

Epigenetic Silencing of *MicroRNA-34b/c* and *B-Cell Translocation Gene 4* Is Associated with CpG Island Methylation in Colorectal Cancer

Minoru Toyota,^{1,2} Hiromu Suzuki,¹ Yasushi Sasaki,² Reo Maruyama,¹ Kohzoh Imai,¹ Yasuhisa Shinomura,¹ and Takashi Tokino²

¹First Department of Internal Medicine and ²Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University, Sapporo, Japan

Abstract

Altered expression of microRNA (miRNA) is strongly implicated in cancer, and recent studies have shown that, in cancer, expression of some miRNAs in cells is silenced in association with CpG island hypermethylation. To identify epigenetically silenced miRNAs in colorectal cancer (CRC), we screened for miRNAs induced in CRC cells by 5-aza-2'-deoxycytidine (DAC) treatment or DNA methyltransferase knockout. We found that miRNA-34b (miR-34b) and miR-34c, two components of the p53 network, are epigenetically silenced in CRC; that this down-regulation of miR-34b/c is associated with hypermethylation of the neighboring CpG island; and that DAC treatment rapidly restores miR-34b/c expression. Methylation of the *miR-34b/c* CpG island was frequently observed in CRC cell lines (nine of nine, 100%) and in primary CRC tumors (101 of 111, 90%), but not in normal colonic mucosa. Transfection of precursor miR-34b or miR-34c into CRC cells induced dramatic changes in the gene expression profile, and there was significant overlap between the genes down-regulated by miR-34b/c and those down-regulated by DAC. We also found that the *miR-34b/c* CpG island is a bidirectional promoter which drives expression of both miR-34b/c and B-cell translocation gene 4 (*BTG4*); that methylation of the CpG island is also associated with transcriptional silencing of *BTG4*; and that ectopic expression of *BTG4* suppresses colony formation by CRC cells. Our results suggest that miR-34b/c and *BTG4* are novel tumor suppressors in CRC and that the *miR-34b/c* CpG island, which bidirectionally regulates *miR-34b/c* and *BTG4*, is a frequent target of epigenetic silencing in CRC. [Cancer Res 2008;68(11):4123–32]

Introduction

MicroRNAs (miRNA) are a group of noncoding small RNAs that negatively regulate the translation and stability of partially complementary target mRNAs (1, 2), and it is now clear that they play pivotal roles in a wide array of biological processes, including cell proliferation and differentiation and apoptosis (1, 2). Consistent with their role in these processes, a number of studies have shown widespread alteration of miRNA expression patterns in

cancer (3–5). Indeed, down-regulation of a subset of miRNAs is a commonly observed feature of cancers, suggesting these molecules may act as tumor suppressors (6). A good example is let-7, which negatively regulates expression of Ras oncogenes (7); its down-regulation in tumors is thought to contribute to activation of the Ras signaling pathway. In chronic lymphocytic leukemia, moreover, expression of miRNA-15 (miR-15) and miR-16, two miRNAs thought to target the antiapoptotic factor BCL2, is frequently diminished (8).

Cancer is fundamentally a genetic and epigenetic disease that requires the accumulation of genomic alterations that inactivate tumor suppressors and activate proto-oncogenes. Although the mechanism underlying miRNA dysregulation in cancer is not yet fully understood, several lines of evidence suggest that an epigenetic mechanism is involved. For example, treatment of a breast cancer cell line with a histone deacetylase (HDAC) inhibitor rapidly induced significant changes in the miRNA expression profile (9). In addition, it was recently shown that pharmacologic unmasking of silenced genes using HDAC and DNA methyltransferase (DNMT) inhibitors restored miR-127 expression in a human bladder cancer cell line (10) and that genetic disruption of *DNMTs* restored expression of miR-124a in a colorectal cancer (CRC) cell line (11). Notably, the DNA encoding both *miR-127* and *miR-124a* is embedded within CpG islands that are hypermethylated in cancer cells. This suggests that, as with other tumor suppressor genes, DNA methylation is a major mechanism by which miRNA expression is silenced in cancer.

To date, we and many others have identified a wide variety of tumor suppressor genes and other tumor-related genes that are inactivated by aberrant DNA methylation in CRC (12–14); however, much remains to be learned about the epigenetic dysregulation of miRNAs in this disease. Our aim in the present study was to identify miRNAs whose expression was epigenetically silenced in CRC. To that end, we used TaqMan reverse transcription-PCR (RT-PCR) to assess the expression of a panel of 157 miRNAs in CRC cell lines and identified 37 miRNAs that were significantly up-regulated by DNMT inhibition in HCT116 cells. Among those, we focused on miR-34b and miR-34c because they were recently reported to be targets of p53 (15–17). We found that the down-regulation of miR-34b/c expression was strongly associated with hypermethylation of its neighboring CpG island, which notably harbors bidirectional promoter activity and also regulates expression of another candidate tumor suppressor gene, B-cell translocation gene 4 (*BTG4*).

Materials and Methods

Cell lines and tissue samples. CRC cell lines were obtained and cultured as described previously (18, 19). HCT116 cells harboring genetic disruptions within the *DNMT1* and *DNMT3B* loci (DKO2; ref. 20) and within

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

M. Toyota and H. Suzuki contributed equally to this work.

Requests for reprints: Hiromu Suzuki, First Department of Internal Medicine, Sapporo Medical University, S1, W16, Chuo-Ku, Sapporo, 060-8543, Japan. Phone: 81-11-611-2111, ext. 3211; Fax: 81-11-618-3313; E-mail: hsuzuki@sapmed.ac.jp.

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the *TP53* locus (21) have been described previously. Cells were treated with 2 $\mu\text{mol/L}$ 5-aza-2'-deoxycytidine (DAC; Sigma) for 72 h, replacing the drug and medium every 24 h. To determine whether miR-34b/c are up-regulated by endogenous p53, wild-type and *p53*^{-/-} HCT116 cells were treated with 0.1 $\mu\text{mol/L}$ DAC for 48 h, replacing the drug and medium 24 h after the beginning of treatment. This was followed by addition of Adriamycin (ADR) to a final concentration of 0.5 $\mu\text{g/mL}$ and incubation for an additional 24 h. Cells were also treated with mock, DAC alone, or ADR alone by using the same amount of drugs and/or same volume of PBS. A total of 111 primary CRC specimens were obtained as described (18, 19). Informed consent was obtained from all patients before collection of the specimens. Samples of adjacent nontumorous colorectal mucosa were also collected from 17 patients. Total RNA was extracted using TRIZOL reagent (Invitrogen) and then treated with a DNA-free kit (Ambion). Small RNA was extracted using a mirVana miRNA isolation kit (Ambion). Genomic DNA was extracted using the standard phenol-chloroform procedure.

Analysis of miRNA expression using TaqMan RT-PCR. A panel of 157 mature miRNAs was analyzed using a TaqMan miRNA Early Access kit (Applied Biosystems). Expression of mature miR-34b/c was analyzed using TaqMan miRNA Assays (Applied Biosystems). Briefly, 5 ng of small RNA or total RNA were reverse transcribed using specific stem-loop RT primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes. The PCR was run in a 7900HT Fast Real-Time PCR System (Applied Biosystems), and SDS2.2.2 software (Applied Biosystems) was used for comparative ΔC_t analysis. U6 snRNA (*RNU6B*; Applied Biosystems) served as an endogenous control.

