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Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer

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The Wnt signaling pathway is a powerful and prominent oncogenic mechanism dysregulated in numerous cancer types. While evidence from transgenic mouse models and studies of human tumors clearly indicate that this pathway is of likely importance in human breast cancer, few clues as to the exact molecular nature of Wnt dysregulation have been uncovered in this tumor type. Here, we show that the Wnt inhibitory factor-1 (*WIF1*) gene, which encodes a secreted protein antagonistic to Wnt-dependent signaling, is targeted for epigenetic silencing in human breast cancer. We show that cultured human breast tumor cell lines display absent or low levels of WIF1 expression that are increased when cells are cultured with the DNA demethylating agent 5-aza-2′-deoxycytidine. Furthermore, the WIF1 promoter is aberrantly hypermethylated in these cells as judged by both methylation-specific PCR and bisulfite genomic sequencing. Using a panel of patient-matched breast tumors and normal breast tissue, we show that WIF1 expression is commonly diminished in breast tumors when compared with normal tissue and that this correlates with *WIF1* promoter hypermethylation. Analysis of a panel of 24 primary breast tumors determined that the *WIF1* promoter is aberrantly methylated in 67% of these tumors, indicating that epigenetic silencing of this gene is a frequent event in human breast cancer. Using an isogenic panel of cell lines proficient or deficient in the DNA methyltransferases (DNMTs) DNMT1 and/or DNMT3B, we show that hypermethylation of the *WIF1* promoter is attributable to the cooperative activity of both DNMT1 and DNMT3B. Our findings establish the *WIF1* gene as a target for epigenetic silencing in breast cancer and provide a mechanistic link between the dysregulation of Wnt signaling and breast tumorigenesis.

Abbreviations: 5-azadC, 5-aza-2′-deoxycytidine; BGS, bisulfite genomic sequencing; DKO, double knockout; DNMT, DNA methyltransferase; KO, knockout; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MSP, methylation-specific PCR; RT–PCR, reverse transcriptase–polymerase chain reaction; TSA, trichostatin A; WIF1, Wnt inhibitory factor-1.

Introduction

Tumorigenesis is a multi-step process in which the activity of cellular growth-promoting genes, termed oncogenes, is increased. Conversely, the activity of genes that normally serve to constrain growth, termed tumor suppressors, is lost or diminished. Collectively, the mechanisms that drive upregulation of oncogene activity and downregulation of tumor suppressor activity stem from genetic and epigenetic changes in the genome (1). *Wnt* was originally identified as a putative proto-oncogene activated by viral insertion in mouse mammary tumors (2). Since this seminal discovery, it is now well documented that Wnt proteins form a family of secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis (3). Binding of Wnt proteins to the frizzled receptors activates the intracellular Dishevelled, which inhibits glycogen synthase kinase 3β (GSK3β) and allows the cytoplasmic accumulation of stabilized β-catenin. Consequently, β-catenin translocates to the nucleus where it engages transcription factors such as T-cell factors (TCFs) and lymphoid-enhancing factors (LEFs). Several growth-promoting genes are transcriptionally activated through this mechanism, including the oncogenic transcription factor c-myc (4). C-myc, in turn, activates a variety of genes involved in cell cycle regulation such as cyclins D1, D2 and E and the phosphatase cdc25A (5,6). Further, c-myc also transcriptionally activates the *hTERT* gene that encodes the catalytic subunit of telomerase (7). This is probably a prominent feature stemming from dysregulation of Wnt signaling since telomere stabilization is an important step in cell immortalization (8).

While no direct evidence linking Wnt to cancer has been uncovered, several of the downstream molecules in the Wnt signaling pathway (i.e. β-catenin, APC, Axin) are dysregulated in a variety of human tumors (9). Moreover, Wnt transgenics have proven to be a powerful model for the study of breast cancer since these mice display high-penetrance mammary adenocarcinomas early in life (10). Additionally, transgenics expressing activated β-catenin display a very similar phenotype (11). Such observations clearly imply that Wnt signaling is of importance in breast tumorigenesis in mice, and evidence collected on human breast tumors (12,13) supports the concept that dysregulation of Wnt signaling is of importance in human breast disease as well. However, mutations in key regulatory molecules of the Wnt signaling cascades are rather infrequent in breast cancer, leaving open the question as to the mechanism(s) that underlie dysregulation of Wnt signaling in this tumor type.

Wnt inhibitory factor-1 (WIF1) is a secreted protein that binds to Wnt proteins and inhibits their activity (14). Recently, the *WIF1* gene was shown to be downregulated in human lung (15), gastrointestinal (16), and breast, prostate and bladder tumors (17). The former two groups observed that decreased WIF1 expression correlated with methylation of CpG dinucleotides within the *WIF1* promoter. Numerous tumor

suppressor or growth regulatory genes have been shown to undergo aberrant *de novo* methylation and transcriptional silencing in human tumors, and epigenetic silencing of tumor suppressor genes is now widely recognized as either a causative or a correlative event in tumor development (18). Thus, it is unsurprising that a list of well-characterized tumor suppressors including *BRCA1*, $p16^{INK4a}$, *14-3-3σ*, *E-cadherin* and *ATM* are known to be targets for epigenetic silencing in breast cancer (19–24). Site-specific DNA methylation is often an early event, as demonstrated in tumor types with a well-defined pattern of progression, such as colon cancer, and is now widely regarded as one of the 'hits' in the Knudsen hypothesis leading to tumor suppressor gene inactivation.

While the *WIF1* gene is a target for epigenetic silencing in some tumor types and its expression is downregulated in breast cancer, it is currently unknown if this gene is subject to epigenetic silencing in breast cancer. In order to determine the frequency and mechanism of WIF1 downregulation in breast tumors, we have analyzed the expression and DNA methylation status of the *WIF1* promoter in a panel of cultured human breast tumor cell lines and surgically obtained human breast tumors. Our results demonstrate that WIF1 inactivation is a frequent event in breast cancer and suggests that dysregulation of Wnt signaling is an important contributor to human breast tumorigenesis.

Materials and methods

Cell culture and drug treatments

The breast tumor cell lines MCF-7, T47D, MDA-MB-231, MDA-MB-435, MDA-MB-468, BT549, ZR75-1 and SK-BR3 were purchased from the American Type Culture Collection. The YYC-B1 cell line was provided by Dr Sun Young Rha (Yonsei Cancer Center, Korea) and the HCT116 colorectal carcinoma cell line and its isogenic derivatives in which the *DNMT1*, *DNMT3B*, and *DNMT1 + DNMT3B* genes were disrupted by homologous recombination (25) were provided by Dr Bert Vogelstein (The Johns Hopkins University). All cell lines were maintained in McCoy's 5-A media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (both from Invitrogen). For drug treatments, 5-aza-2′-deoxycytidine (5-azadC) was added to a final concentration of 5 μM every 24 h. Trichostatin A (TSA) treatments were performed for 24 h using a concentration of 100 nM. All drugs were purchased from Sigma.

