Tumorigenesis and Neoplastic Progression

Gastrin Is an Essential Cofactor for *Helicobacter*-Associated Gastric Corpus Carcinogenesis in C57BL/6 Mice

Shigeo Takaishi,* Shuiping Tu,*
Zinaida A. Dubeykovskaya,* Mark T. Whary,[†]
Sureshkumar Muthupalani,[†] Barry H. Rickman,[†]
Arin B. Rogers,[†] Nantaporn Lertkowitz,[‡]
Andrea Varro,[‡] James G. Fox,[†]
and Timothy C. Wang*

From the Division of Digestive and Liver Disease,* Department of Medicine, Columbia University Medical Center, New York, New York; the Division of Comparative Medicine,[†] Massachusetts Institute of Technology, Cambridge, Massachusetts; and the Physiological Laboratory,[‡] School of Biomedical Sciences, University of Liverpool, Liverpool, United Kingdom

We have previously described a synergistic interaction between hypergastrinemia and *Helicobacter felis* infection on gastric corpus carcinogenesis in FVB/N mice housed under specific-pathogen-free conditions. However, gastrin-deficient (GAS-KO) mice on a mixed C57BL/6/129Sv genetic background maintained in conventional housing were reported to develop spontaneous gastric antral tumors. Therefore, we investigated the role of gastrin in *Helicobacter*-associated gastric carcinogenesis in *H. felis*-infected mice on a uniform C57BL/6 background housed in specific-pathogen-free conditions. Hypergastrinemic transgenic (INS-GAS) mice, GAS-KO mice, and C57BL/6 wild-type mice were infected with *H. felis* for either 12 or 18 months. At 12 months postinfection, INS-GAS mice had mild corpus dysplasia, while B6 wild-type mice had either severe gastritis or metaplasia, and GAS-KO mice had only mild to moderate gastritis. At 18 months postinfection, both INS-GAS and B6 wild-type mice had both severe atrophic gastritis and corpus dysplasia, while GAS-KO mice had severe gastritis with mild gastric atrophy, but no corpus dysplasia. In contrast, both GAS-KO and B6 wild-type mice had mild to moderate antral dysplasia, while INS-GAS mice did not. *H. felis* antral colonization remained stable over time among the three groups of mice. These results point to a distinct effect of gastrin on carcinogenesis of both the gastric cor-

pus and antrum, suggesting that gastrin is an essential cofactor for gastric corpus carcinogenesis in C57BL/6 mice. (*Am J Pathol* 2009, 175:365–375; DOI: 10.2353/ajpath.2009.081165)

Gastric cancer remains the second leading cause of cancer-related mortality in the world, although its incidence and mortality rates have been decreasing in the United States over the past 70 years.^{1–3} The risk of developing gastric adenocarcinoma is strongly associated with *Helicobacter pylori* infection, which is gradually disappearing from western societies. Despite the overall decline in gastric cancer prevalence, the treatment of stomach cancer remains a challenging clinical problem, since most patients who undergo surgical resection develop regional or distant recurrences and the overall 5-year survival rate for gastric cancer patients remains around 20% in western countries.³

H. pylori, first identified in the gastric antrum of patients with active chronic gastritis and peptic ulcers,⁴ is now recognized as the major cause of gastric cancer, and has been classified as a group I carcinogen by World Health Organization.^{5,6} *H. pylori* infection causes persistent chronic gastritis, which in susceptible individuals may progress to atrophy, intestinal metaplasia, dysplasia, and finally, intestinal-type gastric cancer. This sequence, commonly referred to as Correa's cascade, is considered the primary histological pathway for the development of intestinal type of gastric cancer,⁷ and is both initiated and promoted by *H. pylori* infection.

Supported by National Institutes of Health grants R01CA093405 and R01CA120979 (T.C.W.), and R01AI37750, P01CA026731, and P30ES02109 (J.G.F.), the Medical Research Council and the Wellcome Trust and Wolfson Foundation (A.V.).

Accepted for publication March 24, 2009.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Timothy C. Wang, M.D., Division of Digestive and Liver Disease, Department of Medicine, Columbia University Medical Center, 1130 St. Nicholas Avenue, ICRC 923, New York, NY. E-mail: tcw21@columbia.edu.

It has generally been recognized that *H. pylori* infection results in a mild (1.5- to 2-fold elevation) hypergastrinemia that occurs early on in the course of the infection in many individuals. Given the known properties of gastrin as a mucosal growth factor, hypergastrinemia was postulated to be a factor promoting the development of gastric cancer. Indeed, previous studies have suggested a possible association between hypergastrinemia, *Helicobacter* infection, and gastric cancer.^{8–12} Therefore, to study the role of gastrin and the potential mechanisms involved in gastric carcinogenesis, we developed a mouse model of gastric cancer through the generation of insulin-gastrin (INS-GAS) transgenic mice that overexpressed human amidated gastrin. In the absence of *Helicobacter* infection, INS-GAS mice on an FVB/N genetic background exhibited mild hypergastrinemia in association with elevated gastric acid secretion and an increased parietal cell number at 1 to 3 months of age. With increasing age, the INS-GAS mice showed progressive loss of parietal cells and significant changes in the corpus, including hypochlorhydria, gastric atrophy, metaplasia, and dysplasia. At 20 months of age, INS-GAS mice developed invasive gastric cancer.⁹ The gastric cancer phenotype was accelerated by gastric *Helicobacter* spp. infection, and lesion severity was more profound in male INS-GAS mice.¹⁰ The cause of this gender-specific incidence was due in part to ovarian-dependent estrogen production, since *H. pylori* infected ovariectomized female INS-GAS mice also developed severe gastric neoplasia, and 17 β -estradiol treatment significantly suppressed this phenotype.¹²

However, determining the role of gastrin in predisposing individuals to gastric cancer has not been straightforward. Some *H. pylori*-infected patients have lower levels of gastrin and acid secretion relative to non-infected healthy persons, and hypochlorhydria probably plays an important role in the carcinogenic process through altered bacterial colonization along with changes in nitrite levels.¹³ Gastrin-deficient mice on a mixed C57BL/6J 129Sv background developed spontaneous gastric antral tumors when maintained under conventional housing conditions at 12 months of age, while C57BL/6J wild-type and somatostatin-deficient mice did not develop tumors.¹⁴ The authors concluded that neoplastic transformation of the antrum does not require gastrin, and that gastrin may actually suppress the development of gastric antral tumors. In addition, there have been other genetic models reported, such as the gp130^{757F/F} mouse, which do not appear to be dependent on gastrin for tumor development.¹⁵ It has been difficult to reconcile these observations regarding the influence of gastrin on gastric cancer, given the different genetic backgrounds, housing conditions, and *Helicobacter* infection status. Thus, the purpose of this study is to examine the effect of gastrin in *Helicobacter*-associated gastric carcinogenesis using hypergastrinemic (INS-GAS) mice and gastrin deficient (GAS-KO) mice on a uniform C57BL/6 background and housed under SPF conditions.

