

Differential Requirement for DNA Methyltransferase 1 in Maintaining Human Cancer Cell Gene Promoter Hypermethylation

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Abstract

Previous work has shown that DNA hypermethylation of tumor suppressor genes in colorectal cancer cells may be maintained in the absence of the major mammalian methyltransferase, DNA methyltransferase 1 (DNMT1). In an effort to dissect the dependency on DNMT1 to maintain such hypermethylation in different cancer types, we performed a systematic analysis of depletion of DNMT1 in colorectal (SW48), bladder (T24), and breast (T47D) cancer cells by DNMT1-specific small hairpin RNA (shRNA) targeting. We show that although DNMT1-deficient SW48 and T24 cells exhibited no observable growth defects and were able to maintain promoter hypermethylation, DNMT1-deficient T47D breast cells failed to form comparable numbers of colonies when stably selected for the incorporation of the DNMT1-specific shRNA expression vector, suggesting a growth defect with reduced levels of DNMT1. Further treatment of T47D cells with transient transfection of small interfering RNA targeting DNMT1 revealed that severely DNMT1-deficient T47D cells could not fully maintain promoter hypermethylation, and gene silencing was partially reversed at two of the three assayed loci. These observations suggest that human cancer cells may differ in their reliance on DNMT1 for maintaining DNA methylation. (Cancer Res 2006; 66(2): 729-35)

Introduction

Promoter DNA hypermethylation represents an alternative to genetic mechanisms for inactivating genes in human cancers (1, 2). This epigenetic modification of cytosine residues occurs predominantly in a CpG dinucleotide context in the human genome and is catalyzed by three major DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b. Clusters of CpGs, termed CpG islands, are often present at the 5' regulatory regions of genes, and aberrant hypermethylation of CpG islands correlates highly with transcription silencing from the hypermethylated promoter (1, 2). It has been postulated that depletion of DNMTs, specifically DNMT1 because its mouse homologue was characterized as the maintenance methyltransferase, may lead to loss of hypermethylation in cancers, therefore relieving the repression of tumor suppressor expression. This hypothesis was first directly tested in the colorectal cancer cell line HCT116 by knocking out DNMT1 (3). The phenotypes of the DNMT1^{-/-} cells were virtually identical to

the parental wild-type counterpart with only loss of DNA methylation at satellite and juxtacentromeric repeat sequences. This finding was further confirmed in the same cell line and another colorectal cancer cell line, SW480, by using RNA interference-based techniques in both short- and long-term settings (4). These findings in cultured colon cancer cells raised the important question of whether the capacity to maintain hypermethylation in the absence of DNMT1 is a general characteristic of human cancer cells. In this regard, some subsequent studies of other cancer cell types have reported a degree of gene reexpression and promoter demethylation with reduction of DNMT1 by antisense or small interfering RNA (siRNA) methodologies (5-7). In the present study, using DNMT1-specific small hairpin RNA (shRNA) targeting as previously done to deplete DNMT1 in HCT116 and SW480 cells (4), we systematically address the relationship between DNMT1 activity and tumor suppressor promoter hypermethylation in cancer cell lines derived from an additional colorectal cancer (SW48), and from bladder (T24) and breast (T47D) cancers. We find surprising differences in the response of the different cancer cell types to DNMT1 depletion and speculate that these results may have important consequences for clinical management of cancers for which epigenetically based therapies may come to play a significant role.

Materials and Methods

Cell culture. SW48, T24, and T47D cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as directed. shRNA expression vectors, pshDNMT1 and pshMM, were generated previously (4). Cell lines stably expressing pshDNMT1 and pshMM were generated as previously described and were maintained in complete medium supplemented with 0.1 mg/mL hygromycin (Invitrogen, Carlsbad, CA; ref. 4). Hygromycin selection was verified by a No-DNA control plate for each of the cell lines. Colony formation was assessed by visual counting followed by application of the Mann-Whitney test for determination of statistical significance.

Transient siRNA transfection of T47D cells. T47D cells were treated with siDNMT1 (5'-CCAUGAGCACCUGUCUCCTT-3') and siMM (5'-CGAUCAGGACCCUUGUCCTT-3') every 24 hours for 10 days using Lipofectin (Invitrogen) as previously described (4). Cell pellets were harvested every 48 hours for analyses.

Western blotting. Whole cell lysates (5 µg/sample) were analyzed for protein expression by Western blotting using rabbit anti-DNMT1 (1:500 dilution; Santa Cruz, Santa Cruz, CA) and mouse anti-β-actin (1:10,000 dilution; Sigma, St. Louis, MO). The intensity of the DNMT1 bands for each sample was normalized to the intensity of the respective β-actin bands to allow comparison of DNMT1 levels.

