RESEARCH ARTICLE

Regulation of colon gene expression by vitamin B6 supplementation

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Scope: Previous studies have shown that vitamin B6 supplementation suppresses the development of colonic aberrant crypt foci (ACF), precursor lesions of colon cancer, and cell proliferation in mice receiving the colonic carcinogen, azoxymethane (AOM). This study investigated the molecular mechanism of these effects of dietary vitamin B6.

Methods and results: To date, the mechanism by which ACFs develop is not yet fully understood. In a search for factors that play a critical role during ACF development, we examined colon gene expression during early stage of ACF development in AOM-treated mice using DNA microarray analysis. AOM treatment significantly upregulated mRNA closely related to mast cell and cytotoxic T-cell activity. This study also investigated the effect of vitamin B6 supplementation on colon gene expression in AOM-treated mice. We found that vitamin B6 supplementation downregulates *Cd8a* and *Ccl8* mRNA expression, suggesting these candidate genes may play a protective role against colonic ACF development. Furthermore, we examined genomic affects of dietary vitamin B6, and showed that *Reg3*γ mRNA expression in colons is downregulated by vitamin B6.

Conclusion: This study provides an insight into the genomic activities of dietary vitamin B6 that may be protective against colon tumor development.

Keywords:
Aberrant crypt foci / Azoxymethane / Colon / Vitamin B6

1 Introduction

Vitamin B6 is a water-soluble vitamin essential for normal growth, development, and metabolism. Pyridoxal 5′-phosphate is the metabolically active form of vitamin B6 and was originally investigated as a cofactor for numerous proteins that are involved in amino acid metabolism [1, 2], whereas, dietary vitamin B6 is increasingly being recognized as an essential nutrient that may protect against several diseases including atherosclerosis and diabetes [3–5]. Vitamin B6 concentration is potentially affected by cancer cell development as serum vitamin B6 is shown to decrease in cancer patients [6, 7]. Recent epidemiology has indicated an inverse association between vitamin B6 intake and colorectal cancer risk [8, 9], that is, dietary vitamin B6 is potentially protective against colon tumorigenesis. We have previously shown a remarkable effect of vitamin B6 supplementation against colon tumorigenesis in mice receiving azoxymethane (AOM) at a moderate dose [10]. This previous study also indicated that dietary vitamin B6 suppresses protein expression for the proliferation-related genes, c-*myc* and c-*fos*, and reduces oxidative stress in AOM-treated mice [10]. Vitamin B6 also exerts an anti-inflammatory effect via suppressing NF-κB activation in human colon cancer cells and is shown to be anti-angiogenic in an *ex vivo* serum-free matrix culture model [11, 12]. The inhibitory activities of vitamin B6 may be, in part, protective against colon tumor development as chronic inflammation and angiogenesis are considered to be mechanistically linked to tumor development and malignant cell progression.

Importantly, our previous studies demonstrated that vitamin B6 significantly suppressed the development of colonic

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Abbreviations: ACF, aberrant crypt foci; **AOM**, azoxymethane; **Cldn4**, claudin-4; **Mcpt**, mast cell protease; **PBS**, phosphate-buffered saline; **PN**, pyridoxine; **Reg**, regenerating islet-derived gene

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aberrant crypt foci (ACF), precursor lesions of colon cancer, in mice receiving AOM [10]. These observations strongly suggest that dietary vitamin B6 is protective in the early stages of ACF development prior to colon tumorigenesis induced by AOM treatment. AOM is a common model for colorectal cancer, and recent reports on the pathology of colon tumorigenesis have indicated the existence of several signaling pathways that are involved in the development of colon tumorigenesis [13–15]. The mechanistic relationship between these signaling pathways, which include K-ras, β-catenin, and p53 activity, and the pathogenesis has been widely examined at the molecular level [15]. However, limited information is available on the mechanisms by which tumorigenesis develops following AOM treatment.

In this study, we focused on the genomic affects of vitamin B6 supplementation. Previous studies showing the existence of nuclear factors, which functionally interact with vitamin B6, such as steroid hormone receptors [16, 17], hepatocyte nuclear factor 1 (HNF1) [18], receptor interacting protein 140 (RIP140) [19], and NF-κB [11, 20], prompted us to consider that vitamin B6 may be nutritionally effectual via a genomic mechanism. We have recently examined the regulatory effects of dietary vitamin B6 on gene expression in colons using DNA microarray analysis and we showed that pyridoxine supplementation downregulates the inflammatory molecule, serine protease inhibitor clade A mRNA expression in rat colons [11]. As the molecular mechanism by which AOM effects tumorigenicity in early stage of ACF development remains unclear, the first purpose of this study was to gather basic information on colon gene expression in mice receiving AOM treatment using DNA microarray analysis. We isolated candidate genes that were markedly expressed and responded early to AOM treatment. We considered these genes as early marker molecules for an AOM-induced colon pathology and tested whether vitamin B6 supplementation affected the mRNA expression of these genes. Furthermore, this current study also showed the effects of dietary vitamin B6 on genomic activity in normal colons using DNA microarray analysis. Observations reported in this study provide a novel insight into the biological roles of dietary vitamin B6 that may be protective against colon tumorigenesis.

2 Materials and methods

2.1 Animals and diets

The animals were maintained according to the Guide for the Care and use of Laboratory Animals established by Hiroshima University. Male CD-1 (ICR): Crj mice (4-weeks old, Charles River Japan, Hino, Japan) were housed in groups of two or three in metal cages in a room with controlled temperature (24 ± 1°C) and a 12 h light/dark cycle; light from 0800 to 2,000, daily. They had free access to stock diet and deionized water. After consuming a commercial stock diet (MF, Oriental Yeast, Tokyo, Japan) for 1 week, the total of 50 mice were divided into five groups of ten mice (Group A–E). The basal diet was composed of the following components (g/kg diet) [11]: α-cornstarch, 402; casein, 200; sucrose, 200; corn oil, 100; cellulose, 50; AIN-93G mineral mixture, 35; AIN-93 vitamin mixture (vitamin B6 free), 10; and L-cystine, 3. Vitamin B6 (pyridoxine [PN] hydrochloride, Nacalai Tesque, Kyoto, Japan) was supplemented to the basal diet at concentrations of either 1 mg/kg (Groups A–D) or 35 mg/kg (Group E) for 2 weeks (Groups A and B) or 4 weeks (Groups C, D, and E). Food intake and body weight were measured daily (Supporting Information Table S1).

