Induction of Prostaglandin E$_2$ Pathway Promotes Gastric Hamartoma Development with Suppression of Bone Morphogenetic Protein Signaling

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Abstract
Mutations in bone morphogenetic protein (BMP) receptor 1A (BMPRIA) are responsible for a subset of cases of juvenile polyposis (JP) syndrome that develops hamartomatous tumors in the gastrointestinal tract. Mouse genetic studies have shown that suppression of BMP signaling in the intestines causes JP-type hamartoma development. Here, we generated K19-Nog transgenic mice expressing noggin, a BMP antagonist, in gastric epithelium. However, inhibition of BMP signaling did not cause gastric phenotypes. We thus crossed K19-Nog with K19-C2mE mice that expressed Ptgs2 and Ptges in the stomach to generate compound transgenic mice. Expression of Ptgs2 and Ptges results in prostaglandin (PGE$_2$) biosynthesis, and both enzymes are induced in most human gastrointestinal tumors. Importantly, K19-Nog/ C2mE compound mice developed gastric hamartomas that were morphologically similar to those found in JP with mucin-containing dilated cysts and inflammatory infiltration. Notably, treatment of K19-Nog/C2mE mice with a cyclooxygenase-2 inhibitor, celecoxib, significantly reduced tumor size with suppression of angiogenesis, suggesting that induction of the PGE$_2$ pathway together with inhibition of BMP signaling is required for gastric hamartoma development. Moreover, microarray analyses revealed that canonical Wnt signaling target genes were not induced in K19-Nog/ C2mE hamartomas, indicating that BMP inhibition and PGE$_2$ induction lead to gastric hamartoma development independent of the Wnt/β-catenin pathway. These results, taken together, suggest that the PGE$_2$ pathway is an effective preventive target against BMP-suppressed gastric hamartomas, as well as for Wnt/β-catenin–activated adenocarcinomas.

Introduction
Juvenile polyposis (JP) is a hereditary gastrointestinal hamartomatous polyposis syndrome (1). Germline mutations in bone morphogenetic protein (BMP) receptor type IA gene (BMPRIA) have been found in a subpopulation of JP patients (2). BMP ligands bind to a complex of the BMP receptor type II and type I, which leads to phosphorylation of Smad1,5,8, allowing them to form a complex with Smad4 (3, 4). These Smad complexes trans locate to nuclei and function as transcription enhancers. BMP signaling inhibits epithelial cell proliferation and promotes differentiation (5, 6), and suppression of BMP signaling in mice results in intestinal hamartomatous polyp development through activation of the PI3K-Akt pathway (6, 7). Moreover, intestinal epithelial cell–specific deletion of Bmpr1a results in elongated villi and crypt fission (8). These results indicate that BMP signaling promotes intestinal epithelial differentiation, and, thus, suppression of the BMP pathway causes tumorigenesis. Although the main affected site of tumors in JP patients is the colon, gastric polyps have been found in 14% of JP patients, and cancer risk in JP patients increases both in the colon and stomach (9, 10). Recently, it was reported that disruption of Bmpr1a in mouse stomach results in development of tumors in squamous columnar and gastrointestinal transition zones, suggesting that suppression of BMP signaling triggers tumor development also in the stomach (11).

On the other hand, we found that expression of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) is induced simultaneously in gastrointestinal tumor tissues (12). COX-2 and mPGES-1 are functionally coupled for biosynthesis of prostaglandin E$_2$ (PGE$_2$; ref. 13) that plays a critical role in tumorigenesis in the gastrointestinal tract (14–17). However, the role of the PGE$_2$ pathway in hamartomatous tumors is not understood. We constructed transgenic mice expressing Nog encoding noggin in the gastric mucosa and crossed them with another transgenic mice expressing both Ptgs2 and Ptges encoding COX-2 and mPGES-1, respectively (16). We show that inhibition of BMP signaling is not sufficient for gastric tumorigenesis, but that BMP suppression together with PGE$_2$ induction causes development of JP-type gastric hamartoma.

Materials and Methods
Mouse models. K19-C2mE mice expressing Ptgs2 and Ptges; K19-Wnt1 mice expressing Wnt1; and K19-Wnt1/C2mE mice expressing Wnt1, Ptgs2, and Ptges, were described previously (16, 17). pK19-Nog was constructed using keratin 19 gene promoter, mouse Nog cDNA, and SV40 pA (Fig. 1A). The expression vector was microinjected into the fertilized eggs of F1 (C3H and C57BL/6) mice (CLEA) to generate K19-Nog mice. Primer sequences used for genotyping were as follows: F-5'-GTACGCGTGGAATT-GACCTAGG-3', F-5'-GCAAGGTTGGCTACAGACGTC-3'. Transgenic vector constructs are shown in Fig. 1A. K19-Nog and K19-C2mE mice were crossed to generate K19-Nog/C2mE mice. Gastric phenotypes of these mice were examined at age 30 wk. For inhibition of COX-2, mice were given p.o. with celecoxib (Pfizer) at 100 mg/kg/d for 3 wk. All animal experiments were carried out according to the protocol approved by Ethics Committees on Animal Experimentation of Kanazawa University.

