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Kyoto University
Definition of Prostaglandin E₂–EP2 Signals in the Colon Tumor Microenvironment That Amplify Inflammation and Tumor Growth
Xiaojun Ma¹, Tomohiro Aoki¹,², Tatsuaki Tsuruyama³,⁴, and Shuh Narumiya¹,²

Abstract
Inflammation in the colon contributes significantly to colorectal cancer development. While aspirin reduces the colorectal cancer risk, its action mechanism, especially in inflammation in tumor microenvironment, still remains obscure. Here, we examined this issue by subjecting mice deficient in each prostaglandin (PG) receptor to colitis-associated cancer model. Deficiency of PGE receptor subtype EP2 selectively reduced, and deficiency of EP1 and EP3 enhanced, the tumor formation. EP2 is expressed in infiltrating neutrophils and tumor-associated fibroblasts in stroma, where it regulates expression of inflammation- and growth-related genes in a self-amplification manner. Notably, expression of cytokines such as TNFα and IL6, a chemokine, CXCL1, a PG-producing enzyme, COX-2, and Wnt5A was significantly elevated in tumor lesions of wild-type mice but this elevation was significantly suppressed in EP2-deficient mice. Intriguingly, EP2 stimulation in cultured neutrophils amplified expression of TNFα, IL6, CXCL1, COX-2, and other proinflammatory genes synergistically with TNFα, and EP2 stimulation in cultured fibroblasts induced expression of EP2 itself, COX-2, IL6, and Wnt genes. EP2 expression in infiltrating neutrophils and tumor-associated fibroblasts was also found in clinical specimen of ulcerative colitis-associated colorectal cancer. Bone marrow transfer experiments suggest that EP2 in both cell populations is critical for tumorigenesis. Finally, administration of a selective EP2 antagonist potently suppressed tumorigenesis in this model. Our study has thus revealed that EP2 in neutrophils and tumor-associated fibroblasts promotes colon tumorigenesis by amplifying inflammation and shaping tumor microenvironment, and suggests that EP2 antagonists are promising candidates of aspirin-alternative for chemoprevention of colorectal cancer. Cancer Res; 75(14): 2822–32. ©2015 AACR.

Introduction
Colorectal cancer is the third most common cancer and the fourth most common cause of cancer death (1). One major risk factor of colorectal cancer is inflammatory bowel diseases such as ulcerative colitis (2), indicating that the pathogenesis of colorectal cancer is closely associated with inflammatory responses in the colon, and that manipulation of inflammation might prevent colorectal cancer development. In fact, it is well known that the use of aspirin or other NSAIDs is associated with reduction of risk of colorectal cancer (3–5). NSAIDs, including aspirin, exert their effects by inhibiting COX, an enzyme initiating prostaglandin (PG) biosynthesis. Inhibitors of COX-2, an inducible form of COX under inflammatory settings, have also been proved to be effective in the prevention of colorectal cancer (6). However, extensive use of these drugs is precluded due to their adverse effects such as gastrointestinal toxicity and tendency to cardiovascular accidents (7, 8), making essential development of an alternative drug for aspirin. PGs consist of PGD₂, PGE₂, PGF₂α, PGI₂, and thromboxane (TX) A₂, which are formed from arachidonic acid by sequential actions of COX and respective synthases (6). Because PGE₂ is the most abundant PG found in colorectal cancer (9), numerous studies have been done to analyze actions of this PG in colorectal cancer. However, most studies have focused on PGE₂ actions on colorectal cancer cell lines, and some on colon tumors induced either by chemical carcinogen such as azoxymethane (AOM) or Apc gene mutation, both of which themselves induce inflammation in the stroma minimally (10). Up to now, few have analyzed PG actions on stroma components and none, to our knowledge, studied the role of PGs in inflammation in tumor microenvironment of the colon in whole animals. The latter point is essential, because inflammation underlies tumor microenvironment where various types of resident and inflammatory cells interact with tumor cells and promote cancer development in reactive stroma (11). PGs exert their actions through G-protein–coupled receptors specific for each PG, PGD receptor (DP), four subtypes of PGE receptor EP1 to EP4, PGF receptor (FP), PGI receptor (IP), and TXA receptor (TP; ref. 12). Identification of PG receptor responsible for colorectal cancer formation and progression and elucidation of its mechanisms are essential for development of more selective medical.
treatment alternative to aspirin. Here, we have subjected mice deficient in each type or subtype of PG receptor to the AOM/dextran sodium sulfate (DSS)-induced colon tumorigenesis, a model of colitis-associated cancer (CAC; ref. 13), and examined the role of PG receptors in inflammation-associated colon cancer formation and progression. Our study shows that PGE receptor EP2 is essential for colon tumorigenesis in this model, and that administration of an EP2-selective antagonist potently suppresses colon tumor formation.