RT-PCR. Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). The integrity of the cDNA was confirmed by amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR was run in a 50- μL volume containing 100 ng of cDNA, 1 \times Ex Taq Buffer (TaKaRa), 0.3 mmol/L deoxynucleotide triphosphate, 0.25 $\mu\text{mol/L}$ of each primer, and 1 unit of TaKaRa Ex Taq Hot Start Version (TaKaRa). The PCR protocol entailed 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and a 7-min final extension at 72°C. Primer sequences and PCR product sizes are shown in Supplementary Table S1.

Real-time RT-PCR. Real-time RT-PCR was carried out using TaqMan Gene Expression Assays (Applied Biosystems) and a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. SDS2.2.2 software (Applied Biosystems) was used for comparative ΔC_t analysis, and GAPDH served as an endogenous control.

Western blot analysis. Western blot analysis was carried out as described previously (22). Mouse anti-p53 monoclonal antibody (mAb; DO-7; Santa Cruz Biotechnology), mouse anti-p21 mAb (EA10; Oncogene Research), mouse anti-actin mAb (Chemicon), mouse anti-MET mAb (25H2; Cell Signaling Technology), mouse anti-cyclin-dependent kinase 4 (CDK4) mAb (DCS-35; Santa Cruz Biotechnology), and rabbit anti-SC35 polyclonal antibody (Abcam) were all used in accordance with the manufacturers' instructions. The blots were visualized using enhanced chemiluminescence (Amersham Biosciences).

Methylation analysis. Genomic DNA (2 μg) was modified with sodium bisulfite using an EpiTect Bisulfite kit (Qiagen). Methylation-specific PCR (MSP) and bisulfite-sequencing analysis were then performed, as described previously (23, 24). Amplified bisulfite-sequencing PCR products were cloned into pCR2.1-TOPO vector (Invitrogen), and 10 to 12 clones from each sample were sequenced using an ABI3130x automated sequencer (Applied Biosystems). Bisulfite-pyrosequencing was carried out as described previously (25). Primers for pyrosequencing were designed using PSQ Assay Design software (Biotage). After PCR, the biotinylated PCR product was purified, made single-stranded, and used as a template in a pyrosequencing reaction run according to the manufacturer's instructions. Briefly, the PCR products were bound to streptavidin sepharose beads HP (Amersham Biosciences), after which beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 mol/L NaOH solution. After addition of 0.3 $\mu\text{mol/L}$ sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Biotage) and Pyro Q-CpG software (Biotage). Primer sequences and PCR product sizes are shown in Supplementary Table S1.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were carried out as described previously (18, 19). Chromatin was immunoprecipitated for 16 h at 4°C using 10 μL of anti-trimethylated histone H3 lysine 4 (H3K4) mAb (clone MC315; Upstate). In addition, 1/100 of the solution collected before adding the antibody was used as an internal control for the amount of input DNA. Real-time PCR was carried out in a 20- μL volume containing 1/100 of the immunoprecipitated DNA, 10 μL of SYBR Green PCR Master Mix (Applied Biosystems), and 0.5 $\mu\text{mol/L}$ of each primer. The PCR protocol entailed 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescent signals were detected using a 7900HT Real-Time PCR System (Applied Biosystems). Primer sequences and PCR product sizes are shown in Supplementary Table S1.

Promoter reporter assay. Upstream regions of *miR-34b/c* and *BTG4* were amplified by PCR and then cloned into pCR2.1-TOPO (Invitrogen), as described (24). Each PCR primer carried a 5' overhang that contained a *HindIII* recognition site, so that after verifying the sequences, fragments could be cut using *HindIII* and ligated into pGL3-Basic (Promega). In addition, oligonucleotide fragments corresponding to p53-responsive elements were synthesized and inserted upstream of the *miR-34b/c* promoter in the pGL3 vector. Cells (5×10^4 per well in 24-well plates) were transfected with 100 ng of one of the reporter plasmids and 2 ng of pRL-TK (Promega) using Lipofectamine 2000 (Invitrogen). For cotransfection of reporter genes with p53, equal numbers of cells were cotransfected with 100 ng of one of the reporter plasmids, 100 ng of pcDNA-p53 or an empty vector, and 2 ng of pRL-TK (Promega). pGL3-Basic vector without an insert served as a negative control. Luciferase activities were measured 48 h after transfection using a Dual-Luciferase Reporter Assay System (Promega). Primer sequences and PCR product sizes are shown in Supplementary Table S1.

Transfection of precursor miRNA. HCT116 cells (5×10^6) were transfected with 100 pmol of Pre-miR miRNA Precursor Molecules (Ambion) or Pre-miR miRNA Molecules Negative Control 1 (Ambion) using a Cell Line Nucleofector kit V (Amaxa) with a Nucleofector I electroporation device (Amaxa) according to the manufacturer's instructions. For microarray and RT-PCR analysis, total RNA was extracted 48 h after transfection; for Western blot analysis, cell lysate was prepared 72 h after transfection.

Gene expression microarray analysis. HCT116 cells were transfected with control miRNA precursor or miR-34b/c precursor or were treated with mock or DAC, as described above. Total RNA extracted with TRIZOL was cleaned up using an RNeasy Mini Elute Cleanup kit (Qiagen). One color microarray-based gene expression analysis was then carried out according to the manufacturer's instructions (Agilent Technologies). Briefly, 700 ng of total RNA were amplified and labeled using a Low RNA Input Linear Amplification kit (Agilent Technologies), after which the synthesized cRNA was hybridized to the Whole Human Genome Oligo DNA microarray (G4112F; Agilent Technologies). Once hybridized, the array was scanned with an Agilent DNA Microarray Scanner (Agilent Technologies), and the microarray data were processed using Feature Extraction software (Agilent technologies). The data were then further analyzed using Gene Spring GX software (Agilent Technologies). The Gene Expression Omnibus accession numbers of the microarray data are GSM259761, GSM259762, GSM259763, GSM259764, and GSM259765, and the accession number of the series entry is GSE10455.

Expression vectors. A fragment of genomic DNA encoding *miR-34b/c* was amplified by PCR using genomic DNA from HCT116 cells as a template and then cloned into pCR2.1-TOPO (Invitrogen). The forward PCR primer carried a 5' overhang that contained a *BamHI* recognition site and the reverse primer contained a *HindIII* site, so that after verifying the sequences the fragment could be cut using *BamHI* and *HindIII* and ligated into pSilencer 4.1-CMV neo (Ambion). Full-length *BTG4* cDNA was amplified by PCR using cDNA derived from DKO2 cells as a template. RT-PCR was carried out as described above. The amplified PCR product was cloned into pCR2.1-TOPO (Invitrogen), and after the sequence was verified, and the fragment was cut using *EcoRI* and ligated into *EcoRI*-digested pcDNA3.1/HisA (Invitrogen). Primer sequences and PCR product sizes are shown in Supplementary Table S1.

