

Matrix Metalloproteinase-9 Functions as a Tumor Suppressor in Colitis-Associated Cancer

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Abstract

There is a well-documented association of matrix metalloproteinase-9 (MMP-9) and receptor Notch-1 overexpression in colon cancer. We recently showed that MMP-9 is also upregulated in colitis, where it modulates tissue damage and goblet cell differentiation via proteolytic cleavage of Notch-1. In this study, we investigated whether MMP-9 is critical for colitis-associated colon cancer (CAC). Mice that are wild type (WT) or MMP-9 nullizygous (MMP-9^{-/-}) were used for *in vivo* studies and the human enterocyte cell line Caco2-BBE was used for *in vitro* studies. CAC was induced in mice using an established carcinogenesis protocol that involves exposure to azoxymethane followed by treatment with dextran sodium sulfate. MMP-9^{-/-} mice exhibited increased susceptibility to CAC relative to WT mice. Elevations in tumor multiplicity, size, and mortality were associated with increased proliferation and decreased apoptosis. Tumors formed in MMP-9^{-/-} mice exhibited expression of p21^{WAF1/Cip1} and increased expression of β -catenin relative to WT mice. *In vitro* studies of MMP-9 overexpression showed increased Notch-1 activation with a reciprocal decrease in β -catenin. Notch and β -catenin/Wnt signaling have crucial roles in determining differentiation and carcinogenesis in gut epithelia. Despite being a mediator of proinflammatory responses in colitis, MMP-9 plays a protective role and acts as a tumor suppressor in CAC by modulating Notch-1 activation, thereby resulting in activation of p21^{WAF1/Cip1} and suppression of β -catenin. *Cancer Res* 70(2); 792–801. ©2010 AACR.

Introduction

Matrix metalloproteinases (MMP) are zinc-dependent neutral endopeptidases, which participate in degradation of extracellular matrix proteins and are important for normal tissue remodeling but are also involved in several pathologic processes, such as arthritis, atherosclerosis, myocardial infarction, colorectal cancer, tumor invasion, and inflammatory bowel disease (IBD; refs. 1, 2). MMP-9 is a 92-kDa protein, one of the two MMPs known as gelatinases. MMP-9 is absent from most normal adult tissues, including the intestinal epithelial cells. We and several others have shown that MMP-9 is highly expressed during intestinal inflammation in different animal models and human IBD (3, 4). We have also shown that MMP-9^{-/-} mice exposed to dextran sodium sulfate (DSS) or *Salmonella typhimurium* had dramatically reduced inflammation and mucosal injury and showed protection against acute colitis, indicating that MMP-9 is a mediator of inflammation (3). Further, our studies showed

that epithelial-expressed but not immune cell-expressed MMP-9 mediates intestinal inflammation.

In addition to its role in inflammation, recent studies from our laboratory have shown that MMP-9 plays a role in epithelial cell differentiation. We showed that MMP-9^{-/-} mice have increased secretory lineage as evidenced by increased MUC-2 expression and goblet cells and decreased absorptive epithelial cells as shown by carbonic anhydrase. Being a protease, we reasoned that MMP-9 may play a role in cleaving Notch-1, a transcription factor that plays a pivotal role in determining epithelial cell lineages and requires metalloproteinase for its cleavage (5). Indeed, MMP-9 cleaves Notch-1 and activation of Notch-1 is inhibited in MMP-9^{-/-} mice (6). Recent studies have implicated Notch-1 signaling in the pathogenesis of colon cancer, and given the role of MMP-9 in Notch-1 activation and inflammation, in this study, we addressed the role of MMP-9 in colitis-associated colon cancer (CAC).

IBD, which includes ulcerative colitis and Crohn's disease, is a chronic inflammatory disease of the intestine that affects 1 in 200 to 1 in 1,000 individuals in the United States (7). Ulcerative colitis and Crohn's disease are characterized by relapses (acute flare) and remission, which involve immune-mediated tissue injury followed by repair (8, 9). CAC is an important complication of ulcerative colitis or colonic Crohn's disease that results in significant morbidity and mortality (10). Indeed, it causes one of six of all deaths in patients with ulcerative colitis and, thus, is a dreaded complication of the disease (11). The incidence of colon cancer is increased by 2% at 10 years, 8% at 20 years, and 18% at 30 years in patients with ulcerative colitis, and the risk of developing colon

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cancer correlates with the extent, duration, and severity of the disease (12). Although both sporadic colon cancer and CAC are malignancies of the colon, several features make CAC distinct from sporadic colon cancer (13). For example, unlike most sporadic colon cancers that arise from adenomatous polyps, colon cancer develops commonly in flat dysplastic tissue among the individuals preexposed to IBD. In addition, CAC is often multiple, anaplastic, broadly infiltrating, and rapidly growing and occurs at a younger age. Genetic alterations also differ in CAC compared with sporadic colon cancer (14, 15). The pathogenesis of CAC is poorly understood, and remarkably, the role of MMP-9 in CAC has not been explored. The role of MMP-9 in colon cancer is well recognized. MMP-9 has also been implicated to play a role in colon cancer progression and metastasis in animal models as well as in humans (16). It has been documented that MMP-9 regulates metastatic progression in colorectal cancer (17) and was found to be expressed by macrophages, neutrophils, and mast cells. Further, MMP-9 is found to generate angiostatin, which is a crucial factor controlling the growth rate of certain tumors (18).

Although individual and independent roles of MMP-9 during inflammation and cancer in colon or different organs have been documented very well, the function of MMP-9 in mediating inflammation-associated colon cancer has not been studied. The aim of the present study is to understand the role of MMP-9 in an animal model of CAC.

Materials and Methods

Animal models. As described previously (19), wild-type (WT) and MMP-9^{-/-} littermates used in the study were between 9 and 10 wk old at the beginning of the experimental protocol and were maintained on a 12-h dark-light cycle and allowed free access to pelleted, nonpurified diet and tap water.

Induction of azoxymethane + DSS-induced cancer. Age- and sex-matched C57B6 MMP-9^{-/-} mice and their WT littermates were injected i.p. with 7.6 mg/kg azoxymethane (Sigma) on day 0. On day 7, one group (20) of each WT and MMP-9^{-/-} mice was exposed to 3% (w/v) DSS (ICN Biochemicals) by oral administration through their drinking water *ad libitum* for 7 d. On day 14, their water was changed to regular drinking water. On day 28, their drinking water was changed back to 3% DSS for a second cycle of DSS exposure. After a week, these mice were returned to regular drinking water and sacrificed on day 56. Their colons were opened longitudinally. We monitored body weight, stool consistency, and stool occult blood of all the mice during the DSS treatment and recovery phase. One group of mice was assessed for CAC mortality. To perform mortality studies, the same protocol was followed with the exception of one cycle of DSS (21) and animals were followed for 140 d or when the mice developed rectal prolapses and/or >20% body weight loss. For both the long (140 d) and short (56 d) protocols, one group of mice was maintained with regular water and sacrificed after 140 and 56 d, respectively. After macroscopic assessment for polyps, tissue sections were embedded in paraffin. Colon of the WT and MMP-9^{-/-} mice was cut open, and

the number and size of the polyps were determined under the Zeiss microscope (Olympus).

