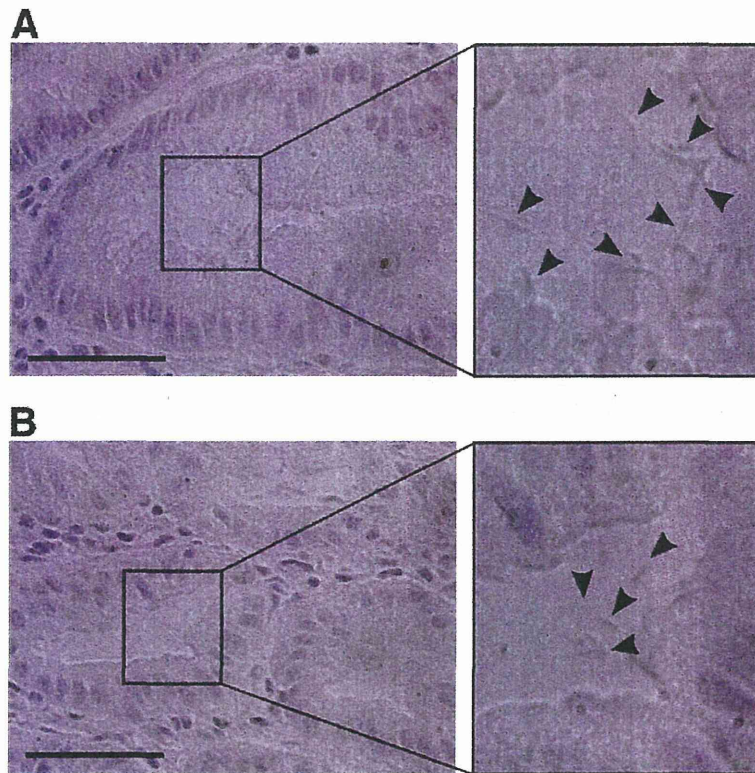
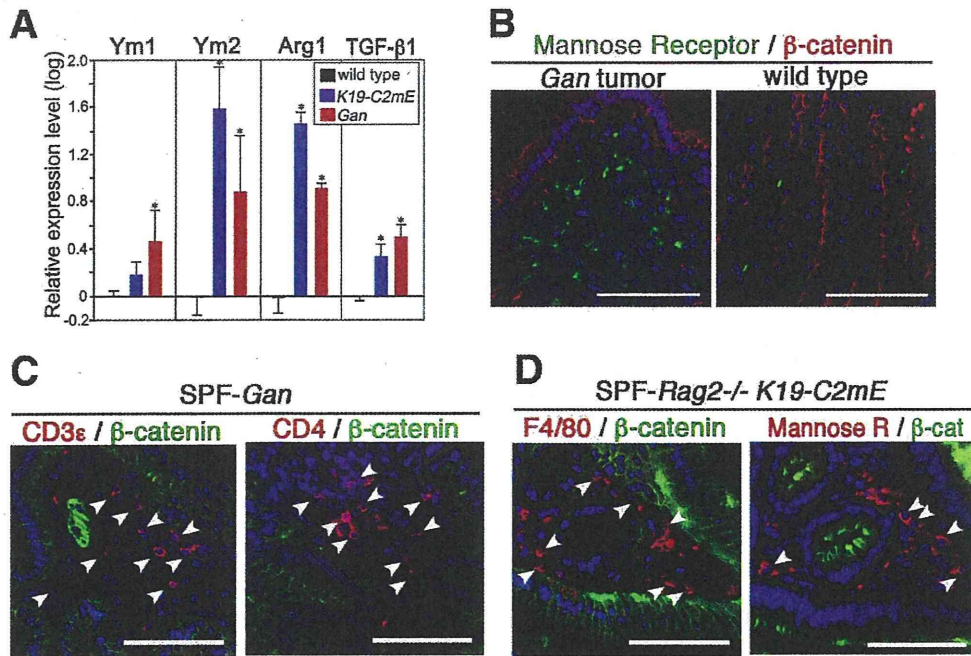


