

formation assay, cells were suspended in culture medium with 0.33% agar, and seeded in a 6-well plate. After 21 days of culture, soft agar was stained with Giemsa solution (Wako, Osaka, Japan).

Histological and immunohistochemical analyses

Tissues were fixed in 4% paraformaldehyde, embedded and sectioned at 4- μ m thickness. Sections were stained with hematoxylin and eosin. Antibodies against Ki-67 (DakoCytomation, Carpinteria, CA, USA) and F4/80 (Serotec, Oxford, UK) were used as the primary antibodies. Immunostaining signals were visualized using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA), and the MOM kit (Vector Laboratories) was used to minimize the background staining signals.

Methylation-specific PCR analysis

The methylation status of miR-7-1 in human gastric cancer and non-tumor tissues was examined by methylation-specific PCR analysis as described previously (Yamashita *et al.*, 2008). Methylation-specific PCR was performed with a primer set specific for the methylated or unmethylated sequence (Me or Un set in Supplementary Table 4). DNA methylated by SssI methylase (New England Biolabs, Ipswich, MA, USA) and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare, Buckinghamshire, UK) were used as methylated and unmethylated controls, respectively.

Chromatin immunoprecipitation analyses

Histone modifications were examined by chromatin immunoprecipitation assay as described previously (Yamashita *et al.*, 2008; Takeshima *et al.*, 2009). In brief, gastric epithelial cells of wild-type, *K19-C2mE* and *Gan* mice ($n=3$ for each genotype) were cross-linked with 1% formaldehyde, and sheared chromatin by Bioruptor UCD-250 (Cosmo Bio, Tokyo, Japan). The samples were incubated with an antibody against H3K27me3 (07-449, Millipore, Billerica, MA, USA), and genomic DNA samples were used for the quantitative chromatin immunoprecipitation-PCR analyses. The primer set was designed for the upstream CpG islands of miR-7-1 (Supplementary Table 4).

Luciferase reporter assay

Constructions of the miR-7 luciferase reporter vector (miR-7 Luc) and control vector (control Luc) are shown in Supplementary Figure 1. Luciferase reporter vectors for the *LPHN2*, *BASPI* and *MAFG* genes were constructed by subcloning 3' untranslated region fragments of the respective human genes into the pGL3 plasmid (Promega, Madison, WI, USA). PCR

primer sequences for cloning 3' untranslated region of the respective genes and amplified fragment lengths are indicated in Supplementary Table 4. The luciferase activity was measured using a Luciferase Assay System (Promega), and the levels were normalized to the total protein levels detected using the Pierce 660 nm Protein Assay (Thermo Scientific, Yokohama, Japan). RAW264 cells (RIKEN BioResource Center) and mouse intraperitoneal macrophages were stimulated with 10 ng/ml lipopolysaccharide (Sigma, St Louis, MO, USA) for 24 h and the conditioned medium was collected as CM(+). The conditioned medium of unstimulated macrophages was collected as CM(-). The conditioned medium of lipopolysaccharide-stimulated and COX-2 inhibitor celecoxib-treated (Pfizer, New York, NY, USA) (10 μ M) RAW264 cells was collected as CM(+ /coxib). CM(+) was fractionated by ultrafiltration using Centricon Plus-70 (Millipore) to prepare CM(+) > 100 kDa, 30–100 kDa, 3–30 kDa and < 3 kDa. Cells were stimulated with CM at a 50% concentration.

Western blotting analysis

Cells were transfected with pre-miR-7 or pre-miR-NC, lysed at 24 h after transfection, and 10 μ g of protein was separated in 7.5% SDS-polyacrylamide gels. An antibody against EGFR (Cell Signaling Technology, Danvers, MA, USA) was used as the primary antibody. Anti- β -actin (Sigma) was used as an internal control. The ECL detection system (GE Healthcare) was used to detect the signals, and band intensities were quantified using the ImageJ application (NIH, Bethesda, MD, USA).

Statistical analysis

The data were analyzed by the unpaired *t*-test using the Microsoft Excel software program (Microsoft). A value of $P < 0.05$ was considered to be statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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The inflammatory network in the gastrointestinal tumor microenvironment: lessons from mouse models

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Abstract Accumulating evidence has indicated that inflammatory responses are important for cancer development. Epidemiological studies have shown that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of colon cancer development. Subsequently, mouse genetic studies have shown that cyclooxygenase (COX)-2, one of the target molecules of NSAIDs, and its downstream product, prostaglandin E₂ (PGE₂), play an important role in gastrointestinal tumorigenesis. Bacterial infection stimulates the Toll-like receptor (TLR)/MyD88 pathway in tumor tissues, which leads to the induction of COX-2 in stromal cells, including macrophages. Induction of the COX-2/PGE₂ pathway in tumor stroma is important for the development and maintenance of an inflammatory microenvironment in gastrointestinal tumors. In such a microenvironment, tumor-associated macrophages express proinflammatory cytokines, including tumor necrosis factor (TNF)- α and interleukin (IL)-6, and these cytokines, respectively, activate the nuclear factor (NF)- κ B and Stat3 transcription factors in epithelial cells, as well as in stromal cells. Recent mouse studies have uncovered the role of such an inflammatory network in the promotion of gastrointestinal tumor development. Genetically engineered and chemically induced mouse tumor models which mimic sporadic or inflammation-associated tumorigenesis were used in these studies. In this review article, we focus on mouse genetic studies using these tumor models, which have contributed to the elucidation of the molecular mechanisms associated with the inflammatory network in gastrointestinal tumors, and we also discuss the

role of each pathway in cancer development. The involvement of immune cells such as macrophages, mast cells, and regulatory T cells in tumor promotion is also discussed.

