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Antibiotics suppress colon tumorigenesis through inhibition of aberrant DNA methylation in an azoxymethane and dextran sulfate sodium colitis model

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Chronic inflammation is involved in the development of colon cancer by inducing mutations and aberrant DNA methylation in colon epithelial cells. Furthermore, there is growing evidence that colonic microbiota modulates the inflammation response in the host and influences colon tumorigenesis. However, the influence of colonic microbiota on aberrant DNA methylation remains unknown. Here, we show the effect of colonic microbes on DNA methylation and tumorigenicity using a mouse model of human ulcerative colitis. Mice treated with azoxymethane (AOM) and dextran sulfate sodium (DSS) showed an increase in degree of colitis, as estimated by body weight, occult blood, and stool consistency/diarrhea at 2 weeks after treatment, but treatment with antibiotics markedly reduced the severity of the colitis. Although mucosal hyperplasia and increased inflammation-related genes were observed in the colonic epithelial cells of the AOM/DSS-treated mice, treatment with antibiotics abrogated these changes. In addition, treatment with antibiotics significantly decreased the number of mucosal nodules from 5.9 ± 5.3 to 0.2 ± 0.6 ($P < .01$) and area of occupancy from 50.1 ± 57.4 to 0.5 ± 1.4 mm^2 ($P < .01$). Aberrant DNA methylation of three marker CpG islands (*Cbln4*, *Fosb*, and *Msx1*) was induced by AOM/DSS treatment in colonic mucosae, but this increase was suppressed by 50%-92% ($P < .05$) with antibiotic treatment. Microbiome analysis showed that this change was associated with a decrease of the *Clostridium leptum* subgroup. These data indicate that antibiotics suppressed tumorigenesis through inhibition of aberrant DNA methylation induced by chronic inflammation.

KEYWORDS
chronic inflammation, colon cancer, DNA methylation, epigenetics, microbiota

1 | INTRODUCTION

Chronic inflammation is involved in the development of a variety of cancers, such as CAC,[1,2] gastric cancer,[3] and liver cancer.[4] It leads to the accumulation of mutations and epigenetic alterations in tissues, and ultimately to the development of cancers.[5,6] Mechanistically, cell proliferation induced by persistent tissue damage leads to increased chance of mutations[7,8] and acceleration of age-associated DNA methylation.[9,10] Reactive oxygen species and reactive nitrogen intermediates are produced by inflammatory cells as well as by up-regulation of inflammation-related genes within epithelial cells, and lead to DNA damage[11] and changes in activity of epigenetic regulators.[12] Micro RNA (miRNA) expression is also altered in response to inflammatory signals, leading to impaired DNA repair activity[13] and altered expression of epigenetic regulatory genes.[14,15]

Especially in the colon, chronic inflammation and resultant tumorigenesis are heavily influenced by the intestinal microbiota,[2,16] which have been investigated mainly using mouse models. T-cell receptor beta ($TCR\beta$) and $p53$ double-knockout ($TCR\beta^{-/-}$ $p53^{-/-}$) mice developed adenocarcinomas, along with mild colitis, under conventional conditions, whereas they did not show inflammatory changes or adenocarcinoma in germ-free conditions.[17] Interleukin-10 knockout ($Il10^{-/-}$) mice developed colitis as a result of microbial-induced activation of effector T cells as well as tumors by administration of a mutagen, AOM, whereas tumor development was suppressed in germ-free conditions.[18] $Apc^{Min/+}$; $Il10^{-/-}$ mice developed colon tumors with inflammation, whereas this was suppressed in germ-free conditions.[19] Furthermore, in the AOM and DSS-induced mouse CAC model, giving vancomycin suppressed tumor development by reducing colon inflammation.[20]

Effects of germ-free conditions or treatment with antibiotics on the decrease in tumor incidence potentially have a large impact on cancer prevention.[21,22] The decreasing effect is considered to be mediated by decreased mutation frequency or levels of aberrant DNA methylation. However, how modulation of colonic microbiota influences levels of aberrant DNA methylation in colonic epithelial cells remains unknown. In the present study, we gave antibiotics to modulate colonic microbiota in a mouse colon cancer model induced by AOM and DSS, and examined the effects of the modulation on inflammation, DNA methylation induction, and colon tumorigenicity.

2 | MATERIALS AND METHODS

2.1 | Animals, treatment with antibiotics, and cancer induction experiments

Male BALB/c mice were purchased from Charles River Laboratories (Yokohama, Japan). To induce colon tumors, 5-week-old mice were i.p. injected with 10 mg/kg body weight of AOM (NARD Institute, Amagasaki, Japan) or PBS (Wako, Osaka, Japan) as control. Antibiotics [6 μg/body ciprofloxacin (LKT Laboratories, Inc., St Paul, MN, USA), 0.2 mg/body metronidazole (Wako), 0.2 mg/body neomycin (AppliChem GmbH, Darmstadt, Germany), 0.1 mg/body vancomycin (Wako)] were given by

gavage for 2 weeks after AOM injection. At 6 weeks of age, DSS (molecular weight, 36 000-50 000; MP Biochemicals, Solon, OH, USA) was given in drinking water at a concentration of 1.5% w/v. Timing and duration of the treatment are shown in Figure 1A. All the animal experiments were approved by the Committee for Ethics in Animal Experimentation at Meiji Co., Ltd and National Cancer Center.

