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CsA treatment; Fig. 5D; Supplementary Fig. S7). These results showed that the CsA treatment suppressed inflammatory responses but not *HP* colonization, and that the suppression of inflammatory responses markedly repressed methylation induction.

Expression analysis of genes with promoter methylation in* HP*-infected GECs. HG2, SC3, and SD2 were located in the promoter regions of *Gpr37, Rnf152*, and *Nptx2*, respectively. Promoter CGIs are generally resistant to DNA methylation (29), and only when genes are transcribed at low levels are they susceptible to DNA methylation (30–32). To confirm the low expression and the effect of methylation on gene expression, we analyzed their expression levels in GECs isolated from gerbils with and without *HP* infection (10 and 50 weeks

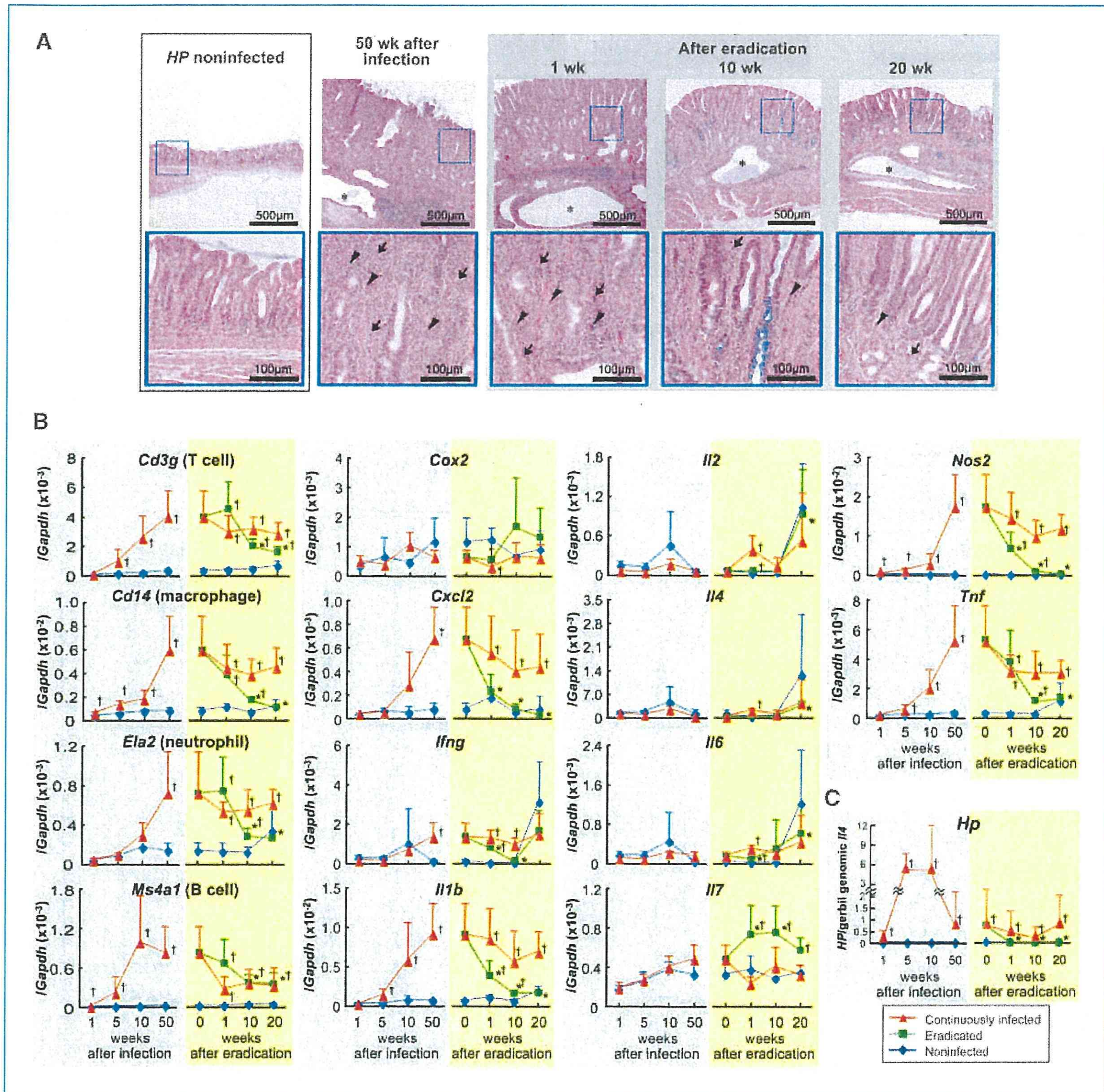

Figure 4. Changes in inflammation after *HP* infection and its eradication. A, histologic changes in gastric mucosa before and after *HP* eradication. Sections were stained with hematoxylin, eosin, and Alcian blue. Infiltration of numerous mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) did not change at 1 wk after eradication but markedly decreased at 10 and 20 wk. However, the presence of fibrosis and heterotopic proliferative glands (*) did not differ. B, temporal profiles of expression of inflammatory cell markers and inflammation-related genes. Red, green, and blue lines, gerbils with continued infection, gerbils with eradication, and those without any *HP* infection, respectively. C, numbers of *HP* in the gerbil stomach. Real-time PCR of *HP*-specific DNA using DNA extracted from gastric tissues containing mucus was done. Values are shown as mean + SD. †, $P < 0.05$, compared with noninfected gerbils; *, $P < 0.05$, compared with the expression level before eradication.

