Bone Morphogenetic Protein Signaling Suppresses Tumorigenesis at Gastric Epithelial Transition Zones in Mice

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Abstract
Bone morphogenetic protein (BMP) signaling is known to suppress oncogenesis in the small and large intestine of mice and humans. We examined the role of Bmpr1a signaling in the stomach. On conditional inactivation of Bmpr1a, mice developed neoplastic lesions specifically in the squamocolumnar and gastrointestinal transition zones. We hypothesized that the regulation of epithelial cell fate may be less well defined in these junctional zones than in the adjacent epithelium and found that the mucosa at the squamocolumnar junction in mice shows a lack of differentiated fundic gland cell types and that foveolar cells at the gastrointestinal junctional zone lack expression of the foveolar cell marker Muc5ac. Precursor cell proliferation in both transition zones was higher than in the surrounding epithelium. Our data show that BMP signaling through Bmpr1a suppresses tumorigenesis at gastric epithelial junctional zones that are distinct from the adjacent gastric epithelium in both cellular differentiation and proliferation. [Cancer Res 2007;67(17):8149–55]

Introduction
The epithelial layer of the gastrointestinal tract is in a state of constant renewal. Stem cells in its basal layers generate descendants that undergo a period of rapid amplification until they withdraw from the cell cycle. Cells are allocated to one of several cell lineages and mature while they migrate to their final destination where they undergo a program of cell death and/or are shed into the lumen of the gut. Cell fate decisions of epithelial cells are taken in a position-dependent, non–cell-autonomous manner (1). Such decisions are governed by information that is perceived from the cellular environment. During development, morphogens form concentration gradients through tissues to specify cell fate in a position-dependent manner. An important role for morphogens has now also been established as regulators of cell fate in the adult gastrointestinal tract. Mutations in the Wnt and bone morphogenetic protein (BMP) pathways are the cause of inherited polyposis syndromes as well as the initiating event in the development of sporadic gastrointestinal cancer development (2, 3).

We have previously shown that BMPs are expressed in the adult stomach (4); however, we still know very little about the role of BMP signaling in the maintenance of homeostasis of the complex epithelial system of the stomach. The BMPs are soluble proteins that are part of the transforming growth factor-β (TGF-β) superfamily. BMP ligands bind to a complex of the BMP receptor type II and a BMP receptor type I (Ia or Ib). This leads to the phosphorylation of the type I receptor that subsequently phosphorylates the BMP-specific Smads (Smad1, Smad5, and Smad8), allowing these receptor-associated Smads to form a complex with Smad4 and move into the nucleus where the Smad complex binds a DNA binding protein and acts as a transcriptional enhancer (5). BMPs regulate apoptosis of intestinal epithelial cells (6) and mutations in the BMP receptor 1a (BMPRIA) have been found in ~20% of families with juvenile polyposis syndrome whereas another 20% of cases of juvenile polyposis syndrome are caused by mutations in SMAD4, the common mediator of BMP and TGF-β signaling (7–9). Mice with a conditional inactivation of the Bmpr1a (10) and mice that overexpress a BMP antagonist in the intestine (11) have an intestinal phenotype that resembles juvenile polyposis with the development of multiple hamartomatous polyps in the intestine. The role of BMP signaling in the adult stomach has not been examined.

The glandular mucosa of the murine stomach is divided into a proximal zymogenic or fundic region and a distal mucous or antral region (12). Stem cells of the gastric mucosa are located approximately halfway down the tubular units that invaginate into the mucosal surface. From this stem cell zone, cells can migrate in two ways. Cells that migrate up to the luminal surface of the stomach form the pit region or foveola. These pit cells secrete mucus (Muc5ac) and are similar throughout the stomach. Cells that migrate downward from the stem cell position form the specialized glandular region of each unit. In the proximal stomach, these glands are fundic or oxyntic-type glands. Fundic glands are composed of acid-producing parietal cells, mucous neck cells, digestive enzyme–producing zymogenic (or chief) cells, and endocrine cells. In the distal stomach, the glands are antral-type glands and they contain a variety of different endocrine cells and mucinous cells. Here, we examine the role of BMP signaling through the Bmpr1a using a conditional knockout approach.