Tumor specimens

Fresh-frozen breast tumors were obtained from the University of Florida Shands Cancer Center Molecular Tissue Bank. All specimens and pertinent patient information were treated in accordance with policies of the Institutional Review Board of the University of Florida Health Sciences Center. Tumors analyzed in this study were examined by a surgical pathologist and identified as invasive breast adenocarcinoma (Stages II or III). All patients were negative for metastasis. Where indicated, matched normal breast tissue samples were obtained from disease-free surgical margin. The tissue samples were divided into two, and one half was pulverized in Trizol (Invitrogen) for RNA purification according to the manufacturer's instructions and the other half was used for DNA preparation using a standard proteinase K, phenol extraction method (24). DNA and RNA from cultured cells were prepared in a similar manner.

Reverse-transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was used in first-strand cDNA synthesis reactions using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers (GE Healthcare). *WIF1* expression was subsequently analyzed by PCR. *WIF1*-specific primers are (F) 5′-CCG AAA TGG AGG CTT TTG TA-3′ and (R) 5′-TGG TTG AGC AGT TTG CTT TG-3′. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as a control for RNA integrity and was amplified using primers (F) 5′-CTG CAC CAC CAA CTG CTT AG-3′ and (R) 5′-AGG TCC ACC ACT GAC ACG TT-3′. PCR thermocycling conditions are 94°C, 9 min, 1 cycle; 94°C, 30 s; 59°C, 1 min; 72°C, 1 min, 32–35 cycles for *WIF1*. PCR conditions for *GAPDH* were the same as for *WIF1* except that an annealing temperature of 58°C was used and PCR was conducted for 20 cycles. A PCR reaction substituting dH_2O for cDNA was conducted as a negative control.

Bisulfite genomic sequencing (BGS) and MSP

BGS was performed as described previously (26) using PCR primers designed to amplify a 500 bp segment of the *WIF1* promoter. Primer sequences are (F) 5′-TTA TTA TTA GTA TTT AGT TAA GTT T-3′ and (R) 5′-ACC TAA ATA CCA AAA AAC CTA-3′ and were used under the following thermocycling conditions: 9 min, 94°C, 1 cycle; 94°C, 30 s; 50°C, 1 min; 72°C, 1 min for 35 cycles and using *Taq* Gold DNA Polymerase (Applied Biosystems). Following PCR, products were cloned using the TOPO-TA cloning kit (Invitrogen), and recombinants were identified by restriction analysis and subsequently sequenced at the University of Florida Center for Mammalian Genetics DNA sequencing facility using a vector-encoded primer (M13-Forw). WIF1 MSP primers are as follows: unmethylated (U) allele-specific primers (F) 5′-TGGT ATT TAG GTT GGG AGG TGA TGT-3′ and (R) 5′-AAC CTC CAC CCA CAA TAC CAA-3′, methylated (M) allele-specific primers (F) 5′-ATT TAG GTC GGG AGG CGA CGC-3′ and (R) 5′-GAC CTC CGC CCG CAA TAC CAA-3′. PCR conditions for MSP are 95°C, 15 min, 1 cycle; 94°C, 30 s; 65°C (M primers) or 56°C (U primers), 30 s; 72°C, 45 s, 40 cycles. PCR reactions were resolved on a 2% agarose gel.

Results

WIF1 downregulation and promoter hypermethylation in breast tumor cell lines

To initially examine if the *WIF1* gene is targeted for epigenetic silencing in breast cancer, we cultured a panel of four breast tumor lines in the presence or absence of the DNA demethylating drug 5-azadC. Subsequently, total RNA was harvested from these cells and used in RT–PCR reactions using WIF1-specific primers (Figure 1A). We observed in all four lines (MCF-7, MDA-MB-231, MDA-MB-468 and BT549) that cells cultured in the presence of 5 μM 5-azadC display elevated levels of WIF1 transcripts, suggesting that genome methylation was repressing WIF1 expression in these cells. To directly test *WIF1* promoter methylation, we designed a set of PCR primers for use in MSP assays (27). When used in PCR reactions containing normal human sperm DNA (generally highly hypomethylated) pre-incubated in either the presence or absence of *Sss*I (CpG) methylase and *S*-adenosyl-L-methionine prior to bisulfite modification, results using designed methylated-specific (M) and unmethylated-specific (U) primers indicate that these MSP primers amplify DNA in a manner that clearly ascertains the methylation status of the *WIF1* promoter (Figure 1B). Sequence analysis confirmed that this amplicon corresponds to the targeted region of the WIF1 promoter (data not shown). When MSP analysis was used on bisulfite-modified genomic DNA harvested from the panel of breast tumor cell lines, we observed amplification with the methylated-specific primer set in all four lines, indicating CpG methylation within the *WIF1* promoter (Figure 1C). Further, genomic DNA harvested from cells cultured with 5-azadC display either complete, or markedly increased, demethylation of the *WIF1* promoter as judged by increases in MSP amplification with unmethylated-specific primers and decreases in amplification with methylation-specific primers. MCF-7 cells, which displayed low levels of *WIF1* transcription before 5-azadC treatment, also displayed amplification with the unmethylated DNA-specific primers. Two of the cell lines (MDA-MB-468 and BT549) showed a lack of *WIF1* expression but some amplification of unmethylated alleles by MSP (Figure 1A and C). This is most probably due to differences in sensitivity of the RT–PCR and the MSP reactions. Increasing the number of PCR cycles for RT–PCR does reveal low-level expression of WIF1 in these cell lines (data not shown).