In an experiment without AOM treatment, 20 mice were divided into two groups of 10 mice, and fed the basal diet. PN hydrochloride was supplemented to the basal diet at concentrations of either 1 or 35 mg/kg for 8 weeks. Body weight and food intake were measured daily (Supporting Information Table S2). Male Wistar rats (4-weeks old) were obtained from Charles River Japan Inc. (Japan). Rats had free access to diets and deionized water. In a vitamin B6-deficient experiment, rats (5 weeks) were fed a PN-deficient basal diet for 3 weeks, after which the rats were divided into two groups ($n = 7$) and fed either a PN-deficient basal diet or a basal diet containing 35 mg/kg PN for 4 days as previously reported [11].

2.2 AOM treatment

After consuming the basal diet containing either 1 or 35 mg/kg PN for 1 week, mice were given saline (Group A and C) or AOM (10 mg/kg body, Sigma) diluted with saline (Group B, D, and E), which was administered via subcutaneous injection once a week (Fig. 1).

2.3 DNA microarray

After removing the rectum from large intestine, the colon was opened longitudinally with fine scissors, and mucus and feces were removed in ice-cold phosphate-buffered saline.

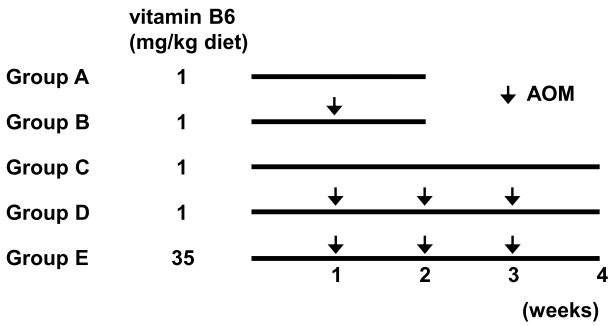

Figure 1. Experimental protocol for AOM treatment. ↓ azoxymethane (AOM), 10 mg/kg body weight, subcutaneous injection.

Table 1. Response of mRNA expressions in the colon to AOM treatment

Gene ID	Gene symbol	Gene description	*P* value	Ratio
Mast cell protease and related protein				
NM_008571	*Mcpt2*	mast cell protease 2	0.000	11.42
NM_010779	*Mcpt4*	mast cell protease 4	0.000	5.78
NM_008570	*Mcptl*	mast cell protease 1	0.000	5.78
NM_007753	*Cpa3*	carboxypeptidase A3, mast cell	0.000	4.84
NM_011246	*Rasgrpl*	RAS guanyl releasing protein 1	0.000	1.79
T cell marker and related protein				
NM_001081110	*Cd8a*	CD8 antigen, alpha chain	0.000	3.41
NM_013487	*Cd3d*	CD3 antigen, delta polypeptide	0.000	2.73
NM_010370	*Gzma*	granzyme A	0.000	2.64
NM_013542	*Gzmb*	granzyme B	0.001	2.35
NM_010689	*Lat*	linker for activation of T cells	0.000	2.61
NM_014194	*Klra7*	killer cell lectin-like receptor, subfamily A, member 7	0.000	2.65
Cytokine				
NM_008337	*Ifng*	interferon gamma	0.000	3.91
NM_021443	*Ccl8*	chemokine (C-C motif) ligand 8	0.000	3.39
NM_008599	*Cxcl9*	chemokine (C-X-C motif) ligand 9	0.000	4.78
NM_011888	*Cell 9*	chemokine (C-C motif) ligand 19	0.000	4.17
NM_009910	*Cxcr3*	chemokine (C-X-C motif) receptor 3	0.000	2.76
NM_019508	*1117b*	interleukin 17B	0.000	0.37
Cytokine-induced protein				
NM_011940	*Ifi202b*	interferon activated gene 202B	0.000	2.21
NM_008330	*Ifi47*	interferon gamma inducible protein 47	0.000	4.37
NM_008332	*Ifit2*	interferon-induced protein with tetratricopeptide repeats 2	0.000	4.08
NM_021792	*Iigpl*	interferon inducible GTPase 1	0.000	3.53
NM_145227	*Oas2*	oligoadenylate synthetase 2	0.000	6.48
Cell defense				
NM_007852	*Defcr6*	defensin related cryptdin 6	0.000	8.06
NM_010031	*Defal*	defensin, alpha 1	0.000	8.01
NM_007850	*Defcr3*	defensin related cryptdin 3	0.000	4.23
NM_007847	*Defcr-rs2*	defensin related cryptdin, related sequence 2	0.000	3.94
Others				
NM_011036	*Pap*	pancreatitis-associated protein	0.001	1.58
NM_010094	*Leftyl*	left right determination factor 1	0.000	3.26
AK090125	*AK090125*	melanoma antigen	0.000	16.39
NM_001045539	*Xlr5d*	X-linked lymphocyte-regulated 5D	0.000	7.57
NM_001081180	*Spink5*	serine peptidase inhibitor, Kazal type 5	0.000	0.59
XM_144765	*Igkv1-135*	immunoglobulin light chain variable region	0.000	0.24
NM_009518	*Wnt10a*	wingless related MMTV integration site 10a	0.000	0.32
NM_021282	*Cyp2e1*	cytochrome P450, family 2, subfamily e, polypeptide 1	0.017	0.72

List of differentially expressed genes grouped into functional categories. DNA microarray analysis was repeated with the Cy3 and Cy5 dyes reversed (a dye swap), the ratio of mRNA expression in AOM-treated mice (group D) to that in control mice (group C) is shown (Ratio).