Materials and Methods

Additional information is provided in Supplementary Materials and Methods.

Animal experiments

All animal experiments were performed in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine (Kyoto, Japan).

Mouse lines deficient in each PG receptor, Ptger1 (EP1), Ptger2 (EP2), Ptger3 (EP3), Ptger (IP), Ptgdr (DP), or Tbxa2r (TP) were previously reported (14). C57BL/6CrSlc mice and Ptges laboratory animals and were approved by the Committee on National Institutes of Health Guide for the care and use of laboratory animals and were approved by the local ethical committee at ATCC.

AOM/DSS model was performed as reported previously with modifications (13). Briefly, 8- to 12-week-old female mice were intraperitoneally injected 10 mg/kg AOM (Sigma) on day 0, received three cycles of DSS treatment, in which DSS (MP Biomedical) was added in drinking water at 2%, and sacrificed on day 80 or 200. An EP2 antagonist, PF-04418948 (15), 1-(4-fluorobenzoyl)-3-[[6-methoxy-2-naphthyl]oxy]methyl-azetidine-3-carboxylic acid, was orally administered to mice by mixing in a chow.

Cells

Primary neutrophils were prepared from mouse bone marrow by discontinuous density gradient centrifugation and CCD-18Co human tumor-associated colon fibroblasts were obtained from ATCC.

Human samples

Human samples were dissected during surgery for diagnosis and used with approval by the local ethical committee at Kyoto University Graduate School of Medicine (Approved number; E1975).

IHC

Fixed colon sections or cells were incubated with primary antibodies described in Supplementary Materials and Methods, followed by incubation with secondary antibodies conjugated with fluorescent dye. Finally, immunofluorescence images were acquired on a confocal fluorescence microscope.

FACS analysis

Inflammatory cells were purified by discontinuous density gradient centrifugation from the colon and incubated with fluorescent-conjugated antibodies described in Supplementary Materials and Methods for FACS analysis (FACSCalibur, BD Biosciences).

qRT-PCR

Total RNA was prepared from the colon or cells and transcribed into cDNA. qRT-PCR was performed with primer described in Supplementary Materials and Methods with Gapdh as an internal control. For quantification, the second derivative maximum method was used.

Statistical analysis

All bars indicate mean±SEM. Statistical comparison between two groups was made using Mann–Whitney U test. Statistical comparisons among more than two groups were conducted using Kruskal–Wallis test followed by post hoc Dunn test.

Results

PGE2–EP2 Signaling is critical for tumorigenesis in mouse CAC model

To identify the PG receptor(s) responsible for CAC, we subjected mice deficient in each PG receptor, EP1 (Ptger1), EP2 (Ptger2), EP3 (Ptger3), IP (Ptger), DP (Ptgdr), or TP (Tbxa2r), to a mouse CAC model of AOM/DSS treatment and examined colon tumorigenesis on day 80 (Fig. 1A). We did not include Ptger4−/− mice in our analysis, because they are available only in mixed genetic background (16). Among the lines tested, Ptger2−/− mice selectively showed significant reduction in the number of macroscopically assessed tumors in the colon compared with wild-type mice, whereas the tumor number significantly increased in four other lines, Ptger1−/−, Ptger3−/−, Ptgdr−/−, and Tbxa2r−/− mice (Fig. 1A and Supplementary Fig. S1A). To verify the involvement of PGE2 in this process, we subjected mice deficient in mPGES-1 (Ptges; ref. 17) to this model. Consistent with the observation in Ptger2−/− mice, Ptges−/− mice also showed significant reduction of the tumor number compared with wild-type mice (Fig. 1A and Supplementary Fig. S1A). However, the tumor number in the Ptges−/− mice was significantly larger than that in the Ptger2−/− mice, possibly reflecting the actions of other PGE receptors (Fig. 1A).

