DNA Methylation Down-regulates CDX1 Gene Expression in Colorectal Cancer Cell Lines*

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CDX1 is a homeobox protein that inhibits proliferation of intestinal epithelial cells and regulates intestine-specific genes involved in differentiation. CDX1 expression is developmentally and spatially regulated, and its expression is aberrantly down-regulated in colorectal cancers and colon cancer-derived cell lines. However, very little is known about the molecular mechanism underlying the regulation of CDX1 gene expression. In this study, we characterized the CDX1 gene structure and identified that its gene promoter contained a typical CpG island with a CpG observed/expected ratio of 0.80, suggesting that the CDX1 gene is a target of aberrant methylation. Alterations of DNA methylation in the CDX1 gene promoter were investigated in a series of colorectal cancer cell lines. Combined Bisulfite Restriction Analysis (COBRA) and bisulfite sequencing analysis revealed that the CDX1 promoter is methylated in CDX1 non-expressing colorectal cancer cell lines but not in human normal colon tissue and T84 cells, which express CDX1. Treatment with 5′-aza-2′-deoxycytidine (5-azaC), a DNA methyltransferase inhibitor, induced CDX1 expression in the colorectal cancer cell lines. Furthermore, de novo methylation was determined by establishing stably transfected clones of the CDX1 promoter in SW480 cells and demethylation by 5-azaC-activated reporter gene expression. These results indicate that aberrant methylation of the CpG island in the CDX1 promoter is one of the mechanisms that mediate CDX1 down-regulation in colorectal cancer cell lines.

DNA methylation occurs in almost all higher eukaryotic organisms and plays a central role in the control of many genetic functions. In the genome of higher eukaryotes, cytosines may be methylated in CpG sequences at locations throughout the genome (1–3). In contrast, small regions of DNA called CpG islands are often unmethylated in normal tissues and are located in the promoter sequences in half of human genes. Recently, many studies have shown that hypermethylation in CpG islands is found in some tumors, which is associated with the inactivation of tumor suppressor genes, including RB (4), p16INK4a (5), von Hippel Lindau (VHL) (6), BRCA1 (7, 8), hMLH1 (9–12), E-cadherin (13), and APC (14).

CDX1, an intestine-specific transcription factor, is a candidate tumor suppressor gene. It directs intestine-specific gene transcription and regulates the intestinal epithelial cell phenotype (15–17). Previously, we demonstrated that murine Cdx1 overexpression in rat normal intestinal epithelial cells (IEC-6) regulates proliferation as a result of inducing cell cycle arrest. This antiproliferative effect may be mediated through down-regulation of the b-type cyclins (18). The Cdx1 gene is expressed in a complex pattern during intestinal development. In mouse embryogenesis, Cdx1 protein is first observed at 12.5 postcoitum in the distal developing intestine. Expression of Cdx1 increases from 13.5 to 14.5 postcoitum during the time of transition from a stratified endoderm to the columnar epithelium of the intestine (19). Cdx1 expression continues in the intestinal epithelium throughout life, predominantly in the crypt. The same pattern of Cdx1 expression was found in the human small intestine and colon (20). Our studies and those of others have demonstrated that CDX1 expression is markedly down-regulated in both adenomas and carcinomas of the colon (20, 21). Little is known about the molecular mechanisms that regulate the developmental and spatial patterns of the CDX1 expression in normal intestine or what induces the down-regulation in colonic adenomas and cancers.

In this study, we hypothesized that CDX1 expression in colorectal cancer cell lines may be in some cases silenced by methylation of the CpG island in the gene promoter. We characterized the CDX1 gene structure and identified the CDX1 gene promoter containing CpG islands. A series of colorectal cancer cell lines was tested for aberrant methylation on the CDX1 gene promoter and associated inactivation of CDX1 expression as a possible mechanism of the down-regulation of CDX1 gene expression in colon cancer. Our results indicate that CDX1 expression is inactivated in association with aberrant methylation in colorectal cancer cell lines.

