF-β2, TGF-βR1, THBS1*, and $p16^{INK4A}$ promoters were all unmethylated in MDAMB453 cells, and TGF-βR2 showed a low level of DNA methylation by direct PCR bisulfite sequencing (Fig. 4*B*). *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* were either unmethylated or only partially methylated in the other nine breast cancer cell lines examined (Fig. 4*C* and Supplementary Fig. S5). Therefore, DNA methylation is not responsible for the suppression of *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* in either the breast cancer cells or post-selection HMECs.

To determine if the epigenetic regulation of *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* in MDAMB453 cells is due to chromatin remodeling rather than DNA hypermethylation, chromatin immunoprecipitation assays were done with dimethylated H3K9, diacetylated H3K9, and trimethylated H3K27 antibodies on untreated MDAMB453 cells versus cells treated with 5-aza-dC in conjunction with TSA (Fig. 5). Treatment with 5-aza-dC/TSA resulted in a loss of dimethylated H3K9 (Fig. 5*A*) and a gain of diacetylated H3K9 (Fig. 5*B*) in all four genes (*TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1*). Interestingly a loss of H3K9 methylation and a gain of H3K9 acetylation were also observed for $p16^{INK4A}$, which is unmethylated in MDAMB453 cells. We also examined the chromatin state of connexin 26 (38) because it is reported to be methylated in MDAMB453 cells. We found that after treatment



Cell line	TGF-β2	TGF-βR1	TGF-βR2	THBS1	ER status
MDAMB453					-
MCF-7					+
T47D					+
MDAMB157					-
SKBR3					-
MCF-10A					-
MDAMB468					-
MDAMB231					-
%Suppression	75	38	88	100	

Figure 3. Gene expression in breast cancer cell lines. *A,* mRNA levels of *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* in eight breast cancer cell lines and Bre-40 and Bre-12 HMECs were determined by quantitative RT-PCR. After normalizing expression to 18S rRNA, the fold change in expression levels was made relative to Bre-40 pre-selection HMECs. *B,* summary table of *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* mRNA expression status in eight breast cancer cell lines. *Dotted boxes,* reduced gene expression relative to expression in Bre-40 pre-selection HMECs. Percentage of cell lines with reduced gene expression is indicated. Estrogen receptor (*ER*) status is also noted (54).

with 5-aza-dC/TSA, there was a substantial loss of H3K9 methylation and a moderate increase in H3K9 acetylation. Interestingly, the four *TGF-β* pathway genes as well as $p16^{INK4A}$ bound more trimethylated H3K27 antibody in MDAMB453 cells treated with 5-aza-dC/TSA, despite these genes being unmethylated in MDAMB453 cells. H3K27 trimethylation was also elevated in the methylated connexin 26 after treatment with 5-aza-dC/TSA. These results suggest that EZH2 polycomb modification could mediate the repression of these genes in the cancer cell, and the polycomb mark is reduced in the cancer cell when the histones are repressed with H3K9 methylation and deacetylation marks regardless of the DNA methylation status. Moreover, the results indicate that there is a similar epigenetic-based repression of the *TGF-β* pathway genes in the post-selection HMECs and the breast cancer cell line MDAMB453, which is associated with histone H3K9 methylation and deacetylation and loss of H3K27 trimethylation.

