Pathology International

Pathology International 2010; **60**: 599–607 doi:10.1111/j.1440-1827.2010.02567.x

Review Article

Mouse models of gastric tumors: Wnt activation and PGE_2 induction

Hiroko Oshima and Masanobu Oshima

Division of Genetics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

Accumulating evidence has suggested that cooperation of oncogenic activation and the host responses is important for cancer development. In gastric cancer, activation of Wnt signaling appears to be a major oncogenic pathway that causes tumorigenesis. In the chronic gastritis caused by *Helicobacter pylori* infection, cyclooxigenase-2 induces prostaglandin E_2 (PGE_2) biosythesis, which plays an important role in tumorigenesis. We constructed a series of mouse models and investigated the role of each pathway in the gastric tumorigenesis. Wnt activation in gastric epithelial cells suppresses differentiation, and induces development of preneoplastic lesions. On the other hand, induction of the PGE_2 pathway in gastric mucosa induces development of spasmolytic polypeptide-expressing metaplasia (SPEM), which is a possible preneoplastic metaplasia. Importantly, simultaneous activation of Wnt and PGE_2 pathways leads to dysplastic gastric tumor development. Moreover, induction of the PGE_2 pathway also promotes gastric hamartoma development when bone morphogenetic protein (BMP) signaling is suppressed. These results indicate that alteration in the Wnt or BMP signaling impairs epithelial differentiation, and the PGE_2 pathway accelerates tumor formation regardless of the types of oncogenic pathways. We review the phenotypes and gene expression profiles of the respective models, and discuss the cooperation of oncogenic pathways and host responses in gastric tumorigenesis.

Key words: gastric cancer, mouse model, PGE_2, Wnt

Gastric cancer is the second most common cause of cancer-related death worldwide.[1] Infection with *Helicobacter pylori* is associated with gastric cancer development, and the International Agency for Research on Cancer (IARC) classified *Helicobacter pylori* as a class I carcinogen.[2] Accumulating evidence has indicated that chronic inflammatory response associated with infectious disease is a critical component of tumor development.[3] Moreover, it has been shown that infections are responsible for more than 15% of all malignant cancers worldwide, including the association between *H. pylori* infection and gastric cancer.[4] Notably, host genetic variants in cytokine genes are related to responsiveness to *H. pylori* infection and the susceptibility to gastric cancer development.[5–7] Specific polymorphisms of interleukin (IL)-1β, an important inflammatory cytokine and a potent inhibitor of gastric acid secretion, contribute to intestinal-type gastric cancer progression.[8] Polymorphisms in tumor necrosis factor (TNF)-α, IL-1 receptor antagonist, and IL-10 also influence gastric cancer development,[8–10] while polymorphisms in the IL-8 promoter have been linked to diffuse-type gastric cancer.[11] These results suggest that the response of the host cytokine network to *H. pylori* infection is an important factor for gastric cancer development.

On the other hand, several somatic alterations that activate oncogenic pathways have been identified in human gastric cancer. For example, allelic loss or mutations in p53 are detected in 60% or 30–50% of gastric cancers, respectively,[12] while mutations in the β-catenin gene is detected in 30% of the Wnt-activated subgroup of gastric cancer.[13] TGF-β type II receptor gene is recognized as a tumor suppressor, and mutations have been found in gastric cancer associated with microsatellite instability (MSI).[14,15] Moreover, about 15% of gastric cancers show expression of both epidermal growth factor (EGF) and EGF receptor (EGFR), suggesting activation of the EGFR signaling pathway.[16] Taken together, these results indicate that both infection-associated inflammatory responses and oncogenic activation by genetic alterations are required for gastric cancer development.

To date, many genetic mouse models have recapitulated some of the developmental stages associated with intestinal-type gastric cancer, such as gastritis, atrophy, mucous cell metaplasia, dysplasia, and invasion.[17] These models are useful for examining the phenotypic changes caused by individual genetic alterations. In addition to these models, we

Correspondence: Masanobu Oshima, DVM, PhD, Division of Genetics, Cancer Research Institute, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. Email: oshimam@kenroku.kanazawa-u.ac.jp

Received 14 Apr 2010. Accepted for publication 10 May 2010.

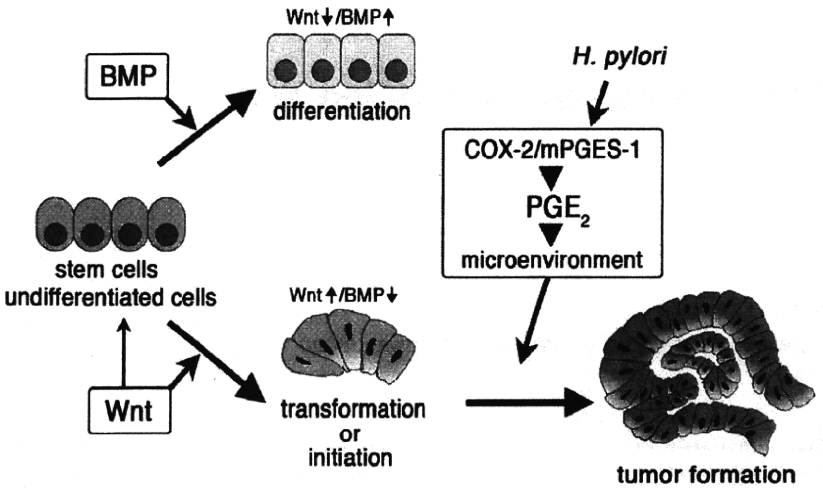

Figure 1 Schematic presentation of the connection of Wnt, BMP, and COX-2/PGE_2 signaling pathways in gastric tumorigenesis. 'Wnt activation' or 'BMP suppression' causes suppression of epithelial differentiation, which leads to transformation of epithelial cells. On the other hand, *Helicobacter pylori* infection induces expression of COX-2 and mPGES-1, resulting in activation of PGE_2 signaling pathway. PGE_2 pathway accelerates proliferation of the transformed cells through formation of tumor microenvironment.

constructed a series of transgenic mouse models to investigate the role of oncogenic and inflammatory pathways, such as Wnt, BMP, and PGE_2 signaling, in gastric epithelial differentiation, inflammation, and tumor development.

ACTIVATION OF WNT SIGNALING IN GASTRIC MUCOSA

Wnt activation in human gastric cancer

Canonical Wnt signaling (Wnt/β-catenin signaling) is a critical pathway in the regulation of development as well as in tumorigenesis.[18] When Wnt signaling is in a resting state, cytoplasmic β-catenin is phosphorylated by GSK-3β within a complex containing APC and Axin, resulting in the degradation of β-catenin through the ubiquitin proteasome pathway.[19] When the pathway is activated, the binding of Wnt ligands to Frizzled receptors leads to the suppression of the phosphorylation of β-catenin, resulting in the to stabilization and nuclear translocation of β-catenin. β-Catenin then interacts with T-cell factor/lymphocyte enhancer factor (TCF/LEF) to induce transcription of Wnt target genes. In the normal intestine, Wnt signaling is important for maintaining the stem cell characteristics and undifferentiated status of the epithelial cells, whereas Wnt signaling is suppressed in the differentiated epithelia (Fig. 1)[19–21] Mutations in the *APC* or β-catenin genes constitutively activate Wnt signaling, which causes tumor development in the intestine.[22,23] In the gastric mucosa, epithelial cells expressing Lgr5, which is a target of Wnt signaling, show stem cell phenotypes, confirming the role of Wnt pathway in normal gastric stem cells.[24] Moreover, nuclear accumulation of β-catenin, a hallmark of Wnt signaling activation, is found in 30–50% of gastric cancers (Fig. 2),[13,25–27] and mutations in the β-catenin gene have also been detected,[13,25,28,29] which suggest that activation of Wnt signaling is a major cause of gastric cancer development. However, *APC* gene mutations are not common in gastric cancer, and β-catenin mutations are present in fewer than 30% of the Wnt-activated gastric cancers.[25] Accordingly, it is possible that other mechanism(s) may also activate Wnt signaling in gastric cancer. For example, it has been reported in gastric cancer cells that downregulation of E-cadherin is associated with β-catenin accumulation,[30] somatic mutations in the ubiquitin ligase β-TrCP causes stabilization of β-catenin,[31] and the expression of the *SFRP1*, *2* and *5* genes encoding the secreted endogenous antagonist of the Wnt ligands is silenced by promoter methylation in gastric cancer cells.[32] All of these alterations contribute to activation of Wnt signaling.

Gastric preneoplastic lesions in *K19-Wnt1* transgenic mice

To examine the role of Wnt signaling in gastric tumorigenesis, we constructed *K19-Wnt1* transgenic mice that express *Wnt1*, one of the canonical Wnt ligands.[27] The *K19* gene promoter was used to express *Wnt1* in gastric epithelial cells, including undifferentiated isthmal cells.[33,34] The number of undifferentiated epithelial cells that express trefoil factor 2 (TFF2) increases significantly in the *K19-Wnt1* mouse glandular stomach, indicating that Wnt signaling functions to maintain the undifferentiated status of the gastric epithelial cells (Fig. 3a,b). Notably, aberrant cryptic foci were found on the mucosal surface of *K19-Wnt1* mice, which consist of dysplastic epithelial cells with irregular branching, and increased cell proliferation and β-catenin accumulation were also detected (Fig. 3c–g). We thus diagnosed these foci as preneoplastic lesions. However, gastric tumors do not

develop in *K19-Wnt1* mice, indicating that activation of Wnt signaling alone is not sufficient for gastric tumor formation. Importantly, macrophages were infiltrated into the preneoplastic lesions, whereas tissue macrophages were sparsely scattered in the normal mucosa of the same mice. It is possible that local inflammatory responses caused by spontaneous physical insult or infection might promote proliferation of Wnt-activated dysplastic cells, resulting in formation of the preneoplastic lesions.

INDUCTION OF PGE_2 PATHWAY IN GASTRIC MUCOSA

Induction of COX-2/PGE_2 pathway in human gastric cancer

Epidemiological studies indicate that the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a decreased incidence of gastric cancer.[35–38] NSAIDs inhibit the enzymatic activity of cyclooxygenases (COXs), which are rate-limiting enzymes for prostaglandin biosynthesis. COX enzymes catalyze synthesis of prostaglandin (PG)H_2, which is subsequently converted to various prostanoids, including PGE_2, by tissue-specific converting enzymes. There are two COX isozymes, COX-1 and COX-2, which share a high degree of structural and enzymatic homology. COX-1 is constitutively expressed in most tissues and is considered to be responsible for maintaining physiological levels of prostaglandin biosynthesis,[39] while COX-2 expression is induced in inflammation and in tumor tissues by cytokines and growth factors.[40–42] Induction of COX-2 is found in approximately 70% of gastric cancer, predominantly in intestinal-type gastric cancer, whereas COX-1 expression is not elevated.[43–45] Moreover, the level of COX-2 expression in gastric cancer correlates with the tumor size, depth of invasion and lymph-node metastasis.[46–48] Microsomal PGE synthase-1 (mPGES-1) is an inducible enzyme that converts PGE_2 from PGH_2, and is functionally coupled with COX-2.[49] Simultaneous induction of COX-2 and mPGES-1 has been observed in a variety of cancers, including gastric cancer, suggesting that the inflammatory PGE_2 pathway is induced in these tumors (Fig. 1).[50–53] Consistently, the PGE_2 level is found to be significantly increased in gastric cancer,[47] and the COX-2 and PGE_2 level is associated with the *H. pylori* infection status,[54,55] indicating that *H. pylori* infection causes induction of the PGE_2 pathway (Fig. 1). These results suggest that the COX-2/PGE_2 pathway plays a key role in *H. pylori* infection-associated inflammation in gastric cancer development.

Suppression of gastric cancer by COX-2 inhibition in animal models

Suppression of gastric tumorigenesis by treatment with COX-2 selective inhibitors (COXIBs) has been examined in several animal model experiments. Growth of gastric cancer cell xenografts was inhibited by treatment with COXIBs in immunodeficient mice.[56,57] Rat gastric cancer induced by carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and mouse gastric tumors induced by *H. pylori* infection or a combination of *H. pylori* with *N*-methyl-*N*-nitrosourea (MNU) were suppressed by treatment with NSAIDs or COXIBs.[58–60] A significant decrease in the PGE_2 level and substantial induction of apoptosis were also found in the tumors of mice treated with NSAIDs or COXIBs. *H. pylori* infection of Mongolian gerbils is an established model to study gastric tumorigenesis by *H. pylori*. Treatment of the *H. pylori*-infected Mongolian gerbils with COXIBs suppressed the development of gastric cancer, as well as intestinal metaplasia.,[61,62] These animal studies indicate that the COX-2 pathway plays a key role in gastric tumorigenesis.

Gastric metaplasia in *K19-C2mE* transgenic mice

To examine the effect of PGE_2 pathway activation in the gastric mucosa, we constructed another transgenic mouse model, *K19-C2mE*, which expresses both COX-2 and mPGES-1 simultaneously in gastric epithelial cells.[34] *K19-C2mE* mice developed hyperplasia in the gastric mucosa, which was suppressed by treatment of the mice with a COX-2 selective inhibitor, NS-398 or meloxicam (Fig. 4a,b).[34,63] Histologically, the major cell type involved in the hyperplasia were mucous cells, which was similar to the findings in the spasmolytic polypeptide/TFF2-expressing metaplasia or SPEM (Fig. 4c,d).[34,64] The SPEM is characterized by the presence of TFF2 immunoreactive cells, which is morphologically similar to those of Brunner's gland. Since SPEM was associated with greater than 90% of the resected gastric cancers analyzed in three studies,[64–66] it is suggested that SPEM is a putative preneoplastic metaplasia in the stomach.[67] In other animal models, SPEM development was also found in the stomach of *Helicobacter*-infected mice, gastrin gene knockout mice, and in Stat3-activating gp130 mutant mice.[68–70] Notably, SPEM in these mouse models was accompanied by inflammatory responses. Importantly, disruption of the TNF-α gene in *K19-C2mE* mice results in the suppression of inflammatory responses and SPEM development, suggesting that TNF-α-associated inflammation plays an essential role in SPEM formation (Fig. 4e–g).[63] In contrast, *Rag2* gene disruption did not suppress SPEM formation in the *K19-C2mE* mice, indicating that acquired immune responses are not involved in SPEM development.

