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Increased Level of Serum Vascular Endothelial Growth Factor by Long-Term Exposure to Hypergravity

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Abstract: *We have previously demonstrated that short-term exposure to hypergravity at 2G for 4 h induces expression of cyclooxygenase-2 (COX-2) in the mouse heart. Moreover, expression of vascular endothelial growth factor (VEGF) is also induced in the heart in a COX-2-dependent manner. Here, we demonstrate that long-term exposure of mice to 2G for 24 h resulted in a significant increase of serum VEGF level, although expression of COX-2 and VEGF in the heart decreased to the 1G-control level. Moreover, increase of serum VEGF was not suppressed by treatment with COX-2 inhibitor, indicating that VEGF was induced in a COX-2-independent manner. These results suggest that gravitational force contributes to maintenance of the serum VEGF level.*

Key words: *hypergravity, vascular endothelial growth factor*

Astronauts experience hypergravity of 3.2G at launch and 1.4G on re-entry. They are also exposed to microgravity during space flights or residence in a space station. For space exploration in the future, it is important to know how gravitational changes affect the physiological and pathological status of the human body in order to prevent unexpected outcomes under altered gravity. To date, several animal experiments have been performed to investigate biological and biochemical responses to hypergravity and microgravity. Since opportunities for conducting animal experiments under microgravity are limited, experiments under hypergravity are important using special centrifugation apparatus (see below). The results from such hypergravity experiments are useful for predicting the effects of microgravity as well as hypergravity on the human body.

We have previously demonstrated that short-term exposure to hypergravity at 2G for 4 h [2G (4H)] induces expression of cyclooxygenase-2 (COX-2; gene symbol, *Ptgs2*) in the heart [7]. COX-2 is a rate-limiting enzyme for prostaglandin biosynthesis, which plays important roles in a variety of pathological responses including tumorigenesis and diabetes [5, 6, 8]. Moreover, we have shown that exposure to hypergravity for 4 h induces expression of vascular endothelial growth factor (VEGF; gene symbol, *Vegfa*) in the heart of wild-type mice but not in COX-2 gene knockout mice [7], suggesting that VEGF expression in the heart under

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hypergravity is dependent on COX-2 induction. However, the role of VEGF in the heart under hypergravity has not been elucidated yet.

To further investigate the role of VEGF expression under hypergravity, mice were exposed to long-term hypergravity at 2G for 24 h [2G (24H)] using the Centrifugal Acceleration Test Facility at the Japan Aerospace Exploration Agency (JAXA, Tsukuba, Japan). Centrifugation with a 7.25-m arm at 15.70 rpm produced a hypergravity condition of 2G. A cage-mounting module was attached at the end of the arm that allowed one-degree freedom, thereby ensuring that the net G field was perpendicular to the floor of the mouse cage. The behavior of the mice was monitored with a CCD camera throughout the centrifugation experiments. Temperature and moisture in the cages were maintained at 24 ± 2°C and 42 ± 2%, respectively. We used eight C57BL/6 female mice at 8 weeks of age (CLEA, Tokyo, Japan) for centrifugation. Four out of 8 mice were treated with a COX-2 inhibitor, meloxicam (Daiichi Pharmaceutical, Tokyo, Japan), at 10 mg/kg/day by oral administration from 3 days before centrifugation. The other 4 mice were used as untreated controls of the 2G (24H) exposure group. Age-matched C57BL/6 females were used as 1G controls under normal gravity. The animal experiments were carried out with approvals of the Committee on Animal Experimentation of Kanazawa University (Kanazawa, Japan) and the Japan Aerospace Exploration Agency (JAXA, Tokyo, Japan).

Immediately after stopping centrifugation, mice were anesthetized with diethylether and euthanized by collection of whole blood by heart puncture. After blood collection, half of the heart was fixed in 4% paraformaldehyde for histological examination and the other half was used for total RNA extraction using ISOGEN solution (Nippon Gene, Tokyo, Japan). Immunostaining for COX-2 was performed as previously described [7]. Rabbit anti-COX-2 polyclonal antibody (Cayman Chemical, USA) was used as the first antibody. Expressions of COX-2, COX-1 (gene symbol, *Ptgs1*), and VEGF were examined by RT-PCR as previously described [7]. The following primer sets were used for the respective genes: COX-2 (F-5'-CAAACTCAA GTTTGACCCAG-3', and R-5'-GCCGGGATCCTTT TACAGCTCAGTTGAACG);COX-1 (F-5'-GAGATGC GCCTAGAGCCCTT-3', R-5'-GCGTCTCACTAAGA CAGACC-3'); and VEGF (F-5'-CTTCCTACAGCACA GCAGATGTGAA-3', R-5'-TGGTGACATGGTTAAT CGGTCTTTC-3'). RNA samples prepared in previous experiments with short-term exposure to 2G for 4 h [2G (4H)] were also used in the expression analysis of this study. The band intensities of RT-PCR results were measured using Image J (NIH, USA). We also measured the serum VEGF levels of the 2G (24H)-exposed and 1G control mice using Mouse VEGF ELISA kit (RayBiotech, Norcross GA, USA).

