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Complete Genetic Suppression of Polyp Formation and Reduction of CpG-Island Hypermethylation in *Apc*$^{Min/+}$ *Dnmt1*-Hypomorphic Mice[1]

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Abstract

Promoter CpG island hypermethylation of critical genes is thought to play an important role in human colorectal tumorigenesis. In this study, we show that low levels of CpG island methylation occur in the normal intestinal mucosa of A*pc*$^{Min/+}$ mice and are increased in Multiple Intestinal Metaplasia (Min) polyps. We examined the interaction between CpG island hypermethylation and tumorigenesis by genetically modulating expression levels of the predominant DNA methyltransferase, Dnmt1, in A*pc*$^{Min/+}$ mice. We show that a combination of *Dnmt1* hypomorphic alleles results in the complete suppression of polyp formation and an accompanying reduction in the frequency of CpG island methylation in both the normal intestinal mucosa and intestinal adenomas. These results suggest that sufficient DNA methyltransferase expression is a prerequisite for polyp formation and that hypomorphic alleles of *Dnmt1* are not merely genetic modifiers but the first identified true genetic suppressors of the Min phenotype.

Introduction

Promoter CpG island hypermethylation leading to transcriptional silencing of critical genes has been documented widely in many types of human cancer (1–4). Three functional DNA methyltransferases have been described in mammals, of which Dnmt1 appears to be the predominant enzyme (5, 6). Colorectal cancer is the second leading cause of death by cancer in the United States (7). The *APC* tumor-suppressor gene is implicated in the majority of colorectal tumors (8). Min[3] mice carry a germ-line mutation in the murine *Apc* ortholog and are predisposed to the development of intestinal neoplasia (9, 10). We have shown previously that the combined effects of heterozygosity for a null mutation of the *Dnmt1* gene and treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine could reduce polyp multiplicity in *Apc*$^{Min/+}$ mice (11). In this previous study, we could not rule out that the reduction in polyp number by 5-aza-2′-deoxycytidine was because of drug toxicity unrelated to its demethylating capability (12). Here, we report that hypomorphic alleles of *Dnmt1* lead to the complete suppression of intestinal polyp formation. Furthermore, we use the sensitive MethyLight assay to show that CpG island hypermethylation occurs at low frequency in the histologically normal mucosa of *Apc*$^{Min/+}$ mice and is increased in intestinal polyps. Finally, we show that *Dnmt1* hypomorphic alleles reduce the frequency of CpG island methylation in the normal mucosa and intestinal polyps.

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[3] The abbreviations used are: Min, multiple intestinal metaplasia; PMR, percentage of methylated reference; Lhx1, Lim1 homeobox protein; *Guca2*, guanylate cyclase activator 2; *Timp3*, tissue inhibitor of metalloproteinase 3; *Mgmt*, O^6-methylguanine methyltransferase; *Itga4*, α-4-Integrin; FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil.

Materials and Methods

Dnmt1-Hypomorphic Mice. The *Dnmt1*^{N} allele has been described previously (5, 13). The *Dnmt1*^{R} allele was generated as follows. A 320-bp *Pvu*II-*Eco*RV fragment from pL1–2LacO (14) was inserted into the unique *Eco*RV site in the same parent plasmid used for the *Dnmt1*^{N} allele (5) to yield plasmid pPWL532. The parent plasmid of this cloning step was later named pBS(10.5) and contains a 10.5-kb fragment spanning exons 2–4 (previously thought to be exon1) cloned into a pSP72 (Promega) vector backbone (5). The *Eco*RV site is located in the third intron of the murine *Dnmt1* gene, 20 bp upstream of the splice acceptor site of the fourth exon. Subsequently, a PGK-TKNeo cassette was introduced adjacent to the vector backbone by ligation of a 12.1-kb partial *Xho*I, complete *Spe*I fragment of pPWL532 with a 3-kb partial *Sal*I and complete *Xba*I fragment of pPGKTKNeo (15) resulting in an insertion-type targeting vector called pPWL540. This insertion-type vector was digested at the unique *Hin*dIII site and transfected by electroporation into J1 murine embryonic stem cells (5). Gene-targeted clones were subjected subsequently to counterselection of the *TKNeo* gene by FIAU as described (15). ES cell clones that had undergone excision of the vector backbone and *TKNeo* gene but had retained the LacO insertion were selected for injection into blastocysts. Chimeric mice with verified germ-line transmission capabilities were bred with 129/svJae animals to obtain pure 129/svJae mice carrying the LacO insertion. This allele was designated the *Dnmt1*^{R} allele for the *Eco*RV insertion site in the third intron.

