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Conditional loss of TGF-β signalling leads to increased susceptibility to gastrointestinal carcinogenesis in mice

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SUMMARY

Background: Downregulation of TGF-β receptors is implicated in colon cancer development. Inactivation of either of the two transmembrane serine/threonine kinases, TGF-β1 types I/II receptors, is now implicated in carcinogenesis, especially gastrointestinal carcinogenesis.

Methods: We generated transgenic mice, called pS2–dnRII or ITF–dnRII, of which the dominant negative mutant of the TGF-β type II receptor was expressed under the control of tissue-specific promoters, the pS2 promoter for stomach and ITF for intestine. They were either infected with *H. pylori* (ATCC 43504 strain, $CagA^+$ and $VacA^+$) or administered with azoxymethane to determine the significance of loss of TGF-β signalling in gastrointestinal carcinogenesis.

Results: Gastric adenocarcinoma developed in pS2–dnRII mice, whereas only chronic active gastritis was noted in wild-type littermates after 36 weeks of *H. pylori* infection. Mice lacking in TGF-β signalling specifically in the stomach showed a significantly higher proliferation cell nuclear antigen-labelling index when infected with *H. pylori* than wild-type littermates ($P < 0.01$). Development of colonic aberrant crypt foci was provoked in mice by intraperitoneal injections of azoxymethane, and ITF–dnRII mice showed significantly higher incidences of ACF and colon cancers than wild-type littermates.

Conclusions: Maintaining normal TGF-β signalling in the gastrointestinal tract seems to be important either for preventing abnormal mucosal proliferation, or for suppressing or retarding carcinogenesis.

INTRODUCTION

Alteration of TGF-β signalling pathways underlies a wide variety of human disorders, including inflammatory diseases, autoimmune diseases and cancers.[1–3] The TGF-β receptors and signalling pathway have been shown to comprise a tumour suppressor pathway in carcinogenesis, and many investigators now believe that the development of resistance to TGF-β by tumour cells represents a key event in the progression of malignancy.[4] It is now known that TGF-β receptors are inactivated by somatic mutation, gene deletion or transcriptional repression in many human malignancies.[5–7] Since the hypothesis was first introduced that defects in the TGF-β receptor system might contribute to the development of resistance to the growth inhibitory effects of TGF-β, correlations between the resistance to growth inhibition by TGF-β and the lack of TGF-β receptor expression have been reported in a variety of human cancers, including gastric and colon cancer, leading to the suggestion that the development of such TGF-β resistance represents a significant step in the process of carcinogenesis.[8] Inactivation of the TGF-β receptor genes and the development of resistance to TGF-β represent relatively late events in the process of

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carcinogenesis and make a key contribution to the transition from late adenoma to frank malignancy.[1, 3, 9]

Although TGF-β1 can suppress the early stages of tumour formation *in vivo*, expression of TGF-β ligand does not correlate with malignancy. Rather, loss of growth inhibitory responses to TGF-β at the cellular level is probably a more important step in malignant progression.[5, 10, 11] Therefore, TGF-β receptors and proteins involved in signalling by TGF-βs might act as tumour suppressors. Inactivation of the type II receptor has been detected in somatic tumour types and may be associated with the replication error (RER+) phenotype in gastrointestinal cancers.[12]

The development of carcinoma in mammalian organisms is now understood to occur in a series of at least three definable stages, which have been termed initiation, promotion and progression. Although the World Health Organisation International Agency for Research on Cancer (WHO-IARC) defines *Helicobacter pylori* infection as a class I carcinogen,[13] debate still exists on the role of *H. pylori* infection in gastric carcinogenesis. Several recent publications state that *H. pylori* enhances carcinogen-induced gastric carcinogenesis; that is, *H. pylori* infection triggers the promotion of gastric carcinogenesis rather than acts as a direct carcinogen.[14, 15] Interestingly, the gastric findings noted in TGF-$\beta^{-/-}$ mice showed similar pathologies to those commonly observed in *H. pylori* infection, such as autoimmune gastritis, hypertrophic gastritis, and gastric dysplasia.[16, 17] Therefore, we hypothesized that the host status of gastric TGF-β signalling might determine the outcome of *H. pylori* infection and that the genetic background of the host, such as loss of TGF-β signalling, might be a critical determinant of why some individuals remain in a symptom-free state despite longstanding *H. pylori* infection, while others suffer from peptic ulcer disease or gastric cancer.

The loss of TGF-β signalling might similarly underlie colon carcinogenesis. Lack of TGF-β-mediated growth inhibition on human colon cancer by downregulation of TGF-β receptors, as well as the effects of TGF-β on stroma formation and angiogenesis, indicate that the status of host TGF-β signalling might be the critical determinant in the initiation or progression of colon cancer after exposure to a colon carcinogen.[18]

In this study we selectively provoked the conditional blocking of TGF-β signalling in the stomach or colon through the dominant negative mutant form of the type II TGF-β receptor (dnRII) under the control of tissue-specific gene expression (Figure 1A,B). We generated pS2–dnRII and ITF–dnRII transgenic mice to explore the significance of TGF-β signalling in gastrointestinal carcinogenesis. We infected pS2–dnRII mice with *H. pylori* to induce gastric carcinogenesis, and induced colon carcinogenesis by the administration of azoxymethane in a mouse model on the basis that azoxymethane, direct and active metabolite of methylating carcinogens such as 1,2-dimethylhydrazine, has been used to induce aberrant crypt foci, a possible preneoplastic lesion, in a murine model.[19]

MATERIALS AND METHODS

Generation of dominant negative mutant TGF-β RII mice

pS2–dnRII mice. The mouse pS2 fragment spans − 2400 bp to + 10 bp relative to the transcriptional start site. The human TGF-β RII fragment spans + 1 to + 911 bp and contains both a C tag sequence and a segment of the mouse protamine 1 gene tract (mP1), which provide an intron and a polyadenylation site.[20] The 3.8kb fragment was micro-injected into fertilized eggs of the FVB/N strain of mouse. Animal care was in accordance with institutional guidelines (NCI/NIH, MD). We obtained four lines of transgenic mice named pS2-1, pS2-2, pS2-3 and pS2-4. Genotyping was performed by Southern hybridization and PCR. For Southern hybridization, DNA obtained from the mouse tail was cut with *Hin*dIII and *Bgl*II overnight, electrophoresed on 1.0% agarose gel and transferred on to a N^+ membrane (Bio-Rad, Hercules, CA, USA). After hybridization with a dnRII probe, the presence of the 3.8kb transgene was confirmed. For PCR genotyping, the following set of primers was used: primer A, 5′-GAATACAAGCTTGGGCTG-3′; and primer C, 5′-CTG CAGTCGCTCATGCAGG-3′. Two lines, pS2-1 and pS2-2, showing high expression of the dnRII transgene, were used for the subsequent experiment.