Materials and Methods

Mice Breeding and *H. felis* Infection

The animal protocol was reviewed and approved by the Columbia University Medical Center Institutional Animal Care and Use Committee. Eight- to twelve-week-old, male and female hypergastrinemic transgenic (INS-GAS) mice, gastrin-deficient (GAS-KO) mice, both backcrossed with C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) more than 10 generations, and C57BL/6J wild-type mice were used in this study.^{9,11,16} Male hypergastrinemic transgenic (INS-GAS) mice in a FVB/N background with or without *H. felis* infection for 9 to 10 months, and FVB/N wild-type mice (Jackson Laboratory, ME), with or without *H. felis* infection for 12 months, were also included in the study for comparison as previously described.⁹ All mice were bred under SPF conditions and thus free from murine-specific pathogens such as Lymphocytic choriomeningitis virus, Sendai virus, Mouse hepatitis virus, Ectromelia virus, *Mycoplasma pulmonis*, *Clostridium piliforme*, *Salmonella* spp., *Pasteurella pneumotropica*, *Corynebacterium kutscheri*, *Citrobacter rodentium*, *Giardia muris*, and *Spironucleus muris*. Mice were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and housed on hard wood bedding in micro-isolator, solid-bottomed polycarbonate cages, and given a commercial rodent diet and water *ad libitum*. Mice were infected by oral gavage with *H. felis* in 0.2 ml trypticase broth three times per week on every other day for a total dose of 100 million colony-forming units per mouse as previously described.⁹ *H. felis*-uninfected mice were sham dosed with 0.2 ml of broth. Mice were euthanized and analyzed at 12 and 18 months post-infection (MPI).

Tissue Collection and Histological Analysis

Following isoflurane inhalation, blood was immediately collected into serum collection vials (BD Biosciences, San Jose, CA) by incision of brachial artery or vein. The mice were then sacrificed by cervical dislocation. The stomach and proximal duodenum were removed and the stomach was incised along the greater curvature. Linear gastric strips from the lesser curvature were fixed overnight in 10% neutral-buffered formalin, embedded, cut to 5- μ m thickness, and stained with H&E. Tissue sections were scored for gastric lesions using previously published criteria by board certified veterinary pathologists (S.M., B.H. R., and A.B.R.) blinded to sample identity.^{17,18} A dysplasia score of 3.0 was considered carcinoma *in situ* or low-grade gastrointestinal intraepithelial neoplasia. Ki-67 immunostaining (Abcam, see immunostaining section) was used to measure epithelial proliferation of gastric mucosa. The ratio of Ki-67-positive to total epithelial nuclei in glands occupying the full length of the proximal corpus was quantified manually for the Ki-67 labeling index, and the results were averaged from two to three mice in each group. The remainder of the gastric tissue was snap-frozen in dry ice and stored at -80°C for mRNA analysis.

Evaluation of *H. felis* Colonization by Quantitative Real-Time PCR

Small pieces of gastric tissue, 1 to 2 mm square in size, from the corpus and antrum respectively, were digested with proteinase K at 55°C for 24 to 48 hours, followed by genomic DNA extraction using DNA isolation kit (Lamda Biotech, St. Louis, MO) based on the manufacturer's instruction. *H. felis* colonization levels in gastric tissue were quantified by real-time PCR assay with *H. felis* flagellar filament B (flaB) primers using QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA) and 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) as previously described.¹⁷ The genomic copies of *H. felis* colonies were normalized by comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level determined by quantitative PCR, which is assumed to represent endogenous stomach genomic DNA quantity. Primer sequences used in this experiment as follows: *H. felis* flaB: forward 5'-TTCGATTGGTCCTACAGGCTCAGA-3', reverse 5'-TTCTTGTTGATGACATTGACCAACGCA-3'; mouse GAPDH: forward 5'-GACATCAAGAAGGTGGTGAAGCA-G-3', and reverse 5'-ATACCAGGAAATGAGCTTGACAA-A-3'. PCR conditions are: 95°C for 15 minutes followed by 40 cycles of 94°C for 10 seconds, 55°C for 20 seconds, and 72°C for 30 seconds. Any sample detecting <10 copies of the *H. felis* genome was considered negative for *H. felis* colonization.

Quantitative Analysis of mRNA Expression

Longitudinal strips of gastric tissue from the anterior wall and the posterior wall were harvested and snap-frozen in dry ice and kept in -80 freezer until processed for analysis. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 4 µg of total RNA with Superscript III First Strand cDNA synthesis kit (Invitrogen, CA). Expression levels of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4), growth factors such as Reg I and Amphiregulin, matrix metalloproteinases (MMPs) such as MMP-9 and -13, were quantified by real-time PCR assays using PCR conditions: 95°C for 15 minutes followed by 40 cycles of 94°C for 10 seconds, 55°C for 20 seconds, and 72°C for 30 seconds, using QuantiTect SYBR Green PCR kit (Qiagen) and 7300 Real Time PCR System (Applied Biosystems, CA). Primer sequences used in this experiment are as follows: TNF-α: forward 5'-TGGCCCA-GACCCTCACACTCAG-3', reverse 5'-ACCCATCGGCTGG-CACCACT-3'; IL-4, forward 5'-ATCGGCATTTTGAACGAG-GTCA-3', reverse 5'-CATCGAAAAGCCCGAAAGAGTCT-3'; Reg I, forward 5'-AAGGAGAGTGGCACTACAGACG-3', reverse 5'-GTATTGGGCATCACAGTTGTCA-3'; Amphiregulin, forward 5'-GGCAAAAATGGAAAAGGCAGAA-3', reverse 5'-CGAGGATGATGGCAGAGACAAA-3'; MMP-9, forward 5'-CGTCATTCGCGTGGATAAGGAG-3', reverse 5'-CCTGGTTCACCTCATGGTCCAC-3'; MMP-13, forward 5'-AGATTATCCCCGCCTCATAGAAGA-3', reverse 5'-GG-AATTGTTGGCATGACTCTCAC-3'; and GAPDH, forward 5'-GACATCAAGAAGGTGGTGAAGCAG-3', reverse 5'-AT-

ACCAGGAAATGAGCTTGACAAA-3'. mRNA level of each gene was normalized to the mRNA level of internal control GAPDH using the $\Delta\Delta C_t$ method.¹⁷

Gastrin Radioimmunoassay

Mouse serum samples were assayed for total amidated gastrin concentrations by radioimmunoassay using antibody L2 (which reacts with G17 and G34, but not progastrin or Gly-gastrins), and L6 for human G17 (which reacts with human G17 only), as previously described.^{12,17}