DNA methyltransferase assay. DNA methyltransferase enzyme activity was assessed using 10 µg of whole cell lysate, 3 µCi S-adenosyl-L-[methyl-³H] methionine, and 0.5 µg poly(deoxyinosinic-deoxycytidylic acid) [poly(dI-dC); ref. 3]. Triplicate reactions were averaged and plotted as dpm/µg after normalization to a parallel reaction containing all components except poly(dI-dC) substrates.

Methylation-specific PCR and bisulfite sequencing. Genomic DNA was extracted with a lysis buffer containing 20 mmol/L Tris-HCl, 20 mmol/L

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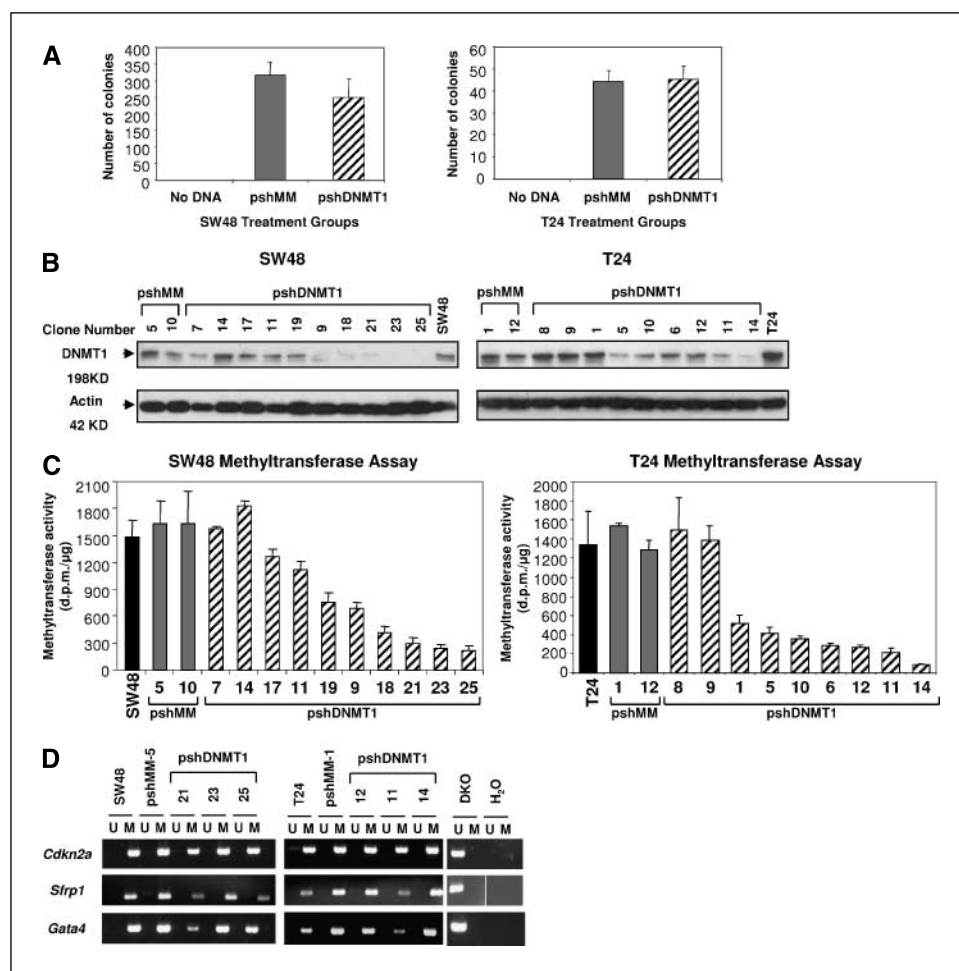


Figure 1. DNMT1-deficient SW48 and T24 cancer cells maintain DNA hypermethylation. **A**, colony formation assay results for SW48 (left) and T24 (right) cells. Cell lines were transfected with No DNA (white columns), pshMM (gray columns), or pshDNMT1 (stippled columns) and selected for expression using hygromycin. Colony number was obtained from visual counting of five sets of pshDNMT1 and pshMM plates for each cell line. **B**, Western blot analyses for DNMT1 and β-actin in randomly selected pshMM and pshDNMT1 clones for both SW48 (left) and T24 (right) cells. **C**, DNA methyltransferase activity assay results for SW48 (left) and T24 (right) clones. **D**, methylation analysis of *Cdkn2a*, *Sfrp1*, and *Gata4* promoters by MSP for SW48 (left) and T24 (right) clones. The parental SW48 and T24 cells contained only methylated (M) alleles for all three genes. This methylation pattern was maintained in all the pshMM and pshDNMT1 clones tested. The DKO cells are unmethylated at all three loci and serve as a positive control for unmethylated (U) allelic amplification.