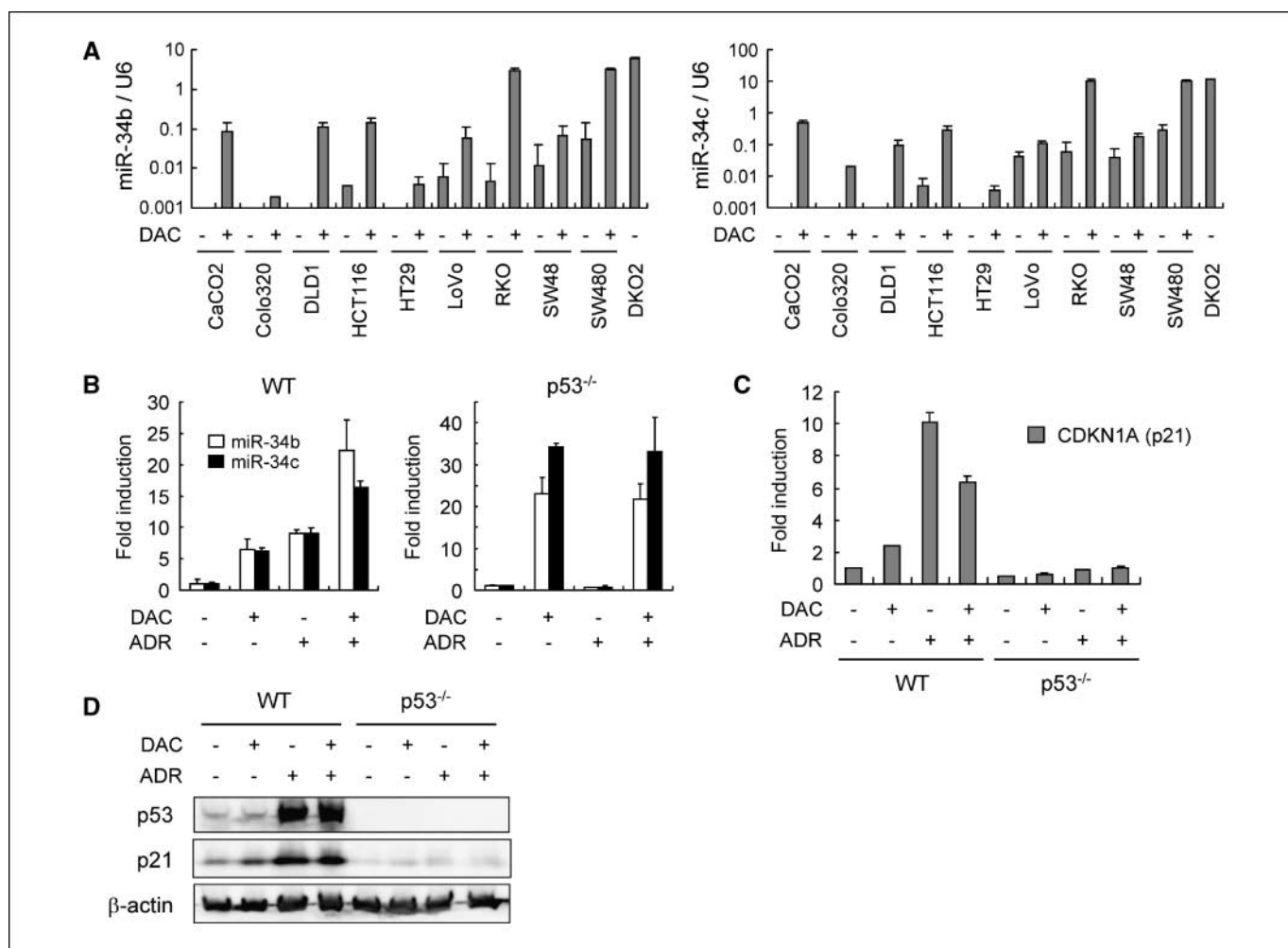


Figure 1. Analysis of miR-34b/c expression in CRC cell lines. **A**, TaqMan RT-PCR results for miR-34b and miR-34c in the indicated CRC cell lines, with (+) and without (-) DAC treatment. Results are normalized to internal U6 snRNA expression. **B**, RT-PCR results for miR-34b/c in wild-type (*WT*) and *p53*^{-/-} HCT116 cells treated with or without a low dose of DAC and/or ADR. Results are shown relative to a value of 1 assigned to mock-treated cells after normalization to internal U6 snRNA expression. *White columns*, miR-34b; *black columns*, miR-34c. **C**, TaqMan RT-PCR results for CDKN1A in wild-type and *p53*^{-/-} HCT116 cells treated as in **B**. Results are shown relative to mock-treated cells after normalization to internal GAPDH expression. **D**, Western blot analysis of p53 and p21 expression in wild-type and *p53*^{-/-} HCT116 cells treated as in **B**. The same blot was reprobed for β -actin as a loading control.

Colony formation assay. Colony formation assays were carried out as described previously (18). Briefly, cells (1×10^5) were transfected with 4 μ g of the expression vector or a control vector using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. A pSilencer 4.1-CMV negative control vector (Ambion) and an empty pcDNA3.1/HisA vector (Invitrogen) served as controls. Cells were then plated on 60-mm culture dishes and selected for 14 d with 0.6 mg/mL G418. Colonies were stained with Giemsa and counted using NIH IMAGE software.

Results

Screening for miRNA up-regulated by DNMT inhibition. To screen for epigenetically silenced miRNAs, we initially examined a panel of 157 miRNAs in three CRC cell lines (HCT116, DLD-1, and RKO) treated with or without DAC. We also analyzed HCT116 cells, in which the DNMT genes *DNMT1* and *DNMT3B* were genetically disrupted (DKO2 cells), thereby abrogating DNA methylation (18, 20). TaqMan RT-PCR analysis revealed that, in HCT116 cells, 37 of the miRNAs were significantly up-regulated (>5-fold) by drug and/or genetic inhibition of DNMTs, although the basal expression of the miRNAs varied (Supplementary Fig. S1). Notably, we were

also able to detect up-regulation of two positive controls, miR-124a and miR-127, which are epigenetically silenced in HCT116 cells (Supplementary Fig. S1; refs. 10, 11). Many of the 37 miRNAs were also up-regulated by DAC in DLD-1 and RKO cells, suggesting epigenetic down-regulation of these miRNAs may be a common feature in CRC (data not shown).

Analysis of miR-34b/c expression in CRC cells. We next focused on miR-34b and miR-34c because recent studies have shown them both to be direct targets of p53. We analyzed a set of CRC cell lines treated with or without DAC and found that miR-34b/c were up-regulated by DAC in all of the cell lines tested (Fig. 1A). To assess the role of endogenous p53 in the expression of miR-34b/c, we next treated HCT116 cells with ADR, an agent known to damage DNA and induce endogenous p53 expression (22). We found that ADR does induce miR-34b/c to some degree (Fig. 1B), although the response was much weaker than that elicited by a high dose of DAC (Fig. 1A). Like ADR, a low dose (0.1 μ mol/L) of DAC also induced miR-34b/c expression only weakly, but we observed synergistic up-regulation upon addition of ADR following the low dose of DAC (Fig. 1B). When we then