Treatment with MNU to the *H. pylori*-infected mice led to the development of gastric tumors. Notably, multiplicity of gastric tumors induced by *H. pylori* infection and MNU treatment was significantly higher in *K19-C2mE* mice compared

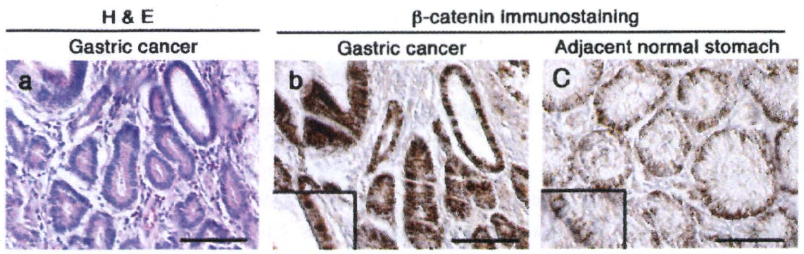

Figure 2 Nuclear localization of β-catenin in human gastric cancer. (**a**) Representative histological sections of gastric cancer (H&E). (**b**) Immunostaining for unphosphorylated (active) β-catenin in the adjoining section of (**a**) showing nuclear β-catenin accumulation. (**c**) Total β-catenin immunostaining in the normal stomach of the same patient showing β-catenin localization on the cell membrane. Scale bars, 200 μm. (Reproduced from Oshima *et al. Gastroenterology* 2006; 131: 1086–1095 with permission from Elsevier).

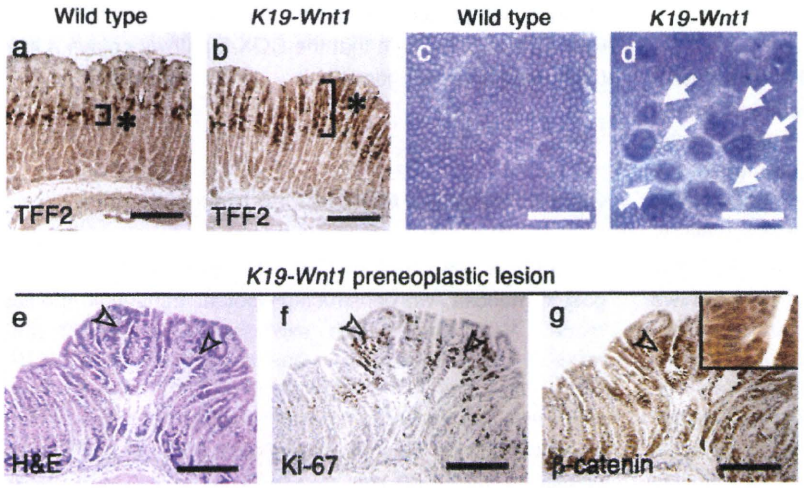

Figure 3 Gastric preneoplastic lesions in the *K19-Wnt1* mouse stomach. (**a,b**) TFF2-expressing undifferentiated epithelial cells (asterisks) in the glandular stomach of wild-type (**a**) and *K19-Wnt1* mice (**b**). Note that the number of TFF2 positive cells was increased in the *K19-Wnt1* mouse. Scale bars, 200 μm. (**c,d**) Toluisdine blue staining of the whole glandular stomach of wild-type (**c**) and *K19-Wnt1* mice (**d**). Arrows in (**d**) indicate preneoplastic lesions. Scale bars in (**c,d**), 0.5 mm. (**e–g**) Histology of preneoplastic lesion (H&E) (**e**), and Ki-67 staining (**f**) and β-catenin immunostaining (**g**) of serial sections. Arrowheads indicate dysplastic epithelial cells. Scale bars in (**e–g**), 100 μm. (Reproduced from Oshima *et al. Gastroenterology* 2006; 131: 1086–1095 with permission from Elsevier).

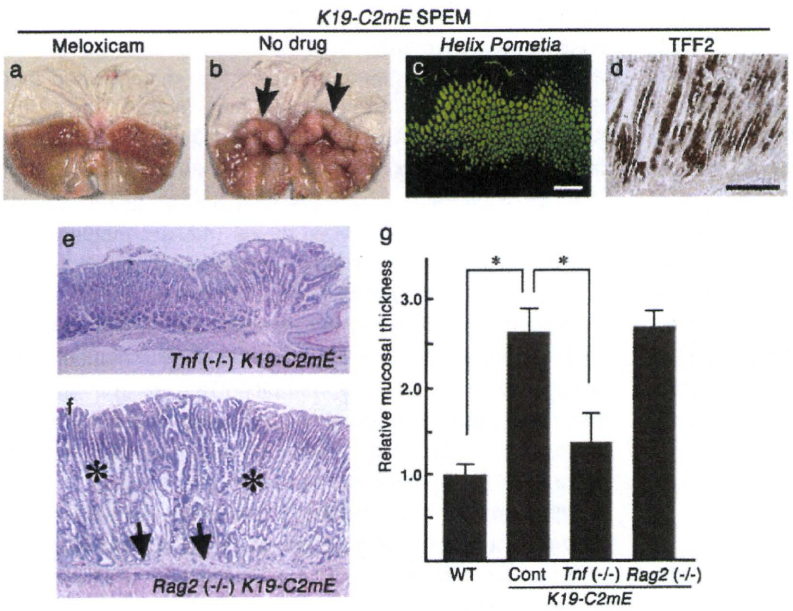

Figure 4 SPEM development in the *K19-C2mE* mouse stomach. (**a,b**) Representative photographs of the gastric mucosa of a meloxicam-treated *K19-C2mE* mouse (**a**) and a no-drug control *K19-C2mE* mouse (**b**). Arrows in (**b**) indicate hyperplastic lesions. (**c,d**) *Helix pometia* lectin staining (**c**) and TFF2 *in situ* hybridization (**d**) of *K19-C2mE* mouse stomach showing expansion of mucous cells expressing TFF2. Scale bars in (**c,d**), 200 μm. (**e–g**) Suppression of SPEM development in *K19-C2mE* mice by TNF-α gene disruption (**e**) but not by Rag2 knockout (**f**). Asterisks and arrows in (**f**) indicate SPEM and submucosal inflammatory infiltration, respectively. Bar graph indicates the mean mucosal thickness of the respective genotypes of mice (**g**). Asterisks, $P < 0.05$. (Reproduced from Oshima M *et al. Cancer Res* 2005; 65: 9147–51 with permission from American Association for Cancer Research, and Oshima H *et al. EMBO J* 2004; 23: 1669–1678 with permission from Nature publishing group).

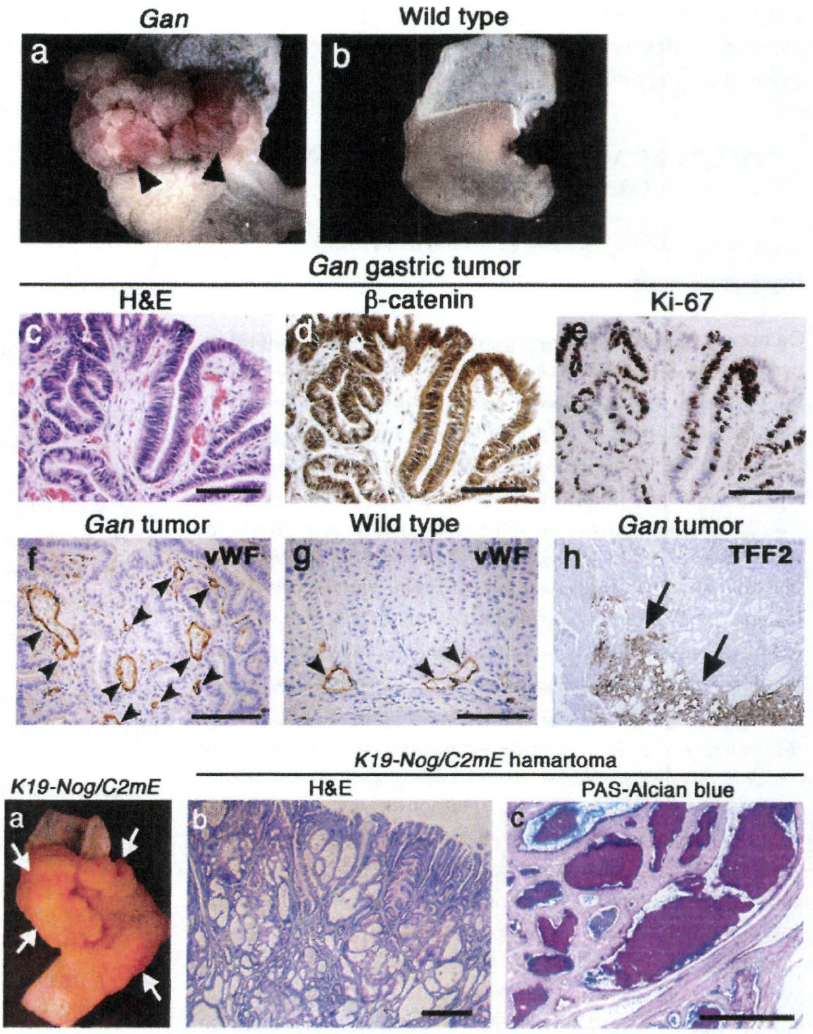

Figure 5 Gastric dysplastic tumors developed in the *K19-Wnt1/C2mE* (*Gan*) mice. (**a**,**b**) Representative photographs of the gastric mucosa of a *Gan* mouse (**a**) and a wild-type littermate mouse (**b**). Arrowheads indicate gastric tumors. (**c–e**) Histology of *Gan* mouse gastric tumors (H&E) (**c**), and β-catenin immunostaining (**d**) and Ki-67 staining (**e**) of the serial sections. Scale bars in (**c–e**), 100 μm. (**f**,**g**) Immunostaining for capillary vessels (arrowheads) using anti-vWF antibody in a *Gan* mouse tumor (**f**) and a wild-type mouse stomach (**g**). Note that the number of vessels increased significantly in *Gan* mouse tumor. Scale bars in (**f**,**g**), 50 μm. (**h**) *In situ* hybridization for TFF2 to detect SPEM (arrows) adjacent to the dysplastic tumor area in the *Gan* mouse stomach. (Reproduced from Oshima *et al. Gastroenterology* 2006; 131: 1086–1095 with permission from Elsevier, and Guo *et al. J Biol Chem* 2008; 283: 19864–71).

Figure 6 Gastric hamartomas developed in the *K19-Nog/C2mE* mice. (**a**) Representative photograph of a *K19-Nog/C2mE* mouse gastric tumor (arrows). (**b**) Histology of a *K19-Nog/C2mE* gastric tumor showing a hamartomatous cystic structure (H&E). (**c**) PAS-Alcian blue staining of a *K19-Nog/C2mE* tumor showing a mucin-containing cystic structure. Scale bars in (**b**,**c**), 200 μm and 100 μm, respectively. (Reproduced from Oshima H *et al. Cancer Res* 2009; 69: 2729–2733 with permission from American Association for Cancer Research).

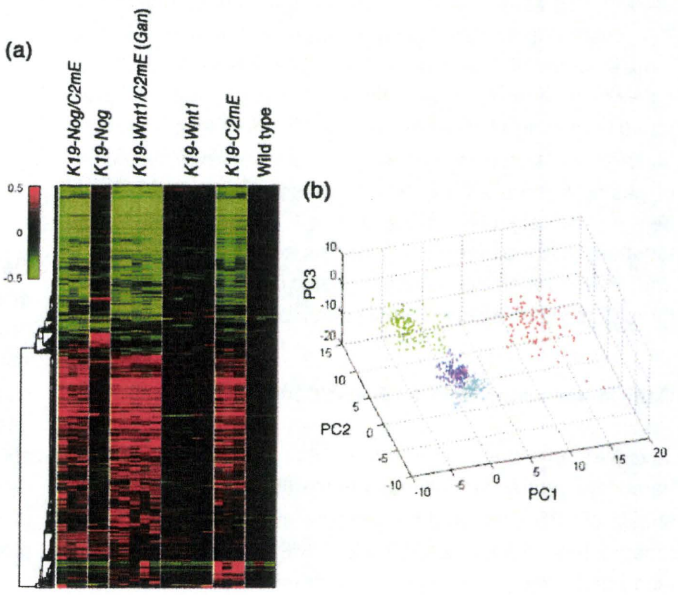

Figure 7 Genome-scale expression pattern of the respective genotype mouse models. (**a**) Clustered in rows are 5440 probe sets selected by genes whose expression levels were over 2-fold greater than the average of wild-type mice. Genotypes of mice are shown on the top. The red-green color scale indicates the log10 ratio of the average of wild-type samples. Red indicates 'upregulated', whereas green indicates 'downregulated'. (**b**) The overall gene expression of human gastric (blue), colon (light blue), breast (green), and lung (red) cancers, and *K19-C2mE*, *K19-Wnt1/C2mE* (*Gan*), *K19-Nog/C2mE* mouse stomach (magenda). The 3D figure was plotted by principal component 1 to 3 calculated using 1925 genes which were altered by more than 2-fold in more than 50 samples. (Reproduced from Itadani H *et al. BMC Genomics* 2009; 10: 615).

with wild-type mice.[71] These results suggest that PGE_2-induced SPEM is a precursor for chemical carcinogen-induced gastric tumors.

