

Smad Signaling Is Required to Maintain Epigenetic Silencing during Breast Cancer Progression

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Abstract

Breast cancer progression is associated with aberrant DNA methylation and expression of genes that control the epithelial-mesenchymal transition (EMT), a critical step in malignant conversion. Although the genes affected have been studied, there is little understanding of how aberrant activation of the DNA methylation machinery itself occurs. Using a breast cancer cell-based model system, we found that cells that underwent EMT exhibited overactive transforming growth factor β (TGF β) signaling and loss of expression of the *CDHI*, *CGN*, *CLDN4*, and *KLK10* genes as a result of hypermethylation of their corresponding promoter regions. Based on these observations, we hypothesized that activated TGF β -Smad signaling provides an "epigenetic memory" to maintain silencing of critical genes. In support of this hypothesis, disrupting Smad signaling in mesenchymal breast cancer cells resulted in DNA demethylation and reexpression of the genes identified. This epigenetic reversal was accompanied by an acquisition of epithelial morphology and a suppression of invasive properties. Notably, disrupting TGF β signaling decreased the DNA binding activity of DNA methyltransferase DNMT1, suggesting that failure to maintain methylation of newly synthesized DNA was the likely cause of DNA demethylation. Together, our findings reveal a hyperactive TGF β -TGF β R-Smad2 signaling axis needed to maintain epigenetic silencing of critical EMT genes and breast cancer progression. *Cancer Res*; 70(3); 968–78. ©2010 AACR.

Introduction

Epigenetic regulation of gene expression is a fundamental feature affecting normal physiologic processes as well as diseases such as cancer. Aberrant global DNA hypomethylation as well as hypermethylation of specific regulatory regions of genes are considered as hallmarks of cancer progression (1). Silencing of tumor suppressor genes by promoter DNA hypermethylation has been associated with the expansion of premalignant cells and acquisition of an invasive phenotype leading to metastatic dissemination (2). Except for the recent implication of the Ras pathway as a potential mediator of epigenetic gene silencing (3), the upstream signaling cascades that control the activity of the DNA methylation machinery remain largely elusive.

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

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doi: 10.1158/0008-5472.CAN-09-1872

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Epithelial-mesenchymal transition (EMT) is a critical process required for the initiation of the metastatic spread of tumor cells to distal tissues (4), and its manifestation has been proposed to involve specific DNA hypermethylation patterns (5). EMT is initiated by a process whereby epithelial cells lose adhesion and cell-cell contacts while undergoing dramatic remodeling of their cytoskeleton. Concurrently, the expression of epithelial marker genes is suppressed, whereas the expression of mesenchymal components becomes increased (6). This process is regulated by factors, such as transforming growth factor β (TGF β), secreted in the tumor microenvironment (5, 7–9). Although this pleiotropic cytokine mediates transcriptional regulation of downstream genes via the formation of Smad2/3-Smad4 complex (10), it also induces the expression of the inhibitory Smad7, a negative feedback regulator of the pathway (11). Interestingly, studies using mutant TGF β RI constructs that are defective in binding Smads, but retained signaling via mitogen-activated protein kinases, revealed that Smads are likely to be involved in the EMT process (12, 13). Additionally, it has been suggested that TGF β could cooperate with other signaling pathways, such as oncogenic Ras, in promoting EMT (9, 14, 15).

TGF β overexpression is commonly observed in advanced breast cancers concomitant with a prevalence of nuclear phosphorylated Smad2 (16), suggesting that overactivation of TGF β signaling might promote metastatic breast cancer. Consistent with this notion, reduction in Smad2/3 signaling or ectopic expression of a Smad-binding-defective TGF β RI mutant has been shown to suppress metastasis of breast cancer cells (17, 18).

Because of the increasing evidence implicating TGF β in EMT and tumor invasion and due to the likely role of the tumor microenvironment in the induction of DNA methylation during conditions of prolonged EMT (5), we hypothesized that the TGF β signaling pathway might be directly involved in epigenetic regulation of cellular plasticity. Here, we describe the use of a breast cancer progression model system (19–21) to elucidate the role of signaling mediators that are critical for the regulation of aberrant DNA methylation patterns during EMT. Our studies show, for the first time, that disruption of the TGF β pathway results in DNA demethylation and reexpression of specific genes accompanied with reversal to epithelial morphology and suppression of the invasive properties of breast cancer cells, suggesting a direct role for this cytokine in the establishment and maintenance of EMT.

Materials and Methods

Cell culture. MCF10A-(MI), MCF10ATk1.cl2-(MII), and MCF10CA1h-(MIII) breast cancer cell lines were obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI) and maintained in DMEM-F/12 medium containing 5% heat-inactivated horse serum, 10 μ g/mL insulin, 20 ng/mL epidermal growth factor, 0.1 μ g/mL cholera enterotoxin, and 0.5 μ g/mL hydrocortisone.

Antibodies. The working dilutions and sources of the antibodies were as indicated: E-cadherin (1:1,000 for Western blot/1:100 for immunofluorescence), β -catenin (1:1,000), and N-cadherin (1:1,000) from BD Biosciences; vimentin (1:1,000), Smad4 (1:1,000), γ -catenin (1:1,000), and fibronectin (1:500) from Santa Cruz Biotechnology; anti-hemagglutinin (1:1,000) from Roche; β -actin (1:15,000) from Sigma; Smad2 (1:1,000) and phosphorylated Smad2 (1:1,000) from Cell Signaling; DNMT1 (1:500) and DNMT3B

(1:500) from Abcam; and anti-5'-methylcytosine (1:50) from EMD Biosciences.

Viral transduction. Stable retroviral and lentiviral transductions were performed using the pBabe and pLKO.1 vectors, respectively, at a multiplicity of infection of 5 plaque-forming units/cell. Additional details can be found in Supplementary Materials and Methods.

Western blotting and immunofluorescence microscopy. Western blotting analysis and immunofluorescence microscopy were performed as previously described (22).

Drug treatments. Cells were grown overnight and then treated with 5 μ mol/L 5'-aza-deoxycytidine (5-Aza), 100 nmol/L trichostatin A (TSA), or 1 mmol/L sodium butyrate (Sigma).

Immunoprecipitation of methylated DNA, methylation-specific PCR, quantitative MSP, and bisulfite sequencing. Genomic DNA from cells was isolated using the DNeasy tissue kit (Qiagen), according to the manufacturer's protocol. Immunoprecipitation of methylated DNA (MeDIP) was performed as previously described (23). EpiTect Bisulfite kit (Qiagen) was used for sodium bisulfite treatment of the genomic DNA. Bisulfite sequencing of the *CDH1* promoter involved TA cloning of the template as described in Supplementary Materials and Methods.