EDTA, 2% SDS, and 0.5 mg/mL proteinase K. Two micrograms of genomic DNA were subjected to bisulfite treatment for subsequent PCR analyses (8). Methylation-specific PCR (MSP) for *Cdkn2a*, *Sfrp1*, and *Gata4* genes were done as previously described, and the products were visualized on 3% agarose gels (9–12). DNMT1^{-/-}, DNMT3b^{-/-} double knockout (DKO) HCT116 cell DNA was included in all reactions as a positive control for the unmethylated allelic amplification (11–13). Bisulfite sequencing of *Cdkn2a* and *Sfrp1* uses primers that do not overlap any CpG sites to amplify both methylated and unmethylated alleles without bias. The PCR products were subsequently cloned into TOPO TA vectors (Invitrogen) and single clones were sequenced to reveal the methylation states of individual CpG sites in each allele.

Reverse transcription-PCR. Total RNA was isolated from cell pellets harvested on the indicated days using the RNeasy Mini kit (Qiagen). An additional DNase treatment (Qiagen, Valencia, CA) was included to remove genomic DNA contamination. One microgram of total RNA from each sample was used for cDNA first-strand synthesis using the Superscript first-strand system (Invitrogen). The concentration of cDNA for each sample was normalized by glyceraldehyde-3-phosphate dehydrogenase reverse transcription results (primers 5'-GAAGTTCGGAGTCAACGGATTT-3' and 5'-ATGGGTGGAATCATATTGGAA-3'). *Cdkn2a* primers were 5'-AGCCTCGGCTGACTGGCTGG-3' and 5'-CTGCCATCATCATGACCTGG-3'. *Sfrp1* primers were 5'-GGCCATCTACCCGTGTCG-3' and 5'-GATGGCCT-CAGATTTCAACTCGT-3'. *Gata4* primers were 5'-GGCCGCCGACACCC-CAATCT-3' and 5'-ATAGTAGCCCGTCCCATCTCG-3'. DKO cDNA was included in all reactions as a positive control for expression of all four genes. No-RT controls were done for each sample and included in the PCR reactions to verify absence of cDNA (data not shown).

Results

To address the role of DNMT1 in maintaining tumor suppressor gene hypermethylation in cancer cells other than colorectal cancer, we first identified cell lines with known epigenetically silenced genes in important cancer types (10–12). A panel of three genes (*Cdkn2a*, *Sfrp1*, and *Gata4*) was selected for the high frequency of inactivation by aberrant hypermethylation and their importance in regulating growth in neoplasia. To establish DNMT1-depleted cell lines, we used the shRNA expression vectors pshDNMT1, which encodes a DNMT1-targeting shRNA, and pshMM, a mismatch control for the DNMT1 shRNA (4). The two vectors were separately introduced into individual cancer cell lines, and transfectants were selected for expression by drug resistance.

Colorectal SW48 and bladder T24 cancer cells maintain DNA hypermethylation with reduction of DNMT1. We first measured the capacity of human cancer cell lines to tolerate chronic DNMT1 depletion using a colony formation assay. As might be expected from previous experiments done in HCT116 and SW480 colon cancer cells, SW48 colorectal cells showed no bias against colony formation in response to selection for DNMT1 shRNA expression (250 ± 54 pshDNMT1 colonies versus 315 ± 39 pshMM colonies; Fig. 1A). Interestingly, identical results were obtained in the bladder cell line T24 (45 ± 6 pshDNMT1 colonies and 44 ± 5 pshMM colonies). There were no statistical differences between the number of pshDNMT1 and pshMM colonies for