ONCOGENIC ACTIVATION AND PGE_2 PATHWAY IN GASTRIC TUMORIGENESIS

Gastric dysplastic tumors in *K19-Wnt1/C2mE* transgenic mice

Compound *K19-Wnt1/C2mE* transgenic mice were generated by crossing *K19-Wnt1* mice and *K19-C2mE* mice, in which both Wnt and PGE_2 pathways are activated simultaneously in the gastric mucosa.[27] *K19-Wnt1/C2mE* mice develop large gastric tumors in the glandular stomach (Fig. 5a,b), while no such tumors are found in either simple *K19-Wnt1* or *K19-C2mE* transgenic mice. Histologically, gastric tumors in the *K19-Wnt1/C2mE* mice (hereafter *Gan* mice for **ga**stric **n**eoplasia) consist of dysplastic epithelial cells with nuclear stratification and irregularly branched tubules (Fig. 5c). Tumor epithelial cells showed increased nuclear accumulation of β-catenin and increased Ki-67 labeling, indicating promotion of Wnt activity and an increased proliferation of tumor cells, respectively (Fig. 5e). Increased capillary vessels were also found in the tumor stroma, which was caused by enhanced angiogenesis through the tumor-stroma interaction (Fig. 5f,g).[72] Interestingly, part of the activated stromal fibroblasts in *Gan* mouse tumors were derived from bone marrow, thus suggesting that the bone marrow cells contribute to the formation of the gastric cancer microenvironment.[72]

Notably, TFF2-expressing SPEM was found adjacent to the dysplastic tumor tissue,[27] showing a similar histology to human gastric cancer (Fig. 5h). *K19-C2mE* mice start to develop SPEM lesions at 5 weeks of age, and the number of TFF-positive metaplastic cells increases with age. In the *Gan* mouse stomach, the same SPEM phenotype is found at 5 weeks of age, however, dysplastic tumor cells are also found beginning at 10 weeks of age. The number of dysplastic tumor cells then increases with age, leading to formation of gastric tumors around 20–30 weeks of age, with SPEM being found adjacent to the tumors. These results, taken together, indicate that the simultaneous activation of the Wnt and PGE_2 pathways causes gastric tumor development through the metaplasia (SPEM)-carcinoma sequence.

Gastric hamartomas in *K19-Nog/C2mE* transgenic mice

Juvenile polyposis syndrome (JPS) is characterized by hereditary gastrointestinal hamartomatous polyposis,[73] and a subset of JPS is caused by germline mutations in the BMP receptor type IA gene (*BMPR1A*).[74] BMP signaling through its type I and II receptors leads to the phosphorylation of Smad 1,5, and 8, resulting in formation of a complex with Smad4, which induces transcription of target genes.[75] BMP signaling inhibits epithelial cell proliferation and promotes differentiation (Fig. 1), and suppression of BMP signaling in the mouse intestine results in JPS-type hamartomatous polyp development,[76–78] elongated villi and crypt fission.[79] Accordingly, it is possible that BMP suppression results in hamartoma formation by impairment of epithelial differentiation. Since the cancer risk in JPS patients increases in the gastrointestinal tract,[80,81] BMP suppression may also contribute to gastric cancer development.

To examine the effect of BMP suppression in gastric epithelial cells, we next constructed *K19-Nog* mice that express noggin, an endogenous BMP antagonist, in the gastric epithelial cells.[82] Although BMP signaling was suppressed in the stomach, *K19-Nog* mice do not develop any gastric lesions, and the histology of the gastric mucosa was normal. To examine the effect of cooperation of BMP suppression and PGE_2 induction, *K19-Nog* mice and *K19-C2mE* mice were crossed to construct compound *K19-Nog/C2mE* mice, in which BMP signaling is suppressed and the PGE_2 pathway is induced in the gastric mucosa. Importantly, the *K19-Nog/C2mE* mice developed large tumors in the glandular stomach (Fig. 6a), suggesting that induction of the PGE_2 pathway is required for tumor formation in the BMP-suppressed gastric mucosa. Histologically, *K19-Nog/C2mE* mouse gastric tumors are not dysplastic, but consist of irregular branching of the epithelial cell layers, combined with the formation of dilated cysts filled with mucin (Fig. 6b,c). Such histological characteristics are distinct from the dysplastic gastric tumors of *Gan* mice (Fig. 5c), but are typical of the hamartomas of JPS patients.[80,81,83] These results indicate that the suppression of BMP signaling associated with PGE_2 induction causes gastric hamartoma development.

These results of compound mutant mice indicate that the type of genetic alteration, such as Wnt activation or BMP suppression, determines the histological type of tumors, such as adenocarcinoma or hamrtoma.[84] On the other hand, induction of the PGE_2 pathway promotes gastric tumor formation regardless of the genetic or histological types.

Gene expression profiles of mouse models and human gastric cancer

Gan mice and *K19-Nog/C2mE* mice develop gastric tumors caused by genetic alterations similar to those found in human gastric cancer and hamartomas, respectively. However, it is still important to compare gene expression profiles of mouse tumors with those of human cancer or hamartomas in order to examine whether these models really recapitulate human gastric tumors. We have measured mRNA expression levels using the Affymetrix GeneChip system, which includes

21 066 Entrez genes and 5324 other sequences.[85] Genome-scale overview of the microarray data revealed that expression changes in the three models, *K19-C2mE* and *K19-Wnt1/C2mE* (*Gan*), and *K19-Nog/C2mE* mice are quite similar, whereas over-expression of *Wnt1* or *Noggin* in *K19-Wnt1* or *K19-Nog* mice, respectively, showed expression changes in a small portion of genes (Fig. 7a). These results suggest that most of the expression changes in *Gan* gastric tumors and *K19-Nog/C2mE* hamartomas are caused by induction of PGE_2 pathway, rather than by Wnt activation or BMP suppression. In other words, a small number of genes that are upregulated or downregulated by Wnt activation or BMP suppression are important for determining the tumor phenotype.

Gene expression signatures of human gastric cancer[86] and breast cancer[87] retrieved from the Stanford Microarray Database,[88] colon cancer[89] from the NCBI GEO (accession GSE5206), and lung tumors[90] retrieved from the United States National Cancer Institute website[91] can be plotted to distinct areas in a 3D figure based on the calculations of principal component analysis using the selected genes (Fig. 7b). Importantly, expression signatures of *Gan*, *K19-C2mE*, and *K19-Nog/C2mE* mice are clustered in a similar area as that of human gastric cancer, but not to the same area as cancers of other organs. These results indicate that *Gan* mouse tumors recapitulate human gastric cancer from the molecular etiology to histology and gene expression profiles. It is also possible that most of the changes in gene expression in human gastric cancer are attributable to *H. pylori* infection-associated inflammatory responses. Taken together, these results indicate that the *Gan* mouse model is a useful tool for studying the effects of oncogenic activation and inflammatory responses in human gastric cancer development and the evaluation of anti-gastric cancer drugs.

CONCLUSION

Wnt signaling functions to maintain the undifferentiated status of gastric epithelial cells. On one hand, activation of Wnt signaling by genetic or epigenetic alteration causes development of preneoplastic lesions. On the other hand, *H. pylori* infection induces expression of COX-2 and mPGES-1, resulting in induction of PGE_2 biosynthesis. Induction of the COX-2/PGE_2 pathway is responsible for SPEM development, which is a possible preneoplastic metaplasia of gastric cancer. Importantly, simultaneous induction of Wnt and PGE_2 pathways causes development of dysplastic gastric tumors (Fig. 1). The results of mouse model studies reported herein suggest that oncogenic activation, such as activation of Wnt signaling by genetic or epigenetic alterations, triggers tumor initiation. However, initiated epithelial cells cannot continue proliferation in the non-inflamed gastric mucosa, thus indicating that Wnt signaling alone is not sufficient for tumor development. In contrast, if the oncogenic pathway is activated in the *H. pylori*-infected (and thus inflamed) stomach, the initiated cells proliferate to develop gastric cancer. Considering the relatively low frequency of somatic oncogenic activation, this hypothesis is consistent with the epidemiology of gastric cancer, in that only a small minority of the *H. pylori*-infected population develops gastric cancer, although *H. pylori* infection is an important risk factor for gastric cancer. It is possible that a similar mechanism underlies the development of hamartoma in the BMP-suppressed gastric epithelial cells (Fig. 1). Further studies using these mouse models will be useful for elucidating the role of oncogenic activation and host responses in gastric tumorigenesis at the molecular level.

ACKNOWLEDGMENTS

We thank Hiraku Itadani and Hidehito Kotani for microarray analyses, and Manami Watanabe for her technical contributions to the gastric tumor mouse model studies.

REFERENCES

1 Correa P. *Helicobacter pylori* infection and gastric cancer. *Cancer Epidemiol Biomarkers Prev* 2003; **12**: 238s–241s.
2 IARC. *Monographs on the Evaluation of Carcinogenic Risks to Humans. Shistosomes, Liver Flukes and Helicobacter Pylori*, Vol. 61. Lyon: International Agency for Research on Cancer, 1994; 177–240.
3 Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860–67.
4 Kuper H, Adami HO, Trichopoulos D. Infections as a major preventable cause of human cancer. *J Intern Med* 2000; **248**: 171–83.
5 Gonzalez CA, Sala N, Capella G. Genetic susceptibility and gastric cancer risk. *Int J Cancer* 2002; **100**: 249–60.
6 Hamajima N, Naito M, Kondo T, Goto Y. Genetic factors involved in the development of *Helicobacter pylori*-related gastric cancer. *Cancer Sci* 2006; **97**: 1129–38.
7 El-Omar EM. Role of host genetic susceptibility in the pathogenesis of gastric cancer. In: Wang TC, Fox JG, Giraud AS, eds. *The Biology of Gastric Cancer*. New York: Springer, 2009; 235–50.
8 El-Omar EM, Carrington M, Chow WH *et al.* Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398–402.
9 Machado JC, Figueiredo C, Canedo P *et al.* A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. *Gastroenterology* 2003; **125**: 364–71.
10 El-Omar EM, Rabkin CS, Gammon MD *et al.* Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* 2003; **124**: 1193–201.
11 Lee WP, Tai DI, Lan KH *et al.* The -251T allele of the interleukin-8 promoter is associated with increased risk of gastric carcinoma featuring diffuse-type histopathology in Chinese population. *Clin Cancer Res* 2005; **11**: 6431–41.
12 Hollstein M, Shomer B, Greenblatt M *et al.* Somatic point mutations in the p53 gene of human tumors and cell lines: Updated compilation. *Nucleic Acids Res* 1996; **24**: 141–6.

13 Clements WM, Wang J, Sarnaik A *et al.* β-catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. *Cancer Res* 2002; **62**: 3503–6.
14 Park K, Kim SJ, Bang YJ *et al.* Genetic changes in the transforming growth factor β (TGF-β) type II receptor gene in human gastric cancer cells: Correlation with sensitivity to growth inhibition by TGF-β. *Proc Natl Acad Sci USA* 1994; **91**: 8772–6.
15 Myeroff LL, Parsons R, Kim SJ. A transforming growth factor β receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. *Cancer Res* 1995; **55**: 5545–7.
16 Tokunaga A, Onda M, Okuda T *et al.* Clinical significance of epidermal growth factor (EGF), EGF receptor, and c-erbB-2 in human gastric cancer. *Cancer* 1995; **75** (6 Suppl): 1418–25.
17 Giraud AS, Judd LM. Genetic models of gastric cancer. In: Wang TC, Fox JG, Giraud AS, eds. *The Biology of Gastric Cancer*. New York: Springer, 2009; 483–512.
18 Taketo MM. Wnt signaling and gastrointestinal tumorigenesis in mouse models. *Oncogene* 2006; **25**: 7522–30.
19 Gregorieff A, Clevers H. Wnt signaling in the intestinal epithelium: From endoderm to cancer. *Genes Dev* 2005; **19**: 877–90.
20 Korinek V, Barker N, Moerer P *et al.* Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet* 1998; **19**: 379–83.
21 van de Wetering M, Sancho E, Verweij C *et al.* The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002; **111**: 241–50.
22 Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C, Taketo M. Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. *Proc Natl Acad Sci USA* 1995; **92**: 4482–6.
23 Harada N, Tamai Y, Ishikawa T *et al.* Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. *EMBO J* 1999; **18**: 5931–42.
24 Barker N, Huch M, Kujala P *et al.* $Lgr5^{+VE}$ stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 2010; **6**: 25–36.
25 Woo DK, Kim HS, Lee HS, Kang YH, Yang HK, Kim WH. Altered expression and mutation of β-catenin gene in gastric carcinomas and cell lines. *Int J Cancer* 2001; **95**: 108–13.
26 Cheng XX, Wang ZC, Chen XY *et al.* Correlation of Wnt-2 expression and β-catenin intracellular accumulation in Chinese gastric cancers: Relevance with tumour dissemination. *Cancer Lett* 2005; **223**: 339–47.
27 Oshima H, Matsunaga A, Fujimura T, Tsukamoto T, Taketo MM, Oshima M. Carcinogenesis in mouse stomach by simultaneous activation of the Wnt signaling and prostaglandin E_2 pathway. *Gastroenterology* 2006; **131**: 1086–95.
28 Park WS, Oh RR, Park YJ *et al.* Frequent somatic mutations of the β-catenin gene in intestinal-type gastric cancer. *Cancer Res* 1999; **59**: 4257–60.
29 Ebert MP, Fei G, Kahmann S *et al.* Increased β-catenin mRNA levels and mutational alterations of the *APC* and β-catenin gene are present in intestinal-type gastric cancer. *Carcinogenesis* 2002; **23**: 87–91.
30 Cheng XX, Wang ZC, Chen XY *et al.* Frequent loss of membranous E-cadherin in gastric cancers: A cross-talk with Wnt in determining the fate of β-catenin. *Clin Exp Metastasis* 2005; **22**: 85–93.
31 Kim CJ, Song JH, Cho YG *et al.* Somatic mutations of the β-*TrCP* gene in gastric cancer. *APMIS* 2007; **115**: 127–33.
32 Nojima M, Suzuki H, Toyota M *et al.* Frequent epigenetic inactivation of *SFRP* genes and constitutive activation of Wnt signaling in gastric cancer. *Oncogene* 2007; **26**: 4699–713.
33 Brembeck FH, Moffett J, Wang TC, Rustgi AK. The keratin 19 promoter is potent for cell-specific targeting of genes in transgenic mice. *Gastroenterology* 2001; **120**: 1720–28.
34 Oshima H, Oshima M, Inaba K, Taketo MM. Hyperplastic gastric tumors induced by activated macrophages in COX-2/mPGES-1 transgenic mice. *EMBO J* 2004; **23**: 1669–78.
35 Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath CW Jr. Aspirin use and risk of fatal cancer. *Cancer Res* 1993; **53**: 1322–7.
36 Zaridze D, Borisova E, Maximovitch D, Chkhikvadze V. Aspirin protects against gastric cancer: Results of a case-control study from Moscow, Russia. *Int J Cancer* 1999; **82**: 473–6.
37 Langman MJ, Cheng KK, Gilman EA, Lancashire RJ. Effect of anti-inflammatory drugs on overall risk of common cancer: Case-control study in general practice research database. *BMJ* 2000; **320**: 1642–6.
38 Akre K, Ekstrom AM, Signorello LB, Hansson LE, Nyren O. Aspirin and risk for gastric cancer: A population-based case-control study in Sweden. *Br J Cancer* 2001; **84**: 965–8.
39 Dewitt DL, Smith WL. Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc Natl Acad Sci USA* 1998; **85**: 1412–16.
40 Xie WL, Chipman JG, Robertson DL, Erikson RL, Simmons DL. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci USA* 1991; **88**: 2692–6.
41 Fletcher BS, Kujubu DA, Perrin DM, Herschman HR. Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. *J Biol Chem* 1992; **267**: 4338–44.
42 Hla T, Neilson K. Human cylooxygenase-2 cDNA. *Proc Natl Acad Sci USA* 1992; **89**: 7384–8.
43 Ristimaki A, Honkanen N, Jankala H, Sipponen P, Harkonen M. Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res* 1997; **57**: 1276–80.
44 Saukkonen K, Rintahaka J, Sivula A *et al.* Cyclooxygenase-2 and gastric carcinogenesis. *APMIS* 2003; **111**: 915–25.
45 Saukkonen K, Nieminen O, van Rees B *et al.* Expression of cyclooxygenase-2 in dysplasia of the stomach in intestinal-type gastric adenocarcinoma. *Clin Cancer Res* 2001; **7**: 1923–31.
46 Murata H, Kawano S, Tsuji S *et al.* Cyclooxygenase-2 overexpression enhances lymphatic invasion and metastasis in human gastric carcinoma. *Am J Gastroenterol* 1999; **94**: 451–5.
47 Uefuji K, Ichikura T, Mochizuki H. Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer. *Clin Cancer Res* 2000; **6**: 135–8.
48 Ohno R, Yoshinaga K, Fujita T *et al.* Depth of invasion parallels increased cyclooxygenase-2 levels in patients with gastric carcinoma. *Cancer* 2001; **91**: 1876–81.
49 Murakami M, Naraba H, Tanioka T *et al.* Regulation of prostaglandin E_2 biosynthesis by inducible membrane-associated prostaglandin E_2 synthase that acts in concert with cyclooxygenase-2. *J Biol Chem* 2000; **275**: 32783–92.
50 Yoshimatsu K, Golijanin D, Paty PB *et al.* Inducible microsomal prostaglandin E synthase is overexpressed in colorectal adenomas and cancer. *Clin Cancer Res* 2001; **7**: 3971–6.
51 Takeda H, Miyoshi H, Tamai Y, Oshima M, Taketo MM. Simultaneous expression of COX-2 and mPGES-1 in mouse gastrointestinal hamartomas. *Br J Cancer* 2004; **90**: 701–4.
52 van Rees BP, Sivula A, Thoren S *et al.* Expression of microsomal prostaglandin E synthase-1 in intestinal type gastric adenocarcinoma and in gastric cancer cell lines. *Int J Cancer* 2003; **107**: 551–6.