Wound-healing assays. Stably transduced cells (1×10^6) were grown overnight in 60-mm dishes to reach confluency, and a wound was introduced using a Q-tip. The cell migration rate in the cell-free area was monitored over indicated times using light microscopy.

Chemotaxis and Matrigel invasion assays. Chemotaxis and Matrigel invasion assays were performed using Transwells containing 8.0- μ m pore membrane (Corning), as described in Supplementary Materials and Methods.

Gene expression analysis. Total RNA was isolated from three biological replicates corresponding to each cell type (MIIpB, MIIIpB, and MIIIpBSmad7) using RNeasy Mini kit

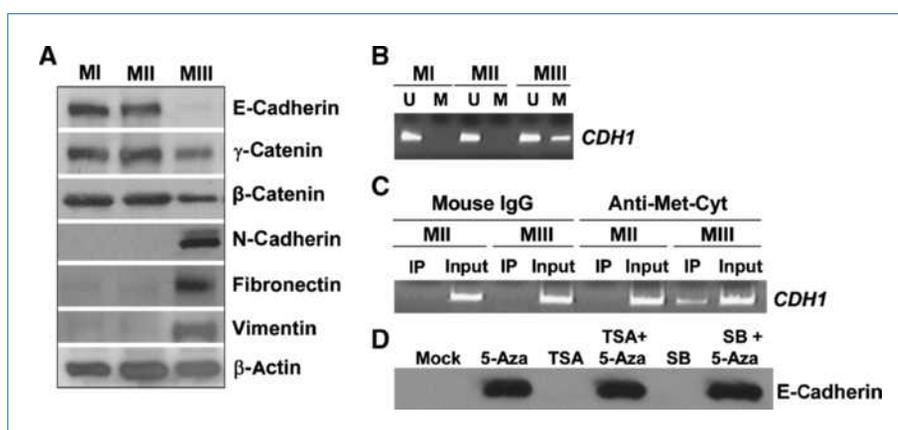


Figure 1. EMT in the MCF10A-based breast cancer progression model correlates with DNA hypermethylation–mediated silencing of E-cadherin expression. A, Western blotting analysis of cell lysates isolated from MI, MII, and MIII cells for detection of epithelial (E-cadherin, β -catenin, and γ -catenin) and mesenchymal (vimentin, fibronectin, and N-cadherin) protein markers. B, MSP analysis of the –160 to +1 bp region of E-cadherin (*CDH1*) promoter using bisulfite-treated genomic DNA isolated from MI, MII, and MIII cells. C, MeDIP using either a mouse IgG (mock) or an anti-methylcytosine (*Met-Cyt*) monoclonal antibody using genomic DNA isolated from MII and MIII cells and PCR analysis of the *CDH1* promoter. D, individual and combinations of 5-Aza, TSA, and sodium butyrate (SB) treatments in MIII cells for 72 h and Western blotting for E-cadherin detection.

