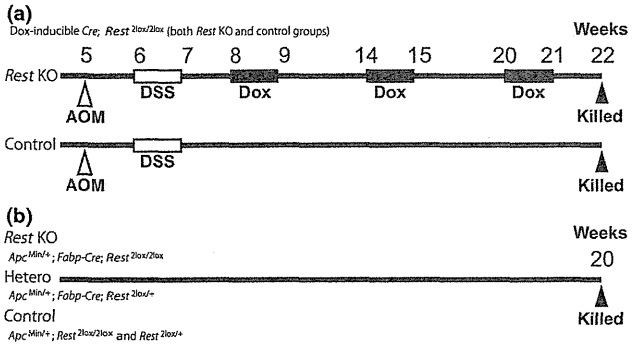

Fig. 1. Schematic drawing of the experimental protocols. The two experiments are different in terms of the timing of the *Rest* recombination. (a) Protocol 1 (*Rest* ablation at the post-initiation phase). (b) Protocol 2 (*Rest* ablation at the pre-initiation phase). Black arrowheads, mice killed; black bars, doxycycline (Dox); white arrowheads, azoxymethane (AOM); white bars, dextran sodium sulfate (DSS). KO, knockout.

Cre-mediated genetic recombination in the intestinal epithelia started before the completion of intestinal morphogenesis, thereby mimicking when the *Rest* recombination occurs before the initiation of carcinogenesis.

In protocol 1, doxycycline-inducible *Cre*; $Rest^{2lox/2lox}$ mice were separated into a *Rest* KO group ($n = 27$) and a control group ($n = 11$). Five-week-old mice were given a single i.p. injection of azoxymethane (15 mg/kg body weight; Wako, Osaka, Japan), a colon-specific carcinogen. One week later, the mice were fed dextran sodium sulfate (20 mg/mL; Wako), a potent tumor promoter for colon tumorigenesis, in their drinking water for 1 week. The mice in the *Rest* KO group were fed 2 mg/mL doxycycline (Sigma, St. Louis, MO, USA) in their drinking water, supplemented with 10 mg/mL sucrose three times per week (at weeks 8, 14, and 20), whereas mice in the control group were fed tap water throughout the experiment. All mice were killed at 22 weeks of age.

In protocol 2, intestine-specific *Rest* KO mice ($Apc^{Min/+}$; $Fabp\text{-}Cre^{+}$; $Rest^{2lox/2lox}$ mice, $n = 23$), heterozygous *Rest* KO mice ($Apc^{Min/+}$; $Fabp\text{-}Cre^{+}$; $Rest^{2lox/+}$ mice, $n = 32$), and control mice ($Apc^{Min/+}$; $Fabp\text{-}Cre^{-}$; $Rest^{2lox/2lox}$ mice and $Apc^{Min/+}$; $Fabp\text{-}Cre^{-}$; $Rest^{2lox/+}$ mice, $n = 26$) were examined for the development of colon tumors. All mice were housed in rooms without any chemical treatment during the experiment and were killed at 20 weeks of age.

In both protocols, the colons were cut open longitudinally, then washed with PBS. Visible tumors (larger than 0.5 mm in their maximum diameters) on the colon mucosa were counted, and their maximum diameters were measured. Tumor samples were fixed in 10% buffered formalin for 24 h and embedded in paraffin. Sections were stained with H&E, then serial sections were used for the immunohistochemical analysis. Immunostaining was carried out using an avidin–biotin immunoperoxidase assay. The primary antibodies used in the immunostaining were anti-β-catenin (1:1000 dilution; BD Biosciences, San Diego, CA, USA), anti-chromogranin A (1:1000 dilution; Dako, Carpinteria, CA, USA), and anti-Ki-67 (1:100 dilution; Dako).

Crypt isolation. In order to examine the effect of *Rest* ablation in the colonic epithelium, we carried out crypt isolation to exclude the contaminating stromal cells in the colonic mucosa, as described previously.[29] The removed colon was cut into three equal segments. The distal segment was used for crypt isolation.

Confirmation of *Rest* recombination. To examine the recombination status of the conditional *Rest* allele in the colon, we carried out a Southern blot analysis of the *Rest* loci. Total DNA was extracted from isolated intestinal crypts of doxycycline-inducible *Rest* knockout mice at the indicated time intervals (Fig. 2a). DNA samples (10 μg each) were digested with Mfe1 (Bio-Rad, Hercules, CA, USA). The digested DNA samples were electrophoresed, transferred onto nylon membranes, and hybridized with a DIG probe against the *Rest* loci in PerfectHyb (Toyobo, Osaka, Japan).[26] Signals were detected by chemiluminescence with LAS-4000 (Fujifilm, Tokyo, Japan). We also carried out a PCR-based analysis to examine the recombination of the *Rest* gene in colon tumors. DNA was isolated from formalin-fixed paraffin-embedding blocks using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA, USA). The PCR was carried out using primers specific for mouse floxed *Rest*. The primers for the $Rest^{2lox}$ allele were forward (F) (5′-CCCTTATGGGTGCAAGTGTT-3′) and reverse (R) (5′-GGGGGACAAAGCCACTCTA-3′). The primers for $Rest^{1lox}$ allele were F (5′-GGGTGCAAGTGTTCTCTTGTCT-3′) and R (5′-CAAGTAACTAAAAATTAGGAACTACCG-3′).

Real-time PCR analysis. Total RNA was extracted from the isolated intestinal crypts as described previously (Fig. 2a), and from the colon tumors of $Apc^{Min/+}$ mice using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA (0.5 μg each) was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR was carried out with the Thermal Cycler Dice Real Time System Single (Takara, Kyoto, Japan) using the SYBR Green (Takara) method. The primer sequences used in the quantitative real-time PCR analyses for *Bdnf*, *Tubb3*, and *Rest* were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). The primers for β-*actin* were F (5′-CATCCGTAAAGACCTCTATGCCAAC-3′) and R (5′-ATGGAGCCACCGATCCACA-3′). The primers for *Gfp* were F (5′-ACCAGCAGAACACCCCCATC-3′) and R (5′-AGCTCGTCCATGCCGAGAGT-3′). The primers for *Syt4* were F (5′-TGCTTTTGGCCTCGTCTTCA-3′) and R (5′-GCGGTTTTACCCTTCACTTCAC-3′).

Statistical analysis. Statistical significance was evaluated using either Student's *t*-test or Welch's *t*-test for paired samples. The results of experiments are presented as the mean ± SEM. $P < 0.05$ was considered to indicate a significant difference.