Supplementary Figure 3. Suppression of gastric tumor growth by antibiotic treatment. (A) The mean tumor volumes calculated from x-ray computerized tomography (CT) images of control *Gan* mice (blue bars, left) and antibiotics-treated *Gan* mice (green bars, right) relative to the level at 0 weeks of treatment (closed bars) (mean \pm standard deviation). * $P < .05$. (B) Representative x-ray CT images of gastric tumors of the same mice from the antibiotics-treated group (top) and control group (bottom) at 0 and 4 weeks of antibiotic treatment. The dashed lines indicate gastric tumors. Note that tumor growth was suppressed by treatment with antibiotics (top).



Supplementary Figure 4. Infection of *Helicobacter felis* in the lumen of the *H felis*-infected GF-Gan mouse gastric glands (A and B). Arrowheads indicate *H felis*. Scale bars, 50 μ m. Original magnification, $\times 1000$.



Supplementary Figure 5. M2 macrophages in *Gan* and *K19-C2mE* mouse stomachs. (A) The relative expression level of M2 macrophage markers—Ym1, Ym2, arginase 1 (Arg1), and transforming growth factor (TGF)- β 1—in the *K19-C2mE* mouse stomach (*blue*) and *Gan* mouse tumors (*red*) in comparison with that in the wild-type mouse stomach (*black*) (mean log₁₀ ratio with standard deviation). **P* < .05 vs wild-type level. (B) Immunostaining of M2 macrophage marker, mannose receptor (*green*) and β -catenin (*red*) with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (*blue*) in *Gan* mouse tumors (*left*) and wild-type mouse stomach (*right*). (C) Immunostaining of CD3 ϵ (*left, red, arrowheads*) or CD4 (*right, red, arrowheads*) and β -catenin (*green*) with DAPI staining (*blue*) of SPF-*Gan* mouse tumors. (D) Immunostaining of F4/80 (*left, red, arrowheads*) or mannose receptor (*right, red, arrowheads*) and β -catenin (*green*) with DAPI staining (*blue*) of the SPF-Rag2^{-/-} *K19-C2mE* mouse stomach. Note that mannose receptor-expressing M2 macrophages were found in the T cell-depleted Rag2^{-/-} *K19-C2mE* mouse inflamed stomach. Scale bars in B–D, 100 μ m.

Activation of epidermal growth factor receptor signaling by the prostaglandin E₂ receptor EP4 pathway during gastric tumorigenesis

Hiroko Oshima, Boryana K. Popivanova, Keisuke Oguma,² Dan Kong, Tomo-o Ishikawa and Masanobu Oshima¹

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

(Received August 11, 2010/Revised December 9, 2010/Accepted December 22, 2010/Accepted manuscript online January 4, 2011/Article first published online February 2, 2011)

Cyclooxygenase-2 (COX-2) plays an important role in tumorigenesis through prostaglandin E₂ (PGE₂) biosynthesis. It has been shown by *in vitro* studies that PGE₂ signaling transactivates epidermal growth factor receptor (EGFR) through an intracellular mechanism. However, the mechanisms underlying PGE₂-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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ in these cells, suggesting indirect mechanism possibly through PGE₂-associated inflammatory responses. These results suggest that PGE₂ 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/PGE₂ 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 E₂ (PGE₂), a downstream product of COX-2, plays a key role in intestinal tumorigenesis,^(3–5) suggesting that the PGE₂ 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 PGE₂ 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 PGE₂ pathway in *in vivo* tumors has not been fully elucidated. Induction of the PGE₂ pathway in the gastric mucosa causes development of inflammatory microenvironment consisting of macrophages and myofibroblasts.^(16,17) It is therefore possible that PGE₂ signaling in such microenvironment contributes to EGFR activation in tumors, and that PGE₂-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 PGE₂ 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 PGE₂ 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 PGE₂ 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

¹To whom correspondence should be addressed.
E-mail: oshimam@kenroku.kanazawa-u.ac.jp

²Research fellow of the Japan Society for the Promotion of Science.