Histologically, tumor lesions in wild-type mice exhibited irregular crypt structure, and enhanced mitosis and loss of polarity of the nuclei in epithelial cells, which corresponds to the high-grade adenoma (Fig. 1B; ref. 18). Massive infiltration of inflammatory cells in stroma and submucosa was also observed in the colon of wild-type mice (Fig. 1B). Although Ptger1−/− or Ptger3−/− mice showed similar histology to wild-type mice (Supplementary Fig. S1B), the colon of Ptger2−/− mice mostly preserved a single-layered columnar epithelium with little inflammatory cell infiltration, and their tumors were microadenomas consisting of multifocal hyperplasia of crypts (Fig. 1B). Histology of the colon of the Ptges−/− mice was similar to that observed in Ptger2−/− mice, but the tumor lesions exhibited more disorganized crypt structure (Supplementary Fig. S1C). These findings suggest that PGE2 formed by mPGES-1 acts on EP2 in cells in the colon and facilitates colon tumorigenesis. Consistently, Ki-67 staining showed positive signals in almost all epithelial cells in the tumors of wild-type mice, but only in the bottom of crypts in Ptger2−/− mice (Fig. 1C). When examined on day 200, the tumor number was still significantly lower in Ptger2−/− mice and the size of their tumors was significantly smaller than that in wild-type mice.
(Supplementary Fig. S1D). Although Ptger2−/− mice at this stage developed high-grade adenomas similar to those observed in wild-type mice on day 80, their phenotypes were apparently less malignant than wild-type counterparts as assessed as irregularity of tissue, cell, and nucleus structures (Supplementary Fig. S1E).

We next analyzed the composition of infiltrating cells in the colon of wild-type and Ptger2−/− mice by FACS. Neutrophils defined as CD45+ CD11b+ Ly-6G+cells (19) were the most abundant population with the highest fold-increase in their number on day 80 in wild-type mice, and their number was significantly smaller in Ptger2−/− mice (Fig. 1D and Supplementary Fig. S1F). No significant difference was found in the numbers of CD4+ (CD45+ CD3+ CD4+), and CD8+ T cells (CD45+ CD3− CD8+), dendritic cells (CD45+ CD11b+ CD11c+) and macrophages (CD45+ CD11b+ F4/80+) between wild-type and Ptger2−/− mice, although the numbers of the latter two populations tended to be larger in wild-type mice than those in Ptger2−/− mice. Notably, acute colitis induced by DSS indeed increased in Ptges−/− mice (Supplementary Fig. S1G) and of similar intensity in wild-type and Ptger2−/− mice (Supplementary Fig. S1H), suggesting that the PGE2−EP2 signaling delays inflammation resolution.

Neutrophils and tumor-associated fibroblasts as two major cell populations expressing EP2 in tumor lesions of the colon

Given the significant reduction of colon tumorigenesis in Ptger2−/− mice, we next used IHC and identified cells expressing EP2 in tumor lesions (Fig. 2A). EP2 signals, the specificity of which was verified in Ptger2−/− mouse colon (Supplementary Fig. S2A), increased remarkably in the colon of wild-type mice after AOM/DSS treatment, and were present as both punctate and linear signals in the stroma and submucosa. No significant EP2 signals were noted in tumors themselves. Coimmunostaining for EP2 and Gr-1 showed that most of the punctate EP2 signals overlapped with Gr-1 signals (Fig. 2A, top), the majority of which represents CD11b+Ly-6G+CD49bLy-6C+ neutrophils (Supplementary Fig. S2B and S2C). Because the remaining linear signals were found in the mesenchyme and mesenchymal cells such as fibroblasts are activated in tumor microenvironment to adopt myofibroblast character and called tumor-associated fibroblasts (TAF; ref. 20), we next stained the colon with antibody to α-smooth muscle actin (α-SMA), a marker of TAFs. Signals for α-SMA indeed increased in the proximity to tumors in the colon following AOM/DSS treatment, and overlapped with linear EP2 signals surrounding the tumor (Fig. 2A, bottom).