2.2 | Sample preparation

Large bowels of 15-week-old mice were cut open longitudinally, and the number of macroscopic colon nodules with major axes >3 mm was counted. Colonic epithelial cells were obtained by the crypt isolation technique after removal of colon nodules from the distal large bowel.[23] To analyze DNA methylation, genomic DNA was extracted using the standard phenol/chloroform method. After collecting the epithelial cells and nodules, distal colon tissue samples were fixed overnight in 10% formalin and paraffin-embedded for histological analysis. For gene expression analysis, the entire colon, including both mucosal and muscle layers, was used, and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan).

2.3 | Estimation of inflammation

At 7 weeks of age, severity of inflammation was monitored by fecal consistency (0, normal; 2, loose stool; 4, diarrhea), bleeding (0, normal; 2, bloody feces; 4, blood around anus), and body weight loss after DSS treatment (0, <3%; 1, 3%; 2, 6%; 3, 9%; 4, >12%). Disease activity index (DAI; from 0 to 12) was obtained as the total of three factors.

2.4 | Histological analysis

Histopathological changes in the colon were analyzed by H&E staining of 4-μm sections. Degree of infiltration of neutrophils and plasma cells was used as an indicator of active inflammation. Mucosal nodules were microscopically examined and categorized as dysplasia, adenoma, or adenocarcinoma.

2.5 | Direct DNA sequencing

Sequence variations at the $Ctnnb1$ exon 2 were detected by amplification of this locus using 20 ng template DNA with primers listed in Table S1. The PCR product was purified using a DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA, USA) and direct cycle sequenced using the Applied Biosystems Big Dye Terminator V3.1 (Thermo Fisher Scientific, Waltham, MA, USA). The sequences were determined with an Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific).

2.6 | Quantitative reverse transcription PCR

Complementary DNA was synthesized from DNase-treated total RNA (2 μg) using Olido-dT$_{20}$ (Thermo Fisher Scientific) and Superscript III reverse transcriptase (Thermo Fisher Scientific). Number of cDNA
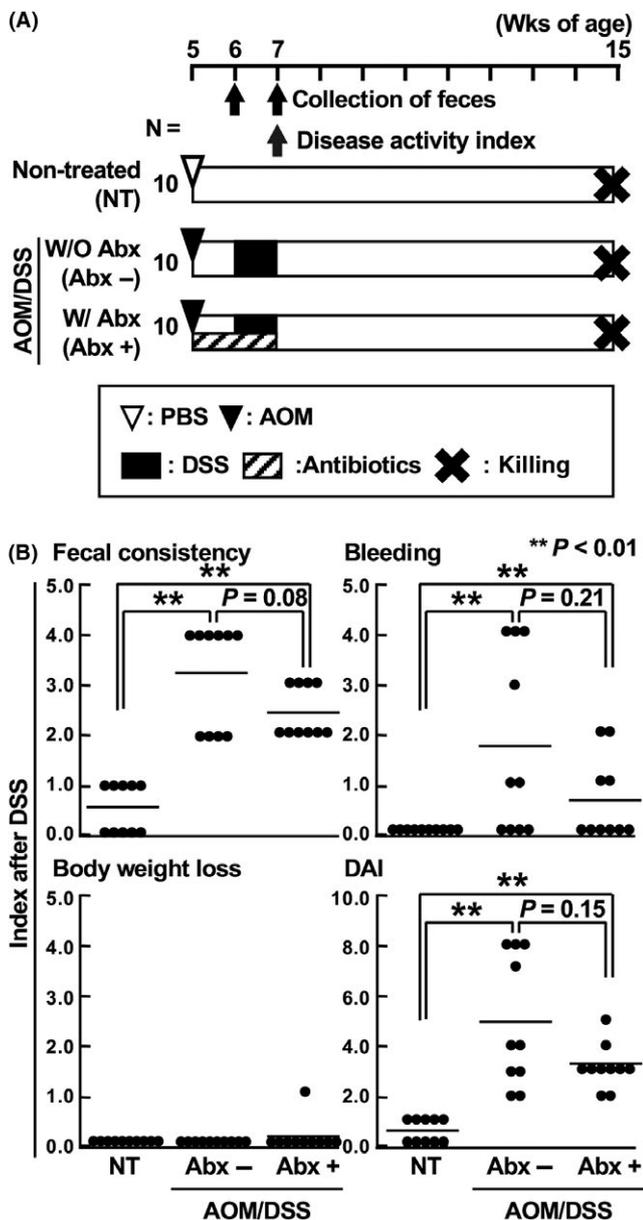
FIGURE 1 Suppression of inflammation by treatment with antibiotics. A, Experimental protocol of tumor induction by azoxymethane (AOM) and dextran sulfate sodium (DSS) administration and treatment with antibiotics. Ten mice (NT) were i.p. injected with PBS(−) at 5 weeks of age. Ten mice (Abx−) were i.p. injected with AOM at 5 weeks of age, and given DSS in drinking water at 6 weeks of age for 1 week. Ten mice (Abx+) were treated with antibiotics in addition to receiving AOM/DSS. Feces were collected at 6 and 7 weeks of age. Severity of inflammation at 7 weeks of age was monitored by disease activity index (DAI). All mice were killed at 15 weeks of age. B, Severity of inflammation at 7 weeks of age monitored by fecal consistency, bleeding, body weight loss, and disease activity index. AOM/DSS-induced inflammation was suppressed by treatment with antibiotics. Bold horizontal bars indicate the average. **P < .01