ITF–dnRII. Using the mouse ITF fragment spanning − 6500 bp to + 10 bp relative to the transcriptional start site, similar procedures were performed to generate ITF–dnRII mice as described above.[21] We obtained four lines of transgenic mice, designated ITF-1 to ITF-4, which possessed high copy numbers of inserted TGF-β dnRII. Genotyping was also performed by Southern hybridization and PCR. For Southern hybridization, DNA obtained from tails was cut with *Bam*H1 and *Xho*I for

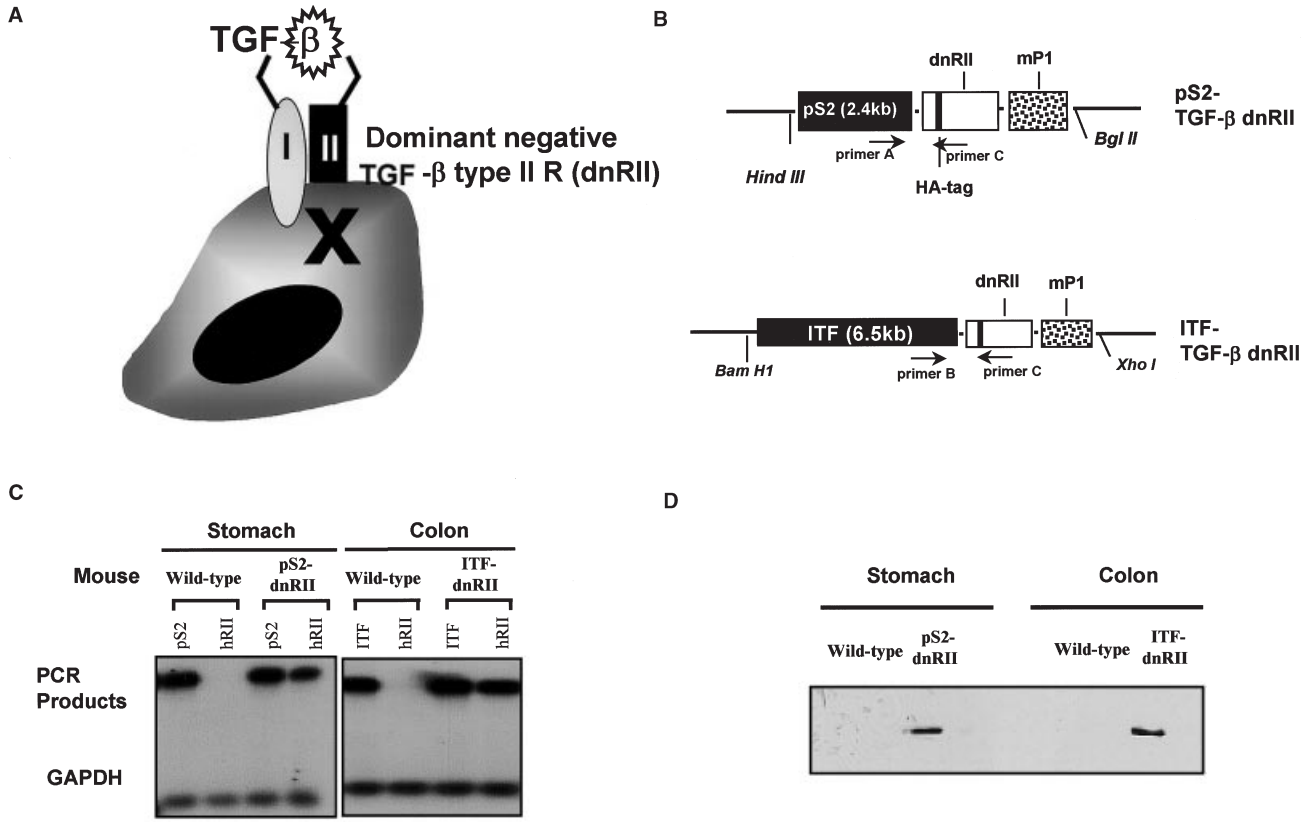


Figure 1. Generation of dominant negative mutant TGF-β RII mice. (A) Dominant negative mutant of TGF-β RII. The dominant negative TGF-β type II receptor did not signal the nucleus after binding of TGF-β to the receptor because of a lack of the cytoplasmic domain containing serine/threonine kinase. (B) Schematic representation of the transgene. TGF-β RII spans + 322 to + 911 and contains a the haemagglutinin tag sequence and a segment of mP1, which provide an intron and polyadenylation site. Transgenic mice were generated using inbred FVB/N zygotes. (C) Genotyping. Using total RNAs isolated from the stomach or intestine of transgenic mice and wild-type littermates, RT–PCR was performed using the specific primer sets for human TGF-β RII, mouse pS2, mouse ITF, and mouse GAPDH. (D) Immunoprecipitation–western blotting analysis of dnRII protein. After immunoprecipitation with haemagglutinin antibody, western blot was performed with TGF-β RII antibody.

ITF–dnRII, electrophoresed on 1.0% agarose gel and transferred on to a N^+ membrane (Bio-Rad, Hercules, CA, USA). After hybridization with a ^{32}P-labelled dnRII probe, the presence of the 8.0kb transgene was confirmed. For PCR genotyping, the following primers were used: primer B, 5′-GGTGGTGATGTGGACAAAG-3′; and primer C, 5′-CTGCAGT-CGCTCATGCAGG-3′. The haemagglutinin sequence of TATGATGTTCCTGATTATGCTAGCCTC was also inserted into the dnRII construct.

RNA isolation and RT–PCR

RNA was isolated from mouse tissue using Trizol (Gibco BRL, Gaithesburg, MD, USA). RT–PCR was performed according to the manufacturer's instructions using a Perkin-Elmer RT–PCR kit (Perkin Elmer, Branchburg, NJ, USA). The primers used were as follows: for TGF-β dnRII, 5′-ACGACATGATAGTCACTGACAACA-3′ and 5′-TTGGGGTCATGGCAAACTGTCTC-3′; for mITF, 5′-GAAGTTTGCGTGCTGCCATGGAG-3′ and 5′-CCGCA-ATTAGAACAGCCT**T**GTG-3′; for mpS2, 5′-CAAGGTGA-TCTGCG-3′ and 5′-ATAGAAGCACCAGGGGAC-3′; and for GAPDH, 5′-GTGGGCCGCTCTAGGCAC CA-3′ and 5′-CGGTTGGCCTTAGGGTT**C**AGGGGGG-3′. The GAPDH served as a positive control and in each sample. Samples were amplified for 25 cycles: 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, in a PCR instrument (GeneAmpTM 9000; Perkin Elmer).