Total RNAs were isolated using RNeasy kit (Qiagen Sciences, Germantown, MD), and pooled RNAs were subjected to cRNA synthesis for a DNA microarray analysis according to the manufacturer's instructions (44 K whole mouse genome 60-mer oligo microarray, Agilent Technologies, Palo Alto, CA). All procedures of fluorescence labeling, hybridization, slide, and image processing were carried out according to the manufacturer's instructions. In this experiment, each comparison was hybridized to two arrays employing a DyeSwap method. DyeSwap method was carried out in order to eliminate the bias between dyes because the difference between Cyanine 3-CTP (Cy-3) and Cyanine 5-CTP (Cy-5) was altered the efficiency of hybridization in case of competitive DyeCoupling assay. Gene expression data were obtained using Agilent Feature Extraction software, using defaults for all parameters except ratio terms, which were changed according to the Agilent protocol to fit the direct labeling procedure. Files and images, including error values and *p*-values, were exported from the Agilent Feature Extraction Program (version 9.5).

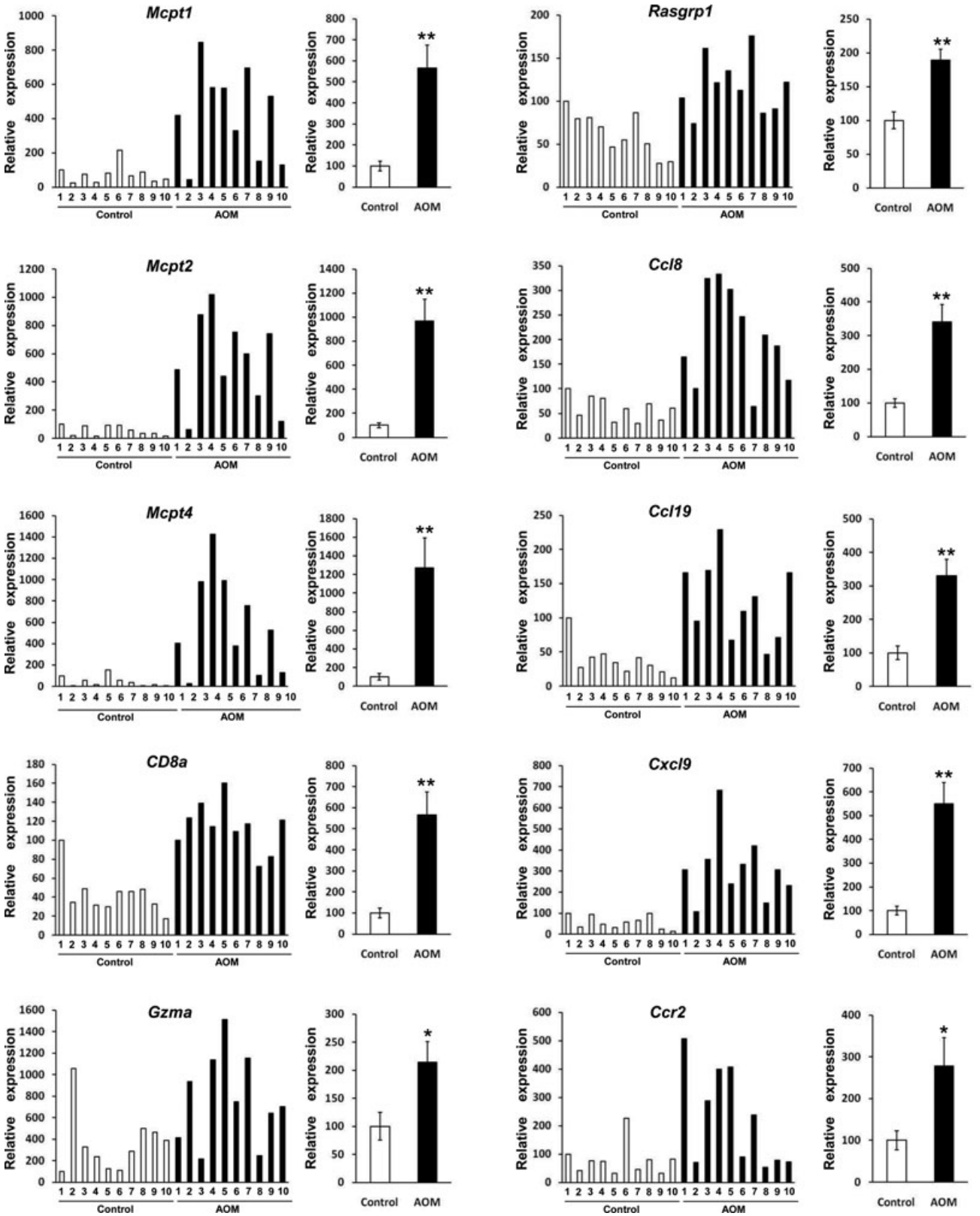

Figure 2. AOM treatment increased mRNA expression of various colon genes. One μg of total RNA from individual mice ($n = 10$) in group C and group D was subjected to quantitative PCR to examine mRNA expression level of selected genes from Table 1. All values are normalized to β-actin levels. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with those of control mice (control).