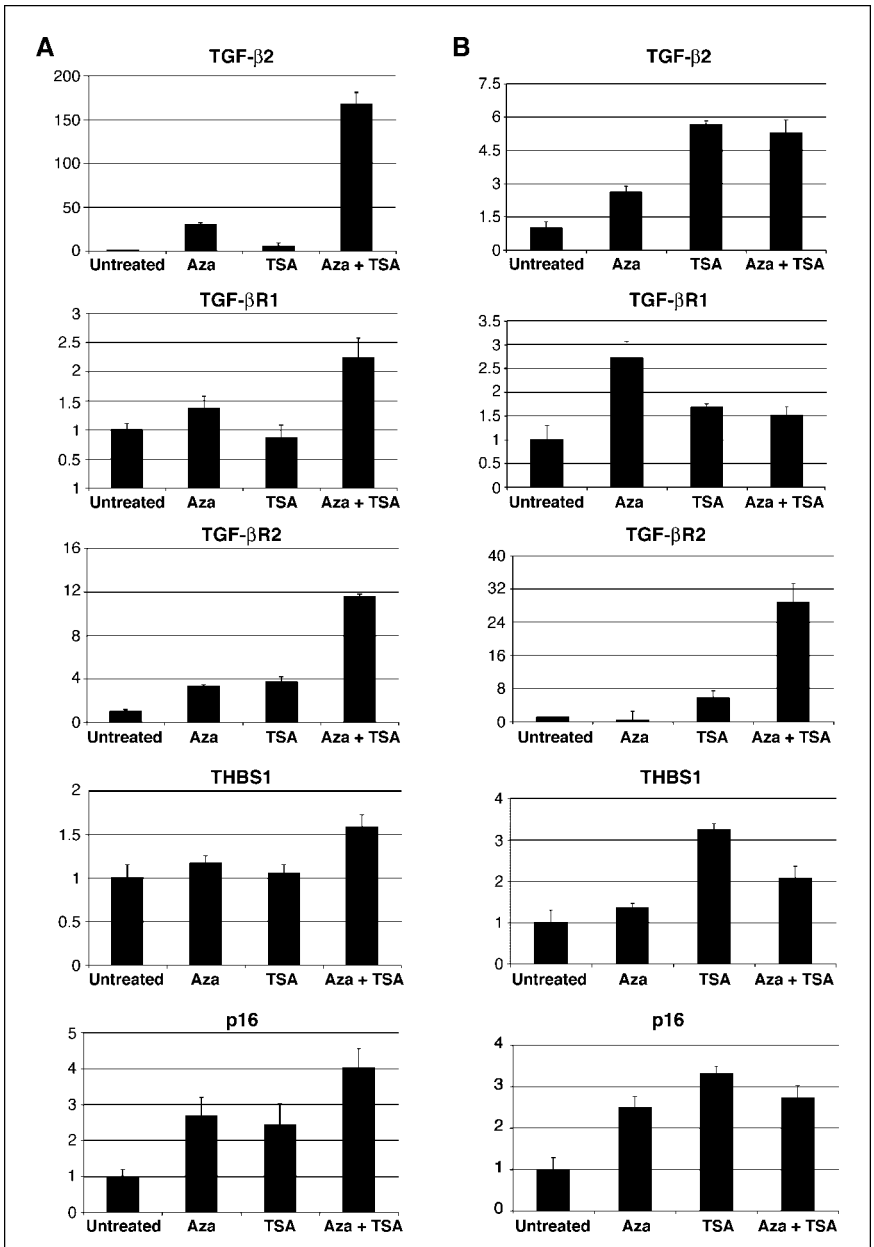
Concordant gene suppression of *TGF-β2, TGF-βR1* and *TGF-βR2* receptors, and *THBS1* in breast cancer. To determine if coordinate suppression of the *TGF-β* pathway genes is common in primary breast cancer cells, we carried out quantitative RT-PCR to measure the expression of *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* in 18 breast tumor samples and compared this with the expression in four normal breast tissue samples (Bre-43, Bre-56, Bre-59, and Bre-67; Fig. 6 and Supplementary Fig. S6). We found that, consistent with the breast cancer cell lines, *TGF-β2, TGF-βR2*, and *THBS1* expression is commonly reduced [$P < 0.03$, $P < 0.002$, and $P < 0.003$, respectively (two-tailed Mann-Whitney U test)] in the cancer samples relative to the normal tissue, whereas *TGF-βR1* expression was more variable (Fig. 6*A*). Levels of expression in the pre-selection HMECs were also consistent with that of the primary normal breast tissue (Fig. 6*B*). Coordinate suppression of all four genes was observed in 10 of 18 (55%) breast tumor samples, and 18 of 18 (100%) showed a reduction of expression of *TGF-β2, TGF-βR2*, and *THBS1* relative to the average normal expression level. We analyzed the CpG island promoters associated with *TGF-β2* in all of the cancer samples and found DNA methylation only in donor 37 (Supplementary Fig. S5*C*). These results support the conclusions from the post-selection HMEC studies that suppression of the *TGF-β* signaling pathway is common in cancer, is an early event, and is associated with chromatin repression rather than DNA methylation.