Immunoprecipitation and Western blotting

Stomach or colon tissues from either control mice or dnRII transgenic mice were homogenized with a dounce homogenizer in lysis buffer. Immunoprecipitation using

anti-haemaglutanin antibody (LCRC, NCI, Bethesda, MD, USA) was carried. For the subsequent anti-TGF-β dnRII western blot, the immunoprecipitated proteins, separated by SDS–PAGE, were transferred on to membrane, incubated with anti-TGF-β RII antibody (Upstate Biotechnology, NY, USA), and antibody-bound proteins were visualized by chemiluminescence (Amersham Pharmacia, Bucks, UK).

Experiment I (pS2–dnRII mice infected with H. pylori*)*

Mice. A total of 132 specific pathogen-free, 6-week-old mice (FVB/N background) were housed in steel cages on hardwood-chip bedding in an air-conditioned biohazard room with a 12-h light/12 h dark cycle. They were given formulated foods (Hae-eun International Co. Ltd., Seoul, Korea) and autoclaved distilled water *ad libitum*. The animals were cared for in accordance with institutional guidelines.

Chemicals and bacteria. H. pylori (ATCC strain 43504, $CagA^+$, $VacA^+$) were inoculated on *Brucella* agar plates (Becton Dickinson, Cockysville, MD, USA) containing 10% heat-inactivated foetal bovine serum and Skirrow medium. They were kept at 37 °C under microaerobic conditions using GasPak jars (Difco, Detroit, MI, USA) and CampyPak (Becton Dickinson). After 24 h of fasting, a 0.1-mL suspension of *H. pylori* containing 10^9 colony-forming units (CFU)/mL was delivered to mice intragastrically using an oral catheter.

Experimental design. A total of 132 mice were divided into three groups as follows: 48 pS2–dnRII mice treated with *H. pylori*, 48 wild-type littermate mice treated with *H. pylori*, and the remaining 36 untreated pS2–dnRII mice.

Histopathological examination. All animals were killed under deep ether anaesthesia at 4, 8, 16, 24, 36 or 50 weeks after the initial treatment, laparotomized and their stomachs excised. The stomachs were opened along the greater curvature, fixed in 10% neutralized formalin in phosphate-buffered saline, processed by standard methods, and embedded in paraffin. Tissues were sectioned at 4 μm for haematoxylin and eosin staining. The slides were coded and examined by two pathologists without knowledge of the groups to which the specimens belonged.

Confirmation of presence of H. pylori *in gastric mucosa.* For confirmation of *H. pylori* infection, approximately 30-mm^2 samples of stomach mucosa from the greater curvature, containing both fundic and pyloric glands, were homogenized in 1 mL *Brucella* broth and cultured for *H. pylori*. Urease activity was measured using a rapid urease test kit (CLO test, Delta West, Australia). The presence of *H. pylori* in gastric pits was further confirmed using Warthin–Starry staining.

Immunohistochemistry. Immunohistochemical identification of proliferating cell nuclear antigen (PCNA) was performed on replicate sections of the stomach tissues. After deparaffinization, the sections were incubated with 3% hydrogen peroxide in methanol for 1 h to block endogenous peroxidase activity. Sections were then washed in PBS, and M.O.M mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA, USA) was applied for 1 h to reduce nonspecific antibody binding. A mouse monoclonal antibody, PC10 (Novocastra, Newcastle, UK), was diluted to 1 : 200 and reacted with the tissue specimen at room temperature for 30 min. After washing, the sections were incubated with M.O.M. biotinylated antimouse IgG (Vector) for 20 min. Sections were then washed and incubated with Vectastatin ABC reagent (Vector) for 5 min. Finally, the sections were reacted with DAB (Vector) for 10 min, counterstained with haematoxylin, and mounted. Normal IgG was substituted for primary antibody as the negative control.

Experiment II (ITF–dnRII mice treated with azoxymethane)

Experimental design. A total of 90 mice aged 6 weeks were included, 45 ITF–dnRII mice and 45 wild-type littermates. Azoxymethane (Sigma, St Louis, MO, USA) was administered at a dose of 5 mg/kg body weight, twice a week for 6 weeks (total azoxymethane administered, 60 mg/kg). Preliminary study had shown that the dose adjustment of azoxymethane was critical because the mice did not tolerate doses over 7 mg/kg. The untreated controls received 0.9% saline in the same volume and at the same time as azoxymethane administration. Ten mice each were killed at 8, 16 and 24 weeks after the last azoxymethane injection. Immunohistochemical identification of PCNA was also performed on replicate sections of colon tissues.

Determination of aberrant crypt foci. The colons of all mice were used to score aberrant crypt foci. At autopsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis and then washed with saline. The colons were cut into three sections (each approximately 2.5 cm in length) starting from the anus, placed between filter paper to reduce mucosal folding and fixed in 10% buffered formalin for at least 24 h. Fixed colon sections were dipped in a 0.2% solution of methylene blue (methylthioninium chloride) in distilled water briefly for less than 30 s, then washed with distilled water. Using a light miscroscope at a magnification of ×10 to ×50, aberrant crypt foci were distinguished by their increased size, their more prominent epithelial cells and their increased pericryptal space compared with surrounding normal crypts. The number of aberrant crypt foci per colon was recorded using a digital camera (Kodak, Rochester, NY, USA) and Image-Pro image analysis system. After scoring, colons were processed for measurement of the PCNA-positive cell labelling index and histological examination.

Statistical analysis

Statistical significance was assessed by means of the Chi-Square test. $P < 0.05$ was considered significant.