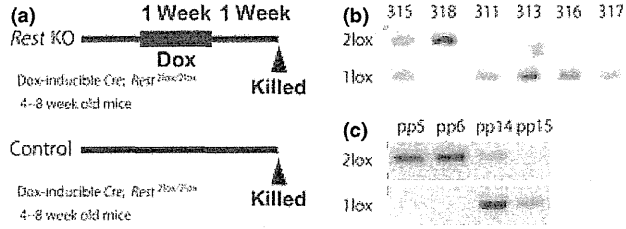

Fig. 2. Genetic recombination of the *Rest* alleles. (a) Doxycycline (Dox)-mediated recombination of *Rest* in protocol 1. A schematic drawing of the experiment. *Rest* conditional knockout (KO) mice with Dox-inducible *Cre* alleles at 4–8 weeks old were treated with 0.2% Dox in their drinking water for 1 week (black bar). Black arrowheads, mice killed. (b) In the Southern blot analysis, in contrast to the control crypts (non-Dox-treated crypts; 315, 318), most of the 2lox alleles in the Dox-treated colonic crypts (311, 313, 316, 317) recombined into 1lox KO alleles. In some cases, the control crypts also contained 1lox alleles (315), probably due to the leaky expression of the *Cre* recombinase. (c) *Rest* recombination in colon tumors in protocol 2. We carried out a PCR-based analysis for *Rest* recombination using colon tumor sections in protocol 2. We could confirm the 1lox KO alleles in five out of six tumors in the *Rest* conditional KO mice with the *Fabp-Cre* allele (pp14 and pp15). However, no recombined *Rest* alleles were observed in any of the nine tumors examined from control mice without the *Fabp-Cre* allele (pp5 and pp6).

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Results

Cre expression induces genetic ablation of *Rest* in colonic mucosa. In order to examine the efficiency of *Rest* recombination in protocol 1, we carried out a Southern blot analysis using genomic DNA from isolated colonic crypts of doxycycline-inducible *Cre*; $Rest^{2lox/2lox}$ mice (Fig. 2a). The Southern blot analysis revealed that the majority of the $Rest^{2lox}$ alleles in the colonic crypts were recombined into $Rest^{1lox}$ alleles in doxycycline-treated mice (the $Rest^{1lox}$ allele was confirmed in seven out of eight colonic crypts). However, partial recombination of the *Rest* allele was also observed in some cases in the non-treated group (5 out of 13 colonic crypts), probably due to the leaky expression of the *Cre* transgene (Fig. 2b).

In protocol 2, *Rest* recombination was examined using a PCR assay with genomic DNA from formalin-fixed paraffin-embedding specimens of *Fabp-Cre*$^+$; $Rest^{2lox/2lox}$ mice. The recombined *Rest* gene ($Rest^{1lox}$ allele) was only detected in mice containing the *Fabp-Cre* allele, and the $Rest^{2lox}$ allele was also detected in these mice, indicating partial recombination, which is consistent with a previous experiment showing a recombination efficiency of approximately 50% in the colonic crypts by the *Fabp-Cre* allele (Fig. 2c).[28] These results indicate that genetic ablation of *Rest* was induced successfully in the colons of two independent *Cre*-expressing mouse models. In both protocol 1 and 2, despite the presence of the $Rest^{1lox}$ allele, the mice were healthy, and no detectable difference was observed in the histological analyses in comparison to the control mice.

Transcript levels of *Rest* and Rest-targeted genes in colonic mucosa. We next examined the expression levels of *Rest* in colonic tumors of $Apc^{Min/+}$ mice. The quantitative real-time PCR analysis revealed that *Rest* expression is not different between the colon tumors and non-neoplastic normal mucosa, suggesting that loss of *Rest* is not involved in the colon tumorigenesis of $Apc^{Min/+}$ mice (Fig. 3a). In order to elucidate the effect of genetic ablation of *Rest* in the colonic mucosa, we measured the mRNA expression levels of the *Rest* gene and Rest-targeted genes in the isolated colonic crypts of doxycycline-inducible *Cre*; $Rest^{2lox/2lox}$ mice by quantitative real-time PCR analysis (Fig. 2a). Consistent with the genetic recombination observed in the Southern blot analysis, the expression of *Rest* in the colonic crypts was significantly decreased in the doxycycline-treated mice in comparison with the non-treated mice (Fig. 3b). In line with the decreased expression of the *Rest* gene, the expression of *Gfp* was also downregulated in the doxycycline-treated crypts (Fig. 3c). In contrast, the expression of *Syt4*, *Bdnf*, and *Tubb3*, which are known to be Rest-targeted genes, were significantly upregulated in the doxycycline-treated crypts (Fig. 3d). These results indicate that the genetic recombination of *Rest* results in the rapid derepression of the Rest-targeted genes, suggesting that *Rest* plays a role in the repression of neuronal genes in the colonic crypts.

Macroscopic analysis of colon tumor development. Macroscopically, polypoid tumors were observed in colonic mucosa of mice during both experiments. In protocol 1, genetic recombination into the $Rest^{1lox}$ allele was only confirmed in *Cre*-induced tumors ($n = 4$). In protocol 2, the recombined *Rest* allele ($Rest^{1lox}$ allele) was detectable in the majority of *Rest* KO tumors (5 out of 6 tumors), whereas all control tumors ($n = 9$) retained non-recombined *Rest* alleles ($Rest^{2lox/2lox}$) (Fig. 2c). In protocol 1, the multiplicity of colon tumors was 0.37 ± 0.14/mouse in the *Rest* KO mice and 0.18 ± 0.18/mouse in control mice (Fig. 4a). In protocol 2, the multiplicity and the maximum diameter of colon tumors were 4.96 ± 0.57/mouse and 3.31 ± 0.12 mm in the *Rest* KO mice, 4.81 ± 0.51/mouse and 3.37 ± 0.12 mm in the *Rest* heterozygous mice, and 4.35 ± 0.71/mouse and 3.68 ± 0.15 mm in the control mice, respectively (Fig. 4b,c). No macroscopic tumors were observed

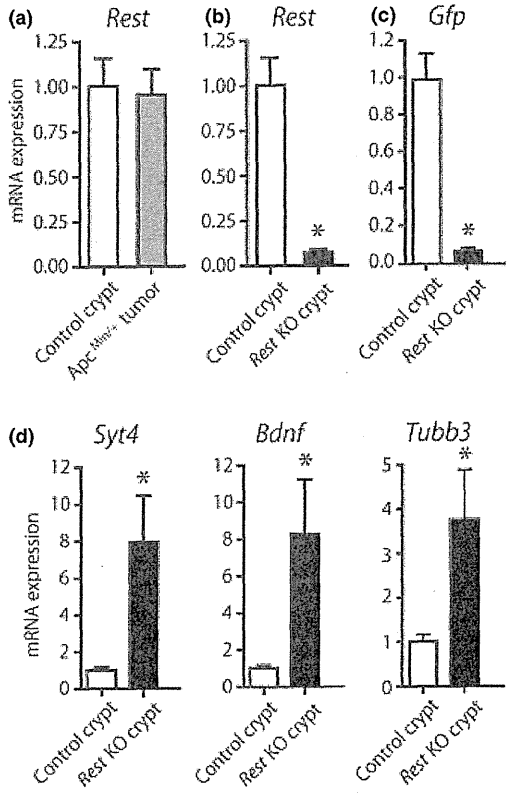

Fig. 3. Transcriptional levels of *Rest* and Rest-targeted neuronal genes in the colon mucosa. (a) $Apc^{Min/+}$ colonic tumors with wild-type *Rest* alleles had nearly the same *Rest* expression level as non-neoplastic crypts. (b–d) Expression levels of *Rest* (b) and *Gfp* (c) were downregulated after the *Rest* ablation in colonic crypts, whereas the expression levels of the Rest-targeted genes (d), *Syt4*, *Bdnf*, and *Tubb3* were significantly upregulated. The mRNA expression levels were analyzed by quantitative real-time PCR and were normalized to the β-actin levels. Data are presented as the mean ± SEM of 13 independent samples. *$P < 0.05$.

