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Smad signaling is required for the maintenance of epigenetic gene silencing during breast cancer progression

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Abstract

Aberrant regulatory DNA methylation patterns have been associated with breast cancer progression. While most efforts have been focused on describing the gene targets for DNA methylation, the molecular events that define the activity of the DNA methylation machinery have remained elusive. Here we describe the use of a breast cancer cell line model system to investigate the mechanisms that regulate epigenetic alterations of gene expression patterns responsible for epithelial-mesenchymal transition (EMT), a critical step during conversion to malignant breast cancer. We found that breast cancer cells which have undergone EMT exhibit overactive TGFβ signaling and loss of expression of genes including *CDH1, CGN, CLDN4* and *KLK10* mediated by DNA hypermethylation of their corresponding promoter regions. Consistent with the notion that activated TGFβ-Smad signaling is involved in “epigenetic memory” to maintain epigenetically silenced state of critical genes, disruption of Smad signaling due to Smad7 overexpression or depletion of Smad2, but not Smad4, in mesenchymal-like breast cancer cells resulted in DNA demethylation and re-expression of the corresponding genes. This reversal of epigenetic changes was accompanied with the acquisition of epithelial morphology and suppression of invasive properties of breast cancer cells. Furthermore, disruption of TGFβ signaling caused a corresponding decrease in DNMT1 binding activity suggesting that failure to maintain methylation of the newly synthesized DNA is the likely cause of demethylation. In summary, our studies reveal for the first time, that hyperactive TGFβ-TGFβR-Smad2 signaling axis is involved in the maintenance of epigenetic silencing of critical target genes to facilitate breast cancer progression.

Keywords

TGFβ; Smad; DNA methylation; EMT; breast cancer; epigenetic memory

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INTRODUCTION

Epigenetic regulation of gene expression is a fundamental feature affecting normal physiological 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 pre-malignant 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 which control the activity of the DNA methylation machinery remain largely elusive.

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 while the expression of mesenchymal components becomes increased (6). This process is regulated by factors, such as TGFβ, secreted in the tumor microenvironment (5,7–9). While 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 TGFβRI constructs that are defective in binding Smads, but retained signaling *via* MAPKs, revealed that Smads are likely to be involved in the EMT process (12,13). Additionally, it has been suggested that TGFβ could cooperate with other signaling pathways, such as oncogenic Ras, in promoting EMT (9,14,15).

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

Because of the increasing evidence implicating TGFβ 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 TGFβ 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 which are critical for the regulation of aberrant DNA methylation patterns during EMT. Our studies show for the first time, that disruption of the TGFβ pathway results in DNA demethylation and re-expression 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 were maintained in DMEM-F/12 medium containing 5% heat-inactivated horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 0.1 ng/ml cholera enterotoxin and 0.5 μg/ml hydrocortisone.

Antibodies

The catalogue numbers, working dilutions and sources of the antibodies were as indicated: E-cadherin (610404-1:1000 for WB/1:100 for IF), β-catenin (610153-1:1000) and N-cadherin (610920-1:1000)-BD Biosciences; vimentin (sc6260-1:1000), Smad4 (sc7966-1:1000), γ-catenin (sc8415-1:1000) and fibronectin (sc9068-1:500)-Santa Cruz; anti-HA (11583816001-1:1000)-Roche; β-actin (A5441-1:15000)-Sigma; Smad2 (3103-1:1000) and P-Smad2 (3101-1:1000)-Cell Signaling; DNMT1 (ab13537-1:500) and DNMT3B (ab13604-1:500)-Abcam; anti-5'-methyl-cytosine (NA81-1:50)-EMD Biosciences.

Viral transduction

Stable retroviral and lentiviral transductions were performed using the pBabe and pLKO.1 vectors, respectively, at a multiplicity of infection (MOI) of 5 pfu/cell. Additional details can be found in supplementary methods.

Western blotting (WB) and immunofluorescence (IF) microscopy

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

Drug treatments

Cells were grown overnight and then treated with 5μM 5'-aza-deoxy-cytidine, 100nM trichostatin A (TSA) or 1mM sodium butyrate (Sigma).

Immunoprecipitation of methylated DNA (MeDIP), methylation-specific PCR (MSP), quantitative MSP (q-MSP) and bisulfite sequencing

Genomic DNA from cells was isolated using the DNeasy tissue kit (Qiagen), according to the manufacturer's protocol. 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 methods.