Genotype Analysis. DNA was isolated from tail biopsies as described previously (16). The genotypes of the *Dnmt1* alleles were determined by multiplex-PCR analysis using primers OL106 (5′-GGGAACTTCCTGACTAGGGG-3′), OL168, (5′CCAACAAACCAGTATGTCTCGT-3′), OL173 (5′-CCCAGTTTC-CAGAAAGCTACC-3′), and OL369 (5′-CAATTCCACACAACATACGAGC-3′). Reactions were carried out in a 15-μl volume with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 0.3 μM each primer, and 0.35 units of Taq polymerase using a cycling condition of 94°C for 4 min, followed by 35 cycles of 94°C for 50 s, 60°C for 50 s, and 72°C for 1 min 30 s, followed by 72°C for 5 min. OL168 and OL173 produced both a 342-bp wild-type-specific band and a 661-bp *Dnmt1*^{R} allele-specific band. OL106 and OL173 produced a 430-bp *Dnmt1*^{N} allele-specific band. OL173 and OL369 produced a second 211-bp *Dnmt1*^{R} allele-specific band. The *Apc* genotype was performed by fluorescence-based, allelic discrimination-PCR (TaqMan; Refs. 17 and 18). The primer and probe sequences are listed below. Forward primer (5′-GCCCAGCTCTTCTTCCTC AAG-3′), Reverse primer (5′-GATGGTAAG-CACTGAGGCCAATAC-3′), *Apc*$^{+/+}$-specific probe (6FAM-TCTCTCTCCAA-ACTTCTGTCTTTCT-TAMRA), and *Apc*$^{Min/+}$-specific probe (6VIC-TCTCTC-TCCTAACTTCTGTCTTTCT-TAMRA). Both probes were synthesized as TurboTaq probes.

Intestinal Polyp Scoring and Size Determination. The entire intestine was removed immediately after euthanasia, washed with 70% ethanol, and fixed in RNA*later* (Ambion, Austin, TX). The entire length of the intestine was measured (cm) and subjected to a careful microscopic screen. Adenomas of at least the size of two villi were included in the counting. The investigator was blind to the genotype while counting. Adenomas and mucosal tissue samples selected for DNA analysis were microdissected from the middle third (cm) of the intestine of male mice. Polyp sizes were determined by measuring the maximum diameter of polyps found in the middle third (cm) of the intestine using calipers with an accuracy of 0.05 mm (MECHANIC Type 6901; Fine Science Tools, Inc., Foster City, CA). Male mice only were used for the size determinations to exclude gender-based differences in polyp size.

DNA Methylation Analysis. Genomic DNA isolation and sodium bisulfite conversion were performed as described previously (16, 19). Agarose beads were incubated for 14 h at 50°C to ensure complete bisulfite conversion. Methylation analysis was performed using the MethyLight assay (20–22). Parallel TaqMan PCR reactions were performed with primers specific for the bisulfite-converted methylated sequence for a particular locus and with two reference primers (Lhx1 and *Guca2*). The ratio between the values obtained using these two reference primers (GENE/Lhx1 and GENE/*Guca2*) was averaged. The PMR at a specific locus was calculated by dividing the *GENE/REFERENCE*-averaged ratio of a sample by the *GENE/REFERENCE*-averaged ratio for *Sss*I-treated (SssI enzyme; New England Biolabs, Beverly, MA) control J1 ES cell DNA and multiplying by 100 (22). CpG islands were defined as being methylated in a particular sample if the PMR value >1. The MethyLight primer and probe sequences are listed below. In all cases, the first primer listed is the forward PCR primer, the second is the TaqMan probe, and the third is the reverse PCR primer: *Apc*, GGGCGTAGGTATACGTGATCGA, 6FAM-AATAACACCCCGACAAACTACGCCAATACAA-TAMRA, CCATTTTCGAACCCGACAA; *Itga4*, CGAGGTGTAGATCGAGGTTTCG, 6FAM-ACAACATCACCGCTTCCCGAAAAACG-TAMRA, CCCGCCTCCTACTCACGTAA; *Mgmt*, CGACACCCTTACGTCACACACT, 6FAM-AACCACGCCCCGCGTACCAA-TAMRA, TAGTTCGAGGGTGTAAAGCGG; *Timp3*, GAGAGGCGGTGGGCGTAG, 6FAM-CGATATACGCTACAACGACGTCCCACGA-TAMRA, CGAAAATATAAACTAAACGCGTCCT; Lhx1, AGAGTGTTTGGAAGTTAGGTGAAGGT, 6FAM-CACAATCAACATCCCAAACATATTCACCCA-TAMRA, CACATTCATAAACACAAATTCACACAAC; and *Guca2*, GGTGTTGTGGTTTAGAAGGTTATGG, 6FAM-TCTCATCATCTTCTACAAACCAAAAC-TAMRA, ACCTTATCCTCAACTTCCAACATACC.