53 Jang TJ. Expression of proteins related to prostaglandin E_2 biosynthesis is increased in human gastric cancer and during gastric carcinogenesis. *Virchows Arch* 2004; **445**: 564–71.
54 Al-Marhoon MS, Nunn S, Soames RW. CagA+ *Helicobacter pylori* induces greater levels of prostaglandin E2 than cagA- strains. *Prostaglandins Other Lipid Mediat* 2004; **73**: 181–9.
55 Sun WH, Yu Q, Shen H *et al.* Roles of *Helicobacter pylori* infection and cyclooxygenase-2 expression in gastric carcinogenesis. *World J Gastroenterol* 2004; **10**: 2809–13.
56 Sawaoka H, Kawano S, Tsuji S *et al.* Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. *Am J Physiol* 1998; **274**: G1061–7.
57 Tang C, Liu C, Zhou X, Wang C. Enhanced inhibitive effects of combination of rofecoxib and octreotide on the growth of human gastric cancer. *Int J Cancer* 2004; **112**: 470–74.
58 Hu PJ, Yu J, Zeng ZR *et al.* Chemoprevention of gastric cancer by celecoxib in rats. *Gut* 2004; **53**: 195–200.
59 Nam KT, Hahm KB, Oh SY *et al.* The selective cyclooxygenase-2 inhibitor nimesulide prevents *Helicobacter pylori*-associated gastric cancer development in a mouse model. *Clin Cancer Res* 2004; **10**: 8105–13.
60 Xiao F, Furuta T, Takashima M, Shirai N, Hanai H. Involvement of cyclooxygenase-2 in hyperplastic gastritis induced by *Helicobacter pylori* infection in C57BL/6 mice. *Aliment Pharmacol Ther* 2001; **15**: 875–86.
61 Magari H, Shimizu Y, Inada K *et al.* Inhibitory effects of etodolac, a selective cyclooxygenase-2 inhibitor, on stomach carcinogenesis in *Helicobacter pylori*-infected mongolian gerbils. *Biochem Biophys Res Commun* 2005; **334**: 606–12.
62 Futagami S, Suzuki K, Hiratsuka T *et al.* Celecoxib inhibits Cdx2 expression and prevents gastric cancer in *Helicobacter pylori*-infected mongolian gerbils. *Digestion* 2006; **74**: 187–98.
63 Oshima M, Ohima H, Matsunaga A, Taketo MM. Hyperplastic gastric tumors with spasmolytic polypeptide-expressing metaplasia caused by tumor necrosis factor-α-dependent inflammation in cyclooxygenase-2/microsomal prostaglandin E synthase-1 transgenic mice. *Cancer Res* 2005; **65**: 9147–51.
64 Schmidt PH, Lee JR, Joshi V *et al.* Identification of a metaplastic cell lineage associated with human gastric adenocarcinoma. *Lab Invest* 1999; **79**: 639–46.
65 Halldorsdottir AM, Sigurdardottrir M, Jonasson JG *et al.* Spasmolytic polypeptide-expressing metaplasia (SPEM) associated with gastric cancer in Iceland. *Dig Dis Sci* 2003; **48**: 431–41.
66 Yamaguchi H, Goldenring JR, Kaminishi M, Lee JR. Identification of spasmolytic polypeptide-expressing metaplasia (SPEM) in remnant gastric cancer and surveillance post-gastrectomy biopsies. *Dig Dis Sci* 2002; **47**: 573–8.
67 Goldenring JR, Nomura S. Insight into the development of preneoplastic metaplasia: Spasmolytic polypeptide-expressing metaplasia and oxyntic atrophy. In: Wang TC, Fox JG, Giraud AS, eds. *The Biology of Gastric Cancer.* New York: Springer, 2009; 361–75.
68 Nomura S, Baxter T, Yamaguchi H *et al.* Spasmolytic polypeptide expressing metaplasia to preneoplasia in *H. felis*-infected mice. *Gastroenterology* 2004; **127**: 582–94.
69 Kang W, Rathinavelu S, Samuelson LC, Merchang JL. Interferon γ induction of gastric mucous neck cell hypertrophy. *Lab Invest* 2005; **85**: 702–15.
70 Judd LM, Alderman BM, Howlett M *et al.* Gastric cancer development in mice lacking the SHP2 binding site on the IL-6 family co-receptor gp130. *Gastroenterology* 2004; **126**: 196–207.
71 Takasu S, Tsukamoto T, Cao XY *et al.* Role of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 expression and β-catenin activation in gastric carcinogenesis in *N*-merhyl-*N*-nitrosourea-treated K19-C2mE transgenic mice. *Cancer Sci* 2008; **99**: 2356–64.
72 Guo X, Oshima H, Kitamura T, Taketo MM, Oshima M. Stromal fibroblasts activated by tumor cells promote angiogenesis in mouse gastric cancer. *J Biol Chem* 2008; **283**: 19864–71.
73 Entius MM, Westerman AM, van Velthuysen ML *et al.* Molecular and phenotypic markers of hamartomatous polyposis syndromes in the gastrointestinal tract. *Hepatogastroenterology* 1999; **46**: 661–6.
74 Howe JR, Bair JL, Sayed MG *et al.* Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. *Nat Genet* 2001; **28**: 184–7.
75 Miyazono K, Maeda S, Imamura T. BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev* 2005; **16**: 251–63.
76 Hardwick JCH, van den Brink GR, Bleuming SA *et al.* Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon. *Gastroenterology* 2004; **126**: 111–21.
77 Haramis AP, Begthel H, van den Born M *et al.* De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* 2004; **303**: 1684–6.
78 He XC, Zhang J, Tong WG *et al.* BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-β-catenin signaling. *Nat Genet* 2004; **36**: 1117–21.
79 Auclair BA, Benoit YD, Rivard N, Mishina Y, Perreault N. Bone morphogenetic protein signaling is essential for terminal differentiation of the intestinal secretory cell lineage. *Gastroenterology* 2007; **133**: 887–96.
80 Chow E, Macrae F. Review of juvenile polyposis syndrome. *J Gastroenterol Hepatol* 2005; **20**: 1634–40.
81 Schreibman IR, Baker M, Amos C, McGarrity TJ. The hamartomatous polyposis syndromes: A clinical and molecular review. *Am J Gastroenterol* 2005; **100**: 476–90.
82 Oshima H, Itadani H, Kotani H, Taketo MM, Oshima M. Induction of prostaglandin E_2 pathway promotes gastric hamartoma development with suppression of bone morphogenetic protein signaling. *Cancer Res* 2009; **69**: 2729–33.
83 Covarrubias DJ, Huprich JE. Best cases from the AFIP. Juvenile polyposis of the stomach. *Radiographics* 2002; **22**: 415–20.
84 Oshima H, Oguma K, Du Y-C, Oshima M. Prostaglandin E_2, Wnt, and BMP in gastric tumor mouse models. *Cancer Sci* 2009; **100**: 1779–85.
85 Itadani H, Oshima H, Oshima M, Kotani H. Mouse gastric tumor models with prostaglandin E_2 pathway activation show similar gene expression profiles to intestinal-type human gastric cancer. *BMC Genomics* 2009; **10**: 615.
86 Chen X, Leung SY, Yuen ST *et al.* Variation in gene expression patterns in human gastric cancers. *Mol Biol Cell* 2003; **14**: 3208–15.
87 Sorlie T, Perou CM, Tibshirani R *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; **98**: 10869–74.
88 Sorlie T. Stanford Microarray Database. (Accessed 2001.) Available from: http://genome-www5.stanford.edu/
89 Kaiser S, Park YK, Franklin JL *et al.* Transcriptional recapitulation and subversion of embryonic colon development by mouse colon tumor models and human colon cancer. *Genome Biol* 2007; **8**: R131.
90 Beer DG, Kardia SLR, Huang C-C *et al.* Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med* 2002; **8**: 816–24.
91 Beer D The United States National Cancer Institute Website. (Accessed 2002.) Available from: https://array.nci.nih.gov/caarray/project/beer-00153

GASTROENTEROLOGY 2011;140:596-607

Prostaglandin E_2 Signaling and Bacterial Infection Recruit Tumor-Promoting Macrophages to Mouse Gastric Tumors

HIROKO OSHIMA,* KYOJI HIOKI,‡ BORYANA K. POPIVANOVA,* KEISUKE OGUMA,* NICO VAN ROOIJEN,§ TOMO-O ISHIKAWA,* and MASANOBU OSHIMA*

*Division of Genetics, Cancer Research Institute, Kanazawa University, Kanazawa; ‡Gnotobiology Laboratory, Central Institute for Experimental Animals, CIEA, Kawasaki, Japan; and §Department of Molecular Cell Biology, Vrije Universiteit, Amsterdam, The Netherlands

BACKGROUND & AIMS: *Helicobacter pylori* infection induces an inflammatory response, which can contribute to gastric tumorigenesis. Induction of cyclooxygenase-2 (COX-2) results in production of prostaglandin E_2 (PGE_2), which mediates inflammation. We investigated the roles of bacterial infection and PGE_2 signaling in gastric tumorigenesis in mice. **METHODS:** We generated a germfree (GF) colony of *K19-Wnt1/C2mE* mice (*Gan* mice); these mice develop gastric cancer. We examined tumor phenotypes, expression of cytokines and chemokines, and recruitment of macrophages. We also investigated PGE_2 signaling through the PGE_2 receptor subtype 4 (EP4) in *Gan* mice given specific inhibitors. **RESULTS:** *Gan* mice raised in a specific pathogen-free facility developed large gastric tumors, whereas gastric tumorigenesis was significantly suppressed in GF-*Gan* mice; reconstitution of commensal flora or infection with *Helicobacter felis* induced gastric tumor development in these mice. Macrophage infiltration was significantly suppressed in the stomachs of GF-*Gan* mice. *Gan* mice given an EP4 inhibitor had decreased expression of cytokines and chemokines. PGE_2 signaling and bacterial infection or stimulation with lipopolysaccharide induced expression of the chemokine C-C motif ligand 2 (CCL2) (which attracts macrophage) in tumor stromal cells or cultured macrophages, respectively. CCL2 inhibition suppressed macrophage infiltration in tumors, and depletion of macrophages from the tumors of *Gan* mice led to signs of tumor regression. Wnt signaling was suppressed in the tumors of GF-*Gan* and *Gan* mice given injections of tumor necrosis factor-α neutralizing antibody. **CONCLUSIONS: Bacterial infection and PGE_2 signaling are required for gastric tumorigenesis in mice; they cooperate to up-regulate CCL2, which recruits macrophage to gastric tumors. Macrophage-derived tumor necrosis factor-α promotes Wnt signaling in epithelial cells, which contributes to gastric tumorigenesis.**

Keywords: Stomach Cancer; Tumor Promotion; Bacterial Infection.

BASIC-ALIMENTARY TRACT

Gastric cancer is the second most common cause of cancer-related death in the world, and *Helicobacter pylori* infection is closely associated with gastric cancer development.[1] Infections are estimated to be related to 15% of malignant cancer development, and infection-associated inflammation is a critical component of cancer development.[2] For example, an inflammatory response promotes tumor cell proliferation, metastasis, and survival, whereas it suppresses antitumor immune responses.[2-4] Moreover, the genetic polymorphisms in genes encoding inflammatory cytokines influence gastric tumorigenesis.[5] These results suggest that an inflammatory cytokine network induced by *H pylori* infection plays a key role in gastric tumorigenesis.