Keywords Gastrointestinal cancer · Inflammation · COX-2 · NF- κ B · Stat3

Introduction

About 150 years ago, Rudolf Virchow described the presence of leukocytes in tumors, and hypothesized that the origin of cancer was at the site of chronic inflammation. It has been reported that chronic infections are associated with 15–20% of malignant cancers [1, 2]. The principal infectious agents are *Helicobacter pylori*, hepatitis B and C viruses, and the human papilloma virus, which are closely associated with gastric cancer, hepatocellular carcinoma, and cervical cancer, respectively. Moreover, about 30% of all cancers have been attributed to smoking and 20% to obesity [3], and it has been shown that both tobacco smoke and obesity can trigger inflammatory responses in the lungs and liver, respectively, which promote tumorigenesis [4, 5]. These results, together with those of other recent studies (reviewed in [6–8]), indicate that inflammation plays an important role in promoting cancer development, and “tumor-promoting inflammation” is now included in the next generation of the criteria considered to be “hallmarks of cancer” [9].

Epidemiological studies have revealed that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) lowers the mortality rate from cancers in the gastrointestinal tract [10, 11]. The target molecules of NSAIDs are cyclooxygenase (COX)-1 and COX-2, and accumulating evidence has indicated that COX-2 and its downstream product, prostaglandin E₂ (PGE₂), play an important role in cancer

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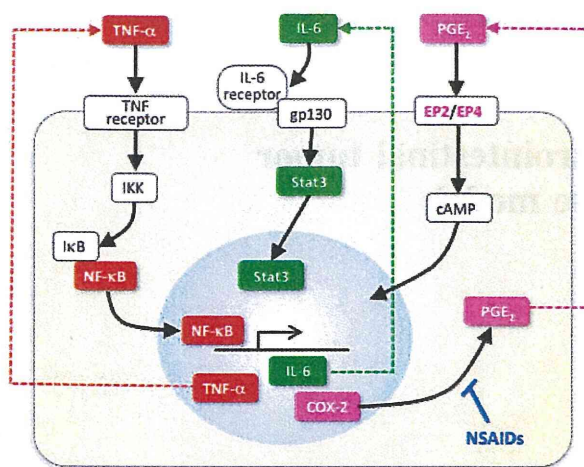


Fig. 1 The interaction of the cyclooxygenase-2 (*COX-2*)/prostaglandin E_2 (PGE_2), tumor necrosis factor- α ($TNF-\alpha$)/nuclear factor- κB ($NF-\kappa B$), and interleukin-6 ($IL-6$)/gp130/Stat3 pathways in the inflammatory environment. *cAMP* cyclic AMP, *IKK* inhibitor of κB kinase, *I κ B* inhibitor of κB , *NSAIDs* non-steroidal anti-inflammatory drugs

development [12, 13]. On the other hand, proinflammatory cytokines are expressed in the tumor microenvironment, and such cytokine signaling is also important for cancer development through the activation of downstream transcription factors [6]. Among them, tumor necrosis factor (TNF)- α and interleukin (IL)-6 activate nuclear factor (NF)- κB and Stat3, respectively, and both the $TNF-\alpha$ / $NF-\kappa B$ and $IL-6$ /Stat3 pathways have been shown to be important for the development of inflammation-associated intestinal tumorigenesis [7, 8]. Moreover, $NF-\kappa B$ induces the expression of *COX-2*, $IL-6$, and $TNF-\alpha$. Accordingly, these signaling pathways construct an inflammatory network in the tumor microenvironment, which plays an important role in tumor promotion (Fig. 1). In this review, we discuss the roles of these inflammatory pathways in gastrointestinal tumorigenesis, which have been identified by a number of mouse model studies, as listed in Table 1.

Mouse models of gastrointestinal cancer

The roles of inflammatory responses in gastrointestinal cancers have been studied using several tumor mouse models (Table 1). *Apc*^{A716} knockout mice and *Apc*^{Min} mice carry heterozygous truncation mutations at codons 716 and 850 of the mouse *Apc* gene, respectively, and somatic deletion of the wild-type *Apc* gene results in the activation of Wnt/ β -catenin signaling, which causes tumor development in the entire intestinal tract [14, 15]. Approximately 80% of colorectal cancers harbor *APC* gene mutations and half of the remainder have mutations in *Cttnb1* gene encoding β -catenin, both of which activate Wnt/ β -catenin signaling [16–18].

Thus, *Apc*^{A716} and *Apc*^{Min} mice recapitulate the molecular mechanism of sporadic colon cancer development. Several other types of *Apc* mutant mice also develop intestinal polyposis (as described in this review and Table 1).