Wound healing assays

Stably tranduced cells (1×10^6) were grown overnight in 60mm 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 8.0μm-pore membrane (Corning), as described in supplementary 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 (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 (FDR) < 0.05 and a greater than 2-fold difference in expression were considered to be differentially expressed. The microarray data generated in this study is available from the NCBI Gene Expression Ommnibus (24) under accession code GSE18070. Real time quantitative RT-PCR (q-RT-PCR) was performed using SYBR Green Power Master Mix (ABI).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the Magna EZ-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: 1) MCF10ATk.cl2 (MII), an H-Ras transformed MCF10A cell line; and 2) MCF10CA1h (MIII), derived from a xenograft of MII cells in nude mice that progressed to carcinoma (19,20). These cell lines were previously reported to exhibit distinct tumorigenic properties when re-implanted in nude mice; MI is non-tumorigenic, 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 non-tumorigenic to carcinogenic state. Interestingly, while 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 (Figure 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, 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 (Figure 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. 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 (Figures 1B & C), consistent with the observed loss of expression (Figure 1A). Moreover, while treatment with the DNA methylation inhibitor 5'-deoxy-azacytidine resulted in a robust increase in E-cadherin expression, treatment with the HDAC inhibitors trichostatin-A (TSA) or sodium butyrate had no effect, indicating that E-cadherin silencing occurs predominantly due to promoter DNA hypermethylation (Figure 1D).

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

While 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. Since TGFβ 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 TGFβ pathway are intact in our model system, we performed luciferase reporter assays using SBE4-luc (27). TGFβ1 treatment significantly induced luciferase activity, which was inhibited upon transient Smad7 overexpression in all three cell lines, indicating the requirement for a functional Smad2/3-Smad4 complex (Figure S1B). Furthermore, this data supported the suitability of our *in vitro* model system to interrogate the role of TGFβ signaling in epigenetic gene silencing.

We disrupted the TGFβ 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 (Figure 2A). As expected, Smad7 overexpression abrogated TGFβ/Smad signaling events as evident from the inhibition of TGFβ-mediated Smad2 phosphorylation (Ser465/467) (Figure 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 morphological changes were accompanied with upregulation of E-cadherin at the adherens junctions, consistent with a role in enhancing the adhesive properties (Figure 2B). It should be noted that while there was increase in the expression of epithelial markers (E-cadherin, γ-catenin), the levels of the mesenchymal markers (vimentin, fibronectin, N-cadherin) were not significantly altered upon Smad7 overexpression (Figure 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 to MIIIpB cells (Figure 2C). These findings were further confirmed by bisulfite sequencing to map CpG methylation sites of the *CDH1* promoter region (Figure 2D, Figure S2).

Smad7 overexpression inhibits migration and invasion of breast cancer cells

Since 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 (Figure 3A) and chemotaxis assays (Figure 3B, Figure S3A) were consistent in exhibiting substantial reduction in migration upon Smad7 overexpression. Furthermore, matrigel invasion assays indicated that Smad7 overexpession significantly inhibited the ability of MIII cells to invade through the matrigel layer (Figure 3C, Figure S3B). In summary, these studies suggested that TGFβ 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

Since 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 (Tables S1, S2 & S3) and 2992 genes between MIIpB and MIIIpB cells (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 (Figure 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 TGFβ-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*, *CCNA1*, *CDH1*, *CGN*, *CLDN1*, *CLDN4*, *DEFB1*, *KLK10/NES1*, *MUC1* and *RARRES1*. We also selected two additional genes, *COBL* and *RNF32* that also belonged to this cluster but with unknown significance to EMT, as potential controls (Figure S4A-II). First, we confirmed the expression patterns of these genes by q-RT-PCR (Figure 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-deoxycytidine, resulted in upregulation of only a fraction of these selected genes (*ABCG2*, *CDH1*, *CGN*, *CLDN4*, *DEFB1*, *KLK10/NES1* and *MUC1*) whereas the others (*CCNA1*, *CLDN1*, *COBL*, *RARRES1* and *RNF32*) remained unaffected (Figure 4B).

Computation of the ratio of unmethylated to methylated (U/M) products in MIII and MIII-Smad7 cells using q-MSP analysis showed that while 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 (Figure 4C). The examination of the −1000 to +1bp 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* knock-down reverses epigenetic gene silencing in MIII cells

Since Smad7 overexpression acts at the level of TGFβR1/R-Smad interaction to abrogate TGFβ 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 shRNAs targeting the respective genes and evaluated the expression patterns of the same candidate genes which were upregulated upon Smad7 overexpression (Figure S4A-II & Figure 4B). Interestingly, knock-down of *SMAD2* (Figure 5A-II), but not *SMAD4* (Figure S5A), led to an increase in the expression of *CDH1*, *CGN*, *CLDN4* and *KLK10/NES1* (Figure 5B, S5B) concomitant with a decrease in the DNA methylation of the respective regulatory regions (Figure 5C). The specificity of this effect upon Smad2 depletion was further substantiated from the observation that Smad2, but not Smad4, knock-down resulted in the cells reverting to a more pronounced epithelial morphology (Figure S5C) phenocopying that of the MIIIpBSmad7 cells (Figure 2B). These findings suggest that intact TGFβ-TGFβR-Smad2 signaling axis is required for the maintenance of epigenetic gene silencing in our model system and that this phenomenon appears 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 chromatin immunoprecipitation 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, TGFβ signaling disruption caused a significant reduction in the amount of DNMT1 bound to these promoters (Figure 5D), without affecting the corresponding protein levels (Figure S6), suggesting that the TGFβ-TGFβR-Smad2 signaling axis regulates DNA methylation maintenance during EMT, perhaps by modulating DNMT1 binding activity.

