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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

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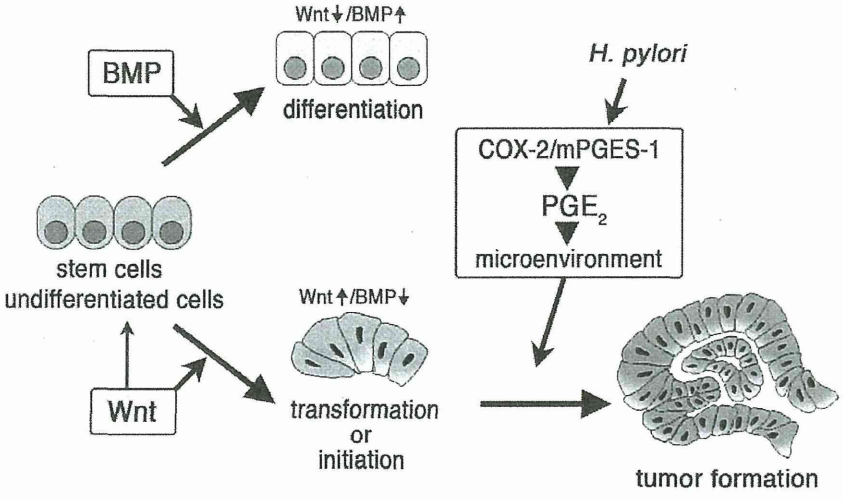

Figure 1 Schematic presentation of the connection of Wnt, BMP, and COX-2/PGE_2 signaling pathways in gastric tumorigenesis. 'Wnt activation' or 'BMP suppression' causes suppression of epithelial differentiation, which leads to transformation of epithelial cells. On the other hand, *Helicobacter pylori* 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

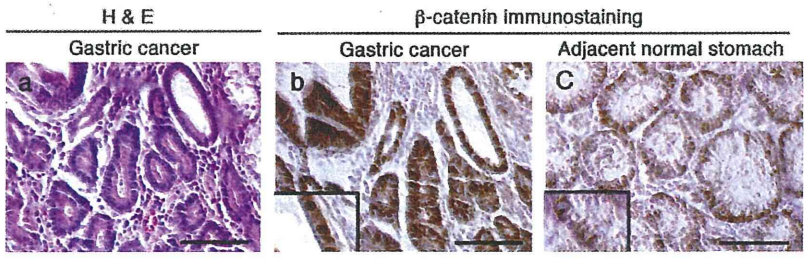

Figure 2 Nuclear localization of β-catenin in human gastric cancer. (**a**) Representative histological sections of gastric cancer (H&E). (**b**) Immunostaining for unphosphorylated (active) β-catenin in the adjoining section of (**a**) showing nuclear β-catenin accumulation. (**c**) Total β-catenin 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).

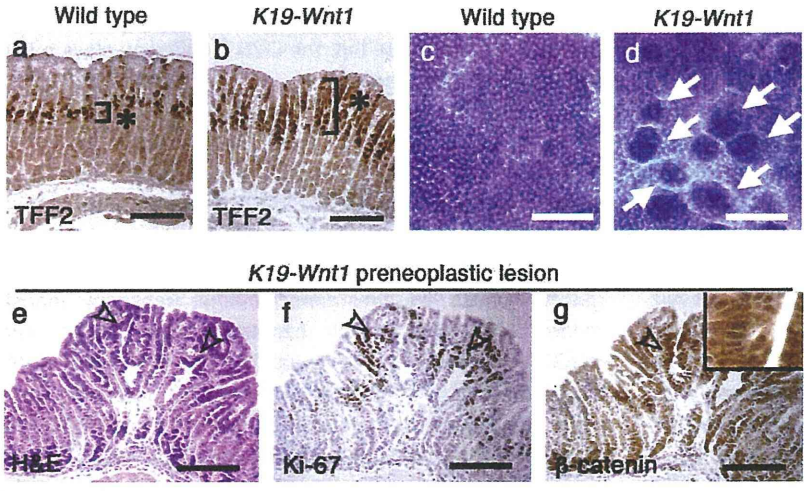

Figure 3 Gastric preneoplastic lesions in the *K19-Wnt1* mouse stomach. (**a,b**) TFF2-expressing undifferentiated epithelial cells (asterisks) in the glandular stomach of wild-type (**a**) and *K19-Wnt1* mice (**b**). Note that the number of TFF2 positive cells was increased in the *K19-Wnt1* mouse. Scale bars, 200 μm. (**c,d**) Toluidine 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).