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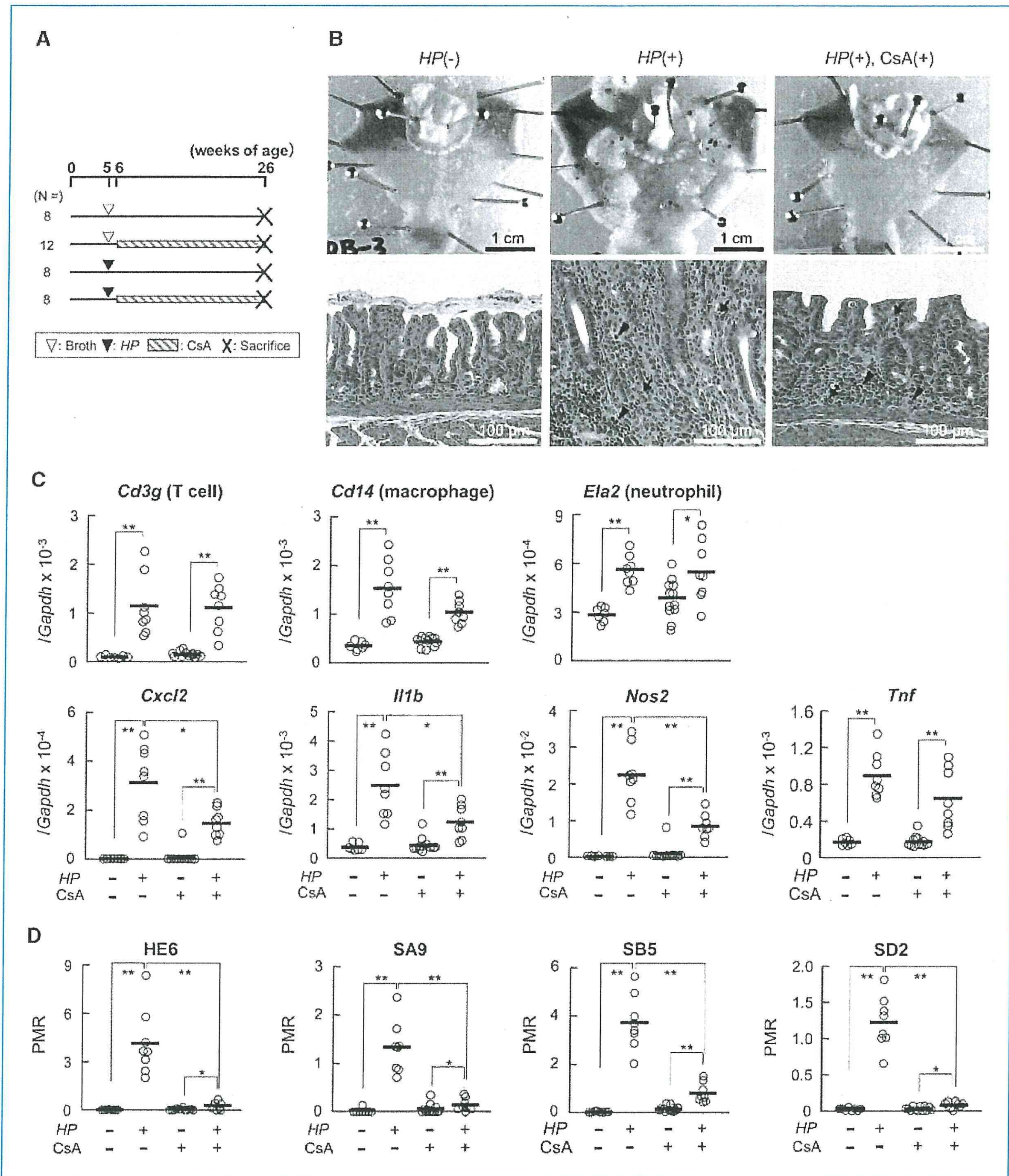

Figure 5. Suppression of inflammation and methylation induction by CsA treatment. A, experimental design for CsA treatment and *HP* infection. B, macroscopic (top) and histologic (bottom) analyses of gastric mucosae. Hyperplastic changes in pyloric area were prominent in *HP*-infected gerbils without the CsA treatment and were markedly suppressed by the CsA treatment. Infiltration of mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) was also severe in *HP*-infected gerbils without the CsA treatment and was repressed in CsA-treated animals. Gastric mucosae of *HP*-negative gerbils with CsA treatment showed no abnormal changes (data not shown). C, expression of inflammatory cell markers and inflammation-related genes. The expression of inflammatory cell markers normalized to *Gapdh* expression was not reduced. However, the expression of three inflammation-related genes (*Cxcl2, Il1b,* and *Nos2*) was significantly reduced by the CsA treatment. D, methylation levels in GECs. The CsA treatment markedly suppressed methylation induction by *HP* infection. Bold horizontal bar, average. *, $P < 0.05$; **, $P < 0.01$.

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after infection) and in gastric cancer cell lines. All the three genes showed low expression levels in the GECs of noninfected and infected gerbils (Supplementary Fig. S8). *Rnf152* expression was significantly decreased in *HP*-infected gerbils compared with noninfected gerbils (44% and 25% at 10 and 50 weeks, respectively, after infection; $P < 0.001$). None of the three genes were expressed in cancer cell lines with complete methylation of these CGIs (Fig. 1B; Supplementary Fig. S8, top).

The absence of DNA methyltransferase upregulation. DNA methyltransferases (Dnmt) are final effectors of maintenance and induction of DNA methylation, and their overexpression is frequently observed in various types of human cancers (33). To analyze possible upregulation of Dnmts by *HP* infection, expression levels of *Dnmt1, Dnmt3a,* and *Dnmt3b* mRNAs were quantified in GECs of gerbils with and without *HP* infection. Contrary to our initial expectation, the expression levels of the three Dnmts were significantly lower in GECs with *HP* infection (1/2 to 1/3) than those without (Supplementary Fig. S8, bottom).

Discussion

Our study using a gerbil model showed that *HP* infection is causally involved in induction of aberrant DNA methylation in GECs. Thus far, a strong association has been shown between the presence of *HP* infection and high methylation levels or high incidence of methylation in human gastric mucosae (5, 10–12). Taking advantage of an animal model, we were able to conduct an experiment by infecting gerbils with *HP* and showed that *HP* infection was the cause of methylation induction.

The critical role of inflammation in methylation induction was shown. Temporal analysis showed that methylation levels were closely associated with infiltration of inflammatory cells, and suppression of inflammation by CsA markedly repressed methylation induction even in the presence of *HP*. These results indicated that *HP* itself was not necessary for methylation induction once inflammation was induced by it. This finding is important because a direct role of *HP* is suggested by the facts that the SHP2 oncoprotein is deregulated by injection of virulent factors such as CagA into GECs (34) and *HP* possesses multiple DNA (cytosine-5) methyltransferases (35).