RESULTS

Generations and phenotypes of dominant negative mutants of TGF-β type II receptor mice

RT–PCR showed the presence of human dominant negative TGF-β type II receptor mRNA in the stomach or colon of transgenic mice, while none was noted in the wild-type littermates (Figure 1C). Immunoprecipitation with haemagglutinin antibody was done using protein homogenates obtained from either stomach or colon. Subsequent western blotting with dnRII antibody showed a positive band only in transgenic mice (Figure 1D). All generated dominant negative mutant TGF-β type II mice showed no congenital abnormalities. Phenotypically, some pS2–dnRII mice showed some lupus-like skin lesions on the dorsum including alopecia, and the body weights of ITF–dnRII mice were typically 30% less than their wild-type littermates. They developed spontaneous pancreatitis or colitis, presenting with haematochezia, malnutrition and exacerbation of skin lesions when fostered under nonspecific pathogen free (SPF) conditions. They showed increased susceptibility to either dextran sulphate sodium-induced ulcerative colitis or cerulein-induced pancreatitis.[20, 21] Interestingly, these transgenic mice showed a high titre of auto-antibodies against pancreatic acinar or colonic epithelia, which triggered autoimmune pancreatitis or ulcerative colitis. No systemic abnormalities were seen when pS2–dnRII mice were infected with *H. pylori* or when ITF–dnRII mice were administered azoxymethane. Because the haemagglutinin sequence was inserted in the trasgene (Figure 1B), immunohistochemical staining with haemagglutinin antibody detected the presence of dnRII proteins (Figure 2Ad,Bd). Wild-type littermates showed negative immunohistochemical staining for haemagglutinin proteins (Figure 2Ac,Bc). Whereas the TGF-β type II receptor is normally expressed in the epithelium and muscle layers, the transgene of dnRII was noted mostly in the epithelia of the stomach and intestine because pS2 or ITF is normally expressed in epithelium (Figure 2Ab,Bb). Similar expression patterns were noted in the stomach.

Pathological findings after H. pylori *infection*

In *H. pylori*-infected wild-type littermate mice, marked infiltration of lymphocytes, macrophages and neutrophils into the lamina propria and submucosa and formation of lymphoid follicles were seen at 24 weeks. Neither gastric or duodenal ulcers nor intestinal metaplasias were observed up to 50 weeks after *H. pylori* infection in the present mouse model (Figure 3). pS2–dnRII mice showed prominent increases in the degree of inflammation compared to wild-type littermates (Figure 3A) and the degree of mucosal proliferation was significantly more severe than in wild-type littermates (Figure 3B). These findings suggested that loss of TGF-β signalling in the stomach resulted in hyperplastic gastritis and severe gastritis, respectively, compared to wild-type littermates maintaining normal TGF-β signalling in the stomach (Table 1).

Proliferation of gastric mucosa after H. pylori *infection*

PCNA staining showed marked expansion of the proliferative zone at the foveolar epithelium in pS2–dnRII mice infected with *H. pylori* compared with similarly infected wild-type littermates (Figure 4A). When the PCNA labelling index was compared according to the time after *H. pylori* infection, *H. pylori*

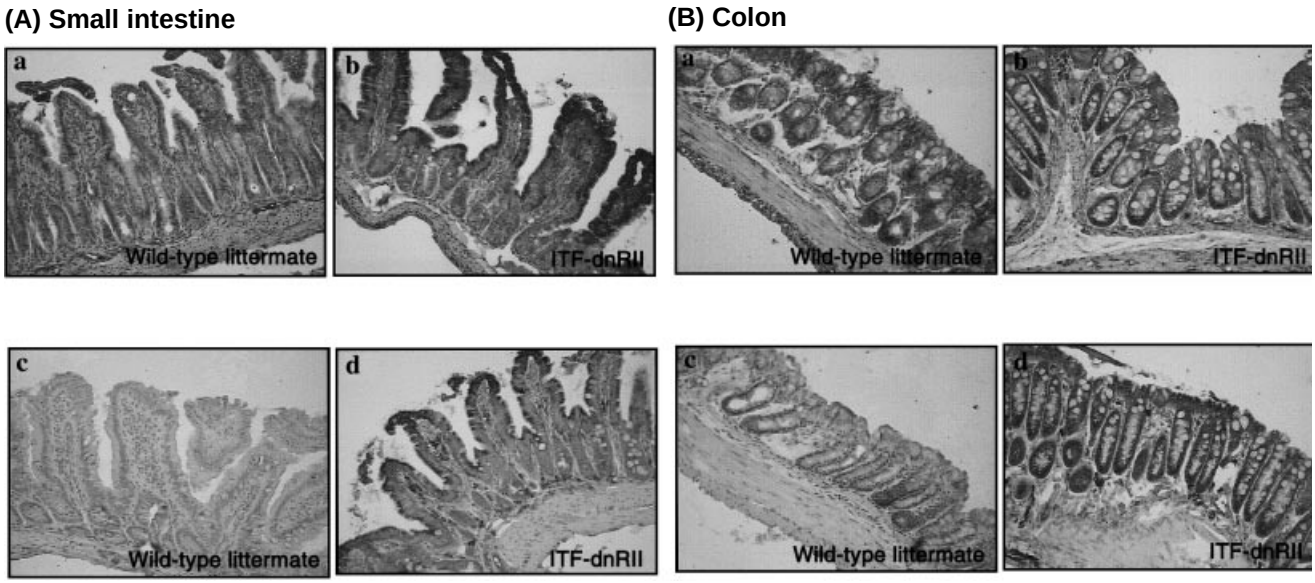


Figure 2. Tissue distribution of dnRII expression. Because only the haemagglutinin tag sequence was inserted, immunohistochemical staining with haemagglutinin antibody showed positive staining only in transgenic mice. Transgenic expression was observed in both small intestine and colon because ITF is expressed at both sites. The expression of dnRII paralleled the expressions of haemagglutinin. (A) small intestine × 100, original magnification; (B) colon (a,b) Immunohistochemical staining of TGF-β RII; (c,d) Immunohistochemical staining of haemagglutinin × 100, original magnification.

infection showed significantly higher mucosal proliferation compared to uninfected mice ($P < 0.01$) (Figure 4B). pS2–dnRII mice infected with *H. pylori* showed a statistically significant increase in PCNA-LI compared with wild-type mice infected with *H. pylori* ($P < 0.05$).

Development of mucosal carcinoma in pS2–dnRII transgenic mice infected with 1 H. pylori

Six of seven pS2–dnRII mice (85.7%) developed either glandular dysplasia or mucosal carcinoma after *H. pylori* infection (Figure 5A). No gastric lesion was found among the uninfected pS2–dnRII transgenic mice (Figure 5C), and wild-type littermates infected with the same strain of *H. pylori* developed only a moderate grade of chronic gastritis (Figure 5B). However, the gastric dysplasia or carcinoma observed in pS2–dnRII transgenic mice at the 36th week after *H. pylori* infection did not progress further, as more invasive carcinoma was not noted at the 50th week after infection. The overall histological findings are summarized in Table 1, which shows that pS2–dnRII mice infected with *H. pylori* developed more severe gastritis, hyperplastic gastritis and mucosal carcinoma than similarly infected wild-type littermates.