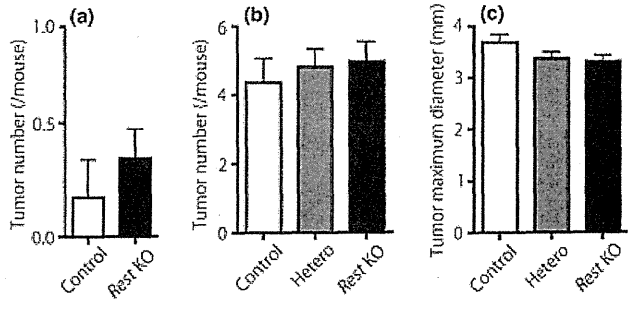

Fig. 4. Macroscopic analysis of colon tumor development. (a) The number of macroscopic tumors in protocol 1. (b,c) The number of macroscopic tumors (b) and the size of tumors (c) in protocol 2. Data are presented as the mean ± SEM.

in the intestine-specific *Rest* KO mice without the Apc^{Min} allele ($Apc^{+/+}$; *Fabp-Cre*$^+$; $Rest^{2lox/2lox}$ mice, $n = 4$). Furthermore, a lack of colon tumor development in *Fabp-Cre*$^+$; $Rest^{2lox/2lox}$ mice was also confirmed in the mice older than 12 months (data not shown), thus suggesting that *Rest* ablation alone is not sufficient to initiate colon tumorigenesis.

Collectively, the multiplicity of colon tumors in the *Rest* KO mice was slightly higher than that in the control mice, but the difference between the *Rest* KO and control mice was not

significantly different (protocol 1: *Rest* KO *vs.* control, $P = 0.46$; protocol 2: *Rest* KO *vs.* control, $P = 0.51$). In addition, no statistically significant difference was observed in the size of the colon tumors, although there was a tendency for the tumor size to correlate with the expression of *Rest* (protocol 2: *Rest* KO *vs.* control, $P = 0.054$).

Histological and immunohistochemical analyses of colon tumors. Colon tumors were processed for histological examinations. As *Rest* recombination occurs partially by the *Fabp-Cre* transgene in protocol 2, the genetic status of *Rest* was determined in each tumor by PCR using primers specific for the $Rest^{2lox}$ and $Rest^{1lox}$ alleles. Regardless of the *Rest* recombination, the histological analysis revealed that all colon tumors consisted of tubular dysplastic glands. There were no detectable histological differences between the colon tumors of *Rest* KO and control mice in either protocol (Fig. 5a).

We further analyzed these tumors by immunostaining for β-catenin, Ki-67, and chromogranin A. The accumulation of β-catenin protein is a critical event that occurs during colon carcinogenesis. Indeed, in the present study, the accumulation of β-catenin was observed in the dysplastic glands in the colonic tumors. However, the β-catenin immunostaining showed no detectable difference between colon tumors in the *Rest* KO and control mice in either protocol (Fig. 5a).

We also carried out Ki-67 immunostaining to compare the proliferative activities of tumor cells with different genetic status of *Rest*. We counted Ki-67 immunopositive tumor cells out of 1000 randomly selected colonic tumor cells from *Rest* KO and control mice, and calculated the ratio of Ki-67 positive tumor cells. The Ki-67 positive cell ratios in the colon tumors of *Rest* KO and control mice were $27.6 \pm 3.39\%$ ($n = 5$) and $31.4 \pm 4.84\%$ ($n = 5$) in protocol 1 ($P = 0.53$), and $44.2 \pm 3.00\%$ ($n = 10$) and $41.2 \pm 2.53\%$ ($n = 11$) in protocol 2 ($P = 0.45$), respectively (Table 1).

Rest has been shown to be a master negative regulator of neuronal differentiation. Indeed, we confirmed that genetic ablation of *Rest* leads to the derepression of neuronal gene expression. Therefore, we next examined the enteroendocrine cell differentiation of the intestinal cells after loss of *Rest* expression. Chromogranin A immunostaining was used to assess the endocrine differentiation of both non-neoplastic and tumor cells in the colon. Chromogranin A immunostaining showed no detectable differences related to the *Rest* recombination in both non-neoplastic and tumor cells (Fig. 5b). The positive cell ratios for chromogranin A in *Rest* KO and control tumors was $0.28 \pm 0.08\%$ ($n = 5$) and $0.10 \pm 0.07\%$ ($n = 5$) in protocol 1 ($P = 0.12$), and $0.07 \pm 0.02\%$ ($n = 10$) and $0.12 \pm 0.02\%$ ($n = 11$) in protocol 2 ($P = 0.17$), respectively (Table 1).

Discussion

Colon carcinogenesis is a multistage process involving genetic and epigenetic alterations of various tumor suppressor genes and oncogenes. Our previous studies identified two distinct stages of colon tumorigenesis in $Apc^{Min/+}$ mice (microadenomas and macroscopic tumors).[30] The formation of microadenomas is accompanied by the activation of the canonical Wnt pathway through the genetic loss of the *Apc* gene, leading to the accumulation of β-catenin. Recent evidence suggests that DNA methylation is closely associated with the progression of microadenomas into macroscopic tumors.[28,31–33] Although the identity of the target genes of DNA methylation involved in colon tumorigenesis remain unclear, these findings suggest that the progression of colonic tumors in $Apc^{Min/+}$ mice requires synchronous alternations in the expression of multiple genes due to global changes of epigenetic modifications.

REST, a novel candidate tumor suppressor gene in colon carcinogenesis, maintains transcriptional silencing of various genes by recruiting multiple co-factors, including Co-Rest,[27] HDAC and Sin3 complex,[34–36] histone H3 K9 methyltransferase G9a,[37] histone H3 K4 demethylase LSD1,[38] and methyl DNA binding protein MeCP2.[39] Previous experiments indicated