Discussion

HMECs grown in culture provide a unique *in vitro* system to dissect the very early molecular pathways leading to carcinogenesis. Post-selection HMECs represent a subpopulation of cells that proliferate rapidly and have $p16^{INK4A}$ silencing and promoter methylation and cyclooxygenase-2 overexpression. In the later stages of post-selection growth, there is an accumulation of

chromosomal abnormalities and expression phenotypes that are conducive to malignant progression (9, 10, 12–15, 34, 39). Recently, it has also been shown that in post-selection HMECs, loss of $p16^{INK4A}$ activity is associated with polycomb-mediated silencing and DNA methylation of the *HOXA9* gene, which raises the possibility that these cells may also share characteristics of stem or progenitor cells (40).

To define early epigenetic lesions in breast cancer, we used primary HMECs grown in culture to generate post-selection HMECs as an *in vitro* model to study mammalian carcinogenesis. We identified concordant epigenetic suppression of multiple members of the *TGF-β* signaling pathway in the post-selection HMECs. This involved lowered expression of the receptors *TGF-βR1* and *TGF-βR2, THBS1* (an activator of *TGF-β2*), and



C

Cell line	TGF-β2	TGF-βR1	TGF-βR2	THBS1	p16
T47D	-	-	++	-	NA
MDAMB453	-	-	+	-	-
MDAMB468	-	-	-	-	-
SKBR3	-	-	-	-	-
KPL1	-	-	-	-	NA
MDAMB231	+	+	-	-	Deleted
DU4475	-	-	-	-	+++
MCF-7	-	+	-	-	Deleted
MDAMB157	-	-	-	-	-
MCF-10A	-	+	-	-	Deleted

Figure 4. Gene reactivation and DNA methylation studies in breast cancer cell lines. mRNA levels of *TGF-β2, TGF-βR1, TGF-βR2, THBS1*, and $p16^{INK4A}$ mRNA in MDAMB453 (*A*) and T47D (*B*) were determined by quantitative RT-PCR in untreated cells and cells treated with 5-aza-dC (*Aza*) alone, TSA alone, or 5-aza-dC/TSA in combination. After normalizing expression to 18S rRNA, the fold change in expression levels was made relative to untreated cells. *C,* summary of DNA methylation status of *TGF-β2, TGF-βR1, TGF-βR2, THBS1*, and $p16^{INK4A}$ in 10 breast cancer cell lines as determined by bisulfite methylation analysis (see Supplementary Fig. S4). −, unmethylated; +, methylated; *NA,* not amplified; *Deleted,* $p16^{INK4A}$ is deleted.