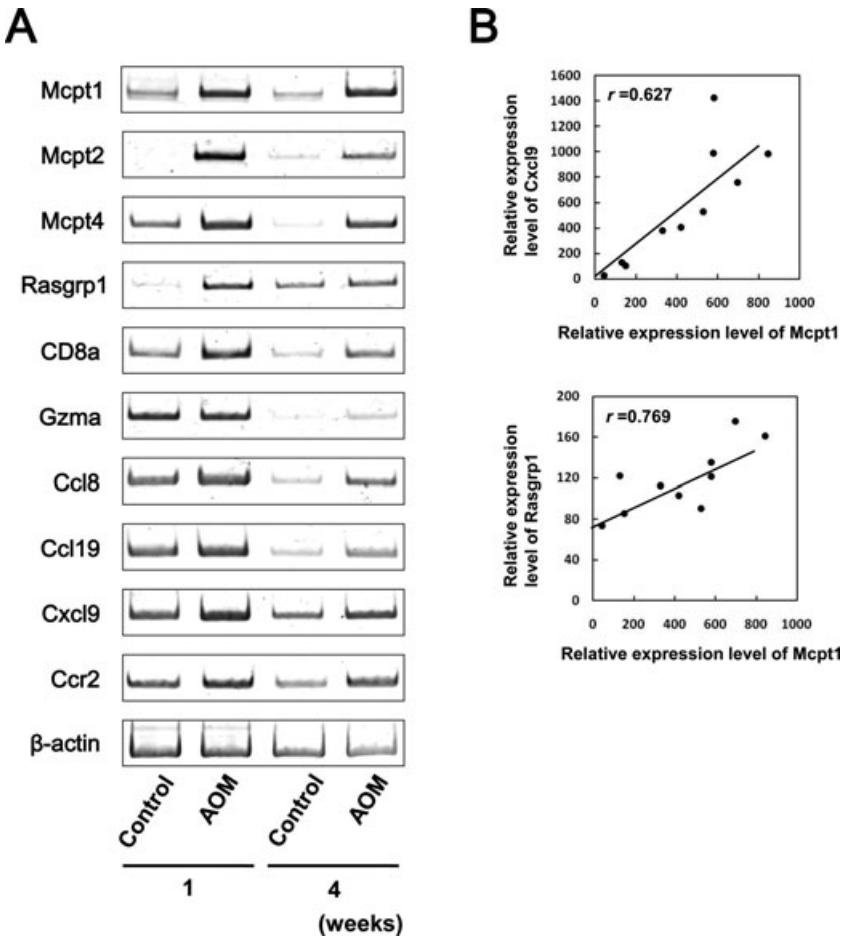

Figure 3. Response of colon mRNA expressions to AOM treatment. (A) Semiquantitative RT-PCR was performed to determine mRNA levels of genes related to mast cell and cytotoxic T cell activity. The level of β-actin (β-actin) transcript was used as a control. (B) The relative mRNA expression level of each gene was determined by quantitative PCR, normalized to β-actin level, and analyzed with Pearson's correlation coefficient.

2.4 RNA analyses

2.4.1 RT-PCR analyses

Semi-quantitative and quantitative PCR analyses were performed on total RNA prepared with an RNeasy kit. The reverse transcriptase reaction was carried out with 1 μg total RNA as a template to synthesize cDNA using SuperScript II reverse transcriptase (Invitrogen) and random hexamers (Invitrogen), according to the manufacturer instructions. For semi-quantitative PCR analysis, cDNA and primers were added to the GoTaq Master Mix (Promega, Madison, WI) to give a total reaction volume of 20 μL. The reactions were sampled after 30 cycles under different PCR conditions, to monitor product accumulation. For quantitative PCR analysis, cDNA and primers were added to the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) to give a total reaction volume of 15 μL. PCR reactions were then performed using an iCycler thermocycler (Bio-Rad). Conditions were set to the following parameters: 10 min at 95°C, followed by 45 cycles each of 15 s at 95°C and 1 min at 60, 62, or 64°C. The primers used for PCR analyses were as follows: Mcpt1, forward, 5′-TGTAATTCCCTTGCC TGGTC-3′, and reverse, 5′-TACAATACCATGGGCCACA C-3′; Mcpt2, forward, 5′-TGTGGTGGGTTTCTCATAG C-3′, and reverse, 5′-CTTTCCTGTTTTCCCCCATC-3′; Mcpt4, forward, 5′-ACTTTATCAAGCCGGGGAAG-3′, and reverse, 5′-ATGAGGAGATTCGGGTGAAG-3′; Rasgrp1, forward, 5′-TGGTGTTCGAGTGCAAGAAG-3′, and reverse, 5′-ACG ATTCTGTTTGGGTGCTC-3′; CD8a, forward, 5′-TTTTCTG CCATGAGGGACAC-3′, and reverse, 5′-ATCACAGGCGA AGTCCAATC-3′; Gzma, forward 5′-ATGTGGCTATCCTT CACCTACC-3′, and reverse, 5′-GCCTCGCAAAATACCATC AC-3′; Ccl8, forward, 5′-TCTACGCAGTGCTTCTTTGC-3′, and reverse, 5′-AACTTCCAGCTTTGGCTGTC-3′; Ccl19, forward, 5′-TTCAGCCTGCTGGTTCTCTG-3′, and reverse, 5′-GCTGTTGCCTTTGTTCTTGG-3′; Cxcl9, forward, 5′-TCCTCTTGGGCATCATCTTC-3′, and reverse, 5′-GGGGTG TTTTGGGTTTTCTG-3′; Ccr2, forward, 5′-TTGACCACCT TCCAGGAATC-3′, and reverse, 5′-TTTACAACCCAACCG AGACC-3′; reg3β, forward, 5′-GGCTTCATTCTTGT CCTCCA-3′, and reverse, 5′-AATTCGGGATGTTTGCTGTC-3′; reg3γ, forward, 5′-TCACCACCATGTCCTGGATGCTG-3′, and reverse, 5′-CTCCACCTCAGAAATCCTGAGGC-3′; claudin-4, forward, 5′-ATGTCATCCCCAAGGGTACA-3′, and reverse, 5′-ACATTGCCTGGGAATCTCCT-3′; β-actin, forward, 5′-TTGGGTATGGAATCCTGTGGCATC-3′, and reverse, 5′-CGGACTCATCGTACTCCTGCTTGC-3′.