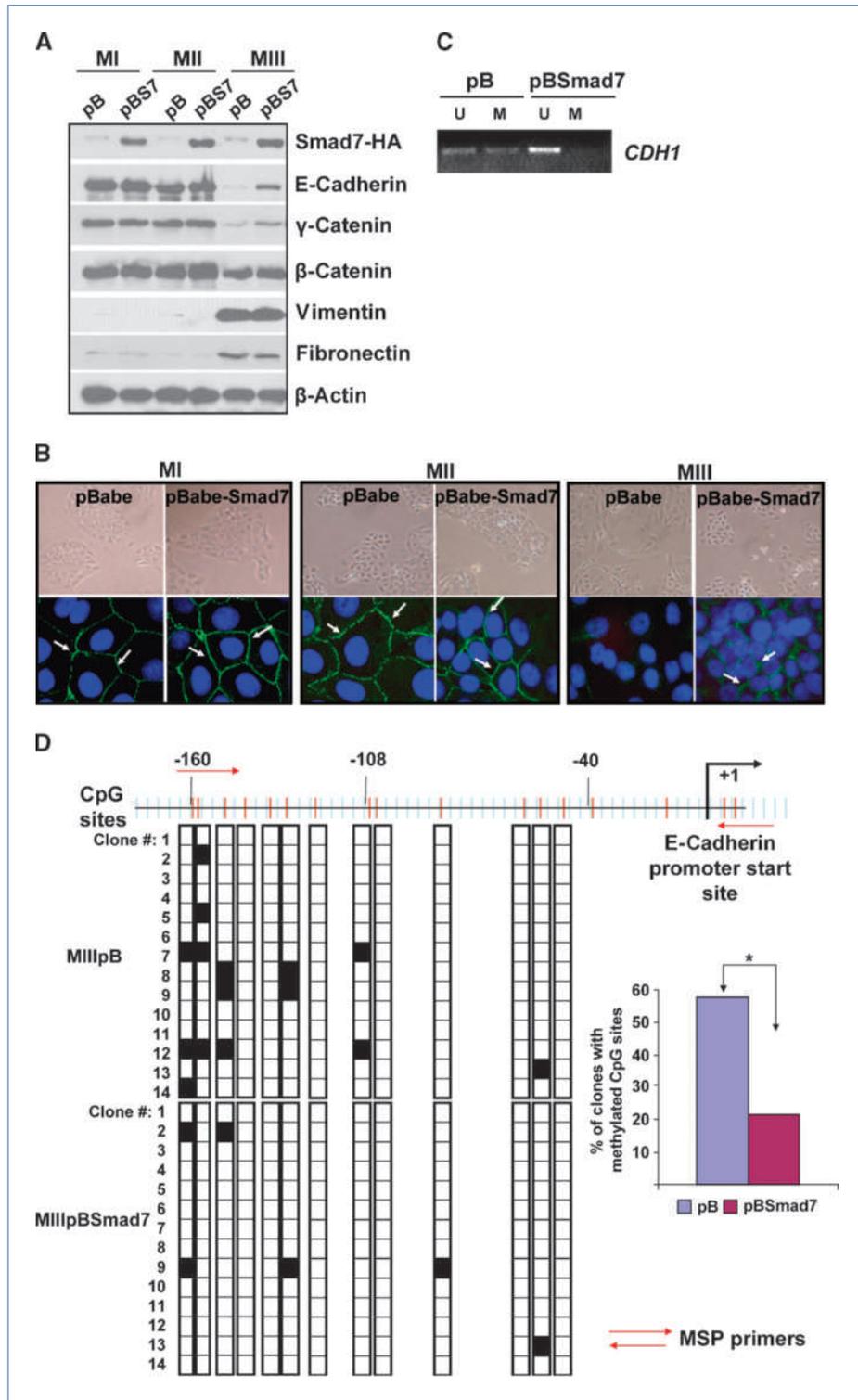


Figure 2. Smad7 overexpression induces promoter DNA demethylation and reexpression of E-cadherin. **A**, Western blotting analysis to detect stable overexpression of exogenous Smad7 and epithelial and mesenchymal markers in MI, MII, and MIII cells. **B**, Smad7 overexpression induces epithelial morphology in mesenchymal-like MIII cells and localization of E-cadherin at the cell-cell junctions. Representative examples of light microscopy (10 \times) and immunofluorescence images (100 \times) generated using an anti-E-cadherin primary and FITC-conjugated secondary antibodies in the MI, MII, and MIII cells stably transduced with either pB or pBSmad7 vectors. Blue, cell nuclei were stained with 4',6-diamidino-2-phenylindole. Localization of E-cadherin (green) at the cell-cell junctions is indicated by white arrows. **C**, semiquantitative MSP analysis of the -160 to +1 bp *CDH1* promoter region using bisulfite-treated genomic DNA isolated from the MIIIpB and MIIIpBSmad7 cells. **D**, mapping of the methylated CpG dinucleotides within the -160 to +1 *CDH1* promoter region by analyzing 14 clones each from the bisulfite-treated templates derived from the MIIIpB and MIIIpBSmad7 cells. White and black squares represent unmethylated and methylated CpGs, respectively. Bar chart shows percentages of clones that exhibited at least one methylated CpG in relation to the total number of clones sequenced. A *p*-value of less than 0.05 (indicated by the * symbol) was considered statistically significant between the compared samples.

(Qiagen), and labeled cRNA fragments were hybridized to human genome U133 Plus 2.0 microarrays (Affymetrix). Gene expression estimates and the measure of sequence specificity of the hybridization intensities were both determined using

standard settings in MAS5 (Affymetrix). Student's *t* test was used to assess differential gene expression. Genes with a false discovery rate of <0.05 and a >2-fold difference in expression were considered to be differentially expressed. The

microarray data generated in this study are available from the National Center for Biotechnology Information Gene Expression Omnibus (24) under accession code GSE18070. Real-time quantitative reverse transcription-PCR (q-RT-PCR) was performed using SYBR Green Power Master Mix (Applied Biosystems).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was performed using the EZ-Magna ChIP G Chromatin Immunoprecipitation kit (Millipore) using chromatin isolated from 1×10^6 cells per condition, according to the manufacturer's protocol.

Results

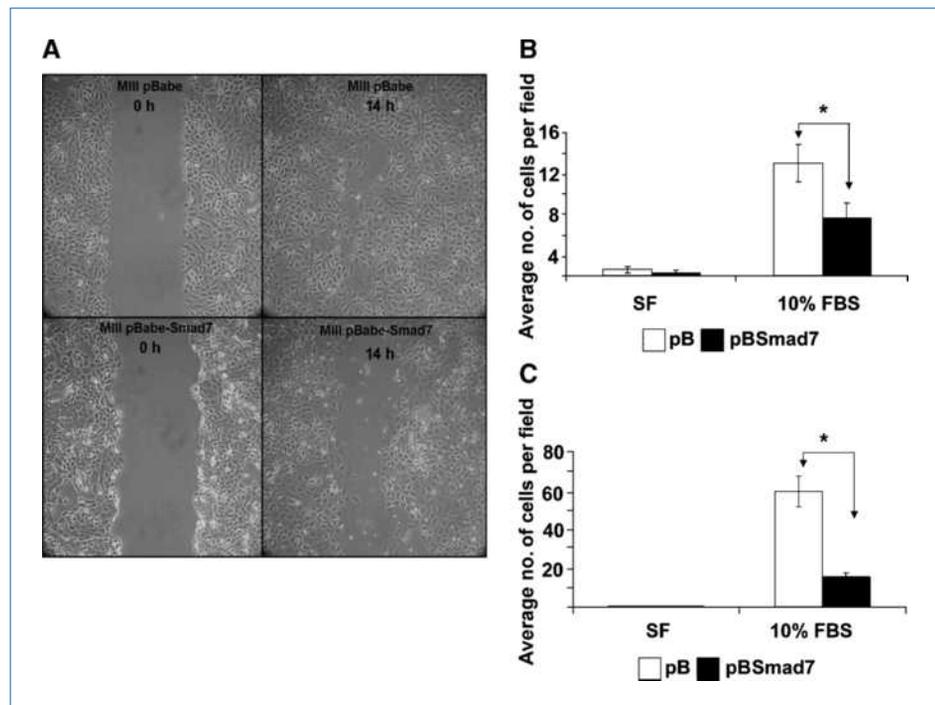
Characterization of the MCF10A-based breast cancer progression model. We took advantage of a previously established cell line model system for breast cancer progression, which consists of a parental spontaneously immortalized mammary epithelial cell line, MCF10A (MI), and two of its derivatives: (a) MCF10ATk.cl2 (MII), an H-Ras-transformed MCF10A cell line, and (b) MCF10CA1h (MIII), derived from a xenograft of MII cells in nude mice that progressed to carcinoma (19–21). These cell lines were previously reported to exhibit distinct tumorigenic properties when reimplanted in nude mice; MI is nontumorigenic, MII forms benign hyperplastic lesions, and MIII forms low-grade, well-differentiated carcinomas (20, 21). The advantage of this system is that these cell lines were derived from a common genetic background (MCF10A) and accumulated distinct genetic/epigenetic alterations *in vivo*, enabling them to acquire properties associated with gradual progression from nontumorigenic to

carcinogenic state. Interestingly, whereas the MI and MII cells exhibited a cobble-shaped epithelial morphology, the MIII cells were spindle shaped with a mesenchymal-like phenotype representing an apparent EMT during progression from MII to MIII (Supplementary Fig. S1A).

To further investigate EMT using this model system, we characterized the expression of epithelial and mesenchymal markers. There was expression of predominantly epithelial markers (E-cadherin, γ -catenin, and β -catenin) in the MI and MII cells and of mesenchymal markers (fibronectin, vimentin, and N-cadherin) in the MIII cells with concomitant downregulation of E-cadherin, β -catenin, and γ -catenin (Fig. 1A). These observations suggested that comparing the features of MII and MIII cells is a logical approach to investigate the molecular events responsible for EMT and the accompanying epigenetic changes during the progression from *in situ* to invasive breast carcinoma.