Serum Titer of *H. felis*-Specific Antibodies

Mouse serum samples collected at necropsy were evaluated for *H. felis*-specific, total IgG, Th2-associated IgG1, and Th1-associated IgG2c, by enzyme-linked immunosorbent assay using an outer membrane protein preparation from *H. felis*, as previously described.¹⁹ In brief, 96-well flat-bottomed plates were coated with 100 µl of antigen (10 µg/ml) overnight at 4°C; sera were diluted to a ratio of 1:100 and added to the wells. Biotinylated secondary goat anti-mouse antibodies, clones A85-1 and 5.7 (BD Biosciences, CA), were used for detecting IgG, IgG1, and IgG2c respectively. Incubation with extravidin peroxidase (Sigma-Aldrich, St. Louis, MO) was followed by treatment with 2,2'-azinobis 3-ethylbenzthiazolinesulfonic acid substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for color development. The absorbance was recorded at A_{405} and A_{595} with a plate reader per manufacturer's protocol (Power WaveX Select, Bio-Tek Instruments, Winooski, VT).

Immunohistochemical Staining

Tissues were fixed in 10% formalin, embedded in paraffin, and processed by standard histological methods. From each selected paraffin block, 5-µm serial sections were cut. Immunohistochemical studies were performed with avidin-biotin-peroxidase complex kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The following primary antibodies were used: anti-Ki-67 (1:100, rabbit polyclonal, Abcam, Cambridge, MA), anti-mouse-trefoil factor (TFF)2 antibody (1:100, rabbit polyclonal antibody, raised in our laboratory), and anti-human MMP-13 antibody (1:100, mouse monoclonal, Chemicon, Billerica, MA). Primary antibodies were incubated at room temperature for 1 hour or overnight, in a humidified chamber. Diaminobenzidine (Vector Laboratories) was used as the chromogen and slides were counterstained with Mayer's hematoxylin.

Statistical Analysis

Gastric lesion scores were compared by Student's *t*-test or Mann-Whitney *U*-test. Expression levels of cytokines and IgG titers were compared using Student's *t*-test.

Figure 1. Macroscopic images and histological analysis of stomachs from *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type (wt) mice at 12 MPI. *H. felis*-infected INS-GAS mice showed accelerated gastric corpus metaplasia and dysplasia (indicated by yellow arrows) at 12 MPI, as compared with infected B6 wild-type and GAS-KO mice. **A:** Macroscopic images of the stomachs at 12 MPI, **left:** *H. felis*-infected INS-GAS mice, **middle:** infected GAS-KO mice, **right:** infected B6 wild-type mice. **B:** H&E images of the gastric corpus. **Left:** *H. felis*-infected INS-GAS mice; **middle:** infected GAS-KO mice; **right:** infected B6 wild-type mice (magnification = original $\times 60$; scale bar = 200 μ m). **C:** H&E images of the gastric antrum. **Left:** *H. felis*-infected INS-GAS mice; **middle:** infected GAS-KO mice; **right:** infected B6 wild-type mice (magnification = original $\times 150$; scale bar = 100 μ m). **D:** The histological scores for gastric corpus of *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type mice. * $P < 0.05$. ** $P < 0.01$. ($n = 6$ for each group).

Statistical analysis was performed using Microsoft Excel or GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA) with significance at $P < 0.01$ or 0.05.

Results

H. felis-Infected INS-GAS Mice Have Accelerated Gastric Corpus Metaplasia and Dysplasia at 12 MPI

We first analyzed the gastric histology of *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type mice at 12 MPI. The stomachs of *H. felis*-infected INS-GAS mice had grossly apparent thickened gastric corpus with pale-appearing mucosa, while GAS-KO and B6 wild-type mice did not exhibit any notable changes (Figure 1A). Histologically, *H. felis*-infected INS-GAS mice had moderate gastritis, foveolar hyperplasia with mild metaplasia, and dysplastic changes in the corpus. GAS-KO or B6 wild-type mice had similar levels of gastritis, but minimal changes were noted for metaplasia and dysplasia in the gastric corpus (Figure 1B). With respect to the gastric antrum, all three groups of mice had mild antral hyperplasia (Figure 1C). These features were confirmed by histological scoring for each parameter. The scores for epithelial defects, oxyntic atrophy, foveolar hyperplasia, intestinal metaplasia, and gastric dysplasia in the corpus were significantly higher in *H. felis* infected INS-GAS mice than those in GAS-KO and B6 wild-type mice (Figure 1D, $P < 0.01$ or 0.05),

while the scores for inflammation were not significantly different among three groups.

H. felis-Infected INS-GAS and B6 Wild-Type Mice Have Severe Gastric Corpus Dysplasia at 18 MPI

Next we analyzed the gastric histology of *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type mice at 18 MPI. Histologically, *H. felis*-infected INS-GAS and B6 wild-type mice both had remarkable foveolar hyperplasia with severe metaplasia, as well as dysplastic changes in the corpus. GAS-KO mice had similar levels of severe gastritis but minimal metaplastic or dysplastic changes in the gastric corpus (Figure 2A). In contrast, *H. felis*-infected GAS-KO and B6 wild-type mice had moderate antral hyperplasia with mild dysplastic changes, while infected INS-GAS mice had mild antral hyperplasia, which was similar with gastric lesions observed at 12 MPI (Figure 2B). The histological scoring for inflammation, foveolar hyperplasia, pseudo-pyloric metaplasia, and gastric dysplasia in the corpus were significantly higher in *H. felis*-infected INS-GAS and B6 wild-type mice than those in GAS-KO mice (Figure 2C, $P < 0.01$ or 0.05) but no significant difference was detected between infected INS-GAS and B6 wild-type mice. However, the histological scores for epithelial defects, antral hyperplasia, and gastric dysplasia in antrum were significantly higher in *H. felis*-infected GAS-KO and B6 wild-type mice com-

Figure 2. Gastric histology and histopathological scores for *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type (wt) mice at 18 MPI. *H. felis*-infected INS-GAS and B6 wild-type mice showed severe gastric corpus metaplasia and dysplasia (indicated by **yellow arrows**) at 18 MPI, while GAS-KO mice only showed severe gastritis. In contrast, GAS-KO and B6 wild-type mice showed moderate antral hyperplasia with mild dysplastic change (indicated by **blue arrows**), while infected INS-GAS mice only showed mild antral hyperplasia. **A:** H&E images of the gastric corpus. **Left:** *H. felis*-infected INS-GAS mice; **middle:** infected GAS-KO mice; **right:** infected B6 wild-type mice (magnification = original $\times 60$; scale bar = 200 μ m). **B:** H&E images of the gastric antrum. **Left:** *H. felis*-infected INS-GAS mice; **middle:** infected GAS-KO mice; **right:** infected B6 wild-type mice (magnification = original $\times 100$; scale bar = 200 μ m). **C, D:** The histological scores for gastric corpus (**C**) and antrum (**D**) of *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type mice. * $P < 0.05$. ** $P < 0.01$. ($n = 6$ for each group).

pared with those noted in INS-GAS mice (Figure 2D, $P < 0.05$), while no significant difference was detected between infected GAS-KO and B6 wild-type mice. In addition, the scores for antral inflammation were nearly identical. Finally, there were no gender differences in the degree of gastric corpus and antral histopathology among the mice in all three groups (see supplemental Figure 1, see <http://ajp.amjpathol.org>).