Among the inflammation-related genes analyzed, the expression levels of *Cxcl2, Il1b, Nos2,* and *Tnf* were upregulated in the stomach with *HP* infection and decreased after eradication, almost paralleling those of methylation levels. In the CsA treatment, in which methylation induction was markedly suppressed, upregulation of *Cxcl2, Il1b,* and *Nos2* by *HP* infection was significantly suppressed and that of *Tnf* also had a tendency to be suppressed. These results suggest that some specific inflammation-related genes are cooperatively involved in methylation induction by *HP* infection. In human ulcerative colitis and hepatitis (cirrhosis), where aberrant methylation is believed to be induced, increased expression of *IL8* (human functional homolog of *Cxcl2*), *IL1B, NOS2,* and *TNF* was also observed (36–39), suggesting that upregulation of these genes is a common feature of methylation-associated inflammation. Especially for human *IL1B*, its allele with a specific single nucleotide polymorphism is known to be associated with increased gastric cancer risk and increased incidence of *CDH1* promoter methylation in gastric cancers (40, 41). Also, increased production of nitric oxide, due to upregulation of a nitric oxide synthase (*NOS2*) by IL1B or administration of nitric oxide donors, induced methylation of *FMR1* and *HPRT* genes *in vitro* (42).

This study also clearly shows that methylation in gastric mucosae with *HP* infection consists of temporary and permanent components, which has been suggested by studies in humans (5, 10). Methylation that disappeared after eradication corresponds to the temporary component, and methylation that did not disappear corresponds to the permanent component. A pyloric gland (mucosal epithelia) is known to be composed of one or a few stem cells, multiple progenitor cells, and a large number of differentiated cells, and it is renewed within 3 to 14 days (43, 44). Temporary methylation is likely to have been induced in progenitor or differentiated cells, which will finally drop off from the gastric epithelium. Permanent methylation is likely to be induced in stem cells, which will remain for life. In humans, methylation levels in gastric mucosae without *HP* infection correlate with gastric cancer risk (5, 10), and this fact is also in line with the hypothesis that permanent methylation in gastric mucosae without *HP* infection reflects methylation in stem cells.

HG2, SC3, and SD2 were methylated in GECs, although they were located in promoter CGIs, which are generally resistant to DNA methylation (29). Among promoter CGIs, those of genes with low transcription are known to be susceptible to methylation (30, 31, 45), and as expected, all the three genes had low transcription levels in GECs. Transcription levels at 10^{-4} to 10^{-3}/*Gapdh* (*GAPDH*) correspond to 1 to 10 copies of mRNA per cell and are less than 35% of the average expression level of all the genes analyzed by expression microarray (46). Because their methylation levels in GECs of gerbils infected with *HP* for 10 and 50 weeks were less than a few percent, their methylation was unlikely to have affected the overall expression levels in gastric mucosae. As a response to *HP* infection, *Rnf152* was downregulated whereas *Gpr37* and *Nptx2* were not.

Promoter CGIs of *GPR37* and *NPTX2* were highly methylated in human gastric mucosae with *HP* infection and were frequently methylated in human gastric cancers. Because their tumor-suppressive functions have not been reported and they are not expressed in normal gastric mucosae (RefExA database[4]), their silencing is unlikely to be causally involved in gastric carcinogenesis, and they are considered to be passengers. Likewise, methylated CGIs that were not associated with genes were likely to be passengers. However, it is now known that a lot of passengers and limited number of drivers are methylated to high and small degrees, respectively, in human gastric mucosae with *HP* infection (5, 45). Therefore, although most methylation identified here was

[4] http://157.82.78.238/refexa/main_search.jsp

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considered to be passenger, it is likely that tumor-suppressor genes are also methylated in association with their methylation. Gastric mucosa with accumulation of silencing of various genes, including both drivers and passengers, is considered to form a field where cancers will develop (epigenetic field for cancerization; refs. 7, 10, 47).

As a final effector of methylation induction, we examined overexpression of *Dnmts*, which are implicated in methylation induction in various human cancers (33). Unexpectedly, all the three *Dnmts* were downregulated by *HP* infection. Our recent data in humans also showed that mRNA levels of Dnmts had decreasing tendencies in *HP*-infected gastric mucosae (45). These results indicate that overexpression of *Dnmts* is not involved in *HP*-induced methylation induction, and suggest that local distribution of Dnmts and/or protective factors, such as the presence of RNA polymerase II (48), might be disturbed by inflammation.

Genome-wide screening to isolate DNA fragments methylated by *HP* infection was done by MS-RDA, which is applicable to any species without genome information. We used cell lines as the driver so that we could avoid heterogeneity of primary samples and aberrant methylation will be present in all the DNA molecules in the driver. This was considered to be essential for a genome-wide screening because most methods cannot detect small differences. Although cell lines might have artificial methylation, we confirmed the presence of specific methylation in GECs, and a high-sensitivity method, qMSP, was used for this. As expected, methylation levels of CGIs identified here were small (i.e., a few percent) in GECs with *HP* infection, showing that the strategy was correct.

In summary, *HP* infection was causally involved in induction of aberrant DNA methylation, and a critical role of inflammation in the induction was indicated. This model is expected to be useful in analyzing detailed molecular mechanisms for induction of aberrant DNA methylation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Suppressive effect of global DNA hypomethylation on gastric carcinogenesis

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Global DNA hypomethylation and concomitant site-specific gene hypermethylation are among the most common molecular alterations in human neoplasia. Although site-specific DNA hypermethylation has been shown to be associated with the development of various tumors accompanied by transcriptional silencing of target genes, the functional significance of global DNA hypomethylation in tumorigenesis remains unclear. Previous studies have revealed that a genetic reduction of the DNA methylation levels leads to opposing effects on tumor development, depending on the tumor cell type and the stage of tumorigenesis. In the present study, we investigated the effect of DNA hypomethylation on gastric carcinogenesis in mice. The genetic reduction of DNA methylation levels suppressed the incidence, number and size of gastric tumors in two different mouse models for gastric tumorigenesis: the *N*-methyl-*N*-nitrosourea-induced model and the *Apc*$^{Min/+}$ mouse model that spontaneously develops gastric tumors with aging. Histological analyses revealed DNA hypomethylation to completely inhibit the development of invasive gastric tumors. These findings indicate that the reduction of DNA methylation levels suppresses gastric carcinogenesis and suggest that DNA methylation is closely associated with gastric tumorigenesis.

