there was a significant reduction of colony formation, suggesting that PRDM5 does indeed function as a tumor suppressor gene.

Epigenetic silencing of PRDM5 in primary colorectal and gastric cancers. Finally, to assess the extent to which PRDM5 is methylated in primary colorectal and gastric cancers, we initially used COBRA to detect methylation of the region around the transcription start site of the gene (Fig. 6A). We found that 4 of 61 primary colorectal and 39 of 78 primary gastric cancers showed such methylation. To examine the methylation status of PRDM5 in more detail, we then carried out bisulfite sequencing using DNA from three primary gastric cancers (Fig. 6B). In the specimens shown by COBRA to be methylated (WG313 and WG314), all of the CpG sites in the region analyzed were densely methylated. Moreover, high quality RNA obtained from 15 of the primary gastric cancers showed diminished expression of PRDM5 in 11 (73%), although expression of PRDM5 also was diminished in a subset of tumors without methylation (Fig. 6C).

Discussion

In the present study, we identified PRDM5 as a target of epigenetic inactivation in both colorectal and gastric cancers; in fact, PRDM5 seems to be the most frequently altered PRDM family gene in gastrointestinal cancer. We also found that expression of PRDM6, PRDM8, PRDM11, and PRDM16 was down-regulated in a subset of colorectal and gastric cancer cell lines, but the fact that their expression was not significantly restored by treating cells with 5-aza-dC indicates that DNA methylation was likely not the principle mechanism by which they were silenced. Earlier studies showed that PRDM2/RIZ1 is silenced in gastrointestinal cancer (25) and that it has an alternative transcript, RIZ2, which lacks the SET domain (24). Our strategy failed to detect down-regulation of PRDM2/RIZ1 in cancer cells because the Taqman primer/probe set we used amplified both PRDM2/RIZ1 and RIZ2. Several other PRDMs, including PRDM1, PRDM3/EVI1, and PRDM16, also express alternative transcripts that lack the SET domain. Epigenetic inactivation of

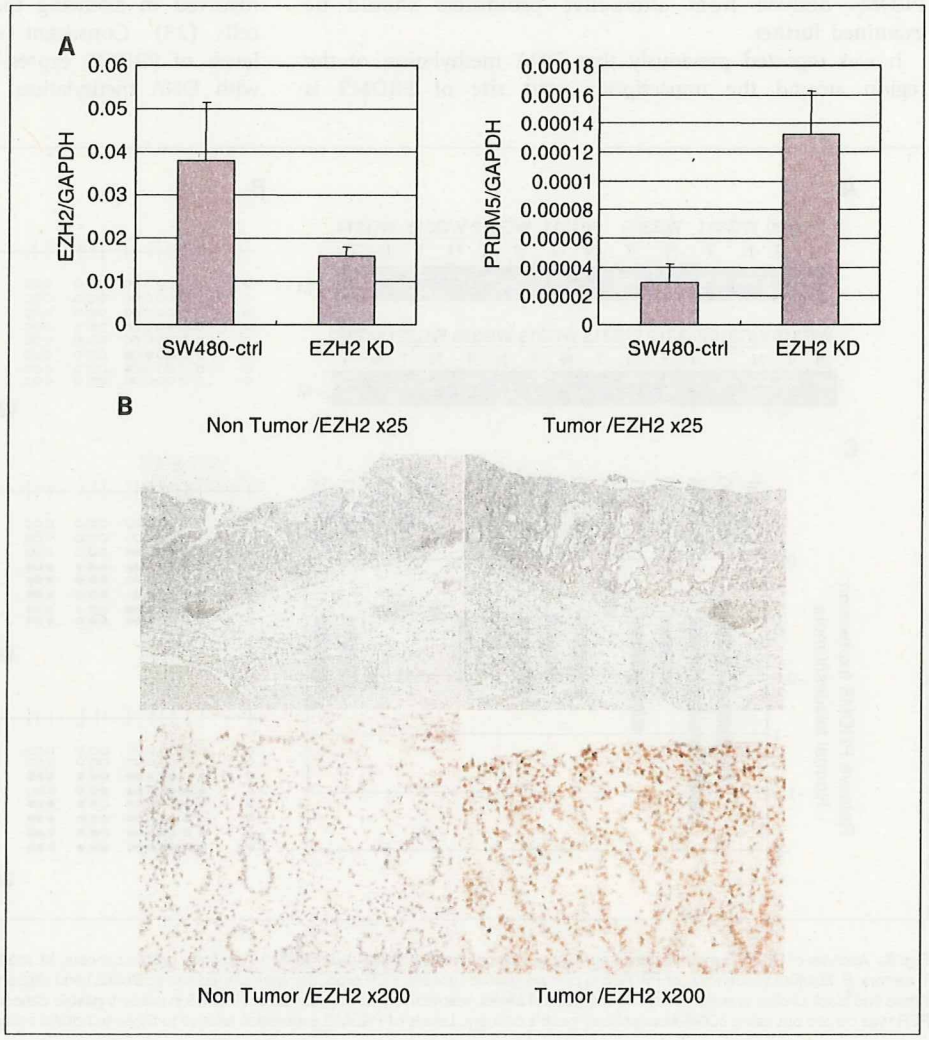

Fig. 4. Role of EZH2 in the silencing of PRDM5. *A*, knocking down EZH2 restored expression of PRDM5 in SW480 cells. An SW480 line stably expressing shEZH2 was established. Columns, averages of five independent PCR analyses of five different clones; bars, SD. *B*, immunohistochemical detection of EZH2 in primary gastric cancers. One representative case. Cancerous tissues showed substantially stronger nuclear staining of EZH2 than noncancerous tissues.

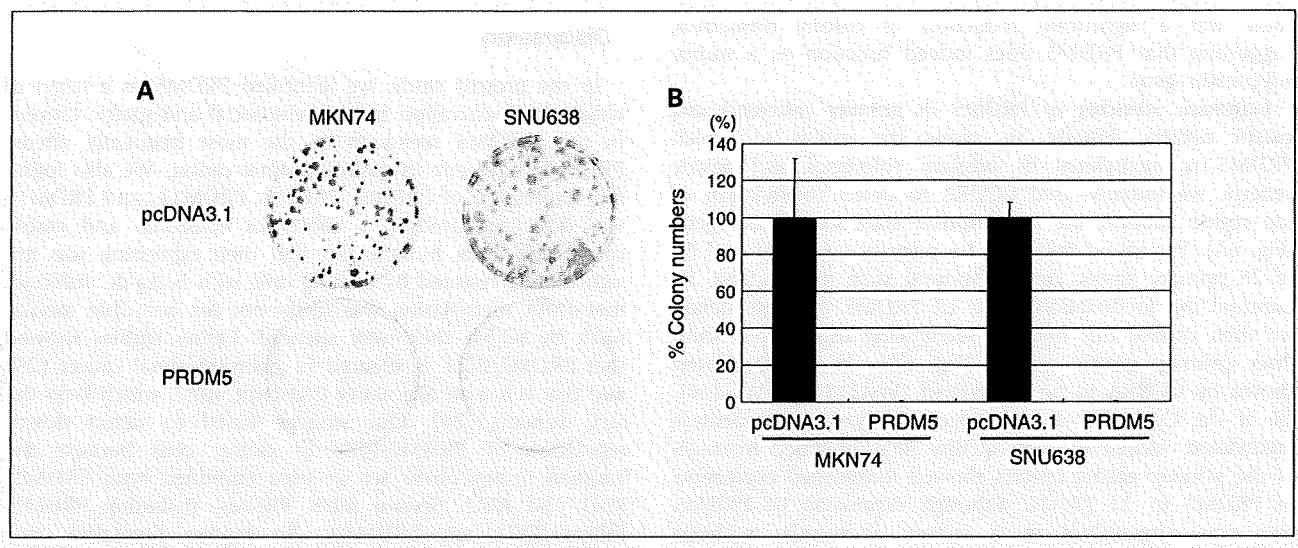

Fig. 5. Suppression of gastric cancer cell growth by PRDM5. *A,* SNU638 and MKN74 cells were transfected with pcDNA3.1 or pcDNA3.1-PRDM5 and incubated in the presence of G418. After 2 wks, the cells were fixed with methanol and stained with Giemsa. *B,* cell counts were obtained after transfecting MKN74 and SNU638 cells with pcDNA3.1 or pcDNA3.1-PRDM5. Each experiment was repeated three times. Columns, average numbers of colonies; bars, SD.

PRDMs derived from alternative promoters should be examined further.

It was reported previously that DNA methylation of the region around the transcription start site of PRDM5 is involved in silencing the gene in breast and liver cancer cells (28). Consistent with that report, we found that levels of PRDM5 expression in MKN7 cells were correlated with DNA methylation, deacetylation of histone H3, and

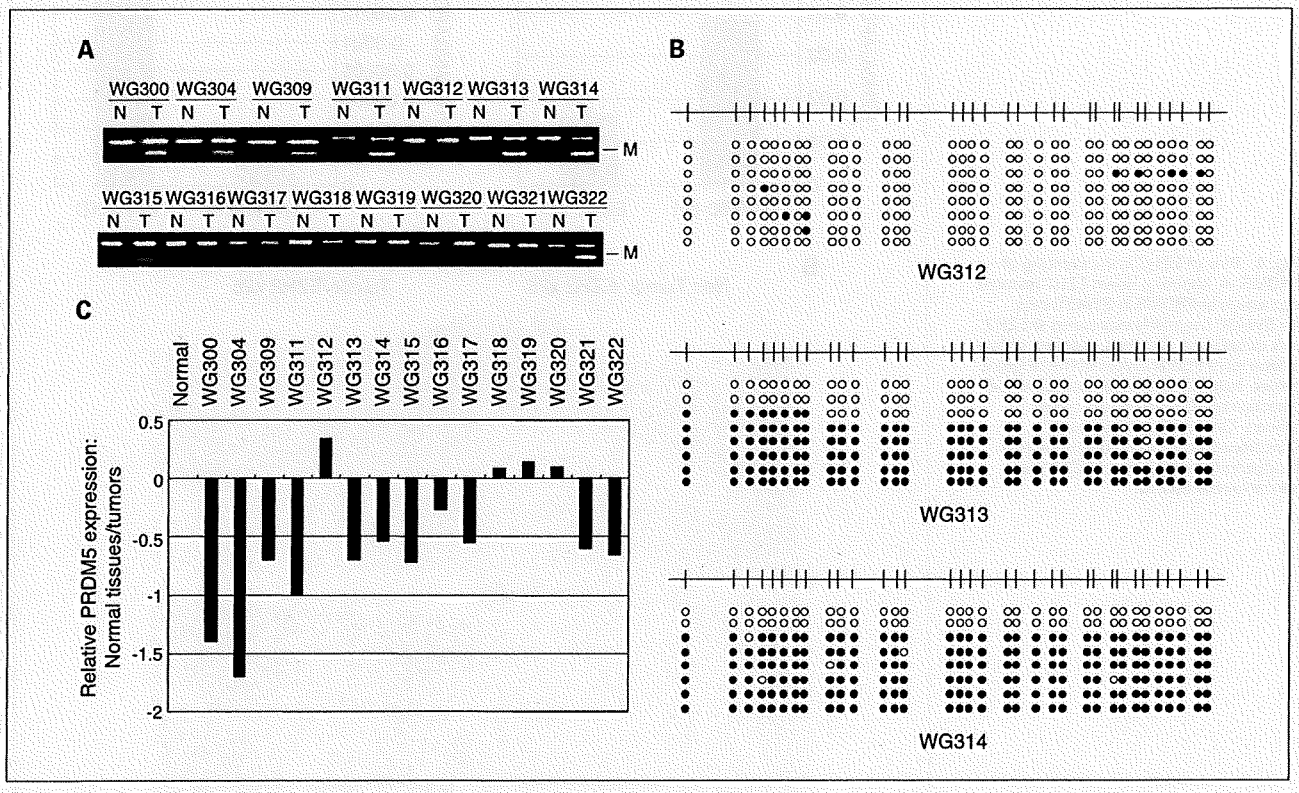

Fig. 6. Analysis of PRDM5 methylation in primary gastric cancers. *A,* COBRA of PRDM5 in primary gastric cancers. M, methylated alleles; N, noncancerous tissues; T, tumors. *B,* bisulfite sequencing of PRDM5 in primary gastric cancers. PCR products were cloned into pcDNA2.1 and sequenced. Eight clones were sequenced in each case. White and black circles, unmethylated and methylated alleles, respectively. *C,* expression of PRDM5 in primary gastric cancers with or without DNA methylation. Real-time PCR was carried out using cDNA from primary gastric cancers. Levels of PRDM5 expression relative to those to normal tissues (*Y axis*).