Table 2. Effect of vitamin B6 supplementation on colon gene expression in AOM-treated mice

Gene ID	Gene symbol	Gene description	*P* value	Fold
Mast cell protease and related protein				
NM_011246	*Rasgrpl*	RAS guanyl releasing protein 1	0.007	0.67
T cell marker and related protein				
NM_001081110	*Cd8a*	CD8 antigen, alpha chain	0.001	0.73
NM 013487	*Cd3d*	CD3 antigen, delta polypeptide	0.002	0.75
NM_010370	*Gzma*	granzyme A	0.000	0.64
NM_010689	*Lat*	linker for activation of T cells	0.000	0.73
NM_010654	*Klrdl*	killer cell lectin-like receptor, subfamily D, member 1	0.017	0.71
NM_019465	*Crtam*	cytotoxic and regulatory T cell molecule	0.006	0.59
Cytokine				
NM_021443	*Ccl8*	chemokine (C-C motif) ligand 8	0.004	0.75
NM_019508	*1117b*	interleukin 17B	0.000	4.29
Cytokineinduced protein				
NM_011940	*Ifi202b*	interferon activated gene 202B	0.004	0.70
NM_008332	*Ifit2*	interferon-induced protein with tetratricopeptide repeats 2	0.003	0.63
NM_025378	*Ifitm3*	interferon-induced transmembrane protein 3	0.002	0.63
NM_029000	*Gvinl*	GTPase, very large interferon inducible1, transcript variant A	0.025	0.72
Secreted protein				
NM_011036	*Pap*	pancreatitis-associated protein	0.000	0.48
NM_011260	*Reg3g*	regenerating islet-derived 3 gamma	0.000	0.61
NM_010094	*Lefty 1*	left right determination factor 1	0.000	0.48
Metabolism				
NM_021282	*Cyp2e1*	cytochrome P450, family 2, subfamily e, polypeptide 1	0.001	1.82
NM_019487	*Hebp2*	heme binding protein 2	0.001	1.98
Others				
AK090125	*AK090125*	melanoma antigen	0.000	0.13
NM_001045539	*Xlr5d*	X-linked lymphocyte-regulated 5D	0.000	0.36
XM_144765	*Igkv1-135*	immunoglobulin light chain variable region	0.000	2.24
NM_009518	*Wnt10a*	wingless related MMTV integration site 10a	0.000	1.89

List of differentially expressed genes grouped into functional categories. DNA microarray analysis was repeated with the Cy3 and Cy5 dyes reversed (a dye swap), and fold change (Fold) represents the average of mRNA expression level in AOM-treated mice with a 35 mg PN HCl/kg diet (group E) relative to a 1 mg PN HCl/kg diet (group D).

2.5 Statistical analyses

Values are presented as means ± SE. Statistical significance was determined by one-way ANOVA and Duncan's multiple-range test. Differences were considered significant for $p < 0.05$ (*) and $p < 0.01$ (**).

3 Results

3.1 Pathological findings of AOM treatment

Both animal and epidemiological studies have shown that vitamin B6 supplementation plays a role in suppressing colon tumorigenesis. Our previous studies have shown that vitamin B6 decreases the incidence of colonic ACF, precursor lesions of colon cancer, in mice receiving AOM [10]. These results suggest that dietary vitamin B6 exerts a critical role in the early stages of colon tumorigenesis. The purpose of this study was to exhibit the potential anti-tumorigenic properties of vitamin B6 at the molecular level with particular emphasis on the biological significance of genomic activity related to dietary vitamin B6. Initially, we used DNA microarray analysis to understand molecular events in the early stage of colonic ACF development in mice receiving AOM. Body weight and food intake were not significantly different between the two groups (group C and D, $n = 10$, Supporting Information Table S1). We isolated total RNA from colon tissues from each group and compared gene expression profiles between the control and AOM-treated mice (group C and D). Microarray data analysis indicated that 538 transcript levels were significantly upregulated in the colon in response to

AOM treatment ($p < 0.02$, Supporting Information Table S3). In particular, AOM treatment increased mRNA expression of genes that are closely related to mast cells and cytotoxic T lymphocytes (CTLs) (Table 1). To confirm the differential expression of these genes, total RNA from individual mice in group C and group D were subjected to quantitative PCR (Fig. 2). mRNA expression of mast cell-specific proteases, *Mcpt1*, *Mcpt2*, and *Mcpt4*, were significantly increased in mice receiving AOM, suggesting mast cell infiltration of the interstitium in response to AOM treatment. CTLs defend against intracellular pathogens by releasing proapoptotic mediators from the cytotoxic granules, granzyme A (Gzma), and B (Gzmb), which are the most abundant granule serine proteases that induce apoptosis of targeted cells. *Cd8a* and granzyme mRNAs are significantly upregulated after AOM treatment, indicating increased infiltration of CD8+ T cells in the colons of mice receiving AOM. Furthermore, several chemokines and chemokine receptor mRNAs are highly expressed in AOM-treated mice, suggesting that these molecules are involved in the migration and infiltration of immune cells. As shown in Fig. 3A, increased mRNA levels, including those for *Mcpts* and *Cd8a*, were observed in response to AOM treatment, 1 week after an AOM injection. *Cd8a*-positive cells were also seen in the colonic mucosa 4 weeks after AOM treatment (Supporting Information Fig. S1). To understand the mechanism of mast cell and CD8+ T cell migration and the interaction between mast cells and CD8+ T cells, mRNA expressions of individual mice were estimated using Pearson's correlation coefficient. Importantly, a striking correlation was observed between *Mcpt1* and *Cxcl9* mRNA expression ($r = 0.627$; $p < 0.01$, Fig. 3B). *Mcpt1* and *Rasgrp1* mRNA levels revealed a positive correlation ($r = 0.769$; $p < 0.01$), however, *Mcpt1* and *Cd8a* mRNA levels were not significantly correlated ($r = 0.353$, Supporting Information Fig. S2), suggesting no mechanistic link between mast cell and CD8+ T cell infiltrations.