To verify the clinical relevance of these findings, we examined the expression of EP2 in the colon from patients with CAC. Numerous punctate EP2 signals were observed in the stroma of the colon, and the majority of these signals were costained with α-SMA (Fig. 2B, top; refs. 21, 22), suggesting the EP2 expression in infiltrating neutrophils also in human CAC. In addition, signals for α-SMA were also detected in the human CAC specimen, and some overlapped with those for EP2 (Fig. 2B, bottom).

Because both infiltrating neutrophils and mesenchymal TAFs express EP2, we wondered how much each cell population contributes to colon tumorigenesis. To clarify this issue, we performed bone marrow transfer experiments between wild-type mice and Ptger2−/− mice. Transplantation of Ptger2−/− bone marrow to irradiated wild-type mice (96.5% reconstitution ratio) significantly reduced the colon tumor number compared with the wild-type recipients transplanted with wild-type bone marrow (Fig. 2C and D), suggesting the significant contribution of EP2 in neutrophils to colon tumorigenesis. Intriguingly, reverse transplantation of wild-type bone marrow to irradiated Ptger2−/− mice (97.9% reconstitution ratio) also significantly suppressed the colon tumorigenesis compared with the control group, suggesting that not only EP2 in infiltrating neutrophils, but also EP2 in the mesenchyme of recipients, possibly that in TAFs, contributes to tumorigenesis and contributions by EP2 in the two populations are interdependent.

EP2 in neutrophils amplifies inflammatory signaling in a positive feedback mechanism

Because proinflammatory molecules such as TNFα, IL6, and COX-2 are critically involved in CAC (10, 23−25), we examined the impact of Ptger2 deficiency on gene expression of these molecules in the colon on day 80 of AOM/DSS treatment. Expression of Tnfα, Il6, and Pges was significantly induced in the colon of wild-type mice and these inductions were significantly attenuated in Ptger2−/− mice (Fig. 3A and Supplementary Fig. S3A). To examine the contribution of neutrophils to expression of these genes, we performed double IHC study to stain these molecules together with Gr-1, and found that the significant proportion of cells expressing TNFα, IL6, and COX-2 in the tumor lesion were Gr-1+ neutrophils (Fig. 3B and Supplementary Fig. S3B). Because NF-kB is the master transcription factor regulating expression of these genes (26−28) and is known to contribute to cancer formation (29, 30), we examined activation of NF-kB in the colon by IHC for NF-kB p65 phosphorylated at Ser276, Ser468, or Ser536 and found that most of these signals were overlapped with those for neutrophil-specific myeloperoxidase (Fig. 3C and Supplementary Fig. S3C). These results suggest that neutrophils recruited to the colon induce expression of proinflammatory genes through NF-kB activation, and contribute to tumorigenesis. Because neutrophils express EP2, and the EP2 deficiency suppresses expression of these genes, we examined the role of EP2 in these gene expressions. We prepared naïve neutrophils from bone marrow as CD11b+Ly-6G+ cells (82.0% purity; Supplementary Fig. S3D), stimulated them with PGE2, an EP2 agonist ONO-AE1-259, and dibutyryl cAMP in the absence or presence of TNFα, and evaluated expression of these genes by qRT-PCR. These cells expressed EP2 (Supplementary Fig. S3E), and the

Figure 1. Suppression of colon tumorigenesis in Ptger2−/− mice. A, protocol of AOM/DSS treatment and the number and size of mice in treated groups. Wild-type (n = 9), Ptger1−/− (n = 5), Ptger2−/− (n = 5), Ptger3−/− (n = 5), Ptger2−/− (n = 4), Ptgir−/− (n = 4), Txaba2r−/− (n = 3), or Pges−/− (n = 7) mice were used. *P < 0.05, **P < 0.01; #P < 0.001. B, hematoxylin and eosin staining of the colon on day 80. Bottom left panels show the tumor region of Ptger2−/− mice. Magnified images corresponding to black or red boxes are shown below. Scale bar, 100 μm in the original images, 20 μm (upper images), or 10 μm (lower images). C, Ki-67 staining of the colon from wild-type or Ptger2−/− mice. Scale bar, 100 μm. Representative images from three specimens are shown. D, inflammatory cell populations in the colon of untreated and AOM/DSS-treated wild-type or Ptger2−/− mice. The numbers of each cell population were analyzed by FACS (n = 3). ***, P < 0.001; n.s., not significant.
addition of PGE2 significantly augmented TNFα-induced expression of Il6 and Ptgs2 (Fig. 3D). This augmenting action of PGE2 was mimicked by ONO-AE1-259, whose specificity on EP2 was confirmed using cells from Ptger2−/− mice (Supplementary Fig. S3F), and by dibutyryl cAMP, a second messenger of EP2 signaling (Fig. 3D). In addition, PCR array analysis revealed that EP2 stimulation enhanced the TNFα-induced expression of various NF-κB–targeted proinflammatory genes in neutrophils (Supplementary Fig. S3G). Because COX-2 (Ptgs2) is the pivotal enzyme producing PGE2, these results indicate the presence of the positive feedback loop consisting of COX-2–PGE2–EP2–NF-κB–COX-2 in neutrophils, which