On the other hand, inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are also risk factors for colorectal cancer [19, 20]. IBD-related colon cancers are associated with a severe inflammatory response, which is an important microenvironment required for inflammation-associated tumor development. The treatment of mice with a genotoxic chemical carcinogen, azoxymethane (AOM), followed by a non-genotoxic agent, dextran sodium sulfate (DSS), induces the development of colitis-associated colon cancer (CAC) [21]. Mutations in *Cttnb1* gene induced by AOM result in the activation of the Wnt/ β -catenin pathway, which is thought to trigger tumor initiation [22]. On the other hand, DSS induces colonic inflammation in rodents, which is required for tumor promotion. Accordingly, the AOM/DSS model is a well-established and widely used mouse model for CAC development, and it mimics IBD-related colon cancer (Table 1). *Rag2* gene-deficient mice lack functional lymphocytes, and are susceptible to infection-induced inflammation in the colon. When *Rag2*^{-/-} mice are infected with the enteric bacterial pathogen, *Helicobacter hepaticus*, the mice rapidly develop CAC [23, 24]. This model system is also used for IBD-related colon cancer (Table 1).

Activation of Wnt/ β -catenin signaling is found in approximately 30–50% of gastric cancers, suggesting a causal role of Wnt signaling in a subpopulation of gastric cancers [25, 26]. On the other hand, *Helicobacter pylori* infection is an important risk factor for gastric cancer [27]. In *H. pylori*-associated gastritis, *COX-2* expression is induced significantly, whereas its level is decreased by *H. pylori* eradication [28, 29]. Two transgenic mouse strains, *K19-Wnt1* mice expressing *Wnt1* in the stomach, and *K19-C2mE* mice expressing *Ptgs2* and *Ptgs1* that encode *COX-2* and microsomal PGE synthase-1 (mPGES-1), mimic Wnt activation and *H. pylori*-induced inflammation, respectively, in the stomach [26, 29–31]. *Gan* mice, which are compound transgenic mice with *K19-Wnt1* and *K19-C2mE*, express *Wnt1*, *Ptgs2*, and *Ptgs1* simultaneously in the gastric mucosa, resulting in the activation of both Wnt signaling and the *COX-2*/ PGE_2 pathways, as found in human gastric cancer. *Gan* mice are thus used as a model of inflammation-associated gastric tumors [26, 30, 31] (Table 1).

The *COX-2*/ PGE_2 /EP2 pathway in gastrointestinal tumorigenesis

It has been demonstrated that treatment of familial adenomatous polyposis (FAP) patients with NSAIDs results in

Table 1 Mouse model studies performed to examine inflammatory networks in gastrointestinal tumorigenesis

Tumor model	Mouse line crossed/treatment	Tumor phenotype changes	References
Sporadic intestinal tumor model (<i>Apc</i> mutant mice)			
<i>Apc</i> ^{Δ716}	<i>Ptgs2</i> ^a knockout mice	Suppression of intestinal polyposis	[34]
<i>Apc</i> ^{Min}	<i>Ptgs1</i> ^b , <i>Ptgs2</i> knockout mice	Suppression of intestinal polyposis	[35]
<i>Apc</i> ^{Min}	<i>Hpgd</i> ^c knockout mice	Increase of colon polyps	[36]
AOM	<i>Ptgs2</i> transgenic mice	Increase of intestinal tumor	[37]
<i>Apc</i> ^{Δ716}	<i>Ptger2</i> ^d knockout mice	Suppression of intestinal polyposis	[38]
		Inhibition of angiogenesis	[39]
<i>Apc</i> ^{Min}	PGE ₂ treatment	Promotion of intestinal polyposis	[40]
<i>Apc</i> ^{Δ14}	<i>Ptges</i> ^e knockout mice	Suppression of intestinal polyposis	[44]
AOM	<i>Ptges</i> knockout mice	Suppression of intestinal polyposis	[45]
<i>Apc</i> ^{Min}	<i>Myd88</i> ^f knockout mice	Suppression of intestinal polyposis	[63, 64]
		Epithelial expression of MyD88 is important	
<i>Apc</i> ^{Δ716}	<i>op/op</i> (macrophage-deficient)	Suppression of intestinal polyposis	[93]
<i>Apc</i> ^{Δ468}	<i>Kit</i> ^{W/W} (mast cell-deficient)	Suppression of intestinal polyposis	[76]
<i>Apc</i> ^{Δ468}	<i>Rag2</i> ^{-/-} (lymphocyte-deficient)	Not affected	
<i>Apc</i> ^{Δ468}	Anti-TNF- α antibody	Suppression of intestinal polyposis	
<i>Apc</i> ^{Min}	CD4 ⁺ CD25 ⁺ T cell transfer	Suppression of intestinal polyposis	[94, 95]
		IL-10 expression in T cells is required	
<i>Apc</i> ^{Min}	<i>Il17a</i> ^g knockout mice	Suppression of intestinal polyposis	[97]
Inflammation-associated colon tumor model (AOM/DSS-CAC model mice)			
AOM/DSS	<i>Tlr4</i> ^h knockout mice	Suppression of CAC development	[60, 62]
		Epithelial expression of TLR4 is important	
AOM/DSS	<i>Ptgs2</i> knockout mice	Exacerbation of CAC development	[68, 69]
AOM/DSS	<i>Ikkb</i> ⁱ conditional KO	Suppression of CAC development	[72]
		Epithelial and myeloid expression is important	
AOM/DSS	<i>Tnfrsf1a</i> ^j knockout mice	Suppression of CAC development	[73]
		Myeloid expression of TNF-Rp55 is important	
AOM/DSS	<i>Ccr2</i> ^k knockout mice	Suppression of CAC development	[74]
		Less macrophage infiltration	
AOM/DSS	<i>Il6</i> ^l knockout mice	Suppression of CAC development	[82]
AOM/DSS	gp130 ^{757F/F}	Suppression of CAC development	[81]
AOM/DSS	<i>Stat3</i> conditional KO	Suppression of CAC development	[81, 82]
		Epithelial expression of Stat3 is important	
<i>H. hepaticus</i> infected <i>Rag2</i> ^{-/-}	CD4 ⁺ CD25 ⁺ T-cell transfer	Suppression of CAC development	[23, 24]
		IL-10 expression in T cells is required	
Gastritis and gastric tumor model			
<i>K19-Wnt1</i>	<i>K19-C2mE</i>	Gastric tumor development (<i>Gan</i> mice)	[26, 30, 31]
<i>Gan</i>	Celecoxib, EP4 inhibitor	Suppression of gastric tumorigenesis	[48, 49]
<i>Gan</i>	Clodronate liposome (macrophage-deficient)	Atrophic changes of tumor cells	[49]
<i>K19-C2mE</i>	<i>Tnf</i> ^m knockout mice	Suppression of gastritis/hyperplasia	[78]
<i>K19-C2mE</i>	<i>Rag2</i> knockout mice	Not affected	
gp130 ^{757F/F}	<i>Il11ra</i> ⁿ knockout mice	Suppression of gastric tumorigenesis	[88]