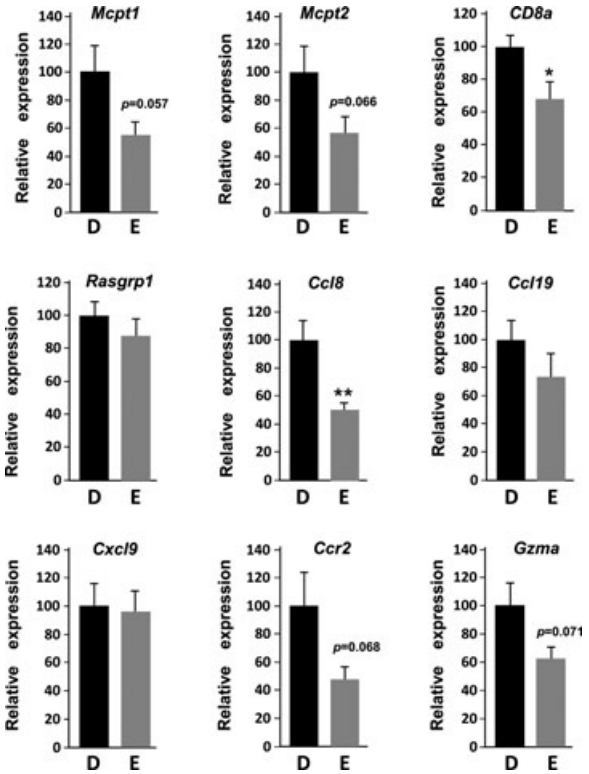

Figure 4. Effects of dietary vitamin B6 on colon gene expression by AOM treatment. One μg of total RNAs from individual mice ($n = 10$) in group D and group E was extracted and subjected to quantitative PCR to examine mRNA expression level of selected genes from Table 2. All values are normalized to β-actin levels. $^*P < 0.05$, $^{**}P < 0.01$ compared with those of group D.

3.2 Effects of dietary vitamin B6 on colon gene expression by AOM treatment

The second aim of this study was to explore the relationship between vitamin B6 activity and colon gene expression in mice receiving AOM. We analyzed the gene expression profiles of two groups that received AOM treatment with either a 1 mg PN HCl/kg diet (group D, $n = 10$) or a 35 mg PN HCl/kg diet (group E, $n = 10$) using DNA microarray analysis. We noted that dietary vitamin B6 downregulated *Cd8a* and granzyme A mRNA expression ($p < 0.05$, Table 2). To determine the expression levels of the mRNA related to CTLs and mast cells, we isolated total RNA from colon tissues from individual mice in group D and group E, and subjected the total RNA to quantitative PCR analyses. As shown in Fig. 4, vitamin B6 supplementation had a tendency to decrease *Mcpt1* and *Mcpt2* mRNA expression ($p = 0.057$ and 0.066, respectively), whereas, *Cd8a* and *Ccl8* mRNA expression was significantly downregulated by dietary vitamin B6.

3.3 Effects of dietary vitamin B6 on colon gene expression in normal animals

The final phase of this study was to determine what effect long-term feeding with vitamin B6 has on genomic activity in colons under normal conditions. Twenty mice were divided into two groups ($n = 10$) and fed either a 1 mg PN HCl/kg diet or a 35 mg PN HCl/kg diet for 8 weeks. According to the results from DNA microarray analysis, 63 transcript levels were significantly upregulated in the colon due to vitamin B6 supplementation, and 41 transcript levels were downregulated ($p < 0.02$, Table 3 and Supporting Information Table S4). Among the upregulated genes, we focused on claudin-4 (*Cldn4*) mRNA expression in colons. We analyzed total RNA from colon tissues from individual mice, because *Cldn4* is well known as a primary barrier component of epithelial cell tight junctions. *Cldn4* mRNA levels were significantly

Table 3. Effect of vitamin B6 supplementation on colon gene expression in normal mice

Gene ID	Gene symbol	Gene description	P value	Fold
Cell cell interaction				
NM_009903	*Cldn4*	Claudin 4	0.000	4.18
NM_008127	*Gjb4*	gap junction membrane channel protein beta 4	0.000	2.07
NM_009866	*Cdh11*	Cadherin 11	0.001	1.61
Cell structure and motility				
NM_026556	*Dynll2*	dynein light chain LC8-type 2	0.000	4.03
NM_022992	*Arl6ip5*	ADP-ribosylation factor-like 6 interacting protein 5	0.000	3.15
NM_130875	*Krtrap16-7*	keratin associated protein 16-7	0.001	1.64
DNA- and RNA-binding				
NM_027427	*Taf15*	TAF15 RNA polymerase II, TATA box binding protein associated factor	0.000	5.02
NM_172434	*Tnrc4*	trinucleotide repeat containing 4	0.000	2.64
Steroid hormone and lipid metabolism				
NM_013478	*Azgpl*	alpha-2-glycoprotein 1, zinc	0.000	2.05
NM_024228	*Gdpd3*	glycerophosphodiester phosphodiesterase domain containing 3	0.000	1.81
NM_008880	*Plscr2*	phospholipid scramblase 2	0.002	0.59
NM_198030	*Hsd17b13*	hydroxysteroid (17-beta) dehydrogenase 13	0.001	0.62
Other metabolism				
NM_021282	*Cyp2e1*	cytochrome P450, family 2, subfamily e, polypeptide 1	0.000	3.05
NM_008630	*Mt2*	metallothionein 2	0.000	2.27
NM_013602	*Mt1*	metallothionein 1	0.006	1.49
Secreted proteins				
NM_011179	*Psap*	prosaposin	0.000	4.37
NM_011036	*PAP*	pancreatitis-associated protein	0.001	0.41
NM_011260	*Reg3y*	regenerating islet-derived 3 gamma	0.002	0.60
Infection and inflammation				
NMJ98193	*Raetle*	retinoic acid early transcript 1E	0.000	3.53
NM_053151	*Klra21*	killer cell lectin-like receptor subfamily A, member 21	0.005	0.50
NM_019508	*1117b*	interleukin 17B	0.000	0.55
NM_008332	*Ifit2*	Interferon-induced protein with tetratricopeptide repeats 2	0.000	0.44