Introduction

Changes in the DNA methylation status, including global DNA hypomethylation and site-specific gene hypermethylation, are concomitantly found in tumors and are the most common molecular alterations in human neoplasia (1). Site-specific DNA hypermethylation has been extensively analyzed and a number of genes have been shown to be hypermethylated and transcriptionally silenced in various tumors. However, the functional significance of global DNA hypomethylation remains unclear, although this alteration was discovered in a wide variety of human cancers >20 years ago. Global DNA hypomethylation, which is frequently observed at the early stages of tumorigenesis in human cancer (2,3), promotes chromosomal instability *in vitro* and accelerates tumor development in several mouse models (4–6). Although the consequences of global hypomethylation and site-specific hypermethylation have been mechanistically connected to chromosome instability and transcriptional silencing, respectively, the cause of aberrant DNA methylation patterns remains unclear.

Abbreviations: *Cdh1*, *Cadherin 1*; *Cdkn2a*, *Cyclin-dependent kinase inhibitor 2A*; *LINE*, long interspersed nuclear element; *Magea2*, *Melanoma antigen family A, 2*; MNU, *N*-methyl-*N*-nitrosourea; *Sfrp*, *Secreted frizzled-related protein*.

†These authors equally contributed to this work.

In human gastric cancers, a variety of aberrant methylations, including global hypomethylation and site-specific DNA hypermethylation, have been identified and the importance of epigenetic alterations in gastric carcinogenesis would be emphasized by the infrequent genetic alterations in gastric cancers (7). Site-specific DNA hypermethylations have been preferentially investigated and it has been revealed that a number of genes are inactivated by promoter hypermethylation in human gastric cancer, including tumor suppressor genes (7–12). On the other hand, global DNA hypomethylation in human gastric cancer has been revealed by quantifications of 5-methylcytosine content (8) and evaluation of methylation status of *long interspersed nuclear elements* (*LINEs*)*-1* repetitive sequences (8,13), but little is known about the role in gastric tumorigenesis.

DNA methylation is catalyzed by a family of three DNA methyltransferases: Dnmt1, Dnmt3a and Dnmt3b (14–16). Although the three Dnmts partially cooperate to establish and maintain genomic methylation patterns, they also have distinctive functions. *Dnmt1* has a preference for hemimethylated DNA and deposits methyl groups on newly synthesized DNA, which results in the faithful replication of methylation patterns. Indeed, a hypomorphic allele of Dnmt1 has been shown to cause global DNA hypomethylation (6). Dnmt1 is therefore considered to be the major maintenance methyltransferase. Using *Dnmt1* hypomorphic alleles as a model for global DNA hypomethylation, previous studies have revealed that global DNA hypomethylation inhibits tumorigenesis in the intestine (17,18), esophagus and tongue (19), whereas it accelerates developments in T-cell lymphomas (6), liver cancers (18) and fibrosarcomas (5), possibly by promoting chromosomal instability. Our previous study also revealed that genetic reduction of DNA methylation eventually suppresses intestinal tumorigenesis but promotes the development of early-stage lesions in the colon of *Apc*$^{Min/+}$ mice (18). These results indicate that the forced reduction of genomic methylation levels leads to opposing effects on tumorigenesis depending on the cell type and the stage of tumorigenesis. Considering the fact that DNA hypomethylating agents have been used for cancer therapy in a subset of cancer (1), it is important to clarify the effect of global DNA hypomethylation on the risk for tumor development in various organs.

In the present study, we investigated the effect of reduced DNA methylation levels on gastric carcinogenesis in *N*-methyl-*N*-nitrosourea (MNU)-induced mice and *Apc*$^{Min/+}$ mice (20–22). We herein report that global DNA hypomethylation significantly suppresses the development of gastric tumors.

Materials and methods

Mice

Two mutant alleles of *Dnmt1* were used: the null *Dnmt1*^{c} allele in the C57BL/6 background (23) and the hypomorphic *Dnmt1*^{chip} allele in the 129Sv4 background (6). *Dnmt1*$^{c/+}$ mice (C57BL/6) were crossed with female *Dnmt1*$^{chip/chip}$ mice (129Sv4) to generate experimental mice in an isogenic F1 hybrid (C57:129) background. A previous study reported that *Dnmt1*$^{chip/+}$ mice have the same levels of genomic methylation as *Dnmt1*$^{+/+}$ mice, whereas *Dnmt1*$^{chip/c}$ mice have reduced DNA methylation contents at pericentromeric satellite repeats (18). We therefore analyzed *Dnmt1*$^{chip/+}$ mice as a control cohort and *Dnmt1*$^{chip/c}$ mice as a DNA hypomethylated cohort in this study. All mice were maintained under specific-pathogen-free conditions with isolated ventilation cages in an air-conditioned room with a 12 h light–dark cycle. They were bred and maintained on a basal diet, CE-2 (CLEA Japan, Tokyo, Japan), until the termination of the study.

Methylation analysis of gastric mucosa

The DNA methylation levels in gastric mucosae were examined by methylation-sensitive Southern blot analysis and bisulfite methylation analysis. Genomic DNA were extracted from the homogenized gastric mucosae of *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice. Firstly, DNA were digested with the methylation-sensitive enzyme HpaII (New England Biolabs, Ipswich, MA) and the digests were analyzed by Southern blotting using a centromeric

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satellite repeat probe as previously reported (6,18,24). Next, the methylation pattern in 5′-noncoding region of *Line-1* was analyzed by bisulfite sequencing in accordance with the previous report (25). Bisulfite treatment of the genomic DNA was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). After polymerase chain reaction amplification using primers for 5′-noncoding region of *Line-1*, the products were cloned into the TOPO vector (Invitrogen, Carlsbad, CA). The inserted polymerase chain reaction fragments of the individual clones obtained from each sample were sequenced with primer for T7 promoter using the ABI Prism Dye Terminator Cycle Sequencing Kit and an ABI Prism 3100 DNA Sequencer. The primers for *Line-1* are shown in supplementary Table 1, available at *Carcinogenesis* Online.

MNU treatment

MNU (Sigma Chemical, St Louis, MO) was dissolved in distilled water at a concentration of 240 p.p.m. and freshly prepared thrice per week for administration in drinking water in light-shielded bottles *ad libitum*. *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ littermates (n = 24 and 18, respectively) from 4 to 6 weeks of age were given drinking water containing 240 p.p.m. MNU on alternate weeks for a total of 10 weeks (total exposure: 5 weeks), according to the protocol described in previous reports (Figure 1A) (20,22). They were thereafter maintained without any further treatment until they were killed at 52 weeks of age.