Northern and Southern Blot Analysis. Total RNA (20 μg) was separated on a 0.03% formaldehyde-denaturing agarose gel buffered with 10 mM sodium phosphate (pH 6.8), blotted, and hybridized with an α-tubulin probe or a *Dnmt1* cDNA probe. Genomic DNA (5 μg) isolated from mouse intestinal tissue was digested with the restriction enzymes *Hpa*II and *Msp*I in parallel reactions (New England Biolabs). Southern blot analysis was performed as described previously (11). Filters were hybridized with a probe containing centromeric minor satellite repeat sequences derived from plasmid pMR150 (23).

Results and Discussion

In previous work, we have shown that $Apc^{Min/+}$ $Dnmt1^{S/+}$ heterozygous mice treated with 5–azaCdR develop fewer intestinal polyps than untreated, $Dnmt1^{+/+}$ $Apc^{Min/+}$ mice (11). This result was substantiated subsequently and expanded on by others (24). One explanation for this suppression of polyp multiplicity by reduced levels of functional Dnmt1 expression could be that CpG island hypermethylation is an important step in polyp formation and requires sufficient Dnmt1 expression. However, CpG island hypermethylation, though widely studied in human colorectal tumors, has never been documented in mouse intestinal tumors. Therefore, we used the sensitive MethyLight assay (20–22) to investigate whether CpG island methylation occurs at all in the mouse intestine and whether it is increased in Min polyps. The high sensitivity of the MethyLight assay was crucial for our analysis because most of our samples had limiting amounts of DNA.

We first used a limited number of mucosal and polyp samples to prescreen 10 CpG islands associated with genes known to be methylated in human tumors or likely to influence intestinal tumorigenesis, if silenced (data not shown). Of the 10 CpG islands, only 3 showed a sufficiently frequent methylation in the samples analyzed and were consequently selected for further analysis. These were the CpG islands associated with the genes *Itga4*, *Timp3*, and *Mgmt*. The CpG island associated with *Cdkn2a* (*p16*) was hypermethylated in a single polyp sample but was not included for additional study because of its very low frequency of methylation. Subsequently, we screened nine normal mucosal samples and 10 intestinal polyps with each of these three MethyLight reactions.

Fig. 1*A* shows an overview of the frequency of CpG island methylation for each of these genes in the 19 samples analyzed. Fig. 1*B* shows the mean percentage of genes methylated for the mucosal samples *versus* the polyp samples. The percentage of these three CpG islands that were methylated in the normal mucosal tissue was 41%, whereas the polyp tissues showed an average of 77% methylation for these same genes. We found substantial variation among mice, *e.g.*, mouse 3 was unmethylated, and mice 5 and 7 were methylated at all three genes in both mucosa and polyp. This is consistent with the variation in methylation profiles observed in human colorectal tumors (20). It is interesting to note that CpG island methylation was detectable in the normal mucosa. This has also been reported for human colorectal tissue and has been shown to increase with age and predispose to subsequent tumorigenesis for some genes (25–27). These results indicate that CpG island methylation occurs in normal mucosa and suggest that cells with an increased frequency of CpG island methylation may be predisposed to tumorigenesis.

