Supporting Information

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SI Text

Immunoblot Analysis. Protein extracts were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed using standard procedures. Mouse monoclonal antibodies against E-cadherin (610181), N-cadherin (610920), and fibronectin (610077) were obtained from BD Biosciences. The mouse monoclonal antibody against β-actin (5441) was obtained from Sigma-Aldrich. The rabbit polyclonal antibodies against the phosphorylated form of Smad2 (3101) and Map kinase (9101) were obtained from Cell Signaling Technology, whereas those against Snail (17732) and MTA3 (A300–160A) were from Abcam and Bethel Lab, respectively.

Methylation-Specific PCR. MSP was performed on bisulfite-modified DNA using previously described primer pairs and PCR cycle conditions for E-cadherin (1), 14–3-3σ (2), ER (3), TWIST (4), CST6 (5), BRCA1 (6), TIMP3 (7), and DKK3 (8). Control templates from human genomic lymphocyte DNA either treated with SssI methylase (methylated control) or untreated (unmethylated control) and a no template (water) control were performed for each experiment. PCR products were electrophoresed on 3% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

AZA and TSA Treatment. Cells were treated with either 1 μM AZA (or PBS vehicle control) or 300 nM TSA (or ethanol vehicle control) for 72 h. For the combination AZA/TSA treatment, cells were treated with 1 μM AZA for an initial incubation period of 48 h. Following this initial incubation, 300 nM TSA and 1 μM AZA were both added to the media for a final 24 h. RNA and genomic DNA were then isolated as above and analyzed via qPCR and MSP, respectively. Experiments were performed twice on independent populations of cells of similar passage number with comparable qPCR and MSP results.

Real-Time Quantitative PCR. Total RNA was isolated from cells and cDNA synthesized using standard methods. Quantitative real-time PCR (TaqMan) was performed on cDNA using the standard curve method with primer/probe sets (Applied Biosystems) for CDH1 (E-cadherin), ESR1 (ER), MTA3, TCF3 (E12/E47), ZEB2 (SIP1), SNAI2 (Slug), and GUSB (external control). The expression of GUSB was used to normalize for variances in input cDNA. Each experiment was performed at least in duplicate with several independent populations of cells. Error bars represent standard deviation.

Fig. S1. Human mammary epithelial cells expressing oncogenic ras maintain their proliferation and morphology after removal of serum. (A) vHMEC-ras0.5 and vHMEC-ras10 cells were switched to serum-free media (vHMEC-ras0.5 → 0 and vHMEC-ras10 → 0), respectively, and continued to proliferate in the absence of serum. (B) Phase contrast photomicrographs of vHMEC-ras cells grown in their original concentration of serum (0.5% and 10%, first and second columns, respectively) or after they were switched to no serum (0.5 → 0 and 10 → 0, third and fourth columns, respectively) in 3D matrigel. Photos of 3D cultures were taken at 10× magnification after 7 days in culture.
Fig. S2. TGFβ-induced EMT is reversible and not associated with epigenetic modifications at the E-cadherin locus. (A) Quantitative real-time PCR (qPCR) analysis of E47, SLUG, and MTA3 transcripts from vHMEC-ras0.5 (0.5), vHMEC-ras10 early- (10E) and late- (10L) passage cells, and negative (−) and positive (+) control breast cancer cell lines. (B) vHMEC-ras0.5 cells were treated with 2 ng/ml TGFβ for 72 h. Photomicrographs (10× magnification) depicting cell morphology at 0, 2, and 4 days post withdrawal of TGFβ. (C) MSP analysis of the E-cadherin and 14–3-3ζ gene loci on bisulfite-treated DNA isolated from vHMEC-ras0.5 untreated (−TGF) or treated (+TGF) with 2 ng/ml TGFβ for 96 h as described in Fig. 3. DNA from MCF7 and MDA-MB-231 cells was used as unmethylated (u) and methylated (m) control templates, respectively for E-cadherin, whereas DNA from MDA-MB-231 and human mammary fibroblasts (HMF) served as unmethylated (u) and methylated (m) controls for 14–3-3ζ.
Fig. S3. Deacetylation and DNA hypermethylation at the E-cadherin promoter contribute to the long-term silencing of E-cadherin expression. (Top) qPCR analysis of E-cadherin expression in late- (Left) and early-passage (Right) vHMEC-ras10 cells treated with AZA and/or TSA, as described in Materials and Methods. (Bottom) MSP analysis of the E-cadherin promoter in the cells treated with AZA and/or TSA using primer sets that specifically amplify either methylated (m) or unmethylated (u) DNA.
Fig. S4. Methylation of the E-cadherin promoter sequences is part of a nonrandom program of targeted epigenetic changes. (A) Immunoblot analysis of DNMT expression was performed on cell lysates prepared from vHMEC-ras0.5 cells (0.5) and vHMEC-ras10 cells (ras10) at different passages following exposure to serum, as indicated. (B) MSP analysis of ER, TWIST, CST6, BRCA1, GATA3, TIMP3, and DKK3 in vHMEC-ras0.5 (0.5), and vHMEC-ras10 early- (10E) and late- (10L) passage cells using primer sets that specifically amplify either methylated (m) or unmethylated (u) DNA. All MSP experiments were performed on at least two independent vHMEC-ras 0.5, 10E, and 10L cells.