expression of miR-34b/c is suppressed by DNA methylation in *H. pylori*-associated gastric cancer cells (Suzuki *et al.*, 2010). Moreover, H3K27me3 leads to tumor-suppressor gene silencing in cancer (Kondo *et al.*, 2008). However, we showed that downregulation of miR-7 is not caused by genomic deletion nor by epigenetic mechanisms, but through stimulation by macrophage-derived factor(s). Several mechanisms for the regulation of miR-7 expression have been reported. For example, miR-7 transcription is directly induced by HoxD10 (Reddy *et al.*, 2008) or c-Myc (Chou *et al.*, 2010). Splicing factor SF2/ASF binds the pri-miR-7 to enhance its cleavage by Drosha (Wu *et al.*, 2010). Accordingly, it is conceivable that macrophage-derived molecule(s) directly downregulate miR-7 expression or indirectly suppress miR-7 expression through modulation of these regulation systems. The identification of responsible macrophage-derived molecule(s) will provide a novel mechanism by which macrophages promote tumorigenesis.

In conclusion, we showed that inflammation simultaneously induces upregulation of oncogenic miRNAs and downregulation of tumor-suppressor miRNAs, which promote tumorigenesis. The expression of miR-7 is induced during differentiation of gastric epithelial cells, suggesting a role for miR-7 in the regulation of epithelial cell differentiation. Accordingly, it is possible that the downregulation of miR-7 contributes to suppression of differentiation, resulting in the promotion of gastric tumorigenesis. Moreover, small molecule(s) expressed by activated macrophages are responsible for miR-7 repression, providing a link between inflammation and cancer. Therefore, miR-7 may be useful for devising a new preventive or therapeutic strategy against gastric cancer through the induction of cancer cell differentiation.

Materials and methods

Mouse models

Construction of *K19-C2mE* and *Gan* (*K19-Wnt1/C2mE*) mice was described previously (Oshima *et al.*, 2004, 2006). In brief, *K19-C2mE* mice express *Ptgs2* and *Ptges* in gastric epithelial cells, whereas *Gan* mice express *Ptgs2*, *Ptges* and *Wnt1*. For expression analyses, *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumor samples, and wild-type mouse stomach tissues were obtained at 30–40 weeks of age. Germfree mouse colonies were constructed as described previously (Oshima *et al.*, 2011), and the histology and miR-7 expression were examined at 55 weeks of age ($n=5$). *H. felis* (American Type Culture Collection 49179, ATCC, Manassas, VA, USA) were inoculated at 10^8 per mouse into wild-type mice at 6–8 weeks of age, and the histology and miRNA expression were examined at 20 weeks after infection ($n=6$). All animal experiments were carried out according to a protocol approved by the Committee on Animal Experimentation of the Kanazawa University.

Microarray analysis

Total RNA was extracted from mouse stomachs ($n=3$) using ISOGEN (Nippon Gene, Tokyo, Japan), pooled with the same genotype mouse RNAs, labeled with Cy3 and hybridized to Mouse miRNA microarray Rel. 12.0 (Agilent Technologies, Santa Clara, CA, USA). The raw data were normalized using the GeneSpring GX software program (Agilent Technologies), and expression levels of miRNAs in gastritis and gastric tumor tissues were compared with those in the wild-type mouse stomach. Transcripts with low signals (less than threefold of the background level) were not used for further analyses. The expression of miRNAs was further examined by real-time RT–PCR using RNA samples independently prepared from a different set of wild-type, *K19-C2mE* and *Gan* mice ($n=5$ for each genotype).

The results of cDNA microarray data sets of *K19-C2mE*, *Gan* and *K19-Nog/C2mE* mice were deposited into the Gene Expression Omnibus, as accession number GSE16902 (Itadani *et al.*, 2009), and were searched for the presence of novel miR-7 target genes using the TargetScan 5.1 program (MIT, Cambridge, MA, USA) (http://www.targetscan.org).

Real-time RT–PCR

Paired gastric cancer and non-tumor stomach tissue samples were obtained from 28 patients during surgery at the Kanazawa University Hospital, Japan. Fresh frozen tissues were used for expression analyses. Clinicopathological data of patients are shown in Supplementary Table 3. Human normal gastric epithelial cells were prepared by isolating the gastric glands from normal stomach tissues ($n=4$) as described previously (Cheng *et al.*, 1984). Approval for this project was obtained from the Kanazawa University Medical Ethics Committee, and written informed consent was obtained before specimen collection. Mouse stomachs were obtained from E15 wild-type mouse embryos, and day 0, day 7, day 14 and adult mice ($n=3$ for each). Total RNAs were extracted from tissues or cells using ISOGEN (Nippon Gene), and cDNAs for miRNAs and mRNAs were constructed using QuantiMir RT kit (System Bioscience, Mountain View, CA, USA) and the PrimeScript RT reagent Kit (Takara, Tokyo, Japan), respectively. Real-time RT–PCR was performed using SYBR Premix Ex TaqII (Takara, Tokyo, Japan) and Stratagene Mx3000P (Agilent Technologies). Sno202 and U44 were used as endogenous miRNA controls for mice and humans, respectively, whereas β-actin was used for endogenous mRNA control. The primer sequences for the miRNAs are shown in Supplementary Table 4. The primers for mRNAs were purchased from Takara.

Cell culture experiments

Mouse glandular stomachs were treated with 0.1% collagenase for 30 min at 37 °C, followed by centrifugation at 20 *g* for 3 min to isolate gastric glands. For the primary culture, isolated gastric glands were digested with trypsin and seeded on collagen-coated dishes as described previously (Oshima *et al.*, 2004). On day 2, the primary cultured cells were treated with 10 mM EDTA-phosphate-buffered saline and passaged. The primary cultured cells on day 2 (P0) and day 6 (P1) were used for the expression analysis. Human gastric cancer cell lines, AGS (ATCC), AZ521, MKN74, MKN45, NUGC4, HCT-111-TC, SH-10-TC (RIKEN BioResource Center, Tsukuba, Japan), Kato-III and MKN7 (Cell Resource Center for Biomedical Research, Tohoku University, Japan) were used in this study.

The pre-miR miRNA-7 (Pre-miR-7), pre-miR negative control (Pre-miR-NC) and anti-miR-7 inhibitor (Ambion, Austin, TX, USA) were used for transfection. The cell proliferation rate was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). For the soft agar colony

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

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

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

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

Luciferase reporter assay
Constructions of the miR-7 luciferase reporter vector (miR-7 Luc) and control vector (control Luc) are shown in Supplementary Figure 1. Luciferase reporter vectors for the *LPHN2*, *BASP1* and *MAFG* genes were constructed by subcloning 3′ untranslated region fragments of the respective human genes into the pGL3 plasmid (Promega, Madison, WI, USA). PCR primer sequences for cloning 3′ untranslated region of the respective genes and amplified fragment lengths are indicated in Supplementary Table 4. The luciferase activity was measured using a Luciferase Assay System (Promega), and the levels were normalized to the total protein levels detected using the Pierce 660 nm Protein Assay (Thermo Scientific, Yokohama, Japan). RAW264 cells (RIKEN BioResource Center) and mouse intraperitoneal macrophages were stimulated with 10 ng/ml lipopolysaccharide (Sigma, St Louis, MO, USA) for 24 h and the conditioned medium was collected as CM(+). The conditioned medium of unstimulated macrophages was collected as CM(−). The conditioned medium of lipopolysaccharide-stimulated and COX-2 inhibitor celecoxib-treated (Pfizer, New York, NY, USA) (10 μM) RAW264 cells was collected as CM(+/coxib). CM(+) was fractionated by ultrafiltration using Centricon Plus-70 (Millipore) to prepare CM(+) > 100 kDa, 30–100 kDa, 3–30 kDa and < 3 kDa. Cells were stimulated with CM at a 50% concentration.

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

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

Conflict of interest

The authors declare no conflict of interest.

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REVIEW

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

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Abstract Accumulating evidence has indicated that inflammatory responses are important for cancer development. Epidemiological studies have shown that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of colon cancer development. Subsequently, mouse genetic studies have shown that cyclooxygenase (COX)-2, one of the target molecules of NSAIDs, and its downstream product, prostaglandin E_2 (PGE_2), play an important role in gastrointestinal tumorigenesis. Bacterial infection stimulates the Toll-like receptor (TLR)/MyD88 pathway in tumor tissues, which leads to the induction of COX-2 in stromal cells, including macrophages. Induction of the COX-2/PGE_2 pathway in tumor stroma is important for the development and maintenance of an inflammatory microenvironment in gastrointestinal tumors. In such a microenvironment, tumor-associated macrophages express proinflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-6, and these cytokines, respectively, activate the nuclear factor (NF)-κB and Stat3 transcription factors in epithelial cells, as well as in stromal cells. Recent mouse studies have uncovered the role of such an inflammatory network in the promotion of gastrointestinal tumor development. Genetically engineered and chemically induced mouse tumor models which mimic sporadic or inflammation-associated tumorigenesis were used in these studies. In this review article, we focus on mouse genetic studies using these tumor models, which have contributed to the elucidation of the molecular mechanisms associated with the inflammatory network in gastrointestinal tumors, and we also discuss the role of each pathway in cancer development. The involvement of immune cells such as macrophages, mast cells, and regulatory T cells in tumor promotion is also discussed.