Serum Gastrin Levels in *H. felis*-Infected INS-GAS Mice Gradually Increased Over Time

We have previously reported that the serum gastrin levels in INS-GAS mice in an FVB/N background gradually in-

creased over time.^{9,17} In this study, we confirmed a similar result of progressive hypergastrinemia in *H. felis*-infected INS-GAS mice on a C57BL/6 background. As shown in Figure 3A and B, total amidated gastrin, as well as human-specific gastrin (G-17) in serum of infected INS-GAS mice on a C57BL/6 background gradually increased at 6, 12, and 18 MPI compared with uninfected INS-GAS/B6 mice. In contrast, serum amidated gastrin levels in *H. felis* infected B6 wild-type mice did not show significant changes during the course of *H. felis* infection (Figure 3C). Similar results were also obtained in *H. felis*-infected INS-GAS mice on an FVB/N background and *H. felis*-infected FVB/N wild-type mice, with progressive hypergastrinemia in the INS-GAS mice and little change

Figure 3. Serum gastrin levels in *H. felis*-infected INS-GAS and B6 mice gradually increased in time course, while those of infected B6 wild-type (wt) mice were stable. Total amidated gastrin as well as human-specific gastrin-17 (G-17) in serum of infected INS-GAS mice gradually increased at 6, 12, and 18 MPI compared with uninfected mice. In contrast, serum amidated gastrin levels in *H. felis*-infected B6 wild-type mice did not show significant changes in time course. **A:** Total (= mouse plus human) amidated gastrin in serum of INS-GAS mice with or without *H. felis* infection. **B:** Human-specific G-17 in serum of INS-GAS mice with or without *H. felis* infection. **C:** Total amidated gastrin in serum of B6 wild-type mice with or without *H. felis* infection (y axis unit: pM; $n = 8$ for each group).

Figure 4. Gastrin overexpression or deficiency does not alter *H. felis* colonization or Th1-Th2 immunobalance. DNA copy numbers of *H. felis* normalized to GAPDH in the corpus were almost the same among three groups, while in the antrum, DNA copy numbers of *H. felis* normalized to GAPDH were the highest in infected GAS-KO and the lowest in infected INSGAS mice (Figure 4A). *H. felis* DNA copy numbers in the antrum were always higher than those in the corpus for each group (Figure 4A). *H. felis*-specific antibody titers did not show significant differences among three groups, which confirmed gastrin-overexpression or gastrin-deficiency did not affect *H. felis* colonization (Figure 4B). Cytokine expression levels such as TNF- α and IL-4 in the stomachs did not show significant differences among three groups (Figure 4C). Serum fractions of IgG1 (representing Th2) and IgG2c (representing Th1) for *H. felis*-specific antibody titers did not show significant differences among three groups (Figure 4D). IgG2c titer was higher than IgG1 titers for all of the mice in each group (except in serum of *H. felis*-infected INSGAS mice at 12 MPI), which also indicated Th1 dominance in *H. felis*-infected mice for all three groups (Figure 4D). **A:** Quantitative real-time PCR analysis of genomic DNAs obtained from corpus and antrum of *H. felis*-infected mice stomachs among three groups at 18 MPI ($n = 4$ for each group). **B:** *H. felis*-specific antibody titers in serum of the mice among three groups at 6, 12, and 18 MPI ($n = 6$ for each group). **C:** Quantitative real-time RT-PCR analysis of cytokine expressions such as TNF- α and IL-4 in the mice stomachs among three groups at 12 MPI ($n = 3$ for each group). **D:** serum IgG1 and IgG2c fractions for *H. felis*-specific antibody titers of the mice among three groups at 6, 12, and 18 MPI ($n = 6$ for each group). * $P < 0.05$ and ** $P < 0.01$.

in the wild-type mice (see supplemental Figure 2, <http://ajp.amjpathol.org>). No gastrin was detected in serum of GAS-KO mice with and without *H. felis* infection (data not shown).

Gastrin Overexpression or Deficiency Does Not Alter *H. felis* Colonization or Th1-Th2 Polarization

For further analysis of the gastric phenotypes observed in *H. felis*-infected INSGAS, GAS-KO and B6 wild-type mice, we investigated *H. felis* colonization status using two distinct methods. First we analyzed *H. felis* colonization by quantitative real-time PCR using genomic DNAs obtained from the corpus and antrum of *H. felis*-infected stomachs among each group at 18 MPI. DNA copy numbers of *H. felis* relative to those of GAPDH in the corpus were similar among three groups, while in the antrum *H. felis* DNA copy numbers relative to those of GAPDH were the highest in infected GAS-KO and the lowest in infected INSGAS mice (Figure 4A). Of note, *H. felis* DNA copy numbers in the antrum were always higher than those in the corpus for each group (Figure 4A). We also analyzed *H. felis*-specific antibody titers in serum of the mice, and no significant differences were detected among three groups at 6, 12, and 18 MPI (Figure 4B). We and other groups have previously reported that Th1-Th2 immunobalance is critical for the progression

of gastric atrophy, as well as metaplastic/dysplastic changes and progression to gastric cancer.^{19,20} Therefore, we investigated whether gastrin overexpression as well as gastrin deficiency influenced the Th1-Th2 immunobalance. First, we analyzed the expression level of Th1 directed cytokine TNF- α , and Th2 directed cytokine IL-4, by quantitative real-time RT-PCR at 12 MPI. We observed no significant differences among the three groups for these two cytokines, although TNF- α levels were much higher than IL-4 levels in each group, consistent with the known Th1 polarized response to *H. felis* infection (Figure 4C). We also measured the serum fractions of IgG1 (representing Th2) and IgG2c (representing Th1) for *H. felis*-specific antibody titers respectively, and we did not detect significant differences among the three groups. Of note, we observed higher titers of IgG2c than IgG1 for all of the mice in each group except in serum of *H. felis* infected INSGAS mice at 12 MPI, consistent with the Th1 dominant response in *H. felis* infected mice for all three groups (Figure 4D).