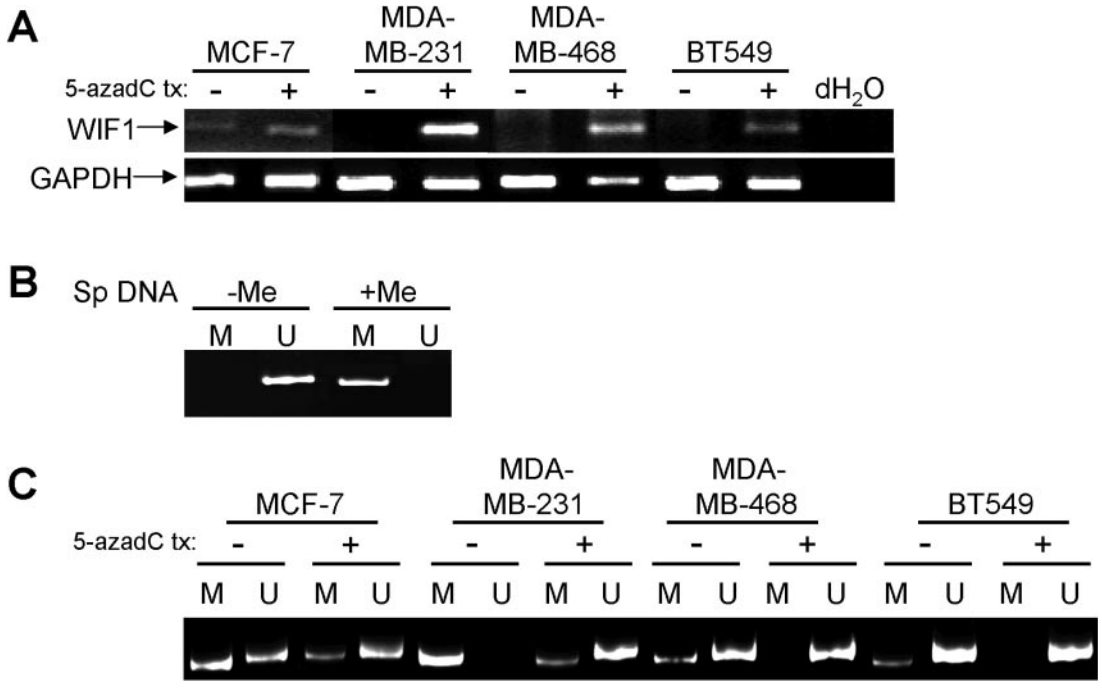

Fig. 1. *WIF1* expression and promoter DNA methylation status in breast cancer cell lines. Cultures of the breast tumor cell lines MCF-7, MDA-MB-231, MDA-MB-468 and BT549 were untreated (−) or treated with 5 μM 5-azadC (+) for 4 days (drug was added fresh every 24 h). Following this treatment, total RNA and genomic DNA were prepared from the cells. (**A**) RT–PCR analysis of *WIF1* expression (top panel) and the housekeeping gene *GAPDH* (bottom panel) as a control for RNA integrity. (**B**) MSP primers for analysis of the methylation status of the *WIF1* promoter were designed and subsequently validated using bisulfite-modified human sperm (Sp) DNA ('−Me', highly hypomethylated) and bisulfite-modified sperm DNA that was methylated *in vitro* with *Sss*I (CpG) methylase ('+Me', fully methylated). 'M' is a PCR reaction using methylated allele-specific primers and 'U' is PCR using unmethylated allele-specific primers. (**C**) Genomic DNA harvested from MCF-7, MDA-MB-231, MDA-MB-468 and BT549 cells before (−) and after (+) 5-azadC treatment was bisulfite-modified as described in Materials and methods. Subsequently, MSP analysis was conducted on these samples using primers and conditions outlined in Materials and methods and validated in part B.

We next examined *WIF1* expression in five additional breast cancer cell lines. RT–PCR analysis showed that MDA-MB-435, YYC-B1, ZR75-1 and SK-BR3 cells display diminished or a complete absence of expression of *WIF1* when compared with normal human breast tissue from an individual without cancer (Figure 2A). Further, MSP analysis indicated that each of these cell lines contains detectable levels of methylated WIF1 promoter. In contrast, T47D cells, which express *WIF1* at levels comparable with the normal breast, did not contain detectable methylated *WIF1* promoter (Figure 2B).

As an independent means of assessing *WIF1* promoter methylation, bisulfite-modified genomic DNA from several breast tumor lines was analyzed by sequencing a 500 bp region of the promoter following PCR with primers that amplify the *WIF1* promoter in a methylation-independent manner. Specifically, PCR primers were designed for regions of the *WIF1* promoter devoid of CpG dinucleotides; thus, amplification proceeds in a manner unbiased by promoter methylation status. Amplicons were subsequently subcloned, and the recombinants were identified and subjected to automated DNA sequencing. Resulting sequences were compared with non-modified *WIF1* promoter sequence and the methylation status of the 40 CpG dinucleotides within this amplicon was determined by characteristic chemical changes associated with cytosines existing in either a methylated or an unmethylated state prior to bisulfite treatment (28). This BGS analysis was conducted on the MCF-7, ZR75-1, MDA-MB-231, YYC-B1 and BT549 breast tumor cell lines (Figure 2C). In ZR75-1, MDA-MB-231, YYC-B1, and to a slightly lesser extent BT549 cells, we observed dense CpG methylation within the *WIF1* promoter in all of the clones analyzed (>70% of all CpG sites methylated). The percent methylation across all CpG sites and all clones is listed in Figure 2C. In MCF-7, reduced CpG methylation was observed, consistent with both the MSP and expression analyses (see Figure 1C and A, respectively) conducted on this cell line. Interestingly, we consistently observed a ∼70 bp region of the *WIF1* promoter (nt no. from −279 to −349, denoted with a horizontal bar with asterisks at the top of Figure 2C) that displays limited CpG methylation even in cell lines displaying overall dense methylation of this region of the genome. Sequence inspection revealed a consensus Sp1 binding site (5′-GGCGGG-3′) within this region, consistent with other reports showing that Sp1 binding to DNA may be able to protect sequences from DNA methylation (29). Nevertheless, observations made on cultured breast tumor cell lines indicate that *WIF1* expression is reduced in these cell lines and, in general, correlates highly with aberrant methylation of CpG dinucleotides within the *WIF1* promoter.

WIF1 downregulation and promoter methylation in primary breast tumors

To determine if epigenetic silencing of the *WIF1* gene occurs in primary breast tumors, we obtained a panel of four surgically removed, snap-frozen breast adenocarcinoma samples and adjacent normal breast tissue from the same patient. Breast tumors and normal tissues were obtained from the University of Florida Shands Cancer Center Molecular Tissue Bank according to Institutional Review Board-approved procedures.