The loss of E-cadherin (*CDH1*) expression, a prominent biomarker for the epithelial state, due to promoter DNA hypermethylation has been associated with EMT and acquisition of invasive properties of breast cancer cells (25, 26). Therefore, we hypothesized that downregulation of E-cadherin expression in MIII cells is mediated by epigenetic silencing. Methylation-specific PCR (MSP) analysis and immunoprecipitation of genomic DNA from the MII and MIII cells using a monoclonal antibody against methylated cytosine residues showed that, in contrast to MI and MII cells, the *CDH1* promoter is hypermethylated in MIII cells (Fig. 1B and C), consistent with the observed loss of expression (Fig. 1A). Moreover, whereas treatment with the DNA methylation inhibitor 5-Aza resulted in a robust increase in

Figure 3. Smad7 overexpression suppresses migration and invasion of MIII cells. A, representative light microscope images of wound-healing assays for MIIIpB and MIIIpBSmad7 cells to evaluate their migration rate into the cell-free area. B, chemotaxis assay. Cells that migrated through the 8- μ m pore-containing membrane of the Transwells were stained with propidium iodide and counted. SF, serum-free; FBS, fetal bovine serum. C, Matrigel invasion assay. Cells that invaded through the Matrigel were stained with trypan blue and counted. All results are presented as the average of cells counted in 10 fields per condition. The results presented are the average of triplicate samples and a *p*-value of less than 0.05 (indicated by the * symbol) was considered statistically significant between the compared samples. Error bars represent \pm standard error (S.E.) values.



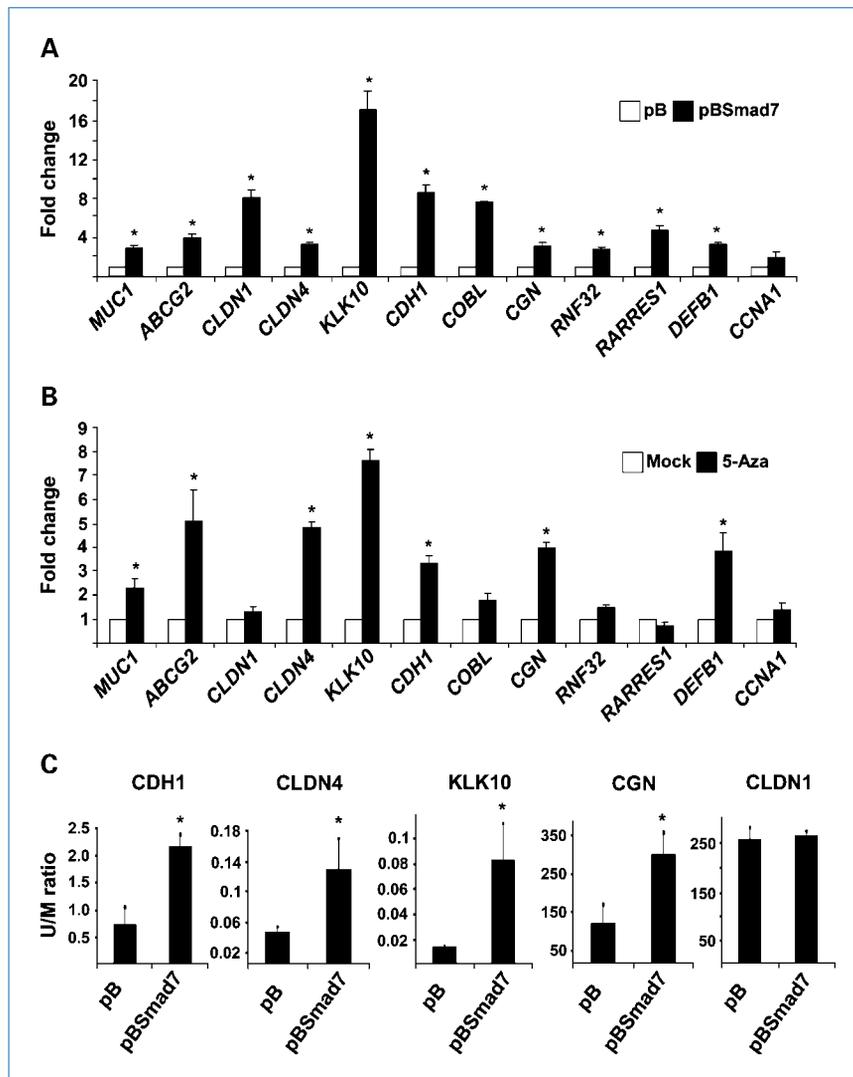


Figure 4. Expression and DNA methylation analysis of candidate genes silenced due to DNA hypermethylation in MIII cells. A, verification of differential expression of indicated genes using q-RT-PCR in MIIIpB and MIIIpBSmad7 cells. B, q-RT-PCR analysis for expression in MIII cells treated with 5 μ mol/L 5-Aza or DMSO (Mock) for 72 h showing induction of *ABCG2*, *CDH1*, *CGN*, *CLDN4*, *DEFB1*, *KLK10*, and *MUC1*. C, q-MSP analysis of *CDH1*, *CGN*, *CLDN1*, *CLDN4*, and *KLK10*. The amount of CpG methylation was quantified based on the unmethylated (U) to methylated (M) product ratio, normalized to β -actin. The results presented are the average of triplicate samples and a *p*-value of less than 0.05 (indicated by the * symbol) was considered statistically significant between the compared samples. Error bars represent \pm standard error (S.E.) values.

E-cadherin expression, treatment with the histone deacetylase inhibitors TSA or sodium butyrate had no effect, indicating that E-cadherin silencing occurs predominantly due to promoter DNA hypermethylation (Fig. 1D).

Smad7 overexpression induces an epithelial morphology in association with CDH1 promoter DNA demethylation.