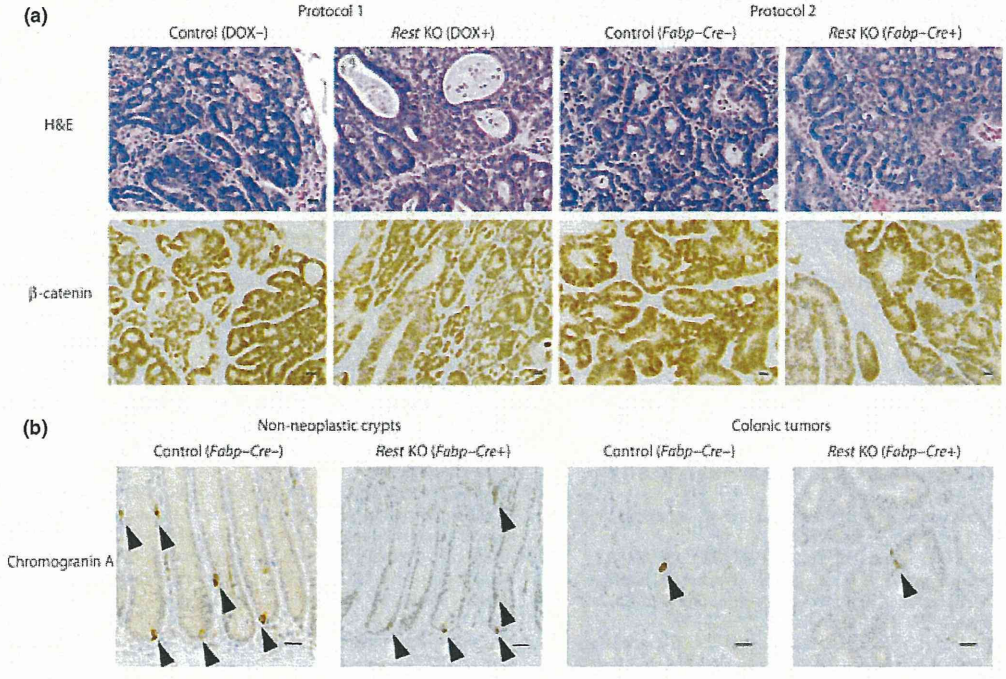

Fig. 5. Histological and immunohistochemical analyses of colonic tumors. (a) There was no detectable difference in the H&E staining (upper panels) and β-catenin immunostaining (lower panels) between the *Rest* knockout (KO) and control tumors in either protocol. (b) Chromogranin A immunostaining of colonic non-neoplastic and tumor cells. Black arrowheads, chromogranin A immunopositive cells, *Rest* KO: $Apc^{Min/+}$; $Fabp\text{-}Cre^{+}$; $Rest^{2lox/2lox}$ mice, control: $Apc^{Min/+}$; $Rest^{2lox/2lox}$ mice. Dox, doxycycline. Scale bars = 20 μm.

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Table 1. Ki-67 and chromogranin A positive tumor cell ratio in colon tumors of *Rest* knockout (KO) and control mice

		Ki-67 (%)	Chromogranin A (%)
Protocol 1	*Rest* KO	27.6 ± 3.39	0.28 ± 0.08
	Control	31.4 ± 4.84	0.10 ± 0.07
Protocol 2	*Rest* KO	44.2 ± 3.00	0.07 ± 0.02
	Control	41.2 ± 2.53	0.12 ± 0.02

Protocol 1, chemically-induced colon carcinogenesis model using doxycycline-inducible *Cre*-expressing mice. Protocol 2, $Apc^{Min/+}$ mouse colon carcinogenesis model combined with *Fabp-Cre* mouse.

thousands of REST-targeted genes in embryonic stem cells and neuronal progenitor cells.[40] REST is thus suggested to alter the chromatin structure in conjunction with its co-factors, while also regulating the transcription of the REST-targeted genes through histone modification, chromatin remodeling, and genomic methylation. Given the fact that DNA methylation, one of the most important epigenetic modifications, plays a critical role in the transition from microadenomas to macroscopic tumors in the colon of $Apc^{Min/+}$ mice, we hypothesized that the global changes in epigenetic modifications caused by *Rest* ablation might affect murine colon tumorigenesis.

In this study, we confirmed that the genetic ablation of *Rest* leads to the decreased expression of *Rest* and increased expression of Rest-targeted genes, suggesting that Rest represses the Rest-targeted genes in the colonic crypts. However, *Rest* ablation at both the pre-initiation and post-initiation phases of colon tumorigenesis showed no significant effect on tumor development in the colon. These findings indicate that loss of *Rest* expression by itself does not promote the development of colon tumors in mice. It is possible that *Rest* deletion alone might not be sufficient for the active changes of epigenetic modifications to induce the progression of colon tumorigenesis.

Rest has been regarded as a master negative regulator of neuronal differentiation in non-neuronal cells. Indeed, a targeted mutation of *Rest* in mice caused derepression of neuron-specific genes in a subset of non-neuronal tissues. Human colonic carcinoma expressing neuroendocrine genes (also called neuroendocrine carcinoma, NEC) is a highly aggressive carcinoma that comprises approximately 0.6% of colonic carcinomas.[41] Importantly, most neuroendocrine genes expressed in NEC are targets of the REST-repressing complex. Considering that the genetic deletion of *Rest* leads to the upregulation of neuronal genes in non-neuronal cells, we hypothesized that *Rest* deletion would induce neuronal differentiation in colonic tumors, thus leading to a NEC-like phenotype. In fact, previous studies have revealed that carcinomas with *REST* dysfunction frequently showed neuroendocrine characteristics.[8,12–16] However, in the present study, colon tumors lacking the *Rest* gene did not show the NEC-like histology. In addition, an increase in chromogranin A-positive cells, which is usually observed in NEC, was not detectable in the *Rest*-deleted tumors. Our results suggest that, although *Rest* ablation leads to the increased mRNA expression of neuronal genes, *Rest* ablation alone is not sufficient to induce neuronal differentiation in the colon. As the incidence of NEC is extremely rare compared to the relatively high incidence of *REST* deletion in colorectal cancer,[24,41] it is still possible that *REST* inactivation, in conjunction with additional genetic and/or epigenetic alterations, may be involved in the development of NEC. In this context, it would be interesting to examine the genetic status of *REST* in NEC in future studies.

In summary, we have shown that the genetic ablation of *Rest* does not affect the development of colon tumors in mice. These findings suggest that other genetic and/or epigenetic alterations might therefore be required to exert the tumor-promoting effect of *Rest* ablation during multistage tumorigenesis of the colon.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Genetic ablation of *Tnfα* demonstrates no detectable suppressive effect on inflammation-related mouse colon tumorigenesis

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ABSTRACT

Colorectal cancer (CRC) is one of the most serious complications of inflammatory bowel disease. Tumor necrosis factor-α (Tnfα) is a major mediator of inflammation and there is increasing evidence that Tnfα/Tnf-receptor-1 (Tnfr1) signaling may act as an endogenous tumor promoter for colon carcinogenesis. In fact, a previous study revealed that mice lacking *Tnfr*1 develop significantly fewer colonic tumors in the inflammation-related CRC model. In addition, antibodies against Tnfα have been shown to inhibit the development of inflammation-related CRC. In the present study, *Apc* Min/+; *Tnfα* −/− mice were treated with 2% dextran sodium sulfate (DSS) and the tumor development was compared with *Apc* Min/+; *Tnfα* +/+ control mice in order to investigate the role of Tnfα by itself in the inflammation-related CRC. Surprisingly, there were no detectable differences in either the severity of colonic inflammation or the expression of DSS-induced chemokines and cytokines (*Ccl*2, *Cxcl*1, *Tnfβ*, *Il*1*β*, *Il*6, and *Cox*-2) that relate to the colonic inflammation and tumorigenesis between these two groups. Furthermore, the genetic ablation of *Tnfα* did not suppress the colon tumorigenesis in comparison to the wild-type mice. Our observations suggest that intricate inflammatory responses promote the inflammation-related mouse colon tumorigenesis.