Apc$^{Min/+}$ *mice model*

Apc$^{Min/+}$ mice in the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). *Apc*$^{Min/+}$ mice were crossed with *Dnmt1*$^{c/+}$ mice (C57BL/6), and male *Apc*$^{Min/+}$; *Dnmt1*$^{c/+}$ mice (C57BL/6) were subsequently crossed with female *Dnmt1*$^{chip/chip}$ mice (129Sv4) to generate experimental mice in an isogenic F1 hybrid (C57:129) background. We analyzed 30 *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice and 21 *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice to quantify gastric lesions at 30–32 weeks of age (Figure 1B). In addition, 9 *Dnmt1*$^{chip/+}$; *Apc*$^{+/+}$ mice and 15 *Dnmt1*$^{chip/c}$; *Apc*$^{+/+}$ mice were analyzed as controls without the *Apc*^{Min} allele. All mice were maintained under the same conditions as those described above.

Preparation of tissue samples for tumor counting and histological analysis

All mice underwent a thorough postmortem examination at the time of killing. The stomach was removed and opened along the greater curvature. The number and the lengths of the major and minor axes of the gastric tumors were determined using a dissecting microscope at ×7 magnification. Tumors >0.5 mm in long axis length were mapped and counted. The sizes of the tumors were determined by multiplying the major axis by the minor axis. To eliminate interobserver error, all counts were performed by a single observer blinded to the genotype of the mice. In addition, all the cases were counted by a second observer to confirm the results of the first observer. After counting the tumors, all the excised stomachs, including the neoplastic nodules, were fixed for 24 h in neutral-buffered 10% formalin and were subsequently cut into eight strips. These strips were processed by standard methods, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. The defining characteristics for adenoma and adenocarcinoma were adapted from both the consensus guidelines on murine models of intestinal cancer (26) and previous reports in the literature (20,27).

Immunohistochemistry

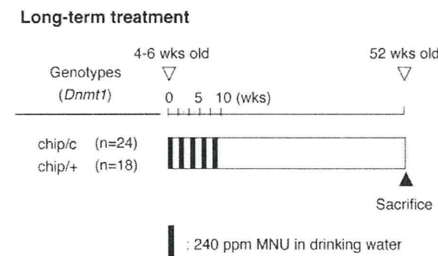
The avidin–biotin–peroxidase complex technique was used for immnohistochemical studies. Five micrometers thick sections were cut, deparaffinized, rehydrated in phosphate-buffered saline, placed in 10 mmol/l citrate buffer (pH 6.0) and heated in a 750 W microwave four times for 6 min. The endogenous peroxidase activity was blocked by incubation for 30 min in 0.3% H_2O_2. After washing three times with phosphate-buffered saline, the sections were then pre-incubated with normal blocking serum for 20 min at room temperature and then were incubated with Ki-67 (1:200; DAKO Corporation, Carpinteria, CA) and cleaved caspase-3 (1:400; Cell Signaling Technology, Danvers, MA) antibody overnight at 4°C. Subsequently, the sections were incubated with biotinylated secondary antibodies (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 30 min, followed by incubation with avidin-coupled peroxidase (Vector Laboratories) for 30 min. The sections were developed with 3,3′-diaminobenzidine using DAKO liquid 3,3′-diaminobenzidine Substrate-Chromogen System (DAKO Corporation) and were then counterstained with hematoxylin. No specific staining was observed in the negative control slides prepared without primary antibody. The number of Ki-67-positive cells per gland was calculated as the Ki67-labeling index.

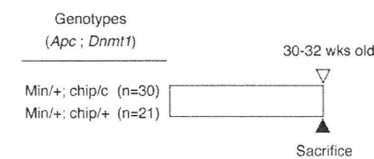
Methylation analysis of MNU-induced gastric tumors

In order to examine the involvement of aberrant site-specific methylations in the development of MNU-induced mouse gastric cancer, the methylation status of the promoter regions of *Cyclin-dependent kinase inhibitor 2A* (*Cdkn2a*), *Cadherin 1* (*Cdh1*), *Secreted frizzled-related protein* (*Sfrp*) *1*, *Sfrp2* and *Melanoma antigen family A, 2* (*Magea2*) were analyzed by bisulfite sequencing. Genomic DNA were extracted from the gastric tumors and the surrounding gastric mucosae of MNU-treated wild-type mouse and bisulfite analysis were performed as described above. The primers for bisulfite sequencing are shown in supplementary Table 1, available at *Carcinogenesis* Online.

Statistical analysis

The incidence of gastric tumors was analyzed using Fisher's exact test. Differences in the number and the size of gastric tumors and Ki-67-positive cell ratio in non-cancerous mucosa were assessed with Mann–Whitney *U*-test.





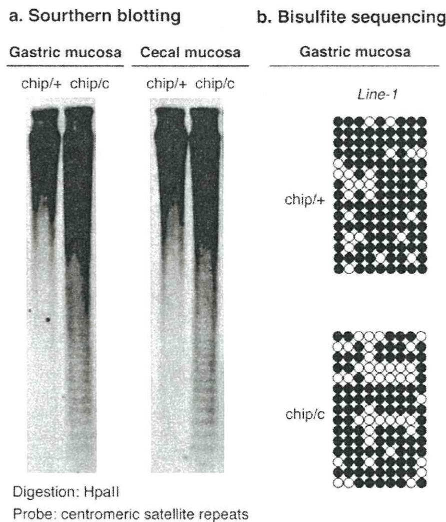


Fig. 1. Experimental design to study the effect of the DNA hypomethylation on gastric carcinogenesis and genomic DNA hypomethylation in *Dnmt1*$^{chip/c}$ mice. (**A**) MNU-induced model. (**B**) *Apc*$^{Min/+}$ mouse model. (**C**) Methylation analysis of gastric mucosa. (a) Methylation-sensitive Southern blot analysis of the pericentromeric regions. Decreased levels of DNA methylation are detectable in both gastric and cecal mucosa of *Dnmt1*$^{chip/c}$ mouse in comparison with those of the *Dnmt1*$^{chip/+}$ mouse. (b) Bisulfite sequencing of the 5′-noncoding region of *Line-1*. A reduction in CpG methylation was observed in the gastric mucosa of *Dnmt1*$^{chip/c}$ mouse as compared with that of the *Dnmt1*$^{chip/+}$ mouse. Filled and open circles represent methylated and unmethylated CpGs, respectively.