Real-time reverse transcription-PCR. Total RNA was reverse transcribed and PCR-amplified. Primer sets used in real-time reverse
transcription-PCR for detection of Nog, Ptgs2, Piger1, Piger2, Piger3, and Piger4 were purchased (TakaraBio).

**Histology.** Tissues were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 4-μm thickness. The following antibodies were used for immunostaining: anti–COX-2 (Cayman Chemical), anti-F4/80 (Serotec), anti-α-smooth muscle actin (Sigma), anti–Ki-67, anti-von Willebrand factor (DakoCytomation), and anti–phosphorylated Smad1,5,8 (Chemicon). Staining signal was visualized using the Vectorstain Elite kit (Vector Laboratories).

**X-ray computed tomography.** K19-Nog/C2mE mice were subjected to X-ray computed tomography using LaTheta LCT-100 (Aloka). Computed tomography analyses were performed 1 wk before celecoxib treatment and at 0, 1, 2, and 3 wk after treatment. Tumor size on computed tomography images was measured using NIH Image software (NIH).

**Immunoblotting.** Tissue samples were homogenized in lysis buffer. Protein samples were separated in a SDS-polyacrylamide gel. Antibody for the active h-catenin (Upstate) was used. The enhanced chemiluminescence detection system (Amersham) was used to detect specific signals.

**Microarray analyses.** Total RNA were prepared from mouse stomach at age 30 wk. Expression profiles of the Wnt target genes,4 cytokines, and chemokines were examined with the Affymetrix GeneChip system and Mouse Genome 430 2.0 Arrays (Affymetrix).

**Statistical analyses.** Statistical analyses were carried out using Student's t test.

### Results and Discussion

**Generation of K19-Nog transgenic mice.** To suppress BMP signaling in the stomach, we constructed K19-Nog mice that expressed Nog encoding noggin in gastric epithelial cells (Fig. 1A). Noggin is a polypeptide that inhibits BMP signaling by binding BMP ligands (4). We confirmed increased levels of Nog mRNA in K19-Nog mouse stomach compared with that in the wild-type by real-time RT-PCR (Fig. 1B). BMP type I receptor phosphorylates Smad1,5,8 upon complex formation with BMP ligand and type II receptor (3). We found phosphorylated Smad1,5,8 by immunohistochemistry in the nuclei of differentiated epithelial cells both at the surface and bottom of the gastric gland (Fig. 1C), which is consistent with a previous report (11). Notably, in the K19-Nog mice, the immunostaining signals of phosphorylated Smad1,5,8 decreased significantly in the upper gastric gland where the K19 promoter is transcriptionally active (Fig. 1C and D). These results indicate that exogenous Nog expression inhibits BMP signaling in the stomach.

**Construction of compound mutant mice.** We previously constructed K19-C2mE transgenic mice expressing both Ptgs2 and Ptges encoding COX-2 and mPGES-1, respectively, in gastric mucosa (Fig. 1A and D). Expression of COX-2 and mPGES-1 leads to increase of PGE2 level in K19-C2mE mouse stomach (16). To investigate the effect of the PGE2 pathway in BMP-suppressed gastric mucosa, we crossed K19-Nog and K19-C2mE mice to generate K19-Nog/C2mE compound mice. We confirmed expression of Ptgs2 by real-time RT-PCR in the stomach of K19-C2mE and K19-Nog/C2mE mice but not in wild-type and K19-Nog mice (Fig. 1B). We also used K19-Wnt1/C2mE mice for this

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4 http://www.stanford.edu/~rnusse/wntwindow.html
study that develop gastric adenocarcinoma caused by simultaneous activation of the Wnt and PGE₂ pathways (17). Genotypes of respective transgenic strains were confirmed by genomic PCR (Supplementary Fig. S1).

**Gastric tumor development in K19-Nog/C2mE mice.** K19-Nog mice did not develop tumorous lesions in the stomach, and the histology of gastric glands was normal (Supplementary Fig. S2; Fig. 2A). In contrast, K19-C2mE mice developed inflammation-associated hyperplasia, and K19-Wnt1 mice developed dysplastic preneoplastic lesions, which were consistent with previous reports (16, 17). Importantly, K19-Nog/C2mE mice develop large tumors in the glandular stomach (Supplementary Fig. S2; Fig. 2A). These results suggest that suppression of BMP signaling is insufficient for gastric tumorigenesis, but that cooperation of BMP inhibition and PGE₂ induction cause gastric tumor development. Such an effect of PGE₂ on tumorigenesis was similar to that found in K19-Wnt1/C2mE mice. Activation of Wnt/β-catenin alone leads to development of small preneoplastic lesions, whereas simultaneous activation of the Wnt/β-catenin and PGE₂ pathways cause gastric adenocarcinoma (Fig. 2A; ref. 17). Therefore, PGE₂ plays an important role in tumorigenesis regardless of the types of genetic alterations.