Figure 2.
Expression of EP2 in neutrophils and TAFs and bone marrow transfer experiment. A, EP2 localization in Gr−1+ cells and α-SMA+ TAFs in the colon of wild-type mice. Immunostaining for EP2 (red), Gr−1 (green; top), or α-SMA (green; bottom) is shown. White arrows, epithelial cells. Representative images from five independent experiments are shown. Right, magnified merged image corresponding to white squares. Scale bar, 50 μm. B, immunostaining for EP2 and hematoxylin and eosin staining of colitis-associated colon cancer of humans. Images of specimen #1 (Supplementary Materials and Methods) are shown as representative. Scale bar, 50 μm (top images) or 200 μm (bottom images). C and D, number of tumors (C) and hematoxylin and eosin staining (D) of the colon of wild-type recipients transplanted with the Ptger2−/− bone marrow (n = 4) or vice versa (n = 8) on day 80. Transplantation of wild-type bone marrow to wild-type mice was served as a control (n = 4). *, P < 0.05; n.s., not significant. Magnified images corresponding to black boxes are shown below. Scale bar, 100 μm.
amplifies expression of proinflammatory genes as observed in other types of cells (31).

Because the Ptger2 deficiency suppressed the infiltration of neutrophils in the colon of AOM/DSS-treated mice, we next examined the role of EP2 in neutrophil recruitment. To address this issue, we first examined expression of various chemokines in the colon, and found that expression of Cxcl1, a chemokine for neutrophils (32), was significantly induced in the colon of wild-type mice and this induction was significantly and selectively suppressed by the Ptger2 deficiency (Fig. 3A and Supplementary Fig. S3H). IHC revealed that CXCL1 was expressed at least in part by Gr-1+ neutrophils infiltrating in the colon of wild-type mice and the Ptger2 deficiency suppressed both infiltration of neutrophils and CXCL1 expression in tissue (Fig. 3E). In vitro experiment using primary culture of neutrophils showed that PGE2, ONO-AE1-259, and dibutyryl cAMP significantly augmented TNFα-induced expression of Cxcl1 as observed for Il6 and Ptgs2 (Fig. 3D and Supplementary Fig. S3F). These results suggest that the PGE2–EP2 pathway forms an autocrine loop for neutrophil recruitment to the colon by inducing CXCL1 expression.

**EP2 in TAFs shapes tumor microenvironment**

We next studied the role of EP2 in TAFs in colon tumorigenesis. Because TAFs secrete growth factors and tissue remodeling factors (20, 33, 34), we examined expression of these factors in the colon of wild-type and Ptger2−/− mice on day 80. We found significant induction of one growth factor, brain-derived neurotrophic factor (BDNF), one matrix protease, matrix metalloprotease 12 (MMP12), and one extracellular matrix protein, osteopontin, in the colon of wild-type mice and significant reduction of their expression in the colon of Ptger2−/− mice (Fig. 4A and 4B).
Supplementary Fig. S4A). Notably, the expression of Spp1, the osteopontin gene, was elevated about 90 folds over day 0 in wild-type mice. The Ptger2 \(-/-\) mouse colon exhibited the lower basal expression and no significant induction (Fig. 4A). IHC showed that signals for these factors were induced and enriched in the mesenchyme and significantly overlapped with those for α-SMA, indicating their main source is TAFs (Fig. 4B and Supplementary Fig. S4B). We then used 18Co human intestinal fibroblasts as a TAF model, and incubated them with ONO-AE1-259 alone or ONO-AE1-259 together with TNFα. ONO-AE1-259 alone induced expression of genes for PTGER2 itself and BDNF and together with TNFα enhanced expression of PTGS2, IL6, and MMP12 (Fig. 4C), indicating that EP2 forms a self-amplification loop and shapes tumor microenvironment. Consistently, coimmunostaining for αSMA and COX-2 showed overlapping signals in the tumor lesions (Fig. 4D and Supplementary Fig. S4B). Notably, COX-2 staining in the mesenchyme was markedly decreased in the colon preparation of Ptger2 \(-/-\) mice transferred with wild-type bone marrow (Fig. 4D), indicating that EP2 in TAFs is required for their activation even in the presence of infiltrating cells.