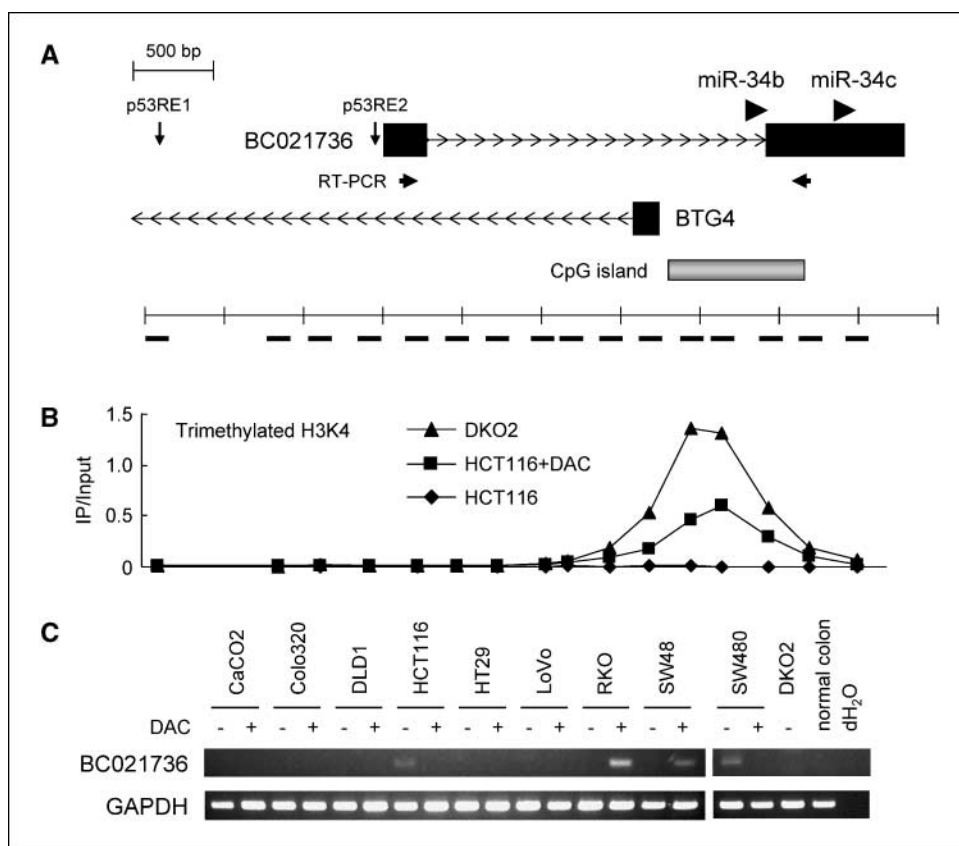


Figure 2. Structure of *miR-34b/c* gene and histone modification. **A**, predicted structure of *miR-34b/c* gene. The locations of the RT-PCR primers for BC021736 (arrows). The two p53 responsive elements, p53RE1 and p53RE2, were reported previously. Exon 1 of *BTG4* is also located in intron 1 of BC021736, but *BTG4* is transcribed in the opposite direction from *miR-34b/c*. The CpG island is indicated by a gray box. Sixteen regions analyzed in ChIP-PCR assays are shown below. **B**, mapping of trimethylated H3K4 upstream of *miR-34b/c*. ChIP assays were performed in HCT116 cells treated with or without DAC and in DKO2 cells. The 16 areas depicted in **A** were examined with real-time PCR, and the results are shown as ratios of immunoprecipitated DNA (IP) to input DNA. **C**, RT-PCR analysis of EST BC021736 in the indicated CRC cell lines, with or without DAC treatment, and in a normal colonic tissue.

treated *p53*^{-/-} HCT116 cells with the same low dose of DAC, we found that it induced somewhat higher levels of *miR-34b/c* expression than we saw in wild-type HCT116 (Fig. 1B). On the other hand, ADR alone did not up-regulate their expression at all, and no synergistic effect was observed upon adding the two together (Fig. 1B). In contrast to *miR-34b/c*, *CDKN1A*, a typical target of p53, was strongly up-regulated by ADR alone in wild-type HCT116 cells (Fig. 1C), but there was little induction of *CDKN1A* by DAC, and no synergistic effect was observed with the combination of DAC and ADR (Fig. 1C). Finally, Western blot analysis of the expression of p53 and p21 protein confirmed that levels of p53 are unaffected by DAC (Fig. 1D). It thus seems that, although *miR-34b/c* are targets of p53 in CRC cells, their expression is suppressed in those cells through an epigenetic mechanism.

Identification of the *miR-34b/c* promoter region. From their gene structure, it can be predicted that *miR-34b* and *miR-34c* are respectively located within intron 1 and exon 2 of the same sequence (EST BC021736) on chromosome 11q23 (Fig. 2A; refs. 15, 16). Earlier studies also identified two putative p53-responsive elements, p53RE1 and p53RE2, upstream of BC021736 (Fig. 2A; refs. 15–17). Our RT-PCR results showed that DAC treatment elicited identical induction of mature *miR-34b/c* (Fig. 1A and B), suggesting these two miRNAs are processed from a single primary transcript. However, RT-PCR revealed that demethylating treatment did not induce BC021736 expression in majority of the CRC cell lines, including HCT116 (Fig. 2C). This discrepancy between the expression of *miR-34b/c* and BC021736 suggests that BC021736 may not be the primary *miR-34b/c* transcript in CRC cells and that a region other than the BC021736 promoter may be responsible for the transcriptional regulation of *miR-34b/c* expression.

Recently, several miRNAs were found to be epigenetically silenced in association with CpG island hypermethylation in cancer cells (10, 11), and we noted that *miR-34b* is embedded within a typical CpG island (Fig. 2A). Given that active promoters are reportedly marked by trimethylation of histone H3K4 (26–28), we carried out ChIP assays and real-time PCR to assess H3K4 trimethylation within an ~4.5-kb region upstream of *miR-34b/c*. In both DAC-treated HCT116 cells and DKO2 cells, we observed significant enrichment of trimethylated H3K4 in the CpG island of *miR-34b/c*, which was completely absent in untreated HCT116 cells (Fig. 2B). This suggests that the CpG island region contains the promoter for *miR-34b/c*; however, examination of the genome database revealed that this region is likely also the promoter for another gene, *BTG4*, which is transcribed in the opposite direction (Fig. 2A). Consistent with that finding, the WWW Promoter Scan³ analysis indicated the presence of promoter activities in both directions in this region (Fig. 3A). To confirm this, we constructed reporter vectors containing the predicted promoter sequences for each direction (Fig. 3A). After transient transfection of two CRC cell lines, we observed high levels of luciferase activity with both pGL3-*miR-34b/c* and pGL3-*BTG4*, although the promoter activity in the *miR-34b/c* direction was almost twice that in the *BTG4* direction (Fig. 3B).

Previous studies showed that p53 directly interacts with a p53RE2 (Fig. 2A; refs. 15, 16). To determine whether the activity of the *miR-34b/c* promoter can be up-regulated by p53, we cloned an

³<http://www.bimas.cit.nih.gov/molbio/proscan/>

oligonucleotide containing the p53RE2 sequence upstream of the pGL3-miR-34b/c reporter (Supplementary Fig. S2). Cotransfection of the reporter with a p53 expression vector markedly up-regulated luciferase expression, confirming that transcription driven from the *miR-34b/c* CpG island can be stimulated by p53 (Supplementary Fig. S2).

Analysis of DNA methylation in the *miR-34b/c* CpG island. To test whether DNA methylation in the *miR-34b/c* CpG island is responsible for silencing, we first carried out MSP analysis and found that the CpG island is significantly methylated in all of the CRC cell lines tested (Fig. 3C). By contrast, DKO2 cells and normal colonic mucosa showed strong signals, indicating the absence of methylation (Fig. 3C). We then verified the MSP results by carrying out bisulfite sequencing in selected samples. This sequencing analysis revealed that the CpG sites in this region were extensively methylated in the three CRC cell lines tested, whereas almost no methylation was seen in DKO2 cells (Fig. 3D).