AOM azoxymethane DSS dextran sodium sulfate, CAC colitis-associated colon cancer, IL interleukin, TNF tumor necrosis factor, COX cyclooxygenase, 15-PGDH 15-hydroxyprostaglandin dehydrogenase, TLR Toll-like receptor, mPGES-1 microsomal PGE synthase-1, KO knockout. Gene symbols used are: ^aCOX-2, ^bCOX-1, ^c15-PGDH, ^dPGE₂ receptor EP2, ^emPGES-1, ^fMyD88, ^gIL-17A, ^hTLR-4, ⁱIKK β , ^jTNF-Rp55, ^kCCR2, ^lIL-6, ^mTNF- α , and ⁿIL-11 receptor- α .

significant regression of colon polyps [32]. Moreover, a large number of animal experiments have shown that treatment with NSAIDs suppressed chemical carcinogen-induced colon tumorigenesis [33]. As a target molecule of NSAIDs, the inducible enzyme COX-2 plays an important role in inflammation and cancer, while COX-1 is expressed constitutively and functions as a house-keeping gene. Importantly, disruption of *Ptgs2* gene in *Apc^{Δ716}* mice and *Apc^{Min}* mice resulted in a significant suppression of intestinal tumorigenesis, thus indicating an essential role of COX-2 in intestinal polyp development [34, 35]. Interestingly, disruption of *Ptgs1* gene encoding COX-1 also suppressed intestinal tumorigenesis [35]. It is possible that COX-1-derived prostaglandins are required for tumor cell proliferation during the initial stage when COX-2 expression is not yet induced [33]. Other lines of genetic evidence also support the role of the COX-2 pathway in intestinal tumorigenesis. Prostaglandins are catalyzed and inactivated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). The number of colon polyps in *Apc^{Min}* mice was markedly increased when *Hpgd* gene encoding 15-PGDH was disrupted, suggesting that PGE₂ has a role in colon tumorigenesis [36]. Moreover, the transgenic expression of *Ptgs2*

in the mouse intestine accelerated chemical carcinogen-induced tumorigenesis [37].

COX-2 catalyzes the synthesis of prostaglandin (PG) H₂, which is then converted to PGE₂. There are four G protein-coupled receptors for PGE₂; EP1, EP2, EP3, and EP4. Notably, disruption of *Ptger2* gene encoding EP2 caused significant suppression of intestinal polyposis in *Apc^{Δ716}* mice, whereas suppression of EP1 or EP3 signaling did not affect tumorigenesis [38]. EP2 signaling increases the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which enhances angiogenesis in intestinal tumors [39]. Moreover, treatment of *Apc^{Min}* mice with PGE₂ increased the development of intestinal tumors through the activation of peroxisome proliferators-activated receptor (PPAR) δ , which promotes the survival of tumor cells [40]. Furthermore, PGE₂ signaling through the EP2 receptor has been shown to activate Wnt/ β -catenin signaling directly in colon cancer cells by the suppression of β -catenin phosphorylation [41]. Accordingly, it is possible that the COX-2/PGE₂ pathway contributes to intestinal tumorigenesis through a variety of PGE₂ functions [12, 13] (Fig. 2).