molecules was quantified by qRT-PCR. Primer sequences and PCR conditions are shown in Table S1. The copy number of each sample was calculated by comparing the amplification curve with those of standard DNA samples of known copy numbers. Number of target cDNA molecules was normalized to that of *Gapdh* cDNA molecules.

2.7 | Quantitative methylation-specific PCR

*Bam*HI-digested genomic DNA (1 μg) was treated with sodium bisulfite as described previously.[24] Bisulfite-treated DNA was resuspended in 40 μL TE, and 1 μL was used for quantitative PCR using primers specific to methylated target loci and B2 SINE repeat (Table S1). DNA methylation levels were expressed as the percentage of methylation reference that was calculated as [(number of molecules methylated at a target region in the sample)/(number of B2 SINE repeat in the sample)]/[(number of molecules methylated at the target region in the fully methylated DNA)/(number of B2 SINE repeat in the fully methylated DNA)]. Genomic DNA treated with *Sss*I methylase (New England Biolabs, Beverly, MA, USA) was used as fully methylated DNA.

2.8 | Fecal DNA extraction and microbe analysis

Feces were collected at 6 and 7 weeks of age and fecal DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA). Fecal DNA was amplified by quantitative PCR (qPCR) using primers specific to the 16S rDNA gene for each group of bacteria species.[25,26] Quantities of each bacteria group were normalized to the number of major bacteria which was amplified by primers for the 16S V2-V3 region.[27] Primer sequences and PCR conditions are shown in Table S2. To calculate the absolute number of bacteria, the amplification curve of a sample was compared with those of a standard DNA sample of known bacterial numbers.

2.9 | Statistical analysis

To evaluate significance in differences in degree of colitis, number of tumors, area of occupancy of tumors, and DNA methylation levels, the Mann-Whitney U test was used using SPSS 13.0J (SPSS Japan Inc., Tokyo, Japan). Differences in mean expression levels and the number of colonic microbiota were analyzed by the Student's t test. Relationships between the number of mucosal nodules, severity of inflammation, level of DNA methylation, and colonic microbiota were estimated by Pearson correlation analysis.

3 | RESULTS

3.1 | Alleviation of colitis by treatment with antibiotics

Azoxymethane was given to 5-week-old male mice, followed by 1-week treatment with DSS with or without antibiotics (vancomycin, neomycin, metronidazole, ciprofloxacin) (Figure 1A). At 7 weeks of age, degree of colitis was monitored by fecal consistency, bleeding, and body weight loss. Fecal consistency and bleeding were significantly affected by giving AOM/DSS, but body weight was not (Figure 1B). Fecal consistency and bleeding tended to be alleviated by treatment with antibiotics (P = .08 for fecal consistency, and P = .21 for bleeding) (Figure 1B).

3.2 | Suppression of mucosal hyperplasia and inflammation-associated gene expression by treatment with antibiotics

At 10 weeks after AOM/antibiotics treatment, microscopic changes of the colonic mucosae were examined. Consistent with previous reports,[20,28,29] diffuse mucosal hyperplasia was observed in the distal colon of AOM/DSS-treated mice. However, no or much less hyperplasia was observed in the colonic mucosae of mice treated with antibiotics (Figure 2A). Expression of *Il1b*, *Il6*, *Il10*, *Nos2*, and *Tnf* was increased in

AOM/DSS-treated mice compared with non-treated mice. Treatment with antibiotics suppressed the increase in inflammation-related genes, especially *Nos2* and *Tnf* (Figure 2B). These data showed that treatment with antibiotics alleviated colonic inflammation and reduced hyperplasia.