We also carried out a quantitative analysis of the methylation of seven CpG sites in the core region of the CpG island using primers designed for bisulfite pyrosequencing (Fig. 3A and

Supplementary Fig. S3A). The average methylation levels in HCT116, RKO, and DLD-1 cells were 85%, 90%, and 67%, respectively, but it was only 4% in DKO2 cells (Supplementary Fig. S3B), which is highly consistent with the bisulfite sequencing analysis (Fig. 3D).

Methylation and expression analysis of *miR-34b/c* in primary CRC. We next analyzed the methylation of the *miR-34b/c* CpG island in a panel of tumor specimens from CRC patients. MSP analysis revealed that the *miR-34b/c* was methylated in 101 of 111 (90%) primary CRCs tested. By contrast, little or no methylation was detected in samples of normal colonic mucosa from the patients, indicating that methylation of the *miR-34b/c* region is a tumor-specific phenomenon (Fig. 4A). We confirmed these results by bisulfite sequencing in selected specimens. In samples of normal colonic tissue, a small number of alleles showed some spotty methylation, but the majority of alleles were completely unmethylated (Fig. 4B and Supplementary Fig. 4). On the other hand, the tumor tissue showed a mixture of entirely or partially methylated alleles and unmethylated alleles, probably reflecting contamination of the sample by normal cells

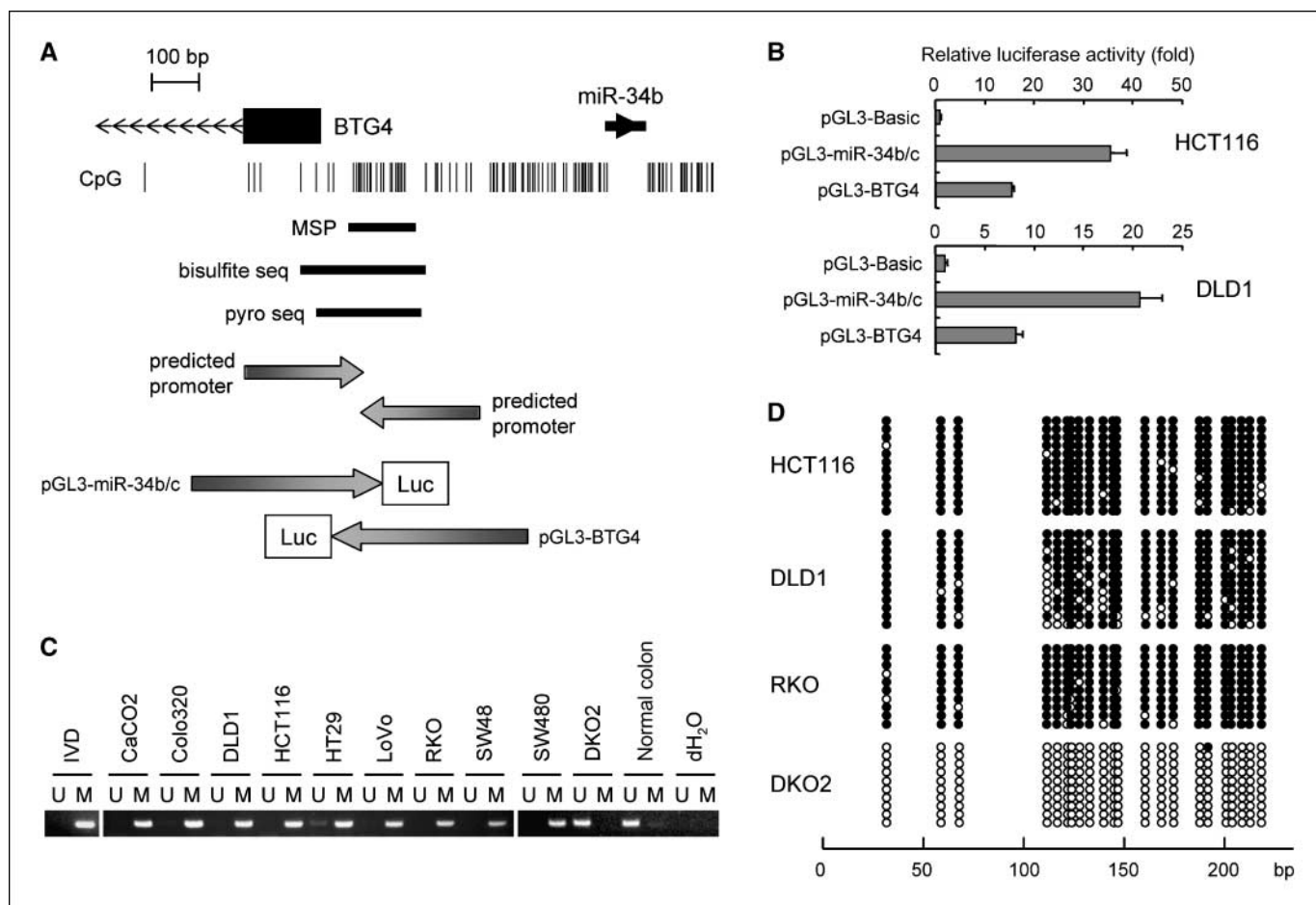


Figure 3. Promoter activity and methylation analysis of the *miR-34b/c* CpG island in CRC cells. **A**, diagram of the 5' CpG island of *miR-34b/c*. Solid box indicates exon 1 of *BTG4*, and an arrow indicates *miR-34b*. The regions analyzed by MSP, bisulfite sequencing, and bisulfite pyrosequencing are indicated by bars below the CpG sites. The predicted promoter regions are indicated by gray arrows. Areas containing the predicted promoter regions were cloned into pGL3-basic. **B**, promoter reporter assay results in indicated CRC cell lines. Normalized luciferase activities are represented. Columns, means of four replications; error bars, SDs. **C**, MSP analysis of the *miR-34b/c* CpG island in a set of CRC cell lines and a sample of normal colonic tissue. *In vitro* methylated DNA (IVD) and DKO2 serve as positive and negative controls, respectively. Bands in the "M" lanes are PCR products obtained with methylation-specific primers; those in the "U" lanes are products obtained with unmethylated-specific primers. **D**, bisulfite sequencing of the *miR-34b/c* CpG island in the indicated CRC cell lines. Open and filled circles represent unmethylated and methylated CpG sites, respectively.

(Fig. 4B). Bisulfite pyrosequencing in 17 normal colonic tissues and 10 CRC tumors in which methylation was not detected by MSP showed only low levels of methylation: the average methylation levels were 11% and 8%, respectively (Fig. 4C). By contrast, CRC tissues with positive MSP results ($n = 101$) showed an average methylation level of 38% (Fig. 4C). Finally, we used TaqMan RT-PCR to assess the expression of miR-34b/c in normal colonic mucosa ($n = 6$) and primary tumor tissues harboring *miR-34b/c* methylation ($n = 14$) and found substantial down-regulation of miR-34b/c expression in the tumor tissues compared with normal colonic mucosa (Fig. 4D).