1. Introduction

The link between carcinogenesis and chronic inflammation has been recognized for certain types of cancer, including colorectal cancer (CRC) [1]. CRC is one of the serious complications of inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease [1,2]. Although previous studies have demonstrated that the link between inflammation and CRC offers a possible strategy to prevent CRC, the underlying molecular processes involved in this interaction still remain poorly understood. Tumor necrosis factor-α (Tnfα) is a key cytokine involved in inflammation, immunity and cellular organization [3]. It was first isolated from the serum of mice infected with *Bacillus-Calmette–Guerin* treated with endotoxin, and shown to replicate the ability of endotoxin to induce haemorrhagic tumor necrosis [4]. Accordingly, it was originally utilized for the treatment of patients with advanced solid tumors [5]. In contrast, recent evidence indicates that Tnfα may act as an endogenous tumor promoter in several tumor tissues. Direct evidence for the involvement of Tnfα in malignancy came from observations that a genetic disruption of the *Tnfα* gene could significantly attenuate chemically induced skin tumor formation [6–8]. In addition, *Tnf-receptor*-1 (*Tnfr*1) deficient mice had reduced oval cell (the putative hepatic stem cell) proliferation during the pre-neoplastic phase of liver carcinogenesis, correlating with fewer liver tumors than wild-type mice [9]. It has therefore been suggested that the Tnfα/Tnfr1 signaling pathway may play an important role in tumor promotion.

The predominant expression of Tnfα in colorectal cancer is observed within tumor-associated macrophages [10]. A previous study reported that Tnfα and Tnfr1 protein are expressed mainly in the infiltrating cells, such as macrophages and neutrophils which are derived from myeloid cells in inflamed colon tissue [11]. These infiltrating cells also express Cox-2 protein which is often up-regulated in colon carcinoma tissues and functionally promotes intestinal tumorigenesis [12]. Furthermore, Greten et al. reported that depleting *Ikkβ* in myeloid cells reduced the expression of pro-

Abbreviations: Tnfα, tumor necrosis factor-α; IBD, inflammatory bowel disease; Tnfr1, tumor necrosis factor-receptor-1; CRC, colorectal cancer; DSS, dextran sodium sulfate; Apc, adenomatous polyposis coli; Min, multiple intestinal neoplasia.

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inflammatory factor genes encoding Tnfα, Il1β, Il6, Kc, Cox-2 and Mmp-9 in the colon, followed by a suppression of colorectal tumor development in inflammation-related colon tumorigenesis [13].

It is noteworthy that the absence of *Tnfr*1 significantly reduces the DSS-induced infiltration of Cox-2-expressing inflammatory cells, thus leading to a reduced incidence of colonic tumors [11]. Moreover, Tnfα can significantly increase Nf-kb activation in various cell types after binding to either Tnfr1 or Tnf-receptor-2 (Tnfr2) [14]. Therefore, it has been thought that endogenous Tnfα may activate Nf-kb signaling in inflammatory cells by interacting with Tnfr1 in an autocrine/paracrine manner and activated Nf-kb increases the expression of pro-inflammatory factors, which could thus lead to the promotion of colorectal tumorigenesis. Given the fact that Tnfr1 signaling is associated with tumorigenesis in the colon, Tnfα itself might be a therapeutic target for the inflammation-related mouse colon tumorigenesis. Indeed, previous studies demonstrated that antibodies against Tnfα inhibit the development of inflammation-related CRC [15].

Several animal models of experimental colitis have been developed for investigating the pathogenesis of IBD and IBD-related CRC and these are often used to evaluate new treatments for IBD [16]. Chemically induced models of intestinal inflammation, such as the dextran sodium sulfate (DSS)-induced model and the trinitrobenzene sulfonic acid (TNBS)-induced model, are the most generally used IBD animal models because the onset of inflammation is immediate and the procedure is relatively straightforward [17,18]. In contrast to the involvement of CD4+ T cells in TNBS-induced colitis, macrophages have been shown to play a central role in DSS-induced colitis [18,19]. Importantly, once *Apc* Min/+ mice, which harbor a germ line mutation in the *Apc* gene, are exposed to DSS, colitis markedly accelerates the development of dysplasia and cancer in the colon of *Apc* Min/+ mice [20]. Therefore, DSS-treated *Apc* Min/+ mouse can be useful for the investigation of inflammation-related colorectal tumorigenesis. The current study examined the effects of the genetic ablation of *Tnfα* on colon tumorigenesis using *Apc* Min/+; *Tnfα* −/− compound mutant mice exposed to DSS in order to determine whether Tnfα by itself could be a target for the prevention/treatment of inflammation-related colon tumorigenesis.

2. Materials and methods

2.1. Animals and diets

Apc Min/+ mice in the C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *Tnfα* −/− mice were maintained in C57BL/6J background [21]. We confirmed that the cell viability of splenocytes from *Tnfα* −/− mice is reduced after the stimulation with phorbol 12-myristate 13-acetate (PMA; 80 nM) and ionomycin (1 μM) when compared with that from *Tnfα* +/+ mice, thus suggesting the distinguishable responses against inflammatory stimuli between *Tnfα* +/+ and *Tnfα* −/− mice. Compound mutant *Apc* Min/+; *Tnfα* −/− mice were generated by breeding *Apc* Min/+; *Tnfα* +/− males to *Apc* +/+; *Tnfα* +/− females. These mice were maintained on a C57BL/6J genetic background to avoid potential strain differences in phenotype. All mice were bred and maintained in a specific pathogen-free animal facility under standard 12:12 h light:dark cycle and fed on a basal diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan), and water *ad libitum* until the termination of the study.