Protein extraction and Western blot analysis. As described previously (19), for Western blot analysis, mucosal strippings were obtained after sacrificing the WT and MMP-9^{-/-} mice ($n = 6$). Antibodies used were MMP-9 (1:1,000; Abcam), inducible nitric oxide synthase (iNOS; 1:2,500; Upstate Biotechnology), cyclooxygenase-2 (COX-2; 1:2,500; Cayman), β -catenin (1:1,000; BD Transduction Laboratories), NF- κ B (p60, 1:500; BD Transduction Laboratories), pCNA (1 μ g/mL; Abcam), caspase-3 (1:1,000; Cell Signaling), Notch intracellular domain (NICD; 1:500; Abcam), and p21^{WAF1/Cip1} (1:1,000; BD Biosciences). For β -catenin, NF- κ B, and p21^{WAF1/Cip1}, the goat anti-mouse secondary antibody (1:4,000; Bio-Rad) was used. For MMP-9, iNOS, COX-2, pCNA, caspase-3, and NICD, the goat anti-rabbit (1:2,500; Bio-Rad) was used as secondary antibody.

Ki67 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining. As described previously (22), paraffin sections of colon were deparaffinized and stained for Ki67 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).

Cell culture and transfection. As described previously (6), Caco2-BBE cells were transfected for 72 h with a pEGFP plasmid with and without the MMP-9 gene. The transfected clones were selected under an antibiotic (geneticin; Life Technologies), and fluorescent cells were isolated using flow cytometry (fluorescence-activated cell sorting) and then used for the detection of MMP-9, β -catenin, iNOS, COX-2, and NICD protein expression.

Statistical analysis. As described previously (19), the data are presented as the mean \pm SE. Statistical analysis was performed using GraphPad Instat 3 software.⁴

Results

MMP-9 is highly expressed in CAC. MMP-9^{-/-} and WT mice were euthanized following short-term protocol (56 days), and protein lysates were prepared from the colon mucosal stripping for Western blot analysis as described in Materials and Methods. Western blot analysis showed that MMP-9^{-/-} mice with or without CAC lacked MMP-9 protein expression (Fig. 1A, lanes 8–15). WT mice given water showed no MMP-9 protein expression (Fig. 1A, lanes 1–4), but WT mice with CAC showed increased MMP-9 protein expression (Fig. 1A, lanes 5–7). Densitometry analysis shown by the graphs revealed a 10.51 \pm 0.21-fold increase in MMP-9 protein expression in WT mice with CAC compared with WT mice treated with water (Fig. 1A).

MMP-9^{-/-} mice show increased susceptibility to CAC. MMP-9^{-/-} and WT mice ($n = 10$ each group) were treated with azoxymethane/DSS to induce CAC and sacrificed after 56 days. Mice were weighed once a week, and the change in body weight shows no significant change in the body weight of MMP-9^{-/-} mice after first and second cycles of 3% DSS,

⁴ <http://www.graphpad.com>

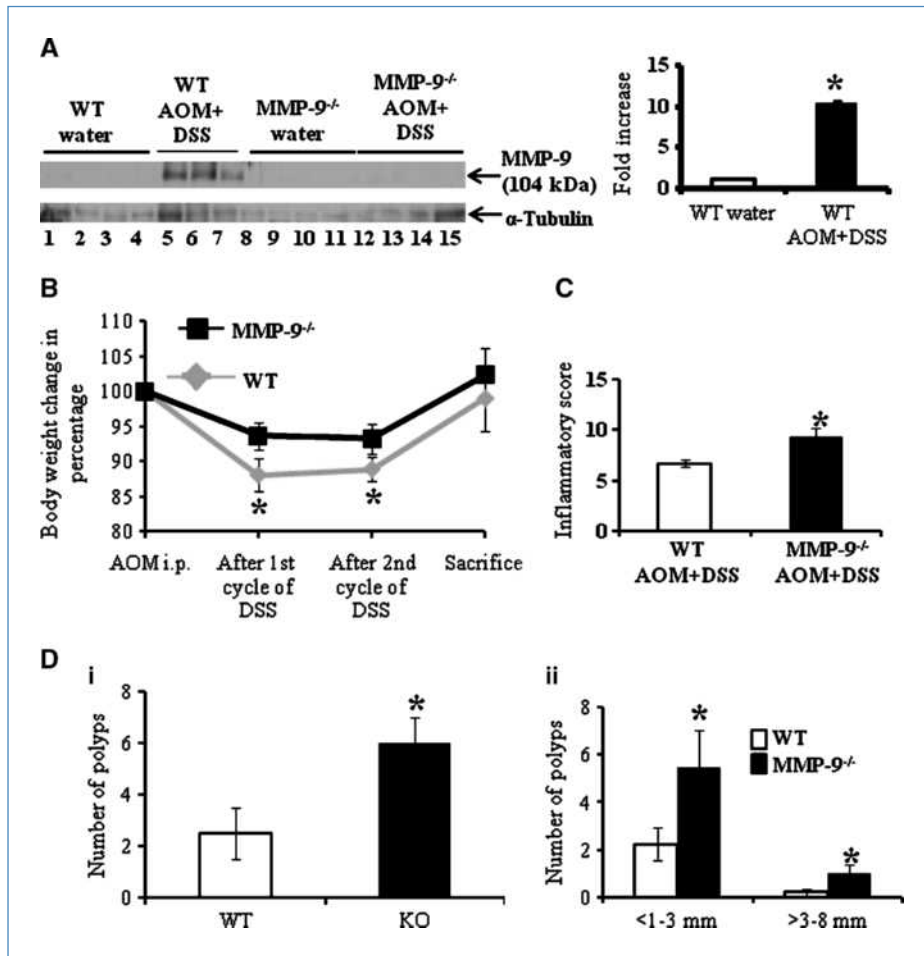


Figure 1. MMP-9 is highly expressed in CAC, and MMP-9^{-/-} mice show increased susceptibility to CAC. WT and MMP-9^{-/-} mice ($n = 6$ each group) were treated once with azoxymethane (AOM) and twice with 3% DSS to induce CAC. Mice were weighed once a week and sacrificed after 56 d. *A*, Western blot of proteins from the mucosal stripping of the colons of WT and MMP-9^{-/-} mice with and without CAC probed with anti-MMP-9. Each lane shows protein (30 μ g/lane) from an individual mouse with or without CAC induction. Western blots were quantitated by scanning densitometry. Values are representative of three experiments. *Columns*, mean; *bars*, SE. *, $P < 0.05$. *B*, change in body weight of WT and MMP-9^{-/-} mice ($n = 10$ each group). Inflammatory score (*C*), polyp count (*D*, *i*), and size of the polyp measured in diameter (*D*, *ii*) as described in Materials and Methods. *Columns*, mean; *bars*, SE. *, $P < 0.05$.