Keywords Gastrointestinal cancer · Inflammation · COX-2 · NF-κB · Stat3

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Introduction

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

Epidemiological studies have revealed that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) lowers the mortality rate from cancers in the gastrointestinal tract [10, 11]. The target molecules of NSAIDs are cyclooxygenase (COX)-1 and COX-2, and accumulating evidence has indicated that COX-2 and its downstream product, prostaglandin E_2 (PGE_2), play an important role in cancer

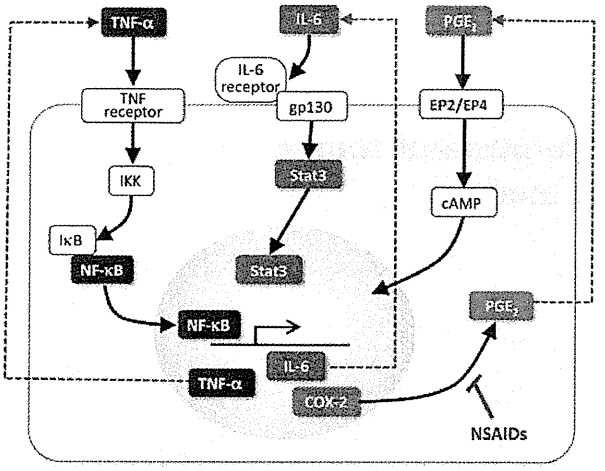

Fig. 1 The interaction of the cyclooxygenase-2 (*COX-2*)/prostaglandin E_2 (*PGE_2*), tumor necrosis factor-α (*TNF-α*)/nuclear factor-κB (*NF-κB*), and interleukin-6 (*IL-6*)/gp130/Stat3 pathways in the inflammatory environment. *cAMP* cyclic AMP, *IKK* inhibitor of κB kinase, *IκB* inhibitor of κB,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

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

Mouse models of gastrointestinal cancer

The roles of inflammatory responses in gastrointestinal cancers have been studied using several tumor mouse models (Table 1). $Apc^{\Delta 716}$ knockout mice and Apc^{Min} mice carry heterozygous truncation mutations at codons 716 and 850 of the mouse *Apc* gene, respectively, and somatic deletion of the wild-type *Apc* gene results in the activation of Wnt/β-catenin signaling, which causes tumor development in the entire intestinal tract [14, 15]. Approximately 80% of colorectal cancers harbor *APC* gene mutations and half of the remainder have mutations in *Ctnnb1* gene encoding β-catenin, both of which activate Wnt/β-catenin signaling [16–18]. Thus, $Apc^{\Delta 716}$ and Apc^{Min} mice recapitulate the molecular mechanism of sporadic colon cancer development. Several other types of *Apc* mutant mice also develop intestinal polyposis (as described in this review and Table 1).

On the other hand, inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are also risk factors for colorectal cancer [19, 20]. IBD-related colon cancers are associated with a severe inflammatory response, which is an important microenvironment required for inflammation-associated tumor development. The treatment of mice with a genotoxic chemical carcinogen, azoxymethane (AOM), followed by a non-genotoxic agent, dextran sodium sulfate (DSS), induces the development of colitis-associated colon cancer (CAC) [21]. Mutations in *Ctnnb1* gene induced by AOM result in the activation of the Wnt/β-catenin pathway, which is thought to trigger tumor initiation [22]. On the other hand, DSS induces colonic inflammation in rodents, which is required for tumor promotion. Accordingly, the AOM/DSS model is a well-established and widely used mouse model for CAC development, and it mimics IBD-related colon cancer (Table 1). *Rag2* gene-deficient mice lack functional lymphocytes, and are susceptible to infection-induced inflammation in the colon. When *Rag2−/−* mice are infected with the enteric bacterial pathogen, *Helicobacter hepaticus*, the mice rapidly develop CAC [23, 24]. This model system is also used for IBD-related colon cancer (Table 1).

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

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

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

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

Tumor model	Mouse line crossed/treatment	Tumor phenotype changes	References
Sporadic intestinal tumor model (*Apc* mutant mice)			
$Apc^{\Delta 716}$	*Ptgs2*[a] knockout mice	Suppression of intestinal polyposis	[34]
Apc^{Min}	*Ptgs1*[b], *Ptgs2* knockout mice	Suppression of intestinal polyposis	[35]
Apc^{Min}	*Hpgd*[c] knockout mice	Increase of colon polyps	[36]
AOM	*Ptgs2* transgenic mice	Increase of intestinal tumor	[37]
$Apc^{\Delta 716}$	*Ptger2*[d] knockout mice	Suppression of intestinal polyposis	[38]
		Inhibition of angiogenesis	[39]
Apc^{Min}	PGE_2 treatment	Promotion of intestinal polyposis	[40]
$Apc^{\Delta 14}$	*Ptges*[e] knockout mice	Suppression of intestinal polyposis	[44]
AOM	*Ptges* knockout mice	Suppression of intestinal polyposis	[45]
Apc^{Min}	*Myd88*[f] knockout mice	Suppression of intestinal polyposis	[63, 64]
		Epithelial expression of MyD88 is important	
$Apc^{\Delta 716}$	*op/op* (macrophage-deficient)	Suppression of intestinal polyposis	[93]
$Apc^{\Delta 468}$	$Kit^{W/W}$ (mast cell-deficient)	Suppression of intestinal polyposis	[76]
$Apc^{\Delta 468}$	*Rag2−/−* (lymphocyte-deficient)	Not affected	
$Apc^{\Delta 468}$	Anti-TNF-α antibody	Suppression of intestinal polyposis	
Apc^{Min}	$CD4^+$ $CD25^+$ T cell transfer	Suppression of intestinal polyposis	[94, 95]
		IL-10 expression in T cells is required	
Apc^{Min}	*Il17a*[g] knockout mice	Suppression of intestinal polyposis	[97]
Inflammation-associated colon tumor model (AOM/DSS-CAC model mice)			
AOM/DSS	*Tlr4* [h] knockout mice	Suppression of CAC development	[60, 62]
		Epithelial expression of TLR4 is important	
AOM/DSS	*Ptgs2* knockout mice	Exacerbation of CAC development	[68, 69]
AOM/DSS	*Ikbkb*[i] conditional KO	Suppression of CAC development	[72]
		Epithelial and myeloid expression is important	
AOM/DSS	*Tnfrsf1a*[j] knockout mice	Suppression of CAC development	[73]
		Myeloid expression of TNF-Rp55 is important	
AOM/DSS	*Ccr2*[k] knockout mice	Suppression of CAC development	[74]
		Less macrophage infiltration	
AOM/DSS	*Il6* [l] knockout mice	Suppression of CAC development	[82]
AOM/DSS	$gp130^{757F/F}$	Suppression of CAC development	[81]
AOM/DSS	*Stat3* conditional KO	Suppression of CAC development	[81, 82]
		Epithelial expression of Stat3 is important	
H. hepaticus infected Rag2−/−	$CD4^+$ $CD25^+$ T-cell transfer	Suppression of CAC development	[23, 24]
		IL-10 expression in T cells is required	
Gastritis and gastric tumor model			
K19-Wnt1	*K19-C2mE*	Gastric tumor development (*Gan* mice)	[26, 30, 31]
Gan	Celecoxib, EP4 inhibitor	Suppression of gastric tumorigenesis	[48, 49]
Gan	Clodronate liposome (macrophage-deficient)	Atrophic changes of tumor cells	[49]
K19-C2mE	*Tnf*[m] knockout mice	Suppression of gastritis/hyperplasia	[78]
K19-C2mE	*Rag2* knockout mice	Not affected	
$gp130^{757F/F}$	*Il11ra1*[n] knockout mice	Suppression of gastric tumorigenesis	[88]

AOM azoxymethane *DSS* dextran sodium sulfate, *CAC* colitis-associated colon cancer, *IL* interleukin, *TNF* tumor necrosis factor, *COX* cyclooxygenase, *15-PGDH* 15-hydroxyprostaglandin dehydrogenase, *TLR* Toll-like receptor, *mPGES-1* microsomal PGE synthase-1, *KO* knockout. Gene symbols used are: [a]COX-2, [b]COX-1, [c]15-PGDH, [d]PGE_2 receptor EP2, [e]mPGES-1, [f]MyD88, [g]IL-17A, [h]TLR-4, [i]IKKβ, [j]TNF-Rp55, [k]CCR2, [l]IL-6, [m]TNF-α, and [n]IL-11 receptor-α

significant regression of colon polyps [32]. Moreover, a large number of animal experiments have shown that treatment with NSAIDs suppressed chemical carcinogen-induced colon tumorigenesis [33]. As a target molecule of NSAIDs, the inducible enzyme COX-2 plays an important role in inflammation and cancer, while COX-1 is expressed constitutively and functions as a house-keeping gene. Importantly, disruption of *Ptgs2* gene in $Apc^{\Delta 716}$ mice and Apc^{Min} mice resulted in a significant suppression of intestinal tumorigenesis, thus indicating an essential role of COX-2 in intestinal polyp development [34, 35]. Interestingly, disruption of *Ptgs1* gene encoding COX-1 also suppressed intestinal tumorigenesis [35]. It is possible that COX-1-derived prostaglandins are required for tumor cell proliferation during the initial stage when COX-2 expression is not yet induced [33]. Other lines of genetic evidence also support the role of the COX-2 pathway in intestinal tumorigenesis. Prostaglandins are catalyzed and inactivated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). The number of colon polyps in Apc^{Min} mice was markedly increased when *Hpgd* gene encoding 15-PGDH was disrupted, suggesting that PGE_2 has a role in colon tumorigenesis [36]. Moreover, the transgenic expression of *Ptgs2* in the mouse intestine accelerated chemical carcinogen-induced tumorigenesis [37].