2.2. Experimental procedures

DSS with a molecular weight of 36,000–50,000 (Wako, Osaka, Japan) was dissolved in distilled water at a concentration of 2% (w/v). Initially, 26 *Apc* Min/+; *Tnfα* +/+ mice (14 males and 12 females) and 25 *Apc* Min/+; *Tnfα* −/− mice (14 males and 11 females) were used for the macroscopic and histological study. The animals of these cohorts were given 2% (w/v) DSS in drinking water for 1 week, starting at 5 weeks of age, according to the protocol described in previous report [20]. The DSS-exposed *Apc* Min/+; *Tnfα* +/+ mice and *Apc* Min/+; *Tnfα* −/− mice were then sacrificed at 10 weeks of age for both the macroscopic inspection and histological analysis (Fig. 1a). Next, 20 male *Tnfα* +/+ mice and 19 male *Tnfα* −/− mice were treated with 2% (w/v) DSS in drinking water for 5 days, starting at 5 weeks of age, and then were sacrificed at day 0 (control, five mice in each group), day 10 (the acute phase of colonic inflammation, seven mice in each group) and day 30 (the chronic phase of colon inflammation, eight mice in *Tnfα* +/+ and seven mice in *Tnfα* −/−) after the exposure of DSS in order to compare the induction of pro-inflammatory factors in the presence or absence of *Tnfα* (Fig. 1b). Trinitrobenzene sulfonic acid (TNBS) was also used for another model of colonic inflammation. TNBS colitis was induced in five male *Tnfα* +/+ and six male *Tnfα* −/− mice according to the method of Wirtz et al. [18] with minor modifications. After the TNBS presensitization, the mice were lightly anesthetized, and an infant feeding catheter (3.5 Fr) was then carefully inserted into the colon such that the tip was 4 cm proximal to the anus. Five percent (w/v) in H_2O TNBS solution dissolved in the same volume of 100% ethanol was then slowly administered into the colon lumen through the catheter. The total injection volume was 100 μl in both groups, thus allowing TNBS to reach the entire colon. After the above administration, the mice were kept upside down while being held by their tails for 60 s and then were returned to their cages. All mice were then sacrificed at day 12 for both macroscopic inspection and histological analysis. At autopsy in each group of mice, their large bowel was flushed with saline, and then was excised. The large bowel from the ileocecal junction to the anal verge was measured, cut open longitudinally along the main axis, and then washed with saline. The total tumor number, tumor localization and the size of each tumor were recorded. The tumor volume was calculated as length × width × width × 0.526 [22]. After macroscopic inspection, it was rolled like a "*Swiss roll*" and then fixed overnight in 10% buffered formalin. Paraffin-embedded sections were made using routine procedures.

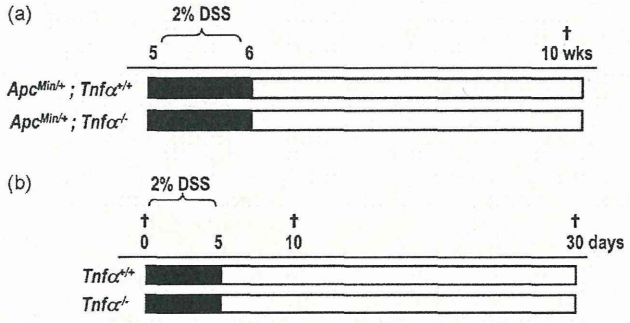

Fig. 1. Experimental protocols for this study. (a) Experimental design to investigate the role of Tnfα in DSS-induced colon tumorigenesis. (■) Basal diet and 2% DSS in drinking water, (□) basal diet and tap water, (†) sacrifice. (b) Experimental design to compare the induction of pro-inflammatory factors in the presence or absence of *Tnfα*. Day 0, day 10 and day 30 represent control, the acute phase of colonic inflammation and the chronic phase of colon inflammation, respectively. (■) Basal diet and 2% DSS in drinking water, (□) basal diet and tap water, (†) sacrifice.

2.3. Histological inflammation score

The histopathological alterations of the colon were examined on hematoxylin and eosin (H&E) stained sections and colon inflamma-

tion was scored according to the following morphological criteria as described previously [23]. Grade 0, normal colon mucosa; Grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation and edema in the mucosa; Grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3, loss of all crypts with severe inflammation in the mucosa, but with retention of the surface epithelium; and Grade 4, loss of all crypts and surface epithelium with severe inflammation in the mucosa.

2.4. Immunohistochemical analysis

The avidin–biotin peroxidase complex technique was used for immunohistochemical studies. Sections (5 μm thick) were made, deparaffinized, rehydrated in PBS, 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 10 min in 0.3% H_2O_2. After washing three times with PBS, the sections were then preincubated with 2% bovine serum albumin in PBS for 40 min at room temperature and then incubated with primary antibodies, anti-β-catenin (1:1000; BD Biosciences PharMingen, San Diego, CA, USA), anti-Cox-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Subsequently, the sections were incubated with biotinylated secondary antibodies against the primary antibodies (1:250; DAKO Corp., Carpinteria, CA, USA) for 30 min followed by incubation with avidin-coupled peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. The sections were developed with 3,3′-diaminobenzidine (DAB) using DAKO Liquid DAB Substrate-Chromogen System (DAKO) and then were counterstained with hematoxylin, dehydrated, and coverslipped.

2.5. Protein extraction and a Western blot analysis

Total protein was extracted from both the normal colon tissues and the colon tumor tissues, which were excised from *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice at autopsy (Fig. 1a), and equivalent amounts of protein (15 μg/lane) were subjected to a Western blot analysis, as described previously [24,25]. The primary antibodies for β-catenin, GAPDH were purchased from BD Biosciences PharMingen (San Diego, CA, USA) and Cell Signaling Technology, Inc. (Danvers, MA, USA), respectively. An antibody against GAPDH served as a loading control.

2.6. Crypt isolation

The excised total colon was washed by PBS several times and cut opened in the longitudinal direction. The total colon was divided into three sections and the distal section was used for crypt isolation. The distal tissue was washed by 1× Hank's Balanced Salt Solution (HBSS; Sigma–Aldrich, St Louis, MO, USA) two times and followed by incubation with 1× HBSS containing 30 mM EDTA at 37 °C for 15 min. After this step, the tissue was dispersed by vortex in the 1× HBSS solution and separated into epithelial crypts and stromal tissues.

2.7. Quantitative real-time reversed transcription-polymerase chain reaction

Total RNA was extracted from the isolated epithelial crypts and stromal tissues of wild-type C57B6/J and Tnfα −/− mice at the indicated time intervals (Fig. 1b) by using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. cDNA was synthesized from 1.0 μg of total RNA by using SuperScript III First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA, USA). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) amplification was performed in a final volume of 20 μl containing 10 μl of 2× SYBR green master mix (Takara, Kyoto, Japan), 1.0 μl of primers (10 μmol/l), 3.0 μl of distilled water and 5.0 μl of cDNA by using a LightCycler 1.0. (Roche Diagnostics, IN, USA) according to the protocols described previously [26]. The reaction conditions included activation at 95 °C for 10 min, denaturation at 95 °C for 10 s, annealing 60 °C for 10 s and extension 72 °C for 6 s. All PCR amplifications were done for 45 cycles. The expression level of each gene was normalized to the β-actin expression level using the standard curve method. The primer sequences used in qRT-PCR analyses were obtained from the PrimerBank (http://pga.mgh.harvard.edu/primerbank/): for *β-actin*, sense 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and antisense 5′-ATGGAGCCACCGATCCACA-3′; for *Tnfα*, sense 5′-CCCTCACACTCAGATCATCTTCT-3′ and antisense 5′-GCTACGACGTGGGCTACAG-3′; for *Tnfβ*, sense 5′-CCACCTCTTGAGGGTGCTTG-3′ and antisense 5′-CATGTCGGAGAAAGGCACGAT-3′; for *Ccl2*, sense 5′-TTAAAAACCTGGATCGGAACCAA-3′ and antisense 5′-GCATTAGCTTCAGATTTACGGGT-3′; for *Cxcl1*, sense 5′-CTGGGATTCACCTCAAGAACATC-3′ and antisense 5′-CAGGGTCAAGGCAAGCCTC-3′; for *Cox-2*, sense 5′-TGAGCAACTATTCCAAACCAGC-3′ and antisense 5′-GCACGTAGTCTTCGATCACTATC-3′; for *Il1β*, sense 5′-GCAACTGTTCCTGAACTCAACT-3′ and antisense 5′-ATCTTTTGGGGTCCGTCAACT-3′; and for *Il6*, sense 5′-TAGTCCTTCCTACCCCAATTTCC-3′ and antisense 5′-TTGGTCCTTAGCCACTCCTTC-3′.