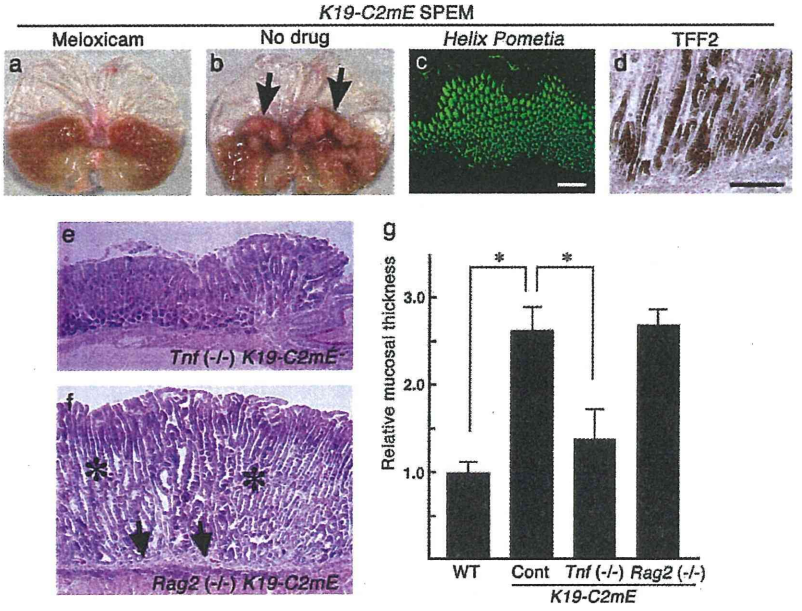

Figure 4 SPEM development in the *K19-C2mE* mouse stomach. (**a,b**) Representative photographs of the gastric mucosa of a meloxicam-treated *K19-C2mE* mouse (**a**) and a no-drug control *K19-C2mE* mouse (**b**). Arrows in (**b**) indicate hyperplastic lesions. (**c,d**) *Helix pometia* lectin staining (**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).