Changes in gene expression profiles induced by miR-34b/c and DAC treatment. We transfected HCT116 cells with miR-34b/c precursor molecules or a negative control, and carried out microarray analyses to assess their global effects on gene transcription. We found that 1,711 probe sets were down-regulated (>1.5-fold) by ectopic miR-34b expression and 3,044 were down-regulated by ectopic miR-34c expression, respectively. Because of the high homology between miR-34b and miR-34c, we found significant overlap between genes down-regulated by miR-34b and

those down-regulated by miR-34c (Fig. 5A and Supplementary Fig. S5). Gene ontology analysis revealed that "cell cycle" genes ($P = 5.30 \times 10^{-19}$) and "M phase" genes ($P = 1.46 \times 10^{-17}$) were the most enriched among the genes down-regulated by miR-34b/c (Supplementary Fig. S5; Supplementary Table S2). To test whether endogenous miRNAs induced by DAC also down-regulate their target genes, we also compared the gene transcription profiles obtained with mock-treated and DAC-treated HCT116 cells. We found that there was significant overlap between genes down-regulated by ectopic expression of miR-34b/c and those down-regulated by DAC treatment (Fig. 5A and Supplementary Fig. S5; Supplementary Table S3).

For selected genes, the microarray data were verified by real-time RT-PCR (Fig. 5B). Hepatocyte growth factor receptor (MET), CDK4, and cyclin E2 (CCNE2) are all reported to be miR-34 targets (15). In addition, we also verified the expression of three other putative targets, caveolin 1 (CAV1), v-myb myeloblastosis viral oncogene homologue (MYB), and splicing factor arginine/serine-rich 2 (SFRS2; Fig. 5B). These genes were also identified in our microarray analysis and contain predicted target sites in their

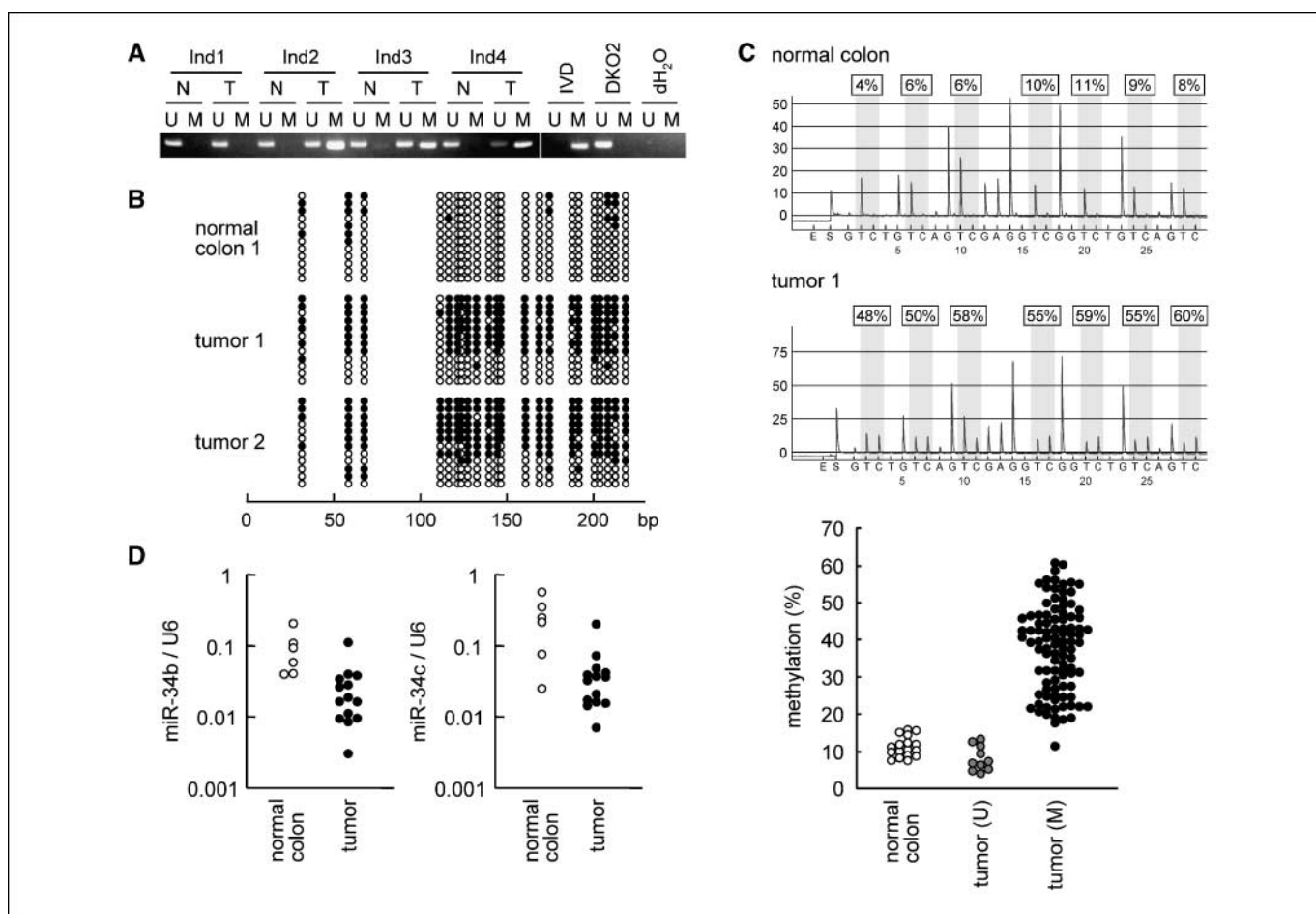


Figure 4. Methylation and expression analysis of the *miR-34b/c* CpG island in primary CRC. **A**, representative results of MSP analysis of *miR-34b/c* in primary CRC (T) and adjacent normal colon from the same patients (N). *In vitro* methylated DNA and DKO2 serve as positive and negative controls, respectively. **B**, bisulfite sequencing of *miR-34b/c* in normal colonic tissue and primary CRC tumors. **C**, representative results of bisulfite pyrosequencing of *miR-34b/c* in normal colon tissue and a primary tumor are shown above. Methylation percentages of seven CpG sites are indicated on the top. Shown below are summarized results of bisulfite pyrosequencing in samples of normal colonic tissue ($n = 17$), primary CRC tumors in which no methylation was detected by MSP (tumor, U; $n = 10$), and primary CRCs in which methylation was detected by MSP (tumor, M; $n = 101$). **D**, summarized results of TaqMan RT-PCR analysis of miR-34b and miR-34c expression in normal colonic tissues ($n = 6$) and primary CRC tumors ($n = 14$). Results are normalized to internal U6 snRNA expression.