2.8. Statistical analysis

The statistical analysis was performed using the GraphPad Prism 4 software program (Graphpad Software, Inc., San Diego, CA, USA). The mean ± SD was calculated for all parameters determined. Statistical significance was evaluated using either Student's *t*-test or Welch's *t*-test for paired samples. p values <0.05 were considered to be statistically significant.

3. Results

3.1. Genetic ablation of Tnfα did not suppress both the colonic inflammation and the expression of pro-inflammatory factors in the colon of mice exposed to DSS

A previous study revealed that *Tnfr1* deficient mice are resistant to the DSS-induced colitis. In the present study, two cohorts of *Apc* Min/+ mice with different *Tnfα* status (*Apc* Min/+; *Tnfα* +/+, *Apc* Min/+; *Tnfα* −/− mice) were exposed to 2% DSS in drinking water for 1 week and sacrificed at the point of week 5 (Fig. 1a). In contrast to the *Tnfr1* deficient mice, mice lacking *Tnfα*, as well as the control mice, exhibited profound body weight loss and bloody diarrhea during the course of DSS treatment. In addition, there were no significant differences in either the physical findings or mortality between *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice. There were no apparent differences in either microscopic appearance of the inflamed colon (data not shown) or the histological inflammation score (1.17 ± 0.83 and 1.25 ± 0.97, respectively, p: 0.82) between these two cohorts.

We also examined the effect of *Tnfα* ablation on intestinal inflammation using the trinitrobenzene sulfonic acid (TNBS)-induced model, a different model of intestinal inflammation. However, we could not detect any differences in the histological inflammation score between *Tnfα* +/+ and *Tnfα* −/− mice (2.75 ± 1.50 and 2.50 ± 1.22, respectively, p: 0.78).

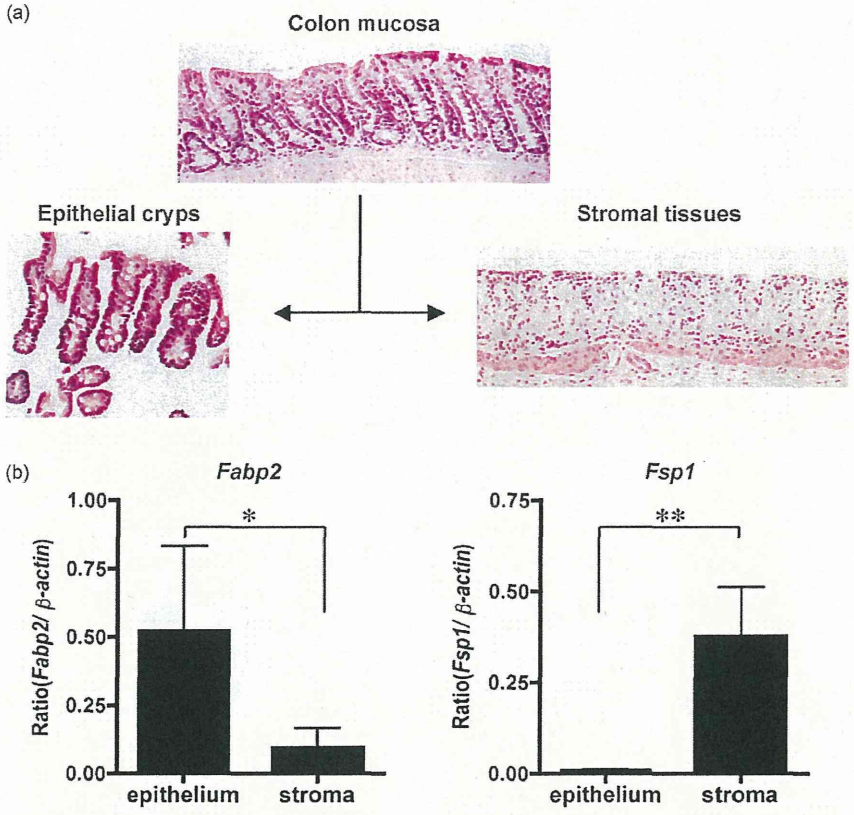

Fig. 2. Crypt isolation and the expression of epithelial or stromal marker gene. (a) Distal colon tissues were separated into epithelial crypts and stromal tissues by crypt isolation as described in Section 2. (b) Quantitative RT-PCR was performed on total RNAs extracted from both epithelial cryps and stromal tissues. The levels of FABP2 and FSP-1 mRNA were normalized to β-actin mRNA levels. Representative results from three independent experiments are shown here. Data represent the mean ± SD. Statistical significance of differences was evaluated by Student's *t*-test with Welch's correction. $^{*}p < 0.05$, $^{**}p < 0.01$.

Both wild-type and *Tnfα* deficient mice were treated with 2% DSS for 5 days and then were sacrificed at day 0 (control), day 10 (the acute phase of colonic inflammation) and day 30 (the chronic phase of colon inflammation; Fig. 1b) in order to compare the induction of pro-inflammatory factors in the presence or absence of *Tnfα*. The crypts were isolated to separate the colons into the epithelial crypts and stromal tissues (Fig. 2a). After crypt isolation, RNA was extracted from these tissue specimens and real-time PCR was used to compare the expression of pro-inflammatory factors between wild-type and *Tnfα* deficient mice. The epithelial marker, *fatty-acid-binding-protein-2* (*Fabp2*) [27–29], was predominantly expressed by RNAs from colonic crypts, whereas the mesenchymal marker, *fibroblast-specific-protein-1* (*Fsp1*), was predominantly expressed at those from stromal tissues (Fig. 2b). These findings indicate that epithelial cells were therefore successfully separated from stromal cells.

The expression of pro-inflammatory factors (*Tnfα*, *Tnfβ*, *Ccl2*, *Cxcl1*, *Il1β*, *Il6*, *Cox-2*) were up-regulated after the DSS exposure in wild-type mice and such altered expression was maintained at a high level until day 30 (Fig. 3a). As expected, during the course of DSS treatment (Fig. 1b), the expression of *Tnfα* was not detectable in either the epithelial or stromal tissues of *Tnfα* deficient mice (Fig. 3b). However, in spite of the lack of *Tnfα* expression, there were no significant differences in the expression of *Tnfβ*, *Ccl2*, *Cxcl1*, *Il1β*, *Il6* and *Cox-2* in either the epithelial or stromal tissues between the wild-type and *Tnfα* deficient mice (Fig. 3c). These results indicate that the genetic ablation of *Tnfα* did not influence either the severity of colitis or the expression of DSS-induced pro-inflammatory factors.