MIII cells exhibit hyperactive TGFβ signaling pathway and resemble Basal-B breast cancers

Since our studies supported that intact TGFβ 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 TGFβ signaling such as *MMP2*, *SERPINE1* and *TGFβ1* in MIII cells. Moreover, we found that the expression of TGFβ1 and the TGFβ-activating proteins LTBP1-4 and THBS1 (29) was also dramatically increased in MIII compared to MII cells (Figure 6A & Figure S7A). Consistent with these observations, ELISA assays confirmed that MIII cells secrete TGFβ1 when cultured in serum-free medium (Figure 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 TGFβ-Smad signaling disruption to a previously published microarray dataset 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 TGFβ-Smad signaling disruption (Cluster 1-Figure S4A-I) exhibit MIII-type expression pattern in the majority of the Basal-B subtype breast cancer cell lines (Figure S8A). On the other hand, the genes with the converse expression pattern (Cluster 4-Figure S4A-I) tend to be expressed at lower levels in the same Basal-B cell lines (Figure 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 TGFβ pathway components (predominantly *LTBP2*, *MMP2*, *SERPINE1*, *TGFBI* and *TGFβ1*) was also higher in Basal-B compared to other subtypes lending further support to the notion that TGFβ pathway overactivation is likely to be an important feature of Basal-B tumors (Figure S8B). Moreover, we also found that a subset of genes including *CDH1*, *DAPK1*, *DSC3*, *GJB2*, *GSTP1*, *KLK6*, *KLK10*, *LATS2*, *PYCARD* and *SFN*, that were upregulated upon disruption of TGFβ pathway in MIII cells, were consistently reported (33) as targets for silencing due to DNA hypermethylation in breast cancers (Figure 6B).

DISCUSSION

To delineate the upstream signaling mechanisms responsible for the maintenance of aberrant promoter DNA methylation patterns during breast cancer progression, we utilized a previously described breast cancer cell line model system. We found that the mesenchymal-like MIII cells, compared to its precursor H-Ras transformed epithelial MII cells (21), harbor hyperactive TGFβ signaling and exhibit an EMT phenotype. Moreover, highly invasive properties of the MIII cells suggesting a pro-metastatic role was substantiated by differential expression of several genes in MIII compared to the MII cells sharing a similar expression pattern with a subset of genes previously identified as mediators of breast cancer metastasis to the lung (34) (Figure S9). 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 employing 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 demonstrate that overactive TGFβ signaling events, mediated by an autocrine feedback loop which maintains high TGFβ1 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 TGFβ-TGFβR-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 TGFβ signaling by either Smad7 overexpression or *SMAD2*, but not *SMAD4*, knock-down in the MIII cells reversed the EMT phenotype and caused re-establishment 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), while 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 TGFβ-TGFβR-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 favors the alternate mechanism which proposes that suppression of the maintenance DNA methyltransferase, DNMT1, results in passive DNA demethylation (40). We found that binding of DNMT1 to *CDH1*, *CLDN4*, *CGN* and *KLK10* promoters was significantly suppressed upon *SMAD2* knock-down (Figure 5D), while DNMT1 and DNMT3B protein levels remain unaffected (Figure S6). Therefore, we propose that reduced DNMT1 binding activity upon disruption of TGFβ-Smad signaling could result in loss of DNA methylation maintenance and passive demethylation of newly synthesized DNA (Figure 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 TGFβ 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 TGFβ signaling-mediated effects during breast cancer progression.