EXPERIMENTAL PROCEDURES
Plasmids—KSBN1 was constructed by subcloning a BamHI fragment containing −1066 + 802 nucleotides relative to the transcriptional start site of the CDX1 gene from 201E12 (22) into Bluescript II KS (Stratagene, La Jolla, CA). A series of luciferase reporter genes containing deletions of the CDX1 promoter was constructed by subcloning a BamHI-SphI, XbaI-SphI, or DraIII-NheI fragment from KSBN1 into pGLBasic (Promega Corp., Madison, WI) to generate −1066CDX1Luc, −600CDX1Luc, or −394CDX1Luc, respectively.

The abbreviations used are: Luc, luciferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 5-azaC, 5-Aza-2′-deoxycytidine; TSA, trichostatin A; RT, reverse transcription; MIN, microsatellite instability.
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Fig. 1. Structure of the CDX1 gene. In A, the cosmid clone 201E12 was used to determine the complete gene structure by restriction enzyme mapping. MCS, multiple cloning site; N, NotI; B, BamHI; E, EcoRI; X, XbaI; H, HindIII. B, primer extension analysis of the human CDX1 gene. An oligonucleotide primer complementary to bases +89 to +127 (PECDX1) was used to prime the reverse transcriptase reaction using RNA isolated from T84 cells. RNA isolated from HCT116 cells was used as a negative control. Lanes A, C, G, and T represent sequencing reactions. Arrowheads depict transcription start sites. In C, the immediate 5’-flanking regions of the mouse and human CDX1 gene are aligned. Shaded areas represent regions of complete homology between the human and mouse genes that extend for at least 5 bases or more. The transcription and translation start sites are indicated. A solid bar indicates the TATA consensus sequence.

Cell Culture—SW480, HCT116, HeLa, DLD1, Colo205, Caco2, and T84 cell lines were obtained from the Johns Hopkins Hospital. SW480 cells were maintained in Kennett’s medium supplemented with 1× l-glutamine and 5% fetal bovine serum. HCT116 cells were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum and T84 cells in 50% Dulbecco’s modified Eagle’s medium and 50% F12 medium supplemented with 5% fetal bovine serum. Colo205, Caco2, and HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, and DLD1 cells were grown in RPMI with 10% inactivated fetal bovine serum.

Western Blot Analysis—Cells (~10⁷ cells) in 100-mm dishes were washed with PBS, lysed with 1 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.05% sodium deoxycholate) was homogenized using a type B pestle. Human tissue was obtained in accordance with institutional regulations. 10 mg of human normal colon tissue was homogenized using a prechilled Dounce homogenizer. Whole cell protein fractions were obtained by centrifuging was homogenized in 1 ml of lysis buffer using a prechilled Dounce homogenizer. Whole cell protein fractions were obtained by centrifuging for 10 min at 4°C and stored at -80°C. 20 μg of whole cell protein was analyzed by 12% SDS-PAGE for 10 min at 4°C and stored at -80°C. 20 μg of whole cell protein was analyzed by 12% SDS-PAGE for 10 min at 4°C and stored at -80°C. 20 μg of whole cell protein was analyzed by 12% SDS-PAGE for 10 min at 4°C and stored at -80°C. 20 μg of whole cell protein was analyzed by 12% SDS-PAGE for 10 min at 4°C.

Drug Treatment with 5-Aza-2’-deoxycytidine (5-azaC) or Trichostatin A (TSA)—Cells were grown in medium containing final concentrations of 1, 3, or 5 μM 5-azaC (Sigma) for 72 h. RNA was isolated from cells that were treated with 3 and 5 μM 5-azaC. If needed, TSA (final concentration, 300 nM) was added to the appropriate dishes, and the incubation was continued for another 24 h.

RT-PCR—RNA from cultured cells was prepared as described previously. Labeled PECDX1 (50,000 cpm) was annealed to 3 μg of poly(A)+ RNA isolated from T8116 and T84 cell lines at 50°C for 3 h. The annealed primer was extended with Superscript II reverse transcriptase (Invitrogen) for 1 h at 37°C followed by hydrolyzing mRNA in 0.1 M NaOH. cDNA was precipitated in ethanol and separated in a 6% denaturing polyacrylamide gel. The sequencing reaction with PECDX1 on a genomic subclone (KSB11) was loaded on the side for size control.