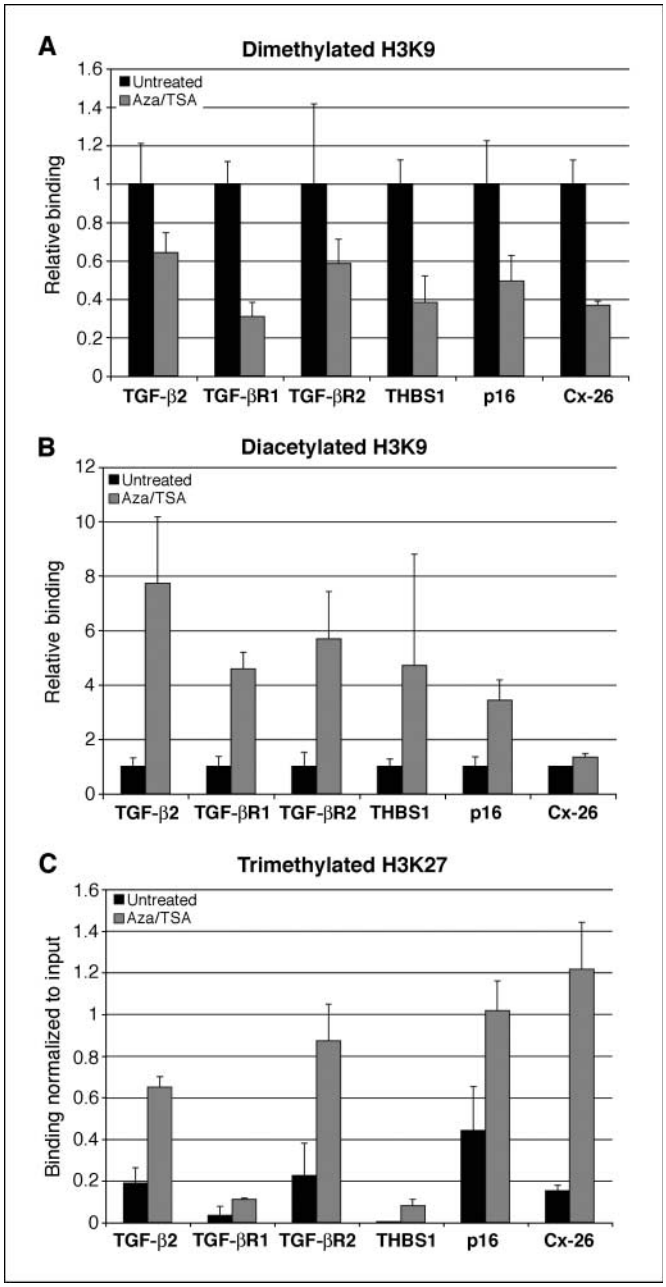


Figure 5. Chromatin immunoprecipitation studies in MDAMB453 cells after 5-aza-dC/TSA treatment. Chromatin from MDAMB453 cells treated with 5-aza-dC/TSA was immunoprecipitated with dimethylated H3K9 (*A*), diacetylated H3K9 (*B*), and trimethylated H3K27 (*C*) antibodies. The amount of immmunoprecipitated DNA (relative binding) was quantified by real-time PCR and was calculated as a ratio of immunoprecipitated DNA to the total amount of input. *A* and *B,* data are relative to untreated cells. *Cx-26,* connexin 26.

the *TGF-β2* gene itself; expression levels of the other TGF-β genes (isoforms), *TGF-β1* and *TGF-β3*, were not suppressed. Functional TGF-β signaling results in nuclear localization of Smad4 in pre-selection HMECs, whereas altered expression of *TGF-β2* and *TGF-β* receptors in post-selection HMECs was associated with a significant reduction in Smad4 nuclear localization, indicating deregulation of a functional TGF-β pathway. Gene suppression of *TGF-β2, TGF-β* receptors, and *THBS1* was also commonly found in breast cancer cell lines and breast cancer tissue samples. Surprisingly, gene suppression was associated with a decrease in polycomb-directed H3K27 histone methylation and an increase in histone H3K9 methylation and deacetylation, but suppression was not associated with DNA methylation. These results indicate that epigenetic suppression of *TGF-β* signaling is an early lesion that promotes cell proliferation in the process of breast oncogenesis.

Contemporary ideas of carcinogenesis envisage a series of stochastic genetic and epigenetic changes that confer a selective growth advantage over healthy cells. These changes collectively lead to the disruption of coordinated networks of intercellular communication and cause a fundamental change in cellular behavior that affects processes such as proliferation, differentiation, and apoptosis, with progressive dysregulation and acquisition of a malignant phenotype. The TGF-β signaling pathway is involved in many different biological processes during embryonic development and also plays an important role in tissue homeostasis (18). TGF-β is an important negative growth regulator that has antiproliferative effects on a range of epithelial cells, and disruption of TGF-β signaling is strongly implicated in many cancers including breast cancer (20, 41). Interestingly, it was previously reported (35, 42) that transformed HMECs commonly showed resistance to TGF-β growth inhibition compared with normal HMECs isolated from reduction mammoplasty, suggesting that suppression of the TGF-β pathway contributes to proliferation of these cells. Genetic lesions of individual members of the *TGF-β* signaling pathway are also frequently reported in human cancer. For example, *TGF-βR1* and *TGF-βR2* are inactivated by mutations in numerous cancers including breast and ovarian cancer (43, 44). In addition to genetic lesions in the *TGF-β* signaling pathway, epigenetic modifications have also been reported in some cancers. For example, *THBS1*, which is an activator of TGF-β (20), is down-regulated in many tumors (28) and methylated in glioblastoma cell lines and in 33% of examined glioblastoma multiforme tumors. The *TGF-β* receptors *TGF-βR1* and *TGF-βR2*, which are essential for *TGF-β* signaling (20), are epigenetically regulated in many cancers; *TGF-βR1* is hypermethylated in gastric cancer (22), and treatment of breast cancer cells with DNA methylation (23) or histone deacetylase (25) inhibitors induces *TGF-βR1* and *TGF-βR2* expression, indicating a role of chromatin remodeling in epigenetic repression. *TGF-β* has been observed to have dual and opposing roles in breast cancer: in healthy tissues it acts as a tumor suppressor gene, but in the advanced stages of cancer it can switch to be a tumor promoter (20). The answer to this apparent paradox may lie in the early stages of epigenetic silencing of the *TGF-β* signaling pathway due to chromatin remodeling and not DNA methylation, therefore permitting later gene reactivation in advanced breast cancer.