In summary, our data suggests 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 TGFβ cascades play a major role in the "epigenetic memory" and maintenance of epithelial gene-specific silencing during EMT mediated by unique DNA methylation patterns (Figure 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 TGFβ/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 TGFβ-TGFβR-Smad2 signaling axis may be a useful therapeutic strategy to target breast cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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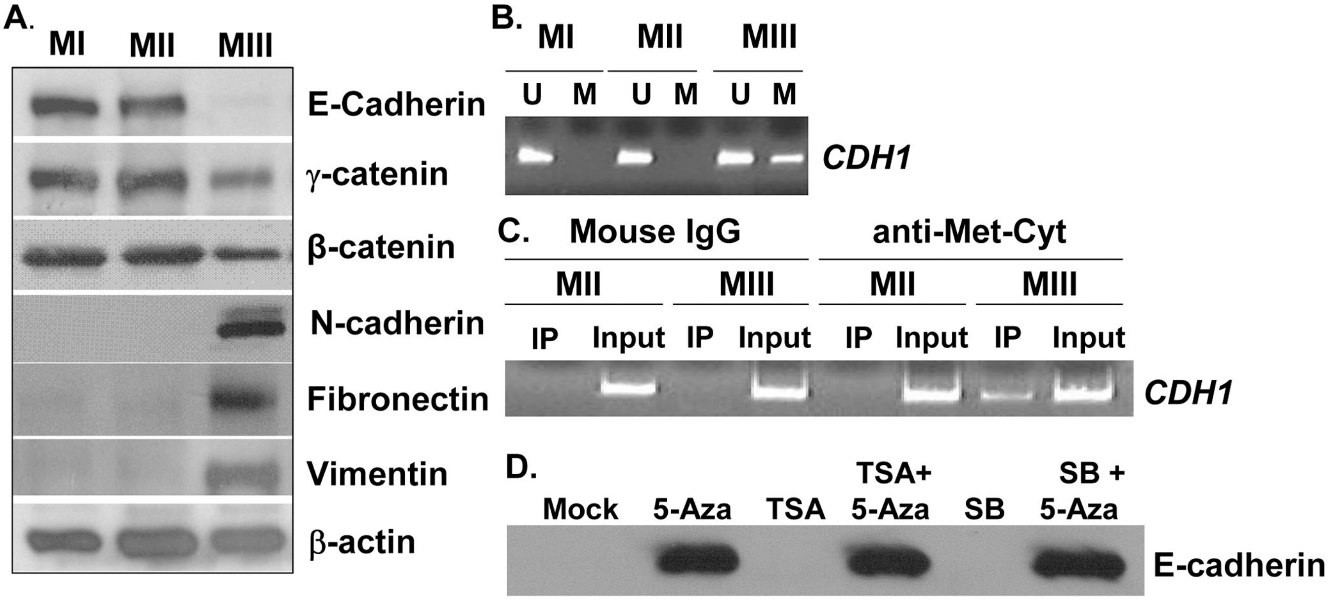

Figure 1. EMT in the MCF10A-based breast cancer progression model correlates with DNA hypermethylation-mediated silencing of E-cadherin expression

A. Western blotting analysis of cell lysates isolated from MI, MII and MIII cells for detection of epithelial (E-cadherin, β-catenin, γ-catenin) and mesenchymal protein markers (vimentin, fibronectin, N-cadherin). **B.** MSP analysis of the −160 to +1 region of E-cadherin (*CDH1*) promoter using bisulfite-treated genomic DNA isolated from MI, MII and MIII cells. **C.** Immunoprecipitation of methylated DNA using either a mouse IgG (mock) or an anti-methyl-cytosine monoclonal antibody using genomic DNA isolated from MII and MIII cells and PCR analysis of the *CDH1* promoter. **D.** Individual and combinations of 5’-deoxyaza-cytidine (5-Aza), trichostatin-A (TSA) and sodium butyrate (SB) treatments in MIII cells for 72h and Western blotting for E-cadherin detection.