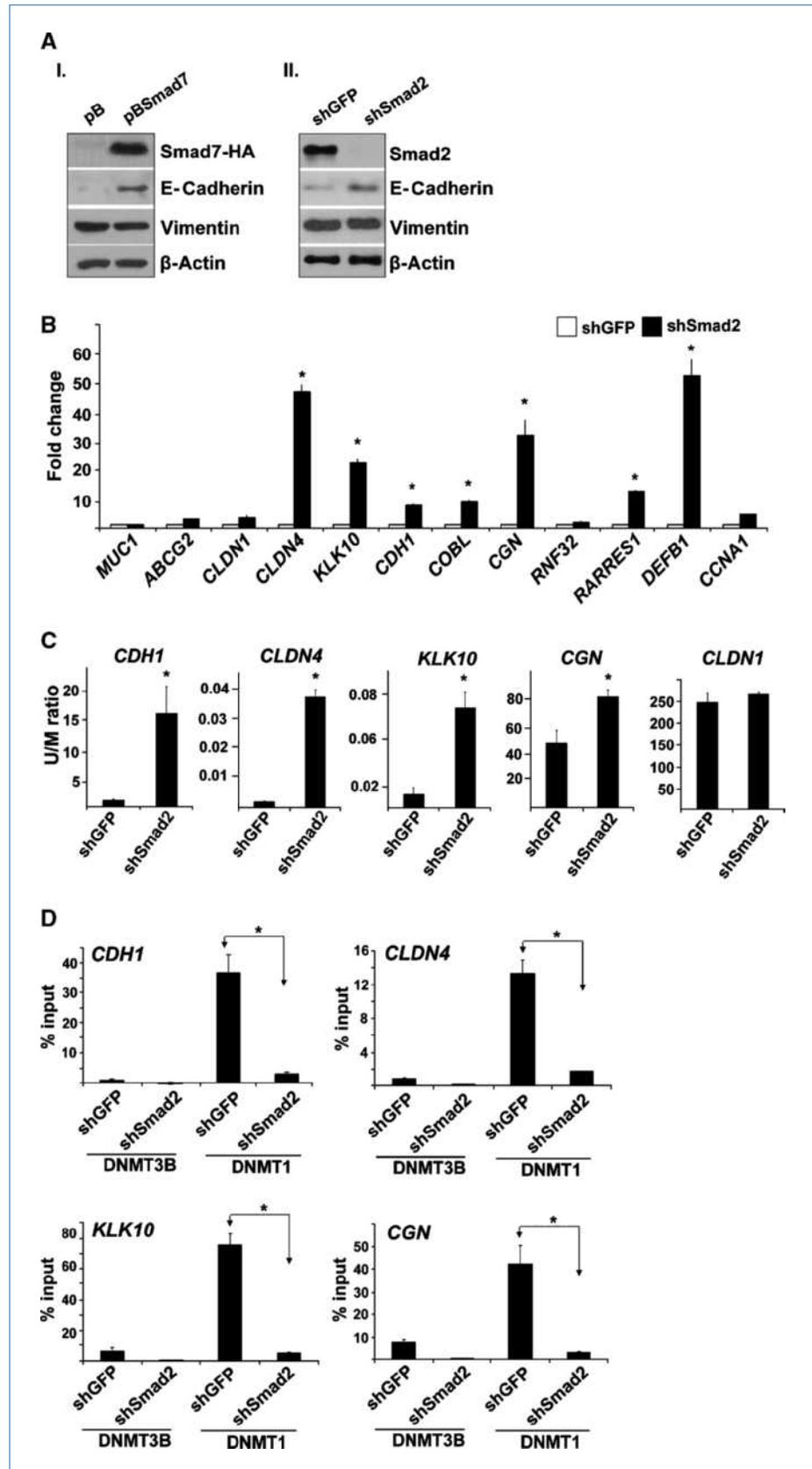
Although a recent report suggested that sustained induction of EMT by the tumor microenvironment induces DNA methylation of genes, including *CDH1* (5), the upstream signaling events that are critical for the acquisition and maintenance of these epigenetic changes remained elusive. Because TGF β signaling has been associated with the manifestation of the EMT phenotype (8), we hypothesized that it might be directly involved in the regulation of the *CDH1* promoter DNA methylation.

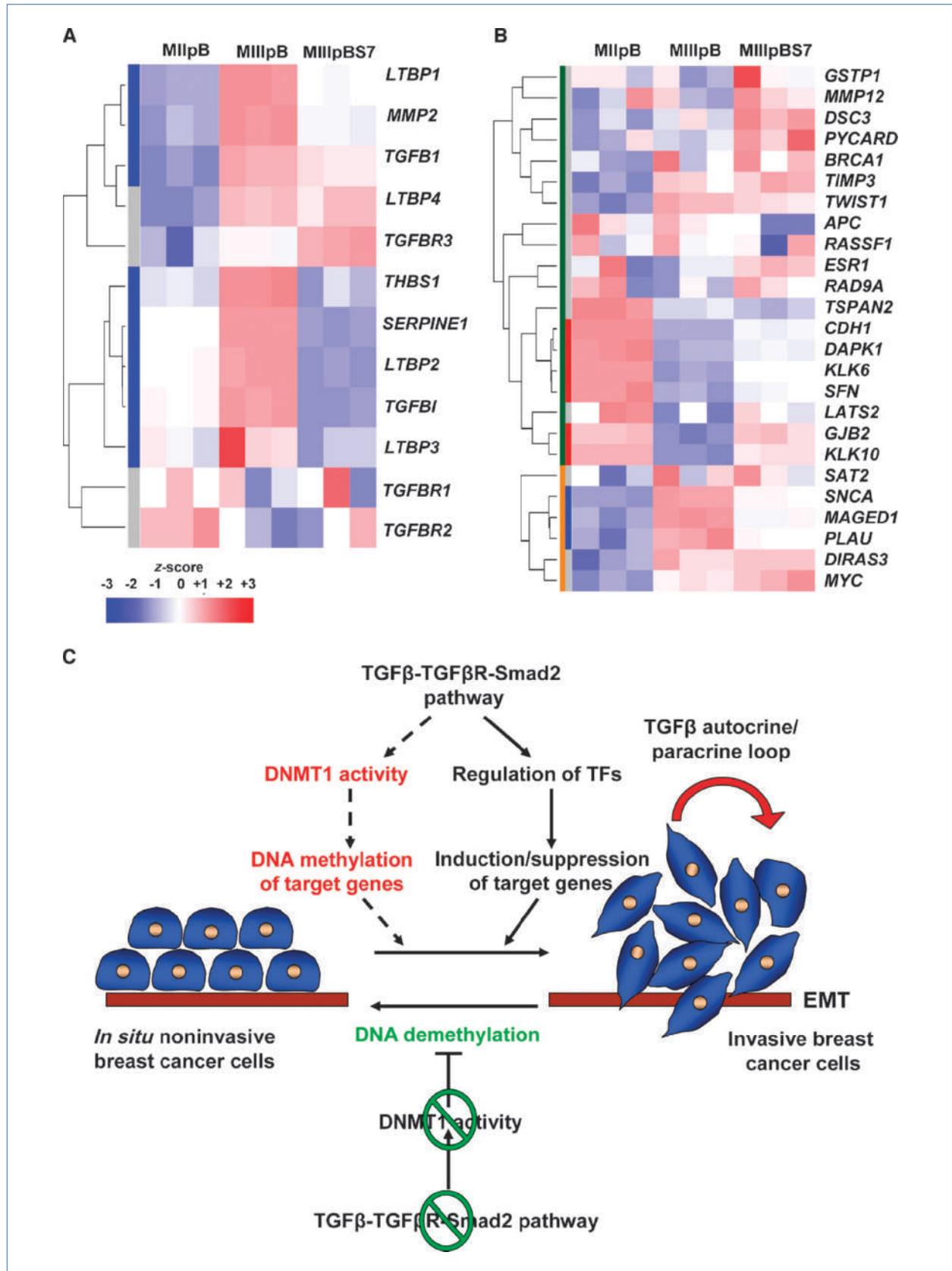
To verify whether all the components of TGF β pathway are intact in our model system, we performed luciferase reporter assays using SBE4-luc (27). TGF β 1 treatment significantly induced luciferase activity, which was inhibited