It is of interest to speculate what triggers chromatin remodeling and repression of *TGF-β2* and associated genes in post-selection HMECs and yet DNA methylation, and therefore irreversible epigenetic silencing, is not triggered, as is the case for $p16^{INK4A}$. Our results shed light on a number of possibilities. First, the degree of expression may be an important factor because $p16^{INK4A}$ is completely inactivated in HMECs whereas expression of *TGF-β2* and related genes is commonly suppressed but not completely silenced. This supports the hypothesis that prior gene silencing is an important factor that initiates aberrant DNA methylation and subsequent chromatin remodeling (32, 45). Second, the degree of chromatin modification in the post-selection HMECs may not be sufficient to promote DNA methylation. For example, the CpG

island promoter region associated with $p16^{INK4A}$ was enriched >40-fold for H3K9 dimethylation after 16 passages in comparison with a 6-fold enrichment for H3K9 dimethylation across the *TGF-β2* CpG island promoter region. Conversely, $p16^{INK4A}$ was associated with a 36-fold enrichment of acetylated H3K9 histones in pre-selection HMECs in comparison with a 2-fold enrichment observed for *TGF-β2*. This observation supports the hypothesis that it is histone methylation and deacetylation that direct subsequent DNA methylation in cancer cells (46). Lastly, an intriguing possibility is that it is the degree of EZH2 polycomb–directed H3K27 methylation in the pre-selection HMECs that plays a crucial role in determining which genes are susceptible to subsequent DNA methylation. For example, there was a 16-fold enrichment in trimethylated H3K27 in the CpG island promoter region associated with $p16^{INK4A}$ in comparison with a 4-fold difference for *TGF-β2* in pre-selection HMEC cells. Recently, Schlesinger et al. (37) reported that genes methylated in cancer are potentially marked in early development by trimethylated H3K27, and in cancer, unlike in normal cells, the presence of the EZH2-containing polycomb complex recruits DNA methyltransferases that lead to *de novo* methylation (47). Ohm et al. (48) and Widschwendter et al. (49) find that genes enriched for trimethylated H3K27 in stem and progenitor cells are also common cancer-specific promoter DNA methylation targets. Members of *TGF-β* signaling pathway also were recently identified as genes in embryonic cells commonly bound by polycomb proteins in a genome-wide screen (50). It is therefore interesting to speculate that post-selection HMECs are derived from a small subset of progenitor cells found in normal mammary epithelial cells and one of the first predisposing steps toward malignancy, which occurs early in post-selection, is associated with a change in expression and concordant chromatin remodeling of polycomb-associated genes, but not all are susceptible to subsequent DNA methylation and permanent repression.