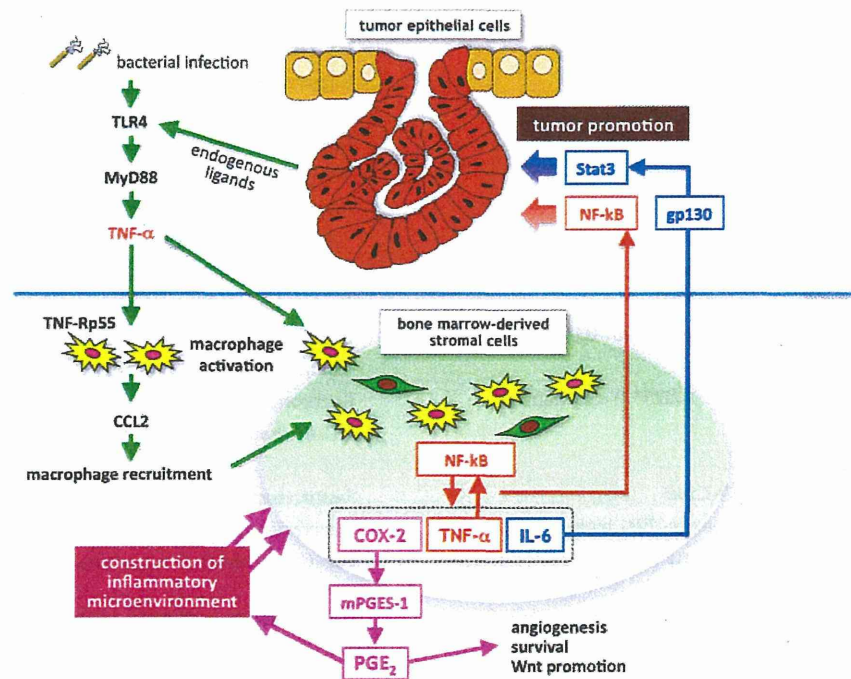


Fig. 2 A schematic diagram of the inflammatory microenvironment in gastrointestinal tumor tissues. Bacterial infection or endogenous ligand activates the Toll-like receptor (TLR)/MyD88 pathway in epithelial cells, which further activate stromal macrophages inducing CCL2. In the activated macrophages, NF- κ B induces the expression of COX-2, IL-6, and TNF- α itself. The COX-2/PGE₂ pathway is important for the construction and maintenance of the inflammatory

network, and PGE₂ accelerates angiogenesis, cell survival, and Wnt activation. TNF- α activates NF- κ B in both epithelial cells and stromal macrophages, whereas IL-6 activates Stat3 in epithelial cells through gp130. NF- κ B and Stat3 play an important role in the promotion of tumorigenesis through suppression of apoptosis and acceleration of the cell cycle. *mPGES-1* Microsomal PGE synthase-1

Microsomal PGE synthase-1 (mPGES-1) is an enzyme that converts PGH₂ to PGE₂, and expression of mPGES-1 is induced in gastric and colon cancers similar to COX-2 [42, 43]. Notably, disruption of *Ptges* gene encoding mPGES-1 in *Apc*^{Δ14} mice or AOM-treated mice resulted in a marked decrease in the PGE₂ level in the intestinal mucosa, which led to a further significant suppression of intestinal tumorigenesis [44, 45]. Taken together, these results indicate that the simultaneous expression of COX-2 and mPGES-1 is required for intestinal tumorigenesis through the induction of PGE₂ signaling.

In gastric cancer tissues, induction of COX-2 is found in approximately 70% of cases [46, 47], and mPGES-1 expression is also induced [42], suggesting that the COX-2/PGE₂ pathway is also important for gastric tumorigenesis. In the *K19-C2mE* transgenic mice, an increased PGE₂ level causes inflammatory infiltration and metaplastic hyperplasia in the gastric mucosa [29]. Although Wnt activation alone is not sufficient for tumor development in *K19-Wnt1* mice, *Gan* mice (compound mutants of *K19-Wnt1* and *K19-C2mE* mice) develop inflammation-associated gastric tumors with 100% incidence [26], indicating that cooperation of the Wnt and PGE₂ pathways can lead to gastric tumorigenesis. Moreover, gastric inflammation and tumorigenesis were significantly suppressed in *Gan* mice when the mice were treated with a COX-2 inhibitor, celecoxib, or an EP4 receptor inhibitor [48, 49]. These results indicate that COX-2/PGE₂/EP4-induced inflammation is involved in the development of gastric cancer. Signaling through both EP2 and EP4 stimulates the intracellular cyclic AMP signaling pathway. It is therefore possible that either EP2 or EP4 receptor signaling plays an important role in gastrointestinal tumorigenesis (Fig. 1).

The Toll-like receptor (TLR)/MyD88 pathway for COX-2 induction in gastrointestinal tumors

Expression of COX-2 and mPGES-1 is detected predominantly in stromal cells, including macrophages and fibroblasts, but not in the epithelial cells of mouse intestinal polyps, and the same is true for human colon polyps [36, 50–52]. Heterozygous mutations in the *Lkb1*, *Smad4*, or *Cdx2* gene lead to the development of gastrointestinal hamartomas, which show histological characteristics distinct from those of dysplastic adenomas developed in *Apc* mutant mice [52–55]. Notably, expression of COX-2 and mPGES-1 is detected in the stromal cells, but not in tumor epithelial cells, in these models [56], thus indicating that the COX-2/PGE₂ pathway is induced in the tumor stroma by a common mechanism, regardless of the type of tumor.