Transfection and Luciferase Assay—Plasmid DNA was transfected using Lipofectin (Invitrogen). For stable transfection, pGLBasic, pGLBasic−600CDX1Luc, or RVCDX1Luc was cotransfected with pLXSP containing a puromycin-resistant gene into SW480 cells. Cells were split and selected in medium containing 5 μg/ml puromycin. For transient transfections, cells were transfected with deletional constructs using Lipofectin (Invitrogen). Transfected cells were washed with phosphate-buffered saline solution and lysed in cell lysis buffer (Promega Corp.), and the supernatant was analyzed for luciferase activity using a microtiter plate Luminometer (Dynex Technologies Inc., Franklin, MA). Transfection efficiency was normalized with β-galactosidase activity.

Primer Extension Analysis—Primer extension was performed as described previously. In brief, oligonucleotide primer (PECDX1: 5’-TCTTGTGTCCAGCATAGC-3’) was 5’ end-labeled with [γ-32P]ATP; 35796
Bisulfite PCR Methylation Analysis—Sodium bisulfite modification of genomic DNA and PCR were performed as described previously (24, 25). Briefly, 2 µg of genomic DNA was denatured with 2 M NaOH for 10 min and then treated with 3 µl sodium bisulfite (Sigma) (pH 5.0) and 10 mM hydroquinone for 20 h at 50 °C. After treatment, DNA was purified using a Wizard DNA Clean-up kit (Sigma) and desulfonated with 0.3 M NaOH. Bisulfite-treated DNA was precipitated with ethanol and resuspended in 20 µl of sterile distilled water. PCR amplification was performed with ~200 ng of treated DNA as the template. The sequence of interest in the bisulfite-treated DNA was amplified with bisulfite-specific primers. PCR was performed as described previously (26). Twenty percent of the PCR products was digested with the appropriate restriction enzymes, precipitated with ethanol, and separated by 6% PAGE. Gels were stained with ethidium bromide, and the intensity was calculated by densitometry using ImageQuant (Molecular Dynamics, Sunnyvale, CA). Bisulfite-specific primers are: bisulfite1F, 5′-TTGTTTTTTA-TTTTAAGTTGTTATTCG-3′; bisulfite1B, 5′-AAAAATAAACCA-AAAACCTAAAAA-3′; bisulfite2F, 5′-TTATTTTTTTAGGTTTTGGTTA- AAACCTAAAAA-3′; bisulfite2B, 5′-CCACCCCAACCTTTTAACACTC-3′.

Bisulfite Sequencing—Amplified bisulfite PCR products were subcloned into the TA vector system (Invitrogen) according to the manufacturer’s instructions. After transformation, DNA was prepared by Qiaprep spin kit (Qiagen, Valencia, CA). DNA sequence analysis was carried out by automated DNA sequencers (Applied Biosystems, Foster City, CA) at the Sequencing Facility of the Department of Genetics at the University of Pennsylvania.

RESULTS

Characterization of CDX1 Gene Structure—The complete genomic structure of CDX1 was characterized by restriction enzyme mapping using a cosmid clone containing human CDX1 genomic DNA (201E12; a generous gift from Dr. Cynthia Bonner (22)). As shown in Fig. 1, all 3 exons of CDX1 are located in the 201E12 that contained 15 kb of 5′-flanking sequences and 8 kb of 3′-flanking sequences relative to exon 1 and exon 3, respectively. The total sequence of the 201E12 is located in the BAC5m9 from chromosome 5 (LBNI H220; Human Genome Center, Lawrence Berkeley National Laboratory). Primer extension analysis was performed to determine the 5′ end of the CDX1 transcript (Fig. 1B). Two transcriptional initiation sites were detected, and the major site was located 97 nucleotides upstream of the translational start site, which is similar to the start site described previously in the mouse Cdx1 gene (27). A TATA consensus sequence (TATAAA) is located 27 nucleotides 5′ to the transcriptional start site. There is 88% homology between the mouse and human CDX1 promoter regions within 200 nucleotides of the transcriptional start site (Fig. 1C).