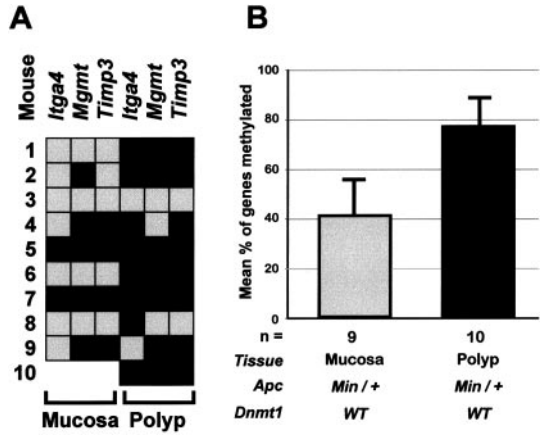

Fig. 1. CpG island methylation in the intestinal mucosa and adenomas. Methylation of CpG islands associated with the *Itga4*, *Mgmt*, and *Timp3* genes was analyzed by MethyLight as described in "Materials and Methods." *A*, schematic overview of all measurements. ■, methylation; ▨, lack of methylation. *B*, summary of the averages obtained for all genes in all mucosal or polyp tissues. *Error bars*, SE.

If a reduction in CpG island methylation is the molecular mechanism by which decreased functional expression of *Dnmt1* suppresses polyp formation in Min mice (11, 24), then one would expect to see a reduced frequency of CpG island methylation in the normal mucosa or polyp tissue of Min mice with reduced Dnmt1 expression. To avoid the use of drug inhibitors, we developed a hypomorphic allele referred to as the $Dnmt1^R$ allele (see "Materials and Methods") and combined this allele with the previously described $Dnmt1^N$ allele (5) to generate mice with varying levels of Dnmt1 expression. Fig. 2*A* shows a schematic representation of the three *Dnmt1* alleles used in this study. Fig. 2*B* shows the relative expression of these alleles in embryonic stem cells. The $Dnmt1^N$ allele does not show detectable levels of gene expression by Northern blot analysis (Fig. 2*B* and Ref. 13) but has been shown to produce low levels of a truncated protein by alternative splicing (13). Quantitation of the normalized RNA expression level of $Dnmt1^{R/+}$ cells *versus* $Dnmt1^{+/+}$ cells indicated that the $Dnmt1^R$ is expressed at ~60% of that of the wild-type allele (Fig. 2*B*). This intermediate level of expression is consistent with the observation that the $Dnmt1^R$ allele resulted in reduced viability in conjunction with the null $Dnmt1^S$ allele but was fully viable in combination with the previously described hypomorphic $Dnmt1^N$ allele (data not shown). We tested whether the combination of these two hypomorphic alleles resulted in detectable hypomethylation *in vivo*. Fig. 2*C* shows that the centromeric minor satellite repeat sequence is significantly hypomethylated in the intestinal mucosa of $Dnmt1^{N/R}$ mice but not in that of any of the other three allelic combinations. Therefore, we conclude