Several studies have suggested the commensal flora to play a role in the homeostasis of the intestinal mucosa. Toll-like receptors (TLRs) are a family of pattern-recognition receptors that detect the molecular products of microorganisms. MyD88 is an adaptor molecule for the TLR-mediated induction of inflammatory cytokines. It has been shown that a disruption of *Tlr2/4* or *Myd88* genes encoding TLR2/4 or MyD88, respectively, in mice results in the impaired mucosal repair of DSS-induced ulcers [57], suggesting that infectious stimulation through the TLR/MyD88 pathway is important for the regeneration process of injured mucosa. Moreover, stromal macrophages are also required for the mucosal repair of DSS-induced ulcers in the colon [58]. Accordingly, it is possible that stromal macrophages are activated by the TLR/MyD88 pathway. In the case of intestinal tumorigenesis, cancer cells may use such a TLR/MyD88-induced regeneration system to increase their proliferation.

The treatment of mice with DSS induces COX-2 expression and PGE₂ production, predominantly in macrophages of the inflamed colon mucosa. However, such COX-2 induction is not found in the mice lacking the TLR4/MyD88 pathway [59], thus indicating the role of bacterial infection in inducing COX-2 expression in colitis tissues. Importantly, AOM/DSS treatment-induced colon tumor development was dramatically suppressed in *Tlr4*^{-/-} mice [60], while exogenous administration of PGE₂ promoted CAC development in the AOM/DSS-treated *Tlr4*^{-/-} mice [61]. Moreover, bone marrow chimera experiments have indicated that TLR4 expression in the intestinal epithelial cells, but not in myeloid cells, is required for CAC development [62]. Taken together, these results suggest that bacterial infection stimulates the TLR/MyD88 pathway in epithelial cells, which leads to activation of stromal macrophages, thus resulting in the induction of the COX-2/PGE₂ pathway in the tumor stroma (Fig. 2).

In contrast to the AOM/DSS model, intestinal polyposis in the *Apc* mutant mice is not associated with UC. It is therefore possible that COX-2 expression is induced by a different mechanism in sporadic colon cancer compared to that in IBD-related tumors. However, *Apc*^{Min} *Myd88*^{-/-} mice showed significant suppression of intestinal polyposis with dramatically decreased mortality compared with control *Apc*^{Min} *Myd88*^{+/+} mice [63, 64]. Moreover, the induction of COX-2 expression was also suppressed in *Apc*^{Min} *Myd88*^{-/-} mouse intestinal tumors [63]. Bone marrow chimera experiments also indicated that MyD88 expression in the epithelial cells was important for intestinal tumorigenesis [64]. These results have demonstrated that activation of the TLR/MyD88 pathway in epithelial cells is also important for COX-2 expression in non-IBD-related colon cancer (Fig. 2).

If bacterial infection is also required for COX-2 induction in the stomach, a low bacterial count due to the acidic environment in the stomach may protect against COX-2 expression in tumorous lesions. Therefore, it is possible that COX-2 induction through the TLR/MyD88 pathway is one of the important mechanisms by which *Helicobacter pylori* infection promotes gastric cancer.

It has also been shown that TLRs can be stimulated with endogenous ligands, including heat shock proteins and various products of the extracellular matrix [65]. Accordingly, it is possible that tumor cell proliferation causes tissue damage, releasing endogenous ligands for TLRs, thus resulting in their activation, which induces COX-2 expression in stromal macrophages. Such “cancer-induced inflammation” may also be one of the mechanisms responsible for the generation of an inflammatory micro-environment, especially in cancers that are not associated with infection [65].

The paradox of the COX-2 pathway in colitis-associated cancer

Although COX-2 inhibition causes suppression of gastrointestinal tumorigenesis, treatment with NSAIDs or a COX-2 inhibitor exacerbates DSS-induced colon injury in rodent models [66]. Consistently, *Ptgs2* gene knockout mice exposed to DSS showed exacerbated phenotypes, such as more severe inflammation, compared with wild-type mice [67]. One of the functions of PGE₂ is to protect the gastrointestinal mucosa. Therefore, COX-2 is important for mucosal protection and regeneration in the DSS-treated mouse colon. Notably, treatment of *Ptgs2* knockout mice with AOM/DSS resulted in a significant increase in tumors, with severe inflammatory responses [68, 69], which appears to be contradictory to the results showing that *Ptgs2* gene disruption causes significant suppression of intestinal polyposis in *Apc* mutant mice [34, 35]. It is possible that in the case of severe UC, cytokine signaling is highly activated and may be sufficient for tumor promotion if the COX-2/PGE₂ pathway is blocked (Fig. 2).

The TNF- α /NF- κ B pathway in gastrointestinal tumorigenesis

TNF- α is one of key regulators of inflammatory responses. Although TNF was originally recognized as a tumor-necrotizing factor, accumulating evidence has indicated that TNF- α has tumor-promoting functions [70]. TNF- α signaling activates NF- κ B, which further induces the expression of inflammatory factors including COX-2, IL-6, IL-8, and TNF- α itself (Fig. 1). Several genetic studies have

demonstrated a link between the TNF- α /NF- κ B pathway and cancer development [71].