Functional Analysis of CDX1 Promoter Elements in Colorectal Cancer Cell Lines In Which CDX1 Expression Is Down-regulated—We examined CDX1 protein expression in a series of colorectal cancer cell lines. Protein expression was analyzed by Western blot. As shown in Fig. 2A, T84 cells expressed high levels of CDX1 protein; however, Caco2, SW480, HCT116, ColoDM, and DLD1 cells contained no expression. Therefore, the majority of colorectal cancer cell lines tested do not express CDX1 protein. HeLa cells, a cervical cancer cell line, was used as a negative non-intestinal control since we assumed that they lacked an intestine-specific transcription machinery that was required for CDX1 gene expression. The level of CDX1 mRNA in each cell line was similar to the protein pattern (data not shown).

Deletion constructs of the CDX1 gene promoter linked to the luciferase reporter were transiently transfected into the colorectal cancer cell lines. As shown in Fig. 2B, T84, Caco2, SW480, and HCT116 cells had a markedly increased transcriptional activity, and ColoDM and DLD1 cells revealed moderate activity of the CDX1 promoter constructs when compared with an inverted promoter construct (RV-3000CDX1Luc). Most of the luciferase activity resided in the construct that contained nucleotides −394 to +75. The result suggests that the CDX1 promoter contained elements that were able to direct transcription in intestinal cell lines. However, there were similar levels of transcriptional activation in all intestinal cell lines although only T84 cells expressed the CDX1 gene. In contrast, HeLa cells that were used for a negative control showed little induction on transcriptional activity of the CDX1 promoter when compared with a RV-3000CDX1Luc.

Hypermethylation of the CDX1 Promoter Correlates with Absence of CDX1 Expression in Colorectal Cancer Cell Lines—Based upon the transfection data, we postulated that CDX1 gene expression could be repressed in non-expressing cells through promoter methylation without affecting the transiently transfected constructs. The CDX1 gene contains a typical CpG island (28) with a CpG observed/expected ratio of 0.80 and a GC content of 73% spanning a region −400 nucleotides to the transcriptional start site. Therefore, we examined the methylation status of specific sites between nucleotides −415 and −16 within this CpG island in normal colon and colorectal cancer cell lines by bisulfite PCR (Fig. 3A). The genomic DNA samples isolated from cell lines as well as normal colon tissue were treated with sodium bisulfite and amplified by PCR. The amplified products were digested with restriction enzymes specific for the methylated alleles. As shown in Fig. 3B, there were very low methylation levels at a few sites in normal colon as well as in T84 and Colo205 cells. By contrast, this same region was highly methylated (70–100%) in HCT116, Caco2, RKO, and ColoDM cells and moderately methylated (30–69%) in SW480 and DLD1 cells. We next employed sodium bisulfite sequencing to examine the methylation status of 33 CpG sites within the CpG island of the CDX1 gene promoter for further
detail using a normal colon sample and representative colorectal cancer cell lines (T84, HCT116, and SW480). In normal colon and T84, which were unmethylated by bisulfite PCR, a slight level of methylation was detected in 26 and 15 of 330 CpG sites in 10 alleles sequenced, respectively. In contrast, HCT116, with 96% of methylation by bisulfite PCR, also showed a high degree of methylation at all the CpG sites examined (327 of 330 sites in 10 alleles). The SW480 cell line showed moderate methylation with 211 of 330 sites in 10 alleles, demonstrating 64% methylation.

5-azaC Derepresses CDX1 in Colorectal Cancer Cell Lines—5-azaC is a DNA methyltransferase inhibitor that can be used as a demethylating agent in replicating cells (29, 30). To further explore the correlation between methylation of the CDX1 gene and silencing of CDX1 gene expression, six colorectal cancer cell lines were grown in the presence of 5-azaC to achieve demethylation of genomic DNA. RT-PCR analysis showed that CDX1 expression was induced in HCT116, Caco2, DLD1, ColoDM, SW480, and RKO cells, whereas untreated samples with high levels of promoter methylation showed no detectable mRNA level. T84 and Colo205 cells expressed CDX1 gene without treatment (Fig. 4). These results suggest that methylation plays a role in silencing CDX1 gene expression in the colorectal cancer cell lines tested.