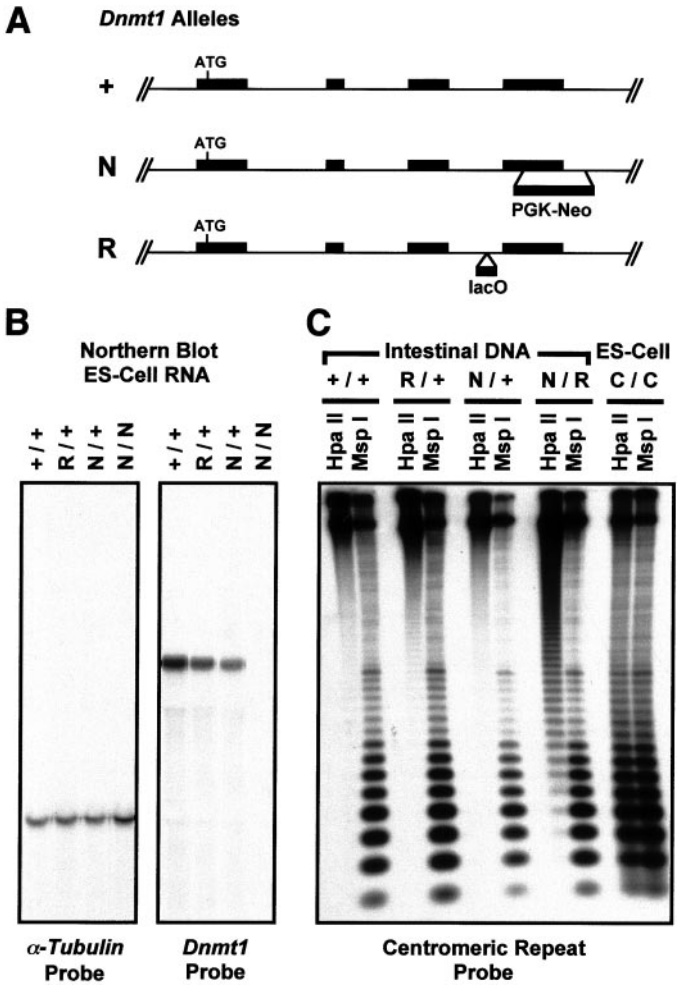

Fig. 2. Structure and characterization of *Dnmt1* hypomorphic alleles. *A*, schematic representation of the structure of the *Dnmt1* hypomorphic alleles, which are described in detail in "Materials and Methods." *B*, Northern blot of total RNA extracted from embryonic stem cells with different hypomorphic alleles, as indicated *above* the *lanes*. *Left panel*, a hybridization with an α-*Tubulin* control probe; *right panel*, a hybridization with a *Dnmt1* cDNA probe. *C*, Southern blot analysis of intestinal DNA derived from 180-day-old mice and from ES cells ($Dnmt1^{C/C}$), hybridized with a centromeric minor satellite repeat probe (23). $Dnmt1^{C/C}$ ES cells are homozygous for a mutation in the catalytic domain of the *Dnmt1* gene and, thus, can be considered nullizygous for *Dnmt1* (13). The *Dnmt1* genotypes and restriction digests are indicated *above* each corresponding *lane*.

that both $Dnmt1^{R}$ and $Dnmt1^{N}$ are hypomorphic alleles with reduced functional expression of *Dnmt1* and together result in significant hypomethylation *in vivo*. Nevertheless, the degree of hypomethylation achieved in these mice is not sufficient to cause an overt phenotype. $Dnmt1^{N/R}$ mice are comparable in size and weight to their wild-type littermates and are fertile.

$Apc^{Min/+}$ *Dnmt1* hypomorphs were generated from crosses between C57BL/6 $Apc^{Min/+}$ $Dnmt1^{N/+}$ males and 129/SvJae $Apc^{+/+}$ $Dnmt1^{R/+}$ females. Mice were euthanized at 180 days and analyzed for polyp multiplicity and polyp size as described in "Materials and Methods." $Apc^{+/+}$ offspring were euthanized on weaning, because we had shown previously that *Dnmt1* mutant mice do not develop intestinal tumors in an $Apc^{+/+}$ background (11). We found a gradual decline in polyp number with diminishing *Dnmt1* expression levels with a drop to 0 in the $Apc^{Min/+}$ $Dnmt1^{N/R}$ mice, which have the lowest levels of *Dnmt1* expression (Fig. 3*A*). The number of polyps was reduced significantly in $Apc^{Min/+}$ $Dnmt1^{N/+}$ mice ($P = 0.001$) and $Apc^{Min/+}$ $Dnmt1^{N/R}$ mice ($P < 0.0001$) compared with wild-type $Apc^{Min/+}$ mice. We did not observe a single polyp in any of the 14 $Apc^{Min/+}$ $Dnmt1^{N/R}$ mice that we analyzed. This suggests that *Dnmt1* hypomorphic alleles act as genetic suppressors of the $Apc^{Min/+}$ phenotype. Other studies have reported genetic modifiers of the Min phenotype that reduce the number of polyps (9, 11, 28–31), but this is the first documentation of complete genetic suppression, to our knowledge.

We also examined the effect of reduced Dnmt1 expression on polyp size. Polyps from $Dnmt1^{R/+}$ ($P = 0.0021$) and $Dnmt1^{N/+}$ ($P < 0.0001$, unpaired *t* test) were significantly smaller than those from $Apc^{Min/+}$ $Dnmt^{+/+}$ siblings at 180 days (Fig. 3*B*), indicating that reduced Dnmt1 expression affects not only polyp formation but also the growth rate of the polyps. This is consistent with the findings of Cormier and Dove (24), who reported a reduced net growth rate and multiplicity of intestinal adenomas in $Dnmt1^{N/+}$ $Apc^{Min/+}$ mice.