Aberrant crypt foci and PCNA-LI after azoxymethane treatment

Mice proved to be highly susceptible to azoxymethane. Initially azoxymethane 8 mg/kg was injected twice a week, which caused massive hepatic central necrosis leading to mortality in all cases. Eventually we adjusted azoxymethane to a dose of 5 mg/kg twice a week, which was repeated for 6 weeks (total dose of azoxymethane 60 mg/kg). The mean number of aberrant crypt foci/whole colon was significantly increased in ITF–dnRII mice compared to wild-type littermates at 8 weeks after the end of azoxymethane injection (25.4 ± 6.7 vs. 8.5 ± 4.5, $P < 0.05$). The mean numbers of aberrant crypt foci/whole colon were also significantly different at 16 weeks after azoxymethane injection (36.5 ± 4.6 vs. 18.3 ± 6.4, $P < 0.01$), and were statistically significantly higher in ITF–dnRII mice than wild-type littermates. In accordance with the increased incidence of aberrant crypt foci/whole colon, the mean PCNA–LI was also significantly increased in ITF–dnRII mice compared to wild-type littermates at 8 weeks after azoxymethane injection (33.7 ± 6.5 vs. 12.3 ± 5.2, $P < 0.05$). The differences in PCNA–LI were more significant at 16 weeks after azoxymethane injection (48.6 ± 6.1

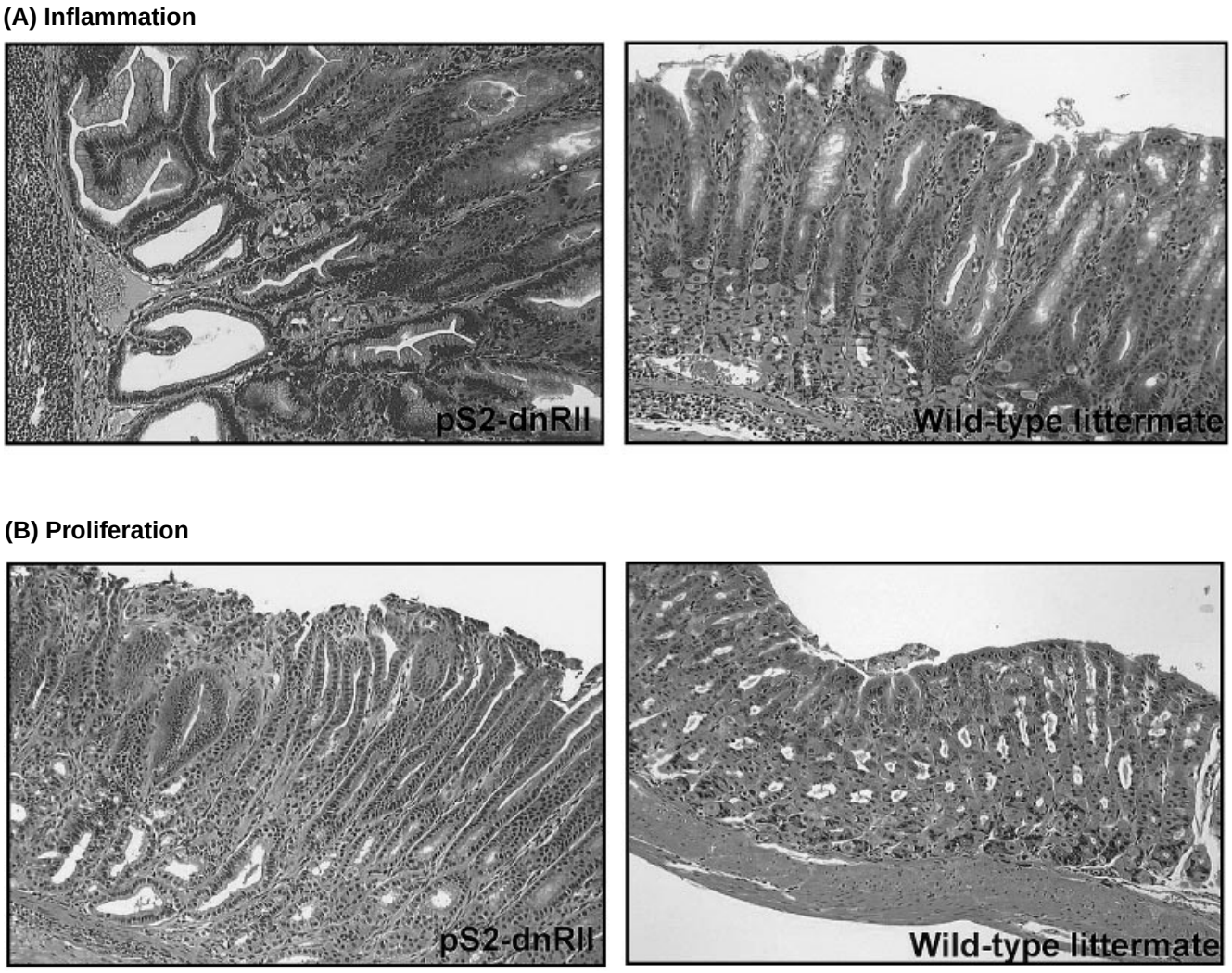


Figure 3. Histopathology of the stomach. (A) Inflammation pS2–dnRII mice showed severe gastritis compared to wild-type littermates infected with *H. pylori* (ATCC 43504 strain, $CagA^+$, $VacA^+$) × 100, original magnification. (B) Proliferation in pS2–dnRII mice showed severe hyperplastic gastritis compared to wild-type littermates infected with *H. pylori* × 100, original magnification.

vs. 23.6 ± 7.5, $P < 0.05$) (Table 2). The incidence of colon adenoma/adenocarcinoma was significantly different between ITF–dnRII transgenic mice and wild-type littermates at 24 weeks after azoxymethane injection. At 24 weeks after azoxymethane injection, 70% of pS2–dnRII mice developed colon adenocarcinoma, while none of the wild-type mice did (Figure 6C). Aberrant crypt foci also progressed to colon adenoma, the precancerous lesion of colon cancer. All pS2–dnRII mice developed colon adenoma, either single or multiple, but only 30% of wild-type littermates did so, and the adenomas in these tended to be fewer in number ($P < 0.001$) (Figure 6B).