Cycooxygenase-2 (COX-2) is an inducible enzyme for prostaglandin biosynthesis, which plays an important role in both inflammation and tumorigenesis.[6,7] Mouse model studies have indicated that induction of the COX-2/prostaglandin E_2 (PGE_2) pathway accelerates intestinal tumorigenesis through the induction of angiogenesis and suppression of apoptosis.[8,9] Among 4 PGE_2 receptors (EP1-EP4), EP4 receptor signaling has been shown to play an important role in intestinal tumorigenesis through the activation of epidermal growth factor receptor.[10] The expression of COX-2 is also found in more than 70% of gastric cancers,[11] which is suppressed by the eradication of *H pylori*,[12] suggesting COX-2 induction by infection in the gastric mucosa. Transgenic mice expressing COX-2 and a PGE_2 converting enzyme, microsomal prostaglandin E synthase-1 (mPGES-1), in the stomach develop hyperplasia with macrophage infiltration, indicating the role of PGE_2 in macrophage recruitment.[13] Tumor-associated macrophages (TAMs) play an important role in tumorigenesis through the enhancement of angiogenesis, migration, and remodeling.[14] Moreover, the simultaneous activation of Wnt and the PGE_2 pathways in the mouse stomach causes dysplastic tumor de-

Abbreviations used in this paper: COX-2, cyclooxygenase-2; *Gan*, Gastric neoplasia; GF, germfree; IL-1β, interleukin-1β; IL-6, interleukin-6; LPS, lipopolysaccharide; mPGES-1, microsomal prostaglandin E synthase-1; NF-κB, nuclear factor-κB; PGE_2, prostaglandin E_2; RT-PCR, reverse transcription-polymerase chain reaction; SPF, specific pathogen free; TAM, tumor-associated macrophage; TNF-α, tumor necrosis factor-α.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.11.007

velopment.[15] Accordingly, it is possible that PGE_2-dependent macrophage recruitment is one of the important mechanisms underlying the *H pylori* infection-associated inflammation in gastric tumorigenesis. However, the relationship between bacterial infection and PGE_2 signaling in gastric tumorigenesis remains unclear.

Commensal bacteria constitutively stimulate the intestinal mucosa, inducing cytokines and chemokines at a basal level, which is important for homeostasis of the intestinal mucosa.[16] The present study shows that indigenous bacteria constitutively stimulate the gastric mucosa, which is required for tumorigenesis in the Wnt-activated and PGE_2-induced gastric mucosa. Bacterial colonization and PGE_2 signaling through EP4 receptor cooperatively induced the expression of CCL2, which was a major pathway for macrophage recruitment in the gastric mucosa. Furthermore, depletion of macrophages caused regressive signs of tumors. These results indicate that bacterial infection and PGE_2 signaling cooperatively recruit macrophages to the gastric mucosa, which promotes gastric cancer development.

Materials and Methods

Animal Experiments

The construction of *K19-Wnt1* [*Tg(Krt19-Wnt1)2Maos*], *K19-C2mE* [*Tg(Krt19-Ptgs2,Krt19-Ptges)8Tko*], and *K19-Wnt1/C2mE* (*Gan* for Gastric neoplasia) [*Tg(Krt19-Wnt1)2Maos/Tg(Krt19-Ptgs2,Krt19-Ptges)8Tko*] transgenic mice was described previously (Supplementary Table 1).[13,15] All mice used in the present study were backcrossed to C57BL/6 mice more than 12 times. Germfree (GF) mouse colonies of all genotypes and wild-type mice were established by cesarean derivation at the Central Institute for Experimental Animals (CIEA, Kawasaki, Japan), and the mice were raised in GF isolators under GF conditions. During the experiments, GF conditions were monitored by cultures of feces, bedding, and swabs in thioglycollate medium or potato dextrose broth every 2–5 weeks (Supplementary Table 2). Specific pathogen free (SPF)-raised mice were maintained in the SPF facility at Kanazawa University. The excluded pathogens in the SPF facility are listed in Supplementary Table 3. Mice were killed and examined at 30 and 55 weeks of age (n = 4–6 mice), and all experimental groups consisted of both female and male mice. Half of the glandular stomach was used for the histologic analysis, and the other half was used for RNA and protein sample preparation. Six GF *Gan* mice were moved to the SPF facility at 7 weeks of age, reconstituted with commensal flora by cohousing with SPF mice and adding dirty bedding from other cages and examined at 30 weeks of age. *Helicobacter felis* (ATCC 49179) were inoculated at 10^8/mouse PO to GF *Gan* mice at 30 weeks of age (n = 3 mice), and gastric phenotypes were examined at 55 weeks of age. The time course and experimental conditions for each group are shown in Supplementary Figure 1. All animal experiments were carried out according to the protocol approved by the Committee on Animal Experimentation of Kanazawa University.

Gastric pH Measurement

Gastric pH was measured as described.[17] Briefly, mice were fasted overnight before necropsy. Sterile water (1.5 mL) was injected into the stomach, the stomach was massaged gently, and the pH of the gastric contents was measured using a pH meter.

Drug Treatment Experiments

The EP4 receptor inhibitor RQ-00015986/CJ-42794[18] was provided from RaQualia Pharma Inc (Taketoyo, Japan). *Gan* mice were treated with RQ-00015986 at 100 mg/kg/day PO from 27 or 52 weeks of age for 3 weeks (n = 4 or 5 mice). For inhibition of tumor necrosis factor (TNF)-α or CCL2, *Gan* mice were injected with a neutralizing antibody against TNF-α (AB-410-NA; R&D Systems, Minneapolis, MN) at 8 mg/kg/day intraperitoneally for 6 days (n = 3 mice) or an antibody against CCL2 (AF-479-NA; R&D Systems) at 1 mg/kg/day intraperitoneally for 3 days (n = 3 mice), respectively. Macrophages were depleted in vivo by injection of 200 μL clodronate (dichloromethylene bisphosphonate)-loaded liposomes intravenously every 3 days for 2 weeks as previously described.[19] Clodronate was a gift from Roche Diagnostics GmbH (Mannheim, Germany) and was encapsulated in liposomes as described previously.[20]

Histology and Immunohistochemistry

Stomach tissues were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 4-μm thickness. These sections were stained with H&E or processed for immunostaining. Tissues were also embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen in liquid nitrogen, and sectioned at 10-μm thickness. The frozen sections were used for CCL2 immunostaining. Antibodies against the proton pump (MBL, Nagoya, Japan), F4/80 (Serotec, Oxford, UK), β-catenin (Sigma, St. Louis, MO), CCL2 (Hycult Biotech, Uden, The Netherlands), EP4 (MBL), CD44 (Millipore, Billerica, MA), EphB3 (Bioworld Technology, St. Louis Park, MN), and Ki-67 (Dako, Carpinteria, CA) were used as the primary antibodies. Staining signals were visualized using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). For fluorescence immunostaining, Alexa Fluor 594 or Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR) was used as the secondary antibody. Apoptosis was examined using the ApopTag Apoptosis Detection Kit (Millipore). The mean index for F4/80 (macrophage), proton pump (parietal cell), Ki-67, or apoptosis was calculated by counting labeled cells per microscopic field (200×) in 5 fields.

Scoring Tumor Volume and Preneoplastic Lesions

The mucosal thickness (tumor height) of the gastric tumors of *Gan* mice and the normal stomach of wild-type mice was measured from histology sections. The mucosal thickness relative to that of wild-type mice was calculated. The number of preneoplastic lesions in the whole glandular stomach of *K19-Wnt1* mice was counted under a dissection microscope after staining with 0.05% toluidine blue. The histologic characteristics of gastric tumors and preneoplastic lesions were described previously.[15] The histology of these lesions was confirmed after scoring.

Cell Culture Experiments

The RAW264 macrophage cells (RIKEN BioResource Center, Tsukuba, Japan) were treated with lipopolysaccharide (LPS) (Sigma) for 24 hours at 1, 10, 50, 100, 1000, or 10,000 pg/mL with or without treatment with a COX-2 inhibitor, celecoxib, or RQ00015986 at 10 μmol/L, and the expression levels of CCL2 and cytokines were examined. Celecoxib was provided by Pfizer (New York, NY). For the primary culture of gastric epithelial cells, the glandular stomach of *K19-Wnt1* mice was treated with 0.1% collagenase followed by trypsin, and epithelial cells were cultured in matrigel (BD Pharmingen, Franklin Lakes, NJ) with epidermal growth factor (EGF) (−) primary culture medium.[13] The primary cultured cells were treated with RQ00015986 at 10 μmol/L for 6 days, and organoid structures consisting of epithelial cells larger than 0.2 mm in diameter were counted.

Immunoblotting Analysis

The tissues were homogenized and sonicated in lysis buffer. Thereafter, the specimens were centrifuged at 20,000*g*, and 10 μg of the supernatant protein sample was separated in a 10% sodium dodecyl sulfate-polyacrylamide gel. An antibody against unphosphorylated active β-catenin (Millipore) was used as the primary antibody. Anti-β-actin (Sigma) was used as the internal control. The ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to detect the signals.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Tumor stroma and epithelial cell samples were separately collected from frozen sections using Laser Microdissection (Leica, Wetzlar, Germany). Total RNA was extracted from the tissues or microdissected samples using ISOGEN (Nippon Gene, Tokyo, Japan), reverse transcribed with PrimeScript RT reagent Kit (Takara, Tokyo, Japan), and polymerase chain reaction (PCR) amplified by Stratagene Mx300P (Agilent Technologies, Santa Clara, CA) using SYBR Premix ExTaqII (Takara). Primers for the real-time reverse-transcription polymerase chain reaction (RT-PCR) were purchased (Takara), and the primer sequences are shown in Supplementary Table 4.

Statistical Analysis

The data were analyzed using the unpaired *t* test and are presented as the means ± standard deviation. A value of $P < .05$ was considered to be statistically significant.

Results

Constitutive Stimulation of Gastric Mucosa by Indigenous Bacteria

To determine whether indigenous bacteria in the stomach stimulate the gastric mucosa, we examined the expression of cytokines and chemokines in the glandular stomach of germfree (GF) wild-type mice and control SPF mice by real-time RT-PCR (Figure 1*A*). Importantly, the expression levels of TNF-α, interleukin (IL)-1β, keratinocyte-derived chemokine (KC), and macrophage inflammatory protein-2 (MIP-2) decreased significantly in the GF mouse stomach compared with the SPF mice, whereas IL-6 expression slightly increased. These results indicate that commensal bacteria constitutively stimulate the gastric mucosa to induce inflammatory cytokines and chemokines at a basal level.

Gan mice were previously constructed by crossing *K19-Wnt1* and *K19-C2mE* mice (Supplementary Table 1).[13,15] We examined the number of parietal cells by immunostaining and measured intragastric pH in these models because bacterial growth is affected by intragastric acidity. The mean parietal cell index was similar in all strains, and the intragastric pH was around 3.5 (Figure 1*B–D*). However, in the dysplastic tumors of *Gan* mice, the number of parietal cells decreased significantly, and intragastric pH increased at 50–55 weeks of age. These results indicate that gastric acidity is not changed in *Gan* mice at early stages of disease compared with other models until large tumors develop at the later stages.

Suppression of Gastric Tumorigenesis in GF Mice

Gan mice raised in an SPF facility (SPF-*Gan*) developed large gastric tumors by 55 weeks of age (Figure 2*A* and *B*), and the mean tumor height increased to approximately 1.5-fold when compared with that at 30 weeks of age. In contrast, gastric tumorigenesis was significantly suppressed in the GF *Gan* (GF-*Gan*) mice, and the mean mucosal thickness of GF-*Gan* mice was less than 40% of the age-matched SPF-*Gan* mice (Figure 2*A* and *B*, Supplementary Figure 2). Approximately 40% of the SPF-*Gan* mice showed a moribund phenotype and thus were killed by 60 weeks of age, whereas all GF-*Gan* mice survived by 55 weeks of age (Figure 2*C*). Importantly, reconstitution of commensal bacteria in GF-*Gan* mice resulted in development of gastric tumors that were significantly larger than those of GF-*Gan* mice (Figure 2*A*, Supplementary Figure 2), and treatment of SPF-*Gan* mice with antibiotics significantly suppressed gastric tu-