Conditional disruption of *Ikkbb* gene encoding IKK β in myeloid cells, which results in specific inhibition of NF- κ B, caused significant suppression of the tumor incidence in AOM/DSS-treated mice, and was associated with decreased expression of cytokines and COX-2 [72]. Conditional deletion of *Ikkbb* in epithelial cells also suppressed AOM/DSS-induced tumorigenesis [72]. Similar results were found in TNF- α receptor gene knockout mice [73]. AOM/DSS treatment in mice lacking *Tnfrsf1a* gene encoding TNF receptor p55 (TNF-Rp55) resulted in attenuated tumor formation, with reduced inflammatory cell infiltration compared with findings in wild-type mice. Moreover, wild-type mice transplanted with *Tnfrsf1a*-deficient bone marrow developed significantly fewer tumors after AOM/DSS treatment [73], indicating that TNF- α stimulation of myeloid cells is important for tumorigenesis. Accordingly, it is possible that the TNF- α -induced NF- κ B activation in myeloid cells is important for CAC development, and NF- κ B activation in epithelial cells also contributes to tumor formation (Fig. 2).

It has also been shown that a disruption of *Ccr2* gene encoding a CCL2-specific receptor, CCR2, led to significantly decreased macrophage infiltration and lower tumor numbers when mice were treated with AOM/DSS [74]. CCL2 is a chemokine that is chemotactic for monocytes and macrophages [75]. Taken together, these findings indicate that the activation of NF- κ B in activated macrophages by TNF- α in an autocrine or paracrine manner is important for the promotion of intestinal tumorigenesis (Fig. 2). The inhibition of TNF- α in *Apc* mutant mice by treatment with an anti-TNF- α antibody also suppressed intestinal polyposis, with the suppression of angiogenesis [76]. Accordingly, it is possible that TNF- α /NF- κ B activation is important in both IBD-related and sporadic colon carcinogenesis.

An important role for the TNF- α /NF- κ B pathway was also discovered in *Mdr2* knockout mice that develop inflammation-associated hepatocellular carcinoma (HCC) [77]. In this mouse model, NF- κ B is activated in the liver by TNF- α signaling, and the inhibition of NF- κ B significantly suppressed the development of HCC after 7 months of age [77]. On the other hand, preneoplastic dysplastic lesions in younger mice were not affected by NF- κ B inhibition. It is possible that the TNF- α /NF- κ B pathway is not required for the initiation step, but it does play a role in the promotion step of HCC development.

The role of the TNF- α /NF- κ B pathway in gastric tumorigenesis in *Gan* mice has not yet been examined. However, *Tnf*-/- *K19-C2mE* mice showed significant suppression of gastritis and hyperplasia compared with control *K19-C2mE* mice, although the COX-2/PGE₂

pathway was still activated by the transgenic expression of COX-2 and mPGES-1 [78]. It is thus conceivable that the TNF- α /NF- κ B pathway is activated in PGE₂-associated gastritis, and that this contributes to inflammation-associated gastric tumorigenesis.

The IL-6/gp130/Stat3 pathway in gastrointestinal tumorigenesis

One of the NF- κ B-inducible cytokines is IL-6, which is important for immune responses, cell survival, apoptosis, and proliferation [79]. The expression of IL-6 is often upregulated in tumor tissues and in the sera of humans and mice with cancers, including colon cancer [80]. The IL-6 cytokine family signals through a common receptor, gp130, which activates Stat3 (Fig. 1). Stat3 plays an important role in the development of a variety of cancers, including CAC [81–84]. AOM/DSS-induced CAC development was significantly suppressed in conditional Stat3-knockout mice that lacked Stat3 in the intestinal epithelial cells and also in *Il6*^{-/-} mice [81, 82]. Survival and proliferation of tumor cells were suppressed in these mutant mice. On the other hand, the number and size of AOM/DSS-induced colon tumors increased significantly in gp130^{757F/F} mice, in which gp130-dependent Stat signaling is constitutively activated [81]. These results indicate that Stat3 activated in epithelial cells plays an important role in the promotion of intestinal tumorigenesis (Fig. 2).

It has been shown that gp130^{757F/F} mice develop gastric tumors with abundant infiltration of inflammatory cells [85]. Moreover, heterozygous mutations of the Stat3 gene in gp130^{757F/F} mice reduced the incidence and multiplicity of gastric tumors, with the suppression of inflammatory responses [86, 87]. These results indicate that Stat3 is also an important tumor-promoting factor in gastric tumorigenesis, and that it is activated by the inflammatory network of the tumor microenvironment. Transforming growth factor (TGF)- β signaling promotes epithelial differentiation, and thus, suppression of the TGF- β signaling pathway has been thought to promote gastrointestinal tumorigenesis. Notably, the activation of Stat3 in gp130^{757F/F} cells desensitizes them to TGF- β by inducing inhibitory Smad7. This may be one of the mechanisms by which Stat3 promotes tumor formation [86]. IL-11 is another member of the IL-6 cytokine family and also signals through gp130. Interestingly, disruption of the IL-11 co-receptor in gp130^{757F/F} mice significantly ablated gastric tumorigenesis [88]. Because IL-11 is upregulated in human and mouse gastric tumors, these results suggest that the IL-11/Stat3 pathway, together with IL-6/Stat3, promotes gastric tumorigenesis.