DISCUSSION

The results of this study clearly demonstrate that the conditional loss of TGF-β signalling selectively in the stomach mucosa contributes to *H. pylori*-associated gastric carcinogenesis and that selective loss of TGF-β signalling in the colon mucosa accelerates azoxymethane-induced aberrant crypt foci formation and the development of colon cancer. Mouse stomach or colon in which TGF-β signalling was defective were found to be highly susceptible to gastrointestinal carcinoma after the initiation of chemical or biological carcinogenic attack, indicating that the maintenance of TGF-β

Table 1. Summary of histopathological findings at 36 weeks in wild-type and pS2-dnRII mice infected with *H. pylori*

	Wild-type littermates	ps2-dnRII transgenic mice
Hyperplasias		
Antrum	2/6	6/7
Fundus	3/6	7/7
Inflammations		
Antrum	0/6	4/7
Fundus	4/6	7/7
Lymphocytic infiltrations		
Antrum	1/6	6/7
Fundus	0/6	6/7
Dysplasia/carcinoma	0/6	7/7

signalling in the stomach or colon may be very important in either preventing carcinogenesis or attenuating inflammation.

Previous studies[20, 21] have shown that the selective loss of TGF-β signalling in the pancreas and intestine renders the affected mice highly susceptible to either autoimmune pancreatitis or ulcerative colitis. The pS2–dnRII transgenic mice showed marked increases in major histocompatibility complex class II (MHC II) molecules and matrix metalloproteinase (MMP) expression in pancreatic acinar cells.[20] pS2–dnRII mice also showed increased susceptibility to cerulein-induced pancreatitis. This pancreatitis was characterized by severe pancreatic oedema, inflammatory cell infiltration, T- and B-cell hyperactivation, IgG-type auto-antibodies against pancreatic acinar cells, and IgM-type auto-antibodies against pancreatic ductal epithelial cells. TGF-β signalling therefore seems to be essential either for maintaining normal immune homeostasis and suppressing autoimmunity or for preserving the integrity of pancreatic acinar cells. Similar phenomena were observed in experiments in ITF–dnRII transgenic mice. ITF–dnRII transgenic mice developed spontaneous colitis presenting with diarrhoea, haematochezia and anal prolapse under non-SPF

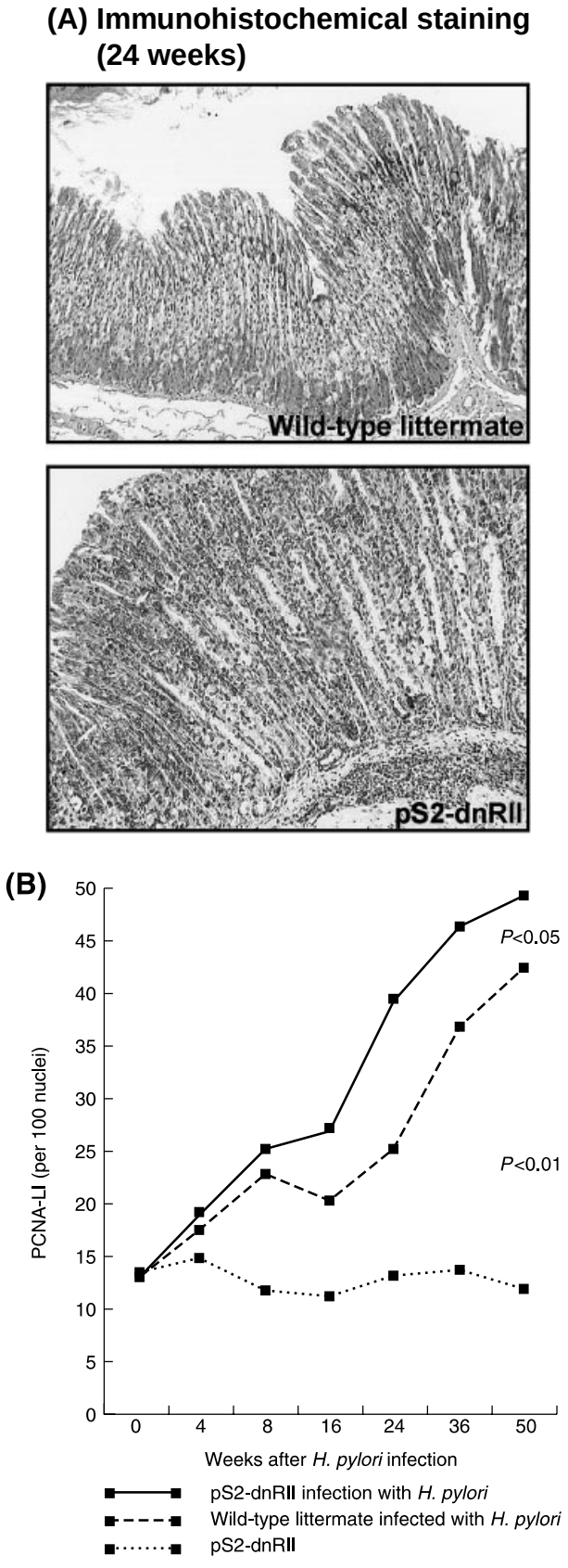


Figure 4. PCNA immunostaining and PCNA-LI. (A) Immunohistochemical staining of PCNA × 100, original magnification. Compared to wild-type littermates infected with *H. pylori*, pS2–dnRII mice showed high numbers of positively stained nuclei in the gastric mucosa. (B) PCNA-LI. Compared to non-infected control mice, *H. pylori* infection caused statistically high PCNA-LI values. PCNA-LI was statistically significantly higher in pS2–dnRII transgenic mice infected with *H. pylori* than wild-type mice infected with the same *H. pylori* strain.