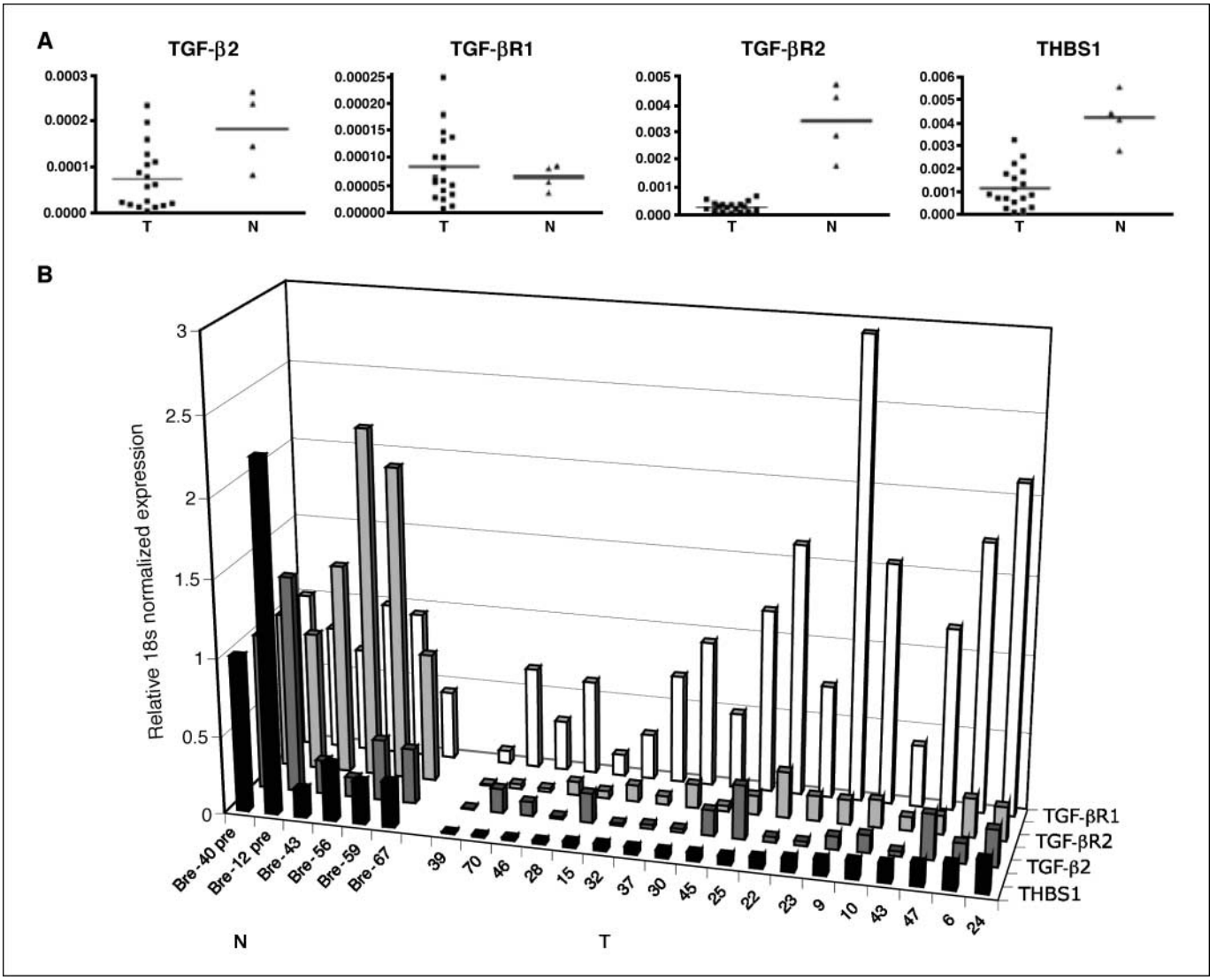


Figure 6. Concordant gene suppression in breast cancer. *A,* scatter plots depicting *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* mRNA expression in 18 breast tumors (*T*; tumors 70, 39, 46, 30, 15, 28, 9, 37, 22, 32, 45, 10, 23, 25, 43, 24, 6, and 47) and four normal breast tissue samples (*N*; Bre-43, Bre-56, Bre-59, and Bre-67). *B,* mRNA expression of *TGF-β2, TGF-βR1, TGF-βR2*, and *THBS1* mRNA expression in the 18 breast tumors and 4 normal breast tissue samples and Bre-40 and Bre-12 pre-selection HMECs was determined by quantitative RT-PCR. Expression levels were normalized to 18S rRNA and made relative to Bre-40 pre-selection HMECs.

Our study is the first to report that multiple members of the *TGF-β* pathway are commonly down-regulated in post-selection HMECs that have acquired an enhanced proliferative capacity in culture and exhibit some of the early events in breast carcinogenesis. We show that repression of these genes is not caused by DNA hypermethylation in either post-selection HMECs or breast cancer cells but by chromatin repression. Interestingly, treatment of breast cancer cells with the demethylation and deacetylation agents was able to induce expression of these pathway genes despite the genes being unmethylated. Gene activation and changes in histone methylation of unmethylated genes in cancer cells after treatment with 5-aza-dC have been previously reported, suggesting that this may be a common phenomenon (30, 51–53). Therefore, the use of epigenetic therapy in cancer for gene reactivation should be approached with caution because the activation of the *TGF-β* pathway may have deleterious effects in stimulating tumor progression. Our findings of coordinate down-regulation of multiple members of a gene pathway through epigenetic remodeling will have major implications in the future understanding of breast cancer initiation and progression.

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