on transient Smad7 overexpression in all three cell lines, indicating the requirement for a functional Smad2/3-Smad4 complex (Supplementary Fig. S1B). Furthermore, these data supported the suitability of our *in vitro* model system to interrogate the role of TGF β signaling in epigenetic gene silencing.

We disrupted the TGF β signaling pathway by stably overexpressing Smad7 in MI, MII, and MIII cells to assess the effects on the DNA methylation status and expression of E-cadherin (Fig. 2A). As expected, Smad7 overexpression abrogated TGF β /Smad signaling events as evident from the inhibition of TGF β -mediated Smad2 phosphorylation (Ser^{465/467}; Supplementary Fig. S1C). Furthermore, Smad7 overexpression caused a profound effect on the morphology of MIII cells elicited by the acquisition of a predominantly cobble-shaped epithelial phenotype as opposed to the spindle-shaped precursor cells. These morphologic changes were accompanied with upregulation of E-cadherin at the adherens junctions, consistent with a role in enhancing the adhesive properties (Fig. 2B). It should be noted that

Figure 5. Retention of the TGF β -TGF β R-Smad2 signaling axis is required for the maintenance of DNA hypermethylation patterns and silencing of epithelial genes. A, Western blotting analysis for detection of Smad7, Smad2, E-cadherin, vimentin, and β -actin levels in MIIIPB and MIIIPBSmad7 cells (I) or MIIISHGFP and MIIISHSmad2 cells (II). HA, hemagglutinin. B, q-RT-PCR expression analysis of the selected target genes (*ABCG2*, *CCNA1*, *CDH1*, *CGN*, *CLDN1*, *CLDN4*, *COBL*, *DEFB1*, *KLK10*, *MUC1*, *RARRES1*, and *RNF32*) in MIIISHGFP and MIIISHSmad2 cells. C, q-MSP analysis of *CDH1*, *CGN*, *CLDN1*, *CLDN4*, and *KLK10* genes in MIIISHGFP versus MIIISHSmad2 cells. The amount of CpG methylation was quantified based on the unmethylated to methylated ratio of PCR products, normalized to β -actin. D, ChIP assays coupled to q-PCR were performed to quantify the binding of DNMT3B or DNMT1 at the *CDH1*, *CGN*, *CLDN4*, and *KLK10* promoters. The results presented are the average of triplicate samples and a *p*-value of less than 0.05 (indicated by the * symbol) was considered statistically significant between the compared samples. Error bars represent \pm standard error (S.E.) values.





whereas there was increase in the expression of epithelial markers (E-cadherin, γ -catenin, and β -catenin), the levels of the mesenchymal markers (vimentin and fibronectin) were not significantly altered upon Smad7 overexpression (Fig. 2A). To determine whether Smad signaling disruption altered the methylation status of the *CDH1* promoter, we performed MSP analysis and found a significant decrease in methylation-specific DNA in MIIIpBSmad7 compared with MIIIpB cells (Fig. 2C). These findings were further confirmed by bisulfite sequencing to map CpG methylation sites of the *CDH1* promoter region (Fig. 2D; Supplementary Fig. S2).

Smad7 overexpression inhibits migration and invasion of breast cancer cells. Because the acquisition of an EMT phenotype has been correlated with the ability of breast cancer cells to acquire properties essential for intravasation through the basement membrane, such as migration and invasion, to initiate the metastatic process (8), we examined whether Smad7 overexpression had any effect on the migratory and invasive properties of MIII cells. Both wound-healing assays (Fig. 3A) and chemotaxis assays (Fig. 3B; Supplementary Fig. S3A) were consistent in exhibiting substantial reduction in migration upon Smad7 overexpression. Furthermore, Matrigel invasion assays indicated that Smad7 overexpression significantly inhibited the ability of MIII cells to invade through the Matrigel layer (Fig. 3C; Supplementary Fig. S3B). In summary, these studies suggested that TGF β signaling disruption due to Smad7 overexpression suppresses the migratory and invasive potential of breast cancer cells.

Smad signaling disruption induces expression of a subset of genes that exhibit silencing by promoter DNA hypermethylation. Because E-cadherin was epigenetically silenced due to DNA hypermethylation in MIII cells, we hypothesized that the establishment of mesenchymal-like properties may require similar epigenetic regulation of other critical genes. To address this possibility, we initially performed a microarray analysis to compare the overall gene expression profiles of MIIpB, MIIIpB, and MIIIpBSmad7 cells. These analyses identified 599 differentially expressed genes between MIIIpB and MIIIpBSmad7 cells (Supplementary Fig. S4A, I and Tables S1–S3) and 2,992 genes between MIIpB and MIIIpB cells (Supplementary Table S4).

To investigate whether Smad signaling abrogation regulates the expression of additional genes due to altered DNA methylation, we focused on differentially expressed

genes that belong to cluster 4 (Supplementary Fig. S4A, I). Based on their expression pattern (downregulated in MIIIpB versus MIIpB and upregulated in MIIIpBSmad7 cells), we hypothesized that a subset of these genes may be induced upon TGF β -Smad signaling disruption due to DNA demethylation. We selected the following genes for further analysis based on previous literature supporting altered epigenetic regulation in cancers and/or due to their involvement in EMT and cell adhesion: *ABCG2*, *CCNAL*, *CDH1*, *CGN*, *CLDN1*, *CLDN4*, *DEFB1*, *KLK10/NES1*, *MUC1*, and *RARRES1*. We also selected two additional genes, *COBL* and *RNF32*, which also belonged to this cluster but with unknown significance to EMT, as potential controls (Supplementary Fig. S4A, II). First, we confirmed the expression patterns of these genes by q-RT-PCR (Fig. 4A), and subsequently, we examined if these genes may also be regulated by DNA hypermethylation. Treatment of MIII cells with a DNA methylation inhibitor, 5-Aza, resulted in upregulation of only a fraction of these selected genes (*ABCG2*, *CDH1*, *CGN*, *CLDN4*, *DEFB1*, *KLK10/NES1*, and *MUC1*), whereas the others (*CCNAL*, *CLDN1*, *COBL*, *RARRES1*, and *RNF32*) remained unaffected (Fig. 4B).