Table 1. Transgenic mouse models and their gastric phenotypes

Transgenic mice	Transgenes	Affected pathway(s)	Gastric phenotype (reference)
<i>K19-C2mE</i>	<i>Ptgs2, Ptges</i>	PGE ₂ induction	Inflammation, hyperplasia ^(16,25)
<i>K19-Wnt1</i>	<i>Wnt1</i>	Wnt activation	Small dysplastic lesion ^(22,25)
<i>K19-Nog</i>	<i>Nog</i>	BMP suppression	No phenotype ^(24,25)
<i>K19-Wnt1/C2mE (Gan)</i>	<i>Wnt1, Ptgs2, Ptges</i>	Wnt activation/PGE ₂ induction	Dysplastic tumor ^(22,25)
<i>K19-Nog/C2mE</i>	<i>Nog, Ptgs2, Ptges</i>	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 BioResource 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 μ M 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 μ g/mL EGF (BD Biosciences)⁽¹⁶⁾ supplemented with 500 ng/mL R-spondin1 (R&D, Minneapolis, MN, USA), 1 μ M 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 μ m in diameter per microscopic field was calculated at day 5.

Immunoblotting analysis. Tissue samples were homogenized and sonicated in lysis buffer. After centrifugation at 2000g, 10 μ g 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, Carpinteria, 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 PGE₂ 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, PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ pathway-dependent EGFR activation (Fig. 1c).

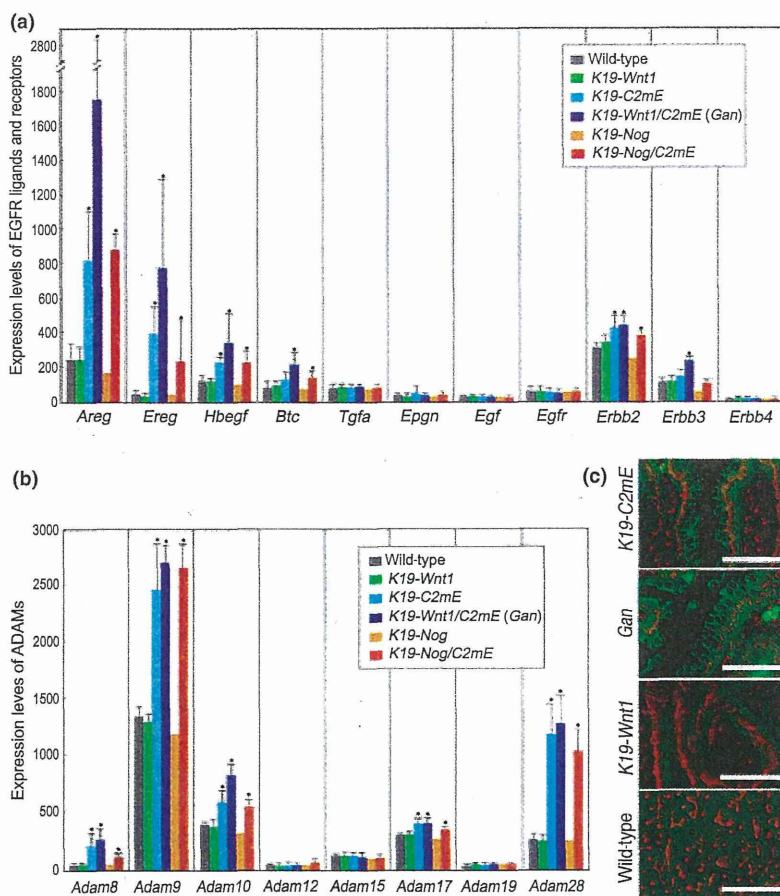


Fig. 1. Gene expression levels of epidermal growth factor receptor (EGFR) ligands, EGFR family members (a) and a disintegrin and metalloproteinases (ADAMs) (b) in the stomach of the respective models (mean \pm SD) calculated from microarray results. Asterisks indicate $P < 0.05$ versus the wild-type 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 μ m.

Induction of EGFR ligands and ADAM proteases by PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂-dependent and independent mechanisms.