Increased Gastric Corpus Cell Proliferation in *H. felis* Infected INSGAS Mice

To further characterize the differences in gastric histopathology in *H. felis*-infected INSGAS, GAS-KO, and B6 wild-type mice described in previous sections, we performed immunohistochemistry with anti-Ki-67 and anti-

A Ki-67 corpus

B Ki-67 antrum

C TFF2 corpus

Figure 5. Immunohistochemical studies with anti-Ki-67 and anti-TFF2 antibodies for *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type (wt) mice at 18 MPI. The numbers of Ki-67 positive cells in corpus were significantly higher in *H. felis*-infected INS-GAS mice than other two groups (Figure 5A), while those numbers in antrum was almost unchanged among three groups (Figure 5B) at 18 MPI. With respect to TFF2 positive cells, which represent spasmolytic peptide expressing metaplasia, we also observed widely and strongly positive areas in the corpus of *H. felis*-infected INS-GAS mice, while minimal spasmolytic peptide expressing metaplasia areas were detected in infected GAS-KO mice. (A) (corpus) and (B) (antrum): immunohistochemistry with anti-Ki-67 antibody. **Left:** The gastric corpus or antrum of *H. felis*-infected INS-GAS mouse, **middle:** infected GAS-KO mouse, **right:** infected B6 wild-type mouse. (18 MPI, magnification = original $\times 150$; scale bar = 100 μm) **C:** immunohistochemistry with anti-TFF2 antibody. **Left:** The gastric corpus of *H. felis*-infected INS-GAS; **middle:** infected GAS-KO; **right:** infected B6 wild-type. (18 MPI, magnification = original $\times 100$; scale bar = 200 μm).

TFF2 antibodies. As shown in Figure 5A, the numbers of Ki-67 positive cells in the gastric corpus were significantly higher in *H. felis*-infected INS-GAS mice than infected GAS-KO or B6 wild-type mice at 18 MPI (Figure 5A). In contrast, the numbers of Ki-67 positive cells in the antrum was similar among the three groups (Figure 5B). With respect to TFF2 positive cells, which represent spasmolytic peptide expressing metaplasia,^{21,22} we also observed strongly positive areas in the corpus of *H. felis* infected INS-GAS mice, while minimal spasmolytic peptide expressing metaplasia-specific staining was noted in the corpus of infected GAS-KO mice (Figure 5C). In contrast, no significant differences were detected in the antrum among the three groups (data not shown).

Reg I, Amphiregulin, and MMP-9 and -13 Genes Were Highly Up-Regulated in *H. felis*-Infected INS-GAS Mice

We have previously performed a microarray analysis of *H. felis* infected INS-GAS (FVB/N) stomachs, and found upregulation of several growth factors such as Reg I and amphiregulin, as well as MMP family members, such as MMP-9, -10, and -13.²³ Based on these earlier observations, we performed quantitative real-time RT-PCR analysis of these genes in the stomachs of three groups of mice. As expected, gene expression for both Reg I and

amphiregulin was significantly up-regulated in infected INS-GAS mice compared with infected GAS-KO and B6 wild-type mice at 12 MPI (Figure 6A). In addition, the expression of MMP-9 and -13 was also higher in infected INS-GAS mice compared with infected GAS-KO and B6 wild-type mice (Figure 6B). MMP-13 upregulation in infected INS-GAS mice was confirmed by immunohistochemistry. MMP-13 positive cells were mainly located in lamina propria of upper one third of metaplastic or dysplastic glands or the submucosa area in the corpus of *H. felis* infected INS-GAS mice, while few MMP-13 positive cells were detected in the corpus of infected GAS-KO mice (Figure 6C).

INS-GAS Mice without *H. felis* Infection Spontaneously Developed Mild Gastric Corpus Dysplasia

We also analyzed INS-GAS, GAS-KO, and B6 wild-type mice without *H. felis* infection. As shown in Figure 7, A–D, INS-GAS mice housed in SPF conditions spontaneously developed mild gastric corpus hyperplasia and dysplasia (both maximum score 1.5) at the age of around 24 months, while GAS-KO and B6 wild-type mice did not show any changes compared with those at younger ages. Of note, gastric antral histology for all mice among the three groups was normal and no tumors were detected. Noteworthy is the observation that GAS-KO mice

Figure 6. The expression of growth factors and MMPs in *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type mice. Quantitative real-time RT-PCR analysis revealed growth factors such as Reg I and Amphiregulin, and MMPs such as MMP-9 and -13, were significantly up-regulated in *H. felis* infected INS-GAS mice compared with infected GAS-KO and B6 wild-type mice (**A–B**). The up-regulation of MMP-13 was confirmed by immunohistochemistry with anti-MMP-13 antibody (**C**). **A:** Real-time RT-PCR analysis of the expression of Reg I and Amphiregulin in the stomachs of *H. felis* infected INS-GAS, GAS-KO and B6 wild-type mice at 18 MPI ($n = 3$ for each group). **B:** Real-time RT-PCR analysis of the expression of MMP-9 and -13 in the stomachs of *H. felis*-infected INS-GAS, GAS-KO, and B6 wild-type mice at 18 MPI ($n = 3$ for each group). **C:** Immunohistochemistry with anti-MMP-13 antibody. **Left:** The gastric corpus of *H. felis*-infected INS-GAS, **right:** *H. felis*-infected GAS-KO. (18 MPI, magnification = original $\times 100$, scale bar = 200 μm).

housed in these conditions did not develop antral gastric cancer (Figure 7D).

Discussion

In this study, we investigated the gastric phenotypes of long-term *H. felis* -infected hypergastrinemic (INS-GAS) mice, gastrin-deficient (GAS-KO) mice, and wild-type (B6 wild-type) mice in a uniform C57BL/6 background under SPF housing conditions. First, we found that infected INS-GAS mice at 12 MPI had accelerated gastric corpus dysplasia, as compared with GAS-KO and B6 wild-type mice, which did not have dysplasia at this time point (Figure 1B). Next, while both of infected INS-GAS and B6 wild-type mice had severe corpus dysplasia at 18 MPI, infected GAS-KO mice did not develop corpus dysplasia in this study (Figure 2A). However, with respect to the gastric antrum, the results were quite different. At 12 MPI, mice in all of the three groups had mild antral hyperplasia (Figure 1C), but at 18 MPI, *H. felis*-infected GAS-KO and B6 wild-type mice had mild to moderate antral dysplasia, while *H. felis*-infected INS-GAS mice did not exhibit pro-

gression of antral pathology (Figure 2B). These results indicate that gastrin may have distinct effects on epithelial homeostasis between the gastric corpus and antrum.