List of differentially expressed genes grouped into functional categories. DNA microarray analysis was repeated with the Cy3 and Cy5 dyes reversed (a dye swap), and fold change (Fold) represents the average of mRNA expression level in mice with a 35 mg PN HCl/kg diet relative to a 1 mg PN HCl/kg diet.

increased in the 35 mg PN group compared to the 1 mg PN group (by 60%; $p < 0.05$, Fig. 5). We also focused on the downregulation of the regenerating islet-derived (Reg) gene family, including *Reg3*β or *Pap* (Pancreatitis-associated protein) and *Reg3*γ mRNA expression in colons in response to dietary vitamin B6 (Fig. 6A and B) as vitamin B6 supplementation also lowered *Pap* and *Reg3*γ mRNA levels in the colons of mice receiving AOM. Interestingly, *Pap* and *Reg3*γ mRNA levels showed a strong correlation ($r = 0.945$, Fig. 6C), suggesting the genomic action of dietary vitamin B6 via a common mechanism. We examined the effect of vitamin B6 supplementation on *Reg3*γ mRNA expression in rat colons. For rats with vitamin B6 repletion after a vitamin B6-deficient diet, *Reg3*γ mRNA expression was actually downregulated in colons (Fig. 6D).

4 Discussion

In the present study, we analyzed colon gene expression in mice receiving AOM. The results show that vitamin B6 supplementation selectively regulates mRNA expression in colons. This study also provides important information about early colon pathology prior to ACF formation induced by AOM treatment. In particular, several mRNAs that are expressed in mast cells or CTLs are significantly upregulated as an acute response to AOM treatment. Mast cells are well known to play important roles in the inflammatory process, and, when activated, mast cells secrete granules, including histamine and several mast cell specific proteases into the interstitium, attracting, and activating leukocytes [21]. There are limited reports on the pathological roles of mast cells

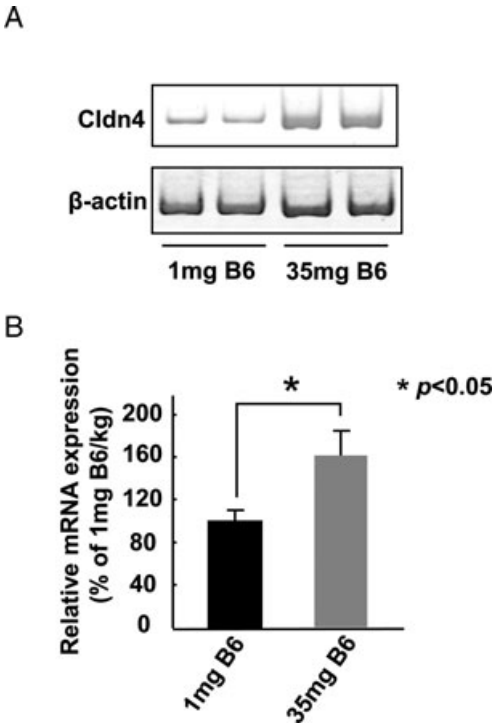

Figure 5. Effects of dietary vitamin B6 on colon *Cldn4* gene expression in normal mice. A, Pooled total RNAs from colons of two groups were subjected to semiquantitative RT-PCR. The level of β-actin (β-actin) transcript was used as a control. B, To confirm the differential expression of the *Cldn4* gene, one μg of total RNAs from individual mice ($n = 10$) was subjected to quantitative PCR. Values are normalized to β-actin levels. $*P < 0.05$ compared with that of 1 mg/kg PN (1mg B6).

in colon carcinogenesis; however, recent reports have suggested that mast cells are involved in the transformation process of several cancer cells [22–24]. Infiltrated mast cells are reportedly found in higher concentrations in the lesions associated with melanoma, mammary carcinoma, and colon cancers, and these mast cells play a role in angiogenesis via vascular endothelial growth factor A secretion and angiopoietin 1. Interestingly, our study shows a strong correlation between *Mcpt1* and *Cxcl9* mRNA levels. *Cxcl9* (mRNA) was previously shown to be highly produced in damaged epithelial cells [25], suggesting that AOM-induced epithelial dysfunction allowed mast cell infiltration with an increased Cxcl9 expression level. In a colon carcinogenesis model using *APCΔ468* mice, mast cells were located in adenomatous polyps and were involved in malignant progression, promoting angiogenesis [26]. Additionally, infiltrated mast cells were observed at the late stage in ACF development in the colons of mice receiving AOM with dextran sodium sulfate [27]. The results of this study also suggest mast cells may be pathologically involved in the process subsequent to ACF formation. However, further experiments to determine the functional significance of mast cells during colon carcinogenesis are needed, using, for example, mast cell-deficient mice, such as W/Wv mice.

In this study, we analyzed colon gene expression in mice receiving AOM and showed that vitamin B6 supplementation significantly downregulates *Cd8a* and *Ccl8* mRNA expression in colons. $CD8^+$ T cells are reportedly involved in intestinal inflammation and relapsing colitis in laboratory animals and human beings [28, 29]. Chronic inflammation and carcinogenesis have been considered to be mechanistically linked. Chronic inflammation leads to upregulation of a series of enzymes and cytokines involved in cell signaling in affected tissues. In particular, inflammatory enzymes, including inducible nitric oxide synthase and cyclooxygenage-2, are shown to play critical roles in the development of colon carcinogenesis from animal experiments using specific inhibitors [30, 31]. The CCl8 protein, identified as monocyte chemoattractant protein 2 (MCP-2), is chemotactic and activates many types of immune cells including mast cells, monocytes, and T cells that are involved in the inflammatory response [32], suggesting a critical role for CCl8 as a chemoattractant at inflamed colon sites following AOM treatment. Green tea and its constituents are well-studied food factors that inhibit colon tumorigenesis in animal models, including mice receiving AOM or 1,2-dimethylhydrazine and $Apc^{Min/+}$ mice [33–35]. A key constituent of green tea, (–)-epigallocatechin-3-gallate (EGCG), was also shown to suppress ACF formation in F344 rats receiving AOM [33]. Mochizuki *et al.* furthermore reported that EGCG protects against colon injury induced by 2,4,6-trinitrobenzene sulfonic acid in rats, partially via lowering the activity of mast cells [36]. Catechins have been shown to inhibit histamine release from mast cells in vitro [37, 38], whereas, an intriguing feature of EGCG is it directly binds to CD11b on $CD8^+$ T cells and modulates the migration of $CD8^+$ T cells to inflammation sites [39]. Although only limited information is available on the pathological roles of these immune cells during the early process of AOM-induced colon tumorigenesis, the suppression of mast cell and CD8+ T cell functions may be a useful target for experiments that aim to discover new food factors that prevent the development of colonic ACF.