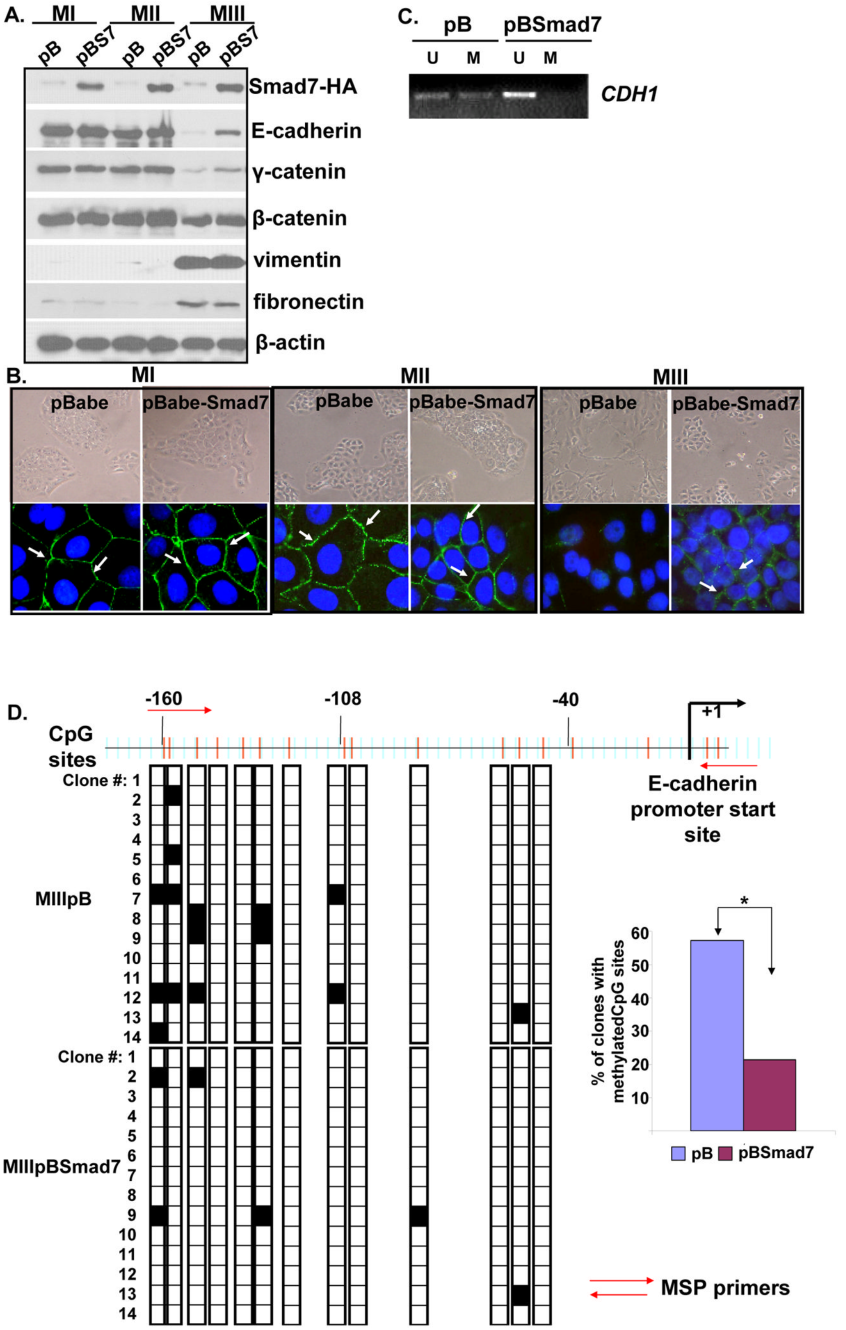

Figure 2. Smad7 overexpression induces promoter DNA demethylation and re-expression of E-cadherin

A. Western blotting analysis to detect stable overexpression of exogenous Smad7, epithelial and mesenchymal markers in MI, MII and MIII cells. **B.** Smad*7* overexpression induces epithelial morphology in mesenchymal-like MIII cells and localization of E-cadherin at the cell-cell junctions. Representative examples of light microscopy (10X) and immunofluorescence images (100X) generated using an anti-E-cadherin primary and FITC-conjugated secondary antibodies in the MI, MII and MIII cells stably transfected with either pB or pBSmad7 vectors. Cell nuclei were stained with DAPI (blue). Localization of E-cadherin (green) at the cell-cell junctions is indicated by white arrows. **C.** Semi-quantitative MSP analysis of the −160 to +1 *CDH1* promoter region using bisulfite-treated genomic DNA. **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.