COX-2 catalyzes the synthesis of prostaglandin (PG) H_2, which is then converted to PGE_2. There are four G protein-coupled receptors for PGE_2; EP1, EP2, EP3, and EP4. Notably, disruption of *Ptger2* gene encoding EP2 caused significant suppression of intestinal polyposis in $Apc^{\Delta 716}$ mice, whereas suppression of EP1 or EP3 signaling did not affect tumorigenesis [38]. EP2 signaling increases the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which enhances angiogenesis in intestinal tumors [39]. Moreover, treatment of Apc^{Min} mice with PGE_2 increased the development of intestinal tumors through the activation of peroxisome proliferators-activated receptor (PPAR) δ, which promotes the survival of tumor cells [40]. Furthermore, PGE_2 signaling through the EP2 receptor has been shown to activate Wnt/β-catenin signaling directly in colon cancer cells by the suppression of β-catenin phosphorylation [41]. Accordingly, it is possible that the COX-2/PGE_2 pathway contributes to intestinal tumorigenesis through a variety of PGE_2 functions [12, 13] (Fig. 2).

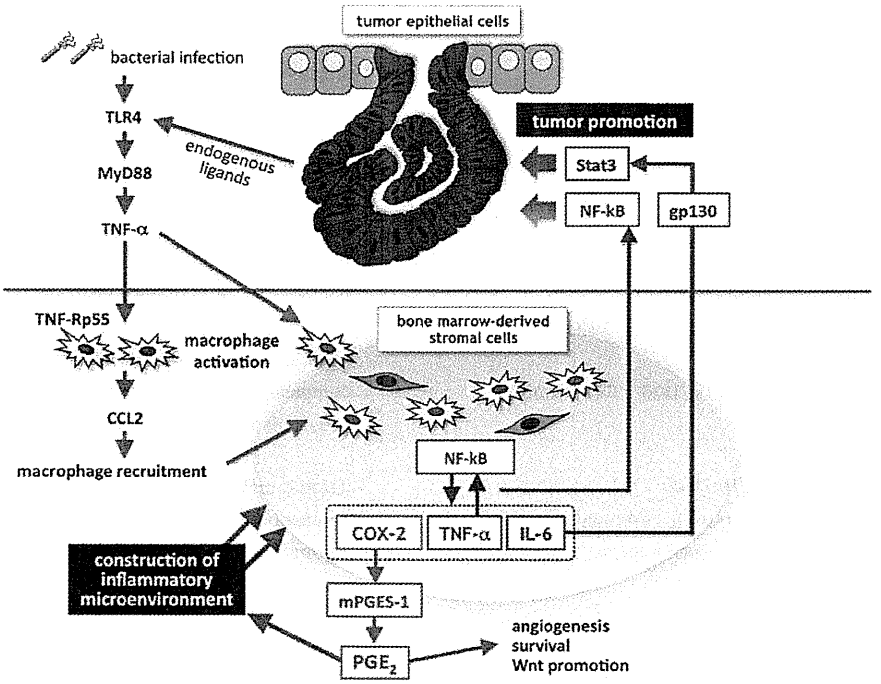

Fig. 2 A schematic diagram of the inflammatory microenvironment in gastrointestinal tumor tissues. Bacterial infection or endogenous ligand activates the Toll-like receptor (*TLR*)/MyD88 pathway in epithelial cells, which further activate stromal macrophages inducing CCL2. In the activated macrophages, NF-κB induces the expression of COX-2, IL-6, and TNF-α itself. The COX-2/PGE_2 pathway is important for the construction and maintenance of the inflammatory network, and PGE_2 accelerates angiogenesis, cell survival, and Wnt activation. TNF-α activates NF-κB in both epithelial cells and stromal macrophages, whereas IL-6 activates Stat3 in epithelial cells through gp130. NF-κB and Stat3 play an important role in the promotion of tumorigenesis through suppression of apoptosis and acceleration of the cell cycle. *mPGES-1* Microsomal PGE synthase-1

Microsomal PGE synthase-1 (mPGES-1) is an enzyme that converts PGH_2 to PGE_2, and expression of mPGES-1 is induced in gastric and colon cancers similar to COX-2 [42, 43]. Notably, disruption of *Ptges* gene encoding mPGES-1 in $Apc^{\Delta 14}$ mice or AOM-treated mice resulted in a marked decrease in the PGE_2 level in the intestinal mucosa, which led to a further significant suppression of intestinal tumorigenesis [44, 45]. Taken together, these results indicate that the simultaneous expression of COX-2 and mPGES-1 is required for intestinal tumorigenesis through the induction of PGE_2 signaling.

In gastric cancer tissues, induction of COX-2 is found in approximately 70% of cases [46, 47], and mPGES-1 expression is also induced [42], suggesting that the COX-2/PGE_2 pathway is also important for gastric tumorigenesis. In the *K19-C2mE* transgenic mice, an increased PGE_2 level causes inflammatory infiltration and metaplastic hyperplasia in the gastric mucosa [29]. Although Wnt activation alone is not sufficient for tumor development in *K19-Wnt1* mice, *Gan* mice (compound mutants of *K19-Wnt1* and *K19-C2mE* mice) develop inflammation-associated gastric tumors with 100% incidence [26], indicating that cooperation of the Wnt and PGE_2 pathways can lead to gastric tumorigenesis. Moreover, gastric inflammation and tumorigenesis were significantly suppressed in *Gan* mice when the mice were treated with a COX-2 inhibitor, celecoxib, or an EP4 receptor inhibitor [48, 49]. These results indicate that COX-2/PGE_2/EP4-induced inflammation is involved in the development of gastric cancer. Signaling through both EP2 and EP4 stimulates the intracellular cyclic AMP signaling pathway. It is therefore possible that either EP2 or EP4 receptor signaling plays an important role in gastrointestinal tumorigenesis (Fig. 1).

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

Expression of COX-2 and mPGES-1 is detected predominantly in stromal cells, including macrophages and fibroblasts, but not in the epithelial cells of mouse intestinal polyps, and the same is true for human colon polyps [36, 50–52]. Heterozygous mutations in the *Lkb1*, *Smad4*, or *Cdx2* gene lead to the development of gastrointestinal hamartomas, which show histological characteristics distinct from those of dysplastic adenomas developed in *Apc* mutant mice [52–55]. Notably, expression of COX-2 and mPGES-1 is detected in the stromal cells, but not in tumor epithelial cells, in these models [56], thus indicating that the COX-2/PGE_2 pathway is induced in the tumor stroma by a common mechanism, regardless of the type of tumor.

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

The treatment of mice with DSS induces COX-2 expression and PGE_2 production, predominantly in macrophages of the inflamed colon mucosa. However, such COX-2 induction is not found in the mice lacking the TLR4/MyD88 pathway [59], thus indicating the role of bacterial infection in inducing COX-2 expression in colitis tissues. Importantly, AOM/DSS treatment-induced colon tumor development was dramatically suppressed in *Tlr4−/−* mice [60], while exogenous administration of PGE_2 promoted CAC development in the AOM/DSS-treated *Tlr4−/−* mice [61]. Moreover, bone marrow chimera experiments have indicated that TLR4 expression in the intestinal epithelial cells, but not in myeloid cells, is required for CAC development [62]. Taken together, these results suggest that bacterial infection stimulates the TLR/MyD88 pathway in epithelial cells, which leads to activation of stromal macrophages, thus resulting in the induction of the COX-2/PGE_2 pathway in the tumor stroma (Fig. 2).

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

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

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

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

Although COX-2 inhibition causes suppression of gastrointestinal tumorigenesis, treatment with NSAIDs or a COX-2 inhibitor exacerbates DSS-induced colon injury in rodent models [66]. Consistently, *Ptgs2* gene knockout mice exposed to DSS showed exacerbated phenotypes, such as more severe inflammation, compared with wild-type mice [67]. One of the functions of PGE_2 is to protect the gastrointestinal mucosa. Therefore, COX-2 is important for mucosal protection and regeneration in the DSS-treated mouse colon. Notably, treatment of *Ptgs2* knockout mice with AOM/DSS resulted in a significant increase in tumors, with severe inflammatory responses [68, 69], which appears to be contradictory to the results showing that *Ptgs2* gene disruption causes significant suppression of intestinal polyposis in *Apc* mutant mice [34, 35]. It is possible that in the case of severe UC, cytokine signaling is highly activated and may be sufficient for tumor promotion if the COX-2/PGE_2 pathway is blocked (Fig. 2).