whereas there was a significant decrease in the body weight of WT mice after first and second cycles of 3% DSS compared with initial body weight (Fig. 1*B*). Both MMP-9^{-/-} and WT mice showed no significant change in body weight at the end of 56 days as shown in Fig. 1*B*. Mice were sacrificed after 56 days, and the number of polyps was counted and measured in diameter. Colons were then processed for histology, and inflammatory score was determined by H&E staining. Figure 1*C* shows that MMP-9^{-/-} mice had significantly higher inflammatory score of 9.4 ± 0.88 compared with WT mice (inflammatory score of 6.7 ± 0.34) induced with CAC (3). Figure 1*D* (*i*) shows that there was significantly higher multiplicity of polyps among MMP-9^{-/-} mice (6 ± 1.65) compared with WT mice (2.5 ± 0.75) with CAC. Figure 1*D* (*ii*) shows that MMP-9^{-/-} mice had significantly more number of polyps in the range of <1- to 3-mm diameter (5.5 ± 1.5) as well as in the range of >3- to 8-mm diameter (1.0 ± 0.38) compared with WT mice (2.25 ± 0.5 in the range of <1- to 3-mm diameter and 0.23 ± 0.15 in the range of >3- to 8-mm diameter).

In another set of experiments, we followed mice for an additional 90 days to assess cancer development and mortality. MMP-9^{-/-} and WT mice ($n = 10$ each group) were induced with CAC as described in Materials and Methods. Mice were

weighed once a week, and their mortality is represented by Fig. 2*A*, which shows the survival curve of MMP-9^{-/-} and WT mice. Ten percent of MMP-9^{-/-} mice given the CAC induction developed rectal prolapse, reflecting the severity of inflammation and significant weight loss starting at 7 weeks. There was 70% mortality in this group at 20 weeks versus 30% mortality for WT mice with CAC. These data indicate higher mortality among MMP-9^{-/-} compared with WT mice. After sacrifice, the colon was cut open and stained with methylene blue, and the number and size of the polyps were assessed (Fig. 2*B* and *C*). Figure 2*B* shows that MMP-9^{-/-} mice had significantly increased multiplicity of tumors (5.4 ± 2.04) compared with WT mice (2.8 ± 0.48), and Fig. 2*C* indicates that MMP-9^{-/-} mice had significantly more number of polyps in the range of <1- to 3-mm diameter (3.9 ± 1.9) as well as in the range of >3- to 8-mm diameter (1.5 ± 1.1), respectively, compared with WT mice (2.8 ± 0.48 in the range of <1- to 3-mm diameter and no polyps in the range of >3- to 8-mm diameter). Figure 2*D* shows the representative gross anatomy of the colon of the MMP-9^{-/-} and WT mice, reflecting the number of polyps among them.

CAC in MMP-9^{-/-} mice is associated with increased proliferation and decreased apoptosis compared with WT

mice. Alteration in cell proliferation is a significant factor in the multistep process of tumorigenesis, and Ki67 is a well-established marker for the assessment of cell proliferation (23). Figure 3A (*left*) shows the Ki67 staining as depicted by brown nuclei staining among the crypts of colonic epithelium of MMP-9^{-/-} and WT mice without the induction of CAC, indicating no significant difference among them, whereas Fig. 3A (*i, right*) suggests an increase in the Ki67 staining among the crypts of MMP-9^{-/-} compared with WT mice having CAC. Figure 3B shows the graphs representing the number of nuclei positive for Ki67 staining per crypt and indicates that MMP-9^{-/-} mice had more proliferation (39.4 ± 9.8) compared with WT mice (18.3 ± 6.9), as indicated by the percentage of the number of Ki67-positive nuclei/crypt. Figure 3C shows the Western blot of pCNA (a marker for proliferation) and supports the immunohistochemistry data of Ki67 staining by showing increased protein expression of pCNA among MMP-9^{-/-} mice compared with WT mice, both induced with CAC (*lanes 10–12 and lanes 4–6, respectively*). Both MMP-9^{-/-} and WT mice showed some constitutive pCNA expression without the induction of CAC, which was higher among MMP-9^{-/-} mice (Fig. 3C, *lanes 7–9 and lanes 1–3, respectively*) compared with WT mice. The adjacent graph shows the quantification of Western blots by scanning densitometry and indicates a 2.2 ± 0.14-fold increase in pCNA expression among MMP-9^{-/-} mice compared with WT mice with CAC. Apoptosis is the intrinsic property of tumor cells, which is modulated by the expression of several oncogenes (24). Figure 4A (*left*) shows the TUNEL staining as depicted by fluorescent green nuclei staining among the crypts of colonic epithelium of MMP-9^{-/-} and WT mice with CAC, Fig. 4A (*middle*) shows the fluorescent blue 4',6-diamidino-2-phenylindole (DAPI)

nuclear staining, and Fig. 4A (*right*) shows the merged image of TUNEL and DAPI, indicating no significant level of apoptosis among MMP-9^{-/-} mice, whereas crypts of WT mice showed some apoptotic cells. Figure 4B showing the Western blot of caspase-3 (another marker for apoptosis required in the cleavage of critical cellular substrates) supports the immunohistochemistry data of TUNEL staining by showing a decreased protein expression of caspase-3 among MMP-9^{-/-} (*lanes 10–12*) mice compared with WT mice (*lanes 7–9*) with CAC. The adjacent graph shows the quantification of Western blots by scanning densitometry and indicates an inhibition of 82.96 ± 16.47% in caspase-3 protein expression among MMP-9^{-/-} mice compared with WT mice with CAC.

CAC in MMP-9^{-/-} mice showed altered levels of p21^{WAF1/Cip1}, β-catenin, and NF-κB. We have previously shown that MMP-9 activates Notch-1 (6). In this study, we examined the expression levels of p21^{WAF1/Cip1}, a “canonical” Notch target, in keratinocytes that has been shown to suppress Wnt signaling and function as a negative regulator of stem cell potential and tumorigenesis (25), consequently suppressing protein levels of β-catenin. Colon of the mice was harvested at 56 days, and mucosal strippings were processed for Western blots. Western blot analysis of p21^{WAF1/Cip1} showed that MMP-9^{-/-} mice with or without CAC had almost negligible protein expression (Fig. 5A, *i, lanes 7–12*) compared with WT mice with or without the induction of CAC (Fig. 5A, *i, lanes 1–6*). CAC induction among the WT mice caused an increase in protein expression of p21^{WAF1/Cip1} compared with WT mice without CAC (Fig. 5A, *i, lanes 4–6 and lanes 1–3, respectively*). Densitometric analysis shown by the graphs revealed a 2.26 ± 0.87-fold increase in p21^{WAF1/Cip1} protein expression among WT mice with CAC (mean ± SE; six mice per group; *P* < 0.05;

Figure 2. MMP-9^{-/-} mice show increased susceptibility to CAC and mortality. Mortality studies were done as described in Materials and Methods. A, mortality among WT and MMP-9^{-/-} mice with CAC and followed for a total of 140 d or sacrificed if they developed rectal prolapse and/or ≥20% body weight loss. Polyp count (B) and size of the polyp measured in diameter (C) as described in Materials and Methods. Columns, mean; bars, SE. *, *P* < 0.05. D, representative gross anatomy of the colon of the MMP-9^{-/-} and WT mice, reflecting the number of polyps among them.