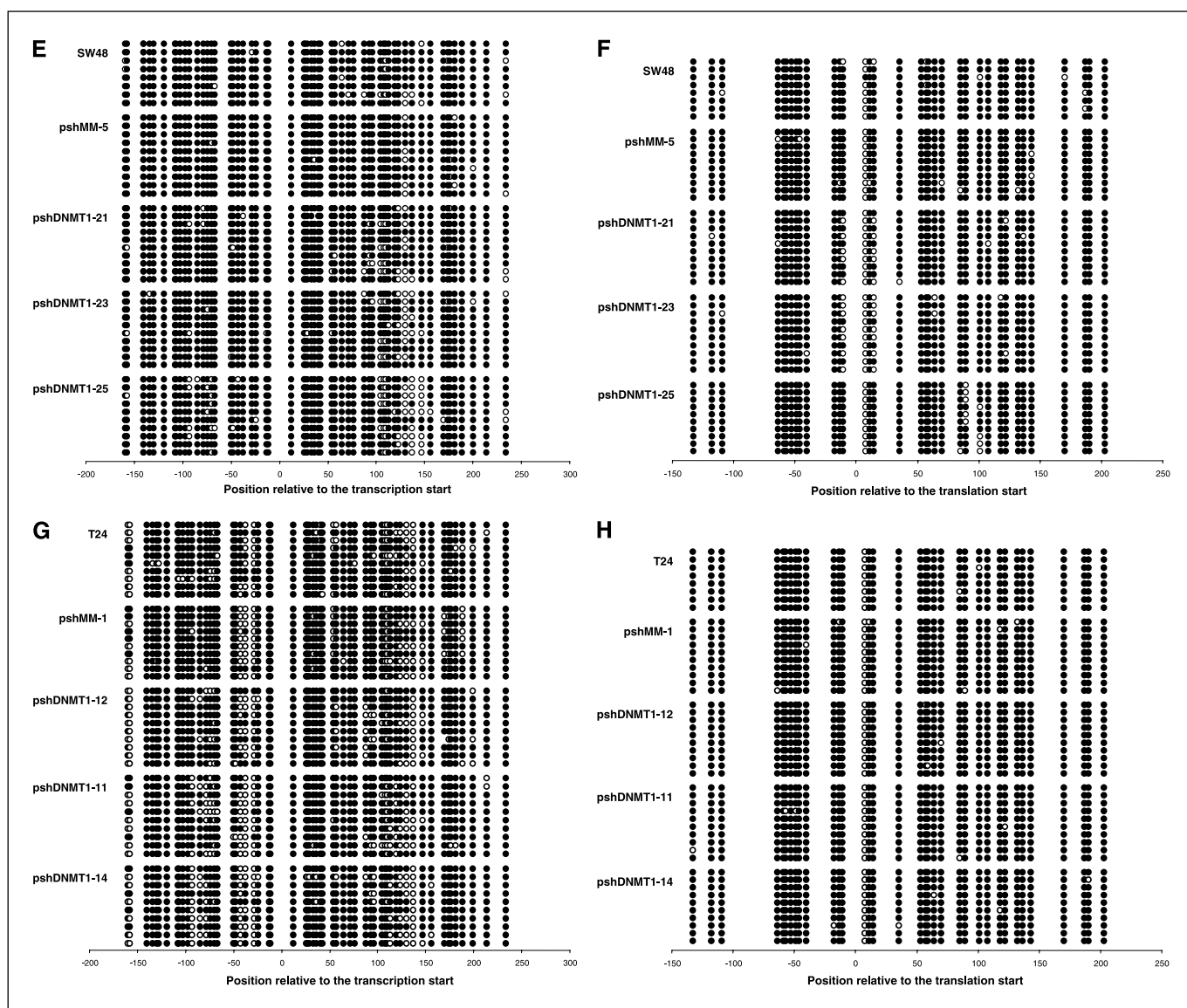


Figure 1 Continued. *E*, bisulfite sequencing data for *Sfrp1* promoters in SW48 and SW48 derived clones. ●, methylated cytosine; ○, unmethylated cytosine. CpG sites are plotted according to their position relative to the transcription start site (marked 0). *F*, bisulfite sequencing data for *Cdkn2a* promoter in SW48 and SW48-derived clones. CpG sites are represented the same way as in (*E*). *G*, bisulfite sequencing data for *Sfrp1* promoters in T24 and T24-derived clones. CpG sites are represented the same way as in (*E*). *H*, bisulfite sequencing data for *Cdkn2a* promoter in SW48 and SW48-derived clones. CpG sites are represented the same way as in (*E*).

SW48 and T24 cells ($U = 8$, $P > 0.20$ for SW48 and $U = 12$, $P > 0.20$ for T24).

Randomly selected pshDNMT1 and pshMM clones for both SW48 and T24 were analyzed for DNMT1 protein and maintenance methyltransferase activity levels. By Western blotting, the pshDNMT1 clones from both cell lines exhibited a range of DNMT1 protein depletion when compared with pshMM or parental cells (Fig. 1*B*). Importantly, these clones also displayed a loss of maintenance methyltransferase activity, which well matched the level of DNMT1 protein depletion (Fig. 1*C*). For example, in SW48 pshDNMT1 clone 25, DNMT1 protein expression was reduced to nondetectable levels as analyzed by Western blotting and the maintenance methyltransferase activity was reduced by 87%. Similar results were observed in T24 pshDNMT1 clone 14, where DNMT1 protein level was barely detectable and only 6% methyltransferase activity remained. Taken together, the results of the colony formation assay

and the phenotypes of the randomly selected pshDNMT1 clones indicated that there was no selection for or against cells lacking DNMT1 for either of the two cell lines.