The TNF-α/NF-κB pathway in gastrointestinal tumorigenesis

TNF-α is one of key regulators of inflammatory responses. Although TNF was originally recognized as a tumor-necrotizing factor, accumulating evidence has indicated that TNF-α has tumor-promoting functions [70]. TNF-α signaling activates NF-κB, which further induces the expression of inflammatory factors including COX-2, IL-6, IL-8, and TNF-α itself (Fig. 1). Several genetic studies have demonstrated a link between the TNF-α/NF-κB pathway and cancer development [71].

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

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

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

The role of the TNF-α/NF-κB pathway in gastric tumorigenesis in *Gan* mice has not yet been examined. However, *Tnf−/− K19-C2mE* mice showed significant suppression of gastritis and hyperplasia compared with control *K19-C2mE* mice, although the COX-2/PGE_2

pathway was still activated by the transgenic expression of COX-2 and mPGES-1 [78]. It is thus conceivable that the TNF-α/NF-κB pathway is activated in PGE_2-associated gastritis, and that this contributes to inflammation-associated gastric tumorigenesis.

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

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

It has been shown that $gp130^{757F/F}$ mice develop gastric tumors with abundant infiltration of inflammatory cells [85]. Moreover, heterozygous mutations of the Stat3 gene in $gp130^{757F/F}$ mice reduced the incidence and multiplicity of gastric tumors, with the suppression of inflammatory responses [86, 87]. These results indicate that Stat3 is also an important tumor-promoting factor in gastric tumorigenesis, and that it is activated by the inflammatory network of the tumor microenvironment. Transforming growth factor (TGF)-β signaling promotes epithelial differentiation, and thus, suppression of the TGF-β signaling pathway has been thought to promote gastrointestinal tumorigenesis. Notably, the activation of Stat3 in $gp130^{757F/F}$ cells desensitizes them to TGF-β by inducing inhibitory Smad7. This may be one of the mechanisms by which Stat3 promotes tumor formation [86]. IL-11 is another member of the IL-6 cytokine family and also signals through gp130. Interestingly, disruption of the IL-11 co-receptor in $130^{757F/F}$ mice significantly ablated gastric tumorigenesis [88]. Because IL-11 is upregulated in human and mouse gastric tumors, these results suggest that the IL-11/Stat3 pathway, together with IL-6/Stat3, promotes gastric tumorigenesis.

Inflammatory cells that promote or suppress tumorigenesis

The major source of inflammatory cytokines and prostaglandins in tumor tissues is macrophages. Tumor-associated macrophages (TAMs) have been shown to promote the progression and metastasis of cancer [89]. TAMs can be classified into several distinct groups by their functions, such as inducing inflammation, invasion, angiogenesis, or metastasis. Macrophages are polarized to either the classical M1 type or the alternative M2 type, and it has been suggested that TAMs are polarized to M2 or M2-like types [90]. It has been shown that CD4+ T cells regulate the polarization of macrophages to the M2 type in mammary tumors [91]. It has recently been shown that COX-2 is important for the M2-polarization of TAMs in Apc^{Min} mouse tumors [92]. Accordingly, it is possible that the COX-2/PGE_2 pathway-induced inflammatory network is important for the education of macrophages into the pro-tumorigenic M2 or M2-like types. Importantly, depletion of functional macrophages in $Apc^{\Delta 716}$ mice by crossing them with op/op mutant mice resulted in significant suppression of intestinal polyposis [93]. Moreover, inhibition of macrophage recruitment in AOM/DSS-treated mice by *Ccl2* gene disruption resulted in the suppression of colon tumor development [74]. Accordingly, macrophages play an important role in both sporadic and IBD-related intestinal tumorigenesis (Fig. 2). In *Gan* mouse gastric tumors, macrophage depletion caused atrophic changes of the tumor cells and apoptosis of stromal cells [49]. Accordingly, it is possible that macrophages play a role in the maintenance of both tumor epithelial cells and stromal cells.

In the $Apc^{\Delta 468}$ mouse intestinal tumors, mast cells are preferentially enriched in the polyp tissues, and these mast cells express TNF-α [76]. Notably, the depletion of mast cells in $Apc^{\Delta 468}$ mice by bone marrow transplantation from $Kit^{Wsh/Wsh}$ mice caused significant suppression of intestinal polyposis, with a decreased level of TNF-α expression. It is therefore possible that both macrophages and mast cells are important components of the inflammatory network in the tumor microenvironment.

Bone marrow transplantation from *Rag2−/−* mice did not affect the intestinal tumorigenesis in $Apc^{\Delta 468}$ mice [76]. Consistently, the gastritis phenotype was not altered in the *K19-C2mE Rag2−/−* mouse stomach [78]. These results indicate that lymphocytes are not required for the construction of the inflammatory network and the promotion of tumorigenesis in the gastrointestinal tract. Importantly, however, the adaptive transfer of $CD4^+$ $CD25^+$ regulatory T cells to Apc^{Min} mice dramatically reduced the number of intestinal polyps, with the induction of necrosis of tumor cells [94, 95]. Moreover, such regression of intestinal

polyps was not found when $CD4^+$ $CD25^+$ cells were prepared from *Il10*−/− mice.

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

Concluding remarks

The development of the inflammatory network in tumor tissues and its possible roles are summarized in Fig. 2. Chronic infection or endogenous ligands derived from tumor cells stimulate the TLRs of epithelial cells, leading to the activation of MyD88. The activation of the epithelial TLR/MyD88 pathway further induces COX-2 expression and PGE_2 production in stromal macrophages through the TNF-α/NF-κB pathway. Epidemiological studies and genetic experiments have demonstrated that COX-2 and its downstream product, PGE_2, play an important role in gastrointestinal tumorigenesis. NF-κB is activated by TNF-α in TAMs, which further induces the expression of TNF-α, IL-6, and COX-2. TNF-α in turn stimulates both tumor epithelial cells and stromal cells, activating NF-κB in these cells, which promotes tumorigenesis. On the other hand, IL-6 activates Stat3 through gp130 in epithelial cells, thus leading to an increase in cell cycling and a decrease of apoptosis. The induction of the COX-2/PGE_2 pathway is important for the development of such an inflammatory tumor microenvironment. When the TNF-α/NF-κB and/or IL-6/Stat3 pathways are activated beyond a threshold, they can promote tumorigenesis if the COX-2/PGE_2 pathway is inhibited. In the inflammatory microenvironment, not only TAMs, but also mast cells and IL-17-expressing T cells, infiltrate and contribute to tumor development. Therefore, targeting the inflammatory network in tumor tissues by the inhibition of PGE_2, NF-κB, Stat3, or downstream pathways may provide an effective preventive or therapeutic strategy against gastrointestinal cancer.

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Conflict of interest None.

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Activation of epidermal growth factor receptor signaling by the prostaglandin E_2 receptor EP4 pathway during gastric tumorigenesis

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Cyclooxygenase-2 (COX-2) plays an important role in tumorigenesis through prostaglandin E_2 (PGE_2) biosynthesis. It has been shown by *in vitro* studies that PGE_2 signaling transactivates epidermal growth factor receptor (EGFR) through an intracellular mechanism. However, the mechanisms underlying PGE_2-induced EGFR activation in *in vivo* tumors are still not fully understood. We previously constructed transgenic mice that develop gastric tumors caused by oncogenic activation and PGE_2 pathway induction. Importantly, expression of EGFR ligands, epiregulin, amphiregulin, heparin-binding EGF-like growth factor, and betacellulin, as well as a disintegrin and metalloproteinases (ADAMs), ADAM8, ADAM9, ADAM10, and ADAM17 were significantly increased in the mouse gastric tumors in a PGE_2 pathway-dependent manner. These ADAMs can activate EGFR by ectodomain shedding of EGFR ligands. Notably, the extensive induction of EGFR ligands and ADAMs was suppressed by inhibition of the PGE_2 receptor EP4. Moreover, EP4 signaling induced expression of amphiregulin and epiregulin in activated macrophages, whereas EP4 pathway was required for basal expression of epiregulin in gastric epithelial cells. In contrast, ADAMs were not induced directly by PGE_2 in these cells, suggesting indirect mechanism possibly through PGE_2-associated inflammatory responses. These results suggest that PGE_2 signaling through EP4 activates EGFR in gastric tumors through global induction of EGFR ligands and ADAMs in several cell types either by direct or indirect mechanism. Importantly, gastric tumorigenesis of the transgenic mice was significantly suppressed by combination treatment with EGFR and COX-2 inhibitors. Therefore, it is possible that inhibition of both COX-2/PGE_2 and EGFR pathways represents an effective strategy for preventing gastric cancer. (*Cancer Sci* 2011; 102: 713–719)

It has been established that induction of cyclooxygenase 2 (COX-2) plays an important role in cancer development.[1,2] Genetic mouse model studies indicated that prostaglandin E_2 (PGE_2), a downstream products of COX-2, plays a key role in intestinal tumorigenesis,[3–5] suggesting that the PGE_2 pathway is a possible target for the chemoprevention. On the other hand, epidermal growth factor receptor (EGFR) signaling is also an important target for cancer prevention.[6] Inhibition of EGFR signaling in Apc^{Min} mice, a model of familial adenomatous polyposis, significantly suppresses intestinal polyposis.[7–9] Importantly, combination treatment using an EGFR inhibitor with non-steroidal anti-inflammatory drugs or a COX-2 inhibitor dramatically suppresses intestinal tumorigenesis.[8,9] It has been shown by *in vitro* experiments that PGE_2 signaling transactivates EGFR through activation of cSrc[10,11] or MMPs[12], as well as induction of amphiregulin, an EGFR ligand[13,14] or tumor necrosis factor-α converting enzyme/a disintegrin and metalloproteinase 17 (TACE/ADAM17), a shedding enzyme for amphiregulin.[15] However, the mechanism responsible for the activation of EGFR by the PGE_2 pathway in *in vivo* tumors has not been fully elucidated. Induction of the PGE_2 pathway in the gastric mucosa causes development of inflammatory microenvironment consisting of macrophages and myofibroblasts.[16,17] It is therefore possible that PGE_2 signaling in such microenvironment contributes to EGFR activation in tumors, and that PGE_2-associated inflammatory responses are also involved in EGFR activation.