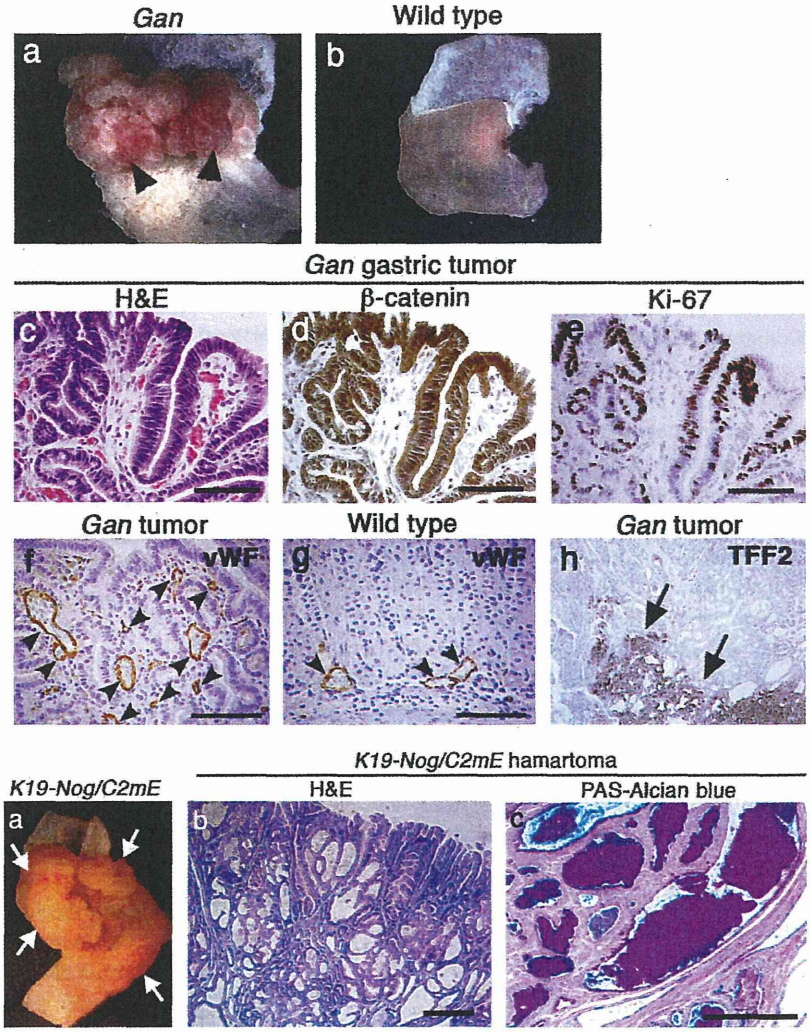

Figure 5 Gastric dysplastic tumors developed in the *K19-Wnt1/C2mE* (*Gan*) mice. (**a,b**) Representative photographs of the gastric mucosa of a *Gan* mouse (**a**) and a wild-type littermate mouse (**b**). Arrowheads indicate gastric tumors. (**c–e**) Histology of *Gan* mouse gastric tumors (H&E) (**c**), 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).

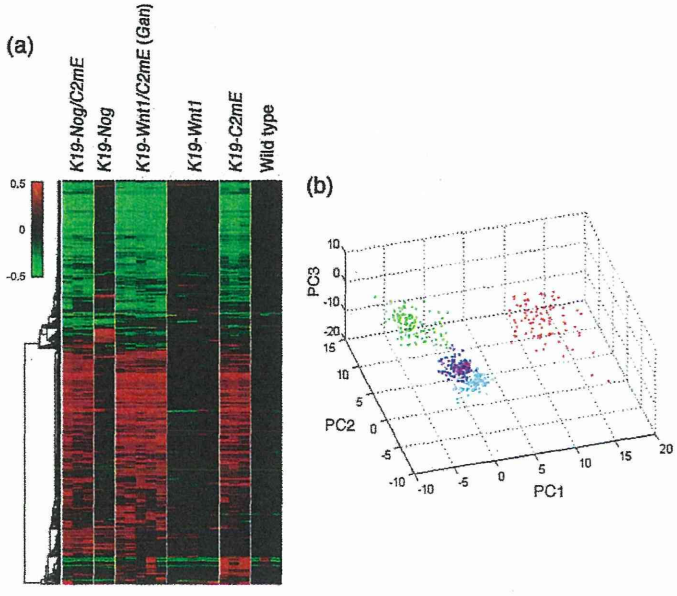

Figure 7 Genome-scale expression pattern of the respective genotype mouse models. (**a**) Clustered in rows are 5440 probe sets selected by genes whose expression levels were over 2-fold greater than the average of wild-type mice. Genotypes of mice are shown on the top. The red-green color scale indicates 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.

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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

Inactivation of chemokine (C-C motif) receptor 1 (CCR1) suppresses colon cancer liver metastasis by blocking accumulation of immature myeloid cells in a mouse model

Takanori Kitamura[a,1], Teruaki Fujishita[a,1], Pius Loetscher[b], Laszlo Revesz[b], Hiroki Hashida[c], Shinae Kizaka-Kondoh[d], Masahiro Aoki[a], and Makoto M. Taketo[a,2]

[a]Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; [b]Novartis Institutes for BioMedical Research, Novartis Pharma AG, CH-4056 Basel, Switzerland; [c]Department of Gastroenterological Surgery and Oncology, Kitano Hospital, Osaka 530-8480, Japan; and [d]Department of Radiation Oncology and Image-Applied Therapy, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

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Recent reports have suggested critical roles of myeloid cells in tumor invasion and metastasis, although these findings have not led to therapeutics. Using a mouse model for liver dissemination, we show that mouse and human colon cancer cells secrete CC-chemokine ligands CCL9 and CCL15, respectively, and recruit CD34$^+$ Gr-1$^-$ immature myeloid cells (iMCs). They express CCL9/15 receptor CCR1 and produce matrix metalloproteinases MMP2 and MMP9. Lack of the *Ccr1*, *Mmp2*, or *Mmp9* gene in the host dramatically suppresses outgrowths of disseminated tumors in the liver. Importantly, CCR1 antagonist BL5923 blocks the iMC accumulation and metastatic colonization and significantly prolongs the survival of tumor-bearing mice. These results suggest that CCR1 antagonists can provide antimetastatic therapies for patients with disseminated colon cancer in the liver.