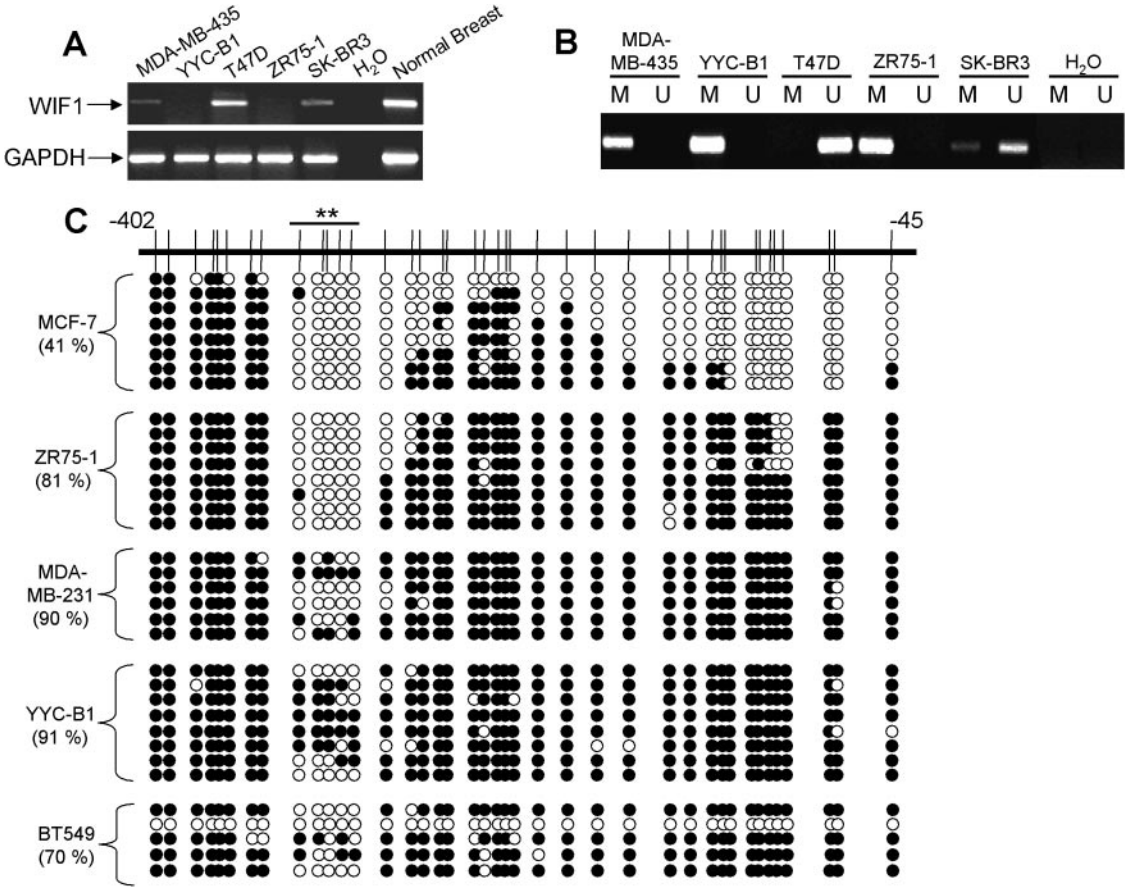

Fig. 2. *WIF1* downregulation and promoter hypermethylation are frequent events in breast tumor cell lines. (**A**) RT–PCR analysis of *WIF1* (top) and *GAPDH* (bottom) expression was conducted on the breast tumor cell lines MDA-MB-435, YYC-B1, T47D, ZR75-1 and SK-BR3 and one normal breast tissue sample from an individual without cancer. (**B**) MSP analysis was conducted on bisulfite-modified genomic DNA harvested from MDA-MB-435, YYC-B1, T47D, ZR75-1 and SK-BR3 cells. (**C**) BGS was conducted on MCF-7, ZR75-1, MDA-MD-231, YYC-B1 and BT549 breast tumor cell lines. Tick marks along the horizontal line at the top represent the location of CpG sites within the region of the WIF1 promoter analyzed [numbering is relative to the transcription start site defined using NCBI Map Viewer (www.ncbi.nlm.nih.gov/mapview)]. Each row of circles represents the sequence analysis of a single clone. Closed circles indicate that the CpG site is methylated; open circles indicate that the CpG site is unmethylated. The per cent methylation across all CpG sites and all clones is shown in parenthesis at the left. The horizontal bar with asterisks indicates the location of a putative Sp1 binding site.

We extracted total RNA from these tissues and subsequently performed RT–PCR to examine *WIF1* transcript abundance. In this panel of matched tumor and normal samples, we observed detectable WIF1 expression in all normal breast tissue samples (Figure 3A). However, WIF1 expression was not detected in three of the matched tumors (BrCa-11,12, and 13) while tumor BrCa-14 showed expression of this transcript at levels consistent with matched normal tissue. We also extracted genomic DNA from these tissue samples, subsequently bisulfite-modified this material and conducted MSP analysis. In concordance with RT–PCR results, the *WIF1* promoter was found to be methylated in tumors BrCa-11, 12 and 13 (Figure 3B) and no methylation of the *WIF1* promoter was detectable in tumor BrCa-14. Of note, MSP analysis revealed that normal breast tissue samples from the MSP-positive BrCa-11 and 13 tumors display low-level but detectable *WIF1* promoter hypermethylation. While this result could be indicative of tumor cell contamination in the adjacent normal tissue sample, it is also consistent with recent studies revealing that aberrant epigenetic events occur in stromal cells during breast tumorigenesis (30). Nevertheless, collectively, these findings indicate that, like cultured breast tumor lines, epigenetic silencing of the *WIF1* gene occurs in primary breast tumors.

To ascertain how commonly this epigenetic event occurs in breast cancer, we conducted MSP analysis on a panel of 20 additional primary breast tumors; Figure 4A shows representative MSP data obtained from eight of these breast tumor samples. In tumor samples BrCa-6, 7, 9, 20 and 21, MSP revealed that these tumors display methylation of the WIF1 promoter. Conversely, tumors BrCa-2, 22 and 24 do not display this aberrant event. Collectively, analysis of 24 primary breast tumors determined that 16 (67%) display aberrant methylation of the *WIF1* promoter as judged by MSP, indicating that this is a common event in breast malignancies.

To independently confirm our MSP results and determine how densely the *WIF1* promoter is methylated in primary breast tumors, we subjected three tumors to BGS analysis. Specifically, we analyzed the methylation-positive tumors BrCa-6 and 20, and the methylation-negative tumor BrCa-2. We observed hypermethylation of the majority of the CpG dinucleotides within the amplified 500 bp region of the *WIF1* promoter in tumors BrCa-6 and 20 (90% methylation across all CpG sites and all clones for both tumors; Figure 4B). In BrCa-2, only a low-level of CpG methylation was observed (7% methylation). BGS analysis of the *WIF1* promoter from normal breast tissue (from an individual without cancer) shows that this region is essentially methylation-free (Figure 4B) and

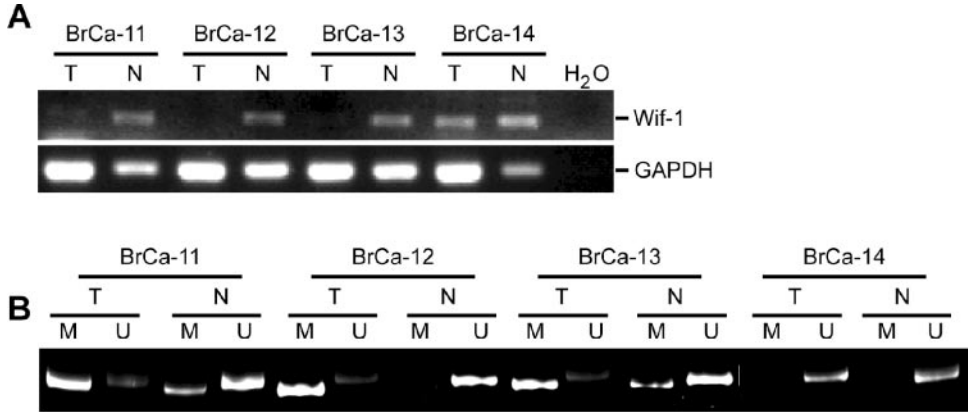

Fig. 3. *WIF1* silencing and promoter hypermethylation in a panel of matched breast tumor and normal tissue. (**A**) RT–PCR analysis of *WIF1* (top) and *GAPDH* (bottom) expression in four snap-frozen breast tumor specimens and adjacent normal breast tissue (BrCa-11–14; 'T' indicates tumor, 'N' is adjacent normal tissue) (top). (**B**) MSP methylation analysis of the *WIF1* promoter in BrCa-11–14 normal and tumor samples using MSP primers specific for methylated (M) and unmethylated (U) DNA.