Materials and Methods
Mice. To obtain normal tissue of the gastric transition zones, C57BL/6 control mice were given a single i.p. injection of 150 mg/mL bromodeoxyuridine (BrdUrd) to label cells in S phase 1 h before being killed by cervical...
dislocation. To allow optimal orientation of the gastric tissue, flat stomachs were prepared according to the method described by Lee et al. (12).

To evaluate the role of Bmpr1a signaling in the gastric mucosa, we used a conditional knockout approach using the Cre/loxP system. We used a mouse with a conditional null allele of Bmpr1a (Bmpr1afx/fx) that was previously generated and characterized (10, 13, 14). In these mice, LoxP sites have been introduced in the first and second introns of the Bmpr1a locus. It was previously shown that when these mice are crossed with CMV-Cre transgenic mice, the phenotype is identical to the Bmpr1a (+/C0/C0) mouse, indicating that deletion of exon 2 is sufficient to completely inactivate Bmpr1a function. For our experiment, the Bmpr1afx/fx transgenic mouse was crossed with the Mx1-Cre transgenic mouse (13) in which expression of the Cre recombinase gene is under control of an IFN-responsive promoter (15) that can be induced on systemic treatment with polyinosinic-polycytidylic acid (PolyI-C). In the resulting Mx1-cre-Bmpr1afx/fx mice, the floxed Bmpr1a is excised on treatment with PolyI-C. Four-week-old Mx1-cre-Bmpr1afx/fx mice were injected i.p. with 250-mg PolyI-C. Because we have previously found that multiple injections with PolyI-C are required for efficient deletion of Bmpr1a, mice were injected with PolyI-C thrice on alternate days (14). Animals were sacrificed at 5 months of age. To confirm that the Mx1 promoter–driven Cre efficiently targets the gastric epithelium as previously described (16), the Mx1-Cre transgenic mouse was crossed with the Z/EG reporter mouse, which expresses green fluorescent protein (GFP) in cells in which efficient Cre-mediated excision of LoxP sites has

Figure 1. Neoplastic change at the gastric epithelial transition zones in PolyI-C–treated Mx1-cre-Bmpr1a^{fx/fx} mice. A to D, immunohistochemistry for GFP expression in PolyI-C–treated Mx1-cre-Z/EG (A and B) and wild-type (C and D) mice used as negative controls shows efficient Mx1-Cre–mediated recombination in the gastric epithelium (A and B). E to K, H&E section of a neoplastic lesion at the squamocolumnar junction showed an area of carcinoma in situ (F, arrows) adjacent to the squamous epithelial layer (enlarged in G). H to K, H&E section of a small polypl at the gastrointestinal junction (I, intestine; P, pyloric ring). I, intestinal epithelium with normal crypt-villus architecture bordered the distal end of the polypl. J, the epithelium of the polypl was of gastric type and histologically mature, consistent with a hamartomatous polypl. K, the epithelium that borders the proximal end of the polypl showed normal gastric antral glands. L to N, immunohistochemical detection of the intestinal brush border protein villin. L, a small polypl at the gastrointestinal junction (P, pyloric ring). M, epithelial cells in the polypl were villin negative, consistent with the gastric type histology. N, villin was expressed by enterocytes in the adjacent intestinal epithelium. A, ×160; B, ×400; C, ×160; D, ×400; E, ×160; F and G, ×400; H, ×80; I to K, ×400; L, ×80; M and N, ×800.
occurred (17). Mx1-Cre-Z/EG mice were injected with PolyI-C thrice on alternate days and sacrificed 18 days after treatment. Stomachs and duodenums were excised, cut longitudinally, fixed in 4% paraformaldehyde, and processed following routine histologic procedures. Animal experiments were approved by the animal ethics review board.