Basal epi-regulin 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 PGE₂ 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).

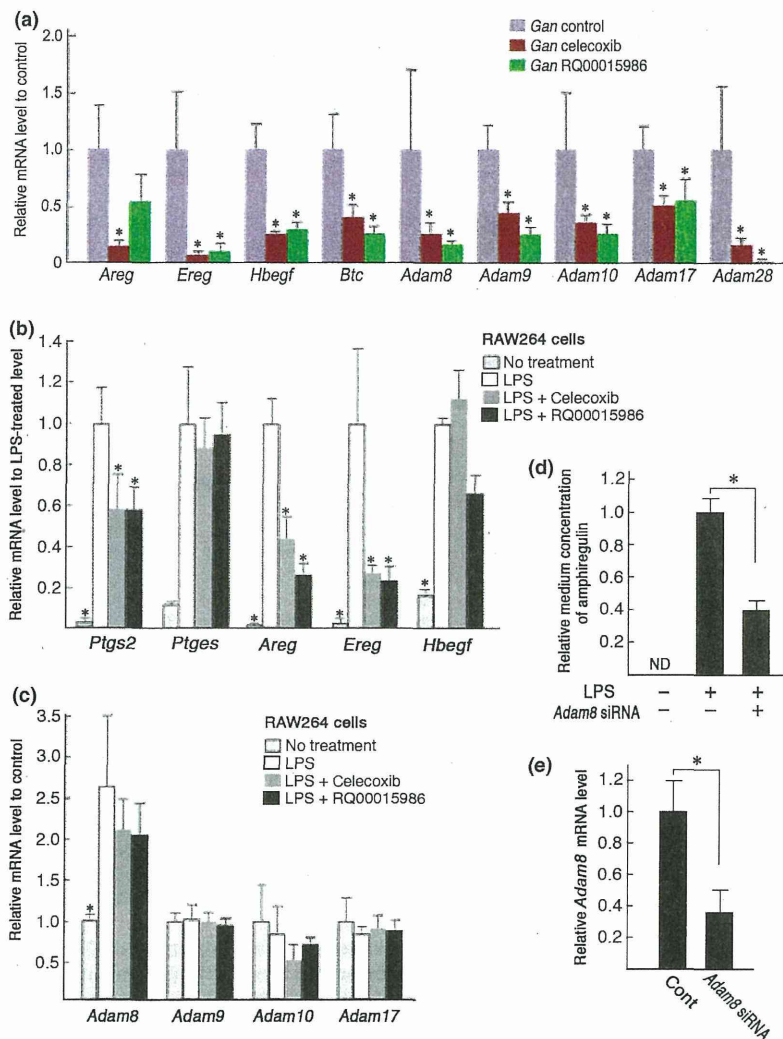


Fig. 2. (a) The mRNA levels of epidermal growth factor receptor (EGFR) ligands and a disintegrin and metalloproteinases (ADAMs) examined by real-time RT-PCR in gastric tumors of celecoxib-treated or RQ00015986-treated *Gan* mice relative to the no-drug control *Gan* mouse level (mean \pm SD). Asterisks 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 \pm 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 \pm 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 \pm 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 \pm 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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂-dependent manner. PGE₂