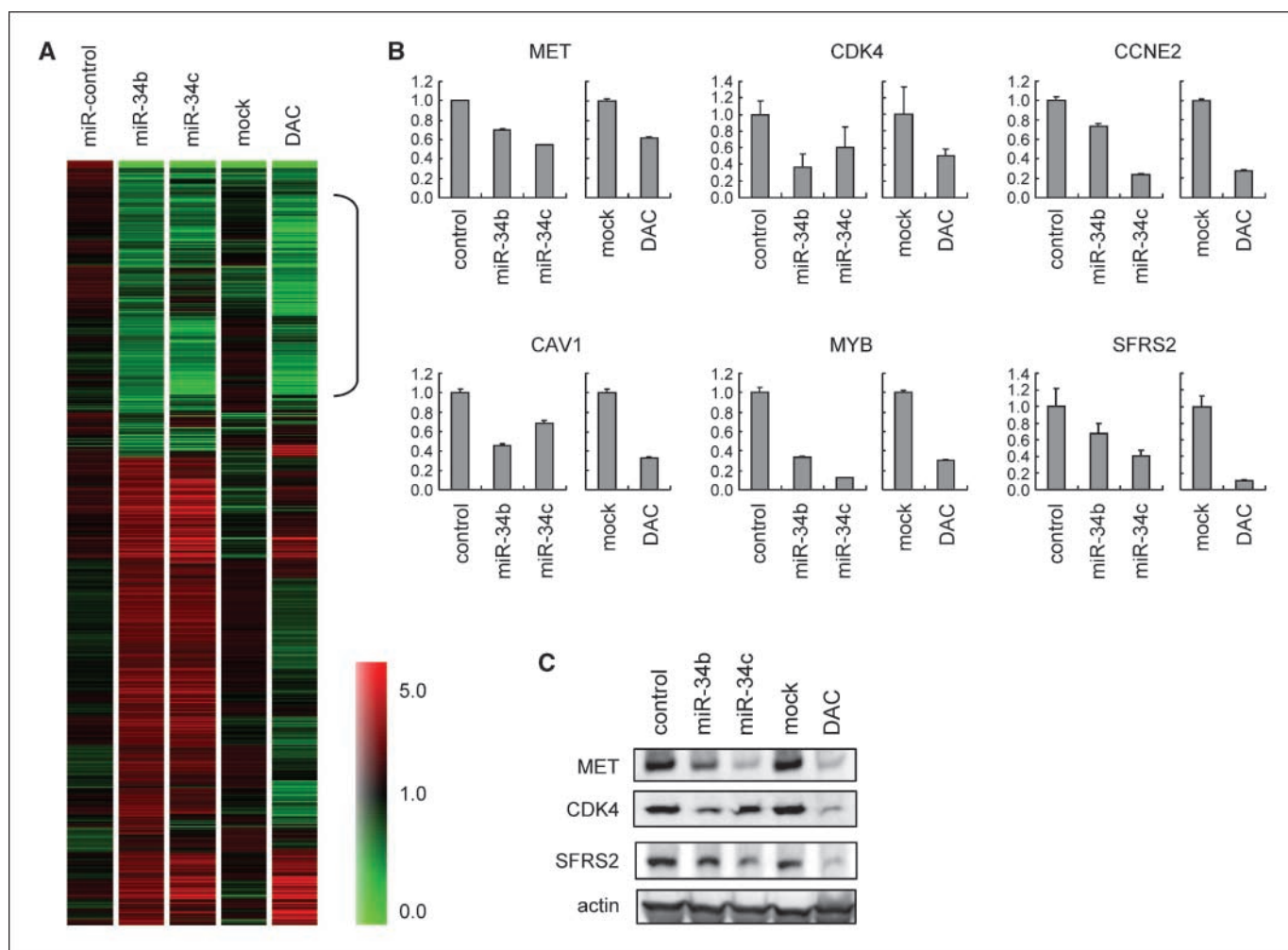


Figure 5. Genes down-regulated by miR-34b/c overlap those down-regulated by DAC. **A**, changes in gene expression after ectopic expression of precursor miR-34b/c or DAC treatment. Genes up-regulated or down-regulated by miR-34b (>1.5-fold) were selected, after which hierarchical clustering was performed by using Gene Spring GX software. Consensus expression signatures that were down-regulated by miR-34b/c and DAC are indicated on the right. **B**, real-time RT-PCR analysis of genes identified by microarray analysis. Results are shown relative to a value of 1 assigned to cells transfected with a negative control or treated with mock after normalization to internal GAPDH expression. *Columns*, means of three replications; *error bars*, SDs. **C**, Western blot analysis of the products of genes identified by microarray analysis. The same blot was reprobed for β -actin as a loading control.

3' untranslated region (UTR; Supplementary Fig. S6). Moreover, Western blot analysis showed that expression of MET, CDK4, and SFRS2 proteins was significantly down-regulated by miR-34b/c or DAC (Fig. 5C).

The results summarized above strongly suggest that miR-34b/c acts as a tumor suppressor in CRC, which prompted us to test whether miR-34b/c can suppress cancer cell growth. We cloned the genomic DNA encoding miR-34b/c and ligated it into the pSilencer 4.1-CMV vector, after which, we verified expression of mature miR-34b/c using TaqMan RT-PCR (Supplementary Fig. S7A). We then carried out colony formation assays after transfecting HCT116 cells with the miR-34b/c expression vector and found that overexpression of miR-34b/c markedly suppressed colony formation (Supplementary Fig. S7B and C).

Analysis of BTG4 in CRC. The bidirectional promoter activity of the *miR-34b/c* CpG island and the frequent hypermethylation of this region in CRC suggest that BTG4 might also be epigenetically silenced in CRC cells. We tested that possibility by analyzing BTG4 expression in a panel of CRC cell lines. As

expected, we found that BTG4 mRNA was completely absent or barely detectable in all of the cell lines tested and DAC treatment rapidly restored its expression (Fig. 6A). We also observed abundant BTG4 expression in DKO2 cells (Fig. 6A). Low levels of BTG4 mRNA were detectable in normal colonic mucosa, suggesting BTG4 is not silenced in normal cells, but its basal expression is relatively limited (Fig. 6A).

Finally, to test whether overexpression of BTG4 would suppress CRC cell growth, we carried out colony formation assays after transfecting DLD-1 and HCT116 cells with a BTG4 expression vector or an empty control vector. After selection with G418 for 2 weeks, we found that introduction of BTG4 markedly suppressed colony formation in all of the CRC cell lines tested (Fig. 6B).

Discussion

In the present study, we found that pharmacologic and/or genetic disruption of DNMT activity in CRC cells up-regulated expression of a substantial number of miRNAs. Among the 37

miRNAs up-regulated by DNMT inhibition, we focused on miR-34b/c because recent studies have shown that miR-34 family members (miR-34a–miR-34c) are direct targets of p53 (15–17, 29–31). Interestingly, among the six groups who have identified miR-34s within the p53 network, Chang and colleagues screened for p53-regulated miRNAs in the HCT116 CRC cell line and found only miR-34a (30). Similarly, Xi and colleagues compared miRNA expression profiles between wild-type and *p53*^{-/-} HCT116 cells, but did not detect miR-34b/c (32). Consistent with those observations, our present findings indicate that miR-34b/c are epigenetically silenced in CRC cell lines and that p53 alone does not induce their expression.

Earlier studies suggest that miR-34b/c are generated by processing a single transcript from a locus on chromosome 11q23 (EST BC021736; refs. 15, 16). However, although significant induction of mature miR-34b/c was observed after demethylation in all of the CRC cell lines tested, a corresponding induction of EST was not observed in a majority of the cell lines, including HCT116 and DKO2. By mapping trimethylation of H3K4 (H3K4me3) in an area ~4.5 kb upstream of *miR-34b/c*, we discovered that demethylation induced significant enrichment of H3K4me3 in a CpG island within which the *miR-34b* coding region is embedded. Levels of H3K4me3 were higher in DKO2 cells than in DAC-treated HCT116 cells, which is consistent with the results of our RT-PCR analysis. Moreover, this CpG island was extensively methylated in all of the CRC cell lines studied, and reporter assays confirmed the

promoter activity in this region. These data support our hypothesis that its CpG island is a region from which miR-34b/c expression is regulated and that hypermethylation of this region is associated with their transcriptional silencing.