Figure 4.
Amplification of inflammation, growth, tissue remodeling, and WNT signaling by EP2 signaling in TAFs. A, mRNA expression of Bdnf, Mmp12, and Spp1 (osteopontin) in the colon of wild-type (\(n = 3\)) or Ptger2 \(-/-\) (\(n = 5\)) mice before (untreated) and on day 80 of AOM/DSS treatment (AOM/DSS). Results by qRT-PCR are shown. **, \(P < 0.01\); ***, \(P < 0.001\); n.s., not significant. B, IHC for α-SMA (green) and BDNF (red) or osteopontin (red) in colon from wild-type or Ptger2 \(-/-\) mice on day 80. Right, merged images. Representative images from five independent experiments are shown. Scale bar, 50 μm. C, induction of PTGER2, BDNF, PTGS2, IL6, and MMP12 in cultured 18Co cells stimulated with vehicle, ONO-AE1-259 (EP2, 0.5 μmol/L), or TNFα (10 ng/mL) for 1 hour (\(n = 3\)). **, \(P < 0.01\); ***, \(P < 0.001\); †, \(P < 0.05\); ‡, \(P < 0.01\). D, IHC for α-SMA (green) and COX-2 (red) in colon from wild-type or Ptger2 \(-/-\) recipients transplanted with the wild-type bone marrow on day 80. Right, merged images. Representative images from five independent experiments are shown. Scale bar, 50 μm. E, induction of WNT2 (top left), WNT2B (top right), and WNT5A (bottom left) in 18Co cells stimulated with vehicle or ONO-AE1-259 (EP2, 0.5 μmol/L) for 3 hours (\(n = 3\)). **, \(P < 0.01\); †, \(P < 0.05\); ‡, impaired induction of Wnt5a in the colon of Ptger2 \(-/-\) mice. **, \(P < 0.05\). G, IHC for β-catenin (green) in the colon of wild-type or Ptger2 \(-/-\) mice on day 80. Right, merged image with DAPI staining (blue). White arrows, β-catenin translocated in part to the nuclei. Representative images from five independent experiments are shown. Scale bar, 5 μm.

Figure 5.
Effects of PF-04418948 on AOM/DSS-induced colon tumorigenesis. A, dose-dependent inhibition of colon tumor formation by PF-04418948. Mice were administered daily with 0, 1, 10, or 100 mg/kg of PF-04418948 (\(n = 4\), each) for 80 days during AOM/DSS treatment and the numbers of tumors in the colon were determined. ***, \(P < 0.001\). B, hematoxylin and eosin staining of the colon of vehicle-treated or PF-04418948-treated (10 mg/kg) mice. Scale bar, 100 μm. Magnified images corresponding to black or red boxes in left panels are shown in middle or right, respectively. Scale bar, 20 μm (top images) or 10 μm (bottom images). C, IHC for Gr-1 (left) and CXCL1 (right) in the colon of mice subjected to AOM/DSS treatment and administered with vehicle (top) or 10 mg/kg PF-04418948 (bottom). Representative images from three independent experiments are shown. Scale bar, 50 μm. D, effects of PF-04418948 administration period on colon tumorigenesis. Mice subjected to AOM/DSS treatment were administered with PF-04418948 (10 mg/kg) from day 0 to 49 (\(n = 3\)), day 29 to 80 (\(n = 9\), day 50 to 80 (\(n = 6\)), or day 0 to 80 (\(n = 4\), and examined on day 80. *, \(P < 0.05\); **, \(P < 0.01\); n.s., not significant.
neutrophils. Intriguingly, EP2 stimulation of 18Co cells also enhanced expression of various Wnt genes (Fig. 4E), and, consistently, both basal and induced expression of Wnt5a in the colon was significantly suppressed in Ptger2−/− mice compared with wild-type mice (Fig. 4F). In line with the Wnt induction, examination for β-catenin signals revealed that, although positive signals were strictly seen in cell–cell adhesion in the hyperplastic crypts in Ptger2−/− mice (Fig. 4G, bottom), β-catenin signals in epithelial cells in the adenoma of wild-type mice spread from the cell–cell boundary to the cytoplasm and translocated partly to the nuclei (Fig. 4G, top).