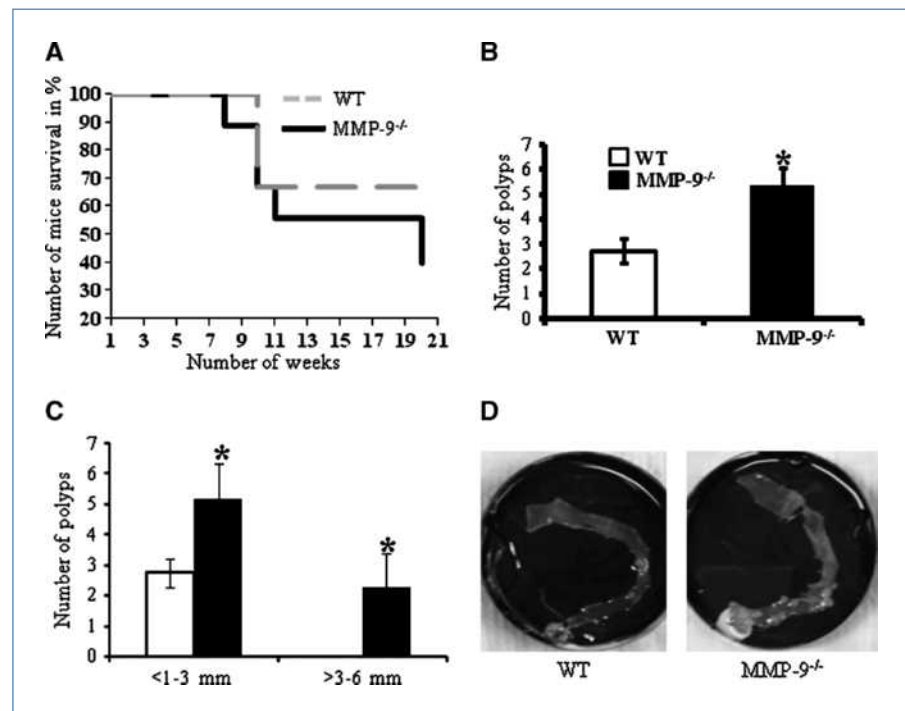


Fig. 5A, *i*). Figure 5A (*ii*) shows that MMP-9^{-/-} mice with CAC had increased expression of β -catenin (*lanes 8–11 and lanes 4–7*, respectively) compared with WT mice. The adjacent graph shows the quantification of Western blots by scanning densitometry and indicates a 2.8 ± 0.14 -fold increase in β -catenin expression among MMP-9^{-/-} mice compared with WT mice with CAC. Figure 5A (*iii*) shows that MMP-9^{-/-} mice compared with WT mice (*lanes 10–12 and lanes 4–6*, respectively) with CAC had decreased expression of NF- κ B, which participates in the control of intestinal homeostasis (26). Both MMP-9^{-/-} and WT mice without CAC showed some constitutive expression of NF- κ B (Fig. 5A, *iii*, *lanes 7–9 and lanes 1–3*, respectively). The adjacent graph shows the quantification of Western blots by scanning densitometry and indicates an inhibition of $57.78 \pm 24.92\%$ in NF- κ B expression among MMP-9^{-/-} mice compared with WT mice with CAC. Together, these data show that CAC in MMP-9^{-/-} is associated with decreased expression of NF- κ B and p21^{WAF1/Cip1} and increased expression of β -catenin, whereas CAC in WT have increased MMP-9, NICD, and p21^{WAF1/Cip1} and a reciprocal decrease in Wnt signaling.

CAC in MMP-9^{-/-} mice showed altered protein expressions of COX-2 and iNOS. COX-2 and iNOS are two proteins well associated with CAC (27, 28). MMP-9^{-/-} and WT mice ($n = 6$ each group) were treated with one dose of azoxymethane and two cycles of 3% DSS to induce CAC and were sacrificed after 56 days. Western blots of protein from the mucosal stripping of the colons were performed as described in Materials and Methods. Figure 5B (*i*) shows that MMP-9^{-/-} mice (*lanes 10–12*) compared with WT mice (*lanes 4–6*) with CAC had increased expression of COX-2, whereas both

MMP-9^{-/-} and WT mice without CAC showed no expression of COX-2 (Fig. 5B, *i*, *lanes 7–9 and lanes 1–3*, respectively). The adjacent graph (Fig. 5B, *i*) shows the quantification of Western blots by scanning densitometry and indicates a 1.6 ± 1.13 -fold increase in COX-2 expression among MMP-9^{-/-} mice compared with WT mice with CAC. Figure 5B (*ii*) shows that MMP-9^{-/-} mice (*lanes 10–12*) compared with WT mice (*lanes 4–6*), both with CAC, had increased expression of iNOS, whereas both MMP-9^{-/-} and WT mice treated with water showed no expression of iNOS (Fig. 5B, *ii*, *lanes 7–9 and lanes 1–3*, respectively). The adjacent graph (Fig. 5B, *ii*) shows the quantification of Western blots by scanning densitometry and indicates a 2.5 ± 0.91 -fold increase in iNOS expression among MMP-9^{-/-} mice compared with WT mice with the treatment of azoxymethane and two cycles of DSS.

MMP-9 overexpression results in altered protein expressions of NICD, β -catenin, COX-2, and iNOS. Our next goal was to determine the effect of MMP-9 overexpression on transcription factors and molecules associated with differentiation and proliferation of colonic epithelium. We used the Caco2-BBE cell line stably transfected with a pEGFP plasmid, with or without the MMP-9 gene, as described in Materials and Methods, as our *in vitro* model to study the effect of MMP-9 on the expression of NICD, β -catenin, COX-2, and iNOS. Figure 6A (*i*) represents the efficiency of transfection confirmed by Western blot of MMP-9. Equal amounts of protein were separated by SDS-PAGE and probed with MMP-9 antibody. β -Tubulin served as the loading control for proteins. The lane marked MMP-9 shows a band at 120 kDa (Fig. 6A, *i*, *lanes 4–6*), consistent with the expression of