3.3 | Suppression of colon tumorigenesis by treatment with antibiotics

To analyze the effect of treatment with antibiotics on colon tumorigenesis, we calculated the number of mucosal nodules and area of
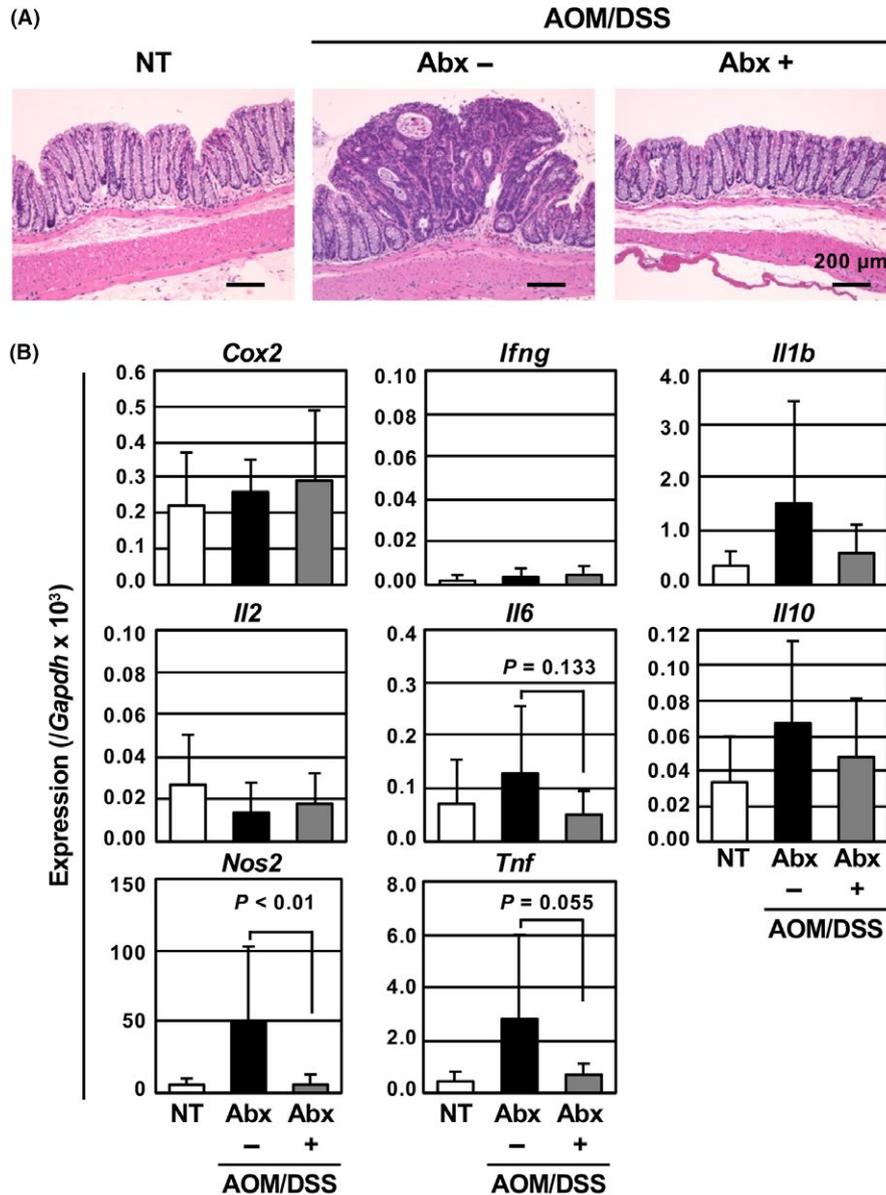

FIGURE 2 Mucosal histopathology and upregulation of inflammation-associated genes, and their suppression by antibiotics. A, Representative microscopic appearance of the colonic mucosae. At 10 weeks after treatment with azoxymethane (AOM)/antibiotics, adenomas/adenocarcinomas and diffuse mucosal hyperplasia were observed in mice without antibiotics (Abx−), but the appearance was suppressed in mice treated with antibiotics (Abx+). The middle photograph shows an adenoma with mucosal hyperplasia in an Abx− mouse. B, mRNA expression levels of inflammation-associated genes 10 weeks after treatment with AOM/antibiotics in colonic tissues. Upregulation of *Nos2* and *Tnf* by AOM/dextran sulfate sodium (DSS) in the mice without antibiotics (Abx−) was not observed in mice with antibiotics (Abx+). Gene expression levels are shown as mean ± SD of 10 mice in each group. Il, interleukin; Ifng, interferon gamma; Nos, nitric oxide synthase; Tnf, tumor necrosis factor

occupancy of nodules in the colon at 10 weeks after AOM/antibiotics treatment. Mucosal nodules were macroscopically observed in the distal region of the colon in 10 of the 10 mice treated with AOM/DSS without antibiotics (Figure 3A). In contrast, only one of the 10 mice treated with antibiotics had multiple mucosal nodules. Histopathologically, the mucosal nodules were identified as adenomas or adenocarcinomas; in addition, several sites of dysplasia were observed (Table 1). Sequence of the *Ctnnb1* gene was analyzed for mutations, specifically in the regions of codons 32, 33, and 34, as adenocarcinomas induced by AOM/DSS are known to display mutations in these sequences.[30] Two out of four adenocarcinomas tested were found to display these *Ctnnb1* mutations (Table 1 and Figure S1). Average number of mucosal nodules was 6.0 in the mice without antibiotics treatment, but was almost zero in the antibiotics-treated mice (Figure 3B). Average of area of occupancy was 50 mm^2 in the mice without antibiotic treatment, and was significantly decreased, to almost zero, in mice treated with antibiotics (Figure 3C). These data clearly showed that antibiotics suppressed AOM/DSS-induced colon tumorigenesis.