If CpG island hypermethylation is an essential feature to polyp formation, and if this is the basis for the suppressive effects of reduced levels of Dnmt1 expression, then we would expect to see a lower CpG island methylation frequency in tissues with reduced DNA methylation levels. The results shown in Fig. 4 show that this is indeed the case. The level (Fig. 4*A*) and frequency (Fig. 4*B*) of CpG island methylation in the intestinal mucosa is substantially diminished in *Dnmt1* hypomorphic mice compared with $Dnmt1^{+/+}$ mice. The most striking reduction is seen in $Dnmt1^{N/R}$ mucosa, where we did not observe a single instance of CpG island methylation >1 PMR threshold ($P = 0.0057$, unpaired *t* test). $Dnmt1^{N/+}$ mice also showed a statistically significant reduction in the frequency of CpG island

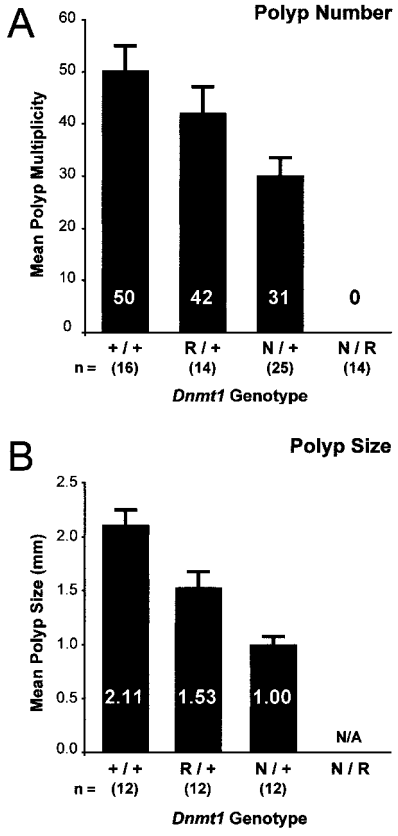

Fig. 3. The mean number and size of intestinal polyps in $Apc^{Min/+}$ mice with different *Dnmt1* genotypes. Mice were euthanized at 180 days of age, and polyps were counted and sized as described in "Materials and Methods." The *Dnmt1* genotype is shown *below* each *bar*. *Error bars*, SE. In *A*, the mean number of polyps for each group is shown as a *bar* with the mean value indicated within the *bar*. Polyps were counted throughout the entire small intestine and colon. *n*, the number of mice in each genotype analyzed. In *B*, the mean polyp size (mm) for each genotype is shown as a *bar* with the mean value indicated within the *bar*. Polyps were measured from the middle third of the intestine of male mice. *n*, the number of polyps analyzed for each genotype.

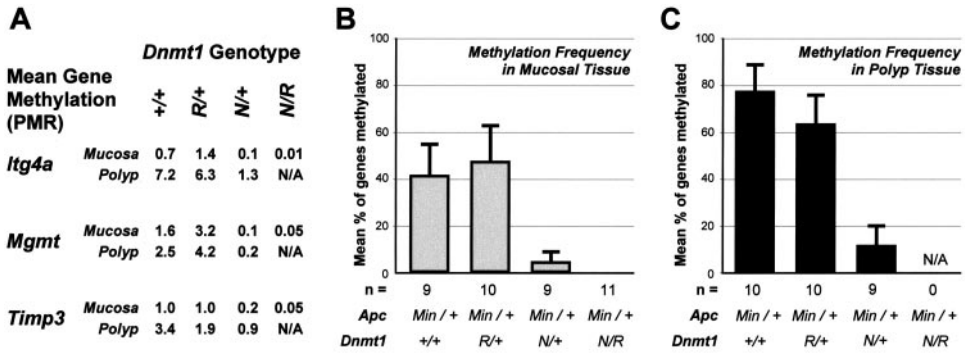

Fig. 4. CpG island methylation in the intestinal mucosa and adenomas of mice with different *Dnmt1* genotypes. Methylation of CpG islands associated with the *Itga4*, *Mgmt*, and *Timp3* genes was analyzed by MethyLight as described in "Materials and Methods." *A*, mean methylation levels of each gene for each type of tissue by genotype. The values are given as PMR (see "Materials and Methods"). *B*, summary of the averages obtained for all genes in all mucosal tissues from each *Dnmt1* genotype as indicated *below* the *bar*. *C*, summary of the averages obtained for all genes in all adenoma tissues from each *Dnmt1* genotype as indicated *below* the *bar*. *n*, the number of mice analyzed for each genotype. *Error bars*, SE.