Next, we tested whether loss of DNMT1 altered hypermethylation patterns in the CpG islands of silenced tumor suppressor promoters. To do this, we used MSP to assess the methylation status at the *Cdkn2a*, *Sfrp1*, and *Gata4* promoters in the pshDNMT1 clones with the most severe depletion of DNMT1 (Fig. 1*D*). The methylation patterns of only methylated alleles were preserved in the parental SW48, pshMM-5, pshDNMT1-21, pshDNMT1-23, and pshDNMT1-25 cells, suggesting that DNMT1-deficient SW48 colorectal cancer cells were able to maintain DNA hypermethylation at the assayed promoters. Similar retention of DNA hypermethylation was observed for DNMT1-deficient T24 clones (pshDNMT1-12, pshDNMT1-11, and pshDNMT1-14) when compared with parental T24 and pshMM-1 cells. Furthermore,

we examined the methylation profiles of *Cdkn2a* and *Sfp1* promoters more closely by bisulfite sequencing and found that DNMT1-deficient SW48 (Fig. 1E and F) and T24 (Fig. 1G and H) cells could maintain hypermethylation at these loci.

T47D breast cancer cell growth is disrupted by loss of DNMT1. The identical strategy used to establish pshDNMT1 and pshMM in SW48 and T24 cells was again used for T47D breast cancer cells. However, contrary to the observations in SW48 and T24, there was a dramatic, significant difference ($U = 25$, $P = 0.01$) in colony formation between pshMM-transfected T47D cells (38 ± 5 colonies) and pshDNMT1 transfectants (1 ± 1 colonies). This suggested the possibility of a selection against DNMT1-deficient T47D cells (Fig. 2A). Indeed, multiple rounds of transfection and selection efforts yielded only six total viable pshDNMT1 T47D clones for analyses. Not unexpectedly, comparison of these clones with parental T47D cells indicated

minimal depletion of DNMT1, with only one clone (pshDNMT1-1) showing a significant reduction ($\geq 50\%$) in DNMT1 protein (Fig. 2B) and a corresponding 49% reduction in enzyme activity (Fig. 2C).

We then examined the promoter hypermethylation in three pshDNMT1 clones with the most DNMT1 depletion (pshDNMT1-4, pshDNMT1-7, and pshDNMT1-1). By MSP analyses, we found that DNA hypermethylation at *Cdkn2a* and *Gata4* promoters was maintained in all of pshDNMT1 clones compared with pshMM-1 and T47D cells (Fig. 2D). We performed bisulfite sequencing to more completely evaluate the *Sfp1* promoter in T47D cells, and this revealed a marginal methylation loss in the pshDNMT1 clones where DNMT1 depletion of $\sim 50\%$ was evident (Fig. 2E). However, bisulfite sequencing data of the *Cdkn2a* promoter confirmed the lack of methylation loss at this locus as measured by MSP (Fig. 2F). The dramatic effects of DNMT1 depletion in