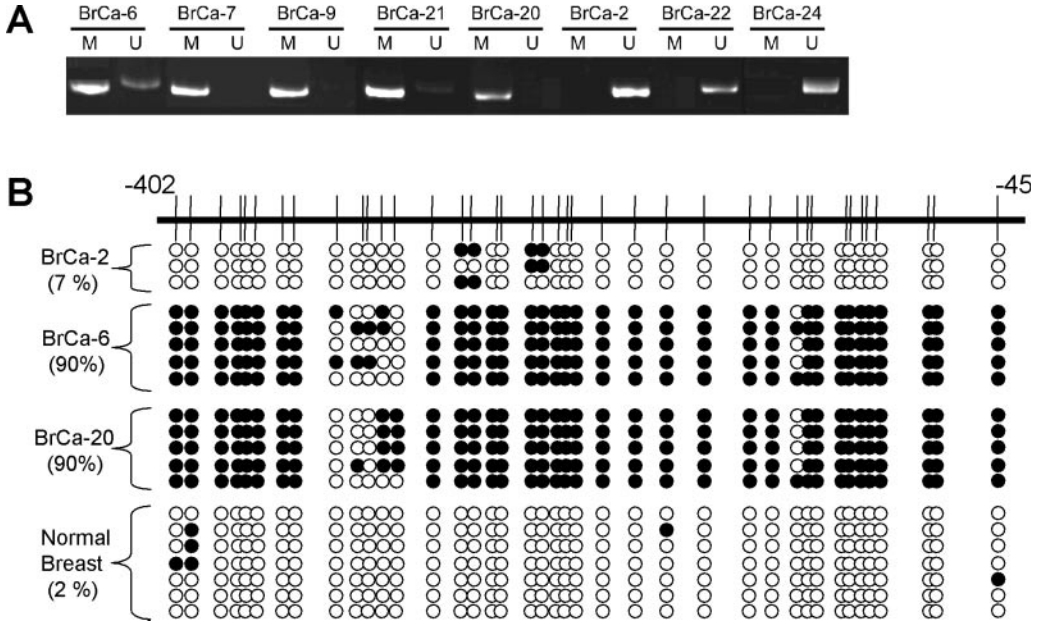

Fig. 4. *WIF1* promoter hypermethylation is a common event in breast tumors. (**A**) Representative MSP results obtained on genomic DNA harvested from eight primary breast adenocarcinoma samples. (**B**) BGS analysis of three breast tumors, two tumors demonstrating *WIF1* hypermethylation by MSP (BrCa-6 and 20) and one MSP-negative (unmethylated) tumor (BrCa-2). One normal breast sample from an individual without cancer is also shown.

further supports the notion that the DNA methylation we observe in the tumor samples results from the disease and is not part of the normal biology of breast tissue. The results of this experiment clearly indicate that large regions of the *WIF1* promoter are subject to dense methylation in primary breast tumors consistent with epigenetic silencing of this gene during breast tumorigenesis.

DNMT1 and DNMT3B cooperate to methylate the WIF1 promoter

Increased expression of one or more of the DNA methyltransferases (DNMTs) has been reported to occur in breast (31) as well as other tumor types (32) and probably contributes to some degree to the aberrant hypermethylation of the *WIF1* promoter we have observed in breast tumors. To assess which of the DNMTs may mediate this aberrant methylation, we employed a model cell line system, namely the HCT116 colon cancer cell line and its isogenic derivatives in which the *DNMT1*, *DNMT3B* and *DNMT1* and *DNMT3B* genes have been genetically disrupted (KO, knockout) (33). We found that untreated parental HCT116 cells, like many of the breast cancer cell lines and tumors, demonstrate a complete lack of WIF1 expression that is upregulated following 5 μM 5-azadC treatment (Figure 5A). Furthermore, BGS analysis determined that this cell line contains a hypermethylated *WIF1* promoter (Figure 5B), supporting our view that these cells are, in general, a valid model for studying methylation of the *WIF1* promoter in cell lines and tumors. Upon disruption of the *DNMT1* gene, and to a lesser extent the *DNMT3B* gene, weak re-expression of WIF1 was detected (weak expression is observable in the *DNMT3B*-deficient cells upon additional PCR cycles; data not shown). In contrast, disruption of both DNMTs (DKO, double knockout), treatment of cells with 5-azadC alone, or a combination of 5-azadC and the histone