Gastric cancer is one of the most frequently diagnosed and lethal malignancies worldwide, with a 5-year survival of only about 20%.[18] COX-2 expression is induced in more than 70% of gastric cancers,[19] and regular use of non-steroidal anti-inflammatory drugs decreases the risk of gastric cancer,[20] suggesting a role of COX-2 pathway in gastric tumorigenesis. In addition to COX-2, activation of Wnt signaling is found in 30–50% of gastric cancers.[21,22] Based on these results, we constructed *K19-Wnt1/C2mE* transgenic mice expressing *Wnt1*, *Ptgs2*, and *Ptges* encoding Wnt1, COX-2, and microsomal prostaglandin E synthase-1, respectively, in gastric mucosa.[22] *K19-Wnt1/C2mE* mice (*Gan* mice for gastric neoplasia) develop gastric tumors caused by the simultaneous activation of Wnt and PGE_2 pathways, although Wnt activation alone results in the development of only small dysplastic lesions. Gene expression profiles of *Gan* mouse tumors were similar to those of human intestinal-type gastric cancer.[23] We also constructed *K19-Nog/C2mE* transgenic mice that express *Nog* encoding noggin, together with *Ptgs2* and *Ptges*.[24] Noggin is an endogenous antagonist for bone morphogenetic protein signaling. *K19-Nog/C2mE* mice develop gastric hamartomas, although *Nog* expression alone does not cause any morphological changes. These results indicate that induction of the PGE_2 pathway plays a key role in the promotion of gastric tumorigenesis, regardless of the types of underlying oncogenic pathway such as Wnt activation or bone morphogenetic protein suppression.[25]

Using these mouse models, we have investigated the mechanism of EGFR activation by the PGE_2 pathway in gastric tumorigenesis. We also examined the role of EGFR signaling in the *in vivo* tumor development by drug dosing experiments.

Materials and Methods

Mouse models. Construction of *K19-Wnt1*, *K19-C2mE*, *K19-Nog*, *K19-Wnt1/C2mE* (*Gan*), and *K19-Nog/C2mE* mice was described previously.[25] Briefly, both *Ptgs2* and *Ptges* are expressed in the *K19-C2mE* mouse stomach, whereas *Wnt1* and *Nog* are expressed in *K19-Wnt1* and *K19-Nog* mice, respectively. Expression of these genes is regulated by the *Krt19* gene promoter that is transcriptionally active in gastric epithelial cells. *Gan* mice and *K19-Nog/C2mE* mice were obtained by

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Table 1. Transgenic mouse models and their gastric phenotypes

Transgenic mice	Transgenes	Affected pathway(s)	Gastric phenotype (reference)
K19-C2mE	*Ptgs2, Ptges*	PGE_2 induction	Inflammation, hyperplasia[16,25]
K19-Wnt1	*Wnt1*	Wnt activation	Small dysplastic lesion[22,25]
K19-Nog	*Nog*	BMP suppression	No phenotype[24,25]
K19-Wnt1/C2mE (Gan)	*Wnt1, Ptgs2, Ptges*	Wnt activation/PGE_2 induction	Dysplastic tumor[22,25]
K19-Nog/C2mE	*Nog, Ptgs2, Ptges*	BMP suppression/PGE_2 induction	Hamartoma[24,25]

BMP, bone morphogenetic protein.

crossing *K19-C2mE* with *K19-Wnt1* or *K19-Nog*, respectively (Table 1). All animal experiments were carried out according to a protocol approved by the Committee on Animal Experimentation of Kanazawa University.

Microarray analyses. We have deposited the results of microarray data sets from a series of mouse models to the Gene Expression Omnibus, as accession GSE16902.[23] Expression profiles of EGFR ligands, EGFR family members, and ADAM family proteases were extracted from the data sets, and the expression levels were compared by using absolute values.

Drug administration. For inhibition of COX-2, mice were fed a diet containing celecoxib (Pfizer New York, NY, USA) at 1500 ppm. For inhibition of EGFR or EP4 receptor, mice were administered orally with ZD1839 (Astra Zeneca, London, UK) or RQ00015986/CJ-42794[26] (RaQualia, Taketoyo, Japan), respectively, at 100 mg/kg/day in 0.5% methylcellulose. Drug-dosing experiments using *Gan* mice were performed for 3 weeks from 47 weeks of age ($n = 5$ for each experiment). The relative gastric tumor volume was calculated by multiplication of tumor height and tumor area measured using the ImageJ application program (NIH, Bethesda, MD, USA). X-ray computed tomography images of gastric tumors in live mice were examined using LaTheta LCT-100 (Aloka, Tokyo, Japan) at weeks 0, 1, 2 and 3 of drug administration.

Reverse transcription-polymerase chain reaction. Total RNA was extracted from mouse stomach or cultured cells using ISOGEN (Nippon Gene, Tokyo, Japan). Extracted RNA was reverse-transcribed with a PrimeScript RT reagent kit (Takara, Tokyo, Japan) and PCR-amplified by ABI prism 7900HT (Applied Biosystems, Carlsbad, CA, USA) using SYBR Premix Ex Taq II (Takara). Primers for real-time RT-PCR were purchased (Takara).

Cell Culture experiments. Mouse macrophage RAW264 cells (RIKEN BioRessource Center, Tsukuba, Japan) were cultured in RPMI1640, and treated with lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA) at 100 ng/mL with or without treatment of celecoxib or RQ00015986 at 10 μM for 24 h. Medium concentration of amphiregulin was measured by using Mouse Amphiregulin ELISA kit (RayBiotech, Norcross, GA, USA). Knockdown of *Adam8* expression was performed using *Adam8* ON-TARGETplus SMARTpool siRNA reagents (Dharmacon, Boulder, CO, USA). For the primary culture of gastric epithelial cells, glandular stomachs of *K19-Wnt1* mice were treated with 0.1% collagenase for 45 min followed by trypsin digestion, and cells were cultured in matrigel (BD Biosciences, Franklin Lakes, NJ, USA) with the primary culture medium with or without 1 μg/mL EGF (BD Biosciences)[16] supplemented with 500 ng/mL R-spondin1 (R&D, Mineapolis, MN, USA), 1 μM of Jagged1 (AnaSpec, Fremont, CA, USA), and 100 ng/mL of Noggin (PeproTech, Rocky Hill, NJ, USA). The primary cultured cells were stimulated with mouse recombinant amphiregulin and epiregulin (R&D) at 20 and 1 ng/mL, respectively, and the mean number of cystic structures >75 μm in diameter per microscopic field was calculated at day 5.

Immunoblotting analysis. Tissue samples were homogenized and sonicated in lysis buffer. After centrifugation at 2000*g*, 10 μg of the supernatant protein was separated in a 10% SDS-polyacrylamide gel. Antibodies for phosphorylated Akt (Ser473) and phosphorylated p44/42 Erk1/2 (cell signaling) were used as the primary antibodies. β-Actin was used as an internal control. The ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to detect specific signals. The band intensities were measured using the ImageJ application (NIH).

Histology and immunohistochemistry. Tissues and the primary cultured cells were paraffin-embedded or frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan), and sectioned. These sections were stained with H&E or processed for immunostaining. Antibody for phosphorylated EGFR (Tyr845) (cell signaling), Ki-67 (DakoCytomation, Carpenteria, CA, USA), or active β-catenin (Millipore, Billerica, MA, USA) was used as the primary antibody. Immunostaining signals were visualized using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA). For fluorescence immunostaining, anti-rabbit IgG Alexa 488 (Molecular Probes, Eugene, OR, USA) was used for the secondary antibody. The mean Ki-67 labeling index of the five independent microscopic fields was calculated.

Statistical analysis. Statistical analyses were performed using the unpaired Student's *t*-test, with *P*-values <0.05 considered significant.