methylation of H3K9. By contrast, SW480 cells showed sparse DNA methylation in the region of the PRDM5 promoter, and trimethylation of H3K27 was correlated with gene silencing. Recently, Abbosh et al. (33) reported that introduction of a dominant-negative H3K27 mutant into cancer cells restores expression of RASSF1, indicating that such histone modification is involved in certain types of gene silencing in cancer. Methylation of H3K27 is catalyzed by EZH2, and overexpression of EZH2 also has been reported in cancers of the prostate, breast, and stomach (17–19). In the breast, for example, overexpression of EZH2 is an early event during tumorigenesis and is involved in preneoplastic progression (34). In the present study, we found that 90% of the gastric cancers tested expressed higher levels of EZH2 than adjacent noncancerous stomach tissues (Fig. 4B), suggesting that down-regulation of PRDM5 cannot be explained solely as the result of overexpression of EZH2. Indeed, Cha et al. (35) reported recently that phospho-AKT can phosphorylate EZH2, thereby preventing its interaction with histone H3, which suggests that overexpression of EZH2 may not always lead to H3K27 trimethylation of genes. Because SW480 cells do not carry a PIK3CA mutation and do not show the phosphorylated form of AKT, we would expect that EZH2 may be active in this cell line (data not shown).

The molecular mechanism by which PRDM5 suppresses cell growth remains unknown. Deng et al. (28) reported that introducing PRDM5 into cancer cells using an adenoviral vector increased the fractions of G_2-M and sub-G_1 cells, suggesting that PRDM5 is involved in cell cycle arrest and apoptosis. Although it remains unclear whether the PR domain of PRDM5 has histone methyltransferase activity, PRDM5 may be involved in transcriptional regulation of genes associated with the cell cycle and apoptosis. Moreover, PRDM5 contains 16 zinc finger domains, which often show sequence-specific DNA binding activity. Thus, a full understanding of the role played by PRDM5 in gene transcription, cell growth, and apoptosis awaits further study.

In summary, we have shown for the first time that PRDM5 expression is often epigenetically silenced in colorectal and gastric cancers. Such silencing of PRDM5 was mediated by either DNA methylation or trimethylation of H3K27. That introduction of PRDM5 into cancer cells suppressed cell growth suggests PRDM5 acts as a tumor suppressor in gastrointestinal cancers. Understanding the precise role played by PRDM5 in gene transcription will not only increase our understanding of the biology of gastrointestinal cancer but may also enable epigenetic silencing of PRDM5 to serve as a useful molecular target for diagnosis and therapy.

Acknowledgments

We thank Dr. T. Takano for excellent assistance with immunohistochemical analysis; Drs. M. Okamoto, T. Maehata, Negishi, A. Miyazaki, Orita, T. Hara, and N. Kato for primary tissue samples; and Dr. William F. Goldman for editing the manuscript.

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Development of Gastric Tumors in *Apc*$^{Min/+}$ Mice by the Activation of the β-Catenin/Tcf Signaling Pathway

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Abstract

Although several lines of evidence suggest the involvement of the Wnt pathway in the development of gastric cancers, the functional significance of the pathway in gastric carcinogenesis is still poorly defined. To examine the role of the Apc/β-catenin signaling pathway in the development of gastric cancers, we investigated the gastric mucosa of the *Apc*$^{Min/+}$ mouse, which is a murine model for familial adenomatous polyposis, carrying a germ-line mutation at codon 850 of *Apc*. We found that aged *Apc*$^{Min/+}$ mice spontaneously develop multiple tumors in the stomach, which are accompanied by loss of heterozygosity of *Apc*. Such tumors consisted of adenomatous glands with strong nuclear accumulation of β-catenin. Even a single adenomatous gland already showed nuclear accumulation of β-catenin, suggesting that Apc/β-catenin pathway is an initiating event in gastric tumorigenesis in *Apc*$^{Min/+}$ mice. Myc and cyclin D1 expressions, which are transcriptional targets of β-catenin/Tcf, increased in the adenomatous lesions. Furthermore, β-catenin/Tcf reporter transgenic mice with *Apc*^{Min} allele showed higher levels of the transcriptional activity of β-catenin/Tcf in the gastric tumors. We also treated *Apc*$^{Min/+}$ and wild-type mice with *N*-methyl-*N*-nitrosourea (MNU), an alkylating agent that induces adenomas and adenocarcinomas in the stomach. Consequently, MNU-treated *Apc*$^{Min/+}$ mice significantly enhanced the tumor development in comparison with *Apc*$^{Min/+}$ mice or MNU-treated wild-type mice. Several gastric tumors in MNU-treated *Apc*$^{Min/+}$ mice showed invasion into the submucosal layer. These results indicate that the Apc/β-catenin pathway may play an important role in at least subset of gastric carcinomas. In addition, *Apc*$^{Min/+}$ mice combined with MNU could be a useful short-term model to investigate multistage carcinogenesis in the stomach. [Cancer Res 2007;67(9):4079–87]

Introduction

Familial adenomatous polyposis (FAP) is regarded as an autosomal dominant disease in which many adenomatous polyps develop in the colon, thereafter progressing to colorectal carcinoma. Genetic linkage studies have shown that the inactivation of the *adenomatous polyposis coli* (*APC*) gene, located on chromosome 5q21, is responsible for such phenotypes in FAP (1, 2). Therefore, inactivating APC is considered to initiate the multistep progression of colorectal cancer (3). The knowledge about how *APC* acts as a tumor suppressor gene was considerably advanced by the demonstration that APC contributes to the degradation of cytosolic β-catenin, thereby linking APC to the Wnt/β-catenin pathway as a negative regulator (4–7). The critical event in the Wnt pathway activation is the elevation of the cytoplasmic pool of β-catenin and its resultant transport to the nucleus (5, 8). Consequently, the inactivation of APC leads to a constitutive accumulation of β-catenin, which triggers an aberrant transcriptional activation of the β-catenin/Tcf target genes, such as *Myc* and *Cyclin D1* (9, 10).

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death in the world (11). Although several lines of evidence suggest the involvement of the Wnt pathway in the development of sporadic gastric cancers, the functional significance of the Apc/β-catenin pathway in gastric carcinogenesis is still poorly defined in comparison with that in colon carcinogenesis. The nuclear accumulation of β-catenin, a hallmark of Wnt activation, has been found in 17% to 54% of gastric adenocarcinomas (12–14), and mutations in *APC* and *β-catenin* are found in a subset of gastric adenocarcinomas. However, the incidence of such mutations varies according to evidence from different studies (0–60% for *APC* mutations and 0–27% for *β-catenin* mutations; refs. 13–18). In addition, FAP patients have been reported to have an increased risk for gastric cancer in Japan but not in Western countries (19–22). Taken together, the functional significance of the Wnt pathway in gastric carcinogenesis therefore remains controversial.

Apc$^{Min/+}$ mouse is a mouse model for FAP and harbors a dominant mutation at the *Apc*, the mouse homologue of the human *APC* gene, resulting in the truncation of the gene product at amino acid 850 (23). Originally, this lineage was established from an ethylnitrosourea-treated C57BL/6J male mouse, and its phenotype is an autosomal dominant trait. Although homozygous *Apc*$^{Min/Min}$ mice die as embryos, *Apc*$^{Min/+}$ mice develop multiple neoplasias in their intestinal tracts within several weeks after birth (23). It is also known that most of the intestinal tumors in the mice that are heterozygous for a mutant allele of *Apc* have lost the *Apc* function due to a loss of heterozygosity (LOH; refs. 24, 25). Although mice generated by gene targeting with a mutation at codon 1638 of the *Apc* have been reported to occasionally develop gastric tumors after 30 weeks of age (26, 27), the relationship between gastric tumorigenesis and the activation of the Apc/β-catenin pathway has not yet been well characterized. *Apc*$^{Min/+}$ mice combined with *Foxl1*$^{-/-}$ mice (28) or *Cdx2* transgenic mice (29) have been shown to develop gastric tumors. However, previous studies have indicated no evidence of a spontaneous development of gastric tumors in *Apc*$^{Min/+}$ mice.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-4025

In the present study, we examined the gastric lesions of $Apc^{Min/+}$ mice and found, for the first time, that aged $Apc^{Min/+}$ mice develop multiple adenomas in the stomach, which are accompanied by *Apc* LOH and the activation of the β-catenin/Tcf signaling pathway. We also treated $Apc^{Min/+}$ mice with *N*-methyl-*N*-nitrosourea (MNU), an alkylating agent that induces adenomas and adenocarcinomas in the stomach, to elucidate the mode of gastric carcinogenesis in $Apc^{Min/+}$ mice. Consequently, MNU-treated $Apc^{Min/+}$ mice were found to strongly promote tumor development, and several gastric tumors showed invasive adenocarcinomas. These results indicate that the Apc/β-catenin pathway plays a significant role in a subset of gastric carcinogenesis, thus suggesting that this model could be a short-term model to investigate multistage carcinogenesis in the stomach.

Materials and Methods

Animals. This study was approved by the Institutional Ethics Review Committee for Animal Experiments at Gifu University. $Apc^{Min/+}$ mice in the C57BL/6J background were obtained from The Jackson Laboratory and maintained by breeding $Apc^{Min/+}$ males to C57BL/6J females. All mice were maintained under specific pathogen-free conditions with isolated ventilation cages in an air-conditioned room with a 12-h light/12-h dark cycle. They were bred and maintained on a basal diet, CE-2 (CLEA Japan, Inc.), until the termination of the study. Heterozygous progeny were identified by a PCR analysis of tail DNA using allele-specific primers (24). To examine gastric lesions, $Apc^{Min/+}$ and age-matched wild-type littermates, 15 to 35 weeks of age, were used in the present study. It is known that the life span of $Apc^{Min/+}$ mice on C57BL/6J background is ~20 weeks (23), whereas the mice in our facility have a better survival as was also observed in several previous studies (30–33). Elderly $Apc^{Min/+}$ mice (i.e., >25 weeks of age) were carefully monitored and sacrificed before become moribund.

Transgenic mice. To generate β-catenin/Tcf reporter mice, Tcf/Lef binding sites plus a minimal thymidine kinase promoter were PCR amplified from the TOPFLASH plasmid (Upstate), ligated to an EGFP-pA cassette (Clontech), and then injected into fertilized eggs from C57BL/6J mice.[5] Next, the β-catenin/Tcf reporter mice were crossed with $Apc^{Min/+}$ mice to generate the reporter mice with the Apc^{Min} allele.

MNU treatment. MNU (Sigma Chemical) was dissolved in distilled water at a concentration of 240 ppm and freshly prepared thrice weekly for the administration in drinking water in light-shielded bottles *ad libitum*. $Apc^{Min/+}$ and wild-type littermates, from 4 to 6 weeks of age, were given drinking water containing 240 ppm MNU on alternate weeks for a total of 10 weeks of exposure according to the protocol described in previous reports (34, 35). The MNU-treated $Apc^{Min/+}$ and wild-type littermates were sacrificed at 15, 20, 25, 30, and 35 weeks of age.