**JP-type hamartoma in K19-Nog/C2mE mouse stomach.** Histologically, gastric tumors in K19-Nog/C2mE mice displayed irregular branching of epithelial cell layers, combined with dilated cysts that were filled with mucin (Fig. 2B and C). Such histological characteristics were different from dysplastic tumors of the K19-Wnt1/C2mE mice (Fig. 2B; ref. 17). We also found abundant α-smooth muscle actin–positive myofibroblasts in stroma. Moreover, we detected infiltration of F4/80-positive macrophages and the accumulation of lymphocytes in the K19-Nog/C2mE tumors (Fig. 2C). These histological characteristics are typical of the hamartoma of JP patients (9, 10, 18), indicating that suppression of BMP signaling associated with PGE₂ induction causes development of JP-type gastric hamartoma. However, tumor incidence in K19-Nog/C2mE mice was 23%, whereas that in K19-Wnt1/C2mE mice was 100% (Supplementary Table). Notably, expression of inflammatory cytokine tumor necrosis factor-α (TNF-α) increased in K19-Nog/C2mE hamartomas as well as in K19-C2mE hyperplasia (Supplementary Fig. S3). However, TNF-α was not induced in nontumor stomach of K19-Nog/C2mE mice, whereas transgenic expression of Ptgs2 stayed at the same level as that in tumor tissues. These results suggest that inflammatory response is also important for hamartoma development together with BMP suppression and PGE₂ induction.

**Suppression of gastric hamartoma by COX-2 inhibition.** To investigate whether the PGE₂ pathway is required for gastric hamartoma development, we treated K19-Nog/C2mE mice with a COX-2 selective inhibitor, celecoxib, at 100 mg/kg/day for 3 weeks. We examined gastric tumor size by X-ray computed tomography scanning, and found that the tumor volume of K19-Nog/C2mE mice decreased significantly by celecoxib treatment (Fig. 3A). The mean relative tumor size on computed tomography images reduced to 58% after celecoxib treatment (Fig. 3B). Histologically, cystic structures were no longer found, and necrotic area was detected in the celecoxib-treated K19-Nog/C2mE tumors (Fig. 3C). The PGE₂ pathway is important for angiogenesis of gastrointestinal tumorigenesis (15, 19). Consistently, the number of capillary vessels decreased significantly in celecoxib-treated K19-Nog/C2mE tumors.
Accordingly, it is possible that angiogenesis is one of the important functions of PGE₂ for hamartoma development.

**Wnt-independent development of gastric hamartoma.** In the intestinal crypt, inhibition of BMP signaling results in activation of the Wnt/β-catenin pathway (7). We thus examined activation of Wnt signaling in K19-Nog/C2mE gastric tumors. The level of the active β-catenin did not increase in K19-Nog/C2mE hamartomas, whereas it elevated markedly in K19-Wnt1/C2mE tumors (Fig. 4A). Consistently, expression of Wnt target genes in K19-Nog/C2mE tumors stayed at the same level as that in wild-type mouse stomach, whereas these genes were up-regulated in K19-Wnt1/C2mE tumors (Fig. 4B). We confirmed that inflammatory cytokines and chemokines were induced in both K19-Nog/C2mE and K19-Wnt1/C2mE tumors. Accordingly, activation of Wnt signaling is not involved in hamartoma development in BMP-suppressed gastric mucosa, although PGE₂ signaling or PGE₂-dependent inflammation may be required for both adenocarcinoma and hamartoma.

We next examined expression of PGE₂ receptors, EP1 to EP4, in tumor tissues. Notably, the expression of Ptgε₁, Ptgε₂, and Ptgε₃ encoding EP1, EP2, and EP3, respectively, decreased significantly in tumors of K19-Nog/C2mE and K19-Wnt1/C2mE mice (Fig. 4C). In contrast, expression of Ptgε₄ encoding EP4 increased dramatically in both K19-Nog/C2mE hamartomas and K19-Wnt1/C2mE adenocarcinomas. These results suggest that PGE₂ signaling through EP4 is important for development of both gastric hamartoma and adenocarcinoma. Namely, it is possible that the type of genetic alterations determines the histological phenotype of tumors, hamartoma or adenocarcinoma, and that the induced PGE₂ pathway promotes tumor growth through EP4 receptor regardless of histological types (Supplementary Fig. S4).

In human stomach, expression of COX-2 is induced in Helicobacter pylori–associated gastric lesions (20). Accordingly, it is conceivable that H. pylori infection contributes to the development of gastric hamartoma through induction of the PGE₂ pathway. Therefore, inhibition of the PGE₂ pathway as well as eradication of H. pylori may be an effective preventive strategy not only for gastric cancer but also for gastric hamartoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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References

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