3.4 | Inhibition of aberrant methylation induction by treatment with antibiotics

Effect of antibiotics treatment on DNA methylation in colonic epithelial cells was analyzed by qMSP of four markers at 10 weeks after AOM/antibiotics treatment. Three (*Fosb*, *Msx1*,

FIGURE 3 Decrease in the incidence of mucosal nodules with antibiotic treatment. A, Representative macroscopic appearance of the colon in the two groups. B, Number and area of mucosal nodules. Horizontal bars indicate the average. **P < .01. Colon mucosal nodules were induced by giving azoxymethane/dextran sulfate sodium (AOM/DSS), but this was suppressed by treatment with antibiotics, Abx, antibiotics

and *Sox11*) of the four markers were reported previously as the regions methylated in colon tumors and in colonic epithelial cells exposed to colitis[28] and one (*Cbln4*) was newly isolated by MBD-chip analysis of colonic epithelial cells in non-treated mice and in AOM/DSS-treated mice (data not shown). First, we analyzed DNA methylation levels of these four markers in colon tumors from four mice bearing apparent nodules and found high levels of methylation (1.6%-6.5% in *Cbln4*, 9.1%-20.8% in *Fosb*, 2.2%-9.0% in *Msx1*, and 1.0%-1.7% in *Sox11*) (Figure 4A). In the colonic epithelial cells of the AOM/DSS-treated mice without antibiotics, methylation levels of three markers (*Cbln4*, *Fosb*, and *Msx1*) were markedly increased (3-6-fold) compared with non-treated mice (0.05%-0.15% in *Cbln4*, 0.2%-0.6% in *Fosb*, and 0.2%-1.2% in *Msx1*) (Figure 4). However, in the mice treated with antibiotics, the increase in DNA methylation levels was suppressed, and the levels remained in the same range as those of the non-treated mice (0.06% in *Cbln4*, 0.3% in *Fosb*, and 0.1% in *Msx1*) (Figure 4). These results showed that treatment with antibiotics suppressed the induction of aberrant methylation, along with inflammation.

3.5 | Change of specific populations of colonic microbiota by treatment with antibiotics

We analyzed changes in the colonic microbiota by qPCR of bacterial 16S rDNA in the feces of the mice with and without antibiotic treatment before and after giving DSS. The number of major bacteria did not change with antibiotics treatment before DSS administration, but slightly increased after DSS administration (Figure S1). Number of *Clostridium leptum* subgroup, anaerobic Gram-positive bacteria, significantly decreased with antibiotics treatment both before and after DSS administration (Figure 5), in agreement with the previous report.[20] The number of *Lactobacillus* group also decreased with antibiotics treatment after DSS administration (Figure 5). In contrast, the number of *Bacteroides fragilis* group, anaerobic Gram-negative bacteria, increased with antibiotics treatment before DSS administration and with or without antibiotics after DSS administration (Figure 5). These data showed that specific populations of microbiota were modulated by antibiotics treatment, and suggested that the changes to the microbiota led to a suppression of inflammation and methylation induction.

3.6 | Correlations between the number of mucosal nodules, DNA methylation levels, and colonic microbiota

Finally, we analyzed the relationships between the number of mucosal nodules, severity of colitis, DNA methylation levels in background epithelial cells, and colonic microbiota focusing only on AOM/DSS-treated mice not in mice treated with antibiotics. There was a significant degree of positive correlation ($r = .82$, $P < .05$) between the number of mucosal nodules and degree of colitis (DAI)
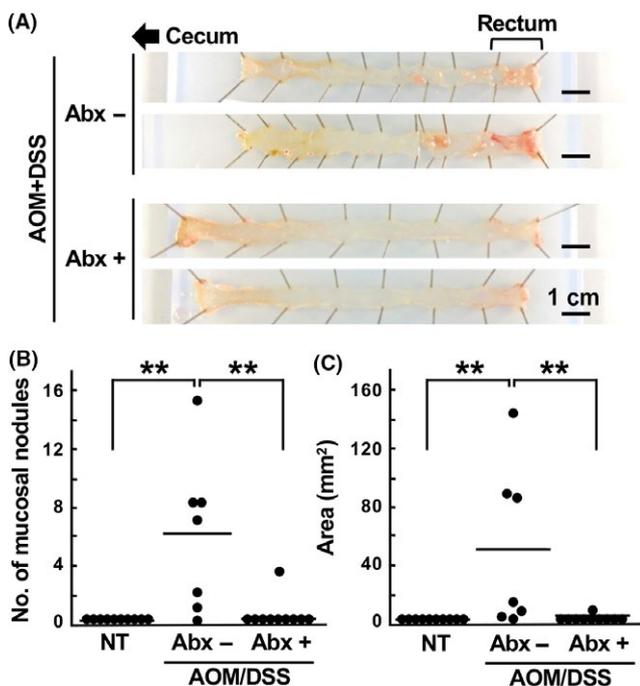
TABLE 1 Incidence of colonic mucosal nodules in AOM/DSS-treated mice without antibiotics