Preparation of tissue samples for tumor counting and histologic analysis. All mice underwent a thorough postmortem examination at the time of sacrifice. The stomach was removed and opened along the greater curvature. The number, as well as the long diameter, of the tumors in the stomach was measured using a dissected microscope at ×7 magnification. Tumors >0.5 mm in diameter were mapped and counted. To eliminate interobserver error, all counts were done by a single observer blinded to the genotype of the mice. All the cases were also counted by a second observer to confirm the results of the first observer. After tumor counting, all of the excised stomachs, including the neoplastic nodules, were fixed for 24 h in neutral-buffered 10% formalin and then cut into eight strips, which were processed by standard methods, embedded in paraffin, sectioned at 5 μm, and stained with H&E. The defining characteristics for adenoma and adenocarcinoma were adapted from both the consensus guidelines on murine models of intestinal cancer (36) and previous reports in the literature (35, 37).

Human tissue samples. A total of 81 formalin-fixed, paraffin-embedded sporadic gastric adenomas (n = 20) and early adenocarcinomas (intramucosal adenocarcinomas; n = 61), dissected in 2003 to 2006 at Gifu University Hospital, was examined for β-catenin expression. The experiments were carried out according to the protocol approved by the Ethics Committee of Gifu University.

Immunohistochemistry. The avidin-biotin peroxidase complex (ABC) technique was used for immunohistochemical studies. Sections (5 μm thick) were cut, 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 30 min in 0.3% H_2O_2. After washing thrice with PBS, the sections were preincubated with normal blocking serum for 20 min at room temperature and then incubated with β-catenin (1:1,000; BD Biosciences PharMingen), c-myc (1:200; Santa Cruz Biotechnology), cyclin D1 (1:200; Santa Cruz Biotechnology), Ki-67 (1:200; DAKO Corp.), AE1/AE3 (1:100; DAKO), and green fluorescent protein (GFP) antibody (1:1,500; Molecular Probes) overnight at 4°C. Subsequently, the sections were incubated with biotinylated secondary antibodies (Vectastain ABC kit, Vector Laboratories) for 30 min followed by incubation with avidin-coupled peroxidase (Vector Laboratories) for 30 min. The sections were developed with 3,3′-diaminobenzidine (DAB) using DAKO Liquid DAB Substrate-Chromogen System (DAKO) and then counterstained with hematoxylin. For immunofluorescence, FITC- or TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) were used and then counterstained with 4′,6-diamidino-2-phenylindole. No specific staining was observed in the negative control slides prepared without primary antibody.

LOH analysis and laser capture microdissection. In the current study, the DNA for the analysis of the *Apc* allelic loss was extracted from the cells isolated with laser microdissected tissue sections as described previously (38, 39). For laser capturing, the slides were put into xylene for 30 min to dissolve the paraffin that otherwise interfered with laser capture microdissection (LCM). Next, the slides were washed for 10 min in 100% ethanol. After staining with H&E, the sections were dehydrated in 100% ethanol, incubated for 2 min in xylene, and then dried at room temperature. Microdissection was done using a laser microdissection system (PALM Microlaser Technologies). The microdissected tissues of normal-appearing epithelium and tumors from $Apc^{Min/+}$ and MNU-treated $Apc^{Min/+}$ mice were used for the analysis. They were digested overnight at 50°C in 20 μL of lysis buffer containing 500 μg/mL proteinase K, 10 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 0.45% NP40, and 0.45% Tween 20. The proteinase K was heat inactivated (10 min at 95°C). LOH of the *Apc* gene was checked using PCR with mismatched primers as described previously (24). Briefly, the amplification of the Apc^{Min} allele resulted in a 155-bp PCR product with one *Hin*dIII site, whereas the 155-bp product from Apc^{+} allele contained two *Hin*dIII sites. *Hin*dIII digestion of PCR-amplified DNA from $Apc^{Min/+}$ heterozygous tissues resulted in a 123-bp product from Apc^{+} allele and a 144-bp product from the Apc^{Min} allele. Therefore, the PCR products from tissue with LOH displayed only one band (144 bp) from the Apc^{Min} allele. The samples were assayed at least twice independently.

Mutation analysis of the *Apc* gene. The mutation cluster region of the mouse *Apc* gene (nucleotides 1,991–5,333; Genbank M88127) was screened for sequence alterations by PCR of four overlapping fragments (segment A, nucleotides 1,991–2,954; segment B, nucleotides 2,919–3,942; segment C, nucleotides 3,862–4,852; segment D, nucleotides 4,807–5,333). PCR products were gel purified using the DNA Gel Extraction kit (Millipore). These samples were also subcloned into pCR 2.1 (Invitrogen) and sequenced using T7 and M13 primers according to the manufacturer's protocol. The standard cycle sequencing was done in the presence of fluorescently labeled dideoxynucleotides by an ABI automated sequencer. Sequencing analyses of all samples were repeated twice to exclude PCR errors.

Quantitative real-time reverse transcription-PCR. Gastric tumors from $Apc^{Min/+}$ mice and MNU-treated $Apc^{Min/+}$ mice were examined for mRNA expression by quantitative real-time reverse transcription-PCR (RT-PCR). RNA was extracted using the RNAqueous-4PCR kit (Ambion) according to the manufacturer's protocol. cDNA was synthesized from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was done in a LightCycler (Roche) with SYBR Premix Ex Taq (TaKaRa). The expression level of each gene was normalized to the β-actin expression level using the standard curve method. Each experiment was done

[5] T. Oyama, et al., manuscript in preparation.

in either duplicate or triplicate, and then, the average was calculated. The primers for amplifications are listed in Supplementary Table S1.

Results

Spontaneous development of gastric tumors in the elderly $Apc^{Min/+}$ mice. We examined the gastric lesions in the $Apc^{Min/+}$ mice of 15, 20, 25, 30, and 35 weeks of age (n = 12, 13, 15, 16, and 13, respectively) and then compared the findings with those in age-matched wild-type littermates (Fig. 1). We found multiple gastric tumors in the $Apc^{Min/+}$ mice >20 weeks of age, whereas no gastric tumors were found in the wild-type littermates at any age. Macroscopically, the gastric tumors in the $Apc^{Min/+}$ mice showed a sessile and/or polypoid morphology (Fig. 1*A*). Although the $Apc^{Min/+}$ mice tended to develop multiple tumors in the small intestine as early as a few weeks after birth, no gastric tumors were found in the stomach of $Apc^{Min/+}$ mice before 20 weeks of age. The incidence of gastric tumors in the $Apc^{Min/+}$ mice at each age is given in Fig. 1*B*. The incidence of gastric tumors in $Apc^{Min/+}$ mice at 35 weeks of age was 100% (n = 13). The number and size of the tumors have increased as it appears from 20 weeks of age by aging (Fig. 1*C* and *D*). When the glandular stomach was divided into the antrum and the corpus, most gastric tumors developed in the pyloric glands of the antrum (Fig. 1*C*).

Nuclear accumulation of β-catenin in the gastric tumors of the $Apc^{Min/+}$ mice. The gastric tumors in $Apc^{Min/+}$ mice were evaluated to determine the histopathologic features (Fig. 2). In H&E staining, such tumors revealed a disturbed glandular architecture, an increased nuclear to cytoplasmic ratio, and nuclear atypia with surrounding hyperplastic glands (Fig. 2*A*). Gastric tumors in the $Apc^{Min/+}$ mice were classified as adenomas with mild to severe cellular atypia.

To clarify the possible involvement of the Apc/β-catenin pathway in the gastric tumors, we determined the subcellular localization of β-catenin by immunohistochemical staining. We found nuclear/cytoplasmic staining of β-catenin in adenomatous lesions (Fig. 2*A*), suggesting that the β-catenin signaling pathway is activated in such tumors. No nuclear/cytoplasmic β-catenin staining was observed in the elongated glands adjacent to these tumors, thus suggesting that these glands are reactive hyperplasia.

In addition to macroscopically detectable tumors, we found small adenomatous lesions in H&E staining (Fig. 2*A*). Such small lesions consisted of a few adenomatous glands or even a single adenomatous gland located near the proliferative compartment of the pyloric glands. β-Catenin immunostaining revealed marked nuclear/cytoplasmic staining even in such a single atypical gland (100%; n = 16), thus suggesting that the accumulation of β-catenin protein is an initiating event in the development of gastric tumors.

To examine the cell proliferative activity in such gastric tumors, we did double immunofluorescence for β-catenin and Ki-67 (Fig. 2*B*). Adenomatous lesions accompanied by β-catenin accumulation showed a high frequency of Ki-67–positive epithelial cells, whereas the adjacent hyperplastic glands showed such activity only in the proliferative compartment.

Increased β-catenin/Tcf transcriptional activity in the gastric lesions. To evaluate the possible activation of the downstream targets of Apc/β-catenin signaling, we investigated the β-catenin/Tcf transcriptional activity. We analyzed the expression of Myc and cyclin D1, both of which are known targets of β-catenin/Tcf transcription. Immunostainings revealed that adenomatous glands with the accumulation of β-catenin showed an increased expression of both Myc and cyclin D1 (Fig. 3*A*). The cyclin D1 mRNA expression in gastric tumors also increased in comparison with the normal epithelium of the stomach by

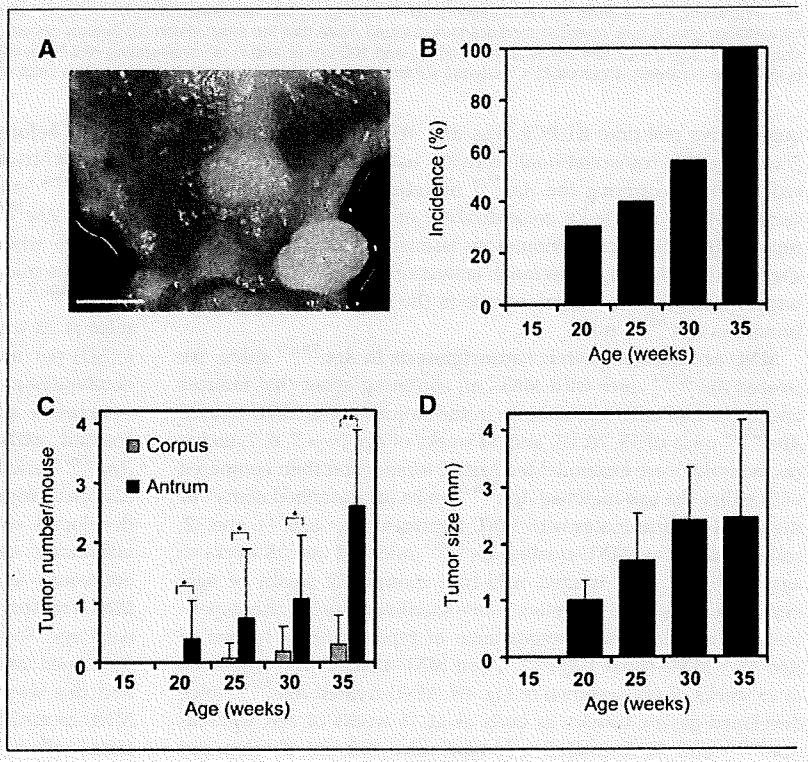

Figure 1. The spontaneous development of gastric tumors in aged $Apc^{Min/+}$ mice. *A*, macroscopic photograph of the grandular stomach of an $Apc^{Min/+}$ mouse at 30 weeks of age. Bar, 2 mm. *B*, incidence of $Apc^{Min/+}$ mice with gastric tumors increased by aging. *C*, number of gastric tumors increased by aging and gastric tumors preferentially developed in the antrum. *Columns*, mean; *bars*, SD. *, $P < 0.05$, Mann-Whitney *U* test; **, $P < 0.01$, Mann-Whitney *U* test. *D*, the size (the maximum long diameter) of gastric tumors in $Apc^{Min/+}$ mice increased over time. *Columns*, mean; *bars*, SD.