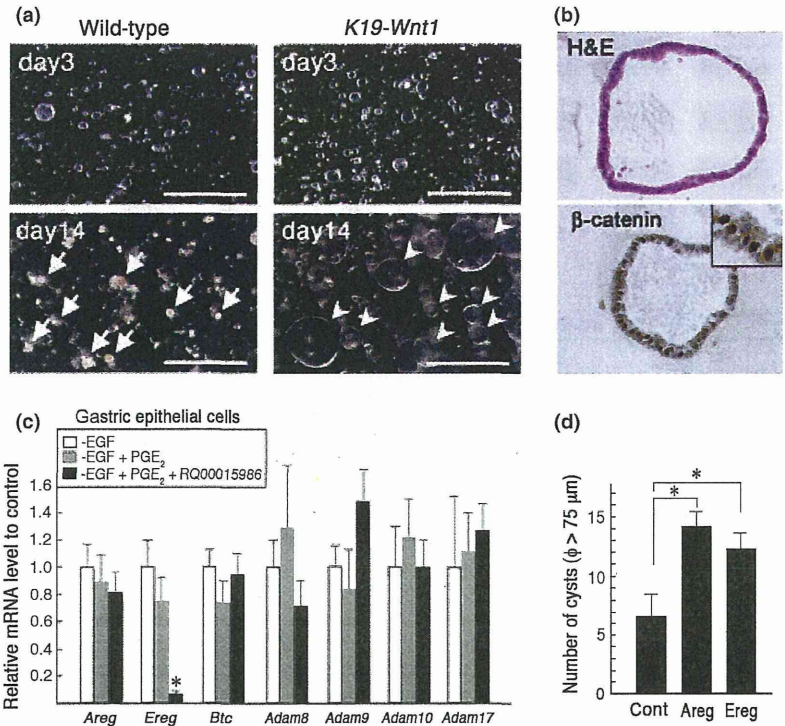


Fig. 3. (a) Representative photographs of the primary cultured gastric epithelial cells in matrigel from wild type (*left*) and *K19-Wnt1* mice (*right*). Arrowheads indicate cystic structures, while arrows indicate clusters of dead cells. Bars indicate 500 μm . (b) Histology (top, H&E) and immunostaining 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 \pm SD). Asterisk indicates $P < 0.05$ versus the level in control cells cultured in EGF (-) medium. (d) The mean number of cystic structures $>75 \mu\text{m}$ in diameter in matrigel of the amphiregulin-treated (*Areg*), epiregulin-treated (*Ereg*) and control (*cont*) gastric epithelial cells (mean \pm SD). Asterisks indicate $P < 0.05$.

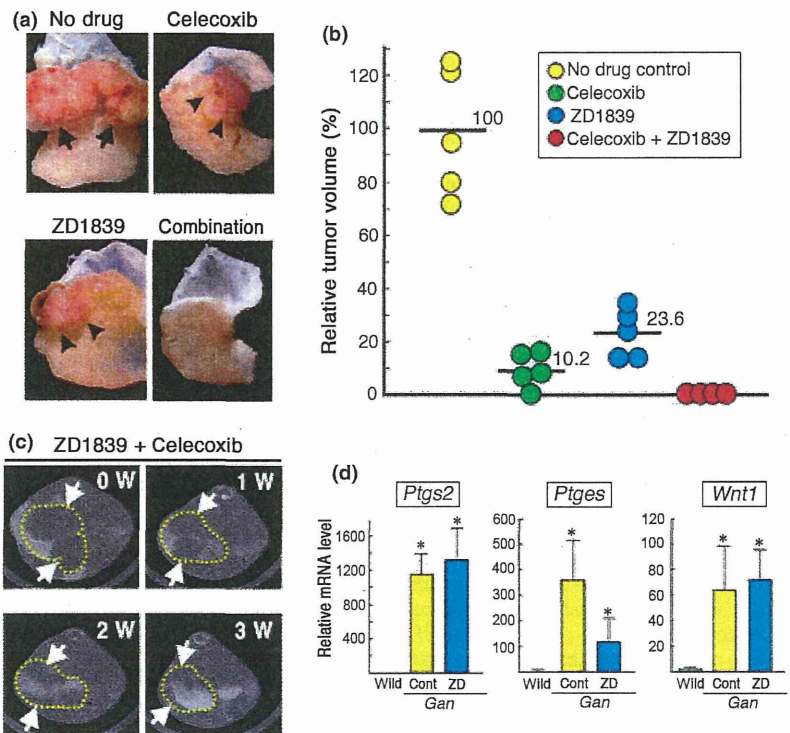


Fig. 4. (a) Representative photographs of *Gan* mouse stomach; no-drug control (top left), celecoxib-treated (top right), ZD1839-treated (bottom left), and treated with a combination of ZD1839 and celecoxib (bottom right). Arrows indicate gastric tumors in control mouse, whereas arrowheads indicate regressed 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 *Ptg2*, *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 \pm 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 PGE₂-independent manner. Accordingly, it is possible that expression of

the respective EGFR ligands is regulated not only by PGE₂ signaling but also by PGE₂-induced inflammation in the different cell types including macrophages and epithelial cells.