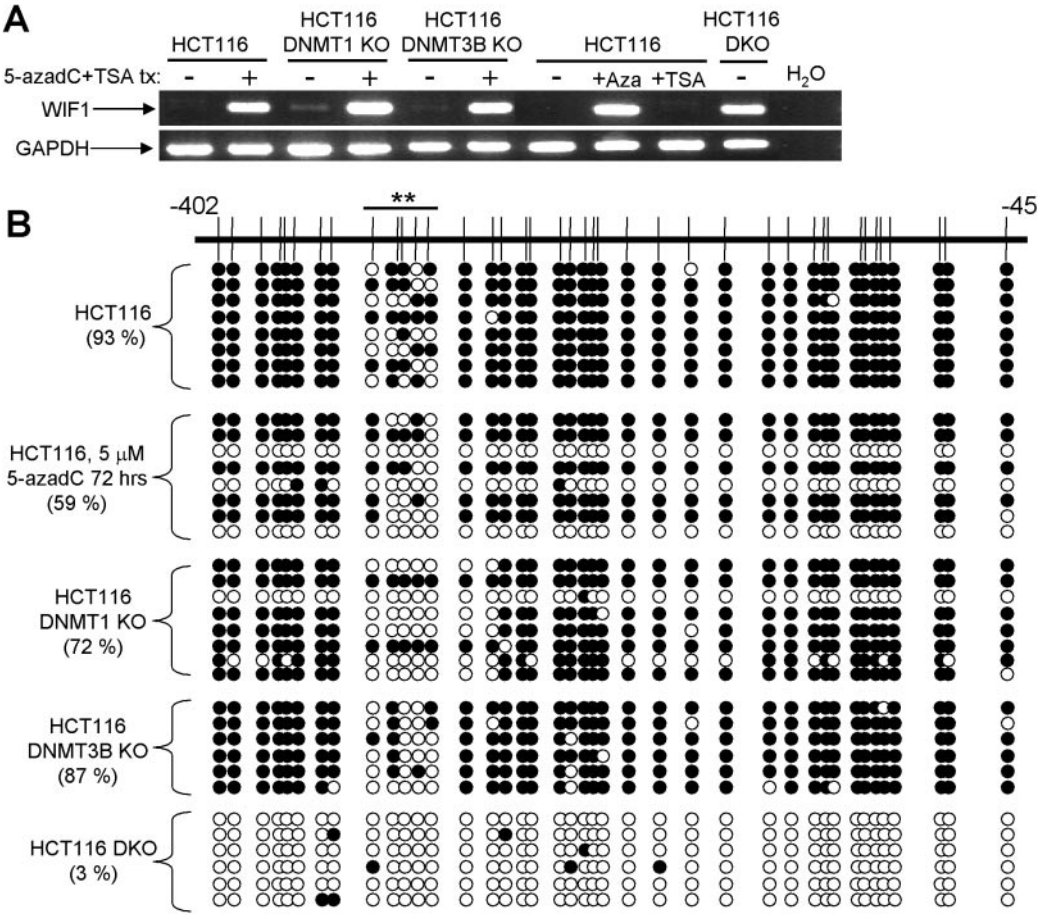

Fig. 5. *DNMT1* and *DNMT3B* cooperatively mediate aberrant *WIF1* promoter hypermethylation. (**A**) RT–PCR analysis of *WIF1* (top) and *GAPDH* (bottom) expression was conducted on the HCT116 colorectal tumor line and its isogenic derivatives with DNMT1 (DNMT1 KO), DNMT3B (DNMT3B KO), or both DNMT1 and DNMT3B (DKO) disrupted by targeted deletion. Cells were harvested for analysis either before (−) or after (+) treatment with 5-azadC and TSA (5 μM 5-azadC for 3 days followed by 100 nM TSA for a final 24 h). Alternatively, HCT116 cells were cultured with 5-azadC (5 μM, 3 days) or TSA alone (100 nM, 24 h). (**B**) BGS analysis of the *WIF1* promoter in each of the cell lines or drug treatments indicated at the left. The percent methylation across all CpG sites and all clones for each of the five conditions is shown in parenthesis at the left. The horizontal bar with asterisks indicates the location of a putative Sp1 binding site.

deacetylase inhibitor, TSA, resulted in robust re-expression of the *WIF1* gene (Figure 5A). DNA methylation was clearly the dominant silencing mechanism since TSA treatment alone did not mediate re-expression of WIF1. BGS DNA methylation analysis of each of the cell lines and drug treatments was highly consistent with the expression data (Figure 5B). Interestingly, the pattern of DNA methylation in the HCT116 *DNMT1* KO cells was most similar to that of the 5-azadC treated cells, in that the demethylation was largely an all-or-none event (i.e. all CpG sites demethylated on a DNA molecule or none changed from the parental pattern). In contrast, the HCT116 *DNMT3B* KO cells displayed a more limited and random pattern of demethylation across most of the region analyzed. The *WIF1* promoter in DKO cells was almost totally hypomethylated and 5-azadC-treated HCT116 cells displayed a significant fraction of completely hypomethylated clones. Collectively, these results indicate that *DNMT1* and *DNMT3B* work in a cooperative fashion to methylate the *WIF1* promoter.

Discussion

Here we demonstrate, using both breast tumor cell lines and primary human breast tumor samples, that the *WIF1* gene is subject to DNA hypermethylation-mediated downregulation in this tumor type. *WIF1* expression was highly correlated with promoter DNA methylation levels as monitored by both MSP and BGS. BGS analysis indicated that DNA methylation spanned a large region of the *WIF1* promoter, with the exception of a small area in the upstream region. Interestingly, this region, which was hypomethylated in all cell lines, primary tumors and the HCT116 colon cancer cell line, contained a consensus Sp1 binding site. Other studies have shown that the binding of Sp1 may be able to block regions of the genome from *de novo* methylation (29) although it remains possible that other DNA binding proteins or aspects of chromatin structure may mediate the hypomethylation of this small region. Lastly, we used the HCT116 cell line and its DNMT-KO derivatives to demonstrate that the enzymatic activity of both *DNMT1* and *DNMT3B* contribute to aberrant *WIF1* promoter DNA methylation.

As a result of this work, we have established the *WIF1* gene as a target for epigenetic silencing in breast adenocarcinoma. Similarly, numerous genes have been characterized in a diverse array of tumor types as subject to silencing through aberrant CpG methylation within their proximal promoter regions (18,34). Breast cancer is certainly no exception as many *bona fide* tumor suppressors have been found to undergo

epigenetic silencing in this tumor type. Of note, Wissman *et al.* (17) found using both immunohistochemical and nucleic acid hybridization approaches that WIF1 expression was downregulated in 63% of breast tumors analyzed. This is in very close agreement with the results of our study that show that 67% (16 out of 24) of the invasive breast tumors investigated display aberrant methylation of the *WIF1* promoter. Taken together, we conclude that epigenetic silencing of the *WIF1* gene is a common event in adenocarcinoma of the breast.

The most compelling evidence for Wnt signaling in promoting mammary tumorigenesis comes from mouse studies. Wnt transgenics display alveolar hyperplasia early in life and nearly 100% of these animals ultimately develop focal mammary carcinomas (10). In humans, activated β-catenin has been observed in a significant percentage of primary breast adenocarcinomas (12), consistent with a role for hyperactivation of Wnt signaling in this tumor type. Indeed, hyperactivation of canonical Wnt signaling resulting from mutations in components in this pathway, such as β-catenin, APC or Axin, are commonly encountered aberrations in many human tumor types (3). However, such mutations are uncommon in breast cancer. Alternatively, dysregulation of extracellular molecules antagonistic to Wnt signaling such as Dkk-1 (35), Frzb (36,37) and WIF1 (14) would predictably result in hyperactivation of Wnt signaling. Interestingly, WIF1 is not the only secreted regulator of the Wnt pathway found to be a target of aberrant epigenetic silencing. The secreted frizzled-related glycoprotein family of proteins (SFRP1–5) competes with the frizzled receptors for Wnt binding, thereby antagonizing Wnt signaling. Aberrant DNA methylation of *SFRP1*, *2* and *4* has been implicated in downregulating these genes in several tumor types (38–40), including *SFRP1* in breast cancer (41). Our finding that the *WIF1* gene is subject to epigenetic silencing in human breast tumors is consistent with this idea and may provide an important mechanistic link between Wnt signaling and human breast disease.