**Antibodies and immunohistochemistry.** A goat polyclonal anti-villin (1:50; C-19) was from Santa Cruz Biotechnology. A mouse monoclonal anti-Muc5ac (1:50; 45M1) was from Novocastra. A mouse monoclonal anti-BrdUrd (1:50) was from Roche. A rabbit polyclonal antibody that recognizes the phosphorylated form of the BMP-specific Smads, Smad1, Smad5, and Smad8 (pSmad 1,5,8, 1:40) was from Cell Signaling Technology. A rabbit anti-β-catenin (1:1,000) was from Transduction Laboratories. A rabbit anti-GFP (1:250) was from Molecular Probes. For all immunohistochemistry except for β-catenin (see below), the following protocol was used: sections were dewaxed and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 1.5% H2O2 in methanol. Antigen retrieval was done by boiling slides for 10 min in 0.01 mol/L sodium citrate (pH 6.0). Nonspecific binding sites were blocked with 10% normal human serum in PBS for 30 min. For unmasking of the BrdUrd epitope, slides were incubated in 2 N HCl at 37°C for 60 min and then washed in boric acid (pH 8.5) before the blocking step. Slides were incubated with the primary antibodies overnight at 4°C in PBS with 0.1% Triton and 1% bovine serum albumin. The second day, slides were washed in PBS and incubated with the appropriate biotinylated secondary antibody at room temperature for 1 h in PBS with 10% normal human serum. Slides were washed in PBS and incubated with streptavidin-biotin-horseradish peroxidase (HRP; DAKO) for 1 h. After washing in PBS, peroxidase activity was detected using the “Fast DAB” system (Sigma) according to the manufacturer's instructions. Finally, sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted in Entellan (Merck) under coverslips.

For double staining of H+K+-ATPase and pSmad1,5,8 sections were first incubated overnight with anti-pSmad1,5,8. The following day, sections were incubated for 60 min with a biotinylated goat anti-rabbit immunoglobulin. Sections were then incubated with streptavidin-biotin-HRP and further processed as described above to detect pSmad1,5,8 expression. Sections were then incubated overnight with anti–H+K+-ATPase. The following day, an alkaline phosphatase–coupled goat anti-mouse immunoglobulin was applied. Alkaline phosphatase was detected with the Fast Red detection method (DAKO), resulting in a red precipitate. Immunohistochemical controls consisted of omission of the primary or secondary antibodies.

For detection of β-catenin, a staining protocol was used that has been developed at the Clevers laboratory (18). Slides were dewaxed and rehydrated. Then, slides were boiled in a pressure cooker for 10 min and...
left to cool down very slowly. Slides were blocked with 2-nitro-5-thiobenzoate (TNB) at room temperature and incubated with the anti–β-catenin antibody at 4°C overnight. Slides were washed thrice in TNS [0.1 mol/L Tris (pH 7.5), 0.15 mol/L NaCl, 0.3% Triton X-100] and incubated for 60 min at room temperature with a biotinylated rabbit anti-mouse antibody from the labeled streptavidin-biotin+ (LSAB+) kit from DAKO. Slides were washed thrice in TNS and incubated for 30 min at room temperature with streptavidin-HRP from the LSAB+ kit diluted 1:100 in TNR. After washing thrice in TNS, slides were incubated for 10 min at room temperature in BT working solution from the LSAB+ kit (diluted 1:50 in amplification diluent from the same kit). Slides were washed thrice in TNS and incubated for 30 min with streptavidin-HRP from the LSAB+ kit diluted 1:100 in TNR at room temperature. Slides were washed thrice in TNS and staining was developed using the DAB substrate-chromogen solution from the LSAB+ kit.