It has been reported that PGE₂ signaling activates MMPs and ADAM17, resulting in shedding of TGF- α or amphiregulin,

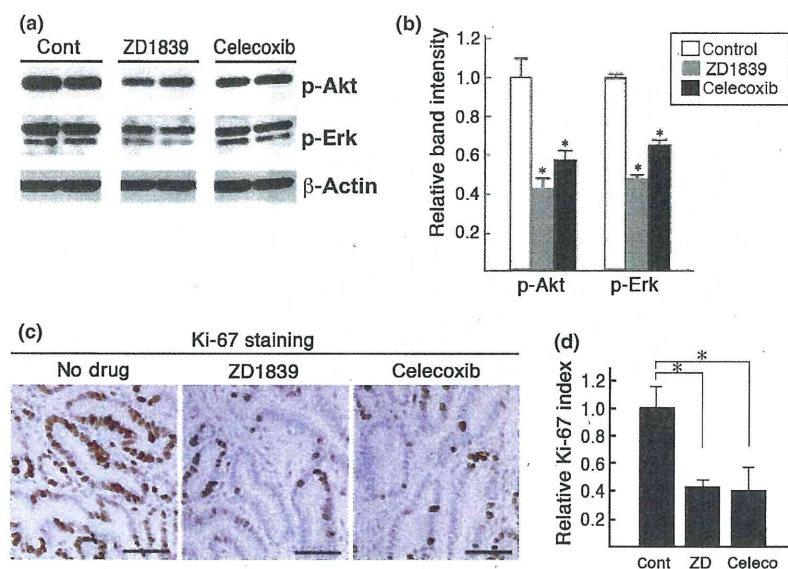


Fig. 5. (a) Western blotting of phosphorylated Akt and phosphorylated Erk1/2 in gastric tumors of two independent no-drug control, ZD1839-treated, and celecoxib-treated *Gan* mice. β -Actin was used as an internal control. (b) Relative band intensities of Western blotting results to the control level (mean \pm SD). Asterisks indicate $P < 0.05$ versus the control level. (c) Immunostaining for Ki-67 of gastric tumors. Bars indicate 100 μ m. (d) Ki-67 labeling index of ZD1839-treated (ZD), and celecoxib-treated (celeco) *Gan* mice relative to that of control *Gan* (cont) mice (mean \pm 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 PGE₂ pathway-dependent manner. Although we could not find direct induction of these ADAMs by PGE₂ in epithelial cells or macrophages, it is possible that PGE₂-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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ pathway. Moreover, disruption of the 15-PGDH gene accelerates intestinal tumorigenesis in mouse models.⁽⁴¹⁾ Accordingly, it is possible that inhibition of both PGE₂ 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 PGE₂ 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.⁽⁴⁵⁾ These results, taken together, suggest that combination treatment with inhibitors of EGFR and EP4 will be an effective strategy for preventing gastric tumorigenesis.

Acknowledgments

We wish to thank M. Watanabe for excellent technical assistance, and H. Itadani and H. Kotani for microarray analyses. This study was supported by Grants-in-Aid for the Third-Term Comprehensive Cancer Control Strategy from the Ministry of Health, Labor, and Welfare, Grant-in-Aids from the Ministry of Education, Science, Culture, and Sports of Japan, and Takeda Science Foundation.

References

- Gupta RA, DuBois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer* 2001; 1: 11-21.
- Wang D, DuBois RN. Eicosanoids and cancer. *Nat Rev Cancer* 2010; 10: 181-93.
- Oshima M, Dinchuk JE, Kargman SL *et al*. Suppression of intestinal polyposis in *Apc*^{A716} knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996; 87: 803-9.
- Sonoshita M, Takaku K, Sasaki N *et al*. Acceleration of intestinal polyposis through prostaglandin receptor EP2 in *Apc*^{A716} knockout mice. *Nat Med* 2001; 7: 1048-51.