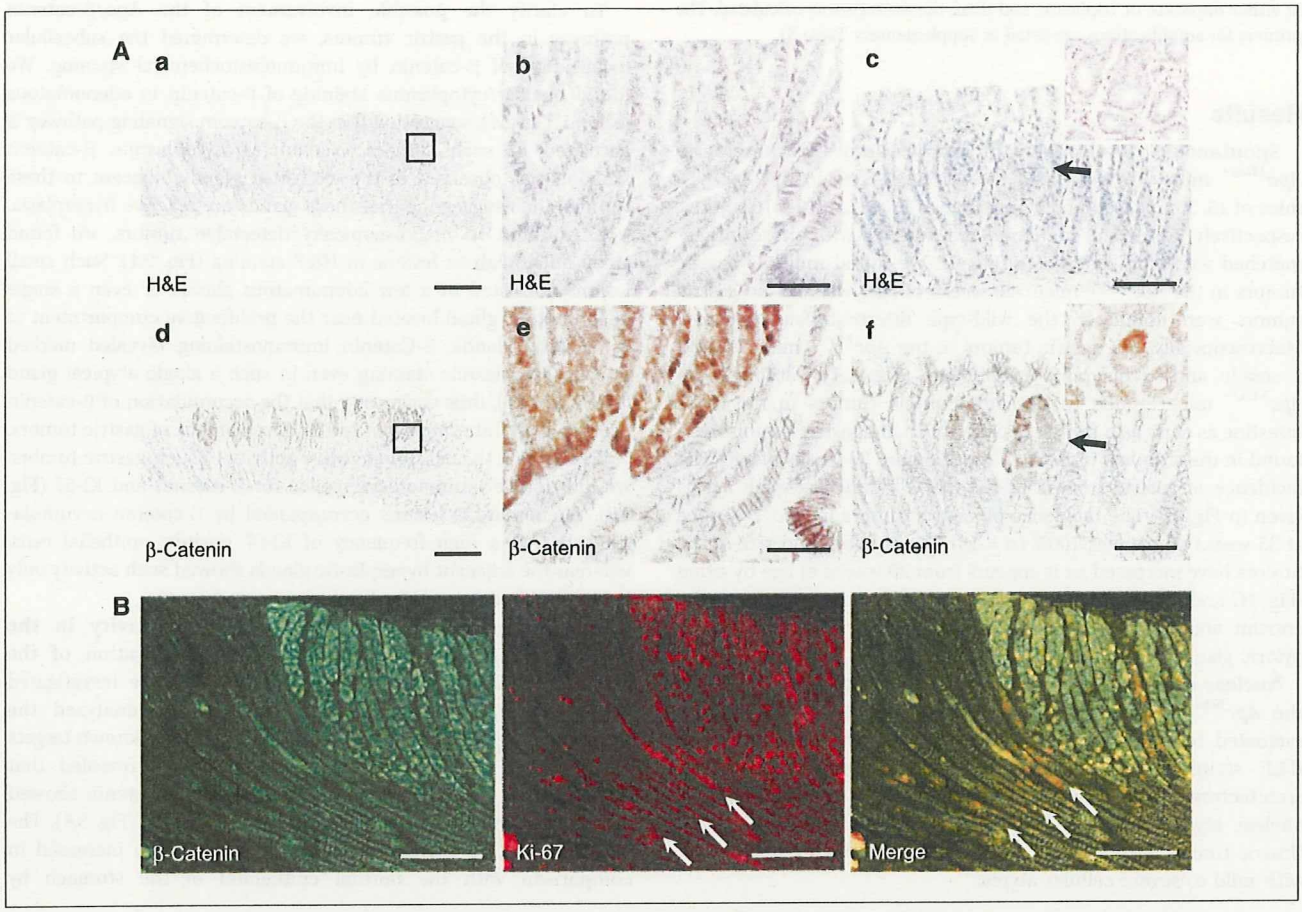

Figure 2. Gastric tumors in $Apc^{Min/+}$ mice show adenomatous lesions with nuclear/cytoplasmic accumulation of β-catenin. *A*, H&E-stained sections (*a–c*) and immunostaining for β-catenin on serial sections (*d–f*). Strong nuclear immunostaining for β-catenin was recognized in the adenomatous glands, whereas the adjacent hyperplastic glands only revealed membranous staining. Note that the small lesion (*c*) already shows β-catenin accumulation (*f*). *c* and *f*, *arrow*, small adenomatous lesion. Bars, 200 μm (*a* and *d*), 50 μm (*b* and *e*), and 100 μm (*c* and *f*). *B*, proliferating Ki-67–positive cells increase in the adenomatous glands with nuclear/cytoplasmic β-catenin accumulation, whereas surrounding hyperplastic glands include positive cells only at proliferative compartment (*arrows*). Bar, 100 μm.

quantitative real-time RT-PCR (Fig. 3*B*). We further examined the β-catenin/Tcf transcriptional activity using the β-catenin/Tcf reporter mice carrying the Apc^{Min} mutation. Immunostaining in serial sections revealed a colocalization of the β-catenin and the reporter (GFP) in such adenomatous lesions (Fig. 3*C*). These results suggest that the transcriptional activation of the β-catenin/Tcf targets thus plays an important role in the pathogenesis of gastric tumors in $Apc^{Min/+}$ mice.

MNU accelerates gastric tumorigenesis in $Apc^{Min/+}$ mice. We treated $Apc^{Min/+}$ mice with MNU, an alkylating agent that induces adenomas and adenocarcinomas in the stomach. The MNU-treated $Apc^{Min/+}$ mice of 15, 20, 25, and 30 weeks of age (n = 7, 6, 7, and 8, respectively) were examined for gastric lesions and then compared with either the age-matched $Apc^{Min/+}$ mice (as described above) or the wild-type littermates with MNU treatment (n = 8, 6, 11, and 12, respectively). Two MNU-treated $Apc^{Min/+}$ mice (22 and 28 weeks of age) and one MNU-treated wild-type mouse (32 weeks of age) became morbid and therefore were sacrificed during the experiment. The macroscopic appearance of typical gastric lesions in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at 25 weeks of age is shown in Fig. 4*A*. MNU-treated $Apc^{Min/+}$ mice developed gastric tumors as early as at 15 weeks of age, whereas $Apc^{Min/+}$ and MNU-treated wild-type mice did not develop any tumors before 20 weeks of age. The incidence of gastric tumors in the MNU-treated $Apc^{Min/+}$ mice was significantly higher than that of $Apc^{Min/+}$ mice or MNU-treated wild-type mice (Fig. 4*B*). MNU-treated $Apc^{Min/+}$ mice also had a greater tumor multiplicity in the stomach when compared with that in $Apc^{Min/+}$ or MNU-treated wild-type mice (Fig. 4*C*). The average number of the gastric tumors in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at 25 weeks of age was 0.8 ± 1.26, 4.3 ± 1.38, and 0.2 ± 0.40 (±SD) per mouse, respectively. These results indicate that MNU accelerates gastric tumorigenesis in $Apc^{Min/+}$ mice.

Invasive adenocarcinomas in the stomach of $Apc^{Min/+}$ mice treated with MNU. Gastric tumors in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice were evaluated for their histopathologic features (Fig. 5). MNU-treated $Apc^{Min/+}$ mice often developed intramucosal adenocarcinomas as well as benign adenomas, although all gastric tumors in $Apc^{Min/+}$ mice were adenomas as described above. The majority of the tumors in MNU-treated wild-type mice were also classified as adenomas at each age. Surprisingly, 3 of 12 (25%) MNU-treated $Apc^{Min/+}$ mice developed invasive adenocarcinomas after 30 weeks of age (Fig. 5*B*), and this was never observed in either the $Apc^{Min/+}$ mice or the MNU-treated wild-type mice during these experiments. Undifferentiated cells in the submucosal layer were confirmed to be invasive

adenocarcinomas using AE1/AE3 staining, which recognizes the epithelial cells (Fig. 5*A*). The histologic grade of gastric tumors in MNU-treated *Apc*$^{Min/+}$ mice progressed over time (Fig. 5*B*), possibly representing the multistage process of the gastric carcinogenesis.

β-Catenin accumulation in gastric tumors of MNU-treated *Apc*$^{Min/+}$ mice. Most of the adenomatous lesions in MNU-treated *Apc*$^{Min/+}$ mice revealed nuclear/cytoplasmic β-catenin staining, similar to the staining in *Apc*$^{Min/+}$ mice (Fig. 6*A*), indicating the involvement of β-catenin activation. However, the majority of small tumors (<1.0 mm) in the stomach of MNU-treated wild-type mice revealed no evidence of β-catenin accumulation, thereby showing only membranous staining and thus suggesting that MNU-induced tumorigenesis is initiated by pathways other than β-catenin activation. The incidences of β-catenin accumulation in gastric tumors (<2.0 mm) of *Apc*$^{Min/+}$, MNU-treated *Apc*$^{Min/+}$, and MNU-treated wild-type mice were 100% (12 of 12), 84.6% (11 of 13), and 12.5% (1 of 8), respectively (Fig. 6*B*). Gastric tumors in MNU-treated *Apc*$^{Min/+}$ mice had a significantly higher frequency of β-catenin accumulation in comparison with the MNU-treated wild-type mice.

Altered gene expressions in gastric tumors of *Apc*$^{Min/+}$ and MNU-treated *Apc*$^{Min/+}$ mice. To examine gene expression patterns on the gastric tumors of *Apc*$^{Min/+}$ mice, we analyzed 18 genes (*Ptgs2, Erbb2, Kras, Ccne1, Cdh1, Cdkn1a, Cdkn1b, Tgfa, Tgfb1, Egf, Egfr, Vegfa, Vegfr2, Fgfr2, Met, Stat3, Runx3*, and *Cdx2*), which have previously been shown to be differentially expressed in human gastric adenomas and carcinomas (15, 40, 41), by quantitative real-time RT-PCR (Fig. 6*C*; Supplementary Fig. S1). The expression of three genes (*Cyclin E1, TGFβ1*, and *STAT3*), in addition to *Cyclin D1* (Fig. 3*B*), significantly increased in the gastric tumors of *Apc*$^{Min/+}$ mice, and five genes (*COX-2, Cyclin E1, TGFβ1, EGFR*, and *K-sam*) were significantly up-regulated in tumors of MNU-treated *Apc*$^{Min/+}$ mice in comparison with the adjacent normal mucosa. Furthermore, the expression of four genes (*COX-2, E-cadherin, TGFα*, and *EGFR*) was significantly higher in tumors of MNU-treated *Apc*$^{Min/+}$ mice in comparison with those in untreated *Apc*$^{Min/+}$ mice. Among these up-regulated genes, the expression of cyclooxygenase-2 (COX-2) mRNA was markedly increased in gastric tumors of both MNU-treated wild-type and *Apc*$^{Min/+}$ mice (Fig. 6*C*).

Frequent LOH of the *Apc* in gastric tumors of *Apc*$^{Min/+}$ mice. To determine whether *Apc* LOH is involved in the development of gastric tumors, we did *Apc* LOH analysis on the adenomatous lesions isolated using the LCM system (Fig. 6*D*). Twenty-seven of 32 (84.3%) dissected tumors from *Apc*$^{Min/+}$ mice showed a predominant signal of the *Apc*^{Min} allele, thus indicating a loss of the wild-type allele. These results suggest that *Apc* LOH is involved in the development of gastric tumors in *Apc*$^{Min/+}$ mice. Gastric tumors in MNU-treated *Apc*$^{Min/+}$ mice showed a reduced frequency of allelic loss at *Apc* (8 of 21, 38.1%; $P < 0.001$ versus *Apc*$^{Min/+}$ mice, by Fisher's exact test). To determine whether mutations of *Apc* are involved in the tumor initiation by MNU, three gastric tumors without allelic loss at *Apc* in MNU-treated *Apc*$^{Min/+}$ mice were examined for somatic mutations at *Apc*. However, no mutation of the *Apc* was detected in these gastric tumors except for a nonsense mutation of *Apc*^{Min} allele (T/A → A/T transversion at nucleotide 2,549 of the *Apc*; ref. 42).