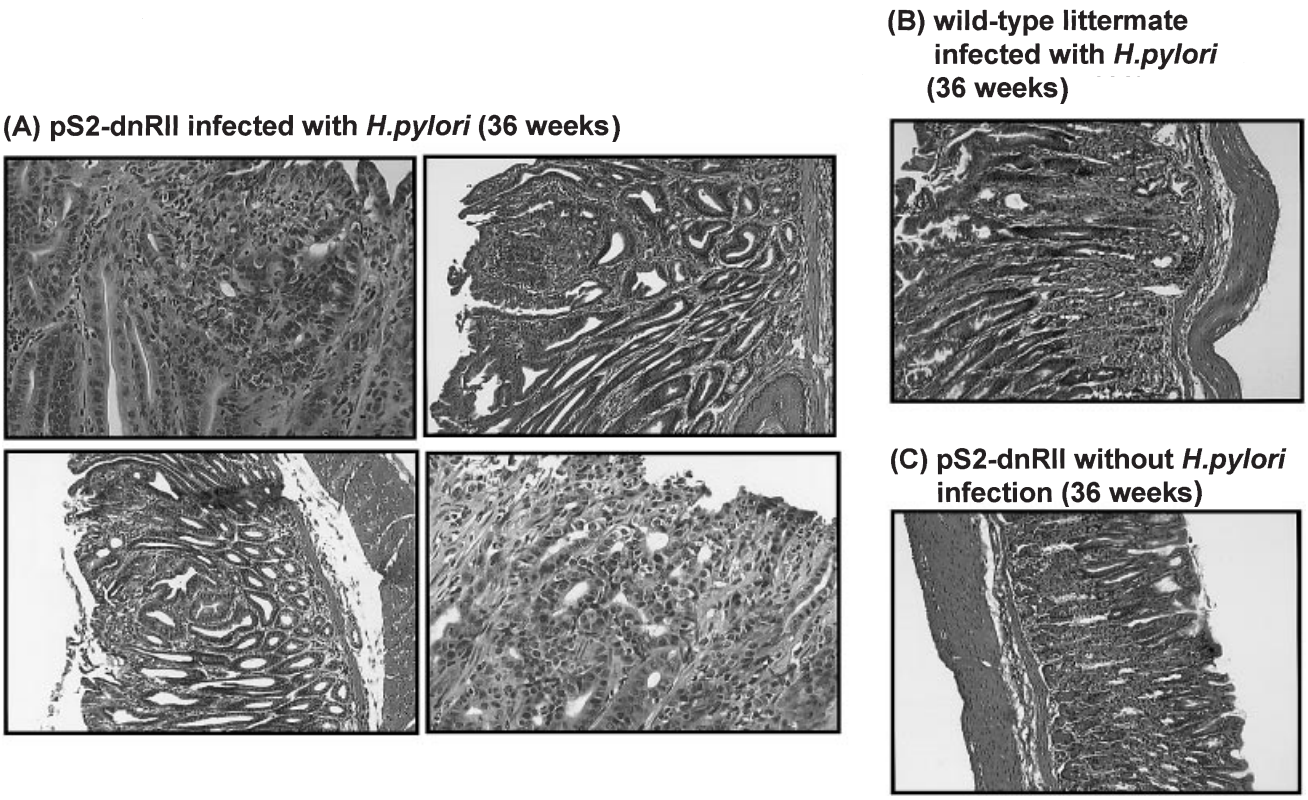


Figure 5. Comparison of gastric pathology. (A) Gastric dysplasia or mucosal carcinoma × 100, original magnification; (B) chronic gastritis × 100, original magnification; (C) normal pathology × 100, original magnification. All experimental mice infected with *H. pylori* developed chronic gastritis, but only the pS2–dnRII mice developed gastric dysplasia or gastric adenocarcinoma after 36 weeks of *H. pylori* infection. No significant pathology was noted in uninfected pS2–dnRII mice.

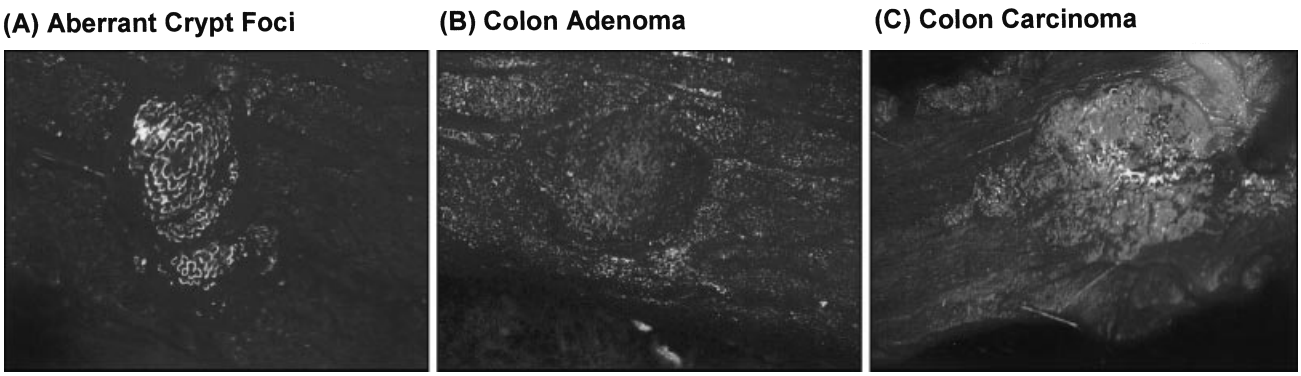
conditions.[21] Under SPF conditions, colitis was induced by dextran sodium sulphate (DSS) mixed in drinking water to examine the significance of the loss of TGF-β signalling in the pathogenesis of ulcerative colitis. Transgenic mice showed increased susceptibility to DSS-induced ulcerative colitis, and elicited increased expression of MHC class II, generation of auto-antibodies against intestinal goblet cells, and increased MMP activity in intestinal epithelial cells compared to wild-type littermates challenged with DSS. The specific deficiency of TGF-β signalling in the intestine contributes to the development of inflammatory bowel disease. The maintenance of TGF-β signalling may be important in regulating the immune homeostasis in the intestine.

Based on these previous observations, we infected mice with *H. pylori* to compare *H. pylori*-associated gastritis and carcinogenesis between pS2–dnRII and wild-type littermates. pS2–dnRII transgenic mice showed exaggerated and more severe inflammatory reactions compared to wild-type littermates, indicating that the loss of TGF-β signalling selectively in the stomach resulted in the loss of anti-inflammatory regulators, in turn inducing a more extensive degree of gastritis. Another feature noted in this animal model was that pS2–dnRII

Table 2. PCNA-LI and occurrence of aberrant crypt foci in wild-type littermates and ITF-dnRII mice treated with azoxymethane

Time after last azoxymethane	PCNA-LI†		Ocurrence of aberrant crypt foci	
	Wild-type	ITF-dnRII	Wild-type	ITF-dnRII
8 weeks (n = 10)	12.3 ± 5.2	33.7 ± 6.5	8.5 ± 4.5	27.4 ± 6.7*
8 weeks (n = 10)	23.6 ± 7.5	48.6 ± 6.1	18.3 ± 6.4	36.5 ± 4.6*

†Proliferating nuclear antigen-labelling index (%, mean ± S.D.); *occurrence of aberrant crypt foci (no. of aberrant crypt foci/whole colon). $P < 0.05$ (vs. wild-type littermates).