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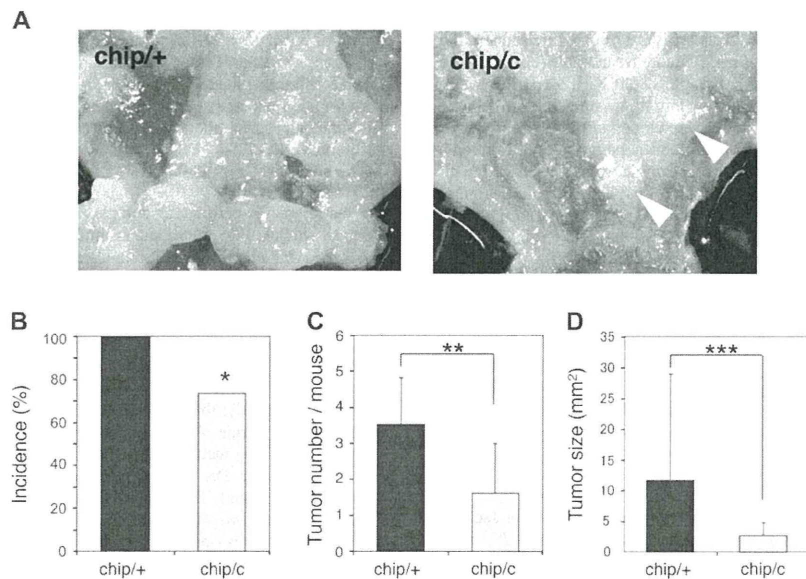

Fig. 2. DNA hypomethylation suppresses gastric tumorigenesis in MNU-treated mice. (**A**) Macroscopic photographs of the glandular stomach in MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice at 52 weeks of age. (**B**) Incidence of gastric tumors in MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice (n = 24 and n = 18). The incidence of MNU-treated *Dnmt1*$^{chip/c}$ mice with gastric tumors was significantly lower than that of MNU-treated *Dnmt1*$^{chip/+}$ mice. $^{*}P < 0.05$, by Fisher's exact test. (**C**) Number of gastric tumors per mouse in MNU-treated *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice. MNU-treated *Dnmt1*$^{chip/c}$ mice developed significantly fewer gastric tumors than MNU-treated *Dnmt1*$^{chip/+}$ mice. Columns, mean; bars, SD. $^{**}P = 0.0001$, by Mann–Whitney *U*-test. (**D**) Sizes of gastric tumors in MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice. The tumor size of gastric tumors in the MNU-treated *Dnmt1*$^{chip/c}$ mice was significantly smaller than that of the MNU-treated *Dnmt1* $^{chip/+}$mice. Columns, mean; bars, SD. $^{***}P < 0.0001$, by Mann–Whitney *U*-test.

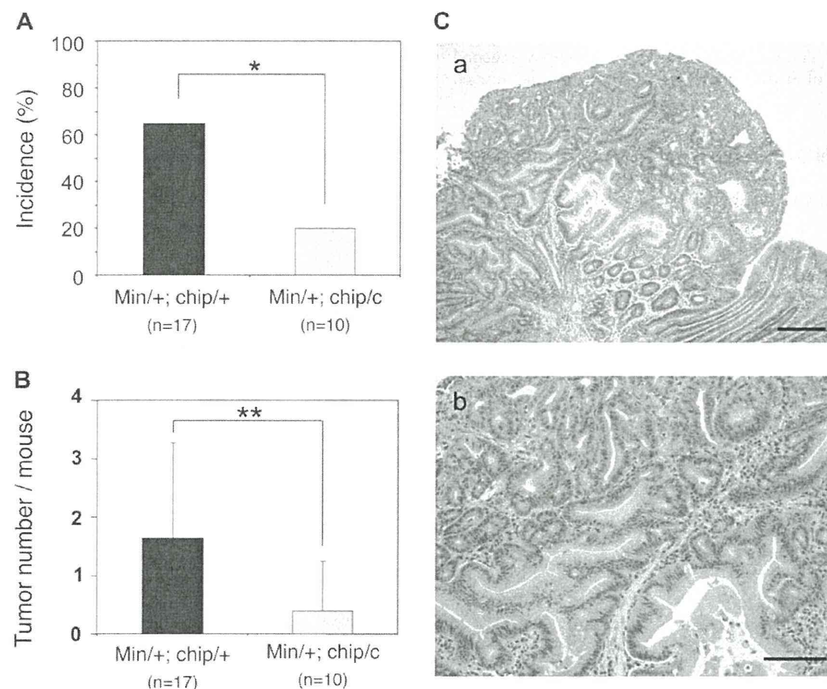

Fig. 3. DNA hypomethylation suppresses the development of gastric tumors in *Apc*$^{Min/+}$ mice model. (**A**) Incidence of gastric tumors in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice and *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice (n = 17 and n = 10). The incidence was significantly smaller in *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice than in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice. $^{*}P < 0.05$, by Fisher's exact test. (**B**) Number of gastric tumors per mouse in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice and *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice. *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice developed significantly fewer gastric tumors than *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice. Columns, mean; bars, SD. $^{**}P < 0.05$, by Mann–Whitney *U*-test. (**C**) Histopathology of the gastric tumors in *Apc*$^{Min/+}$ mice. (a) Adenoma in *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice; bar, 200 μm. (b) Higher magnification in (a); bar, 100 μm.