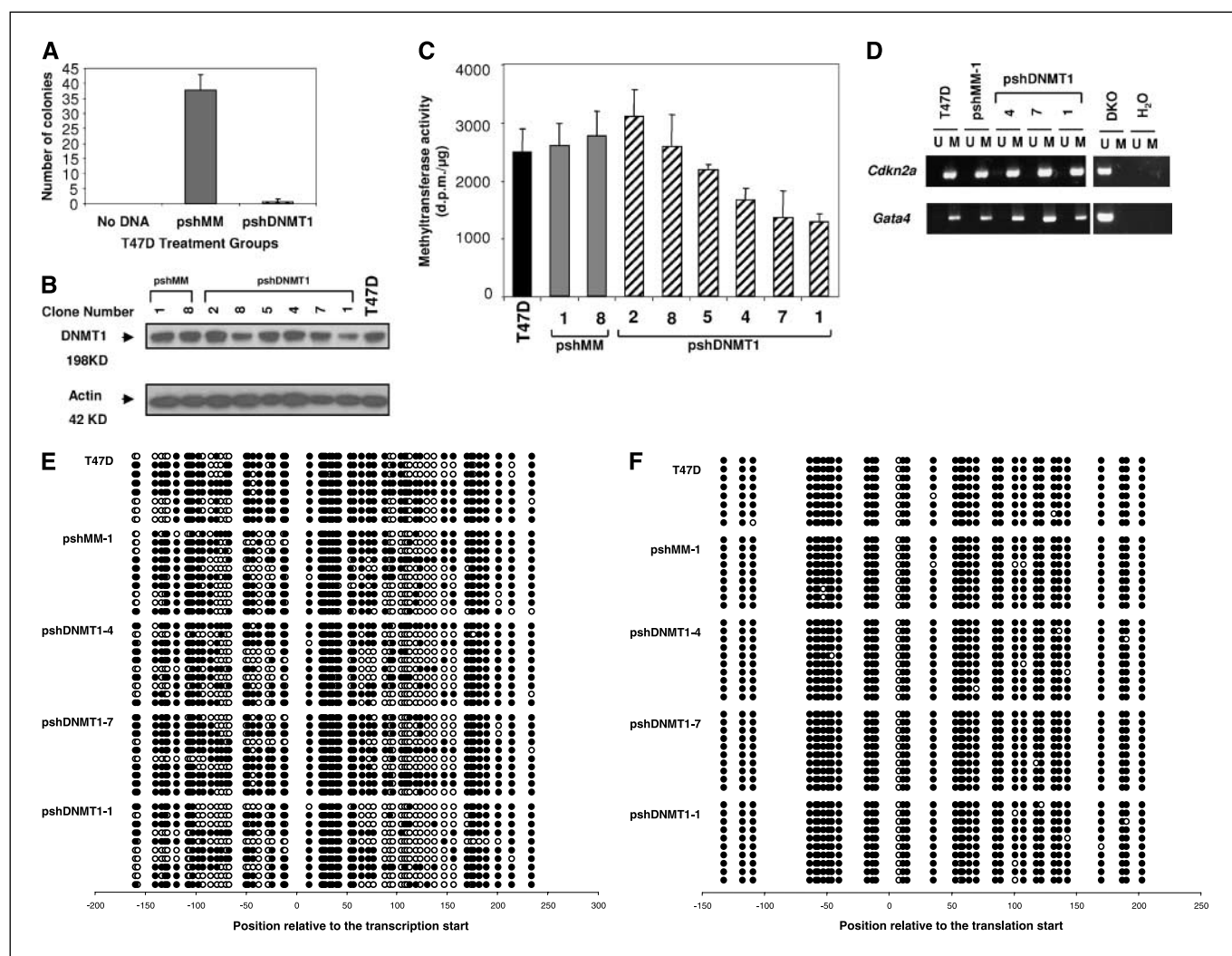


Figure 2. T47D breast cancer cells display growth defects and demethylation following depletion of DNMT1. *A*, colony formation assay results for T47D-derived cells. Cells were transfected with No DNA (white columns), pshMM (gray columns), or pshDNMT1 (stippled columns) and subjected to hygromycin selection. Colony numbers were obtained from visual counting of five sets of pshDNMT1 and pshMM plates for each cell line. *B*, Western blot analyses for DNMT1 and β -actin in randomly selected pshMM and all pshDNMT1 clones for T47D cells. *C*, DNA methyltransferase activity in pshMM (solid gray columns) and pshDNMT1 (stippled columns) T47D breast cancer clones. *D*, MSP results on *Cdkn2a* and *Gata4* promoters in T47D parental and stable clones. In the parental T47D cells, both promoters are hypermethylated. DKO cells were used as controls for unmethylated allelic amplification. *E*, bisulfite sequencing data for *Sfp1* promoters in T47D and T47D-derived clones. ●, methylated cytosine; ○, unmethylated cytosine. CpG sites are plotted according to their position relative to the transcription start site. *F*, bisulfite sequencing data for *Cdkn2a* promoter in T47D and T47D-derived clones. CpG sites are represented the same way as in (*E*).