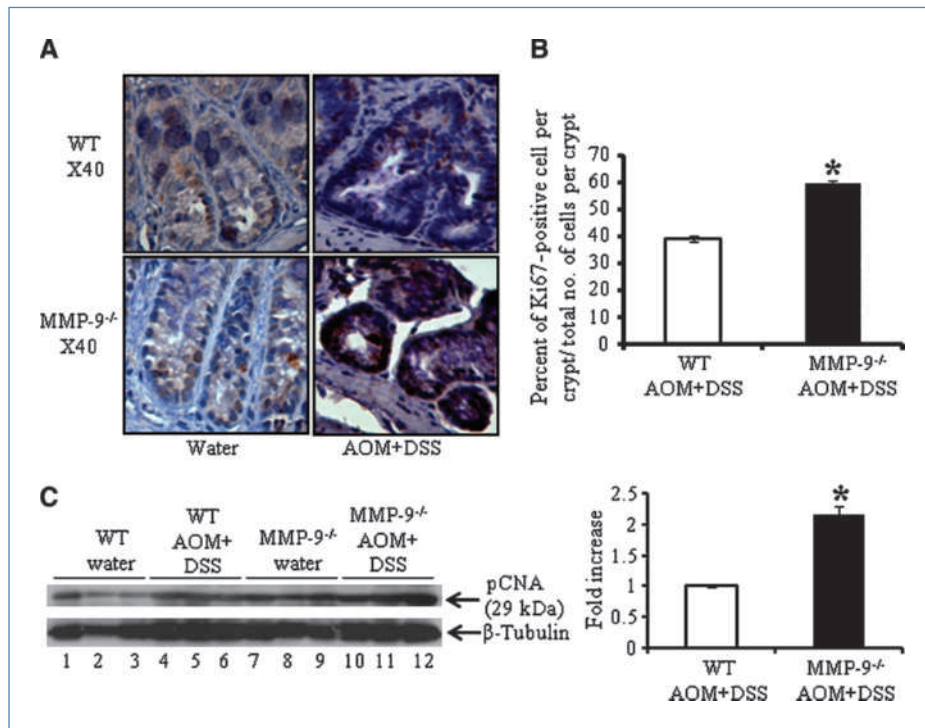
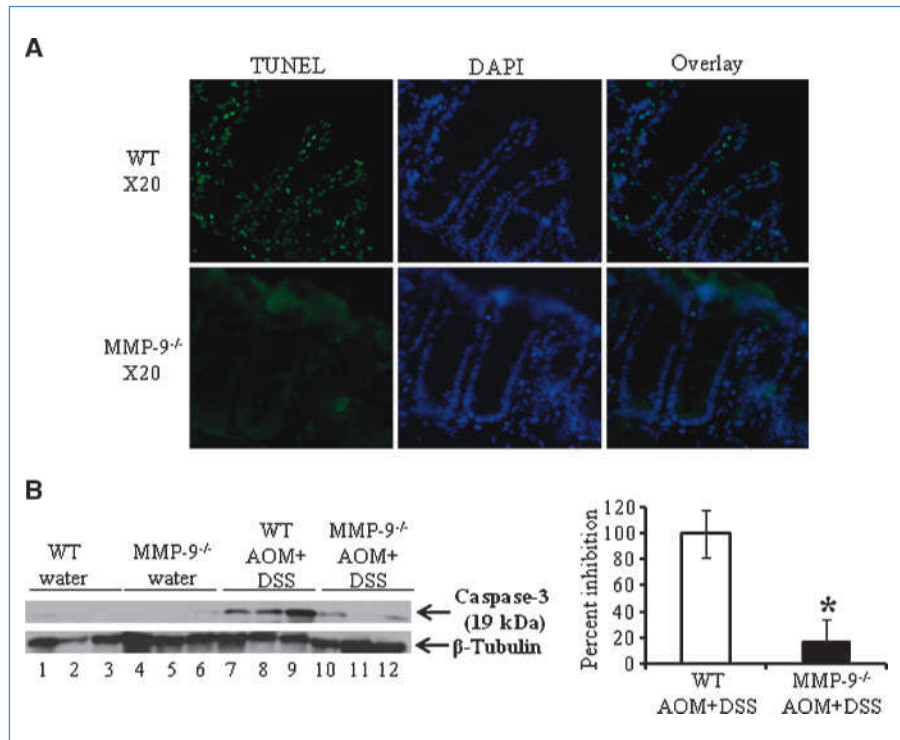


Figure 3. CAC in MMP-9^{-/-} is associated with increased proliferation. WT and MMP-9^{-/-} mice ($n = 6$ each group) with induced CAC and sacrificed after 56 d. Colon of the mice was processed for immunohistochemistry using anti-Ki67 antibody as described in Materials and Methods. *A*, representative sections of MMP-9^{-/-} and WT mice with CAC. Proliferating cells are indicated by brown nuclei of the crypts with a counterstaining of hematoxylin. *B*, number of nuclei positive for Ki67 staining per crypt. *Columns*, mean; *bars*, SE. *, $P < 0.05$. *C*, Western blot of protein from the mucosal stripping of the colons of WT and MMP-9^{-/-} with and without induction of CAC probed with anti-pCNA. Each lane shows protein (30 μ g/lane) from an individual mouse. Western blots were quantified by scanning densitometry, and densities of MMP-9^{-/-} mice relative to WT mice, both induced with CAC, are graphed adjacently. *Columns*, mean; *bars*, SE. *, $P < 0.05$.

Figure 4. CAC in MMP-9^{-/-} mice is associated with decreased apoptosis. **A**, TUNEL (left) and DAPI (middle) staining and apoptotic cells (right). **B**, Western blot of whole tissue lysates of the colons of WT and MMP-9^{-/-} mice with and without CAC probed with anti-caspase-3. Each lane shows protein (30 µg/lane) from an individual mouse. Adjacent graph shows the quantification of Western blots by scanning densitometry and plotting the densities of MMP-9^{-/-} mice relative to WT mice both induced with CAC. Values are representative of three experiments. Columns, mean; bars, SE. *, *P* < 0.05.



GFP-MMP-9. MMP-9 is undetectable in the vector-transfected cells (Fig. 6A, *i*, lanes 1–3), similar to normal colonic epithelia. Western blot using anti-NICD, anti-β-catenin, anti-COX-2, and anti-iNOS-specific antibodies [Fig. 6A (*ii* and *iii*) and B (*i* and *ii*), lanes 4–6, respectively] showed a significant increase in NICD and a decrease in β-catenin, COX-2, and iNOS in MMP-9-overexpressing cells compared with vector-transfected cells [Fig. 6A (*ii* and *iii*) and B (*i* and *ii*), lanes 1–3, respectively]. Densitometric analysis revealed a 1.61 ± 0.02-fold increase in NICD protein expression and an inhibition of 37.17 ± 3.93%, 48.98 ± 30.30%, and 64.11 ± 3.11% in protein expression of β-catenin, COX-2, and iNOS in MMP-9-overexpressing cells compared with vector. Together, our *in vitro* data using the stably transfected Caco2-BBE cell line show that MMP-9 downregulates β-catenin, COX-2, and iNOS but activates NICD expression, which are all key factors in regulating differentiation and proliferation of the gut epithelium.