Based on earlier studies, the likely mechanisms for the effect of amidated gastrin on corpus carcinogenesis include up-regulation of growth factors^{9,17} in combination with induction of apoptosis in gastric epithelial cells, particularly parietal cells,^{24,25} both of which may trigger Correa's cascade and progression to gastric dysplasia. Gastrin has been reported to have a growth factor-like effect on gastric cells, and we have previously reported that Reg I, amphiregulin, and some of MMP family members, such as MMP-7, -9, -10, and -13 were up-regulated in INS-GAS mice of FVB/N background.^{23,26,27} In the current study, we confirmed up-regulation of these genes except MMP-7 and -10 as shown in Figure 6, A–C. Previously we have reported that *H. felis* or *H. pylori* infection of INS-GAS male mice in an FVB/N background rapidly leads to corpus gastric cancer at 7 to 8 MPI.^{8,9} In contrast to these findings in FVB/N mice, the current study demonstrated that *H. felis* infected INS-GAS mice on a C57BL/6 background do not develop gastric corpus neoplasia even after 18 MPI. This notably different outcome most likely derives from the difference in the genetic background of the mice in these studies. FVB/N mice have been reported to be cancer-prone compared with some other strains, particularly C57BL/6 mice. Recently Wakabayashi et al reported that the increased susceptibility to Ras-mediated skin carcinomas by FVB/N mice is the result of a carboxy-terminal polymorphism in the mouse *Patched* (*Ptch1*) gene, an inhibitory receptor for ligands of the Hedgehog gene family.²⁸ Therefore, it seems likely that the increased susceptibility of the INS-GAS mice on an FVB/N background to gastric cancer is related to the same *Ptch1* gene polymorphism. Indeed, recent studies suggest that Sonic Hedgehog signals are likely involved in gastric cell lineage homeostasis and gastrointestinal cancers.^{29,30} Another difference observed in *H. felis* -infected INS-GAS mice on a C57BL/6 background was the gender-independence for gastric dysplasia phenotype. In our previous study, gastric carcinogenesis occurred only in male INS-GAS mice on an FVB/N background,¹⁰ while on a C57BL/6 background, previous studies indicated that *H. felis* infection led to gastric dysplasia in both male and female animals.^{31,32} As shown in supplemental Figure 1, (see <http://ajp.amjpathol.org>), male- and female-specific histological scores exhibited the same pattern in both the gastric corpus and the gastric antrum. Therefore, in this study, we have confirmed that the gastric phenotype of *H. felis*-infected mice (INS-GAS, GAS-KO, wild-type) on a C57BL/6 background was gender-independent.

From the results for antral histological changes in this study, as well as from previous reports by other groups, we would concur that gastrin may have a protective effect on gastric antral carcinogenesis. Zavros et al previously reported that their GAS-KO mice on a B6/129Sv mixed background and maintained in conventional housing conditions developed antral cancer at the age of 12 months.¹⁴ While the mechanism for protective effects of gastrin on antral tumorigenesis have not been defined, it

Figure 7. Gastric corpus histology of INS-GAS, GAS-KO, and B6 wild-type (wt) mice without *H. felis* infection at 24 months of age. INS-GAS mice without *H. felis* infection spontaneously developed mild gastric corpus dysplasia (indicated by yellow arrow) at the older age, while no changes were detected in uninfected B6 wild-type and GAS-KO mice. **A** and **B**: H&E images of the gastric corpus of INS-GAS mice (**A**) and B6 wild-type mice (**B**) without *H. felis* infection of 24 month age. **C** and **D**: H&E images of the gastric corpus (**C**) and the gastric antrum (**D**) of GAS-KO mice without *H. felis* infection of the same age as **A** and **B**. Magnification= original $\times 100$ (**A**, **B**, **C**); and $\times 200$ (**D**). Scale bar = 100 μm .

has been observed that gastrin can up-regulate trefoil family members such as TFF1 and TFF2.^{33,34} TFF1 has been regarded as a tumor suppressor gene for the stomach, and both TFF1 and TFF2 can protect gastric epithelial cells from injury and apoptosis induced by chronic gastric *Helicobacter* infection. TFF1 knockout mice have been shown to develop spontaneous antral gastric tumors,³⁵ while TFF2 knockout mice (B6/129Sv mixed background) show increased susceptibility to *H. pylori*-induced dysplasia that shows a preference for the gastric antrum.^{36,37}

However, the most notable feature regarding the GAS-KO mice is their hypochlorhydria, and the precise role of bacterial colonization in this model remains to be investigated. The absence of gastric cancer in GAS-KO mice in our study, compared with previous reports, could be due in part to differences in genetic background but is most likely due to difference in housing conditions and thus results in differences in the complexity of colonization of the gastrointestinal tract. In our study, all mice were bred and housed under SPF conditions, as opposed to conventional housing. The interaction between the host and bacterial flora is now considered to be a critical factor for most inflammation-associated cancers, and microbes other than *Helicobacter spp.* may have a causal link to gastric carcinogenesis.³⁸ For example, it is previously reported that *Acinetobacter lwoffii* infection can induce many of the same histological changes, including atrophy and metaplasia, as gastric *Helicobacter* infection.³⁹ In the stomachs of GAS-KO or omeprazole-treated C57BL/6 wild-type mice under conventional housing, chronic gastritis due to bacterial overgrowth was observed and attributed to hypochlorhydria, and then antibiotic treatment successfully resolved the gastric inflammation in these mice.⁴⁰ Microarray analysis of the gastric mucosa of these mice revealed that immune defense genes, interferon-regulated response genes, and intesti-

nal metaplasia-related genes such as Cdx-2 were significantly up-regulated in these hypochlorhydric animals.⁴¹ Analogous to the studies in mice, hypochlorhydria in human patients with atrophic gastritis or following an antrectomy has also been reported result in an increased risk for developing gastric cancer, with most of the risk attributed to bacterial overgrowth as well as generation of carcinogenic substances such as *N*-nitrosamines, which can be found in increased amounts in hypochlorhydric patients.^{42,43} We also recently reported that antibiotics treatment of *H. pylori* infected INS-GAS mice on an FVB/N background had an effect on arresting progression of gastric lesions.¹⁸

Overall, the data taken together are consistent with a model in which gastrin serves as a rheostat for the stomach. Gastrin plays a central role in the safety network for the protection from mucosal damages caused by gastric acid secretion induced by gastrin itself, and thus either too much or too little gastrin can predispose to carcinogenesis.¹ While this study supports the notion that alterations in gastrin physiology can contribute to neoplastic transformation of the stomach, it is clear that host factors other than gastrin are also at least as important for *Helicobacter*-associated gastric carcinogenesis. *Helicobacter spp.* infection induces a wide variety of pro-inflammatory cytokines, such as IL-1 β , IL-6, and IL-11, that have been linked to cancer.^{44–47} IL-1 β , in particular, has been linked genetically to gastric cancer in *H. pylori*-infected patients, and when IL-1 β is overexpressed in a mouse stomach, it can induce a gastric cancer that mimics closely human gastric cancer.⁴⁸ In the current study, IL-1 β , IL-6, and IL-11 were all up-regulated in infected INS-GAS, GAS-KO, and B6 wild-type mice, and not significantly different among the three groups (data not shown), suggesting that gastrin may not be involved in the regulation of these cytokines.