- 5 Seno H, Oshima M, Ishikawa T *et al.* Cyclooxygenase 2- and prostaglandin E₂ receptor EP₂-dependent angiogenesis in *Apc*^{D716} mouse intestinal polyps. *Cancer Res* 2002; **62**: 506–11.
- 6 Dannenberg AJ, Lippman SM, Mann JR, Subbaramaiah K, DuBois RN. Cyclooxygenase-2 and epidermal growth factor receptor: pharmacologic targets for chemoprevention. *J Clin Oncol* 2005; **23**: 254–66.
- 7 Roberts RB, Min L, Washington MK *et al.* Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis. *Proc Natl Acad Sci USA* 2002; **99**: 1521–6.
- 8 Torrance CJ, Jackson PE, Montgomery E *et al.* Combinatorial chemoprevention of intestinal neoplasia. *Nat Med* 2006; **6**: 1024–8.
- 9 Buchanan FG, Holla V, Katkuri S, Matta P, DuBois RN. Targeting cyclooxygenase-2 and the epidermal growth factor receptor for the prevention and treatment of intestinal cancer. *Cancer Res* 2008; **67**: 9380–8.
- 10 Buchanan FG, Wang D, Bargiacchi F, DuBois RN. Prostaglandin E₂ regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem* 2003; **278**: 35451–7.
- 11 Buchanan FG, Gorden DL, Matta P, Shi Q, Matrisian LM, DuBois RN. Role of β -arrestin1 in the metastatic progression of colorectal cancer. *Proc Natl Acad Sci USA* 2006; **103**: 1492–7.
- 12 Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E₂ transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* 2002; **8**: 289–93.
- 13 Shao J, Lee SB, Guo H, Evers BM, Sheng H. Prostaglandin E₂ stimulates the growth of colon cancer cells via induction of amphiregulin. *Cancer Res* 2003; **63**: 5218–23.
- 14 Subbaramaiah K, Benezra R, Hudis C, Dannenberg AJ. Cyclooxygenase-2-derived prostaglandin E₂ stimulates *Id-1* transcription. *J Biol Chem* 2008; **283**: 33955–68.
- 15 Al-Salihi MA, Ulmer SC, Doan T *et al.* Cyclooxygenase-2 transactivates the epidermal growth factor receptor through specific E-prostanoid receptors and tumor necrosis factor- α converting enzyme. *Cell Signal* 2007; **19**: 1956–63.
- 16 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.
- 17 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.
- 18 Stadlander CT, Waterbor JW. Molecular epidemiology, pathogenesis and prevention of gastric cancer. *Carcinogenesis* 1999; **20**: 2195–207.
- 19 Saukkonen K, Rintahaka J, Sivula A *et al.* Cyclooxygenase-2 and gastric carcinogenesis. *APMIS* 2003; **111**: 915–25.
- 20 Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath CW Jr. Aspirin use and risk of fatal cancer. *Cancer Res* 1993; **53**: 1322–7.
- 21 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.
- 22 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 E₂ pathway. *Gastroenterology* 2006; **131**: 1086–95.
- 23 Itadani H, Oshima H, Oshima M, Kotani H. Mouse gastric tumor models with prostaglandin E₂ pathway activation show similar gene expression profiles to intestinal-type human gastric cancer. *BMC Genomics* 2009; **10**: 615.
- 24 Oshima H, Itadani H, Kotani H, Taketo MM, Oshima M. Induction of prostaglandin E₂ pathway promotes gastric hamartoma development with suppression of bone morphogenetic protein signaling. *Cancer Res* 2009; **69**: 2729–33.
- 25 Oshima H, Oguma K, Du YC, Oshima M. Prostaglandin E₂, Wnt and BMP in gastric tumor mouse models. *Cancer Sci* 2009; **100**: 1779–85.