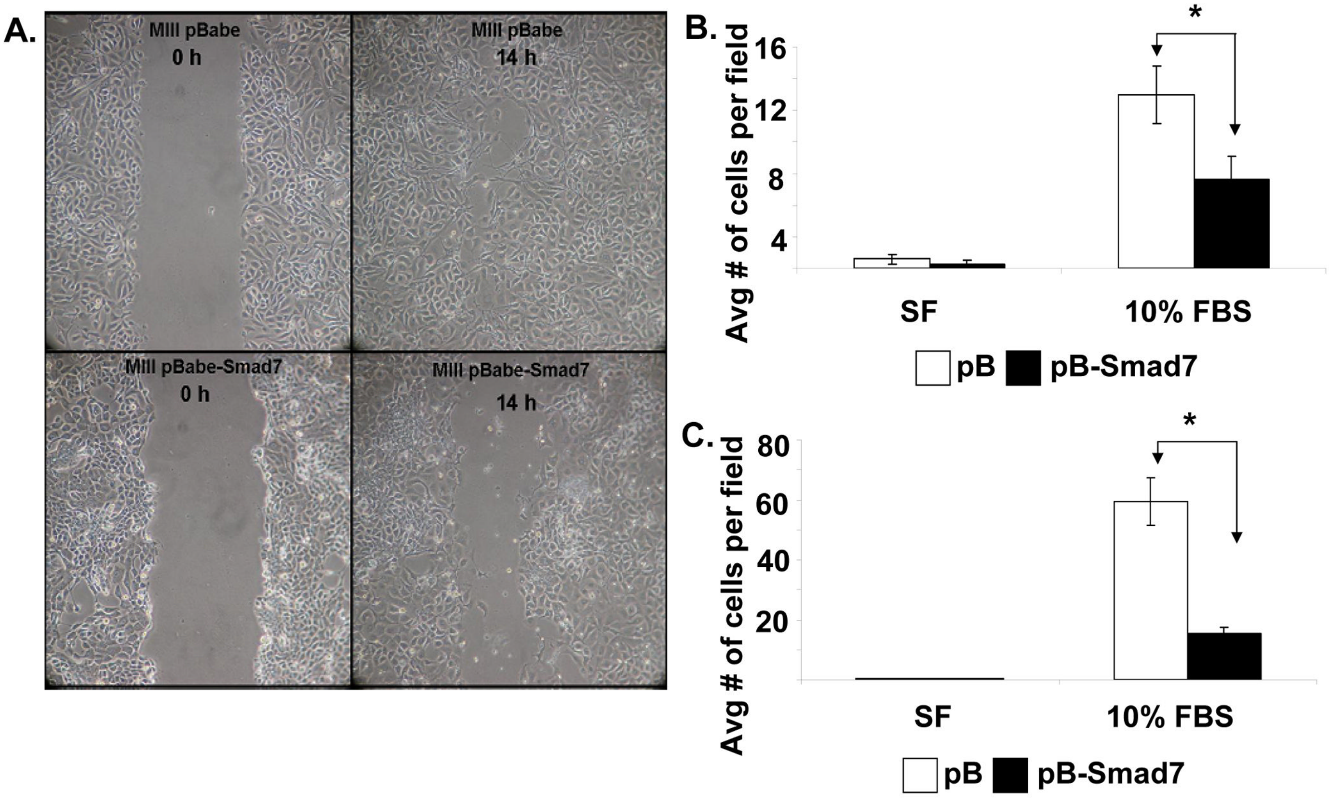

Figure 3. Smad7 overexpression suppresses migration and invasion of MIII cells

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 8µm pore-containing membrane of the transwells were stained with propidium iodide (PI) and counted. **C.** Matrigel invasion assay. Cells that invaded through matrigel were stained with Trypan blue and counted. All results are presented as the average of cells counted in ten fields per condition.