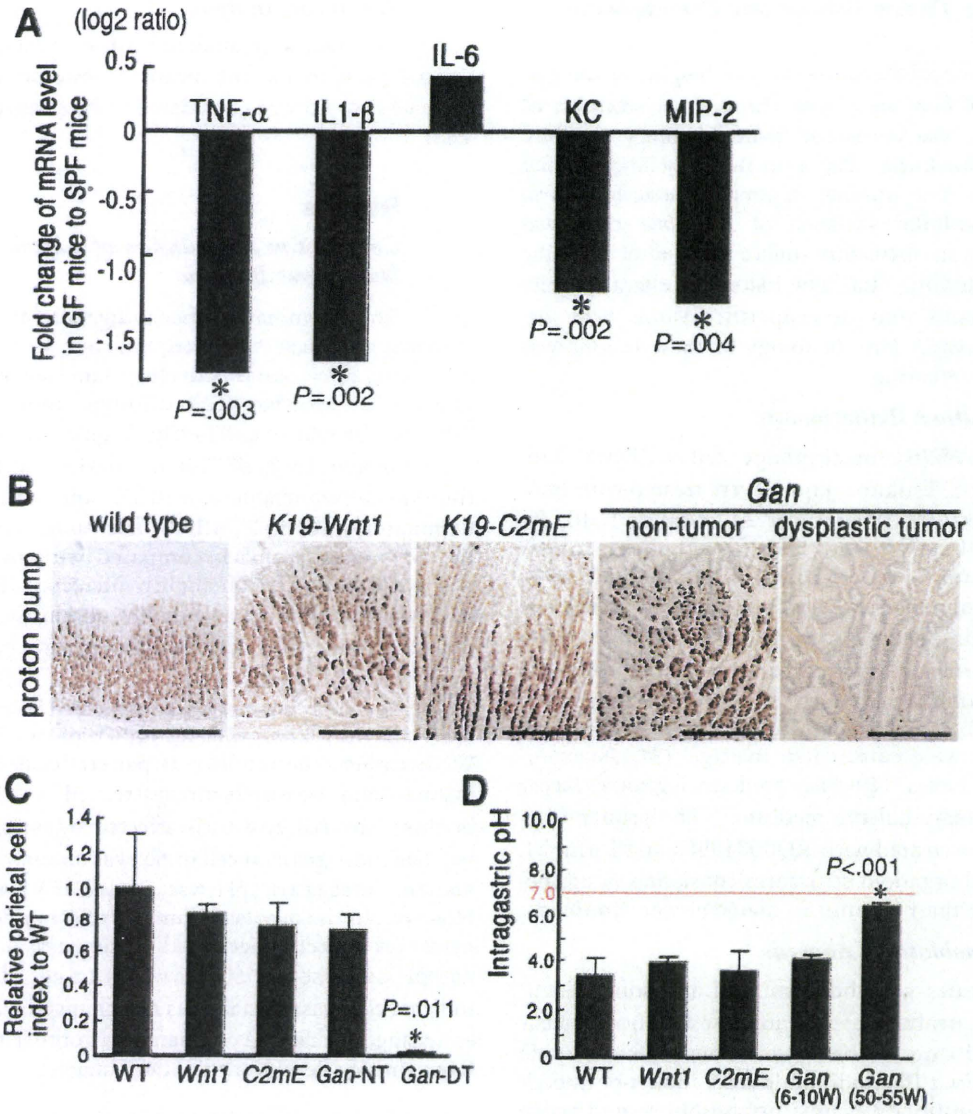

Figure 1. Stimulation of normal gastric mucosa by indigenous bacteria. (*A*) Messenger RNA (mRNA) levels of cytokines and chemokines in the gastric mucosa of the germfree (GF)-wild-type mice (mean log2 ratio to the SPF mouse level). *$P < .05$. (*B*) Immunostaining for proton pumps (parietal cells) in the gastric mucosa of the indicated strains. *Scale bars*, 100 μm. (*C*) The parietal cell index relative to the wild-type mouse level (mean ± standard deviation [SD]). *$P < .05$ vs the wild-type level (*WT*). *Gan*-NT, *Gan* nontumor; *Gan*-DT, *Gan* dysplastic tumor. (*D*) Intragastric pH of the indicated mouse strains (mean ± SD). *$P < .001$ vs the wild-type level (*WT*).

mor growth (Supplementary Figure 3). These results indicate that colonization of indigenous bacteria is required for gastric tumor development.

Moreover, infection with *Helicobacter felis*, separate species of *Helicobacter pylori*, in the GF-*Gan* mouse stomach at 30 weeks of age (GF->*H felis* mice) induced the development of gastric tumors by 55 weeks of age (Figure 2*A* and *B*). The infection of *H felis* in gastric glands was confirmed by microscopy of histology sections (Supplementary Figure 4). These results also indicate the role of infection in gastric tumorigenesis.

Apoptotic cells were found only on the mucosal surface of gastric tumors (Figure 2*D*), and the mean apoptosis index was 42.4% and 40.0% on the mucosal surface in SPF-*Gan* and GF-*Gan* mice, respectively. On the other hand, the number of Ki-67-labeled proliferating cells was significantly lower in the GF-*Gan* mouse stomach (Figure 2*E* and *F*), suggesting that bacterial colonization contributes to the tumor cell proliferation.

Suppression of Gastric Tumorigenesis by Inhibition of the PGE$_2$ Receptor EP4

There are 4 PGE$_2$ receptors (EP1–EP4), and the expression level of EP4 was increased significantly in *Gan* mouse tumors.[21] SPF-*Gan* mice were thus treated with a specific EP4 inhibitor, RQ-00015986, for 3 weeks from 52

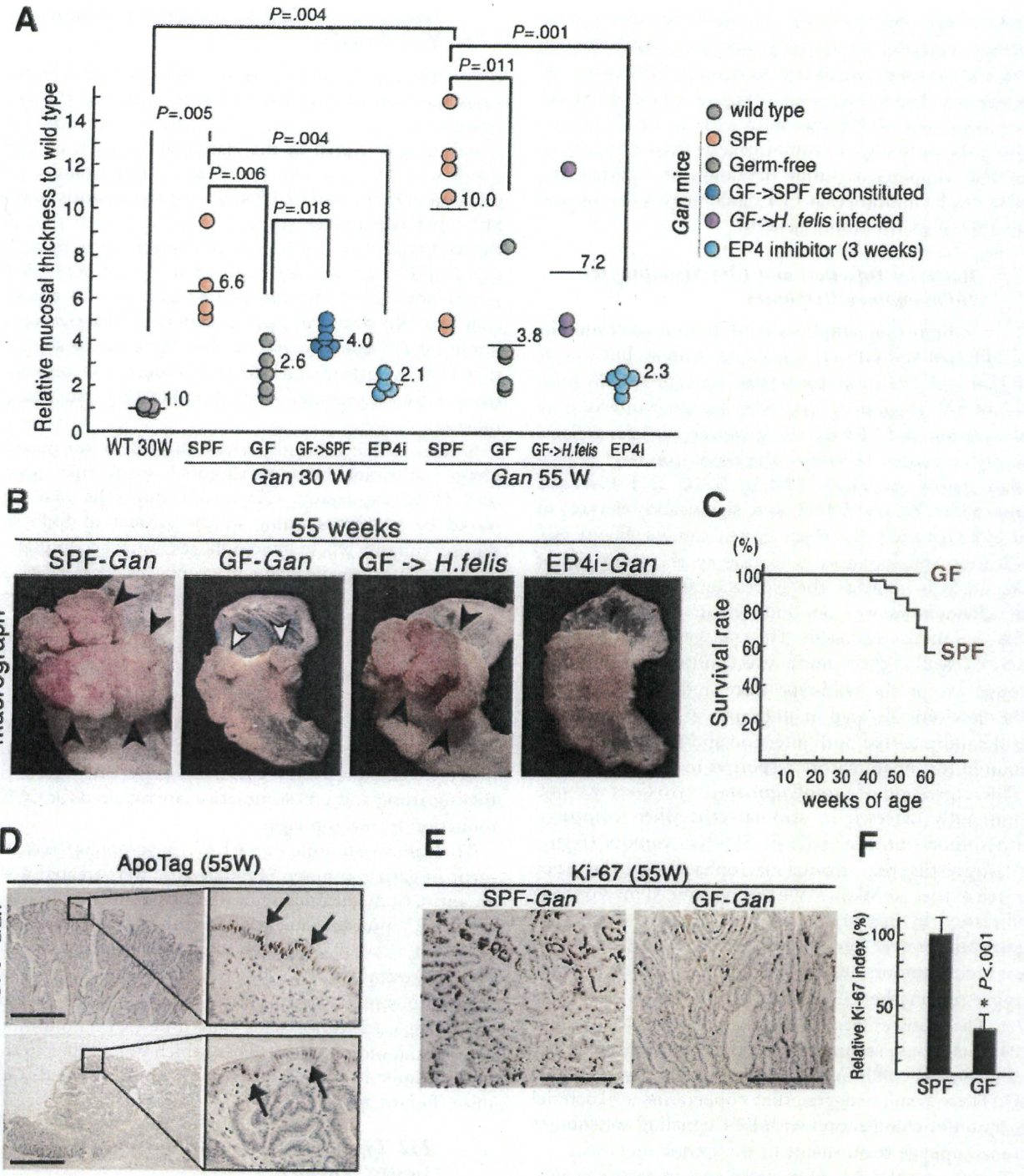

Figure 2. Suppression of gastric tumorigenesis in germfree (*GF*) *Gan* mice. (*A*) The gastric mucosal thickness (tumor height) of SPF-*Gan* (*SPF*), GF-*Gan* (*GF*), commensal flora-reconstituted GF-*Gan* (*GF->SPF*), *H felis*-infected GF-*Gan* (*GF->H felis*), and EP4 inhibitor-treated-*Gan* (*EP4i*) mice at 30 and 55 weeks of age relative to wild-type mice (*WT*). (*B*) Representative macroscopic photographs of the stomach of the indicated group of *Gan* mice at 55 weeks of age. *Black arrowheads* indicate tumors, whereas the *white arrowheads* indicate suppressed tumorous lesions in the *GF*-Gan mouse. (*C*) The survival rate of the SPF-*Gan* and GF-*Gan* mice. All GF-*Gan* mice were used for experiments at 55 weeks of age. (*D*) Apoptosis analyses of SPF-*Gan* and GF-*Gan* mouse gastric tumors. *Arrows* indicate apoptotic cells on the mucosal surface of tumors. (*E*) Ki-67 immunostaining in the gastric tumors of SPF-*Gan* and GF-*Gan* mice. *Scale bars* in *D* and *E*, 100 μm. (*F*) Relative mean Ki-67 labeling index (mean ± standard deviation). *$P < .001$ vs SPF level.

weeks of age (Supplementary Figure 1). Notably, gastric tumors regressed significantly following inhibition of EP4, and the mean tumor size decreased to 23% of that of the age-matched SPF-*Gan* mice (Figure 2*A* and *B*). Moreover, treatment of SPF-*Gan* mice with an EP4 inhibitor during the early stage of tumorigenesis from 27 weeks of age also suppressed tumor development significantly. These results indicate that EP4 signaling plays an important role in gastric tumorigenesis.

Bacterial Infection and EP4 Signaling for Inflammatory Responses

Submucosal lymphocyte infiltration was found in the SPF-*Gan* and GF->*H felis* mouse tumors but not in GF-*Gan* and EP4 inhibitor-treated *Gan* (EP4i-*Gan*) mice (Figure 3*A*), suggesting that both bacterial infection or colonization and EP4 signaling are required for inflammatory responses. Moreover, the expression level of proinflammatory cytokines, TNF-α, IL-1β, and IL-6 and chemokines, KC and MIP-2, were significantly elevated in the SPF-*Gan* and GF->*H felis* gastric tumors (Figure 3*B*), indicating inflammatory responses in the stomachs of these mice. In contrast, the expression of these cytokines and chemokines was not induced in the GF-*Gan* and EP4i-*Gan* mouse stomachs. The transgenic expression of COX-2 (*Ptgs2*) in the stomach was confirmed in all mouse groups except the wild-type mice, indicating that the PGE$_2$ level was elevated in all groups of *Gan* mice. These results indicate that both infection and EP4 signaling are required for inflammatory responses in the stomach.

The expression of proinflammatory cytokines was predominantly detected in stromal cells when compared with tumor epithelial cells in SPF-*Gan* tumors (Figure 3*C*), suggesting that stromal macrophages were the major source of these cytokines. We thus examined macrophage infiltration by immunostaining. As expected, numerous macrophages were found in the SPF-*Gan* and GF->*H felis* *Gan* mouse tumors, whereas macrophage infiltration was suppressed in the GF-*Gan* and EP4i-*Gan* mice (Figure 3*D*). The number of macrophages in the GF-*Gan* and EP4i-*Gan* mouse stomachs decreased significantly to 11% and 33% of the SPF-*Gan* mouse level, respectively (Figure 3*E*). These results suggest that cooperation of bacterial infection or colonization with EP4 signaling contributes to macrophage recruitment to the gastric mucosa.

To examine the role of macrophages in gastric tumorigenesis, SPF-*Gan* mice were treated with clodronate liposomes to deplete macrophages in vivo. Macrophage-depleted areas were found by immunostaining in the clodronate liposome-injected *Gan* mouse tumors (Figure 3*F*). In the macrophage-depleted area, tumors showed regressive signs with atrophic changes of tumor cells or peel-off of tumor epithelial cells from the mucosal surface, suggesting that macrophages are important for the maintenance or survival of tumor epithelial cells.

Induction of CCL2 by Bacterial Infection and EP4 Signaling

The expression level of macrophage-tropic chemokines was examined in the SPF-*K19-C2mE* and GF-*K19-C2mE* mouse stomach. The PGE$_2$ pathway but not Wnt signaling is activated in the glandular stomach of *K19-C2mE* mice (Supplementary Table 1). Importantly, expression of CCL2 and CCL8 increased significantly in the SPF-*K19-C2mE* mouse stomach compared with that in the wild-type mice (Figure 4*A*). In contrast, expression of CCL2 and CCL8 was not induced in the GF-*K19-C2mE* mouse stomach. Induction of CCL2 and CCL8 expression was also found in gastric tumors of SPF-*Gan* mice but not in GF-*Gan* mice (Figure 4*B*). These results suggest that PGE$_2$ signaling and bacterial colonization cooperatively induce expression of CCL2 and CCL8 in the gastric mucosa.

Because CCL2 is an important chemokine for macrophage infiltration in colon tumors,[22] we further examined CCL2 expression. CCL2-expressing cells were detected by immunostaining in the stroma of SPF-*Gan* mouse tumors where macrophages were accumulated (Figure 4*C*), suggesting that macrophages express CCL2. The stimulation of RAW264 macrophage cells with LPS induced the expression of CCL2 in a dose-dependent manner (Figure 4*D*). A low concentration of LPS was used for further experiments to examine the effect of low counts of indigenous bacterial colonization in the stomach. Importantly, treatment of RAW264 macrophages with an EP4 inhibitor significantly suppressed LPS-induced CCL2 expression (Figure 4*E*), suggesting that both EP4 signaling and LPS stimulation are required for CCL2 induction in macrophages.

To examine the role of CCL2 in macrophage recruitment in gastric tumors, SPF-*Gan* mice were treated with an anti-CCL2 neutralizing antibody. Notably, inhibition of CCL2 suppressed macrophage infiltration in tumors, although a few macrophages were still detected (Figure 4*F*), suggesting that CCL2 is a major chemokine that recruits macrophages to gastric tumors. In the macrophage-depleted tumor areas caused by CCL2 inhibition, tumors showed regressive signs, which was similar to the observations in the clodronate liposome-treated *Gan* mice (Figure 3*F*).