Results

Induction of EGFR ligands and ADAM proteases in gastric tumors by PGE_2 pathway. We examined gene expression profiles of EGFR ligands and EGFR members in the stomach or gastric tumors of the all mouse models listed in Table 1. Among these models, PGE_2 pathway is induced in the stomach of *K19-C2mE*, *Gan*, and *K19-Nog/C2mE* mice by expression of *Ptgs2* and *Ptges*. Hereafter, these three strains are termed the *C2mE* group. Interestingly, expression of amphiregulin (*Areg*), epiregulin (*Ereg*), HB-EGF (*Hbegf*), and betacellulin (*Btc*), as well as Her2 (*Erbb2*), and Her3 (*Erbb3*) increased significantly in the stomach of the *C2mE* group mice (Fig. 1a). In contrast, such induction was not observed in the stomach of *K19-Wnt1* and *K19-Nog* mice, indicating that induction of PGE_2 pathway is responsible for upregulation of these genes.

ADAMs activate EGFR signaling through ectodomain shedding of EGFR ligands, and are induced in a variety of cancer tissues.[27] Notably, expression of *Adam8*, *Adam9*, *Adam10*, *Adam17*, and *Adam28* was increased significantly in the stomach of the *C2mE* group mice but not in other strains, indicating the PGE_2 pathway-dependent induction of these ADAMs (Fig. 1b). It has been shown that ADAM8, ADAM 10 and ADAM17 can cleave and activate amphiregulin, epiregulin, HB-EGF, or betacellulin.[28–30] It is thus possible that EGFR is activated in the gastric mucosa of *C2mE* group mice through induction of both EGFR ligands and ADAM proteases. Induction of *Erbb2* may also contribute to EGFR activation by increasing the heterodimerization of EGFR and HER2. Consistently, the immunostaining intensity of phosphorylated EGFR increased significantly in the gastric epithelial cells of *K19-C2mE* and *Gan* mice but not in those of WT and *K19-Wnt1* mice, indicating PGE_2 pathway-dependent EGFR activation (Fig. 1c).

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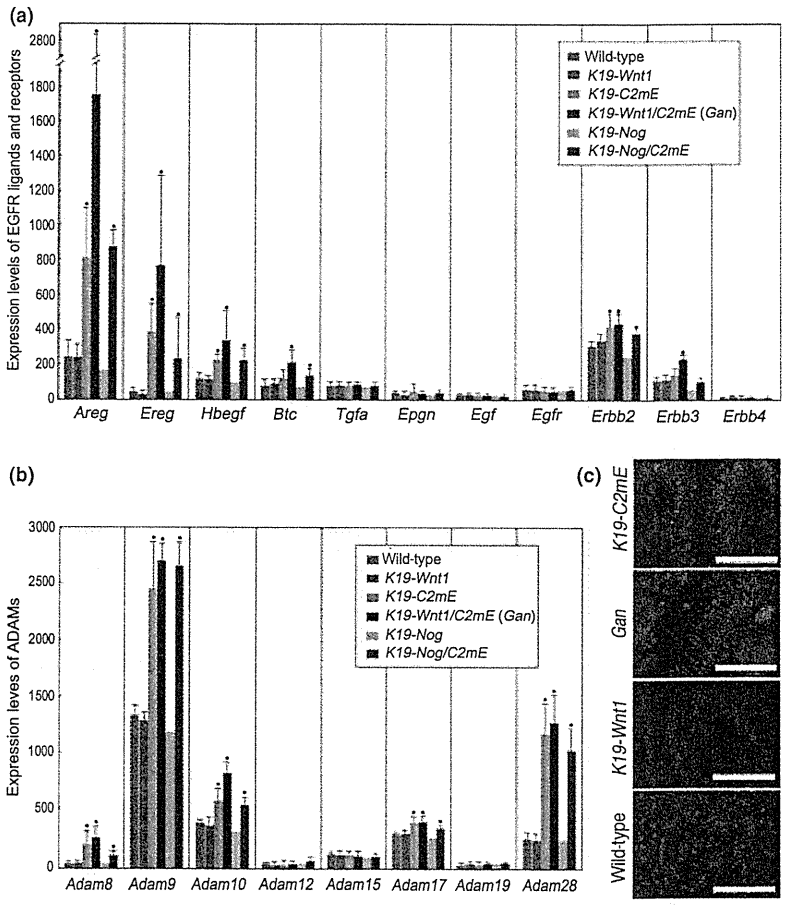

Fig. 1. Gene expression levels of epidermal growth factor receptor (EGFR) ligands, EGFR family members (a) and a disintegrin and metalloproteinases (ADAMs) (b) in the stomach of the respective models (mean ± SD) calculated from microarray results. Asterisks indicate $P < 0.05$ versus the wild-type level. (c) Fluorescence immunostaining for phosphorylated EGFR at Tyr845 (green) in the gastric mucosa of the indicated genotype mice. DAPI staining for nuclei is visualized in red. Bars indicate 100 μm.

Induction of EGFR ligands and ADAM proteases by PGE_2 receptor EP4 signaling. We next treated *Gan* mice with a COX-2 inhibitor, celecoxib, and found that expression of *Areg*, *Ereg*, *Hbegf*, and *Btc* as well as *Adam8*, *Adam9*, *Adam10*, *Adam17* and *Adam28* in gastric tumors decreased significantly (Fig. 2a). Among the four PGE_2 receptors, EP1-EP4, expression of EP4 was significantly increased in gastric tumors of *C2mE* group mice.[24] We thus treated *Gan* mice with an EP4-specific inhibitor, RQ00015986. Importantly, inhibition of the EP4 receptor caused a decrease in the expression of these EGFR ligands and ADAMs to a similar level to that in the celecoxib-treated mice (Fig. 2a). These results indicate that PGE_2 signaling through EP4 is required for induction of EGFR ligands and ADAMs in gastric tumor tissues.

Induction of EGFR ligands by EP4 signaling in activated macrophages. Macrophages are infiltrated in the gastric mucosa in the *C2mE* group mice,[16,22] and tumor-associated macrophages play an important role in tumorigenesis through expression of growth factors.[31] We thus examined induction of EGFR ligands and ADAMs in macrophages using the RAW264 cells. Stimulation of macrophages with LPS induced expression of *Ptgs2* and *Ptges*, resulting in an increased PGE_2 level in the cell culture medium (Fig. 2b and not shown). In the LPS-activated macrophages, expression of *Areg*, *Ereg*, and *Hbegf*, as well as *Adam8* increased significantly, while expression of other ADAM members did not (Fig. 2b,c). Notably, inhibition of COX-2 or the EP4 receptor by treatment with celecoxib or RQ00015986, respectively, significantly suppressed induction of *Areg* and *Ereg* in the LPS-stimulated macrophages. These results suggest that EP4 signaling induces expression of *Areg* and *Ereg* in the activated macrophages in an autocrine or paracrine manner. In contrast, expression of *Hbegf* and *Adam8* was not decreased by inhibition of COX-2 or EP4, suggesting that other factors from activated macrophages induced these genes. Expression of *Btc* was not detected in the LPS-stimulated or control RAW264 cells (data not shown).

We confirmed that medium concentration of the cleaved amphiregulin increased significantly in the LPS-stimulated RAW264 cells (Fig. 2d). To examine the role of Adam8 in shedding of amphiregulin, we used *Adam8* siRNA that successfully decreased *Adam8* mRNA level in macrophages (Fig. 2e). Importantly, transfection of *Adam8* siRNA reduced amphiregulin concentration significantly (Fig. 2d). These results indicate that LPS stimulation induces amphiregulin secretion from macrophages through induction of *Areg* and *Adam8* in a PGE_2-dependent and independent mechanisms.

Basal epiregulin expression by EP4 signaling in gastric epithelial cells. To examine gene expression in gastric epithelial cells, we established the primary culture system in matrigel. Although gastric epithelial cells from WT mice proliferated for 3–5 days in matrigel forming small cystic structures (Fig. 3a), they could not continue proliferation. In contrast, gastric epithelial cells from *K19-Wnt1* transgenic mice continued proliferation in matrigel forming large cystic structures. These structures consisted of monolayer of epithelial cells with nuclear accumulation of β-catenin (Fig. 3b), suggesting that Wnt activation increases self-renewal activity of gastric epithelial cells. Expression of EGFR ligands and ADAMs was not increased by PGE_2 stimulation in the primary cultured epithelial cells. However, EP4 inhibition resulted in a significant decrease of *Ereg* expression level, suggesting that EP4 signaling is required for basal expression of *Ereg* (Fig. 3c). Expression of *Hbegf* was not detected in the gastric epithelial cells (data not shown).