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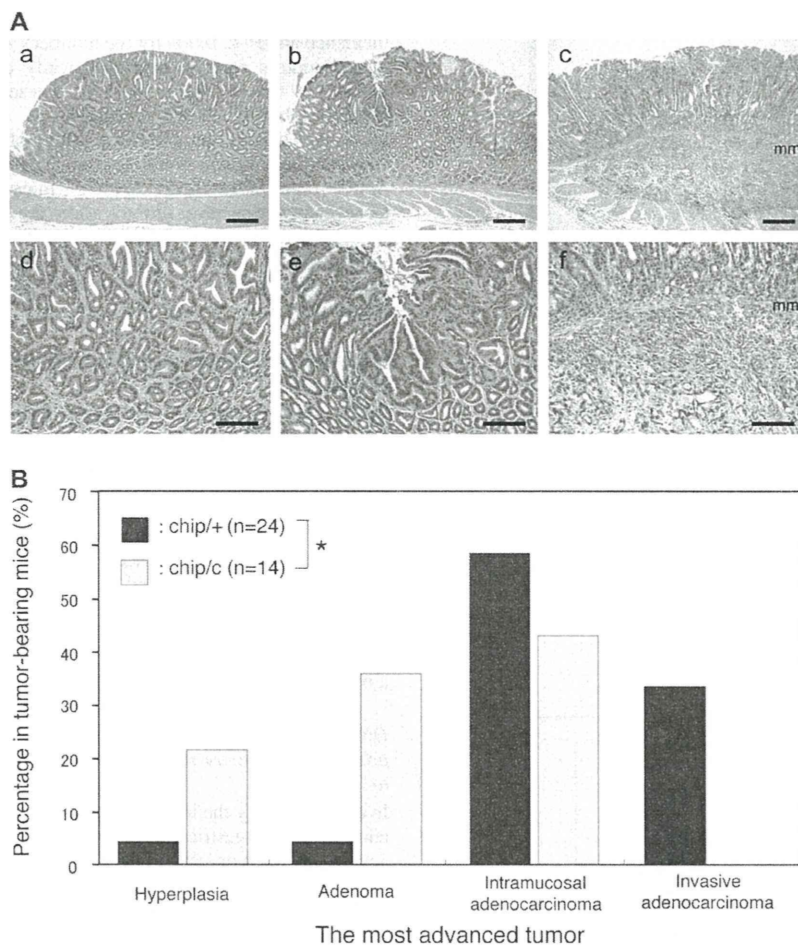

Fig. 4. DNA hypomethylation suppresses the progression of gastric carcinogenesis. (**A**) Histopathologic features of gastric tumors in MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice at 52 weeks of age. (a) Adenoma in MNU-treated mice. (b) Intramucosal adenocarcinoma. (c) Invasive adenocarcinoma. (d–f) Higher magnification in (a–c). mm, muscularis mucosae; bars, 200 µm in (a–c) and 100 µm in (d–f). (**B**) Histologic grade of the most advanced tumor in tumor-bearing MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice (n = 24 and n = 14). The development of malignant gastric cancer was significantly decreased in *Dnmt1*$^{chip/c}$ mice as compared with *Dnmt1*$^{chip/+}$ mice. Columns, mean; bars, SD. $^{*}P$ = 0.0005, by Spearman's rank correlation test.

Spearman's rank correlation test was used to determine the relationship between *Dnmt1* genotype and tumor malignancy.

Results

Dnmt1 hypomorphic alleles induce global DNA hypomethylation in gastric mucosa

To determine whether the mutant *Dnmt1* alleles definitely affect DNA methylation in the gastric mucosa, the DNA methylation levels in the gastric mucosae were examined. The methylation-sensitive Southern blot analysis revealed that the gastric mucosa of the *Dnmt1*$^{chip/c}$ mouse was significantly hypomethylated at pericentromeric regions in comparison with that of the *Dnmt1*$^{chip/+}$ mouse (Figure 1C), consistent with both our previous findings and the present observations in the colonic mucosa (18). Additionally, bisulfite analysis showed the reduced CpG methylation of the *Line-1* regulatory region in the *Dnmt1*$^{chip/c}$ mouse gastric mucosa, whereas this region was highly methylated in that of the *Dnmt1*$^{chip/+}$ mouse (Figure 1C). When compared the methylation frequency at each CpG in the *Line-1* regulatory region, the values in the *Dnmt1*$^{chip/c}$ mouse were lower than those in the *Dnmt1*$^{chip/+}$ mouse at almost all CpGs except for the second CpG. These findings indicate that *Dnmt1* hypomorphic alleles lead to the global DNA hypomethylation in the mouse gastric mucosa.

DNA hypomethylation suppresses gastric tumorigenesis in MNU-treated mice

We administered MNU, an alkylating agent that induces the formation of adenomas and adenocarcinomas in murine glandular stomach tissue, to *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice, which express different levels of DNA methyltransferase *Dnmt1*. The MNU-treated *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice (n = 24 and 18, respectively) were examined for gastric lesions at 52 weeks of age. Macroscopically, most tumors developed in the pyloric antrum and showed a sessile and/or polypoid morphology in both *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice (Figure 2A). Both the incidence and the multiplicity of macroscopic gastric tumors in the MNU-treated *Dnmt1*$^{chip/c}$ mice were significantly decreased as compared with those in the MNU-treated *Dnmt1*$^{chip/+}$ mice (Figure 2B and C). The incidence of gastric tumors was 77.8% (14/18) in *Dnmt1*$^{chip/c}$ mice, whereas it was 100% (24/24) in *Dnmt1*$^{chip/+}$ mice ($P < 0.05$). The average numbers of the gastric tumors in *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice were 1.7 ± 1.47 and 3.8 ± 1.33 (±SD) per mouse, respectively (P = 0.0001). Furthermore, the tumor size in the MNU-treated *Dnmt1*$^{chip/c}$ mice was significantly smaller than that of the MNU-treated *Dnmt1* $^{chip/+}$mice ($P < 0.0001$, Figure 2D). Large tumors, namely >5 mm in the longest diameter, were only observed in MNU-treated *Dnmt1*$^{chip/+}$ mice (data not shown). These results clearly indicate that a genetic

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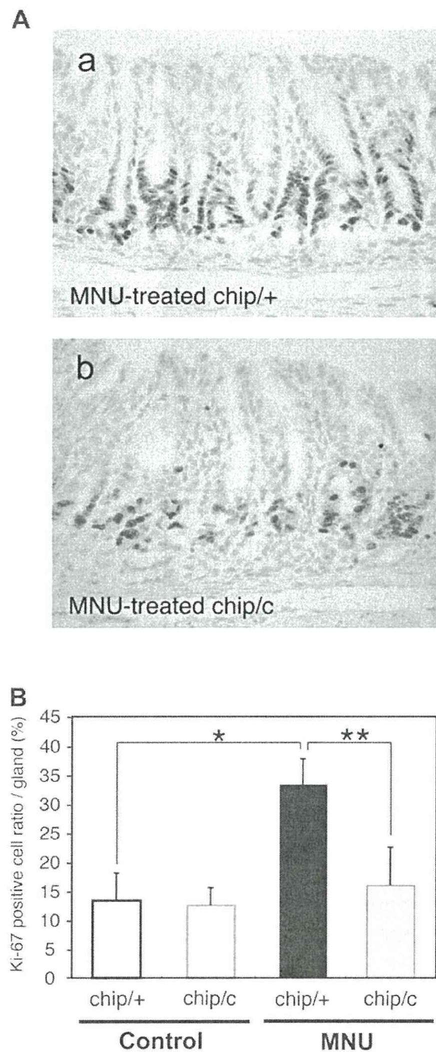