The *cyclin D1* gene (*CCND1*) is one of the targets of Wnt signaling in breast epithelium (12). Furthermore, overexpression of *cyclin D1* is quite common in breast cancer, with numerous groups reporting overexpression in >50% of invasive breast tumors (42,43). While increased *cyclin D1* expression is often traced to amplification of the *CCND1* gene, gene amplification is not required to result in overexpression (44). Moreover, overexpression of *cyclin D1* is quite common in ductal carcinoma *in situ* (DCIS) lesions (44), indicating that increased *cyclin D1* abundance occurs early in the disease process. Similarly, the human telomerase catalytic subunit *hTERT* gene is both upregulated by Wnt signaling (7) and expressed early during breast cancer progression (45). Should epigenetic silencing of the *WIF1* gene be a contributing event in driving cyclin D1 and/or *hTERT* expression through dysregulation of Wnt signaling, then this aberrant epigenetic event would be expected to occur early in tumor progression as well. This possibility is supported by immunohistochemical data indicating that *WIF1* expression is reduced in early-stage breast adenocarcinomas (17). While here we document that the *WIF1* gene is aberrantly methylated in invasive breast adenocarcinoma, we are currently examining earlier cancerous and pre-cancerous stages (i.e. usual and atypical ductal hyperplasia and DCIS) to determine when during tumor progression aberrant methylation of the *WIF1* promoter occurs during breast cancer progression.

Studies using the HCT116 parental and DNMT-KO derivatives revealed that both *DNMT1* and *DNMT3B* must be inactivated in order to achieve efficient *WIF1* promoter demethylation and robust re-expression of the gene. We observed low-level re-expression of *WIF1* in the *DNMT1* KO cells, and consistent with this we found a 21% reduction in DNA methylation levels by BGS. This is in contrast to the 5-azadC-treated and DKO (*DNMT1* + *DNMT3B*) HCT116 cells, where high-level *WIF1* re-expression and 34 and 90% reductions in methylation levels, respectively, were observed. Interestingly, the pattern of demethylation in the DKO, 5-azadC-treated and *DNMT1* KO cells was similar in that the demethylation was largely all-or-none on an individual DNA strand. This is probably due to *DNMT1* operating at replication foci in a processive manner (46). *DNMT3B* KO HCT116 cells displayed only a low level of DNA demethylation and very limited re-expression of WIF1 (visible with a higher number of PCR cycles than used in Figure 5A, not shown). The limited demethylation in the *DNMT3B* KO cells was, however, more random and no completely demethylated strands were observed. Our findings that both *DNMT1* and *DNMT3B* are needed to mediate full *WIF1* promoter hypermethylation is consistent with recent findings that the maintenance function of *DNMT1* is less efficient than previously thought, and that continued *de novo* activity from *DNMT3B* is probably necessary to maintain the full complement of cellular DNA methylation patterns (47,48). Our results also indicate that any therapy aimed at pharmacologically reactivating expression of *WIF1* in tumors would have to target both DNMTs in order to be effective.

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References

1. Weinberg,R.A. (1996) How cancer arises. *Sci. Am.*, **275**, 62–70.
2. Nusse,R. and Varmus,H.E. (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell*, **31**, 99–109.
3. Logan,C.Y. and Nusse,R. (2004) The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.*, **20**, 781–810.
4. He,T.C., Sparks,A.B., Rago,C., Hermeking,H., Zawel,L., da Costa,L.T., Morin,P.J., Vogelstein,B. and Kinzler,K.W. (1998) Identification of c-MYC as a target of the APC pathway. *Science*, **281**, 1509–1512.
5. Pelengaris,S., Khan,M. and Evan,G. (2002) c-MYC: more than just a matter of life and death. *Nat. Rev. Cancer*, **2**, 764–776.
6. Adhikary,S. and Eilers,M. (2005) Transcriptional regulation and transformation by Myc proteins. *Nat. Rev. Mol. Cell Biol.*, **6**, 635–645.
7. Wu,K.J., Grandori,C., Amacker,M., Simon-Vermot,N., Polack,A., Lingner,J. and Dalla-Favera,R. (1999) Direct activation of TERT transcription by c-MYC. *Nat. Genet*, **21**, 220–224.
8. Maser,R.S. and DePinho,R.A. (2002) Connecting chromosomes, crisis, and cancer. *Science*, **297**, 565–569.
9. Polakis,P. (2000) Wnt signaling and cancer. *Genes Dev.*, **14**, 1837–1851.
10. Li,Y., Hively,W.P. and Varmus,H.E. (2000) Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer. *Oncogene*, **19**, 1002–1009.