Nuclear accumulation of β-catenin in human gastric adenomas and early adenocarcinomas. To clarify roles of Wnt/β-catenin pathway in the early stage of human gastric carcinogenesis, we investigated the β-catenin expression in sporadic adenomas and

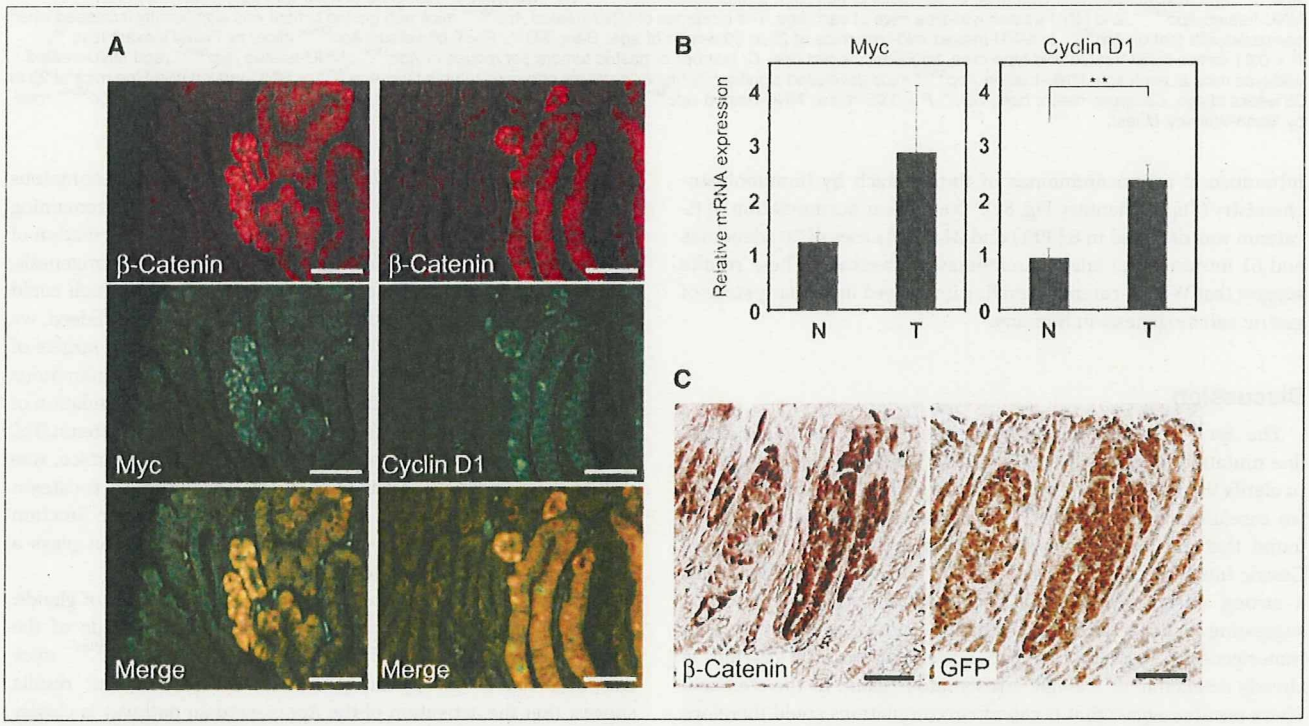

Figure 3. Increased β-catenin/Tcf transcriptional activity in the adenomatous lesions of the stomach in *Apc*$^{Min/+}$ mice. *A*, double immunofluorescent staining for β-catenin, Myc, or cyclin D1. β-Catenin accumulation (*red*) is colocalized (*yellow*) with Myc (*left*; *green*) and cyclin D1 (*right*; *green*) overexpressions in the adenomatous glands within the tumor. *B*, increased mRNA expression of *Myc* and *cyclin D1* in the gastric tumors of *Apc*$^{Min/+}$ mice. The expression of each gene was examined by quantitative real-time RT-PCR and normalized to *β-actin* expression. *N*, normal epithelium ($n = 10$); *T*, tumor ($n = 10$). *Columns*, mean of three independent experiments; *bars*, SE. **, $P < 0.01$, Mann-Whitney *U* test. *C*, β-catenin/Tcf reporter mice show GFP (*right*) expression in adenomatous glands with β-catenin accumulation (*left*). Bar, 100 μm (*A* and *C*).

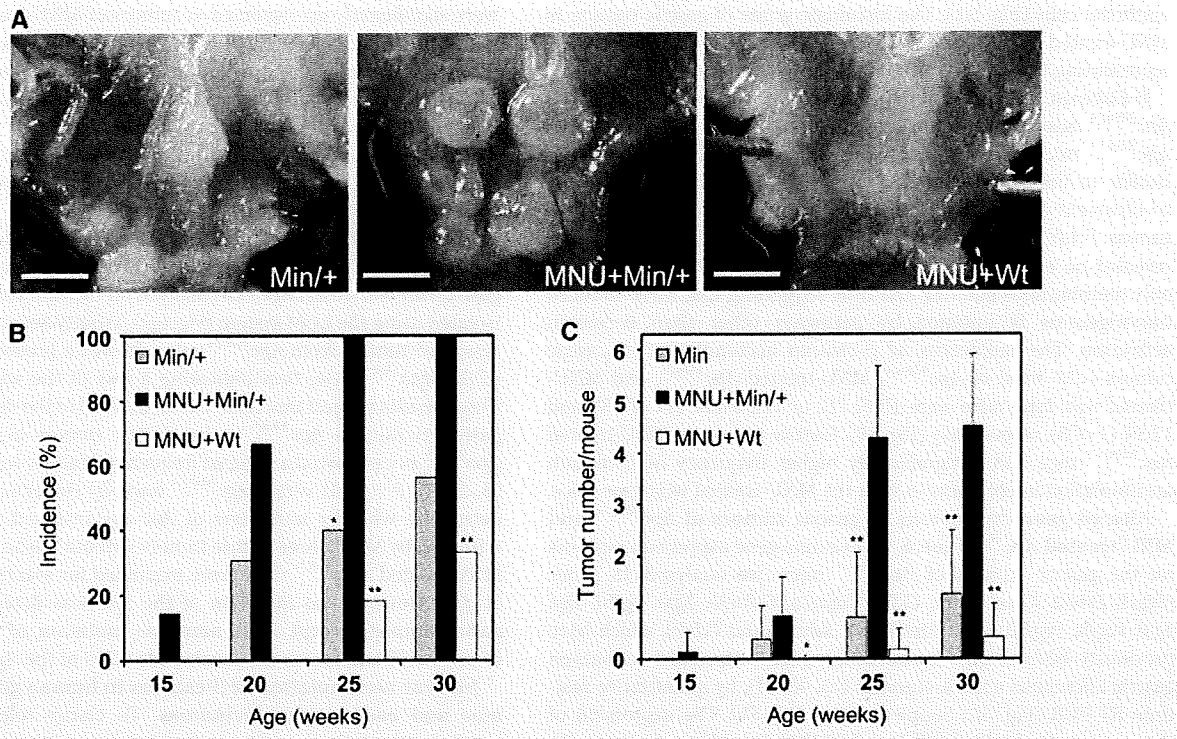

Figure 4. MNU treatment accelerates gastric tumorigenesis in $Apc^{Min/+}$ mice. *A,* representative macroscopic photographs of the stomach in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at 25 weeks of age. Bar, 2 mm. *Min/+*, $Apc^{Min/+}$; *Wt*, wild-type. *B,* incidence of mice with gastric tumors in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at each age. The incidence of MNU-treated $Apc^{Min/+}$ mice with gastric tumors was significantly increased when compared with that of $Apc^{Min/+}$ or MNU-treated wild-type mice at 25 to 30 weeks of age. *Bars,* SD. *, $P < 0.05$ versus $Apc^{Min/+}$ mice, by Fisher's exact test; **, $P < 0.01$ versus MNU-treated wild-type mice, by Fisher's exact test. *C,* number of gastric tumors per mouse in $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice at each age. MNU-treated $Apc^{Min/+}$ mice developed significantly higher numbers of gastric tumors than $Apc^{Min/+}$ or MNU-treated wild-type mice at 20 to 35 weeks of age. *Columns,* mean; *bars,* SD. *, $P < 0.05$ versus MNU-treated $Apc^{Min/+}$ mice, by Mann-Whitney *U* test; **, $P < 0.01$ versus MNU-treated $Apc^{Min/+}$ mice, by Mann-Whitney *U* test.

intramucosal adenocarcinomas of the stomach by immunohistochemistry (Supplementary Fig. S2). The nuclear accumulation of β-catenin was detected in 8 (40%) and 24 (39%) cases of 20 adenomas and 61 intramucosal adenocarcinomas, respectively. These results suggest that Wnt/β-catenin signaling is involved in the early stage of gastric carcinogenesis in humans.

Discussion

The $Apc^{Min/+}$ mouse, a murine model for FAP, carrying a germline mutation at codon 850 of *Apc* (23), has been used extensively to clarify the pathogenesis of intestinal tumorigenesis. In this study, we carefully monitored $Apc^{Min/+}$ mice over 25 weeks of age and found that aged $Apc^{Min/+}$ mice develop multiple gastric tumors. Gastric tumors in $Apc^{Min/+}$ mice showed adenomatous lesions with a strong nuclear/cytoplasmic accumulation of β-catenin, thus suggesting that an altered β-catenin expression is involved in tumorigenesis. Furthermore, the accumulation of β-catenin is already detectable at a single adenomatous gland in the stomach. These results suggest that β-catenin accumulations could therefore be an initiating event in gastric carcinogenesis. We consistently found the nuclear accumulation of β-catenin in ~40% of adenomas and early carcinomas in human gastric tissues.

β-Catenin accumulation is caused by a loss of the *Apc* function through LOH in intestinal tumors in mice that are heterozygous for a mutant allele of *Apc* (24). In the present study, adenomatous lesions in the stomach were also found to have lost the remaining allele of *Apc*, indicating that a loss of *Apc* leads to the formation of such adenomatous glands. Similar to the intestinal tumorigenesis, *Apc* LOH may lead to the accumulation of β-catenin, which could activate its downstream pathway in the gastric tumors. Indeed, we showed that *Myc* and *Cyclin D1* expressions, which are targets of β-catenin/Tcf transcription (9, 10), also increased in adenomatous lesions, and they were colocalized with the nuclear accumulation of β-catenin. In addition, the transcriptional activity of β-catenin/Tcf, which is assessed by the use of β-catenin/Tcf reporter mice, was also found to increase in the adenomatous glands with β-catenin accumulation. These results suggest that a loss of the *Apc* function followed by the activation of β-catenin/Tcf transcription plays a causal role in the development of gastric cancers.

The glandular stomach consists of two different types of glands: the pyloric glands of the antrum and the fundic glands of the corpus. The highest frequency of gastric tumors in $Apc^{Min/+}$ mice was observed in the pyloric glands of the antrum. Our results suggest that the activation of the Apc/β-catenin pathway is closely associated with gastric tumorigenesis in the pyloric glands. The biological characteristics of the pyloric glands in the antrum have recently been shown to be developmentally similar to those of the small intestinal epithelium (40, 43). In addition to the frequent development of tumors in both the small intestine and the antrum

of the stomach in $Apc^{Min/+}$ mice, our results suggest that the mechanisms of gastric tumorigenesis in the pyloric glands may be similar to those of intestinal tumorigenesis. In contrast, we did not detect any fundic gland polyps that are often associated with FAP patients. The importance of the Apc/β-catenin pathway in the development of fundic gland polyps in mice remains to be elucidated.