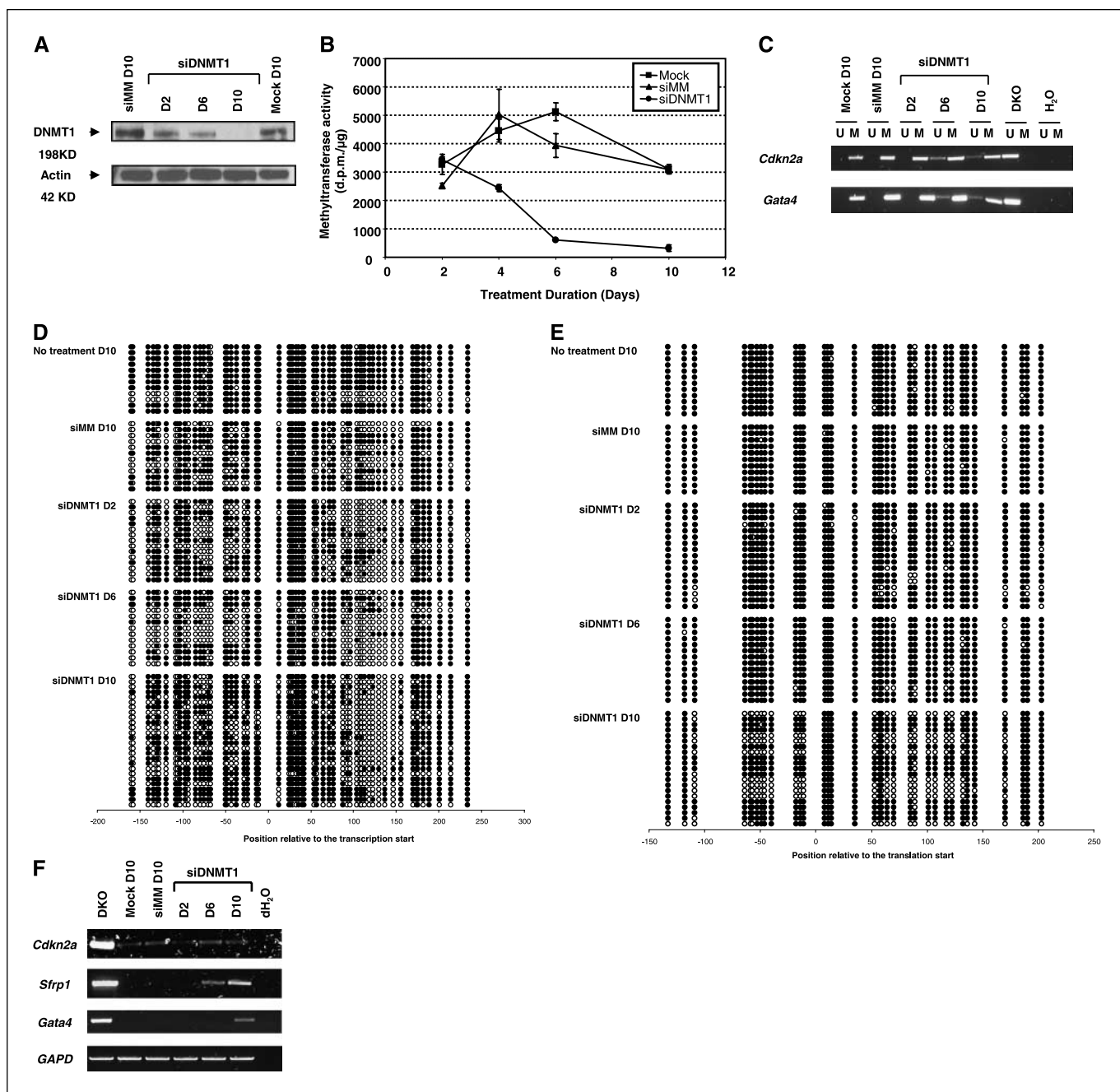


Figure 3. Transient depletion of DNMT1 by siRNA in T47D breast cancer cells. *A*, Western blot analyses for DNMT1 and β -actin for T47D cells treated every 24 hours with siMM, siDNMT1, or mock transfection. The cells were harvested for analysis on the indicated days. *B*, methyltransferase activity assay results on days 2, 4, 6, and 10. ■, mock-treated cells; ▲, siMM-treated cells; ●, siDNMT1-treated cells. *C*, MSP analyses of *Cdkn2a* and *Gata4* promoters. Mock- and siMM-treated T47D cells contain only methylated alleles for both genes, yielding only the methylated band. siDNMT1-treated cells showed partial demethylation at both promoters on treatment days 6 and 10. *D*, bisulfite sequencing of *Sfrp1* promoter. ○, unmethylated cytosine at each CpG site; ●, methylated cytosine at each CpG site. The CpG sites were plotted relative to the transcription start site (marked 0). Obvious demethylation could be seen as early as day 2 in the siDNMT1-treated T47D cells. *E*, bisulfite sequencing of *Cdkn2a* promoter. CpG sites are represented the same way as in (*D*). *F*, RT-PCR analyses of *Cdkn2a*, *Sfrp1*, and *Gata4*. Accompanying promoter demethylation, *Sfrp1* and *Gata4* cDNA were detected in the siDNMT1-treated cells, whereas these genes remained silent in the mock- and siMM-treated cells.

T47D cells, in contrast with those observed in colorectal or bladder cancer cells, suggested a critical role for this enzyme in mediating cancer cell growth in this cell line. We investigated this possibility further in studies below.

Full promoter CpG hypermethylation in T47D breast cancer cells requires DNMT1. Having observed that T47D cells did not tolerate chronic high level depletion of DNMT1, we treated cells

transiently with siDNMT1 or siMM. After repeated transfections over a 10-day period, we were able to reduce DNMT1 protein to virtually nondetectable levels (Fig. 3*A*) and eliminate 90% of maintenance methyltransferase activity (Fig. 3*B*). We then examined the methylation status of *Cdkn2a*, *Sfrp1*, and *Gata4* in these siDNMT1-treated cells by both MSP and bisulfite sequencing. First, MSP data on *Cdkn2a* and *Gata4* clearly