Discussion

Chronic inflammation is linked to cancer development in several organs (14). CAC advances through a series of genetic alterations that endow cells with growth advantages over normal cells. As advantaged cells proliferate, they progressively acquire even more DNA damage that further favors their proliferation (14); however, molecules released by inflammatory cells may either suppress or enhance tumor progression. MMPs have the ability to cleave various bioactive substrates and thereby can either promote or suppress tu-

mor progression, resulting in a delicate balance of protumor and antitumor activity determining the fate of the tumors (18). During inflammation progressing to cancer, MMPs might be involved at different steps, starting from the growth of the primary tumor to the support of the tumor growth in the metastatic site. Some studies have shown that the chronic inflammation associated with some cancers can further stimulate cancer progression due to the release of MMPs from the inflammatory cells (29, 30). There are only a few elaborate studies on the role of MMPs in colitis-associated tumorigenesis (31), although some studies have shown that inhibition of gelatinases results in regression of tumor growth (18, 32). The gelatinases, especially MMP-9, can also stimulate tumor growth (18). Recently, Sinnamon and colleagues (33) have shown that APC-Min mice genetically deficient in MMP-9 expression had fewer tumors than littermate controls, and suggested the alteration of proliferation by MMP-9 among APC-Min adenoma cells. Burg-Roderfeld and colleagues (34) have shown that MMP-9 hemopexin domain has an inhibitory effect on migration and adhesion of colorectal carcinoma cells. Another study by Pozzi and colleagues (35) showed that reduction of plasma levels of MMP-9, in either normal or integrin α1-null mice, leads to decreased synthesis of angiostatin and consequent increased tumor growth and vascularization, and was also supported by the study of Bjorklund and Koivunen (18). In melanoma, increased expression of MMP-9 is found initially, but at the later stage, it is reversed (18, 36). It has been reported that in breast and colon cancer, MMP-9 expression has been correlated with both increased and decreased survival and formation of distant metastasis (18, 37).

In the present study, we observed that MMP-9^{-/-} mice showed increased susceptibility to inflammation-associated colon cancer/polyps as indicated by their number, size of polyps, and the mortality rate, although we have shown previously (3) that MMP-9^{-/-} mice are protected from inflammation. Interestingly, MMP-9^{-/-} mice induced with inflammation-associated colon cancer/polyps exhibited higher proliferation and lower apoptosis compared with WT mice inflammation. This implies the underlying role of MMP-9 in regulating proliferation and apoptosis during the carcinogenic process. This fact was further supported by the altered protein expressions of different inflammatory and transcription factors, which are well known to be involved in regulating the cell proliferation and differentiation or apoptosis.

Based on our data that MMP-9 functions as the protease that cleaves and activates Notch-1 (6), we explored the role of MMP-9-mediated Notch-1 activation in CAC. In the present study, we observed the activation of p21^{WAF1/Cip1} expression due to activation of Notch-1 signaling in CAC in WT mice, which was clearly inhibited in CAC in MMP-9^{-/-} mice. The *in vitro* results using our overexpressing MMP-9 cell line also corroborated with *in vivo* data supporting the hypothesis that despite being a mediator of proinflammatory response, MMP-9 plays a protective role in CAC by modulating carcinogenesis through its effect on Notch signaling.

Intestinal epithelium is a very dynamic tissue involving key cellular processes such as proliferation and differentiation. It has been known that there are many genes that are involved in the normal development of intestine and also play a

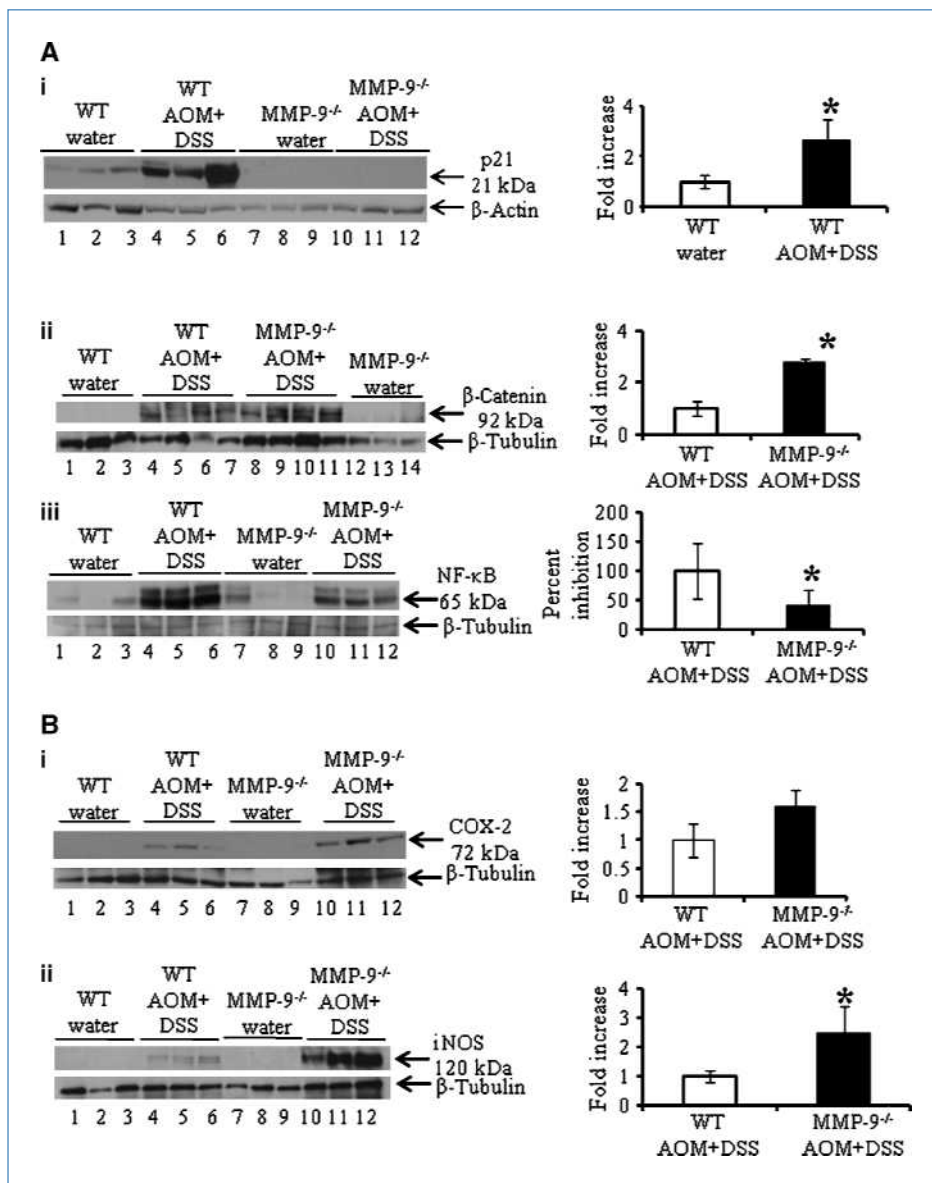


Figure 5. CAC in MMP-9^{-/-} mice showed altered protein expressions of p21^{WAF1/Cip1}, β-catenin, NF-κB, COX-2, and iNOS. WT and MMP-9^{-/-} mice (n = 6 each group) with CAC were sacrificed after 56 d. Each lane shows protein (30 μg/lane) from an individual mouse with or without induction of CAC. A, i to iii, Western blots of protein from the mucosal stripping of the colons of WT and MMP-9^{-/-} mice with and without CAC probed with anti-p21^{WAF1/Cip1}, anti-β-catenin, and anti-NF-κB, respectively. Adjacent graphs show the quantification by scanning densitometry. B, i and ii, Western blots of protein from the mucosal stripping of the colons of WT and MMP-9^{-/-} mice with and without CAC probed with anti-COX-2 and anti-iNOS, respectively. Western blots were quantitated by scanning densitometry and graphed adjacently. Values are representative of three experiments. Columns, mean; bars, SE. *, P < 0.05.