chemokine | metalloproteinase | stromal cell

Colon cancer is one of the leading causes of cancer-related deaths (1). Although most primary tumors can be resected surgically, colorectal cancer frequently spreads to the liver, which is responsible for the high mortality of the disease (2). For successful metastasis, cancer cells need to invade surrounding tissues, penetrate microvessels, survive in circulation, disseminate to distant organs, form micrometastases, and expand into macrometastases. To progress through these steps, tumor cells often acquire the capability of survival and invasion by activating metastatic signaling pathways or inactivating metastasis suppressor genes (2, 3). In addition to these cell autonomous changes, tumor stromal cells, especially bone marrow-derived myeloid cells, actively participate in early steps of the metastatic cascade in some mouse models (4). For example, tumor-associated macrophages (TAMs) promote migration and intravasation of mammary tumor cells (5, 6). Bone marrow-derived cells that express myeloid cell marker CD11b and granulocyte marker Gr-1 (CD11b$^+$ Gr-1$^+$) also promote metastasis of breast cancer cells, likely through promotion of intravasation and suppression of immune responses (7). Furthermore, CD11b$^+$ myeloid cells that express vascular endothelial growth factor receptor 1 (VEGFR1) accumulate at the metastatic sites before the arrival of lung cancer and melanoma cells and foster the dissemination of the cancer cells (8). These reports suggest that bone marrow-derived myeloid cells can help cancer epithelium in early steps of metastasis. It remains to be determined whether therapeutics targeting such myeloid cells can prevent cancer metastasis (9).

As a model for invasive colon cancer, we previously constructed *cis-Apc*$^{+/\Delta 716}$ *Smad4*$^{+/-}$ (*Apc*/*Smad4*) mice that develop intestinal adenocarcinomas with marked invasions by loss of *Apc* and *Smad4* tumor suppressor genes in the intestinal epithelium (10, 11). In the *Apc*/*Smad4* tumors, we reported that the invading cancer epithelium is associated with immature myeloid cells (iMCs) that express myeloid progenitor cell marker CD34 and CD11b (12). Because these iMCs do not express Gr-1 or VEGFR1, they belong to a different subclass from the Gr-1$^+$ iMCs in breast cancer (7) or VEGFR1$^+$ myeloid cells in pre-metastatic niches (8). Using the *Apc*/*Smad4* mice, we further showed that the CD34$^+$ Gr-1$^-$ iMCs promote colon cancer invasion into the adjacent tissues (12).

However, whether colon cancer cells in the metastasizing sites can recruit the iMCs like those in the primary sites has not been investigated. Because the intestinal tumors in *Apc*/*Smad4* mice do not metastasize to the liver or lung during their short lifespan (11), and because no practical models are available whose endogenous (i.e., nontransplant) tumors progress and metastasize to the liver (or lung), here we have resorted to a transplantation model to determine whether colon cancer cells can recruit the iMCs in the metastasizing sites.

Results

Mouse Colon Cancer Cells Disseminated to the Liver Are Associated with iMCs. To investigate possible roles of the CD34$^+$ Gr-1$^-$ iMC subclass in colon cancer metastasis, we injected CMT93 mouse colon cancer cells (Table S1) into the spleen of syngeneic C57BL/6 mice, which allowed efficient dissemination of tumor cells to the liver. We found massive accumulations of stromal cells that expressed CD34, CD11b, and CD45, but not Gr-1 or VEGFR1 (Fig. 1*A* and Fig. S1*A*). We further confirmed that they did not express CD31 (a marker for endothelial cells), CD14 (monocytes), B2.20 (B-cells), CD3ε (T cells), or αSMA (myofibroblasts). These characteristics fit those of the iMC subclass in the primary colon cancer of *Apc*/*Smad4* mice (12). Of note, some of these stromal cells in the liver expressed macrophage marker F4/80 and dendritic cell marker CD11c that were absent in the *Apc*/*Smad4* primary tumors (Fig. S1*A*). We also verified their bone marrow-origin by transplanting bone marrow cells containing GFP into irradiated recipients (Fig. S1 *B* and *C*). Based on these results, we concluded that the cancer-associated stromal myeloid cells in the liver belong to the CD34$^+$ Gr-1$^-$ iMC subclass. Further analyses showed that the iMCs started to accumulate at day 7 postinjection when disseminated cancer cells began to form tumor glands. The iMCs accumulated further by day 14 with expansion of the tumor glands in the liver (Fig. 1*B*). By day 21, the

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Conflict of interest statement: P.L. and L.R. are employees of Novartis Pharma, AG that holds patents for BL5923.

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[1]T.K. and T.F. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: taketo@mfour.med.kyoto-u.ac.jp.

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