Pharmacologic inhibition of colon tumorigenesis by a selective EP2 antagonist, PF-04418948

To examine the potential of EP2 as a therapeutic target for CAC, we orally administered a selective EP2 antagonist, PF-04418948 (15), to wild-type mice during AOM/DSS treatment. PF-04418948 administered from the beginning of the experiment significantly suppressed the colon tumor formation in a dose-dependent manner, and no tumor was found with the daily dose of 100 mg/kg (Fig. 5A). Histologically, only crypt hyperplasia with apparently normal morphology of epithelial cells was observed in mice administered 10 mg/kg PF-04418948 daily compared with high-grade adenoma in the vehicle-treated group (Fig. 5B). Furthermore, treatment with PF-04418948 remarkably suppressed the infiltration of inflammatory cells and expression of CXCL1 in the colon (Fig. 5B and C). To identify the time window for the inhibitory action of PF-04418948, we administered this compound at 10 mg/kg/day from day 0 to 49, from day 50 to 80, and from day 29 to 80 and examined the number and size of tumors. Treatment with PF-04418948 from day 0 to 49 and from day 29 to 80 but not from day 50 to 80 significantly suppressed the formation of colon tumors to the extent seen in the mice treated over an entire period from day 0 to 80 (Fig. 5D). However, the PF-04418948 treatment affected little the size of tumors as observed in Ptger2−/− mice (Fig. 5D). These results suggest the potential of an EP2 antagonist in chemoprevention for initiation and recurrence of colorectal cancer in high-risk patients.

Discussion

Tumor growth is not only determined by tumor cells themselves, but also by the environment surrounding the tumor. Inflammation underlies this microenvironment, where resident mesenchymal cells and inflammatory cells interact in the stroma to promote tumor growth through the actions of cytokines, growth factors, growth modulators, extracellular matrix proteins, and matrix proteases (11). The present study examined actions of PGs in tumor microenvironment in colon tumorigenesis by subjecting mice deficient in each type or subtype of PG receptor to the CAC model. Among the lines tested, only the Ptger2−/− mice exhibited significant suppression of colon tumor formation with reduced inflammatory responses and attenuated inflammatory cell infiltration (Figs. 1, Fig. 3, and Fig. 4). A similar reduction was also observed in Ptges−/− mice, verifying involvement of PGE2 (Fig. 1A). EP2 is expressed in both infiltrating neutrophils and TAFs in the mesenchyme (Fig. 2). EP2 stimulation in neutrophils synergizes with TNFα to produce COX-2, cytokines, including IL6, and a neutrophil chemokine, CXCL1. We have preliminarily examined the underlying mechanism of this synergy, and found that EP2 and TNFα synergistically activate NF-kB (unpublished observation). Thus, the synergistic action of EP2 and TNFα makes dual amplifying cycles, one for amplification of NF-kB activation by the COX-2–PGE2–EP2–NF-kB–COX-2 cycle and the other for amplification of neutrophil recruitment by autocrine production of CXCL1 (Fig. 3). EP2 stimulation also triggers signaling amplification in TAFs by inducing EP2 expression and producing growth factors such as BDNF, various WNT molecules, osteopontin, and MMP12 (Fig. 4). Intriguingly, the bone marrow transfer experiment showed that EP2 in the two cell populations is equally
important for tumorigenesis, suggesting that EP2 signaling in these cell populations functions in an interdependent manner to drive crypt hyperplasia, disorganize crypt structure to the high-grade adenoma, then convert it to adenocarcinoma (Fig. 6).