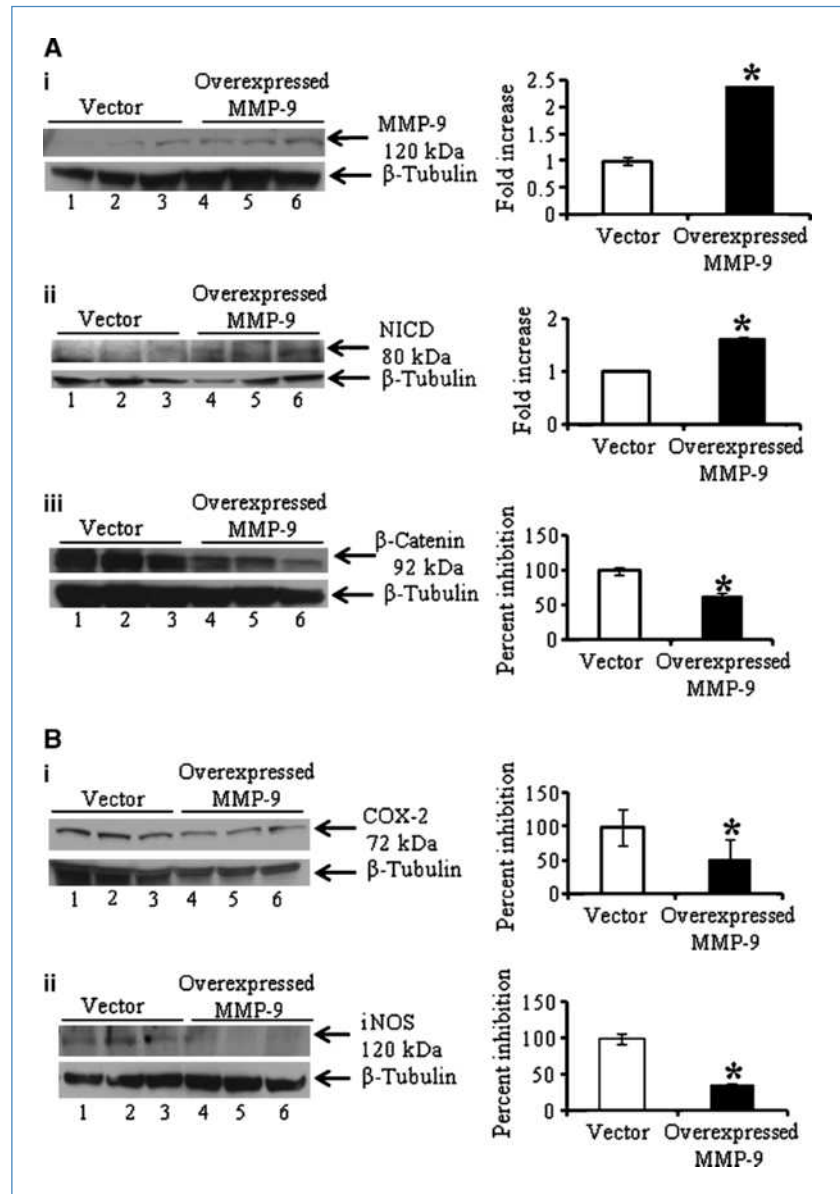


Figure 6. MMP-9 overexpression results in altered levels of NICD, β -catenin, COX-2, and iNOS. Caco2-BBE cells stably transfected with a pEGFP plasmid, with or without the MMP-9 gene, were plated on six-well filters at confluency and cell lysates were collected and immunoblotted for MMP-9 (A, i), NICD (A, ii), β -catenin (A, iii), COX-2 (B, i), and iNOS (B, ii). β -Tubulin was used as the loading control. Western blots were quantitated by scanning densitometry and graphed adjacently. Each lane shows protein (30 μ g/lane). Values are representative of three experiments. Columns, mean; bars, SE. *, $P < 0.05$.

critical role in the process of human carcinogenesis (38–41). Continuous renewal of the intestinal epithelium requires coordinated regulation between different signaling pathways and molecules to maintain the balance between proliferation and differentiation of epithelial stem cells and immature progenitor cells. One of the important signaling systems regulating the intestinal epithelium homeostasis is the Wnt signaling pathway (42), which involves dephosphorylation and stabilization of β -catenin and its nuclear translocation, and activation of target genes by the complex consisting of β -catenin and the T cell factor family of transcription factors (43). Another important pathway is the highly conserved Notch signaling pathway, which controls selective cell fate decisions and subsequent differentiation in the intestine (44, 45) with oncogenic and growth-promoting roles. It can also function

as a tumor suppressor (39, 45). It has been reported that Notch-1 deficiency results in increased β -catenin-mediated signaling in hyperproliferative skin and primary tumor lesions, indicating that Notch might suppress Wnt signaling (45, 46). This is mediated by Notch by indirect activation of p21, which subsequently binds to the Wnt4 promoter and downregulates its expression (25, 45). Further, overactivation of the Wnt pathway due to mutations in β -catenin causes many of the epithelial cells to enter into a proliferative state and display a failure of the differentiation programs in these cells (44, 47). Additionally, Wnt signaling probably integrates with other stem cell niche-derived signals such as BMP, Hedgehog, and Notch (44, 48). Irrespective of whether these signals have a stromal or an epithelial origin, they reflect a constant cross-talk among them, which is very important

for the maintenance of normal epithelium (48, 49). A few studies have shown that repeated exposure to DSS causes the appearance of dysplasia and/or cancer in mice due to repeated mucosal erosion and regeneration of the colonic epithelium, causing the increase in the susceptibility of mucosal epithelia for dysplasia and/or cancer development (50). Although underlying mechanisms are still unknown, increased proliferative activity preceded dysplasia, which results in the increase of proliferating cells to apoptotic cells. In the present study, our results have shown increased proliferation among the MMP-9^{-/-} mice induced with inflammation-associated colon cancer as well as increased β -catenin expression. Similar to keratinocytes but in contrast to colonic epithelial cells, our data show that MMP-9-mediated Notch signaling is associated with inhibition of Wnt signaling and mediates cell survival.

In conclusion, we show that MMP-9 acts as a tumor suppressor in CAC, likely through its effect on the Notch signaling pathway. The absence of MMP-9 is associated with defective Notch-1 activation, suppressed p21^{WAF1/Cip1} expression, and reciprocal activation of Wnt signaling and increased proliferation.