Demethylation Activates the CDX1 Gene Promoter in SW480 Stable Clones—To obtain further functional evidence of the importance of promoter methylation, we tested de novo methylation by using established stably transfected clones with the CDX1 gene promoter. The construct containing −600 to +75 of the CDX1 gene linked to the luciferase reporter (−600CDX1Luc) was stably transfected into SW480 cells. Constructs without the promoter (vector) or an inverted −600 to +75 of the CDX1 linked to the luciferase reporter (RVCDX1Luc) were also transfected as controls. Individual clones were selected, grown in the presence or absence of 5-azaC, and analyzed for luciferase expression. As shown in Table I, luciferase activity of the clones containing −600CDX1Luc had similar levels as the clones containing RVCDX1Luc. This result differed from transient transfections, which demonstrated 20-fold higher activity of −600CDX1Luc when compared with the RVCDX1Luc (data not shown). Furthermore, the clones containing −600CDX1Luc gave an 11-fold induction in promoter activity due to demethylation after treatment with 5-azaC, whereas clones containing RVCDX1Luc showed a background level of induction when compared with clones transfected with pGL2 basic vector (Fig. 5). The result provided functional evidences on inactivation of the CDX1 gene promoter by de novo methylation, which could be reversed by demethylation with 5-azaC.

DISCUSSION

There is accumulating evidence that DNA methylation plays a role in cancer pathophysiology and that promoter hypermethylation is often an early event in tumor progression. Earlier
studies suggested that hypomethylation was involved in carcinogenesis through the activation of oncogenes, such as ras (31). More recently, however, it is increasingly apparent that aberrant hypermethylation of CpG islands in the promoters of certain tumor suppressor genes leads to loss of gene function in certain tumors. In colorectal cancers, APC, p16INK4A, THBS1, HIC1, N33, COX2, CACNA1G, MYOD, and VERSICAN are hypermethylated in CpG islands in their promoters (32).

Several lines of evidence indicate that CDX1 inhibits cell proliferation, and its expression is reduced in the majority of colon cancers. In this study, we show that the CpG islands of the CDX1 gene promoter are a target of aberrant methylation, resulting in silencing the expression in CDX1 in the human colorectal cancer cell lines tested. These results suggest that methylation plays a significant role in the modulation of CDX1 transcription in colon-derived cancer cell lines and possibly in colon cancer.

There are several possibilities to explain methylation-mediated gene repression in colorectal cancer cell lines. First, methylation can directly interfere with the DNA binding of specific transcription factors. Sequence analysis of the 5' flanking region of the CDX1 gene shows that there are a number of potential cis-regulatory elements including SP1, AP1, CDX, and TCF4 binding sites. However, our study shows that the frequency of methylation within the 5' flanking region of the CDX1 gene is not sequence-specific but region- and cell type-specific. Second, methylation may result from the accessibility of promoter accessible to methyltransferase or methyl-CpG-binding proteins (MBDs) as a secondary event due to the lack of transcription factors. However, this is unlikely to be true in the repression of the CDX1 expression since our data from transient transfection demonstrated that all necessary transcription factors were present to activate the minimal promoter of the CDX1 gene in the colorectal cancer cell lines tested. Third, methylation may play a role in transcriptional repression

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**FIG. 4.** Expression analysis of CDX1 by RT-PCR. Re-expression of CDX1 by 5-azaC treatment was assessed by RT-PCR. HCT116, Caco2, DLD1, RKO, SW480, and ColoDM cell lines were treated with 3 μM 5-azaC for 3 days. RNA was isolated and reverse-transcribed. PCR was performed using primers located in exon 3 of CDX1. Human 36B4 or GAPDH was used as a control. Corresponding negative controls are shown as an RT negative.

**FIG. 5.** Induction of CDX1 promoter activity in SW480 stable clones. Each bar represents average fold induction of CDX1 promoter activity from Six individual clones after treatment with 5-azaC. Values are expressed as mean and standard deviation (n = 6).