M2 Type Polarization of Macrophages in Gastric Tumors

TAMs play a pivotal role in tumor development.[14] Macrophage activation is classified as "classically" activated (M1) or "alternatively" activated (M2) type, and TAMs generally express characteristics of M2-polarized macrophages.[23] Interestingly, the expression of M2 macrophage markers, Ym1, Ym2, arginase 1 (Arg1), and transforming growth factor-β, increased significantly in both the SPF-*K19-C2mE* stomach and SPF-*Gan* tumors (Supplementary Figure 5*A*). Consistently, expression of

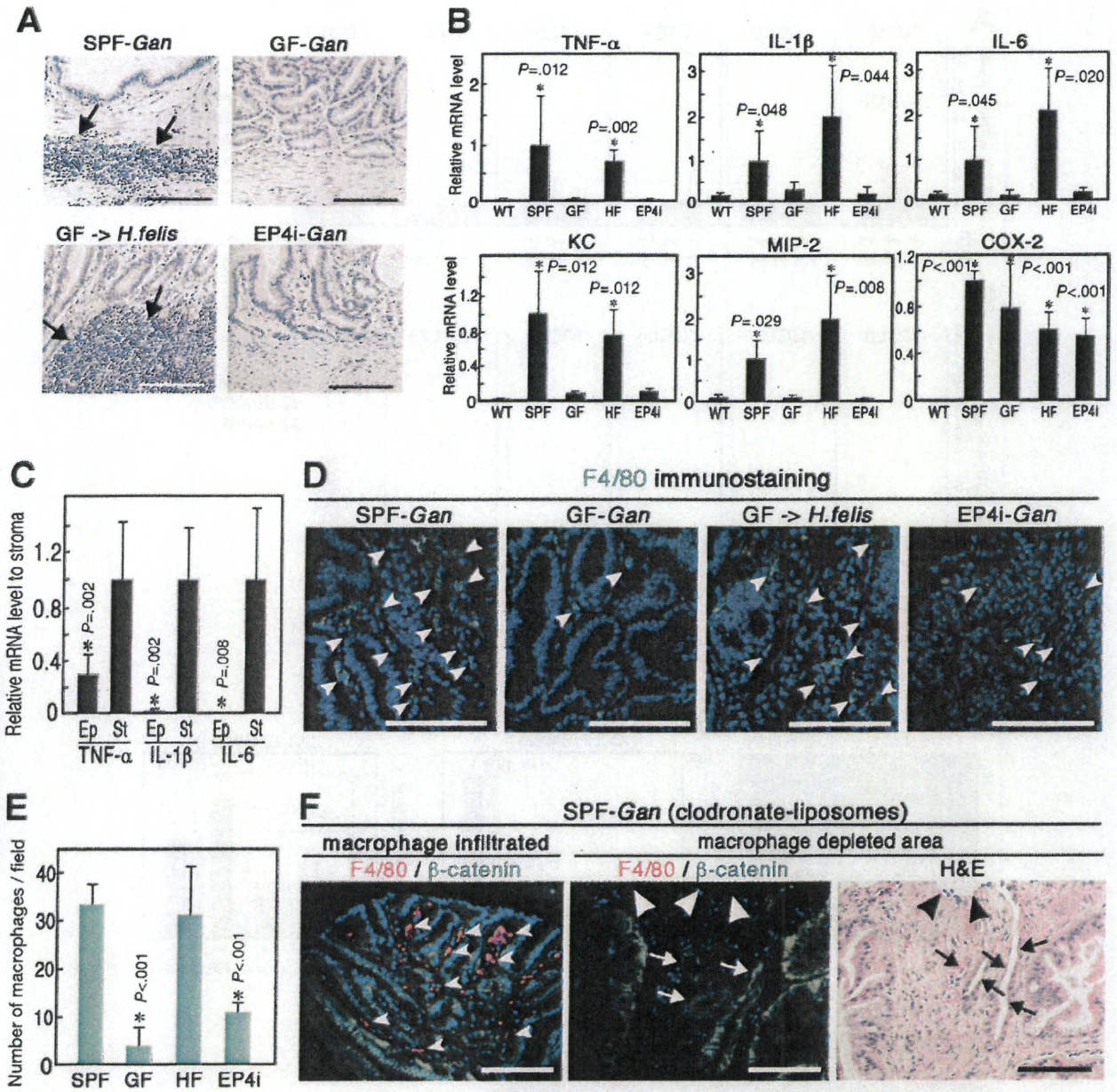

Figure 3. Macrophage recruitment in the *Gan* mouse tumors. (*A*) H&E staining of the SPF-*Gan*, GF-*Gan*, *H felis*-infected GF-*Gan* (GF->*H felis*), and EP4 inhibitor-treated *Gan* (EP4i-*Gan*) mouse stomachs. *Arrows* indicate lymphocyte infiltration. *Scale bars*, 100 μm. (*B*) Relative messenger RNA (mRNA) levels of inflammatory cytokines, chemokines, and COX-2 in wild-type mouse stomach (*WT*) and SPF-*Gan* (*SPF*), GF-*Gan* (*GF*), *H felis*-infected GF-*Gan* (*HF*), and EP4i-*Gan* (*EP4i*) mouse gastric tumors (mean ± standard deviation [SD]). *$P < .05$ vs wild-type level. (*C*) The mRNA level of inflammatory cytokines in the microdissected tumor epithelial cells (*Ep*) relative to the level in the tumor stroma (*St*). *$P < .01$. (*D*) Immunostaining of F4/80 (*green*) with 4',6-diamidino-2-phenylindole staining (*blue*) in gastric tumors of the indicated groups. *Arrowheads* indicate F4/80-positive macrophages. *Scale bars*, 100 μm. (*E*) The mean number of F4/80-positive macrophages per microscopic field (mean ± SD). *$P < .001$ vs SPF level. (*F*) Immunostaining of F4/80 (*red*) and β-catenin (*green*) in gastric tumors of a control *Gan* mouse (*left*) and clodronate liposome-treated *Gan* mouse (*center*). H&E staining of serial section of a clodronate liposome-treated *Gan* mouse tumor (*right*). *Scale bars*, 100 μm. *Arrowheads* (*left*) indicate macrophages. *Arrowheads* and *arrows* (*center* and *right*) indicate the mucosal surface of the tumor and tumor cells, respectively, in the macrophage-depleted area.

an M2 marker, mannose receptor, was detected by immunohistochemistry in the SPF-*Gan* mouse tumors (Supplementary Figure 5*B*). It has been reported that $CD4^+$ T cells regulate the M2 properties of macrophages.[24] CD4+ T cells infiltrated into the SPF-*Gan* mouse tumors (Supplementary Figure 5*C*). However, M2 macrophages were also found in SPF-*Rag2*$^{-/-}$ *K19-C2mE* mouse stomachs (Supplementary Figure 5*D*), suggesting

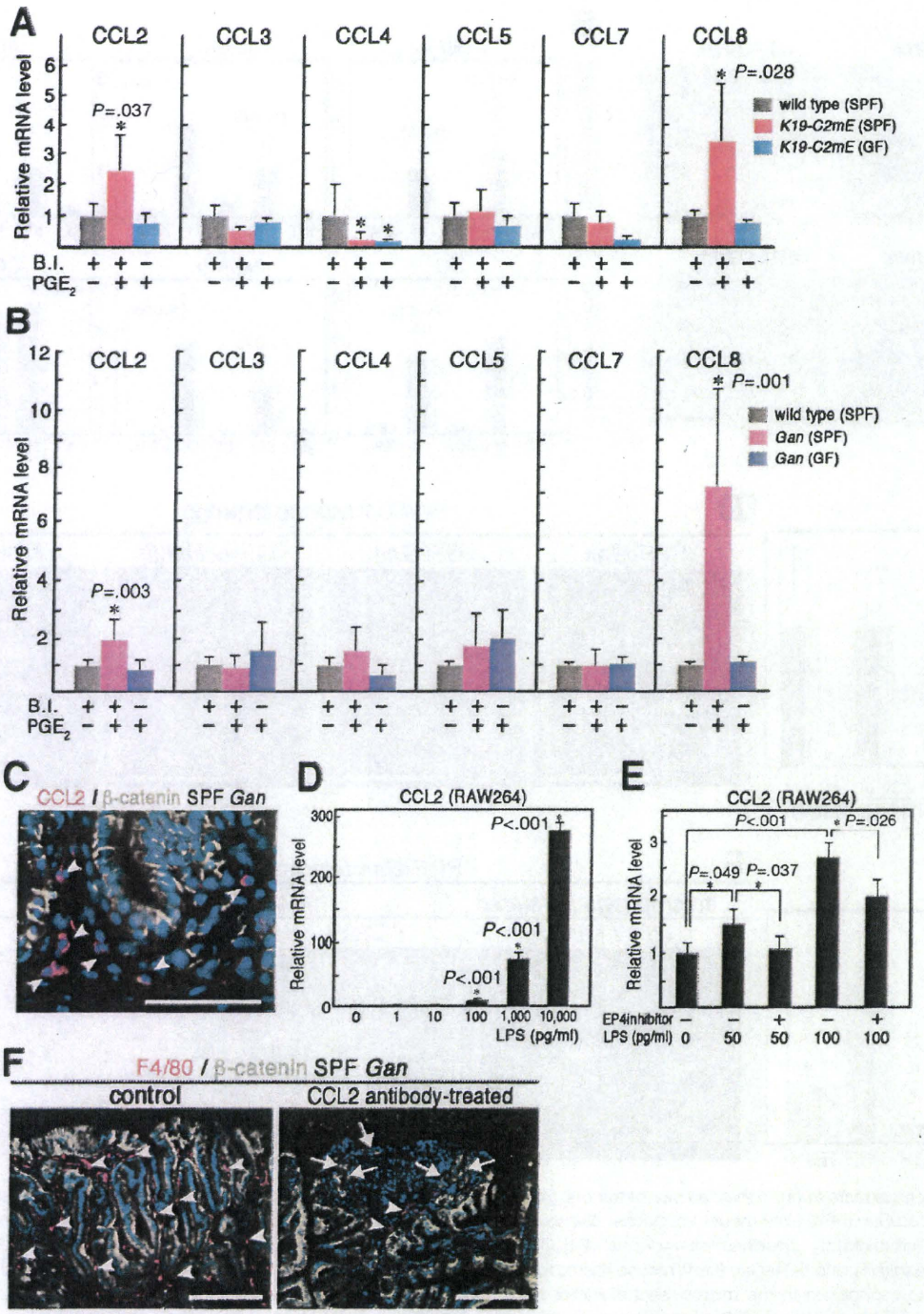

Figure 4. Chemokine induction by bacterial colonization and PGE$_2$ signaling. (*A* and *B*) The messenger RNA (mRNA) levels of the indicated chemokines in SPF-*K19-C2mE* (*red*) and GF-*K19-C2mE* (*blue*) gastric mucosa (*A*) and SPF-*Gan* (*pink*) and GF-*Gan* (*purple*) gastric tumors (*B*) relative to that in the control SPF wild-type mouse stomach (*gray*) (mean ± standard deviation [SD]). The indigenous bacterial colonization (*B.I.*) and PGE$_2$ transgenic status (*PGE$_2$*) are indicated at the bottom. *$P < .05$ vs wild-type level. (*C*) Immunostaining of CCL2 (*red*, *arrowheads*) and β-catenin (*green*) with 4′,6-diamidino-2-phenylindole (DAPI) staining (*blue*) in SPF-*Gan* gastric tumor. *Scale bar*, 100 μm. (*D*) The mRNA levels of CCL2 in the LPS-stimulated RAW264 cells relative to that in the unstimulated control cells (mean ± SD). *$P < .001$ vs control level. (*E*) The mRNA levels of CCL2 in RAW264 cells with indicated treatment relative to the level in the unstimulated RAW264 cells (mean ± SD). *$P < .05$. (*F*) Immunostaining for F4/80 (*red*, *arrowheads*) and β-catenin (*green*) with DAPI staining (*blue*) in tumors of control SPF-*Gan* (*left*) and CCL2 antibody-treated SPF-*Gan* mice (*right*). *Scale bars*, 100 μm. *Arrows* in *right panel* indicate regressed tumors in the macrophage-depleted area.

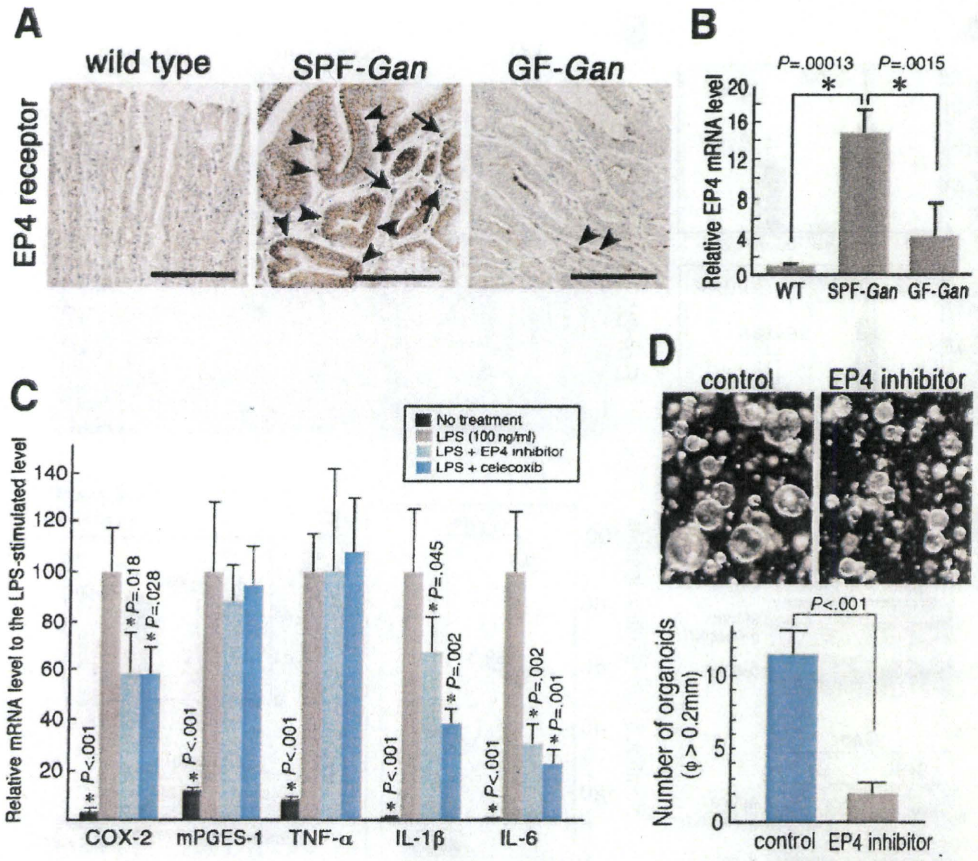

Figure 5. EP4 signaling on macrophages and epithelial cells. (*A*) Immunostaining of EP4 in the normal gastric mucosa of wild-type mouse and gastric tumors of SPF- and GF-*Gan* mice. *Arrowheads* and *arrows* indicate EP4 expression in the tumor epithelia and tumor stromal cells, respectively. *Scale bars*, 100 μm. (*B*) The messenger RNA (mRNA) levels of EP4 in gastric tumors of SPF-*Gan* and GF-*Gan* mice relative to the wild-type mouse level (mean ± standard deviation [SD]). *$P < .01$. (*C*) The mRNA levels of inflammatory cytokines in the control or drug-treated RAW264 cells relative to the level of LPS-stimulated RAW264 cells (mean ± SD). *$P < .05$ to the LPS-stimulated level. (*D*) Representative photographs of organoid structures formed by the primary cultured gastric epithelial cells in matrigel with EP4 inhibitor treatment (*top right*) and no-treatment control (*top left*). The mean number of organoids larger than 0.2 mm in diameter in the microscopic field on day 6 of culture (*bottom*) (mean ± SD). *$P < .001$.

that macrophages in the PGE_2-induced inflammation can be polarized to the M2 type in the absence of T cells.