**BrdUrd-positive nuclei.** To assess proliferation of epithelial cells in the gastric mucosa, gastric specimens from BrdUrd-injected control mice were stained with antibodies against BrdUrd. Pictures of each transition zone and the adjacent gastric mucosa were taken at ×100 magnification and positive nuclei counted with the use of an image analysis program (Image-ProPlus, MediaCyberNetics). In each zone, five well-oriented vertical units were counted per mouse. The average number of positive nuclei per vertical unit was compared between groups. To be able to compare the results between animals, it was ensured that all sections visualized the entire axis of the superficial epithelium to the muscularis mucosa.

**Statistical analysis.** Data are presented as mean ± SE. Comparisons between groups of data were made using the Student t test. P < 0.05 was considered statistically significant.

**Results**

**Bmpr1a signaling suppresses tumorigenesis at gastric epithelial junctional zones.** To address the role of BMP signaling in the gastric mucosa, we examined mice in which the Bmpr1a can be conditionally inactivated on treatment with PolyI-C. We first confirmed the previously reported targeting of gastric epithelial cells by the Mx1 promoter–driven Cre (16). Mx1-Cre-Z/EG reporter mice were treated with PolyC and the expression of GFP was detected with an anti-GFP antibody. As can be seen in Fig. 1A to D, strong staining was detected of epithelial cells throughout the gastric glands in PolyC-treated Mx1-Cre-Z/EG mice (Fig. 1A and B) whereas no staining was detected in the stomachs of wild-type mice (Fig. 1C and D). This experiment confirmed the efficient Mx1-Cre–driven recombination in gastric epithelial cells that was previously reported by others. PolyC-treated Mx1-Cre-Bmpr1aflox/flox mice showed a phenotype of epithelial hyperplasia in the proximal, fundic region (data not shown). The most remarkable phenotype was observed at the squamocolumnar and gastrointestinal transition zones. Bmpr1a mutant mice developed tumors specifically at both gastric epithelial junctional zones (Fig. 1E–K). Five of seven mice showed intraepithelial neoplasia, varying from mild dysplasia to carcinoma in situ specifically at the squamocolumnar junction (Fig. 1E–G). Six of seven mice examined showed formation of a polyp of variable size specifically at the gastrointestinal transition zone (Fig. 1H–K). Histologically, the smaller polyps showed normal differentiation of the epithelial cells and recruitment of inflammatory cells to the lamina propria, consistent with a hamartomatous polyp (Fig. 1H–K). In the larger polyps, we observed foci of dysplastic epithelial cells, indicating adenomatous transformation of the hamartomatous polyp (Fig. 1E–G). Histologically, all polyps consisted of gastric type columnar epithelial cells. At the gastrointestinal transition zone, the polyps bordered directly on the small intestine; epithelial cells in the polyps were negative for the intestinal enterocyte marker villin (Fig. 1L–N).

Comparative analysis of epithelial cells in mucosal layers of conditional Bmpr1a mutant mice (arrows). C and D, no nuclear accumulation is detected in a gastric tumor that was present in the same specimen as the intestinal tumor shown in (B). Original magnification: A, ×400; B, ×200; C, ×100; D, ×400.

**Figure 3.** Expression of β-catenin. A, in normal small intestinal crypts, nuclear β-catenin can be detected in a few cells at the base of the crypt (arrows). B, extensive nuclear accumulation of β-catenin can be detected in the small intestinal tumors of conditional Bmpr1a mutant mice (arrows). C and D, no nuclear accumulation is detected in a gastric tumor that was present in the same specimen as the intestinal tumor shown in (B). Original magnification: A, ×400; B, ×200; C, ×100; D, ×400.