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**TABLE I**

Re-activation of CDX1 promoter in SW480 stable clones after treatment with 5-azaC

| Clone*  | Light unit  | Induction  | Clone  | Light unit  | Induction  | Clone  | Light unit  | Induction  |
|---------|-------------|------------|---------|-------------|------------|---------|-------------|------------|
| G4 (−)  | 3.692       |            | R1 (−)  | 330.77      |            | C5 (−)  | 118.28      |            |
| G4 (+)  | 27.306      | 7.39       | R1 (+)  | 1440.54     | 4.36      | C5 (+)  | 1513.122    | 12.79      |
| G5 (−)  | 28.851      |            | R4 (−)  | 202.46      |           | C6 (−)  | 149.289     |            |
| G5 (+)  | 91.533      | 3.17       | R4 (+)  | 1065.94     | 5.3       | C6 (+)  | 1476.009    | 9.88       |
| G6 (−)  | 4.485       |            | R5 (−)  | 158.53      |           | C11 (−) | 926.306     |            |
| G6 (+)  | 28.218      | 6.29       | R5 (+)  | 595.34      | 3.76      | C11 (+) | 13,108.194  | 14.15      |
| G10 (−) | 21.87       |            | R6 (−)  | 330.61      |           | C15 (−) | 185.995     |            |
| G10 (+) | 23.45       | 1.17       | R6 (+)  | 1372.41     | 4.15      | C15 (+) | 1762.189    | 9.13       |
| G11 (−) | 12.07       |            | R8 (−)  | 690.31      |           | C18 (−) | 1542.36     |            |
| G11 (+) | 15.25       | 1.26       | R8 (+)  | 1844.33     | 2.67      | C18 (+) | 23,861.48   | 15         |
| G12 (−) | 62.96       | 0.64       | R9 (−)  | 288.06      | 5.14      | C23 (−) | 635.387     |            |
| G12 (+) | 40.67       |            | R9 (+)  | 1482.91     |           | C23 (+) | 6312.598    | 9.93       |

* SW480 clones transfected with the 600 nucleotides of the 5'-flanking sequence of the CDX1 promoter linked to the luciferase reporter genes (clone series C), the inverted promoter construct (R), and reporter construct without promoter (G). Individual stable clones were selected and grown in the medium with (+) or without (−) 5-azaC for 3 days.

* Light units were normalized with total protein amount (μg) per assay plate (n = 6).

* Fold induction = light unit with 5-azaC/light unit without 5-azaC.
through alteration of the chromatin structure, which influences gene accessibility to the necessary transcription machinery. Recently, the methyl-CpG-binding proteins (i.e. MBDs and MeCP2) have been shown to participate in protein complexes that recruit active corepressor complexes containing histone deacetylases (33, 34). We tested this possibility by treating cells with TSA, a histone deacetylase inhibitor. In our preliminary experiment, we treated non-CDX1-expressing colorectal cancer cells with TSA alone, but it failed to activate CDX1 expression in colorectal cancer cell lines (data not shown). Moreover, treatment of TSA following demethylation with 5-azaC did not increase the level of CDX1 expression activated by 5-azaC alone, suggesting that methylation-mediated repression might be sufficient to silence CDX1 gene expression. It has been reported that mammalian DNA methyltransferase (DMNT1) can bind transcriptional co-repressors and directly repress gene transcription (35) in addition to the fact that DNA methyltransferases can bind histone deacetylases (36, 37). Therefore, the transcriptional silencing mechanism through methylation may be complex, and it is likely that more than one mechanism is involved in silencing CDX1 gene expression in colorectal cancer cell lines.

Promoter hypermethylation-mediated loss of gene function appears to be essential for certain genetic events that derive from tumor progression. For example, MLH1 hypermethylation may occur prior to the onset of microsatellite instability (MIN+) (12). The MIN phenotype can be seen in almost all colon cancer samples from hereditary nonpolyposis colorectal cancer and in 15–20% of sporadic colorectal cancers (2). In addition, MIN+ is one of the fundamental pathways of chromosomal instability in colon cancers that hasten the process of tumorigenesis. Previous studies have revealed that the MIN phenotype has a high level of methylation of genes (38). Our study did not find an association of methylation with the MIN phenotype. A group of genes, including p16INK4a, MLH1, and others, appear to be coordinately methylated in colon cancer (25), which suggests that a subset of colon tumors including MIN+ arises through pathways that involve a certain predisposition to epigenetic gene silencing. However, it remains to be determined whether hypermethylation of the CDX1 gene promoter occurs independently or is predisposed by certain factors during colon tumorigenesis. In summary, our data demonstrate that the CDX1 promoter is aberrantly hypermethylated in colorectal cancer cell lines and suggest that it may be an important mechanism in the pathogenesis of colon cancers.

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