The MNU-induced rodent models for gastric carcinogenesis have been widely used to study carcinogenesis of the stomach. These animal models have been used not only for investigating the pathogenesis of gastric carcinogenesis but also for identifying possible tumor promoters and chemopreventive agents (34, 35, 44, 45). However, mice have been known to be relatively resistant to MNU, and therefore, previous studies showed that 48 to 52 weeks were required to induce gastric carcinoma in C57BL/6J mice (35, 37, 46, 47). In the present study, we treated $Apc^{Min/+}$ mice with MNU and found MNU to strongly promote tumor development in the stomach of $Apc^{Min/+}$ mice in comparison with MNU-treated wild-type mice. Furthermore, MNU-treated $Apc^{Min/+}$ mice developed invasive adenocarcinomas, which were not detectable in either $Apc^{Min/+}$ mice or MNU-treated wild-type mice at the same age. These results indicate that this model could be a short-term model for gastric carcinogenesis with β-catenin accumulation in mice.

It is interesting to note that the majority of small gastric tumors (<1.0 mm) induced by MNU in wild-type mice showed no evidence of β-catenin accumulations, whereas all tumors in $Apc^{Min/+}$ mice showed an accumulation of the protein even in a single adenomatous gland. These findings suggest that an initiating event in the stomach of an MNU-induced model is independent of Wnt activation. This notion is consistent with previous findings in rodents, which suggest that β-catenin accumulation may be a later event of gastric carcinogenesis in MNU-induced models (48–50). The fact that $Apc^{Min/+}$ mice treated with MNU tend to develop more aggressive tumors in the stomach may be attributable to the combined activation of different oncogenic pathways in both models. It is noteworthy that the expression of COX-2 mRNA specifically increased in gastric tumors of MNU-treated mice. It has recently been reported that the simultaneous activation of Wnt signaling and the COX-2 pathway leads to the development of gastric cancers in mice (12). The cross-talk between Wnt and the COX-2 pathway may contribute to the rapid development of gastric tumors in MNU-treated $Apc^{Min/+}$ mice. In this context, the MNU-treated $Apc^{Min/+}$ mice could be a useful model to investigate multistage carcinogenesis of the stomach.

In the current study, most of the adenomatous lesions in MNU-treated $Apc^{Min/+}$ mice revealed nuclear accumulations of β-catenin, similar to those in $Apc^{Min/+}$ mice (Fig. 6*A* and *B*), indicating the involvement of β-catenin activation. However, allelic loss at *Apc* in such tumors was less frequent than that in $Apc^{Min/+}$ mice (Fig. 6*D*). In addition, no mutation at the *Apc* was

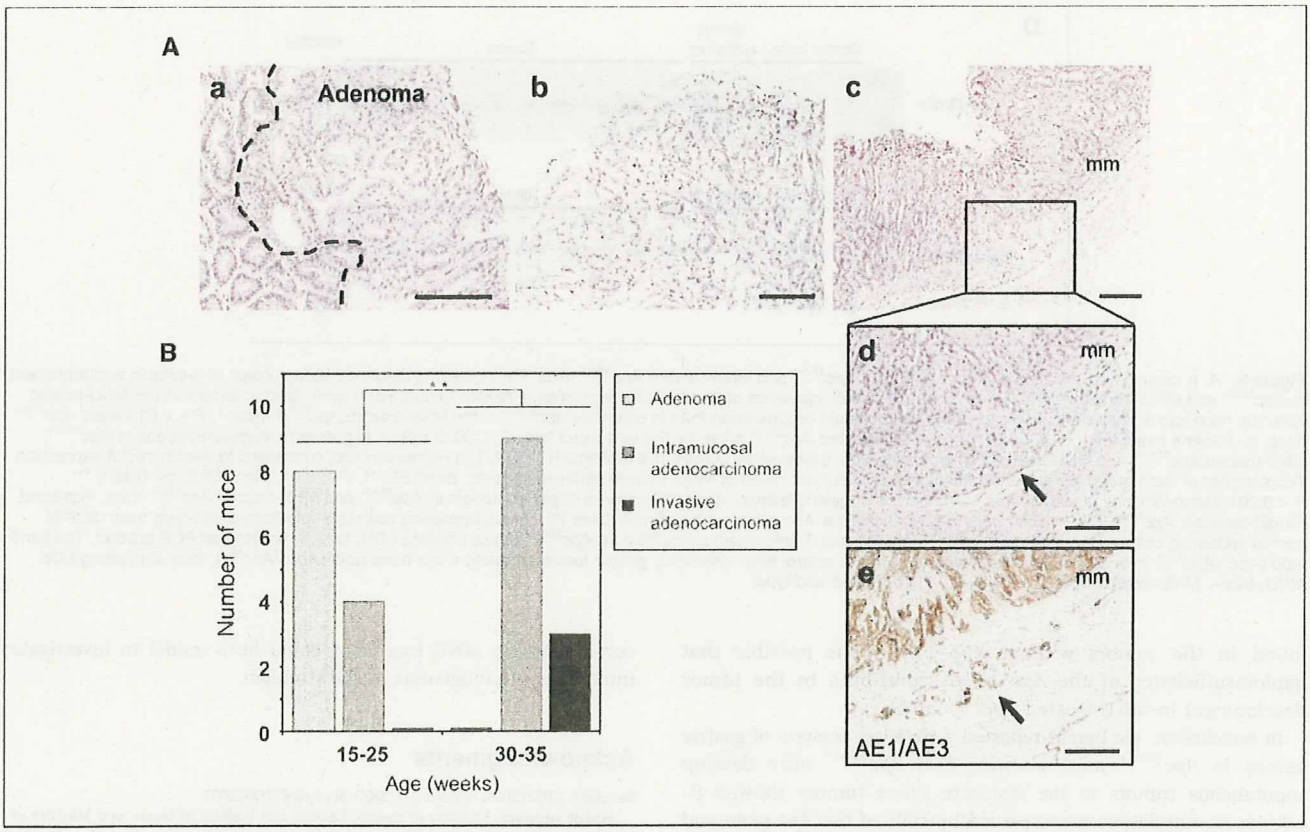

Figure 5. Gastric tumors in MNU-treated $Apc^{Min/+}$ mice show more aggressive lesions. *A*, histopathologic features of gastric tumors in MNU-treated $Apc^{Min/+}$ mice. *a*, adenomas in MNU-treated $Apc^{Min/+}$ mice were similar to that in $Apc^{Min/+}$ mice. *Dotted line*, border between hyperplastic and adenomatous (*Adenoma*) lesion. *b*, intramucosal adenocarcinomas with distinctive cellular and structural atypia. *c*, several gastric adenocarcinomas showed invasion into the submucosa. *d*, higher magnification of the boxed section in (*c*). *e*, invasive undifferentiated cells expressed an epithelial marker, AE1/AE3. *mm*, muscularis mucosae. Bar, 100 μm. *B*, histologic grade of the most advanced tumor in tumor-bearing MNU-treated $Apc^{Min/+}$ mice. The histologic grade of the tumors progressed over time. *Columns*, number of mice with each histologic type. **, $P < 0.01$, Spearman's rank correlation test.

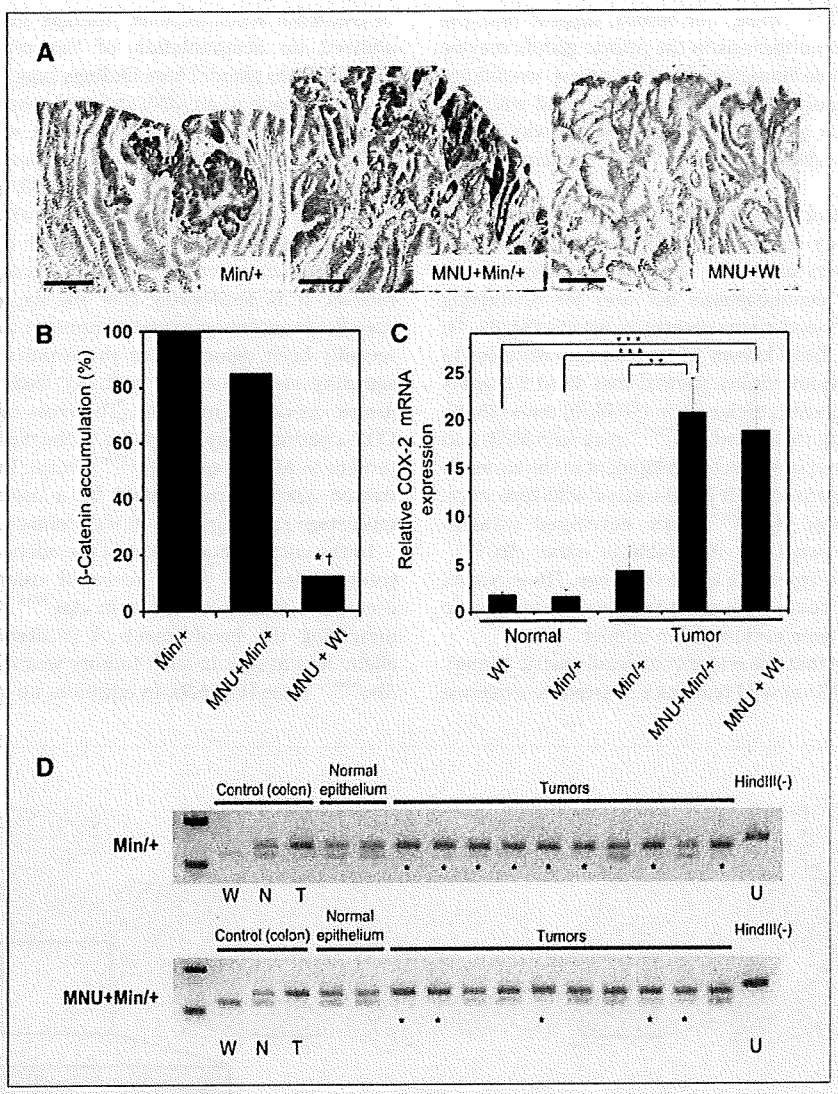

Figure 6. *A,* β-catenin accumulation of gastric tumors in $Apc^{Min/+}$ and MNU-treated $Apc^{Min/+}$ mice. The nuclear/cytoplasmic accumulation of β-catenin was prominent in $Apc^{Min/+}$ and MNU-treated $Apc^{Min/+}$ mice. Bar, 100 μm. *B,* incidence of β-catenin accumulation in gastric tumors (<2.0 mm). Gastric tumors in the MNU-treated wild-type mice had a significantly lower frequency of β-catenin accumulation than in either the $Apc^{Min/+}$ or the MNU-treated $Apc^{Min/+}$ mice. *, $P < 0.01$ versus $Apc^{Min/+}$ mice, by Fisher's exact test; †, $P < 0.01$ versus MNU-treated $Apc^{Min/+}$ mice, by Fisher's exact test. *C,* COX-2 mRNA expressions in gastric lesions of $Apc^{Min/+}$, MNU-treated $Apc^{Min/+}$, and MNU-treated wild-type mice were assessed by quantitative real-time RT-PCR. The expression was normalized to β-actin mRNA expression. Ten samples of each group were analyzed in triplicate. *Columns,* mean of three independent experiments; *bars,* SE. **, $P < 0.01$, Mann-Whitney *U* test; ***, $P < 0.001$, Mann-Whitney *U* test. *D, Apc* LOH analysis of gastric lesions. *Apc* LOH analysis of gastric tumors in $Apc^{Min/+}$ and MNU-treated $Apc^{Min/+}$ mice. *Top band, Hin*dIII-resistant Apc^{Min} PCR product; *bottom band,* wild-type *Apc* allele cut by *Hin*dIII. *Lane W,* normal-appearing colonic crypts from a wild-type mice; *lane N,* normal-appearing colonic crypts from an $Apc^{Min/+}$ mouse; *lane T,* intestinal tumors from an $Apc^{Min/+}$ mouse showing LOH; *lane U,* undigested PCR product. The band ratio (Apc^{+}/Apc^{Min}) in each sample was compared with the control lane. *Asterisks,* gastric tumors showing a low band ratio (Apc^{+}/Apc^{Min}), thus suggesting LOH. *MNU+Min/+,* MNU-treated $Apc^{Min/+}$; *MNU+Wt,* MNU-treated wild-type.

found in the tumors without *Apc* LOH. It is possible that haploinsufficiency of the *Apc* might contribute to the tumor development in MNU-treated $Apc^{Min/+}$ mice.