showed distinct demethylation at both loci by day 6 of the treatment (presence of U amplicons) and was sustained until day 10 (Fig. 3C). By bisulfite sequencing analysis, the *Sfrp1* promoter in the parental T47D cells has a somewhat heterogeneous hypermethylation pattern. However, partial but pronounced demethylation was observed for the *Sfrp1* promoter in siDNMT1-treated cells on all of the assayed days (Fig. 3D), especially in the +50 to +100 region and for sets of alleles in the -50 to -150 region. The *Cdkn2a* promoter, on day 10, showed substantial demethylation in subsets of alleles around the transcription start site (-50 to +125; Fig. 3E). Clearly, DNMT1-deficient T47D cells could not maintain the full DNA hypermethylation patterns that were present in the parental and mock-treated T47D cancer cells.

The most important functional consequence of promoter hypermethylation is the corresponding tumor suppressor gene silencing in cancers. Therefore, we examined whether the partial demethylation observed at the three tumor suppressor promoters was sufficient to induce gene reexpression in the DNMT1-deficient T47D cells. *Cdkn2a* had been shown previously to be expressed at extremely low levels in parental T47D cells despite the heavy DNA methylation at its promoter (10). Reverse transcription-PCR (RT-PCR) results in the present study displayed this low-level basal expression of *Cdkn2a* in all the control and DNMT1-deficient cells. This basal expression may have obscured the detection of small changes in *Cdkn2a* expression in the DNMT1-deficient cells. However, RT-PCR analyses showed that both *Sfrp1* and *Gata4* became distinctly reexpressed with time in the DNMT1-depleted cells, whereas these two genes are completely silent in the control cells (Fig. 3F). The expression data for these latter two genes indicate a good correlation between promoter demethylation after DNMT1 depletion and relief of gene silencing at the assayed loci in T47D cells.

Discussion

The apparent lack of selection against DNMT1 deficiency and the obvious retention of DNA hypermethylation of tumor suppressor genes in SW48 and T24 cancer cells support the previous observations in HCT116 and SW480 colorectal cancer cells that some tumor cells can maintain DNA hypermethylation in the absence of, or severely reduced levels of, DNMT1. Given the similar observations in HCT116, SW480, and SW48 colorectal cancer cells, our data suggest that uncoupling of DNMT1 enzymatic activity and maintenance of DNA hypermethylation may be a general feature of some cancer cell types. Genetic knockout studies in HCT116 revealed that DNMT3b, a conventionally recognized *de novo* DNA methyltransferase, has overlapping functions to DNMT1 in the cancer setting with regard to the maintenance of promoter hypermethylation (13). This same compensatory relationship between DNMT1 and DNMT3b was

also documented in ovarian cancer cell line CP70, where DNMT1 knockdown alone produced little or no promoter demethylation, whereas DNMT1 and DNMT3b cosuppression by siRNA resulted in significant demethylation and gene reexpression (5). It is formally possible that a similar cooperativity between DNMT1 and DNMT3b can account for the dispensability of DNMT1 in both SW48 and T24 cells. It should be noted, however, that such cooperativity may not be absent from the breast cancer cells studied in our present work. The levels of demethylation and gene reexpression in the T47D cells transiently depleted of DNMT1, although significant, are not nearly as dramatic as in HCT116 cells, which are simultaneously genetically disrupted for DNMT1 and DNMT3b (10). Hence, even in the T47D breast cancer cells, there may be interactions between DNMT1 and DNMT3b. The discrepancies in DNMT1 dependency between the cell lines, then, could be a result of different expression levels of DNMT3b.

The poor colony formation by DNMT1-deficient T47D cells might have been contributed by a time-dependent reexpression of tumor suppressor genes that subsequently stalled cell cycle or initiated apoptosis. These observations are consistent with reports of gene reexpression following DNMT1 reduction in MDA-MB-231, Hs578t, and HCC1954 breast cancer cells previously by others (6, 7). Alternatively, breast cancer cells could be differentially dependent on the presence of DNMT1 for other key cellular functions, such as direct maintenance of proper DNA replication (14). Whichever is the case, the present systematic comparison together with other published reports lead us to conclude that there may be a distinctive differential dependency on DNMT1 among cancer cell types for maintaining DNA hypermethylation and for long-term survival. These findings underscore a necessity to better understand the functional roles of DNMTs in different cancer types. Importantly, the notion of exclusively targeting DNMT1 to achieve some degree of tumor suppressor gene reexpression may be feasible in certain cancer types, such as breast cancers, whereas the same strategy alone may be insufficient to amount to any therapeutic value in, for instance, colorectal cancers. This would also suggest that a close examination and understanding of mechanisms underlying these cell-specific changes may reveal a cellular defect that may then be used to identify which patients are responsive and which are not. We hope that our data serves as a constructive step in this direction.

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