Our findings are consistent with previous studies examining the involvement of cytokines, growth factors, chemokines, and tissue remodeling factors in CAC. For example, TNF receptor subunit p55 (Rp55)-deficient mice or wild-type mice treated with etanercept showed reduced tumor formation in the same AOM/DSS model (24). TNFα is a well-known pathogenic factor in human inflammatory bowel diseases (35), and TNFα polymorphisms are associated with ulcerative colitis-associated colorectal cancer (36). Similarly, Il6−/− mice developed less number of tumors in the AOM/DSS model (23). Accumulation of CD11b+Ly-6G− neutrophils to the colon and involvement of CXCL1 in their recruitment in the AOM/DSS model were also reported previously (37, 38). The CD11b+ Ly-6G− cells we found could be granulocytic myeloid-derived suppressor cells (MDSC) as suggested by Katoh and colleagues (37). Our preliminary FACS analysis confirmed on involvement of TAFs and TAF-derived factors in CAC (23). Accumulation of CD11b+Ly-6G− cells was positive for CD115, indicating that this population could be MDSCs (data not shown). Furthermore, much has been suggested on involvement of TAFs and TAF-derived factors in CAC (20, 34). Here, we found that osteopontin was induced in an EP2-dependent manner in the colon undergoing tumorigenesis. This was consistent with the previous findings (39) that osteopontin expression was induced in intestinal polyps of Apc−/− mice and downregulated by treatment with a COX-inhibitor. These authors suggested that osteopontin induction was mediated by a nuclear factor NotA2, which we previously found is quickly induced downstream of EP2 through the CAMP-PKA-CREB/CRTC2 pathway in T cells (40). Experimentally, osteopontin in tumor microenvironment has been shown to significantly affect tumor outcome (41), and, clinically, osteopontin is recognized as a colorectal cancer progression marker (42). Our study demonstrates that PG𝐸2−EP2 signaling amplifies the actions of all of these molecules through amplifying expression of genes and recruitment of responsible cells.

Our findings of the EP2 expression in TAFs in the mesenchyme are consistent with our previous finding that EP2 was expressed in stromal region in Apc−/− mice and Pgter2 deficiency suppressed intestinal polyph formation (43). Furthermore, several studies reported expression of EP2 in various types of cancers (44–46) and aspirin lowers the risk of solid tumors other than colorectal cancer (4, 5). It is interesting to test how widely the EP2 actions we found here operate in microenvironment of cancers other than colorectal cancer.

In this study, we did not examine contribution of one PGE receptor subtype, EP4, to colon tumorigenesis, because Pgter4−/− mice are alive only in a mixed genetic background. Previously, studies using cell lines, chemical carcinogens or Apc mutation implicated EP4 in colon tumorigenesis (47, 48). These studies suggest that PGE2−EP4 signaling functions directly in intestinal epithelial cells to promote tumorigenesis. Consistent with this idea, our preliminary study shows that administration of an EP4 antagonist does not completely suppress inflammation but reduces the number of tumor formed in mice subjected to the AOM/DSS model (unpublished observation).

Finally, this study has shown that administration of an EP2-selective antagonist potently suppresses colon tumor formation. Pgter2−/− mice exhibit impaired fertilization but otherwise develop normally (49). EP2-selective antagonists are therefore supposed to be free of gastrointestinal toxicity of NSAIDs and free of adverse tendency to cardiovascular accidents of COX-2-selective inhibitors (8). EP2-selective antagonists also appear to be superior to mPGES-1-selective inhibitors, because tumor formation was significantly less in Pgter2−/− mice than Pgter−/− mice. EP2 was also shown to be responsible for PGE2-mediated induction of MDSCs (50). EP2-selective antagonists can thus be a good alternative to aspirin in chemoprevention of colorectal cancer, exploiting its potent tumor-suppressing action without its adverse effects.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Naraumiya
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Ma, T. Aoki
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Ma, T. Aoki, T. Tsunamiya
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Ma, T. Tsunamiya
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Definition of Prostaglandin E$_2$–EP2 Signals in the Colon Tumor Microenvironment That Amplify Inflammation and Tumor Growth

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