methylation in the normal mucosa ($P = 0.025$) and polyps ($P = 0.0002$; Fig. 4*C*). We could not measure the CpG island methylation frequency in $Dnmt1^{N/R}$ adenomas, because these mice did not develop any polyps.

These results are consistent with a model in which CpG island methylation occurs in the normal intestinal mucosa in a small minority of cells, which then become predisposed to undergo neoplastic transformation. We do not suggest that the three individual genes that we analyzed are necessarily implicated in tumorigenesis but just that they are representative of CpG island hypermethylation occurring at many CpG islands throughout the genome. Presumably, some of these CpG island methylation events occur in growth-controlling genes and contribute to polyp formation and additional growth. This model is supported by the observation that substantially reduced expression of *Dnmt1* in the intestinal mucosa results in the complete suppression of detectable polyp formation. One caveat to our results is that we used a binocular stereo microscope to scan the intestine for adenomas, rather than relying on serial sections analyzed with an upright microscope. Therefore, microadenomas or aberrant crypt foci in $Dnmt^{N/R}$ mice may have been missed. Nevertheless, if such early stage adenomas did arise in these mice, they did not progress further to a stage at which they would be detectable using a dissecting microscope. We found that the RNA*later* fixative that we used to allow for subsequent nucleic acid analysis rendered the intestines too fragile for subsequent embedding and analysis at high magnification. With these caveats in mind, we tentatively conclude that CpG island hypermethylation, mediated at least in part by Dnmt1, is an essential and rate-limiting step in intestinal adenoma formation in $Apc^{Min/+}$ mice and that *Dnmt1* hypomorphic alleles are the first identified true genetic suppressors of the $Apc^{Min/+}$ phenotype.