Wild-type littermate (24 weeks)	**3/10 (30%)**	**1/10 (10%)**
ITF-dnRII transgenic mice (24 weeks)	**10/10 (100%)**	**7/10 (70%)**
P value	***<0.001***	***<0.001***

Figure 6. Methylene blue staining of (A) aberrant crypt foci × 40, original magnification; (B) colon adenoma × 30, original magnification; and (C) colon adenocarcinoma × 20, original magnification. The incidence of colon adenoma or adenocarcinoma at 24 weeks after azoxymethane administration was significantly different between ITF–dnRII and wild-type littermates ($P < 0.01$).

mice infected with *H. pylori* showed severe hyperplastic gastritis and gastric epithelial dedifferentiation.

Our observations were consistent with those of other groups. To study the role of interleukin-10 (IL-10), a potent anti-inflammatory and immune regulatory cytokine, in the host response to gastric *Helicobacter* infection, Berg *et al.*[22] colonized the stomachs of IL-$10^{-/-}$ and wild-type mice with *H. felis*, as a animal model of human *H. pylori* infection. While wild-type mice develop a mild, focal chronic gastritis, *H. felis*-infected IL-$10^{-/-}$ mice develop severe hyperplastic gastritis, characterized by a dense, predominantly mononuclear cell inflammation of the mucosa and submucosa and epithelial cell proliferation and dedifferentiation. Taken together, these and our findings indicate that, in the absence of TGF-β or IL-10, the inflammatory and immunological responses of the murine host to gastric colonization with *Helicobacter* is a rapidly evolving pathological process with features that mimic those associated with *H. pylori* infection in humans.

Confirmation of our finding that the loss of anti-inflammatory cytokine augments the degree of gastritis raises the question of whether the opposite is true. For example, is the inflammatory response against *Helicobacter* infection attenuated when the host loses inflammatory cytokines? The contribution of gamma interferon (IFN-γ) to the immune response to *Helicobacter* infection, especially to the induction of gastric inflammation, was investigated in IFN-$\gamma^{-/-}$ mice.[23] *H. pylori*-infected wild-type mice developed severe infiltration of mononuclear cells in the lamina propria and erosions in the gastric epithelium 15 months after infection, whereas *H. pylori*-infected IFN-$\gamma^{-/-}$ mice showed no inflammatory reaction at all. These results clearly demonstrated that inflammatory cytokines induced by *H. pylori* infection play an important role in the induction and propagation of gastric inflammation caused by *H. pylori* infection, and that the loss of inflammatory cytokines attenuates *H. pylori*-associated gastritis.

In spite of much epidemiological evidence indicating a significant relationship between *H. pylori* infection and gastric adenocarcinoma,[24, 25] doubt still exists as to whether the risk of stomach cancer is raised by *H. pylori* infection. Against this, some epidemiological results have shown no, or even a negative, relationship between *H. pylori* infection and gastric carcinogenesis.[26–28] Therefore, it is essential to have an appropriate animal model to clarify the role of *H. pylori* in gastric carcinogenesis. The Mongolian gerbil model has been commonly employed for this purpose. Although after long-term infection, about 40% of Mongolian gerbils were found to develop gastric adenocarcinoma,[29, 30] other groups found no carcinoma in repeated experiments, considering instead that *H. pylori* infection enhanced glandular stomach carcinogenesis in Mongolian gerbils treated with chemical carcinogens.[31–33] On

the basis of these findings, WHO-IARC defined *H. pylori* infection as a class I carcinogen. Our previous observation also suggested the promoting role of *H. pylori* infection in gastric carcinogenesis in a mice model.[34] Moreover, the fact that only some patients develop symptomatic chronic atrophic gastritis or gastric cancer, despite the high prevalence of *H. pylori*, suggests that host genetic status, for instance the loss of TGF-β signalling, might determine the outcome of *Helicobacter* infection.

Aberrant crypt foci were first identified microscopically in methylene blue-stained whole-mounted preparations of colonic mucosa from carcinogen-treated rodents.[35] Aberrant crypts are distinguished from normal crypts in these preparations by their asteroid- or oval-shaped lumen and elevated thick epithelium[35] (Figure 6A). Pathological features of aberrant crypt foci found during carcinogenesis in these rodents seem to be heterogeneous, from mildly atypical to dysplastic crypts. Aberrant crypt foci have been considered to be neoplastic due to the high incidence of K-*ras* mutation, p53 mutation, high expression of iNOS and COX-2, increased cell proliferation due to decreased expression of p16, and altered expression of β-catenin.[36–39] We are currently studying the status of β-catenin and iNOS in our model and the chemopreventative role of specific iNOS inhibitors such as S,S′-1,4-phenylene-*bis* (1,2-ethanediyl)*bis*-isothiourea (PBIT).[40] Results to date have clearly shown that increased PCNA expression occurs in conjunction with an increased incidence of aberrant crypt foci and colon carcinogenesis, which is aggravated due to the loss of TGF-β signalling in the colon. The incidence of aberrant crypt foci was significantly increased in azoxymethane-injected ITF–dnRII transgenic mice compared to wild-type littermates in our study. In accordance with this increased aberrant crypt foci, colonic adenoma and adenocarcinoma were significantly increased in mice with conditional loss of TGF-β signalling, signifying the important role of loss of TGF-β signalling in colon carcinogenesis (Figure 5).

TGF-β signals are mediated both by TGF-β receptors and by their signalling molecules, Smad proteins. Inactivation of either of the two transmembrane serine/threonine kinases, termed TGF-β type I and type II receptors, is now known to underlie a wide variety of human pathologies, in particular carcinogenesis.[41] Numerous studies have demonstrated that the TGF-β receptor complex and its downstream signalling intermediates constitute a tumour suppressor pathway.[42, 43] The common mechanism of loss of expression of the TGF-β type II receptor involves transcriptional repression or mutational inactivation resulting from microsatellite instability. We published the first clear evidence of transcriptional repression of TGF-β type II in the tumorigenesis of Ewing's sarcoma, based on the finding that the transcriptional repression of TGF-β type II receptor is a major target of the EWS-FLI1 oncogene.[44] Mutational inactivation of TGF-β type II receptor is common among human colon cancers with microsatellite instability. BAT-RII frame shift mutations were identified in 100 of 111 RER-positive colon cancers.[45] Loss of heterozygosity of the TGF-β type II receptor gene was detected by analysing a polymorphic site in intron 2 in three of nine gastric cancers.[46]

In conclusion, conditional loss of TGF-β signalling increased the susceptibility of affected organ to carcinogens. Host genetic factors may determine the outcome of *H. pylori* infection or carcinogen treatment. Maintaining normal TGF-β signalling in the gastrointestinal tract is important either for avoiding abnormal mucosal proliferation or for suppressing carcinogenesis, because even heterozygous loss of the TGF-β type II receptor itself plays a role as a tumour suppressor.

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