3.2. Genetic ablation of *Tnfα* did not suppress the infiltration of *Cox-2*-expressing inflammatory cells and the accumulation of β-catenin protein in the inflamed colorectal tumor tissues

The coordinated activation of the Apc/β-catenin pathway and the Cox-2 signaling pathway plays an important role in colon tumor formation and progression [30]. A previous study revealed a lack of *Tnfr1* to lead to the decreased expression of Cox-2 in the stromal tissues and the decreased expression of nuclear β-catenin in the colonic tumor cells [11]. Therefore, the infiltration of Cox-2-expressing inflammatory cells and the accumulation of nuclear β-catenin was examined by immunostaining. In contrast to previous data, no significant differences were observed in the infiltration index of Cox-2-positive inflammatory cells between *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice (42.62 ± 6.65 and 45.73 ± 2.90 cells per field at 400× magnification, respectively, *p*: 0.32) (Fig. 4a). The accumulation of nuclear β-catenin protein in colon tumor cells was not different between the two cohorts (Fig. 4b). In addition, Western blotting revealed the expression of β-catenin protein in colonic tumors to not change between *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice (Fig. 4c).

3.3. The genetic ablation of *Tnfα* did not suppress the inflammation-related colon tumorigenesis

Tnfr1 deficient mice markedly attenuate tumor formation induced by azoxymethane (AOM) and DSS in comparison to wild-type mice [11]. In the present study, as in a previous report [20], colon tumors were detectable only in *Apc* Min/+ mice exposed to

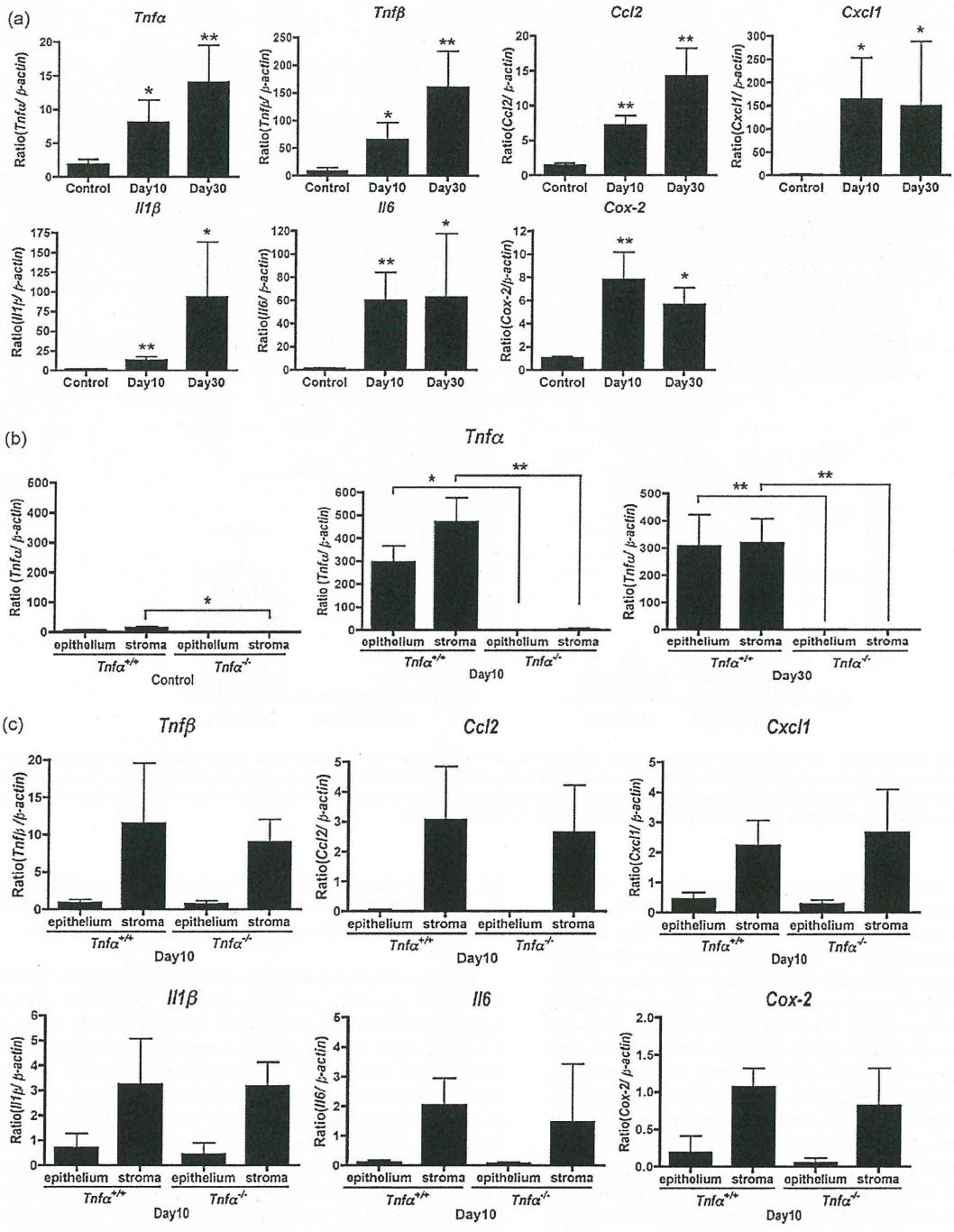

Fig. 3. Pro-inflammatory factor gene expression in the colon. Quantitative RT-PCR was performed on total RNAs extracted from stromal tissues of wild-type mice (a), epithelial and stromal tissues of both wild-type and Tnfα$^{-/-}$ mice (b and c) at the indicated time intervals as indicated. The levels of each pro-inflammatory factor were normalized to β-actin mRNA levels. Representative results from three independent experiments are shown here. Data represent the mean ± SD. Statistical significance of differences was evaluated by Student's *t*-test with Welch's correction (a–c). $^{*}p < 0.05$, $^{**}p < 0.01$.

DSS, whereas no tumors were found in *Apc* +/+ mice with the DSS exposure. The incidence and multiplicity of colonic tumors were 100% and 16.15 ± 5.84/mouse in *Apc* Min/+; *Tnfα* +/+ mice and 100% and 14.27 ± 7.71/mouse in *Apc* Min/+; *Tnfα* −/− mice, respectively. The tumor volumes were 3.90 ± 2.55 mm^3 and 3.81 ± 2.49 mm^3 in *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice, respectively (Table 1). Importantly, no significant differences were observed in either the tumor incidence and multiplicity or the tumor volumes between *Apc* Min/+; *Tnfα*+/+ and *Apc* Min/+; *Tnfα*−/− mice. Microscopic examinations also failed to detect any histological differences in colon tumors between *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice (data not shown). In addition, the incidence and multiplicity of small intestinal tumors did not alter regardless of the *Tnfα* status (100% and 47.0 ± 2.16/mouse in *Apc* Min/+; *Tnfα* +/+ mice and 100% and 48.8 ± 3.59/mouse in *Apc* Min/+; *Tnfα* −/− mice, respectively).