**No nuclear accumulation of β-catenin in gastric tumors of Bmpr1a mutant mice.** Overactivity of the Wnt pathway with nuclear accumulation of β-catenin plays a critical role in the formation of intestinal tumors. Nuclear accumulation of β-catenin has been observed in intestinal tumors of mice that overexpress the BMP inhibitor Noggin in their intestine and intestinal tumors of conditional Bmpr1a mutant mice. These observations suggest that BMP signaling negatively regulates Wnt signaling in the intestine. We examined nuclear expression of β-catenin in normal small intestinal crypts (Fig. 3A), in intestinal tumors of Bmpr1a mutant mice (Fig. 3B), and in gastric tumors of Bmpr1a mutant mice (Fig. 3C and D). As expected, we detected nuclear β-catenin in a few cells at the base of the normal small intestinal crypt (Fig. 3A; ref. 18). Similarly, we detected abundant nuclear accumulation of β-catenin in intestinal tumors of conditional Bmpr1a.
mutant mice (Fig. 3B) as described (10). In contrast, no such accumulation was observed in gastric tumors of Bmpr1a mutant mice (Fig. 3C and D). A few cells with nuclear β-catenin could be detected in some gastric tumors but most of the tumors were negative. The intestinal tumor shown in Fig. 3 was in the same specimen as the gastric tumor shown in the same figure. The intestinal tumor, therefore, served as an internal positive control for the staining.

The murine squamocolumnar and gastrointestinal transition zones. Because differentiating cells at epithelial junctional zones are exposed to conflicting extrinsic signals from two different epithelial tissues, we hypothesized that the regulation of epithelial cell fate may be less well defined in these zones than in the adjacent epithelium. We have therefore examined the squamocolumnar epithelial transition zone of the proximal stomach and the gastrointestinal junction in normal adult mice (n = 7). Similar to the gastric epithelium immediately adjacent to the squamous epithelium of the esophagus in humans (the cardia; ref. 19), gastric epithelium at the squamocolumnar epithelium in mice was characterized by one to three gastric glands that are mainly composed of mucous cells (Fig. 4A and B). The cardiac glands lacked the specialized cell types of the fundic glands of the proximal stomach as they contained no zymogenic cells and only an occasional parietal cell (Fig. 4A and B). This lack of parietal cells was confirmed by immunohistochemistry for H⁺K⁺-ATPase (Fig. 4C).

The gastrointestinal transition zone is localized at the pyloric ring. Here, the glandular region of the distal gastric epithelium (antrum) borders the crypt-villus structure of the small intestine (Fig. 4D and E). We examined epithelial differentiation of pit cells at the gastrointestinal junction using the expression of Muc5ac. Muc5ac is a mucin that is uniformly expressed by gastric pit cells in the gastric antrum (Fig. 4F; ref. 20). At the gastrointestinal transition zone, however, expression of Muc5ac was patchy and most pit cells were actually Muc5ac negative (Fig. 4G). These data supported the idea that cells at the squamocolumnar and gastrointestinal junction lack some of the signals that specify cell fate in the adjacent epithelium. Because epithelial cells in the

Figure 4. The gastric junctional zones in the normal mouse. A to C, the squamocolumnar junction. A, H&E section showed pink-staining triangular parietal cells in the fundic mucosa (arrows), whereas the cardiac glands (enlarged in B) at the junction between the squamous epithelium and the fundic mucosa lacked parietal cells and were composed entirely of mucous cells (B, asterisk). C, immunohistochemistry for the parietal cell marker H⁺K⁺-ATPase confirmed lack of parietal cells in the cardiac glands (asterisk) in the mouse. D and E, H&E staining of the gastrointestinal junction (arrow, intestinal villus; P, pyloric ring). E, higher magnification of the gastrointestinal transition zone. F and G, Muc5ac immunohistochemistry. F, uniform expression of Muc5ac in pit cells of the gastric antrum in the mouse. G, at the transition zone, most epithelial cells were Muc5ac negative compared with the pits of the distal antral mucosa (arrows). A, ×100; B and C, ×400; D, ×80; E to G, ×200.
gastric transition zones may be exposed to conflicting signals that regulate their cell fate, we hypothesized that the cell cycle of epithelial precursor cells in these zones may be less tightly regulated and that these cells may have a higher rate of proliferation than those in the surrounding epithelium. We therefore examined epithelial BrdUrd incorporation in normal mice as a marker of the regulation of cell fate at the epithelial junctional zones. Indeed, we found that proliferation was increased at both gastric epithelial transition zones compared with the adjacent gastric epithelium (Fig. 5A–H).