References

- Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2:161–74.
- Okamoto R, Watanabe M. Cellular and molecular mechanisms of the epithelial repair in IBD. *Dig Dis Sci* 2005;50 Suppl 1:S34–8.
- Castaneda FE, Walla B, Vijay-Kumar M, et al. Targeted deletion of metalloproteinase 9 attenuates experimental colitis in mice: central role of epithelial-derived MMP. *Gastroenterology* 2005;129:1991–2008.
- Baugh MD, Perry MJ, Hollander AP, et al. Matrix metalloproteinase levels are elevated in inflammatory bowel disease. *Gastroenterology* 1999;117:814–22.
- Brou C, Logeat F, Gupta N, et al. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell* 2000;5:207–16.
- Garg P, Ravi A, Patel NR, et al. Matrix metalloproteinase-9 regulates MUC-2 expression through its effect on goblet cell differentiation. *Gastroenterology* 2007;132:1877–89.
- Loftus EV, Jr. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology* 2004;126:1504–17.
- Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003;3:521–33.
- Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998;115:182–205.
- Langholz E, Munkholm P, Davidsen M, Binder V. Colorectal cancer risk and mortality in patients with ulcerative colitis. *Gastroenterology* 1992;103:1444–51.
- Prior P, Gyde S, Cooke WT, Waterhouse JA, Allan RN. Mortality in Crohn's disease. *Gastroenterology* 1981;80:307–12.
- Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001;48:526–35.
- Choi PM, Zelig MP. Similarity of colorectal cancer in Crohn's disease and ulcerative colitis: implications for carcinogenesis and prevention. *Gut* 1994;35:950–4.
- Feagins LA, Souza RF, Spechler SJ. Carcinogenesis in IBD: potential targets for the prevention of colorectal cancer. *Nat Rev Gastroenterol Hepatol* 2009;6:297–305.
- Fukata M, Abreu MT. Role of Toll-like receptors in gastrointestinal malignancies. *Oncogene* 2008;27:234–43.
- Mook OR, Frederiks WM, Van Noorden CJ. The role of gelatinases in colorectal cancer progression and metastasis. *Biochim Biophys Acta* 2004;1705:69–89.
- Bernhard EJ, Gruber SB, Muschel RJ. Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. *Proc Natl Acad Sci U S A* 1994;91:4293–7.
- Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta* 2005;1755:37–69.
- Garg P, Vijay-Kumar M, Wang L, Gewirtz AT, Merlin D, Sitaraman SV. Matrix metalloproteinase-9-mediated tissue injury overrides the protective effect of matrix metalloproteinase-2 during colitis. *Am J Physiol Gastrointest Liver Physiol* 2009;296:G175–84.
- Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* 2003;94:965–73.
- Okayasu I, Ohkusa T, Kajjura K, Kanno J, Sakamoto S. Promotion of colorectal neoplasia in experimental murine ulcerative colitis. *Gut* 1996;39:87–92.
- Kolachala V, Ruble B, Vijay-Kumar M, et al. Blockade of adenosine A2B receptors ameliorates murine colitis. *Br J Pharmacol* 2008;155:127–37.
- Saleh HA, Jackson H, Khatib G, Banerjee M. Correlation of bcl-2 oncoprotein immunohistochemical expression with proliferation index and histopathologic parameters in colorectal neoplasia. *Pathol Oncol Res* 1999;5:273–9.
- Hickman JA. Apoptosis induced by anticancer drugs. *Cancer Metastasis Rev* 1992;11:121–39.
- Devgan V, Mammucari C, Millar SE, Brisken C, Dotto GP. p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation. *Genes Dev* 2005;19:1485–95.
- Inan MS, Tolmacheva V, Wang QS, Rosenberg DW, Giardina C. Transcription factor NF- κ B participates in regulation of epithelial cell turnover in the colon. *Am J Physiol Gastrointest Liver Physiol* 2000;279:G1282–91.
- Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994;107:1183–8.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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28. Ambis S, Merriam WG, Bennett WP, et al. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. *Cancer Res* 1998;58:334–41.
29. Coussens LM, Raymond WW, Bergers G, et al. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev* 1999;13:1382–97.
30. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000;103:481–90.
31. Newell KJ, Matrisian LM, Driman DK. Matrilysin (matrix metalloproteinase-7) expression in ulcerative colitis-related tumorigenesis. *Mol Carcinog* 2002;34:59–63.
32. Lubbe WJ, Zhou ZY, Fu W, et al. Tumor epithelial cell matrix metalloproteinase 9 is a target for antimetastatic therapy in colorectal cancer. *Clin Cancer Res* 2006;12:1876–82.
33. Sinnamon MJ, Carter KJ, Fingleton B, Matrisian LM. Matrix metalloproteinase-9 contributes to intestinal tumorigenesis in the adenomatous polyposis coli multiple intestinal neoplasia mouse. *Int J Exp Pathol* 2008;89:466–75.
34. Burg-Roderfeld M, Roderfeld M, Wagner S, Henkel C, Grotzinger J, Roeb E. MMP-9-hemopexin domain hampers adhesion and migration of colorectal cancer cells. *Int J Oncol* 2007;30:985–92.
35. Pozzi A, LeVine WF, Gardner HA. Low plasma levels of matrix metalloproteinase 9 permit increased tumor angiogenesis. *Oncogene* 2002;21:272–81.
36. van den Oord JJ, Paemen L, Opendakker G, de Wolf-Peeters C. Expression of gelatinase B and the extracellular matrix metalloproteinase inducer EMMPRIN in benign and malignant pigment cell lesions of the skin. *Am J Pathol* 1997;151:665–70.
37. Pacheco MM, Mourao M, Mantovani EB, Nishimoto IN, Brentani MM. Expression of gelatinases A and B, stromelysin-3 and matrilysin genes in breast carcinomas: clinico-pathological correlations. *Clin Exp Metastasis* 1998;16:577–85.
38. Guo RJ, Suh ER, Lynch JP. The role of Cdx proteins in intestinal development and cancer. *Cancer Biol Ther* 2004;3:593–601.
39. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* 2003;3:756–67.
40. Pasca di Magliano M, Hebrok M. Hedgehog signalling in cancer formation and maintenance. *Nat Rev Cancer* 2003;3:903–11.
41. Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta* 2003;1653:1–24.
42. Fodde R, Brabletz T. Wnt/ β -catenin signaling in cancer stemness and malignant behavior. *Curr Opin Cell Biol* 2007;19:150–8.
43. Morin PJ, Sparks AB, Korinek V, et al. Activation of β -catenin-Tcf signaling in colon cancer by mutations in β -catenin or APC. *Science* 1997;275:1787–90.
44. Nakamura T, Tsuchiya K, Watanabe M. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. *J Gastroenterol* 2007;42:705–10.
45. Koch U, Radtke F. Notch and cancer: a double-edged sword. *Cell Mol Life Sci* 2007;64:2746–62.
46. Nicolas M, Wolfer A, Raj K, et al. Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* 2003;33:416–21.
47. Su LK, Kinzler KW, Vogelstein B, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 1992;256:668–70.
48. Sancho E, Battle E, Clevers H. Signaling pathways in intestinal development and cancer. *Annu Rev Cell Dev Biol* 2004;20:695–723.
49. Brabletz S, Schmalhofer O, Brabletz T. Gastrointestinal stem cells in development and cancer. *J Pathol* 2009;217:307–17.
50. Inoue T, Murano M, Kuramoto T, et al. Increased proliferation of middle to distal colonic cells during colorectal carcinogenesis in experimental murine ulcerative colitis. *Oncol Rep* 2007;18:1457–62.