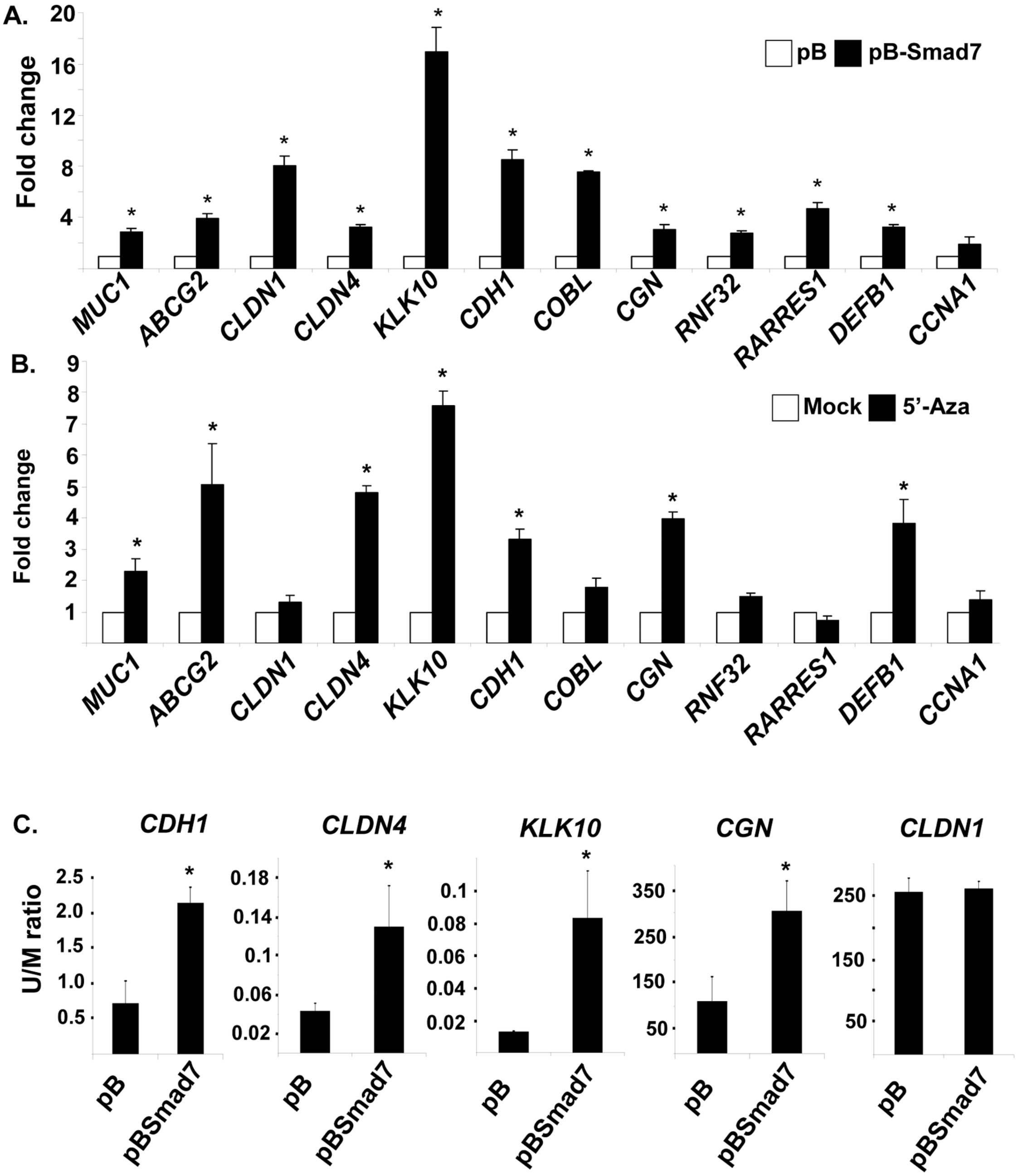

Figure 4. Expression and DNA methylation analysis of candidate genes silenced due to DNA hypermethylation in MIII cells

A. Verification of differential expression of indicated genes using q-RT-PCR. **B.** Q-RT-PCR analysis for expression in MIII cells treated with 5μM 5'-aza-deoxycytidine(5-Aza) or DMSO (mock) for 72h 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.

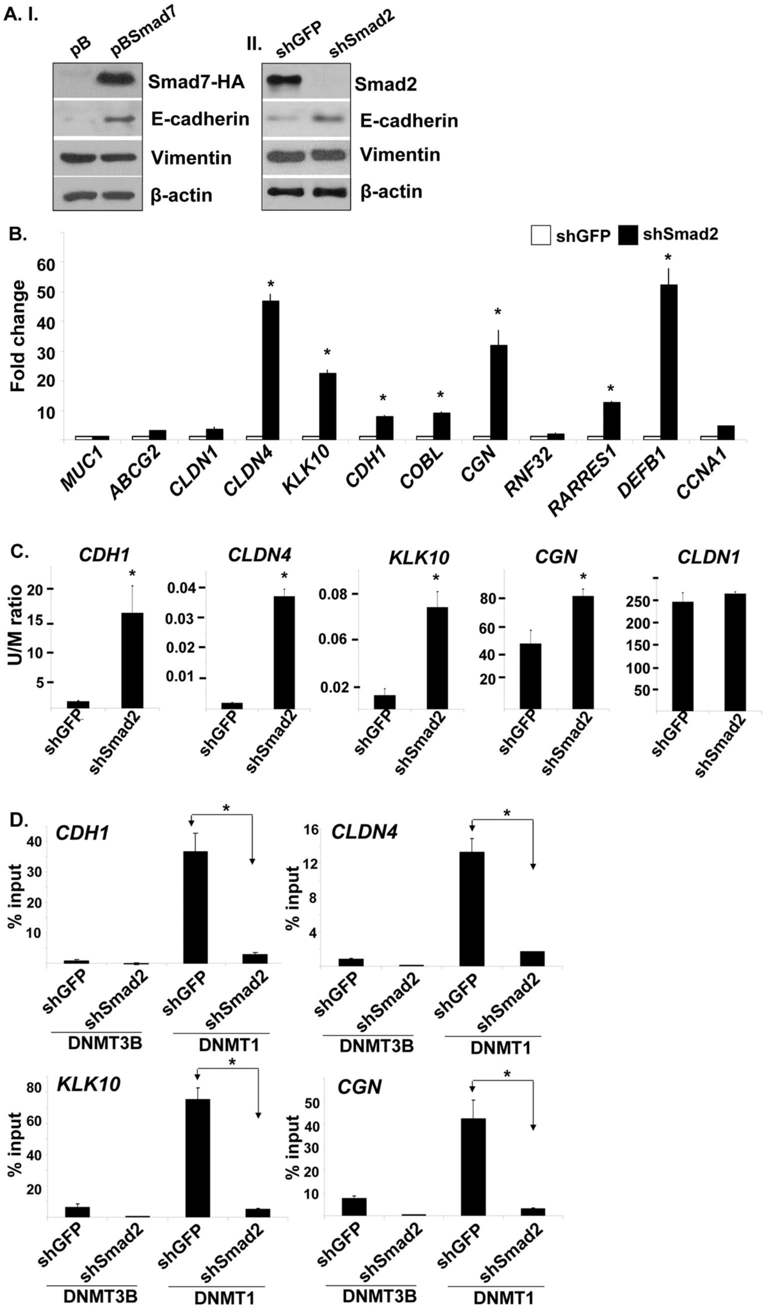

Figure 5. Retention of the TGFβ-TGFβR-Smad2 signaling axis is required for the maintenance of DNA hypermethylation patterns and silencing of epithelial genes

A. Western blotting analysis for detection of Smad7, Smad2, E-cadherin, Vimentin and β-actin levels in MIIIpB and MIIIpBSmad7 cells **(A-I)** or MIIIshGFP and MIIIshSmad2 **(A-II)**. **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 (U) to methylated (M) ratio of products, normalized to β-actin. **D.** Chromatin immunoprecipitation (ChIP) assays coupled to q-PCR were performed to quantify the binding of DNMT3B or DNMT1 at the *CDH1*, *CGN*, *CLDN4* and *KLK10* promoters.