			No. of neoplasms		Mutation status of *Ctnnb1* exon 2	
Sample	No. of nodules examined	No. of dysplasias	Adenoma	Adenocarcinoma	Sequence	Amino acid substitution
#9	8	0	2	2	Wild-type	–
#12	0	0	0	0	NA	–
#13	15	0	3	2	Wild-type	–
#14	7	1	3	2	GAT → AAT	Asp → Asn
#33	2	3	3	1	NA	–
#46	1	0	0	2	NA	–
#47	8	1	0	3	GGA → GAA	Gly → Glu

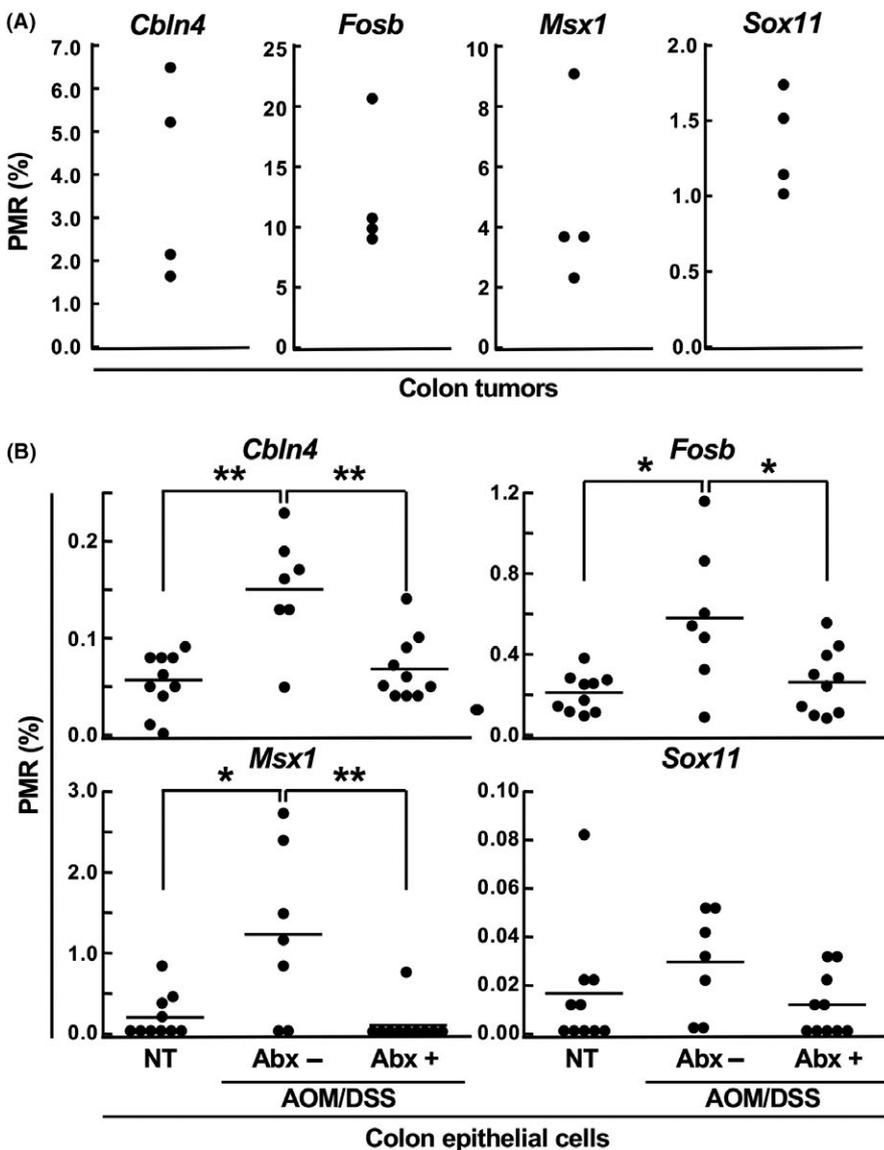AOM/DSS, azoxymethane and dextran sulfate sodium; NA, not analyzed.

FIGURE 4 Suppression of aberrant DNA methylation induction by treatment with antibiotics. A, DNA methylation levels were analyzed by quantitative methylation-specific PCR (qMSP) of four marker genes in colon tumors. High levels of methylation were observed. B, DNA methylation levels at 10 weeks after treatment with azoxymethane (AOM)/antibiotics were analyzed by qMSP of four marker genes in colonic epithelial cells. Giving AOM/dextran sulfate sodium (DSS) induced aberrant methylation in three markers, which was suppressed by treatment with antibiotics. Horizontal bars indicate the average. *$P < .05$; **$P < .01$. Abx, antibiotics; PMR, percentage of methylation reference

that was monitored by fecal consistency, bleeding, and body weight loss (Figure 6). A positive correlation was also observed with the levels of DNA methylation in colonic mucosae as observed at the *Msx1* locus ($r = .86$, $P < .01$; Figure 6); a tendency toward elevated levels of DNA methylation was also observed at the *Fobs* locus ($r = .68$, $P = .096$). After DSS treatment only, number of the

B. fragilis group showed a significant negative correlation ($r = -.83$, $P < .01$) whereas that of the *Lactobacillus* group showed a positive correlation ($r = .73$, $P = .065$) with the number of mucosal nodules (Figure 6).