In conclusion, we herein reported a detailed analysis of gastric lesions in $Apc^{Min/+}$ mice, showing that $Apc^{Min/+}$ mice develop spontaneous tumors in the stomach. These tumors showed β-catenin accumulation accompanied by LOH of the *Apc* gene and the activation of the β-catenin/Tcf signaling pathway. Our results suggest that the Wnt pathway thus plays a causal role in the development of gastric cancer, and this animal model could provide a useful means to investigate the human gastric tumorigenesis in relation to Wnt activation. Furthermore, $Apc^{Min/+}$ mice combined with MNU treatment could be a model to investigate multistage carcinogenesis in the stomach.

Acknowledgments

Received 10/31/2006; revised 2/7/2007; accepted 2/14/2007.

Grant support: Ministry of Health, Labour and Welfare of Japan, and Ministry of Education, Culture, Sports, Science and Technology of Japan.

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We thank Drs. Satoshi Yamashita and Akihiro Hirata for helpful discussions and Kyoko Takahashi, Ayako Suga, and Yoshitaka Kinjyo for technical assistance and animal care.

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Review Article

Multistep carcinogenesis of the colon in *Apc*$^{Min/+}$ mouse

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(Received July 27, 2006/Revised September 3, 2006/Accepted September 5, 2006/Online publication October 19, 2006)

Colon cancer arises through different histological stages representing different genetic and epigenetic alterations. The *Apc*$^{Min/+}$ mouse has a point mutation at the *Apc* gene, and it is considered to be a model for human familial adenomatous polyposis. Our previous studies have revealed the presence of a number of intramucosal microadenomas in the colons of *Apc*$^{Min/+}$ mice, in which only a few macroscopic tumors were recognized. These observations suggest that there are two distinct stages for colon carcinogenesis in *Apc*$^{Min/+}$ mouse, and the *Apc*$^{Min/+}$ mouse is regarded as a good model to study multistage colon carcinogenesis. A number of genes that modify intestinal tumorigenesis have been identified using *Apc* mutant mice combined with other mutant mice. It has become apparent that epigenetic modification strongly affects intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. We herein describe the different stages of colon tumorigenesis and their modifiers, and discuss the possible application of *Apc* mutant mice in order to better understand the molecular mechanisms of multistage carcinogenesis in the large bowel of humans. (*Cancer Sci* 2007; 98: 6–10)

Apc$^{Min/+}$ and *Apc* mutant mice

The loss of *APC* function has been proven to play a pivotal role in colorectal carcinogenesis.[1] *APC* is now recognized as a recessive tumor suppressor gene, and inactivation of both alleles is necessary for tumor formation. The mutant mouse lineage is considered to be predisposed to multiple intestinal neoplasms (Min) and is thus regarded as one of the models for colorectal tumorigenesis. Originally, this lineage was established from an ethylnitrosourea-treated C57BL/6 male mouse, and its phenotype is an autosomal dominant trait.[2] The dominant mutation is known to be located in *Apc*, the mouse homolog of the human *APC* gene, resulting in truncation of the gene product at amino acid 850.[3] Although homozygous *Apc*$^{Min/Min}$ mice die as embryos, *Apc*$^{Min/+}$ mice develop multiple intestinal neoplasias in their intestinal tracts within several weeks of birth. It is also known that most of the intestinal tumors in mice that are heterozygous for a mutant allele of *Apc* have lost *Apc* function by LOH, especially in mice with a C57BL/6 genetic background.[4] Using gene targeting strategies in ES cells, several lines of genetically engineered *Apc* mutant mice, including mice with conditional inactivating alleles, have been established.[5–7] Although the onset, severity and location of tumors vary among these lines, they all develop multiple tumors in their intestine.

A number of the genes that modify intestinal tumorigenesis have been identified using *Apc* mutant mice. Deletions in the genes related to arachidonic acid metabolism, such as *Ptgs2*, *cPLA*[2] and *EP2*, have been shown to suppress intestinal tumorigenesis,[8–11] whereas deletions in genes related to genomic stability increases tumorigenesis in *Apc* mutant mice.[12,13] Such experiments greatly expand our understanding of intestinal tumorigenesis while also helping to clarify the molecular mechanisms of carcinogenesis in organs other than the intestine.

Small intestine and colon

The intestinal tract consists of the small intestine (duodenum, jejunum and ileum) and the large intestine or colon. There are a number of anatomical and physiological differences between the small intestine and the colon. Developmentally, the midgut forms the distal part of the duodenum, all of the small intestine, cecum, appendix and the ascending and transverse colon, whereas the hindgut develops the remaining colon and rectum.

In the small intestine, the stem and progenitor compartment is believed to reside in crypts. Stem cells in the small intestine are suggested to be located on the fourth or fifth position from the bottom of the crypts and then differentiate into four different cell types: absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Enterocytes, enteroendocrine cells and goblet cells occupy the villi whereas the Paneth cells reside at the bottom of the crypts and secrete antimicrobial agents.[14] In contrast, the mucosa of the colon has a flat surface epithelium instead of villi and consists only of a crypt compartment. The proliferative stem and precursor cells in the colon occupy the bottom two-thirds of the crypts, whereas differentiated cells constitute the surface epithelium and top third of the crypts. The stem and progenitor cells in the colonic crypts differentiate into enterocytes, enteroendocrine cells and goblet cells, whereas Paneth cells are absent in the colon under normal physiological conditions.

Given such differences between the small intestine and colon, it should be noted that the major location of intestinal tumors in *Apc* mutant mice is the small intestine, whereas most human bowel cancers tend to arise in the colon. In addition, there is also evidence indicating that tumor formation in the colon does not always correlate with that in the small intestine in *Apc*$^{Min/+}$ mice. For example, *Apc*$^{Min/+}$;*BubR1* compound mice develop tumors preferentially in the colon, whereas they demonstrate fewer tumors in the small intestine than control *Apc*$^{Min/+}$ mice.[15] These findings imply that tumorigenesis in the colon is different from that in the small intestine. It is therefore important to differentiate between tumorigenesis in the small intestine and large intestine.

Small dysplastic crypts as pretumoral lesions in the colon of *Apc*$^{Min/+}$ mouse

Colon carcinogenesis is regarded as a multistep event with genetic and epigenetic alterations. In humans, the adenomatous polyposis coli, β-catenin, Ki-*ras* oncogene and *p53* genes are thought to play important roles at different stages of colorectal carcinogenesis.[1,16,17] In previous studies, we have shown the presence of small dysplastic crypts in the colonic sections of

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Abbreviations: *Apc*/*APC*, adenomatous polyposis coli; ES, embryonic stem; LOH, loss of heterozygosity.

doi: 10.1111/j.1349-7006.2006.00348.x

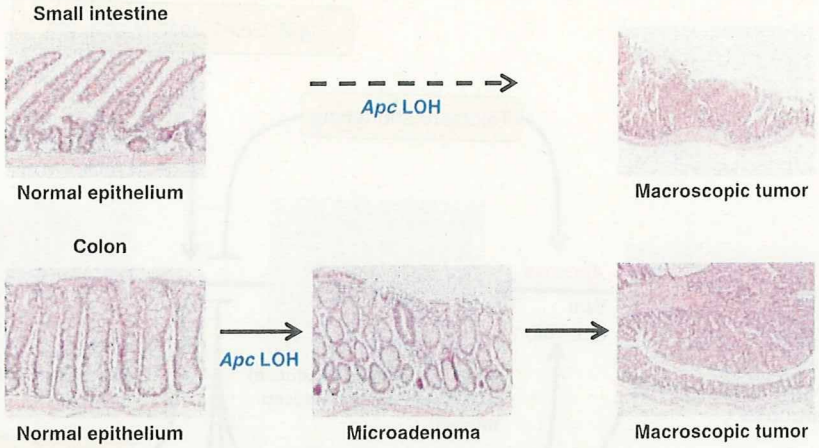

Fig. 1. Intestinal tumorigenesis in $Apc^{Min/+}$ mice. A previous study indicated that the process of tumorigenesis in the small intestine is different from that in the colon. There are two histologically distinguishable lesions in the colon of the $Apc^{Min/+}$ mouse.

rodents that are treated with colon-specific carcinogens.[18,19] These small dysplastic crypts were shown to harbor frequent β-catenin mutations, which are also detectable in most colon tumors.[20] Because of the accumulation of β-catenin protein, such crypts were designated as β-catenin accumulated crypts (BCAC). A series of studies have demonstrated that BCAC are likely to be direct precursor lesions of carcinogen-induced colon tumorigenesis.[21]

We revealed the presence of a number of intramucosal microadenomas in the colon of $Apc^{Min/+}$ mice.[22] Micoradenomas in the colon of $Apc^{Min/+}$ mice consisted of one to six dysplastic crypts. Importantly, such microadenomas in the colon were found to have lost the remaining allele of *Apc*, thus indicating a loss of the *Apc* function to have already occurred in such crypts.[22] Accordingly, it seems to be reasonable to apply Knudson's 'two-hit' theory to the formation of the microadenomas.[23] In agreement with the presence of *Apc* LOH, the accumulation of β-catenin is observed in all microadenomas in the colon of the $Apc^{Min/+}$ mouse, suggesting that microadenomas are identical lesions to BCAC.[21] Together with the fact that colon tumors in $Apc^{Min/+}$ mice harbor frequent *Apc* LOH and the accumulation of β-catenin, microadenomas may therefore be direct precursors of colon tumors in $Apc^{Min/+}$ mice (Fig. 1).

Aberrant crypt foci (ACF) were first described by Bird *et al.*[24] and a number of studies, including a molecular analysis, have emphasized the significance of ACF as preneoplastic lesions in colon carcinogenesis.[25] Therefore, ACF are now used to evaluate potential chemopreventive agents against colon carcinogenesis.[26,27] Nevertheless, there is increasing evidence indicating the lack of any correlation between tumor development and the formation of ACF.[28,29] Interestingly, previous observations showed a lack of classical ACF in the surface of the colonic mucosa of $Apc^{Min/+}$ mice with a number of miroadenomas,[21,30] thus suggesting that microadenomas are independent lesions of ACF. K-*ras* mutations are recognized in the majority of classical ACF in rats and humans,[31] indicating that K-*ras* mutations are closely associated with the formation of ACF. Consistent with this notion, mouse strains carrying oncogenic alleles of K-*ras* develop ACF in the colon.[32] These findings suggest the activation of Wnt and K-*ras* pathways to be responsible for the formation of BCAC and ACF, respectively. Interestingly, the contribution of BCAC (or microadenomas in $Apc^{Min/+}$ mouse) and ACF to colon carcinogenesis was suggested by examining the phenotype of $Apc^{Min/+}$ mice and mice carrying oncogenic alleles of K-*ras*. It is important to note that $Apc^{Min/+}$ mice develop colonic tumors as well as microadenomas, whereas activated K-*ras* mice develop only ACF but no tumors in their colon.[32] It seems to be true that the activation of Wnt signaling is important as an initiating event and BCAC are direct precursors of colon tumors. The significance of ACF with a K-*ras* mutation in premalignant lesions remains to be resolved (Fig. 2). It is possible that activated K-*ras* may cause oncogenic stress, which may thus induce cells to initiate apoptosis, eventually leading to the elimination of this cell population.