Fig. 5. DNA hypomethylation suppresses abnormal cell proliferation in the non-cancerous gastric mucosa induced by MNU treatment. (**A**) Ki-67 immunostaining of the non-cancerous gastric mucosa in MNU-treated *Dnmt1*$^{chip/+}$ (a) and *Dnmt1*$^{chip/c}$ (b) mice at 52 weeks of age. (**B**) Percentage of Ki-67-positive cells in non-cancerous gastric mucosa of non-treated and MNU-treated *Dnmt1*$^{chip/+}$ and *Dnmt1*$^{chip/c}$ mice (n = 10 for each group). The Ki-67-positive cell ratio in MNU-treated *Dnmt1*$^{chip/c}$ mice was equivalent to that in non-treated *Dnmt1*$^{chip/c}$ mice, whereas MNU-treatment significantly elevated the Ki-67-positive cell ratio in *Dnmt1*$^{chip/+}$ mice. Columns, mean; bars, SD. $^{*}P < 0.0005$ and $^{**}P < 0.0005$, by Mann–Whitney *U*-test.

reduction of DNA methylation levels suppresses the initiation and promotion stage of MNU-induced gastric carcinogenesis.

DNA hypomethylation suppresses the development of gastric tumors in Apc$^{Min/+}$ mice

We also investigated the effect of reduced DNA methylation levels on gastric carcinogenesis using a different mouse model. We have previously reported that *Apc*$^{Min/+}$ mice spontaneously develop gastric tumors with aging (21). *Apc*$^{Min/+}$ mice were crossed with *Dnmt1* hypomorphic mice to generate *Apc*$^{Min/+}$ mice that express different levels of *Dnmt1*. Consistent with the results in the MNU-induced gastric tumor model, both the incidence and the number of the gastric tumors were significantly smaller in *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice than in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ mice (Figure 3A and B, $P < 0.05$ for the incidence and $P < 0.005$ for the number). As previously reported, the gastric tumors were histopathologically classified as adenomas in the *Apc*$^{Min/+}$ mice (Figure 3C) (21). These results provide additional evidence that a genetic reduction of DNA methylation levels suppresses gastric tumorigenesis. Although we assessed the cell proliferative activities in non-cancerous gastric mucosa of *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ and *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice, genetic reduction of the DNA methylation level did not affect the cell proliferative activities in the gastric mucosa of *Apc*$^{Min/+}$ mice. The Ki-67-positive cell ratios in *Dnmt1*$^{chip/+}$; *Apc*$^{Min/+}$ and *Dnmt1*$^{chip/c}$; *Apc*$^{Min/+}$ mice (n = 10 for each animals) were 22.3 ± 5.8 and 19.4 ± 5.4 (average ± SD), respectively.

DNA hypomethylation suppresses the progression stage of gastric carcinogenesis

MNU-induced gastric lesions were evaluated for their histopathologic features and classified as hyperplasia, adenoma, intramucosal adenocarcinoma or invasive adenocarcinoma (Figure 4A). Histopathlogical analyses clearly demonstrated significant decreases in the development of intramucosal and invasive adenocarcinomas in MNU-treated *Dnmt1*$^{chip/c}$ mice as compared with *Dnmt1*$^{chip/+}$ mice (Figure 4B), thus indicating that the reduced DNA methylation levels suppressed the progression into advanced gastric tumors. It is noteworthy that the MNU-treated *Dnmt1*$^{chip/+}$ mice often developed invasive adenocarcinomas, whereas the MNU-treated *Dnmt1*$^{chip/c}$ mice did not (Figure 4B). These findings suggest that DNA methylation may play a role in the progression stage of gastric tumorigenesis.

DNA hypomethylation reduces the persistent increase in the cell proliferative activity in the non-cancerous gastric mucosa exposed to MNU

In order to clarify the inhibitory mechanisms of reduced DNA methylation levels on gastric tumorigenesis, we assessed cell proliferative activity in the non-cancerous gastric mucosa of MNU-treated and non-treated *Dnmt1*$^{chip/c}$ and *Dnmt1*$^{chip/+}$ mice by immunostaining for Ki-67, a marker for proliferating cells (Figure 5A). The Ki-67-positive index of gastric epithelium was significantly higher in MNU-treated *Dnmt1*$^{chip/+}$ mice than in non-treated *Dnmt1*$^{chip/+}$ mice ($P < 0.005$), thus suggesting that MNU induces abnormal cell proliferation even in non-cancerous gastric epithelium to provide favorable conditions for tumor development (Figure 5B). In contrast, the Ki-67-positive cell ratio in MNU-treated *Dnmt1*$^{chip/c}$ mice was equivalent to that in non-treated *Dnmt1*$^{chip/c}$ mice and it was significantly lower than that in the MNU-treated *Dnmt1*$^{chip/+}$ mice ($P < 0.005$), thus indicating that reduced levels of genomic methylation suppress the abnormal cell proliferation induced by MNU (Figure 5B). We also performed cleaved caspase-3 in order to examine the effect of DNA hypomethylation on the induction of apoptosis. However, no significant differences were observed in the induction of apoptosis between *Dnmt1*$^{chip/c}$ mice and *Dnmt1*$^{chip/+}$ mice (data not shown).

The aberrant site-specific methylations are not found in MNU-induced mouse gastric tumors unlike in the case of human gastric cancers

We cannot rule out the possibility that the genetic reduction of DNA methylation suppress gastric tumorigenesis by blocking the aberrant site-specific methylation of specific genes that play a crucial role in gastric tumorigenesis, but there is no available information on hypermethylated or hypomethylated genes in MNU-induced mouse gastric tumors. In order to examine the involvement of aberrant site-specific methylations in the development of MNU-induced mouse gastric tumors, the methylation status of the promoter regions of *Cdkn2a*, *Cdh1*, *Sfrp1*, *Sfrp2* and *Magea2* were analyzed. Though it has been reported that the CpG islands in the promoter regions of *Cdkn2a*, *Cdh1*, *Sfrp1* and *Sfrp2* are highly methylated and those of *Magea2* are frequently demethylated in human gastric tumors (7–12), altered methylation patterns were not found in MNU-induced mouse gastric

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