4 | DISCUSSION

The present study shows, for the first time, that treatment with antibiotics inhibited aberrant methylation in mouse colonic epithelial cells induced by giving AOM/DSS, and that the inhibition was associated with a marked reduction in colon tumors. Based upon the well-established role of chronic inflammation in induction of aberrant methylation,[28,31] the suppression of chronic inflammation by antibiotics was considered as the mechanism of inhibition of methylation induction.

Among the eight inflammation-associated genes analyzed in the present study, *Nos2* expression was significantly repressed in mice treated with antibiotics, and *Tnf* tended to be repressed, in line with previous studies.[20,32] Inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α) are known to be key mediators of colitis. Elevated TNF-α is associated with both human inflammatory bowel disease (IBD) and murine colitis,[33] and upregulation of iNOS is involved in human ulcerative colitis and murine colitis.[34,35] In the mouse colitis model, epithelial cells have been found to show increased levels of Cox2 and Nos2 proteins, whereas inflammatory cells are known to express Tnf-α.[36,37] At the same time, increased expression of *Nos2* and *Tnf* are known to be associated with aberrant methylation induction, not only in the colon of DSS-treated mice but also in the stomach of *Helicobacter pylori*-infected Mongolian gerbils.[28,31,38,39] Increased production of nitric oxide enhances enzymatic activity of DNA methyltransferases.[12] Thus, treatment with antibiotics was considered to suppress chronic inflammation characterized by *Nos2* and *Tnf* elevation, and inhibit induction of aberrant methylation.

The number of *C. leptum* subgroup significantly decreased with antibiotics both before and after giving DSS, which was in line with a previous report[20] and suggested a role of the *C. leptum* subgroup in induction of inflammation and DNA methylation. In humans, the number of *C. leptum* subgroup was decreased in IBD patients,[40] but was not different between colon cancer patients and healthy volunteers.[41] In contrast, the number of *B. fragilis* group was increased by treatment with antibiotics before giving DSS and with or without antibiotics after DSS treatment. This suggested that it can contribute to inflammation and DNA methylation only in cooperation with the *C. leptum* subgroup. The promoting role of *B. fragilis* is supported by a study that subsequent risk of colorectal cancer increased among patients with bacteremia from *B. fragilis*,[42] and also by a study showing that enterotoxigenic *B. fragilis* triggered colon carcinogenesis in a mouse model.[43] Naturally, these bacteria include various genera and species, and a more detailed analysis is necessary
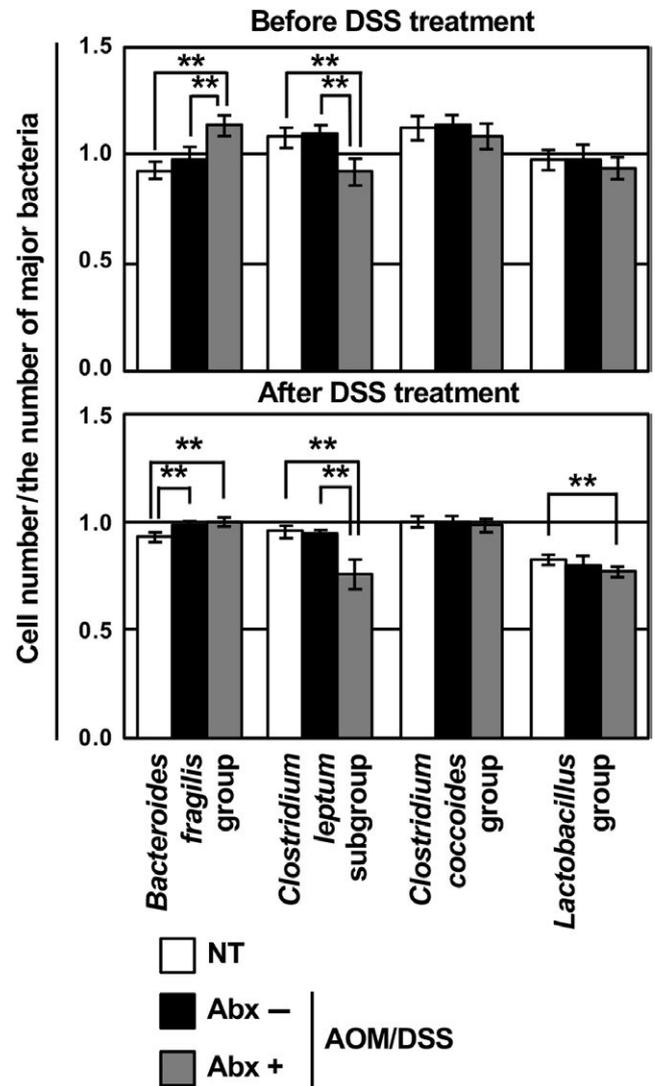

FIGURE 5 Changes in colonic microbiota with antibiotic treatment. Cell numbers of bacteria belonging to the four groups in the feces collected before and after dextran sulfate sodium (DSS) administration. Number of bacteria was analyzed by qPCR using primers for the 16S rDNA of each type of bacteria species. Quantities of each bacteria group were normalized to the number of major bacteria. Treatment with antibiotics increased the quantity of *Bacteroides fragilis* group and decreased *Clostridium leptum* subgroup and *Lactobacillus* group. Number of bacteria is shown as mean ± SD of 10, nine, and 10 mice for non-treatment (NT), treatment without antibiotics (Abx−), and treatment with antibiotics (Abx+), respectively. **$P < .01$. AOM, azoxymethane

to identify bacteria that suppress colitis and aberrant DNA methylation.