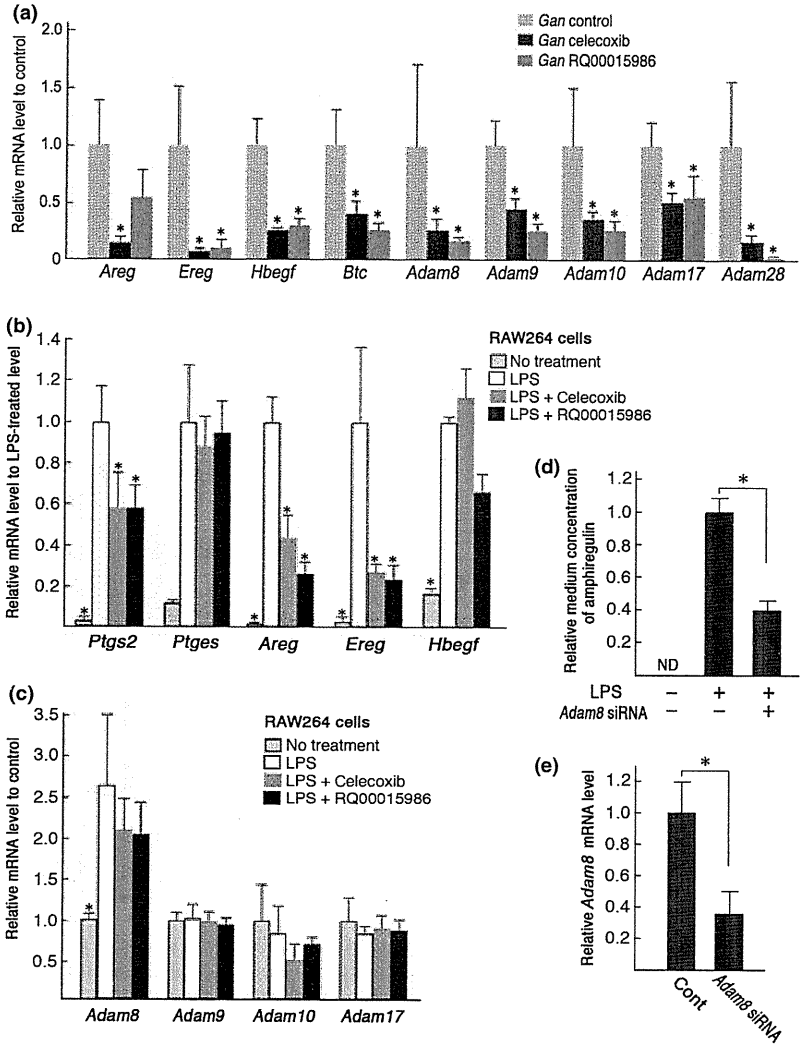

Fig. 2. (a) The mRNA levels of epidermal growth factor receptor (EGFR) ligands and a disintegrin and metalloproteinases (ADAMs) examined by real-time RT-PCR in gastric tumors of celecoxib-treated or RQ00015986-treated *Gan* mice relative to the no-drug control *Gan* mouse level (mean ± SD). Asterisks indicate $P < 0.05$ versus the control level. (b) The mRNA levels of *Ptgs2*, *Ptges*, and EGFR ligands in the control and the drug-treated lipopolysaccharide (LPS)-stimulated RAW264 cells relative to that of LPS-stimulated RAW264 cells (mean ± SD). Asterisks indicate $P < 0.05$ versus the level of LPS-stimulated cells. (c) The mRNA levels of *Adams* in no-drug and drug-treated LPS-stimulated RAW264 cells relative to that of control RAW264 cells (mean ± SD). Asterisk indicates $P < 0.05$ versus the level of LPS-stimulated cells. (d) Concentration of amphiregulin in the culture medium of the LPS-stimulated and *Adam8* siRNA-transfected RAW264 cells relative to that of LPS-stimulated RAW264 cells (mean ± SD). Asterisk indicates $P < 0.05$. (e) The *Adam8* mRNA level in the *Adam8* siRNA-transfected RAW264 cells relative to that of control cells (cont) (mean ± SD). Asterisk indicates $P < 0.05$. [Correction added after online publication on March 18, 2011. *Areg* mRNA is changed to *Adam8* on Fig. 2(e).]

Notably, stimulation of the gastric epithelial cells either by amphiregulin or epiregulin increased the size of cystic structures in matrigel, indicating that these EGFR ligands accelerate proliferation of gastric epithelial cells (Fig. 3d). These results support the idea that induction of amphiregulin and epiregulin by PGE_2 pathway promotes gastric tumorigenesis through activation of epithelial EGFR.

Suppression of *Gan* mouse gastric tumorigenesis by EGFR inhibition. Treatment of *Gan* mice with celecoxib decreased gastric tumor volume to 10.2% of the no-drug control mice, confirming that COX-2 pathway is important for gastric tumorigenesis (Fig. 4a,b). Importantly, treatment of *Gan* mice with an EGFR inhibitor, ZD1839, also reduced the gastric tumor volume to 23.6% of the control mice. Moreover, combination treatment with ZD1839 and celecoxib resulted in complete regression of *Gan* mouse gastric tumors. We confirmed the dramatic regression of gastric tumors by combination therapy with celecoxib and ZD1839 in the same mice by chronological examinations using X-ray computed tomography (Fig. 4c). The transgenic expression of *Ptgs2* and *Wnt1* in the ZD1839-treated *Gan* mice stayed at a similarly high level as that in the control *Gan* mice (Fig. 4d). On the other hand, expression of *Ptges* decreased significantly by ZD1839 treatment, suggesting that endogenous *Ptges* was induced by activation of EGFR signaling in gastric tumors. However, *Ptges* expression level in the ZD1839-treated *Gan* mice was still at the high level compared with WT mice. These results collectively indicate that EGFR activation is required for gastric tumorigenesis, even if the Wnt and PGE_2 pathways are activated.

Suppression of tumor cell proliferation by EGFR inhibition. Two major pathways downstream of EGFR signaling are the MAPK and PI3K/Akt pathways.[32] The levels of phosphorylated Akt and Erk1/2 were significantly decreased by ZD1839 treatment in the *Gan* mouse gastric tumors (Fig. 5a,b). Notably, celecoxib treatment also suppressed the phosphorylation of Akt and Erk1/2 to a similar level as in the ZD1839-treated mice, suggesting that induction of PGE_2 pathway is a major mechanism for activation of EGFR in gastric tumors.

Most tumor cells were immunostained for Ki-67 in the control *Gan* mice, while the number of Ki-67 positive cells was significantly decreased both in the ZD1839-treated and celecoxib-treated mice (Fig. 5c,d). Accordingly, it is possible that the PGE_2 pathway accelerates tumor cell proliferation through EGFR activation.

Discussion

We found that there was simultaneous gene upregulation of EGFR ligands, *Areg*, *Ereg*, *Hbegf* and *Btc*, in the mouse gastric tumors, which occurred in a PGE_2-dependent manner. PGE_2

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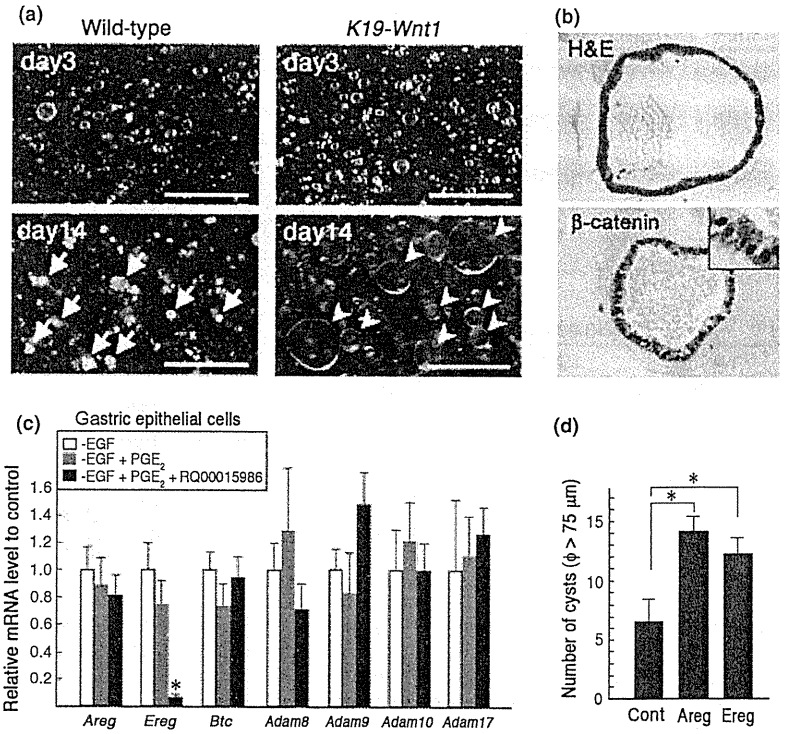

Fig. 3. (a) Representative photographs of the primary cultured gastric epithelial cells in matrigel from wild type (*left*) and *K19-Wnt1* mice (*right*). Arrowheads indicate cystic structures, while arrows indicate clusters of dead cells. Bars indicate 500 μm. (b) Histology (top, H&E) and immunostaining with anti-active β-catenin antibody (bottom) of cystic structures. Inset indicates nuclear accumulation of active β-catenin in the epithelial cells. (c) Relative expression of epidermal growth factor receptor (EGFR) ligands and a disintegrin and metalloproteinases (ADAMs) in gastric epithelial cells cultured in matrigel with the indicated treatment (mean ± SD). Asterisk indicates $P < 0.05$ versus the level in control cells cultured in EGF (–) medium. (d) The mean number of cystic structures >75 μm in diameter in matrigel of the amphiregulin-treated (*Areg*), epiregulin-treated (*Ereg*) and control (*cont*) gastric epithelial cells (mean ± SD). Asterisks indicate $P < 0.05$.