Computation of the ratio of unmethylated to methylated products in MIII and MIII-Smad7 cells using quantitative MSP analysis showed that whereas the degree of DNA methylation observed in the promoter regions of *CDH1*, *CGN*, *CLDN4*, and *KLK10/NES1* was significantly decreased, it was unaffected in the *CLDN1* promoter upon Smad7 overexpression (Fig. 4C). The examination of the $-1,000$ to $+1$ bp promoter DNA sequences of *ABCG2*, *DEFB1*, and *MUC1* did not reveal the regulatory CpG residues of these genes. Further studies will be necessary to identify the relevant differentially methylated CpG residues.

SMAD2 but not SMAD4 knockdown reverses epigenetic gene silencing in MIII cells. Because Smad7 overexpression acts at the level of TGF β R1/R-Smad interaction to abrogate TGF β signaling (11, 28), we wanted to confirm whether downstream mediators, Smad2 and/or Smad4, are also critical components required for the epigenetic regulation of target genes. To test this possibility, we independently depleted *SMAD2* and *SMAD4* expression in MIII cells using short hairpin RNAs targeting the respective genes and evaluated the expression patterns of the same candidate genes that were upregulated upon Smad7 overexpression [Supplementary Fig. S4A, II]. Interestingly, knockdown of *SMAD2*

Figure 6. Hyperactivation of TGF β signaling in MIII cells inversely correlates with the expression of a subset of genes that are epigenetically silenced in breast cancers. A, heat map for the expression of genes involved in the activation of TGF β 1 as well as of downstream targets of TGF β signaling in MIIpB, MIIIpB, and MIIIpBSmad7 cells. B, heat map for the expression of the most frequently silenced genes in breast cancers due to DNA hypermethylation (33) in MIIpB, MIIIpB, and MIIIpBSmad7 cells. Green and orange bars on the left represent hypermethylated and hypomethylated genes, respectively. Red and blue bars indicate which of the hypomethylated or hypermethylated genes, respectively, changes in expression to the MII-like levels upon Smad7 overexpression. Heat map colors indicate the z-score for the expression of each gene (red, highest expression; blue, lowest expression). C, a model for epigenetic regulation of EMT mediated by overactive TGF β signaling pathway. Hyperactivation of TGF β /Smad signaling cascade due to increase in TGF β in the local microenvironment via secretion by the cancer and/or stromal cells mediates epigenetic regulation and/or induces a transcriptional program leading to EMT of breast cancer cells. Sustained EMT requires intact TGF β signaling pathway to regulate the DNA methylation machinery, leading to the maintenance of epigenetic gene silencing. Disruption of TGF β -TGF β R-Smad2 signaling events results in inhibition of DNMT1 binding activity, leading to passive demethylation of newly synthesized DNA and reexpression of genes involved in cell adhesion. Reversal of the silenced epithelial gene expression patterns promotes the establishment of epithelial morphology and suppression of the invasive behavior of breast cancer cells.

(Fig. 5A, II), but not *SMAD4* (Supplementary Fig. S5A), led to an increase in the expression of *CDH1*, *CGN*, *CLDN4*, and *KLK10/NES1* (Fig. 5B; Supplementary Fig. S5B) concomitant with a decrease in the DNA methylation of the respective regulatory regions (Fig. 5C). The specificity of this effect upon Smad2 depletion was further substantiated from the observation that *SMAD2*, but not *SMAD4*, knockdown resulted in the cells reverting to a more pronounced epithelial morphology (Supplementary Fig. S5C) phenocopying that of the MIIIpBSmad7 cells (Fig. 2B). These findings suggest that intact TGF β -TGF β R-Smad2 signaling axis is required for the maintenance of epigenetic gene silencing in our model system and that this phenomenon seems to be Smad4 independent.

To determine if the changes in the promoter methylation status are due to a passive or active demethylation process, we performed ChIP assays to measure the binding of DNMT1 and DNMT3B to the promoter of the target genes. We found that the maintenance methyltransferase DNMT1 was the predominant methyltransferase bound to the promoters of *CDH1*, *CGN*, *CLDN4*, and *KLK10* in the MIIIshGFP cells. Interestingly, TGF β signaling disruption caused a significant reduction in the amount of DNMT1 bound to these promoters (Fig. 5D) without affecting the corresponding protein levels (Supplementary Fig. S6), suggesting that the TGF β -TGF β R-Smad2 signaling axis regulates DNA methylation maintenance during EMT, perhaps by modulating DNMT1 binding activity.

MIII cells exhibit hyperactive TGF β signaling pathway and resemble basal B breast cancers. Because our studies supported that intact TGF β signaling is required for EMT and DNA methylation maintenance during breast cancer progression, we compared the gene expression profiles between the invasive mesenchymal-like MIII cells and the non-invasive epithelial MII cells. We found that there were relatively high expression levels of the downstream targets of TGF β signaling, such as *MMP2*, *SERPINE1*, and *TGF β I*, in MIII cells. Moreover, we found that the expression of TGF β 1 and the TGF β -activating proteins LTBP1, LTBP2, LTBP3, LTBP4, and THBS1 (29) was also dramatically increased in MIII compared with MII cells (Fig. 6A; Supplementary Fig. S7A). Consistent with these observations, ELISA assays confirmed that MIII cells secrete TGF β 1 when cultured in serum-free medium (Supplementary Fig. S7B).