References

1. Baylin, S. B., and Herman, J. G. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet., *16:* 168–174, 2000.
2. Herman, J. G., and Baylin, S. B. Promoter-region hypermethylation and gene silencing in human cancer. Curr. Top. Microbiol. Immunol., *249:* 35–54, 2000.
3. Costello, J. F., and Plass, C. Methylation matters. J. Med. Genet., *38:* 285–303, 2001.
4. Jones, P. A., and Laird, P. W. Cancer epigenetics comes of age. Nat. Genet., *21:* 163–167, 1999.
5. Li, E., Bestor, T. H., and Jaenisch, R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell, *69:* 915–926, 1992.
6. Okano, M., Bell, D. W., Haber, D. A., and Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. Cell, *99:* 247–257, 1999.
7. Ries, L. A., Wingo, P. A., Miller, D. S., Howe, H. L., Weir, H. K., Rosenberg, H. M., Vernon, S. W., Cronin, K., and Edwards, B. K. The annual report to the nation on the status of cancer, 1973–1997, with a special section on colorectal cancer. Cancer (Phila.), *88:* 2398–2424, 2000.
8. Fearnhead, N. S., Britton, M. P., and Bodmer, W. F. The ABC of APC. Hum. Mol. Genet., *10:* 721–733, 2001.
9. Moser, A. R., Dove, W. F., Roth, K. A., and Gordon, J. I. The Min (multiple intestinal neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. J. Cell Biol., *116:* 1517–1526, 1992.
10. Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene [Published erratum in Science (Wash. DC), *256:* 1114, 1992]. Science (Wash. DC), *256:* 668–670, 1992.
11. Laird, P. W., Jackson-Grusby, L., Fazeli, A., Dickinson, S. L., Jung, W. E., Li, E., Weinberg, R. A., and Jaenisch, R. Suppression of intestinal neoplasia by DNA hypomethylation. Cell, *81:* 197–205, 1995.
12. Juttermann, R., Li, E., and Jaenisch, R. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc. Natl. Acad. Sci. USA, *91:* 11797–11801, 1994.
13. Lei, H., Oh, S. P., Okano, M., Juttermann, R., Goss, K. A., Jaenisch, R., and Li, E. *De novo* DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development, *122:* 3195–3205, 1996.
14. Labow, M. A., Baim, S. B., Shenk, T., and Levine, A. J. Conversion of the lac repressor into an allosterically regulated transcriptional activator for mammalian cells. Mol. Cell. Biol., *10:* 3343–3356, 1990.
15. Chan, M. F., van Amerongen, R., Nijjar, T., Cuppen, E., Jones, P. A., and Laird, P. W. Reduced rates of gene loss, gene silencing, and gene mutation in dnmt1-deficient embryonic stem cells. Mol. Cell. Biol., *21:* 7587–7600, 2001.
16. Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. Simplified mammalian DNA isolation procedure. Nucleic Acids Res, *19:* 4293, 1991.
17. Gibson, U. E., Heid, C. A., and Williams, P. M. A novel method for real time quantitative RT-PCR. Genome Res., *6:* 995–1001, 1996.
18. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. Real time quantitative PCR. Genome Res., *6:* 986–994, 1996.
19. Olek, A., Oswald, J., and Walter, J. A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic Acids Res., *24:* 5064–5066, 1996.
20. Eads, C. A., Danenberg, K. D., Kawakami, K., Saltz, L. B., Danenberg, P. V., and Laird, P. W. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression (Published erratum appears in Cancer Res., *59:* 5860, 1999). Cancer Res., *59:* 2302–2306, 1999.
21. Eads, C. A., Danenberg, K. D., Kawakami, K., Saltz, L. B., Blake, C., Shibata, D., Danenberg, P. V., and Laird, P. W. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res., *28:* E32, 2000.
22. Eads, C. A., Lord, R. V., Wickramasinghe, K., Long, T. I., Kurumboor, S. K., Bernstein, L., Peters, J. H., DeMeester, S. R., DeMeester, T. R., Skinner, K. A., and Laird, P. W. Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res., *61:* 3410–3418, 2001.
23. Chapman, V., Forrester, L., Sanford, J., Hastie, N., and Rossant, J. Cell lineage-specific undermethylation of mouse repetitive DNA. Nature (Lond.), *307:* 284–286, 1984.
24. Cormier, R. T., and Dove, W. F. Dnmt1N/+ reduces the net growth rate and multiplicity of intestinal adenomas in C57BL/6-multiple intestinal neoplasia (Min)/+ mice independently of p53 but demonstrates strong synergy with the modifier of Min 1(AKR) resistance allele. Cancer Res., *60:* 3965–3970, 2000.
25. Issa, J. P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., and Baylin, S. B. Methylation of the oestrogen receptor CpG island links aging and neoplasia in human colon. Nat. Genet., *7:* 536–540, 1994.
26. Nakagawa, H., Nuovo, G. J., Zervos, E. E., Martin, E. W., Jr., Salovaara, R., Aaltonen, L. A., and de la Chapelle, A. Age-related hypermethylation of the 5′ region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. Cancer Res., *61:* 6991–6995, 2001.
27. Issa, J. P. CpG-island methylation in aging and cancer. Curr. Top. Microbiol. Immunol., *249:* 101–118, 2000.
28. Dietrich, W. F., Lander, E. S., Smith, J. S., Moser, A. R., Gould, K. A., Luongo, C., Borenstein, N., and Dove, W. Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse. Cell, *75:* 631–639, 1993.
29. Shoemaker, A. R., Moser, A. R., Midgley, C. A., Clipson, L., Newton, M. A., and Dove, W. F. A resistant genetic background leading to incomplete penetrance of intestinal neoplasia and reduced loss of heterozygosity in Apc*Min/+* mice. Proc. Natl. Acad. Sci. USA, *95:* 10826–10831, 1998.
30. Shoemaker, A. R., Gould, K. A., Luongo, C., Moser, A. R., and Dove, W. F. Studies of neoplasia in the Min mouse. Biochim. Biophys. Acta, *1332:* F25–F48, 1997.
31. Bilger, A., Shoemaker, A. R., Gould, K. A., and Dove, W. F. Manipulation of the mouse germline in the study of Min-induced neoplasia. Semin. Cancer Biol., *7:* 249–260, 1996.

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