The present study shows an indirect association of colonic microbiota with chronic inflammation. These findings can be further investigated by giving certain bacterial subgroups to mice; it has, however, been reported that the outcome of such a study may be influenced by conditions including bacterial strain, bacterial preparation, timing of dosage, and condition or state of the mouse.[44] Such a transplantation-based experiment would be required to show a direct causal relationship.

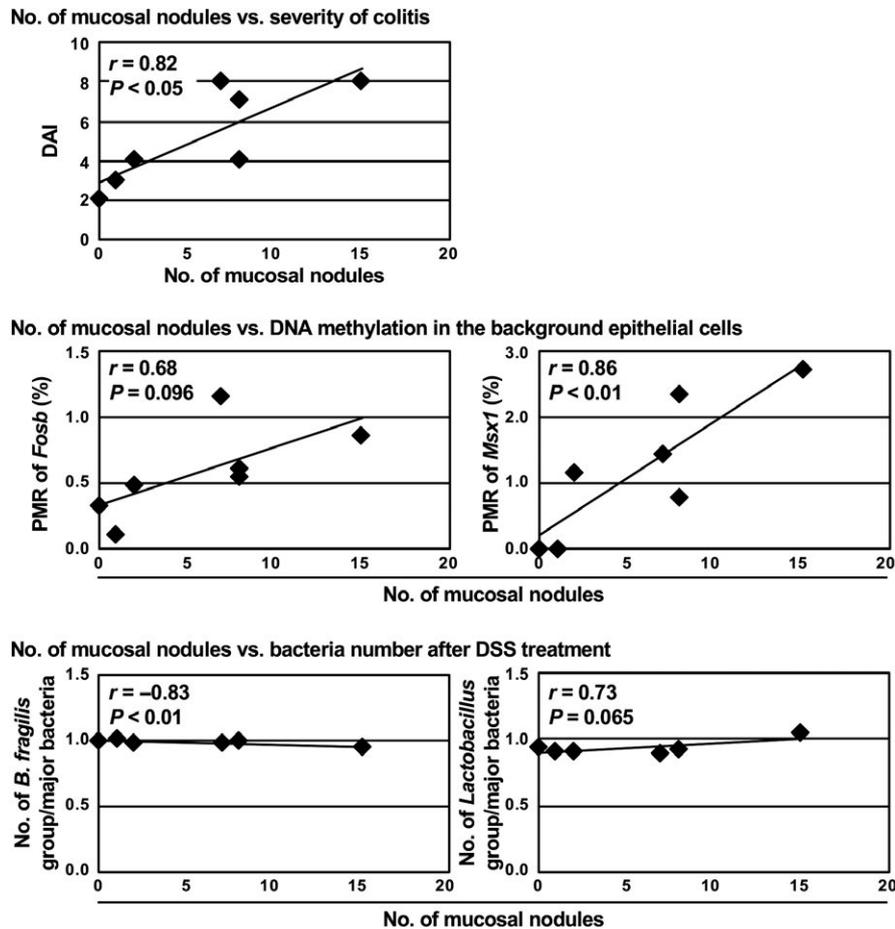## No. of mucosal nodules vs. severity of colitis

FIGURE 6 Correlations between the number of mucosal nodules, severity of colitis, DNA methylation, and colonic microbiota. Relationships between the number of mucosal nodules, severity of colitis, DNA methylation levels in the background epithelial cells, and colonic microbiota were examined in azoxymethane/dextran sulfate sodium (AOM/DSS)-treated mice not treated with antibiotics. Number of mucosal nodules was positively correlated with the degree of colitis ($r = .82$, $P < .05$) and with DNA methylation levels at the *Msx1* locus ($r = .86$, $P < .01$); a significant negative correlation was observed in the number of the *Bacteroides fragilis* group ($r = −.83$, $P < .01$) after treatment with DSS only. DAI, disease activity index; PMR, percentage of methylation reference

Aberrant DNA methylation is known to be accumulated in non-cancerous or precancerous tissues exposed to environmental factors, producing an "epigenetic field for cancerization".[45,46] The level of epigenetic field for cancerization is a promising cancer risk marker, as evidenced by the multicenter prospective cohort study for metachronous gastric cancer.[47,48] The present study also supported that this epigenetic marker is well associated with tumor development, and indicated that it is useful to monitor the effect of modulation of the intestinal microbiome.

In summary, antibiotics suppressed tumorigenesis by inhibiting aberrant DNA methylation induction by chronic inflammation. Modulation of the intestinal microbiome targeting chronic inflammation represents a strategy to prevent chronic inflammation-associated cancers.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.