Discussion

Here, we show that Bmpr1a conditional null mice developed neoplasia specifically at both gastric junctional zones. A small lesion at the gastrointestinal junctional zone showed that the process of neoplasia seems to start with an accumulation of immature epithelial cells that were partly positive for BrdUrd, indicating that an expansion of the precursor cell compartment is a primary event in the genesis of these polyps. Intestinal tumors of conditional Bmpr1a mutant mice (10) and mice that overexpress the BMP antagonist Noggin in the intestine (11) develop intestinal tumors that show intense nuclear accumulation of β-catenin. This indicates that BMP signaling acts as a break on tumorigenic Wnt signaling activity in the intestine. No evidence was found for a similar molecular mechanism in the genesis of gastric polyps in the Bmpr1a conditional mutant mouse as we observed very little nuclear β-catenin in the gastric polyps. This indicates that the mechanism of tumor suppression by BMP signaling in the stomach may be distinct from that in the intestine.

Enhanced sensitivity to oncogenesis at the gastrointestinal junctional zone was previously described in mice. Mice treated with the carcinogen N-nitroso-N-butilurea specifically develop polypoid tumors at the junction of the gastric and intestinal epithelial layers (21). Additionally, mice with a mutation in Smad4 develop tumors specifically at the junction of the stomach and the intestine (22). Enhanced sensitivity to oncogenesis at the junction of two different epithelial tissues is known to exist in humans but is a poorly understood phenomenon. The best known clinical examples of tumors associated with epithelial transition zones in humans are carcinomas that arise at the esophagogastric junction and at the squamocolumnar junction of the uterine cervix (23, 24). Other examples are tumors that arise at the anorectal squamocolumnar junction and adenomas and carcinomas of the ampulla of Vater (25–27).

Epithelial cells that have to make cell fate choices at the junction of two distinctive epithelial tissues are prone to receive conflicting extrinsic information from the two different epithelial environments. At this point, morphogen gradients involved in the regulation of cell fate of one epithelial tissue type may contrast...
with those that specify another epithelial phenotype. In support of this notion, we found that cellular differentiation in both gastric transition zones is distinct from the surrounding epithelium. This is clearly seen at the squamocolumnar junction where differentiation of epithelial cells in the cardiac glands is less complex than in the adjacent fundic glands. The conflicting signals perceived by cells in a transition zone may not only influence cellular differentiation but also compromise the usually stringent controls of the cell cycle and predispose these cells to oncogenesis. We have indeed found some preliminary evidence that the regulation of the cell cycle in the gastric epithelial transition zones may be distinct from the adjacent epithelium, but further experiments are needed to support this concept.

It was recently proposed that the juvenile polyposis phenotype in Smad4 mutant mice results from loss of Smad4 in T cells and not in the epithelium (28). Because Smad4 is a common mediator mechanism may exist for Bmpr1a signaling, this suggests that a similar mechanism may exist for Bmpr1a mutations. We find little evidence in the current study that tumor formation is mediated via loss of Bmpr1a from mesenchymal cell types as we observe loss of pSmad1,5,8 staining in the epithelium of polyps whereas the underlying inflammatory infiltrate is strongly positive. In addition, in the Mx1-Cre Z/EG reporter mice, we observe efficient recombination in the gastric epithelium but few GFP-positive cells in the mesenchyme of the stomach. This is a very interesting avenue of research, however, and we cannot exclude that Bmpr1a mutant cells in mesenchyme play a role in our model. Further research will have to address this possibility.

In conclusion, our data show that the squamocolumnar and gastrointestinal junctional zones in mice are epithelial areas that are distinct from the surrounding epithelium in both cellular differentiation and cell cycle regulation. We find that these areas show an enhanced propensity to oncogenesis that is suppressed by Bmpr1a signaling.

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