In summary, the present study shows that *H. felis*-infected INS-GAS and wild-type mice on a C57BL/6 background and housed under SPF conditions progressed to severe gastric corpus dysplasia, while *H. felis*-infected GAS-KO mice had only severe gastritis at 18 MPI. In contrast, infected GAS-KO and B6 wild-type mice had mild to moderate antral dysplasia, while infected INS-GAS mice did not have progressive antral disease at this time point. These results demonstrate gastrin has a distinct effect on the gastric corpus and antrum in the setting of chronic gastric *Helicobacter* infection. While gastrin is possibly an essential cofactor for gastric corpus carcinogenesis, gastrin deficiency can predispose animals to antral tumorigenesis, and thus any imbalances in gastrin physiology may represent a risk for gastric transformation. This study provides novel insights for the elucidation of the mechanism of *Helicobacter*-associated gastric cancer.

Acknowledgments

We thank Wataru Shibata for *H. felis* culture, and Kelly S. Betz, Shanisha A.K. Gordon, and Anthony Mitchell for mouse colony maintenance and technical assistance.

References

- Wang TC: Gastrin transgenes and gastric cancer. Edited by Merchant JL, Buchan AMJ, Wang TC. Los Angeles, CURE foundation, 2004, pp. 235–252
- Fox JG, Wang TC: Inflammation, atrophy, and gastric cancer. *J Clin Invest* 2007, 117:60–69
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ: Cancer statistics, 2008. *CA Cancer J Clin* 2008, 58:71–96
- Marshall BJ, Warren JR: Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984, 1:1311–1315
- Schistosomes, liver flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Lyon, 7–14 June 1994. IARC Monogr Eval Carcinog Risks Hum 1994, 61:1–241
- Fox JG, Wang TC: *Helicobacter pylori*—not a good bug after all! *N Engl J Med* 2001, 345:829–832
- Correa P, Houghton J: Carcinogenesis of *Helicobacter pylori*. *Gastroenterology* 2007, 133:659–672
- Fox JG, Wang TC, Rogers AB, Poutahidis T, Ge Z, Taylor N, Dangler CA, Israel DA, Krishna U, Gaus K, Peek RM Jr: Host and microbial constituents influence *Helicobacter pylori*-induced cancer in a murine model of hypergastrinemia. *Gastroenterology* 2003, 124:1879–1890
- Wang TC, Dangler CA, Chen D, Goldenring JR, Koh T, Raychowdhury R, Coffey RJ, Ito S, Varro A, Dockray GJ, Fox JG: Synergistic interaction between hypergastrinemia and *Helicobacter* infection in a mouse model of gastric cancer. *Gastroenterology* 2000, 118:36–47
- Fox JG, Rogers AB, Ihrig M, Taylor NS, Whary MT, Dockray G, Varro A, Wang TC: *Helicobacter pylori*-associated gastric cancer in INS-GAS mice is gender specific. *Cancer Res* 2003, 63:942–950
- Wang TC, Koh TJ, Varro A, Cahill RJ, Dangler CA, Fox JG, Dockray GJ: Processing and proliferative effects of human progastrin in transgenic mice. *J Clin Invest* 1996, 98:1918–1929
- Ohtani M, Garcia A, Rogers AB, Ge Z, Taylor NS, Xu S, Watanabe K, Marini RP, Whary MT, Wang TC, Fox JG: Protective role of 17 beta-estradiol against the development of *Helicobacter pylori*-induced gastric cancer in INS-GAS mice. *Carcinogenesis* 2007, 28:2597–2604
- McColl KE, el-Omar EM, Gillen D: Alterations in gastric physiology in *Helicobacter pylori* infection: causes of different diseases or all epiphenomena? *Ital J Gastroenterol Hepatol* 1997, 29:459–464
- Zavros Y, Eaton KA, Kang W, Rathinavelu S, Katukuri V, Kao JY, Samuelson LC, Merchant JL: Chronic gastritis in the hypochlorhydric gastrin-deficient mouse progresses to adenocarcinoma. *Oncogene* 2005, 24:2354–2366
- Judd LM, Alderman BM, Howlett M, Shulkes A, Dow C, Moverley J, Graill D, Jenkins BJ, Ernst M, Giraud AS: Gastric cancer development in mice lacking the SHP2 binding site on the IL-6 family co-receptor gp130. *Gastroenterology* 2004, 126:196–207
- Koh TJ, Goldenring JR, Ito S, Mashimo H, Kopin AS, Varro A, Dockray GJ, Wang TC: Gastrin deficiency results in altered gastric differentiation and decreased colonic proliferation in mice. *Gastroenterology* 1997, 113:1015–1025
- Takaishi S, Cui G, Frederick DM, Carlson JE, Houghton J, Varro A, Dockray GJ, Ge Z, Whary MT, Rogers AB, Fox JG, Wang TC: Synergistic inhibitory effects of gastrin and histamine receptor antagonists on *Helicobacter*-induced gastric cancer. *Gastroenterology* 2005, 128:1965–1983
- Lee CW, Rickman B, Rogers AB, Ge Z, Wang TC, Fox JG: *Helicobacter pylori* eradication prevents progression of gastric cancer in hypergastrinemic INS-GAS mice. *Cancer Res* 2008, 68:3540–3548
- Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN, Nagler-Anderson C: Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces *Helicobacter*-induced gastric atrophy. *Nat Med* 2000, 6:536–542
- Stoicov C, Whary M, Rogers AB, Lee FS, Klucsevsk K, Li H, Cai X, Saffari R, Ge Z, Khan IA, Combe C, Luster A, Fox JG, Houghton J: Coinfection modulates inflammatory responses and clinical outcome of *Helicobacter felis* and *Toxoplasma gondii* infections. *J Immunol* 2004, 173:3329–3336
- Nomura S, Baxter T, Yamaguchi H, Leys C, Vartapetian AB, Fox JG, Lee JR, Wang TC, Goldenring JR: Spasmolytic polypeptide expressing metaplasia to preneoplasia in *H. felis*-infected mice. *Gastroenterology* 2004, 127:582–594
- Nomura S, Yamaguchi H, Ogawa M, Wang TC, Lee JR, Goldenring JR: Alterations in gastric mucosal lineages induced by acute oxyntic atrophy in wild-type and gastrin-deficient mice. *Am J Physiol Gastrointest Liver Physiol* 2005, 288:G362–G375
- Takaishi S, Wang TC: Gene expression profiling in a mouse model of *Helicobacter*-induced gastric cancer. *Cancer Sci* 2007, 98:284–293 review of 41 cases. *Toxicol Pathol* 2003, 31:304–309
- Chen D, Zhao CM, Dockray GJ, Varro A, Van Hoek A, Sinclair NF, Wang TC, Koh TJ: Glycine-extended gastrin synergizes with gastrin 17 to stimulate acid secretion in gastrin-deficient mice. *Gastroenterology* 2000, 119:756–765
- Cui G, Takaishi S, Ai W, Betz KS, Florholmen J, Koh TJ, Houghton J, Pritchard DM, Wang TC: Gastrin-induced apoptosis contributes to carcinogenesis in the stomach. *Lab Invest* 2006, 86:1037–1051
- McCaig C, Duval C, Hemers E, Steele I, Pritchard DM, Przemeck S, Dimaline R, Ahmed S, Bodger K, Kerrigan DD, Wang TC, Dockray GJ, Varro A: The role of matrix metalloproteinase-7 in redefining the gastric microenvironment in response to *Helicobacter pylori*. *Gastroenterology* 2006, 130:1754–1763
- Varro A, Kenny S, Hemers E, McCaig C, Przemeck S, Wang TC, Bodger K, Pritchard DM: Increased gastric expression of MMP-7 in hypergastrinemia and significance for epithelial-mesenchymal signaling. *Am J Physiol Gastrointest Liver Physiol* 2007, 292:G1133–G1140
- Wakabayashi Y, Mao JH, Brown K, Girardi M, Balmain A: Promotion of Hras-induced squamous carcinomas by a polymorphic variant of the Patched gene in FVB mice. *Nature* 2007, 445:761–765
- Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, Parker AR, Shimada Y, Eshleman JR, Watkins DN, Beachy PA: Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* 2003, 425:846–851
- Zavros Y: The adventures of sonic hedgehog in development and repair. IV Sonic hedgehog processing, secretion, and function in the stomach. *Am J Physiol Gastrointest Liver Physiol* 2008, 294:G1105–G1108
- Cai X, Carlson J, Stoicov C, Li H, Wang TC, Houghton J: *Helicobacter felis* eradication restores normal architecture and inhibits gastric cancer progression in C57BL/6 mice. *Gastroenterology* 2005, 128:1937–1952
- Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR, Wang TC: Gastric cancer originating from bone marrow-derived cells. *Science* 2004, 306:1568–1571
- Khan ZE, Wang TC, Cui G, Chi AL, Dimaline R: Transcriptional reg-