The current study explored the effects of dietary vitamin B6 on genomic activity in colons under normal conditions using DNA microarray analysis. We showed that *Cldn4* mRNA levels were significantly upregulated by vitamin B6 supplementation. Recent studies on flavonoids have shown quercetin to have a remarkable effect on intestinal barrier functions [40]. Quercetin reportedly enhances the barrier function via regulating transcriptional expression of the tight junction protein CLDN4 [41, 42]. These studies suggest that increased *Cldn4* mRNA levels represent an important protective effect of food components against barrier disturbance in intestinal inflammation. We further focused on the two genes, *Reg3*γ and *Pap* (also known as *Reg3*β), which are downregulated in colons by vitamin B6 supplementation. Dietary vitamin B6 also decreases *Reg3*γ and *Pap* mRNA expression in mice receiving AOM. The Reg gene family was

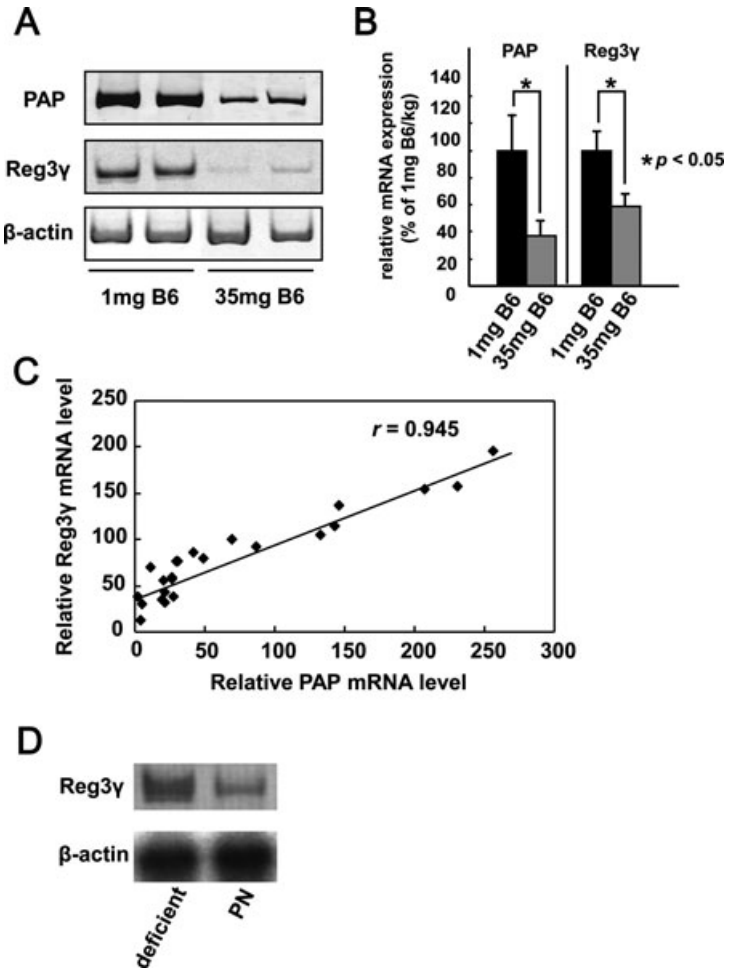

Figure 6. Effects of dietary vitamin B6 on Reg family gene expression in normal animals. *A*, Pooled total RNAs from colons of two groups (n = 5) were subjected to semiquantitative RT-PCR. The level of β-actin (β-actin) transcript was used as a control. *B*, To confirm the differential expression of the *Cldn4* gene, one μg of total RNAs from individual mice ($n = 10$) was subjected to quantitative PCR. Values are normalized to β-actin levels. $^*P < 0.05$ compared with that of 1 mg/kg PN (1mg B6). *C*, The relative mRNA expression level of each gene was determined by quantitative PCR, normalized to β-actin level, and analyzed with Pearson's correlation coefficient. *D*, After being fed a PN-deficient basal diet for 3 weeks, the rats were divided into two groups ($n = 7$), and fed PN-deficient basal diet (−PN group, deficient) or basal diet containing 35 mg/kg PN (+PN group, PN) for 4 days. One microgram of pooled total RNAs from colons of two groups was subjected to semi-quantitative RT-PCR. The level of β-actin (β-actin) transcript was used as a control.

originally isolated and characterized as lectin-like secretion proteins that play a role in antimicrobial host defense. Interestingly, several Reg mRNAs are reportedly expressed in cancer cells including pancreatic and colorectal cancers [43–45], whereas, serum PAP levels were significantly elevated in patients with gastric, colorectal, or pancreatic cancers compared with healthy subjects [46–48]. Also, *Reg3*γ and *Pap* mRNA expression was shown to be upregulated in chronic colitis, suggesting that Reg mRNA expression is upregulated in colonic inflammation [49]. Because dietary vitamin B6 has been shown to have anti-inflammatory effects, including prevention of contact dermatitis and stomatitis, this study also suggests vitamin B6 may be nutritionally effectual in cases of chronic inflammatory diseases.

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