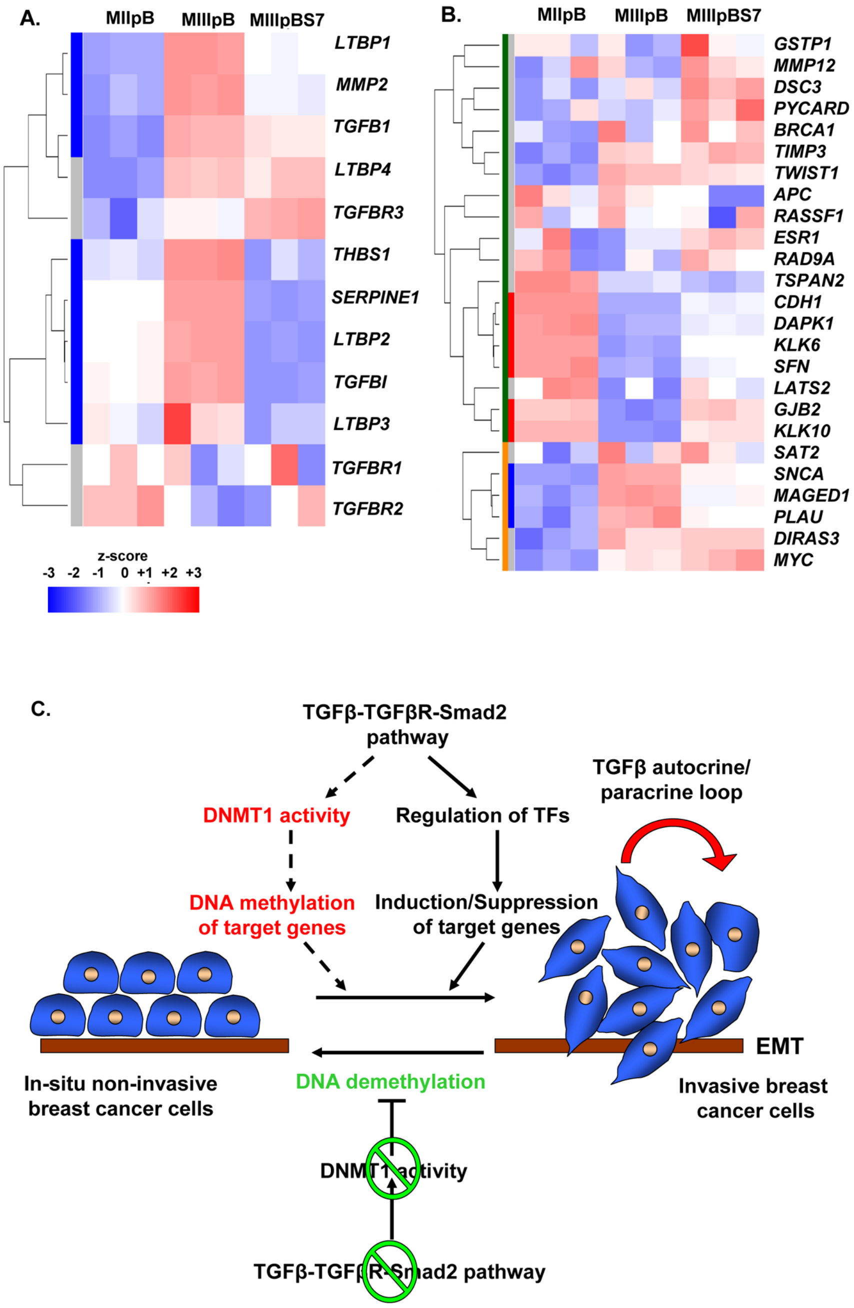

Figure 6. Hyperactivation of TGFβ signaling in MIII cells inversely correlates with the expression of a subset of genes that are epigenetically silenced in breast cancers

A. Heat map for the expression of genes involved in the activation of TGFβ1 as well as of downstream targets of TGFβ 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 represent hypermethylated and hypomethylated genes, respectively. Red and blue bars indicate which of the hypo- or hypermethylated genes' expression, respectively, change to MII-like levels upon Smad7 overexpression. Heat map colors indicate the z-score for each gene's expression (red=highest and blue=lowest expression). **C.** A model for epigenetic regulation of

EMT mediated by overactive TGFβ signaling pathway. Hyperactivation of TGFβ/Smad signaling cascade due to increase in TGFβ 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 TGFβ signaling pathway to regulate the DNA methylation machinery leading to the maintenance of epigenetic gene silencing. Disruption of TGFβ-TGFβR-Smad2 signaling events results in inhibition of DNMT1 binding activity, leading to passive demethylation of newly-synthesized DNA and re-expression 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.