Inflammatory cells that promote or suppress tumorigenesis

The major source of inflammatory cytokines and prostaglandins in tumor tissues is macrophages. Tumor-associated macrophages (TAMs) have been shown to promote the progression and metastasis of cancer [89]. TAMs can be classified into several distinct groups by their functions, such as inducing inflammation, invasion, angiogenesis, or metastasis. Macrophages are polarized to either the classical M1 type or the alternative M2 type, and it has been suggested that TAMs are polarized to M2 or M2-like types [90]. It has been shown that CD4⁺ T cells regulate the polarization of macrophages to the M2 type in mammary tumors [91]. It has recently been shown that COX-2 is important for the M2-polarization of TAMs in *Apc*^{Min} mouse tumors [92]. Accordingly, it is possible that the COX-2/PGE₂ pathway-induced inflammatory network is important for the education of macrophages into the pro-tumorigenic M2 or M2-like types. Importantly, depletion of functional macrophages in *Apc*⁴⁷¹⁶ mice by crossing them with op/op mutant mice resulted in significant suppression of intestinal polyposis [93]. Moreover, inhibition of macrophage recruitment in AOM/DSS-treated mice by *Ccl2* gene disruption resulted in the suppression of colon tumor development [74]. Accordingly, macrophages play an important role in both sporadic and IBD-related intestinal tumorigenesis (Fig. 2). In *Gan* mouse gastric tumors, macrophage depletion caused atrophic changes of the tumor cells and apoptosis of stromal cells [49]. Accordingly, it is possible that macrophages play a role in the maintenance of both tumor epithelial cells and stromal cells.

In the *Apc*⁴⁴⁶⁸ mouse intestinal tumors, mast cells are preferentially enriched in the polyp tissues, and these mast cells express TNF- α [76]. Notably, the depletion of mast cells in *Apc*⁴⁴⁶⁸ mice by bone marrow transplantation from *Kit*^{Wsh/Wsh} mice caused significant suppression of intestinal polyposis, with a decreased level of TNF- α expression. It is therefore possible that both macrophages and mast cells are important components of the inflammatory network in the tumor microenvironment.

Bone marrow transplantation from *Rag2*^{-/-} mice did not affect the intestinal tumorigenesis in *Apc*⁴⁴⁶⁸ mice [76]. Consistently, the gastritis phenotype was not altered in the *K19-C2mE Rag2*^{-/-} mouse stomach [78]. These results indicate that lymphocytes are not required for the construction of the inflammatory network and the promotion of tumorigenesis in the gastrointestinal tract. Importantly, however, the adoptive transfer of CD4⁺ CD25⁺ regulatory T cells to *Apc*^{Min} mice dramatically reduced the number of intestinal polyps, with the induction of necrosis of tumor cells [94, 95]. Moreover, such regression of intestinal

polyps was not found when CD4⁺ CD25⁺ cells were prepared from *Il10*^{-/-} mice.

The transfer of CD4⁺ CD25⁺ T cells to *Helicobacter hepaticus*-infected *Rag2*^{-/-} mice, another IBD-related colon cancer model, resulted in suppression of colitis and tumor development, but *Il10*-disrupted CD4⁺ CD25⁺ T cells could not suppress the development of CAC [23, 24]. IL-10 suppresses inflammatory responses, thus indicating that regulatory T cells expressing IL-10 suppress intestinal tumorigenesis by inhibiting the formation of the inflammatory network. Although CD25⁺ Foxp3⁺ T cells are found in *Apc*^{Min} mouse polyp stroma, they no longer express IL-10, and instead switch to the production of IL-17 [95]. It is therefore possible that anti-inflammatory regulatory T cells (Foxp3⁺ IL-10⁺ IL-17⁻) shift to pro-inflammatory T cells (Foxp3⁺ IL-10⁻ IL-17⁺) in polyp tissues [96]. Moreover, the ablation of *Il17a* gene in *Apc*^{Min} mice significantly suppressed the development of intestinal polyps and inflammatory cytokine expression, indicating that T-cell-derived IL-17 plays an important role in intestinal tumorigenesis [97].

Concluding remarks

The development of the inflammatory network in tumor tissues and its possible roles are summarized in Fig. 2. Chronic infection or endogenous ligands derived from tumor cells stimulate the TLRs of epithelial cells, leading to the activation of MyD88. The activation of the epithelial TLR/MyD88 pathway further induces COX-2 expression and PGE₂ production in stromal macrophages through the TNF- α /NF- κ B pathway. Epidemiological studies and genetic experiments have demonstrated that COX-2 and its downstream product, PGE₂, play an important role in gastrointestinal tumorigenesis. NF- κ B is activated by TNF- α in TAMs, which further induces the expression of TNF- α , IL-6, and COX-2. TNF- α in turn stimulates both tumor epithelial cells and stromal cells, activating NF- κ B in these cells, which promotes tumorigenesis. On the other hand, IL-6 activates Stat3 through gp130 in epithelial cells, thus leading to an increase in cell cycling and a decrease of apoptosis. The induction of the COX-2/PGE₂ pathway is important for the development of such an inflammatory tumor microenvironment. When the TNF- α /NF- κ B and/or IL-6/Stat3 pathways are activated beyond a threshold, they can promote tumorigenesis if the COX-2/PGE₂ pathway is inhibited. In the inflammatory microenvironment, not only TAMs, but also mast cells and IL-17-expressing T cells, infiltrate and contribute to tumor development. Therefore, targeting the inflammatory network in tumor tissues by the inhibition of PGE₂, NF- κ B, Stat3, or downstream pathways may provide an effective preventive or therapeutic strategy against gastrointestinal cancer.

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Conflict of interest None.

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