- 26 Takeuchi K, Tanaka A, Kato S, Aihara E, Amagase K. Effect of (S)-4-(1-(5-Chloro-2-(4-fluorophenoxy)benzamido)ethyl) benzoic acid (CJ-42794), a selective antagonist of prostaglandin E receptor subtype 4, on ulcerogenic and healing responses in rat gastrointestinal mucosa. *J Pharmacol Exp Ther* 2007; **322**: 903–12.
- 27 Mochizuki S, Okada Y. ADAMs in cancer cell proliferation and progression. *Cancer Sci* 2007; **98**: 621–8.
- 28 Sahin U, Weskamp G, Kelly K *et al.* Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol* 2004; **164**: 769–79.
- 29 Sanderson MP, Erickson SN, Gough PJ *et al.* ADAM10 mediates ectodomain shedding of the betacellulin precursor activated by *p*-aminophenylmercuric acetate and extracellular calcium influx. *J Biol Chem* 2005; **280**: 1826–37.
- 30 Horiuchi K, Gall SL, Schulte M *et al.* Substrate selectivity of epidermal growth factor-receptor ligand sheddases and their regulation by phorbol esters and calcium influx. *Mol Biol Cell* 2007; **18**: 176–88.
- 31 Pollard JW. Trophic macrophages in development and disease. *Nat Rev Immunol* 2009; **9**: 259–70.
- 32 Yarden Y, Sliwkowski MX. Untangling the ErbB signaling network. *Nat Rev Mol Cell Biol* 2001; **2**: 127–37.
- 33 Tanida S, Joh T, Itoh K *et al.* The mechanism of cleavage of EGFR ligands induced by inflammatory cytokines in gastric cancer cells. *Gastroenterology* 2004; **127**: 559–69.
- 34 Mazzocca A, Coppari R, De Franco R *et al.* A secreted form of ADAM9 promotes carcinoma invasion through tumor-stromal interactions. *Cancer Res* 2005; **65**: 4728–38.
- 35 Fogel M, Gutwein P, Mechtersheimer S *et al.* L1 expression as a predictor of progression and survival in patients with uterine and ovarian carcinomas. *Lancet* 2003; **362**: 869–75.
- 36 Romano M, Ricci V, Di Popolo A *et al.* *Helicobacter pylori* upregulates expression of epidermal growth factor-related peptides, but inhibits their proliferative effect in MKN28 gastric mucosal cells. *J Clin Invest* 1998; **101**: 1604–13.
- 37 Keates S, Sougioultzis S, Keates AC *et al.* *cag+* *Helicobacter pylori* induce transactivation of the epidermal growth factor receptor in AGS gastric epithelial cells. *J Biol Chem* 2001; **276**: 48127–34.
- 38 Wallasch C, Crabtree JE, Bevec D *et al.* *Helicobacter pylori*-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. *Biochem Biophys Res Commun* 2002; **295**: 695–710.
- 39 Yan F, Cao H, Chaturvedi R *et al.* Epidermal growth factor receptor activation protects gastric epithelial cells from *Helicobacter pylori*-induced apoptosis. *Gastroenterology* 2009; **136**: 1297–307.
- 40 Mann JR, Backlund MG, Buchanan FG *et al.* Repression of prostaglandin dehydrogenase by epidermal growth factor and snail increases prostaglandin E₂ and promotes cancer progression. *Cancer Res* 2006; **66**: 6649–56.
- 41 Myung SJ, Rerko RM, Yan M *et al.* 15-hydroxyprostaglandin dehydrogenase is an in vivo suppressor of colon tumorigenesis. *Proc Natl Acad Sci USA* 2006; **103**: 12098–102.
- 42 Doherty GA, Byrne SM, Molloy ES *et al.* Proneoplastic effects of PGE₂ mediated by EP4 receptor in colorectal cancer. *BMC Cancer* 2009; **9**: 207.
- 43 Oshima H, Hioki K, Popivanova BK *et al.* Prostaglandin E₂ signaling and bacterial infection recruit tumor-promoting macrophages to mouse gastric tumors. *Gastroenterology* 2010 Nov 9. [Epub ahead of print]