Two distinct stages for colon tumorigenesis in the $Apc^{Min/+}$ mouse: micoradenomas and macroscopic tumors

In $Apc^{Min/+}$ mice, the number of colonic microadenomas per area was higher than that of adenomatous lesions in the small intestine, suggesting that the loss of *Apc* occurs frequently in colonic crypts as well as in the epithelium of the small intestine.[22] Despite the frequent development of microadenomas, the number of macroscopic colonic tumors is much less than that of small intestinal tumors. Interestingly, the size of microadenomas in the colon of $Apc^{Min/+}$ mice does not increase with time, suggesting that microadenoma itself is a self-limiting lesion (Y Yamada and H Mori, unpublished data). Based on the multistep carcinogenesis theory in the colon, the findings indicate that there are at least two distinct stages for colon tumorigenesis in $Apc^{Min/+}$ mice and additional events are thus required for transition from microadenomas to macroscopic tumors (Fig. 1). In contrast to colonic lesions, the adenomatous lesions in the small intestine show various sizes, and the mean size of the lesions is significantly larger than in the colon.[22] As the loss of *Apc* is also involved in the earliest lesions in the small intestine,[5] it is possible that, in the small intestine, the loss of function of *Apc* results in the formation of microadenomas that could develop directly into intestinal tumors by aging (Fig. 1). These findings may explain why the $Apc^{Min/+}$ mouse develops intestinal tumors preferably in the small intestine, again suggesting that the mechanisms of tumorigenesis involved in the small intestine may differ from those in the colon.

Alterations required for transition from microadenomas to macroscopic tumors in the colon

It remains unclear which event is mainly responsible for the transition from microadenomas to macroscopic tumors in the colon of $Apc^{Min/+}$ mice. For example, *p53*, K-*ras* and B-*raf* mutations or microsatellite instabilities, which are observed frequently in human colon cancers, are not detectable in colon tumors of $Apc^{Min/+}$ mice (Y Yamada and H Mori, unpublished data). In our previous study using a rat model, specific β-catenin mutations at the residues that regulate β-catenin levels directly

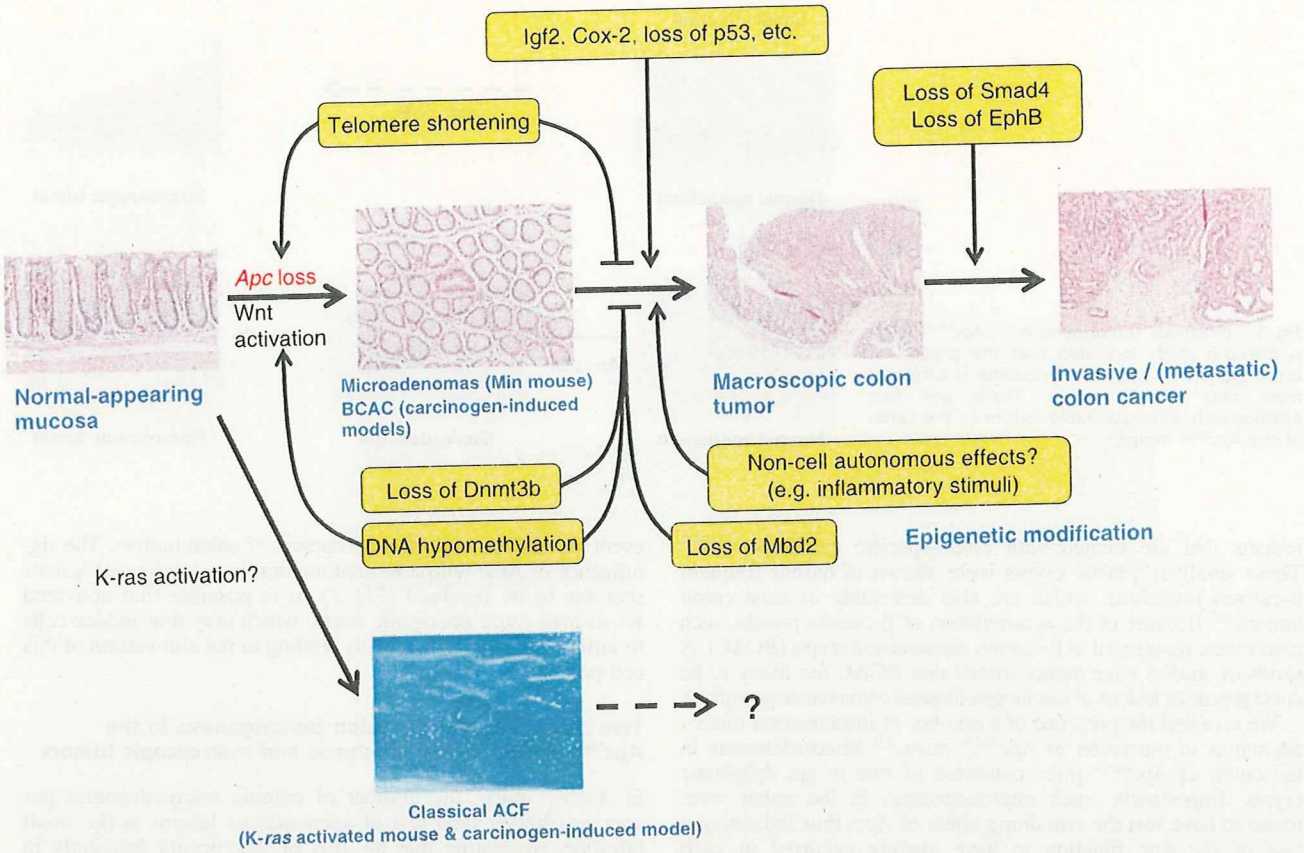

Fig. 2. A model for multistage colon carcinogenesis in mice and its possible modifiers. Genetically engineered mice have shown a number of modifiers for different stages of colon carcinogenesis.

were selected from a wide spectrum of mutations during the development of colon tumors from intramucosal small lesions.[20] This finding suggests that an increased level of oncogenic β-catenin is therefore required for tumor development in the colon, and activation of the β-catenin/Tcf pathway, which is involved in not only the initiation stages but also the promotion stages. Consistent with this assumption, the expression of nuclear β-catenin has been shown to correlate with the size of colon neoplasms in humans.[33] In addition, a recent study indicated that β-catenin/Tcf signaling can be further activated by upstream signals regardless of any constitutive activation of the pathway by downstream mutations in colon cancer cells.

Together with the critical contribution of the microenvironment (stem cell niche) in maintaining the self-renewal of stem cells,[34] recent findings that cancer-associated fibroblasts play a significant role in the tumor promotion of breast cancer[35] have shed some new light on the involvement of the non-cell autonomous effect on tumor formation. In *Apc* mutant mice, fibroblasts and endothelial cells adjacent to neoplastic cells are reported to express COX-2, which is suggested to enhance tumorigenesis[36] and to be a molecular target of cancer prevention.[37] Furthermore, several immune-deficient mice with accompanying colonic inflammation are reported to develop proliferative lesions that occasionally have the potential to progress into adenocarcinomas.[38] A recent study has also shown that the inflammatory stimuli induced by dextran sodium sulfate strongly promotes colon tumorigenesis in the $Apc^{Min/+}$ mouse.[39] Importantly, these findings suggest the existence of a non-cell autonomous effect on colorectal carcinogenesis.

Effects of epigenetic modifications on intestinal tumorigenesis in the $Apc^{Min/+}$ mouse

Changes in DNA methylation status are one of the most common molecular alterations in human neoplasia.[40] The role of aberrant hypermethylation in the silencing of tumor suppressor genes has been well documented.[40] In contrast, the functional significance of genome-wide DNA hypomethylation is still unclear, although this alteration has been reported in a wide variety of human cancers.[41,42] In colon carcinogenesis, DNA hypomethylation has been observed in both adenomas and adenocarcinomas,[42] suggesting that such hypomethylation is associated with the early stages of carcinogenesis.

Dnmt1 has been demonstrated as a maintenance DNA methyltransferase that is essential to maintain global DNA methylation levels,[43,44] and therefore Dnmt1 hypomorphic mice express global DNA hypomethylation.[45,46] It was shown recently that Dnmt1 hypomorphic mice develop an increased number of microadenomas, whereas a significant reduction in the number of macroscopic colonic tumors in $Apc^{Min/+}$ mice was also revealed.[46] Such observations indicate that the forced reduction of DNA methylation levels has dual effects on intestinal carcinogenesis, suggesting that global DNA hypomethylation can promote early events in tumorigenesis. Because microadenomas in DNA hypomethylated mice harbor frequent *Apc* LOH,[46] the increased incidence of microadenomas may be attributable to an elevated rate of loss of the wild-type *Apc* allele, which is also consistent with previous studies demonstrating DNA hypomethylation-dependent LOH events.[47,48]

doi: 10.1111/j.1349-7006.2006.00348.x

Although the precise mechanism by which the reduced DNA methylation level suppresses the transition from microadenomas to macroscopic tumors remains to be elucidated, a recent study showed the targeted deletion of *Dnmt3b*, de novo DNA methyltransferase, to suppress colon tumorigenesis in $Apc^{Min/+}$ mice, whereas no such effect was observed on the formation of microadenomas.[49] DNA hypermethylation accompanied by aberrant gene silencing has been shown to be associated with neoplastic progression in many tumors, including colon cancers.[50,51] Dnmt3b is involved in regional DNA methylation[52] and therefore, it is expected to play a role in DNA hypermethylation in cancers. Because site-specific DNA hypermethylation at several genes has also been reported in the intestinal tumors of $Apc^{Min/+}$ mice,[53] it is possible that such regional hypermethylation plays an important role in the transition from microadenomas to macroscopic tumors. In addition, mice lacking DNA methyl-binding protein (MBD2) also consistently develop a smaller number of intestinal tumors in $Apc^{Min/+}$ mice.[54]

Biochemical evidence indicates that DNA methylation is one component of a wider epigenetic program that includes other postsynthesis modifications of chromatin, and site-specific DNA hypermethylation is associated with the inactive states of chromatin.[55] It is interesting to note that *Dnmt3b* is just involved in the transition of microadenomas to macroscopic tumors but it is not necessary to maintain tumor cell growth.[49] Site-specific DNA hypermethylation may play a role in the fixation of silenced chromatin, and once tumor growth is initiated, Dnmt1 may maintain such heterochromatic states. This notion is also consistent with the decreased tumor formation in $Apc^{Min/+}$ mice with Dnmt1 hypomorphs. A global understanding of how DNA hypermethylation is associated with transcriptional repression, which is also linked with chromatin structure, is therefore necessary to elucidate the molecular mechanisms by which DNA hypomethylation suppresses the transition from microadenomas to macroscopic tumors.

Progression to invasive colon cancer

Most tumors in the $Apc^{Min/+}$ mouse are benign adenomas and do not demonstrate either aggressive invasion or metastasis, which are critical characteristics of human cancers.[56] Although a loss of p53 function is considered to play important a role in the conversion of adenomas to adenocarcinomas in humans,[16] $Apc^{Min/+}$;$p53^{-/-}$ mice indicate no evidence of malignant transformation.[57] However, some genes are suggested to be molecular determinants involving tumor invasions in *Apc* mutant mice. $Smad4^{+/-}$;$Apc^{\Delta 716/+}$ mice develop larger tumors, although the tumor number does not change in comparison with $Apc^{\Delta 716/+}$ mice.[58] In addition, $Ephb3^{-/-}$;$Apc^{Min/+}$ mice consistently showed larger colorectal polyps than their control littermates.[59] Around half of $Ephb3^{-/-}$;$Apc^{Min/+}$ animals develop carcinomas that invade the muscle layer, which is thus a feature of malignancy that is not present in $Ephb3^{+/+}$;$Apc^{Min/+}$ tumors. Although species-specific requirements for cellular transformation have been suggested,[60] *Apc* mutant mice are useful models for investigating malignant transformation in colon carcinogenesis. Further analyses are thus needed to determine how intestinal cancer cells metastasize to the liver and/or lung, and to establish practical mouse models for cancer metastasis.

Acknowledgments

This research was supported by grants from the Ministry of Health, Labour and Welfare of Japan, and grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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doi: 10.1111/j.1349-7006.2006.00348.x