To further assess the relevance of this phenomenon to EMT, we compared the differential gene expression patterns in the MIII cells with and without TGF β -Smad signaling disruption to a previously published microarray data set from 51 breast cancer cell lines (30). Interestingly, the genes that are highly expressed in MIII cells relative to MII cells and reverted to MII-like levels upon TGF β -Smad signaling disruption (cluster 1; Supplementary Fig. S4A, I) are found to be similar to the expression pattern observed in the majority of the basal B subtype breast cancer cell lines (Supplementary Fig. S8A). Furthermore, the genes with the converse expression pattern (cluster 4; Supplementary Fig. S4A, I) tend to be also expressed at lower levels in the same basal B-cell lines (Supplementary Fig. S8A). Overall, these results suggest that MIII

cells exhibit a similar expression pattern as the basal B subtype cell lines, a subtype associated with acquisition of EMT (31, 32). Additionally, the expression of some TGF β pathway components (predominantly *LTBP2*, *MMP2*, *SERPINE1*, *TGF β I*, and *TGF β 1*) was also higher in basal B compared with other subtypes, lending further support to the notion that TGF β pathway overactivation is likely to be an important feature of basal B tumors (Supplementary Fig. S8B). Moreover, we also found that a subset of genes, including *CDH1*, *DAPK1*, *DSC3*, *GJB2*, *GSTP1*, *KLK6*, *KLK10*, *LATS2*, *PYCARD*, and *SFN*, which were upregulated upon disruption of TGF β pathway in MIII cells, have been consistently reported (33) as targets for silencing due to DNA hypermethylation in breast cancers (Fig. 6B).

Discussion

To delineate the upstream signaling mechanisms responsible for the maintenance of aberrant promoter DNA methylation patterns during breast cancer progression, we used a previously described breast cancer cell line model system. We found that the mesenchymal-like MIII cells, compared with its precursor H-Ras-transformed epithelial MII cells (21), harbor hyperactive TGF β signaling and exhibit an EMT phenotype. Moreover, highly invasive properties of the MIII cells suggesting a prometastatic role were substantiated by differential expression of several genes in MIII compared with the MII cells sharing a similar expression pattern with a subset of genes previously identified as mediators of breast cancer metastasis to the lung (Supplementary Fig. S9; ref. 34). Overall, these results indicate that the MCF10A-based breast cancer cell line model system is an attractive and highly relevant model to study the molecular mechanisms responsible for epigenetic regulation of EMT during transition from *in situ* to invasive breast carcinoma.

By using gene expression profiling and by examining the epigenetic regulation of differentially expressed genes in this breast cancer model system, we found that there was DNA hypermethylation-mediated silencing of genes involved in cell adhesion and tight junction formation, including *CDH1*, *CGN*, and *CLDN4*, as well as the epithelial protease *KLK10/NES1* in basal B-like breast cancer cells that have undergone EMT. These observations are also consistent with a recent report showing that suppression of *CDH1* expression during sustained EMT is mediated by the establishment of promoter DNA hypermethylation (5).

Furthermore, our studies show that overactive TGF β signaling events, mediated by an autocrine feedback loop that maintains high TGF β 1 levels in the microenvironment, are responsible for sustaining the altered epigenome and the invasive properties of breast cancer cells. Moreover, our studies provide direct evidence for the involvement of intact hyperactive TGF β -TGF β R-Smad2 signaling axis in orchestrating a specific DNA methylation pattern that favors EMT and the invasive behavior of breast cancer cells. Several observations support this conclusion. First, disruption of TGF β signaling by either Smad7 overexpression or *SMAD2*, but not *SMAD4*, knockdown in the MIII cells reversed the EMT phenotype

and caused reestablishment of the epithelial morphology. Second, the observed mesenchymal to epithelial transition was accompanied by the upregulation of transcripts for the *CDH1* gene, encoding a key cell-cell adhesion molecule and negative regulator of WNT signaling cascade (35), the tight junction genes *CLDN4* and *CGN*, as well as the protease *KLK10/NES1*. *CDH1* levels have been directly correlated with epithelial phenotype and metastatic properties of cancer cells (36), whereas the *KLK10/NES1* protease was shown to be specifically expressed in epithelial cells and suppress breast tumor growth *in vivo* (37). Finally, significant decreases in promoter DNA methylation of the critical target genes upon TGF β -TGF β R-Smad2 signaling disruption strongly support a direct involvement of this axis in modulating the functionality of the DNA methylation machinery to maintain the epigenetically silenced state.

Despite the identification of putative DNA demethylase enzymes and evidence for the involvement of a DNA repair pathway in this process (38), the existence of active DNA demethylation mechanisms in mammals has been elusive (39). Our data favor the alternate mechanism which proposes that suppression of the binding of maintenance DNA methyltransferase, DNMT1, to the target DNA sequences results in passive DNA demethylation (40). We found that binding of DNMT1 to *CDH1*, *CLDN4*, *CGN*, and *KLK10* promoters was significantly suppressed upon *SMAD2* knockdown (Fig. 5D), whereas DNMT1 and DNMT3B protein levels remained unaffected (Supplementary Fig. S6). Therefore, we propose that reduced DNMT1 binding activity on disruption of TGF β -Smad signaling could result in loss of DNA methylation maintenance and passive demethylation of newly synthesized DNA (Fig. 6C). The passive demethylation in the absence of intact Smad2, but not Smad4, suggests that Smad2 may play a role in loading DNMT1 onto specific gene promoters to modulate DNA methylation when TGF β signaling becomes overactive. Alternatively, Smad2 may interact with other factors to transcriptionally regulate target genes or control DNMT1 activity via post-translational modifications. Finally, it is also likely that DNMT1 binding is regulated by remodeling of localized

chromatin in response to TGF β signaling-mediated effects during breast cancer progression.

In summary, our data suggest that increased TGF β levels in the breast tumor microenvironment promote hyperactive Smad signaling to enable the acquisition of EMT-like properties. Furthermore, we propose that overactive TGF β cascades play a major role in the “epigenetic memory” and maintenance of epithelial gene-specific silencing during EMT mediated by unique DNA methylation patterns (Fig. 6C). To our knowledge, this is the first report to provide conclusive evidence that the reversal of the DNA hypermethylation status of gene promoters occurs as a result of a signaling pathway perturbation, in this case the TGF β /Smad cascade. By extension, our study provides a framework for uncovering genes that are coordinately regulated by epigenetic mechanisms in response to specific signaling events commonly deregulated during cancer progression. Finally, our findings provide additional credence to the idea that inhibition of TGF β -TGF β R-Smad2 signaling axis may be a useful therapeutic strategy to target breast cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Didier Trono, Bert Vogelstein, Robert Weinberg, and Jeff Wrana for generously providing reagents.

Grant Support

Susan G. Komen for the Cure Investigator-Initiated Research Award KG081435 and NIH grant CA101773 (S. Thiagalingam).

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Received 5/22/09; revised 11/19/09; accepted 11/22/09; published OnlineFirst 1/19/10.

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Smad Signaling Is Required to Maintain Epigenetic Silencing during Breast Cancer Progression

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Cancer Res 2010;70:968-978. Published OnlineFirst January 19, 2010.

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