11. Michaelson,J.S. and Leder,P. (2001) Beta-catenin is a downstream effector of Wnt-mediated tumorigenesis in the mammary gland. *Oncogene*, **20**, 5093–5099.
12. Lin,S.Y., Xia,W., Wang,J.C., Kwong,K.Y., Spohn,B., Wen,Y., Pestell,R.G. and Hung,M.C. (2000) Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc. Natl Acad. Sci. USA*, **97**, 4262–4266.
13. Ozaki,S., Ikeda,S., Ishizaki,Y., Kurihara,T., Tokumoto,N., Iseki,M., Arihiro,K., Kataoka,T., Okajima,M. and Asahara,T. (2005) Alterations and correlations of the components in the Wnt signaling pathway and its target genes in breast cancer. *Oncol. Rep.*, **14**, 1437–1443.
14. Hsieh,J.C., Kodjabachian,L., Rebbert,M.L., Rattner,A., Smallwood,P.M., Samos,C.H., Nusse,R., Dawid,I.B. and Nathans,J. (1999) A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature*, **398**, 431–436.
15. Mazieres,J., He,B., You,L., Xu,Z., Lee,A.Y., Mikami,I., Reguart,N., Rosell,R., McCormick,F. and Jablons,D.M. (2004) Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. *Cancer Res.*, **64**, 4717–4720.
16. Taniguchi,H., Yamamoto,H., Hirata,T., Miyamoto,N., Oki,M., Nosho,K., Adachi,Y., Endo,T., Imai,K. and Shinomura,Y. (2005) Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. *Oncogene*, **24**, 7946–7952.
17. Wissmann,C., Wild,P.J., Kaiser,S. *et al.* (2003) WIF1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer. *J. Pathol.*, **201**, 204–212.
18. Jones,P.A. and Baylin,S.B. (2002) The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.*, **3**, 415–428.
19. Dobrovic,A. and Simpfendorfer,D. (1997) Methylation of the *BRCA1* gene in sporadic breast cancer. *Cancer Res.*, **57**, 3347–3350.
20. Esteller,M., Silva,J.M., Dominguez,G. *et al.* (2000) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J. Natl Cancer Inst.*, **92**, 564–569.
21. Ferguson,A.T., Evron,E., Umbricht,C.B. *et al.* (2000) High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proc. Natl Acad. Sci. USA*, **97**, 6049–6054.
22. Nass,S.J., Herman,J.G., Gabrielson,E., Iversen,P.W., Parl,F.F., Davidson,N.E. and Graff,J.R. (2000) Aberrant methylation of the estrogen receptor and E-cadherin 5′ CpG islands increases with malignant progression in human breast cancer. *Cancer Res.*, **60**, 4346–4348.
23. Holst,C.R., Nuovo,G.J., Esteller,M., Chew,K., Baylin,S.B., Herman,J.G. and Tlsty,T.D. (2003) Methylation of p16(INK4a) promoters occurs *in vivo* in histologically normal human mammary epithelia. *Cancer Res.*, **63**, 1596–1601.
24. Vo,Q.N., Kim,W.J., Cvitanovic,L., Boudreau,D.A., Ginzinger,D.G. and Brown,K.D. (2004) The *ATM* gene is a target for epigenetic silencing in locally advanced breast cancer. *Oncogene*, **23**, 9432–9437.
25. Rhee,I., Bachman,K.E., Park,B.H. *et al.* (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*, **416**, 552–556.
26. Qiu,G.H., Tan,L.K., Loh,K.S., Lim,C.Y., Srivastava,G., Tsai,S.T., Tsao,S.W. and Tao,Q. (2004) The candidate tumor suppressor gene *BLU*, located at the commonly deleted region 3p21.3, is an E2F-regulated, stress-responsive gene and inactivated by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma. *Oncogene*, **23**, 4793–4806.
27. Herman,J.G., Graff,J.R., Myohanen,S., Nelkin,B.D. and Baylin,S.B. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA*, **93**, 9821–9826.
28. Frommer,M., McDonald,L.E., Millar,D.S., Collis,C.M., Watt,F., Grigg,G.W., Molloy,P.L. and Paul,C.L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl Acad. Sci. USA*, **89**, 1827–1831.
29. Macleod,D., Charlton,J., Mullins,J. and Bird,A.P. (1994) Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. *Genes Dev.*, **8**, 2282–2292.
30. Hu,M., Yao,J., Cai,L., Bachman,K.E., van den Brule,F., Velculescu,V. and Polyak,K. (2005) Distinct epigenetic changes in the stromal cells of breast cancers. *Nat. Genet.*, **37**, 899–905.
31. Agoston,A.T., Argani,P., Yegnasubramanian,S., DeMarzo,A., Ansari-Lari,M.A., Hicks,J.L., Davidson,N.E. and Nelson,W.G. (2005) Increased protein stability causes DNA methyltransferase 1 dysregulation in breast cancer. *J. Biol. Chem.*, **280**, 18302–18310.
32. Robertson,K.D., Uzvolgyi,E., Liang,G., Talmadge,C., Sumegi,J., Gonzales,F.A. and Jones,P.A. (1999) The human DNA methyltransferases (DNMTs) 1, 3a, and 3b: Coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res.*, **27**, 2291–2298.
33. Rhee,I., Bachman,K.E., Park,B.H. *et al.* (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*, **416**, 552–556.
34. Esteller,M. (2005) Aberrant DNA methylation as a cancer-inducing mechanism. *Annu. Rev. Pharmacol. Toxicol.*, **45**, 629–656.
35. Glinka,A., Wu,W., Delius,H., Monaghan,A.P., Blumenstock,C. and Niehrs,C. (1998) Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature*, **391**, 357–362.
36. Leyns,L., Bouwmeester,T., Kim,S.H., Piccolo,S. and De Robertis,E.M. (1997) Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell*, **88**, 747–756.
37. Wang,S., Krinks,M., Lin,K., Luyten,F.P. and Moos,M., Jr (1997) Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell*, **88**, 757–766.
38. Liu,T.-H., Raval,A., Chen,S.-S., Matkovic,J.J., Byrd,J.C. and Plass,C. (2006) CpG island methylation and expression of the secreted frizzled-related protein gene family in chronic lymphocytic leukemia. *Cancer Res.*, **66**, 653–658.
39. Fukui,T., Kondo,M., Ito,G., Maeda,O., Sato,N., Yoshioka,H., Yokoi,K., Ueda,Y., Shimokata,K. and Sekido,Y. (2005) Transcriptional silencing of secreted frizzled related protein 1 (SFRP1) by promoter hypermethylation in non-small-cell lung cancer. *Oncogene*, **24**, 6323–6327.
40. He,B., Lee,A.Y., Dadfarmay,S., You,L., Xu,Z., Reguart,N., Mazieres,J., Mikami,I., McCormick,F. and Jablons,D.M. (2005) Secreted frizzled-related protein 4 is silenced by hypermethylation and induces apoptosis in beta-catenin-deficient human mesothelioma cells. *Cancer Res.*, **65**, 743–748.
41. Lo,P.K., Mehrotra,J., D'Costa,A., Fackler,M.J., Garrett-Mayer,E., Argani,P. and Sukumar,S. (2006) Epigenetic suppression of secreted frizzled related protein 1 (SFRP1) expression in human breast cancer. *Cancer Biol. Ther.*, **5,** [Epub] ahead of print] PMID: 16410723.
42. Gillett,C., Smith,P., Gregory,W., Richards,M., Millis,R., Peters,G. and Barnes,D. (1996) Cyclin D1 and prognosis in human breast cancer. *Int. J. Cancer*, **69**, 92–99.
43. Zhang,S.Y., Caamano,J., Cooper,F., Guo,X. and Klein-Szanto,A.J. (1994) Immunohistochemistry of cyclin D1 in human breast cancer. *Am. J. Clin. Pathol.*, **102**, 695–698.
44. Gillett,C.E., Lee,A.H., Millis,R.R. and Barnes,D.M. (1998) Cyclin D1 and associated proteins in mammary ductal carcinoma *in situ* and atypical ductal hyperplasia. *J. Pathol.*, **184**, 396–400.
45. Yashima,K., Milchgrub,S., Gollahon,L.S., Maitra,A., Saboorian,M.H., Shay,J.W. and Gazdar,A.F. (1998) Telomerase enzyme activity and RNA expression during the multistage pathogenesis of breast carcinoma. *Clin. Cancer Res.*, **4**, 229–234.
46. Hermann,A., Goyal,R. and Jeltsch,A. (2004) The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites. *J. Biol. Chem.*, **279**, 48350–48359.
47. Liang,G., Chan,M.F., Tomigahara,Y., Tsai,Y.C., Gonzales,F.A., Li,E., Laird,P.W. and Jones,P.A. (2002) Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Mol. Cell. Biol.*, **22**, 480–491.
48. Jackson,M., Krassowska,A., Gilbert,N., Chevassut,T., Forrester,L., Ansell,J. and Ramsahoye,B. (2004) Severe global DNA hypomethylation blocks differentiation and induces hyperacetylation in embryonic stem cells. *Mol. Cell. Biol.*, **24**, 8862–8871.

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