- ulation of the human trefoil factor TFF1, by gastrin. *Gastroenterology* 2003, 125:510–521
34. Tu S, Chi AL, Lim S, Cui G, Dubeykovskaya Z, Ai W, Fleming JV, Takaishi S, Wang TC: Gastrin regulates the TFF2 promoter through gastrin-responsive cis-acting elements and multiple signaling pathways. *Am J Physiol Gastrointest Liver Physiol* 2007, 292:G1726–G1737
35. Lefebvre O, Chenard MP, Masson R, Linares J, Dierich A, LeMeur M, Wendling C, Tomasetto C, Chambon P, Rio MC: Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein. *Science* 1996, 274:259–262
36. Farrell JJ, Taupin D, Koh TJ, Chen D, Zhao CM, Podolsky DK, Wang TC: TFF2/SP-deficient mice show decreased gastric proliferation, increased acid secretion, and increased susceptibility to NSAID injury. *J Clin Invest* 2002, 109:193–204
37. Fox JG, Rogers AB, Whary MT, Ge Z, Ohtani M, Jones EK, Wang TC: Accelerated progression of gastritis to dysplasia in the pyloric antrum of TFF2 $-/-$ C57BL6 x Sv129 *Helicobacter pylori*-infected mice. *Am J Pathol* 2007, 171:1520–1528
38. Selgrad M, Malfertheiner P, Fini L, Goel A, Boland CR, Ricciardiello L: The role of viral and bacterial pathogens in gastrointestinal cancer. *J Cell Physiol* 2008, 216:378–388
39. Zavros Y, Rieder G, Ferguson A, Merchant JL: Gastritis and hypergastrinemia due to *Acinetobacter lwoffii* in mice. *Infect Immun* 2002, 70:2630–2639
40. Zavros Y, Rieder G, Ferguson A, Samuelson LC, Merchant JL: Genetic or chemical hypochlorhydria is associated with inflammation that modulates parietal and G-cell populations in mice. *Gastroenterology* 2002, 122:119–133
41. Friis-Hansen L, Rieneck K, Nilsson HO, Wadstrom T, Rehfeld JF: Gastric inflammation, metaplasia, and tumor development in gastrin-deficient mice. *Gastroenterology* 2006, 131:246–258
42. Friis-Hansen L: Achlorhydria is associated with gastric microbial overgrowth and development of cancer: lessons learned from the gastrin knockout mouse. *Scand J Clin Lab Invest* 2006, 66:607–621
43. Kodama K, Sumii K, Kawano M, Kido T, Nojima K, Sumii M, Haruma K, Yoshihara M, Chayama K: Gastric juice nitrite and vitamin C in patients with gastric cancer and atrophic gastritis: is low acidity solely responsible for cancer risk? *Eur J Gastroenterol Hepatol* 2003, 15:987–993
44. El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS: Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000, 404:398–402
45. Judd LM, Bredin K, Kalantzis A, Jenkins BJ, Ernst M, Giraud AS: STAT3 activation regulates growth, inflammation, and vascularization in a mouse model of gastric tumorigenesis. *Gastroenterology* 2006, 131:1073–1085
46. Ernst M, Najdovska M, Grail D, Lundgren-May T, Buchert M, Tye H, Matthews VB, Armes J, Bhathal PS, Hughes NR, Marcusson EG, Karras JG, Na S, Sedgwick JD, Hertzog PJ, Jenkins BJ: STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice. *J Clin Invest* 2008, 118:1727–1738
47. Giraud AS, Jackson C, Menheniott TR, Judd LM: Differentiation of the Gastric Mucosa IV: Role of trefoil peptides and IL-6 cytokine family signaling in gastric homeostasis. *Am J Physiol Gastrointest Liver Physiol* 2007, 292:G1–G5
48. Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, Betz KS, Penz-Oesterreicher M, Bjorkdahl O, Fox JG, Wang TC: Overexpression of interleukin-1 β induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 2008, 14:408–419