EP4 Signaling on Macrophages and Epithelial Cells

Expression of the EP4 receptor was detected by immunostaining in both tumor epithelial cells and stromal cells of SPF-*Gan* mice, whereas it was rarely detected in the GF-*Gan* or wild-type mouse stomach (Figure 5*A*). Induction of EP4 in SPF-*Gan* tumors was confirmed by real-time RT-PCR (Figure 5*B*). When RAW264 macrophages were stimulated with LPS, expression of COX-2 and mPGES-1, as well as proinflammatory cytokines, was elevated (Figure 5*C*). Importantly, treatment of LPS-stimulated macrophages with an EP4 inhibitor or celecoxib suppressed the induction of COX-2, IL-1β, and IL-6. Moreover, inhibition of EP4 suppressed proliferation of the primary cultured gastric epithelial cells in matrigel (Figure 5*D*). These results suggest that EP4 signaling is also important for macrophage activation and epithelial cell proliferation.

Wnt Promotion by Bacterial Infection and TNF-α Stimulation

Expression of Wnt-target genes, CD44 and Eph receptor B3 (EphB3), was significantly down-regulated in the GF-*Gan* and EP4i-*Gan* mouse stomachs (Figure 6*A*). In the wild-type mouse stomach, expression of CD44 was found only in the neck of the gastric gland, whereas EphB3 was not detected (Figure 6*B*). Notably, expression of CD44 and EphB3 was significantly induced in tumor epithelial cells of SPF-*Gan* mice, which was suppressed in GF-*Gan* mice. Consistently, the active β-catenin level was decreased in the GF-*K19-Wnt1* stomach and GF-*Gan* mouse gastric tumors compared with SPF mice (Figure 6*C*), indicating that Wnt signaling activity is suppressed under GF conditions. We previously showed that macrophage-derived TNF-α promotes Wnt signaling in gastric

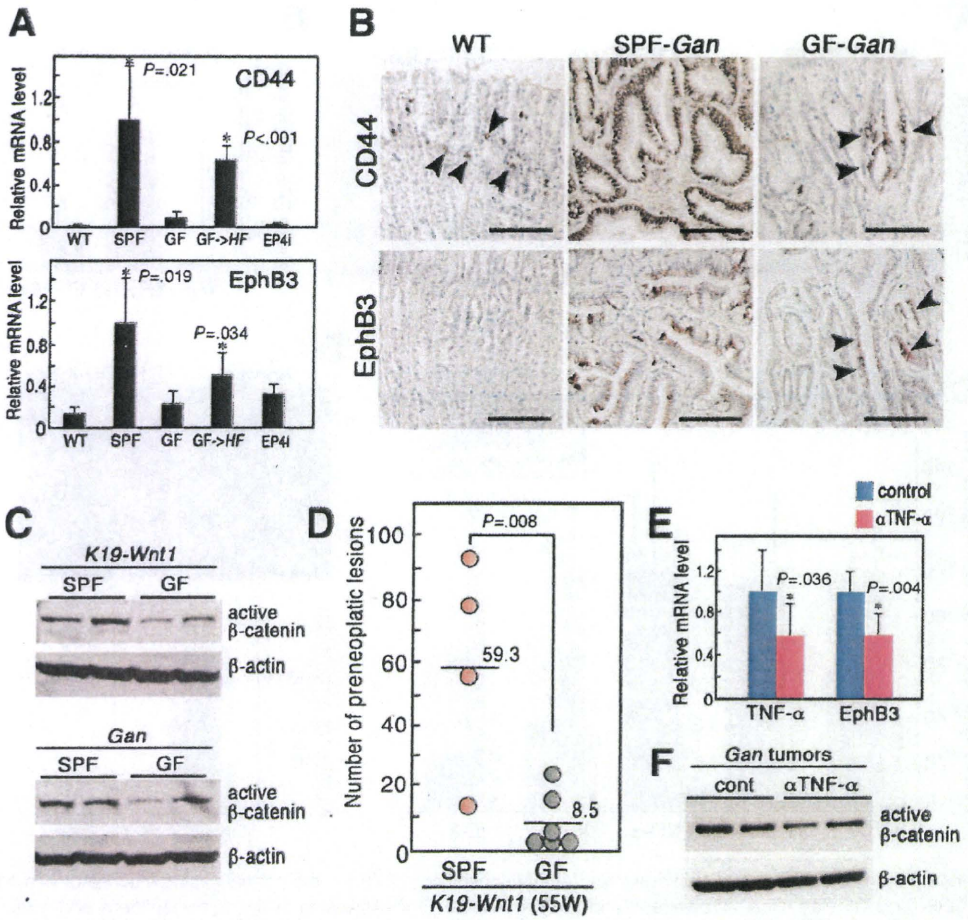

Figure 6. Wnt promotion by bacterial infection and TNF-α. (*A*) Relative messenger RNA (mRNA) levels of Wnt-target genes, CD44 and EphB3, in the wild-type mouse stomach (*WT*) and SPF-*Gan* (*SPF*), GF-*Gan* (*GF*), *H felis*-infected GF-*Gan* (*GF->HF*), and EP4 inhibitor-treated-*Gan* (*EP4i*) mouse gastric tumors (mean ± standard deviation [SD]). *$P < .05$ vs wild-type level. (*B*) Immunostaining of CD44 (*top*) and EphB3 (*bottom*) in the wild-type mouse stomach (*left*) and SPF-*Gan* (*center*) and GF-*Gan* (*right*) mouse tumors. *Arrowheads* indicate immunostained epithelial cells in the wild-type (*WT*) and GF-*Gan* mice. *Scale bars*, 100 μm. (*C*) Immunoblotting of active β-catenin in the SPF-*K19-Wnt1* and GF-*K19-Wnt1* mouse stomach (*top*) and SPF-*Gan* and GF-*Gan* mouse gastric tumors (*bottom*). β-Actin was used as an internal control. (*D*) The number of preneoplastic lesions developed in SPF-*K19-Wnt1* (*SPF*) and GF-*K19-Wnt1* (*GF*) mice. The mean numbers are indicated. (*E*) The mRNA levels of TNF-α and EphB3 in the gastric tumors of the anti-TNF-α neutralizing antibody-treated *Gan* (*αTNF-α*) relative to those of untreated control *Gan* mice (control) (mean ± SD). *$P < .05$ vs control. (*F*) Immunoblotting of active β-catenin in control (*cont*) and anti-TNF-α antibody-treated (*αTNF-α*) *Gan* mouse tumors.

cancer cells.[25] It is therefore possible that the decreased level of TNF-α in the GF-*Gan* and EP4i-*Gan* mouse stomachs resulted in suppression of Wnt signaling.

K19-Wnt1 mice develop preneoplastic lesions caused by promotion of Wnt signaling by macrophage-derived TNF-α[25] (Supplementary Table 1). Importantly, the number of preneoplastic lesions decreased significantly in the GF-*K19-Wnt1* mice compared with the SPF-*K19-Wnt1* mice (Figure 6*D*), suggesting that bacterial colonization is important for macrophage recruitment, which triggers TNF-α-induced Wnt promotion.

We next investigated whether TNF-α promotes Wnt signaling in the gastric tumor tissues. Treatment of the SPF-*Gan* mice with an anti-TNF-α neutralizing antibody resulted in down-regulation of TNF-α in tumors, possibly caused by suppression of macrophage activation (Figure 6*E*). Importantly, expression of EphB3, a Wnt-target gene, was also down-regulated. Consistently, the active β-catenin level decreased in gastric tumors by TNF-α inhibition (Figure 6*F*). These results indicate that macrophage-derived TNF-α enhances Wnt activity in gastric tumors, which may promote gastric tumorigenesis.

Discussion

Accumulating evidence has indicated that infection-associated inflammation plays an important role in cancer development.[2] Bacterial infection stimulates toll-like receptors, which induces activation of the nuclear factor-κB (NF-κB) pathway. The activation of NF-κB causes tumor promotion through induction of growth factors and suppression of apoptosis.[26] NF-κB activation

also induces COX-2 expression, which is followed by induction of the PGE_2 pathway. The COX-2/PGE_2 pathway plays a key role in intestinal tumorigenesis.[8,9] These results indicate that infection plays a key role in the activation of the NF-κB and PGE_2 pathways, which promotes tumorigenesis. In the present study, we infected *H felis* at 30 weeks of age to examine the effect of infection in tumorigenesis because we found a significant suppression of tumorigenesis in GF-*Gan* stomach at 30 weeks of age. Importantly, *H felis* infection in GF-*Gan* mice induced gastric tumor development by 55 weeks. Accordingly, the present study indicates that bacterial infection is still required for tumorigenesis even after the induction of the PGE_2 pathway.

It has been shown that PGE_2 signaling through EP4 receptor is important for intestinal tumorigenesis through the activation of epidermal growth factor receptor.[10] The current results also showed EP4 to play an important role in tumorigenesis. Bacterial colonization and EP4 signaling cooperatively induce expression of macrophage-tropic chemokine CCL2. It has been shown that CCL2 signaling is important for macrophage infiltration in colon cancers in the intestinal tumorigenesis.[22] Accordingly, it is possible that expression of CCL2 induced by bacterial colonization and EP4 signaling is important for macrophage recruitment in gastric tumorigenesis.

Intestinal commensal bacteria stimulate the toll-like receptors in the mucosa, which is important for the proliferation of undifferentiated epithelial cells.[16] Moreover, macrophages are an important niche component for the proliferation of intestinal progenitor cells in the tissue repair process.[27] Accordingly, it is possible that the innate immune response to commensal bacteria is important for the proliferation of tumor epithelial cells through macrophage recruitment. On the other hand, acquired immunity by T cells is essential for *H felis*-associated gastric pathology.[28] It is thus possible that T cells play a role in gastric tumorigenesis in *H felis*-infected GF-*Gan* mice. However, hyperplasia still developed, and the macrophages were polarized to M2, in the SPF-*Rag2*$^{-/-}$ *K19-C2mE* mouse stomach[29] (Supplementary Figure 5*D*), suggesting that increased PGE_2 levels and commensal flora can trigger these gastric phenotypes without T-cell response.

We previously showed that macrophage-derived TNF-α promotes Wnt signaling activity in gastric cancer cells, which contributes to gastric tumorigenesis.[25] Moreover, Wnt activation levels correlate with the incidence of intestinal tumorigenesis in *Apc* knockout mice,[30] and promotion of Wnt signaling activity may play an important role in malignant progression.[31] In the present study, inhibition of TNF-α resulted in a decrease in Wnt signaling activity in gastric tumors, confirming that TNF-α functions as a Wnt promoting factor in vivo. Notably, Wnt activity in the gastric tumors of GF-*Gan* mice was lower than that of SPF-*Gan* mice, which may have been caused by a decreased level of macrophage-derived TNF-α. Accordingly, it is possible that TNF-α-dependent Wnt promotion is one of the important mechanisms by which macrophages induce tumorigenesis, which is triggered by bacterial colonization and EP4 signaling.

The COX-2/PGE_2 pathway has been shown to suppress the T helper 1 immune response in the *H pylori*-infected stomach.[32] Notably, nonsteroidal anti-inflammatory drug treatment suppresses gastric carcinogenesis in the insulin-gastrin transgenic (INS-GAS) gastric tumor model mice. However, nonsteroidal anti-inflammatory drug treatment enhances gastritis in *Helicobacter*-infected INS-GAS mice, which may promote gastric tumorigenesis.[17] These results suggest that the PGE_2 pathway suppresses infection-associated carcinogenesis, which appears to be inconsistent with the present results. However, the present results indicate that commensal flora with low bacterial counts can elicit gastritis when the mucosal PGE_2 level is increased, and such commensal flora and PGE_2-dependent inflammation are important for gastric tumorigenesis. It is therefore possible that the role of PGE_2 for immune responses and tumorigenesis varies according to the level of infection status, such as exogenous aggressive infection by *Helicobacter* or commensal colonization, although this remains to be investigated.

In conclusion, bacterial infection or colonization, in cooperation with PGE_2 signaling through the EP4 receptor, induces expression of CCL2, resulting in macrophage recruitment to gastric mucosa. TNF-α produced by macrophages promotes Wnt signaling in the tumor cells, which may promote gastric tumorigenesis. Accordingly, the eradication and inhibition of the PGE_2 pathway may be an effective strategy for preventing gastric cancer development.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010

References

1. Correa P, Camargo MC, Piazuelo MB. Overview and pathology of gastric cancer. In: Wang TC, Fox JG, Giraud AS, eds. The biology of gastric cancer. New York: Springer, 2008:1–24.
2. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002; 420:860–867.
3. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow. Lancet 2001;357:539–545.
4. Mantovani A, Allavena P, Sica A, et al. Cancer-related inflammation. Nature 2008;454:436–444.
5. El-Omar EM, Carrington M, Chow WH, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404:398–402.