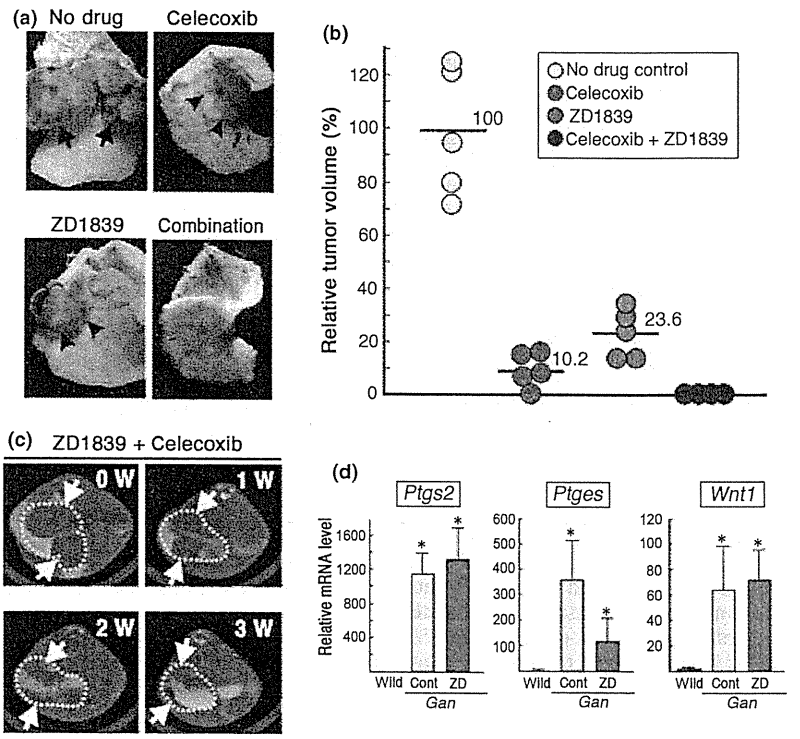

Fig. 4. (a) Representative photographs of *Gan* mouse stomach; no-drug control (top left), celecoxib-treated (top right), ZD1839-treated (bottom left), and treated with a combination of ZD1839 and celecoxib (bottom right). Arrows indicate gastric tumors in control mouse, whereas arrowheads indicate regressed tumors in the drug-treated mice. (b) Tumor volumes of *Gan* mice treated with celecoxib, ZD1839, and a combination of ZD1839 and celecoxib relative to control mice. Each filled circle indicates the value of individual mice, and the means of the respective groups are indicated. (c) X-ray computed tomography images of the same *Gan* mouse treated with a combination of ZD1839 and celecoxib at weeks 0, 1, 2 and 3 after starting drug administration. Yellow dashed lines indicate the stomach. Arrows indicate gastric tumors. (d) The mRNA levels of *Ptgs2*, *Ptges*, and *Wnt1* examined by real-time RT-PCR in the gastric tumors of no-drug control (Cont) and ZD1839-treated (ZD) *Gan* mice relative to wild-type level (wild) (mean ± SD). Asterisks indicate $P < 0.05$ versus wild-type level.

signaling through the EP4 receptor is required for basal expression of *Ereg* in epithelial cells, whereas both *Areg* and *Ereg* are induced by EP4 signaling in macrophages. On the other hand, *Hbegf* is induced in the activated macrophages in a PGE_2-independent manner. Accordingly, it is possible that expression of the respective EGFR ligands is regulated not only by PGE_2 signaling but also by PGE_2-induced inflammation in the different cell types including macrophages and epithelial cells.

It has been reported that PGE_2 signaling activates MMPs and ADAM17, resulting in shedding of TGF-α or amphiregulin,

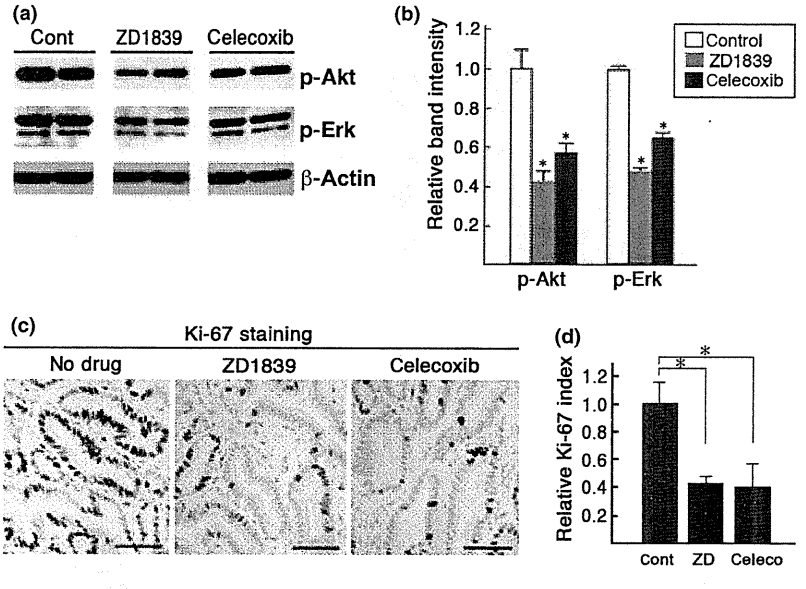

Fig. 5. (a) Western blotting of phosphorylated Akt and phosphorylated Erk1/2 in gastric tumors of two independent no-drug control, ZD1839-treated, and celecoxib-treated *Gan* mice. β-Actin was used as an internal control. (b) Relative band intensities of Western blotting results to the control level (mean ± SD). Asterisks indicate $P < 0.05$ versus the control level. (c) Immunostaining for Ki-67 of gastric tumors. Bars indicate 100 μm. (d) Ki-67 labeling index of ZD1839-treated (ZD), and celecoxib-treated (celeco) *Gan* mice relative to that of control *Gan* (cont) mice (mean ± SD). Asterisks indicate $P < 0.05$.

which causes EGFR activation.[12,15] However, the present results indicate that the expression of ADAM8, ADAM9, ADAM10, ADAM17, and ADAM28 are induced in gastric tumors in a PGE_2 pathway-dependent manner. Although we could not find direct induction of these ADAMs by PGE_2 in epithelial cells or macrophages, it is possible that PGE_2-associated inflammation induces these ADAMs indirectly. It has consistently been shown that the inflammatory cytokine IL-8 induces ADAM10-dependent shedding of HB-EGF and amphiregulin.[33] Notably, all of these induced ADAMs have been shown to be important in tumorigenesis.[27] In addition to shedding of EGFR ligands, they induce tumor cell migration, invasion and dissemination.[27,34,35] Therefore, it is conceivable that induction of such ADAM functions by activated PGE_2 pathway contributes to gastric tumorigenesis and malignant progression.

To examine the role of macrophages for induction of EGFR ligands and ADAMs, we used RAW264 mouse macrophage cell line because it was technically difficult to prepare macrophages from the *in vivo* gastric tumors. Therefore, it remains to be confirmed the induction of EGFR ligands and ADAMs in the tumor-infiltrated macrophages.

Helicobacter pylori infection induces expression of HB-EGF and amphiregulin in gastric cancer cells.[36–38] *H. pylori* infection in mice carrying the kinase-defective mutant EGFR allele ($EGFR^{wa2}$) showed increased apoptosis of gastric epithelial cells, suggesting that EGFR activation by *H. pylori* infection is important for protection from apoptosis.[39] Moreover, *H. pylori* infection to *Adam17*-disrupted gastric epithelial cells failed to activate the EGFR, suggesting that ADAMs play a role in *H. pylori* infection-induced EGFR activation.[39] Importantly, we previously showed that *H. felis* infection caused induction of *Ptgs2* and *Ptges* in gastric epithelial cells.[16]

Accordingly, it is possible that *H. pylori* infection induces PGE_2 pathway, which further activates EGFR through global induction of EGFR ligands and ADAMs, similar to the effects observed in the *C2mE* group mice. It is therefore possible that inhibition of the PGE_2 pathway, as well as eradication of *H. pylori* infection, can suppress EGFR activation in the *H. pylori*-infected gastric mucosa, thereby preventing gastric carcinogenesis.

The level of PGE_2 is regulated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which inactivates prostaglandins. Importantly, expression of 15-PGDH is downregulated by EGFR signaling in colon cancer cells,[40] indicating that EGFR signaling activates PGE_2 pathway. Moreover, disruption of the 15-PGDH gene accelerates intestinal tumorigenesis in mouse models.[41] Accordingly, it is possible that inhibition of both PGE_2 and EGFR pathways represents an effective therapeutic strategy for gastrointestinal tumorigenesis by suppression of both the individual signaling pathways and the positive feedback loop between two signaling pathways. Among the four PGE_2 receptors, EP4 is the most abundant receptor in mouse gastric tumor models[24] and in human colon cancer tissues.[42] We have shown here that EP4 signaling is responsible for global induction of EGFR ligands and ADAMs through direct or indirect mechanisms, and macrophages are major source of EGFR ligands. Moreover, we have recently demonstrated that inhibition of EP4 signaling significantly suppressed gastric tumorigenesis in *Gan* mice.[43] These results, taken together, suggest that combination treatment with inhibitors of EGFR and EP4 will be an effective strategy for preventing gastric tumorigenesis.

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