Recent studies have shown that miRNAs located within CpG islands can be transcriptionally regulated by DNA methylation and that the patterns of methylation can vary in normal and cancer cells (33). For example, *miR-124a* is hypermethylated in a cancer-specific manner whereas *miR-127* is methylated in both normal tissues and tumors (10, 11), and *let-7a-3* is methylated in normal tissues and hypomethylated in some lung adenocarcinomas (34). In this regard, perhaps the most striking finding of the present study is that the *miR-34b/c* CpG island is methylated in the great majority of primary CRC tumors and that the methylation is tumor-specific. Both nonquantitative MSP analysis and quantitative bisulfite pyrosequencing revealed that 90% of primary CRCs exhibit hypermethylation of the *miR-34b/c* CpG island. The high frequency of their methylation in CRC and their contribution to the p53 network imply that miR-34b/c function as important tumor suppressors in response to colorectal tumorigenesis. Notably, several other p53 target genes are also reported to be epigenetically silenced in cancer. For example, we reported that *14-3-3σ* is methylated in both gastric and liver cancer (35, 36) and that *DFNA5* is also frequently methylated in gastric cancer (37). We therefore suggest that the silencing of p53 target genes impairs tumor suppression by p53, particularly for cancer cells not exhibiting *p53* mutation.

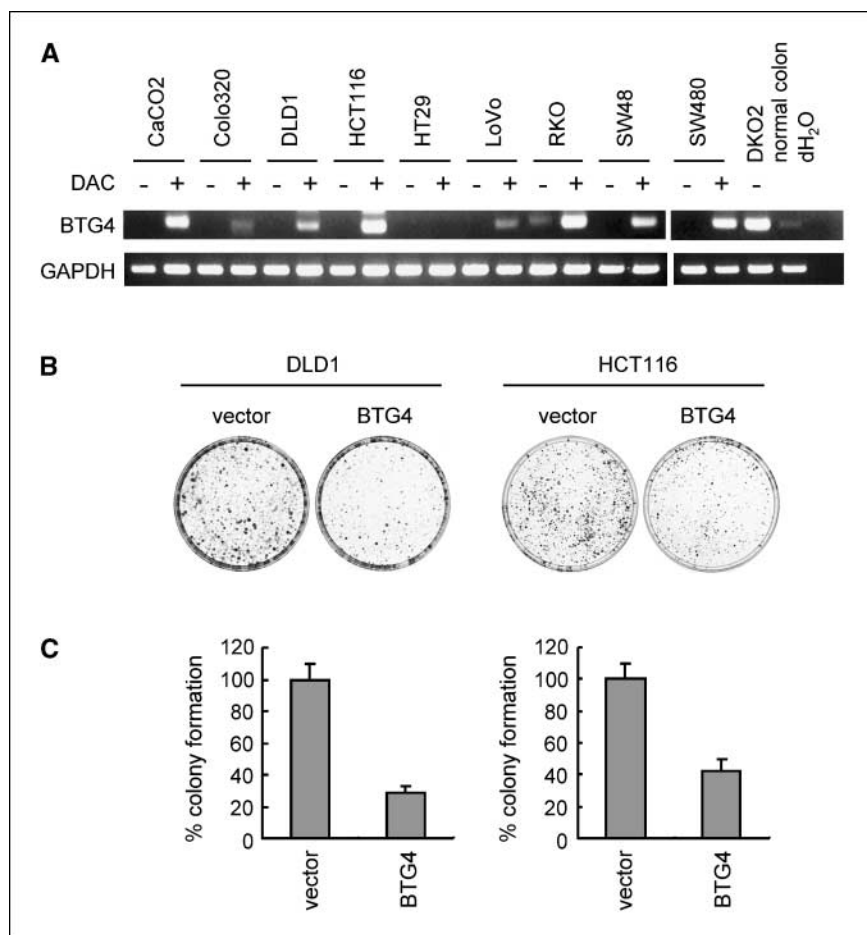


Figure 6. Analysis of BTG4 in CRC cells. **A**, RT-PCR analysis of *BTG4* expression in the indicated CRC cell lines, with or without DAC treatment, and in a normal colonic tissue. **B**, colony formation assays with CRC cell lines transfected with *BTG4* or control plasmid (*vector*). Representative results from a colony formation assay carried out using the indicated CRC cell lines are shown above, and relative colony formation efficiencies are presented below. Shown are means of three replications; error bars represent standard deviations.

The functions of miR-34s have recently come under intensive study. Induction of cell cycle arrest and/or apoptosis by miR-34s in cancer cell lines, including HCT116 cells, has been shown by several groups and is indicative of the tumor suppressor function of miR-34s. In addition, two groups have used microarray analysis to show that ectopic expression of miR-34s induces dramatic changes in the gene expression profiles of cancer cells (15, 30). He and colleagues found significant enrichment of cell cycle genes (based on gene ontology classification) among the genes down-regulated by miR-34a, miR-34b, or miR-34c in four cancer cell lines (15). On the other hand, Chang and colleagues, who also performed a microarray analysis of HCT116 cells with forced miR-34a expression, found that the most highly enriched category among up-regulated genes was cell cycle genes (30). When we carried out a gene expression analysis after transfection of miR-34b or miR-34c into HCT116 cells in the present study, our results were similar to those of He and colleagues, who identified *MET*, *CCNE2*, and *CDK4* as putative miR-34 targets. We also found that there is significant overlap between the genes down-regulated by miR-34b/c and those down-regulated by DAC. Because DAC treatment induces expression of a number of miRNA-coding and protein-coding genes in cancer cells, the results we obtained here cannot be explained solely by reexpression of miR-34b/c. Nevertheless, our results provide compelling evidence that restoration of silenced miRNAs can lead to down-regulation of target oncogenes and that miRNAs could be important targets for epigenetic anticancer therapy.

We also found that the *miR-34b/c* CpG island is a bidirectional promoter, and its methylation is associated with the silencing of both *miR-34b/c* and *BTG4*. Bidirectional gene organization is defined as two genes arranged head-to-head on opposite strands with <1,000 bp between their transcription start sites (38). Housekeeping genes are relatively enriched around such bidirectional promoters, especially DNA repair genes (38). Because many important genes are organized in this way and many bidirectional promoters are associated with CpG islands, hypermethylation of bidirectional promoters is of particular interest with respect to tumor development (39).

BTG4 is a novel member of the PC3/BTG/TOB family of growth inhibitor genes (40). The *BTG* family genes exert antiproliferative effects and have the ability to induce cell cycle arrest and are thus

thought to act as tumor suppressors (41). *BTG2* (*PC3*) was the first identified member of this family and was originally isolated as a gene induced by nerve growth factor during neuronal differentiation of rat PC12 cells (41). Rouault and colleagues showed that p53 regulates the expression of *BTG2* and that its function is likely relevant to cell cycle control and the cellular response to DNA damage (42). In addition, Boiko and colleagues identified *BTG2* as an important downstream effector of p53-dependent arrest of proliferation in mouse and human fibroblasts transduced by oncogenic Ras (43). Recently, *BTG3* was shown to be a target of p53, and it negatively regulates cell proliferation by binding to and inhibiting E2F1 (44). Although much remains unclear about the function of *BTG4*, deletion of 11q is a common abnormality in chronic lymphocytic leukemia, and a recent study suggested that inactivation of *BTG4* may contribute to the disease's pathogenesis (45).

In summary, we have shown frequent epigenetic silencing of novel p53 target miRNAs in CRC. The CpG island of *miR-34b/c* is a bidirectional promoter that regulates expression of distinct tumor suppressor genes and is a frequently methylated region in CRC. The high frequency and tumor-specificity of *miR-34b/c* methylation suggests that it could serve as a useful tumor marker. We also present evidence that restoration of silenced miRNAs leads to down-regulation of target oncogenes in cancer, and thus, miRNAs could be important targets of anticancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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