As shown in Fig. 1A, expression of COX-2 was not found in the heart of 2G (24H) exposed mice nor that of the 1G-control mice. This result contrasts sharply with induction of COX-2 in the heart by short-term exposure to 2G (4H) (Fig. 1B, C). Consistently, COX-2 expression was not detected by immunostaining in the hearts of 2G (24H)-exposed mice nor in the hearts of 1G control mice (Fig. 2A, B). In the previous study, however, COX-2 expression was detected in vessels of 2G (4H)-exposed mouse hearts [7]. Although COX-1 is a constitutively expressed enzyme in most tissues, its expression level was significantly increased in the 2G (4H)-exposed mouse heart (Fig. 1B, C). However, we found the same amount of COX-1 mRNA level in the hearts of 2G (24H)-exposed mice as in the 1G control mice (Fig. 1A, C). Consistent with the results of COX-2 expression, VEGF was at the basal level in the 2G (24H)-exposed mouse hearts and in the 1G control mice, although its level was slightly but significantly increased in the 2G (4H)-exposed mouse hearts. Treatment with meloxicam did not affect the expression level of these genes in the 2G (24H) group. Accordingly, it is possible that COX-2, COX-1 and VEGF were transiently induced in the heart by hypergravity as an acute response, and then the expression decreased to the normal level within 24 h. It has been reported that induction of COX-2 plays an important role in protection of the heart against ischemic stress and infarction [9]. In the heart of the 2G (24H)-exposed mice, however, we did not find any ischemic changes, such as necrosis or infarction (Fig. 2C, D). These results, taken together, suggest that long-term exposure to hypergravity does not cause ischemic stress in the heart, and thus induction of COX-2 and VEGF are not required for heart function under hypergravity. Expression of COX-2 is induced not only by ischemia but also by mechanical stress, serum stimuli, and inflammatory responses. It is

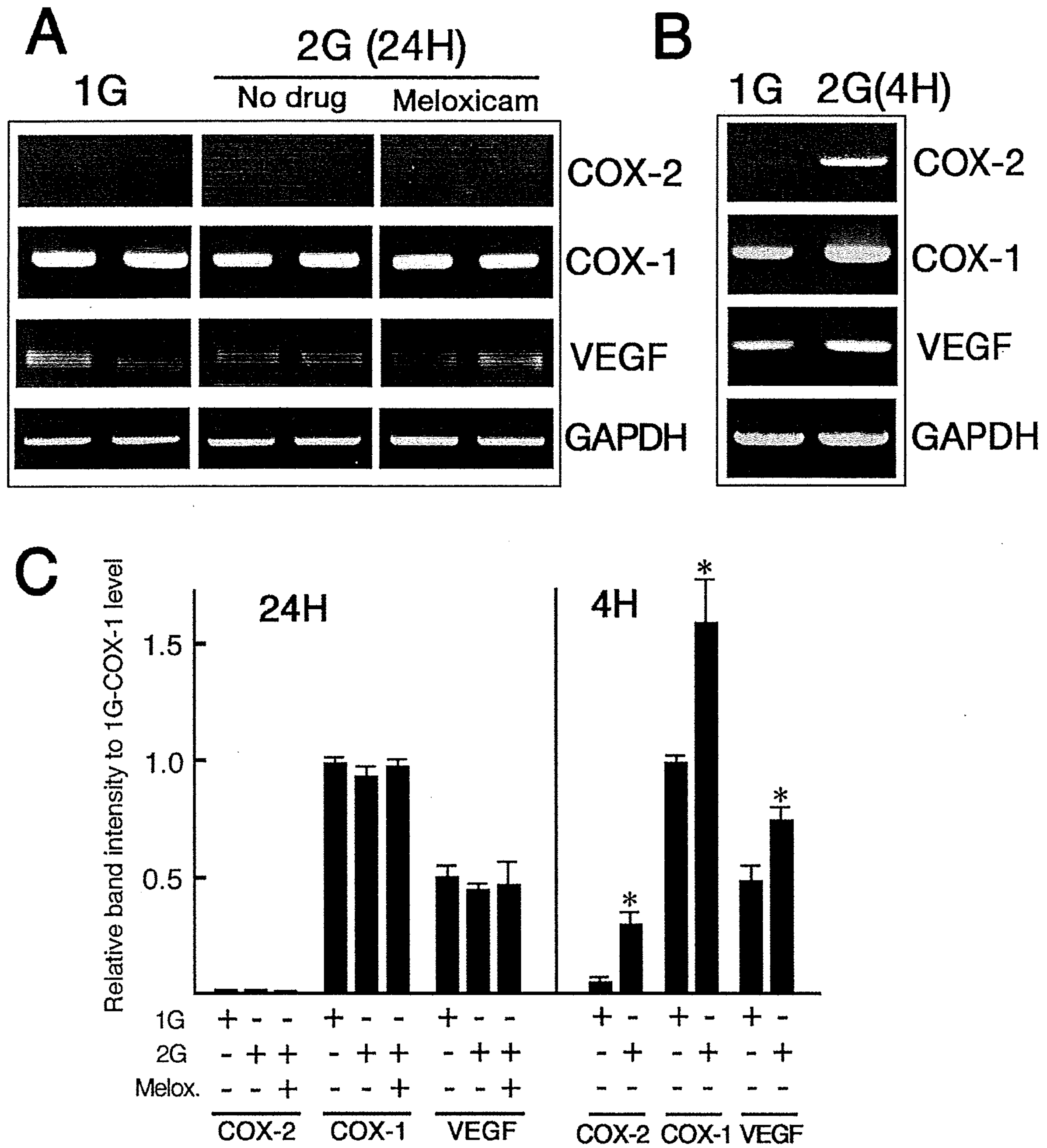

Fig. 1. Representative RT-PCR analysis of COX-2, COX-1, and VEGF in the heart of 1G control, 2G (24H)-exposed, and meloxicam-treated 2G (24H)-exposed mice (A), and 1G control and 2G (4H)-exposed mice (B). GAPDH was used as an internal control. (C) Relative band intensities of RT-PCR results to that of COX-1 level at 1G is shown as mean ± SD. "Meloxicam" indicates meloxicam-treated mice. Asterisks indicate significant difference versus respective 1G control (4H). Note that expression levels of COX-2, COX-1 and VEGF are increased only in the 2G (4H)-exposed mouse heart tissues.

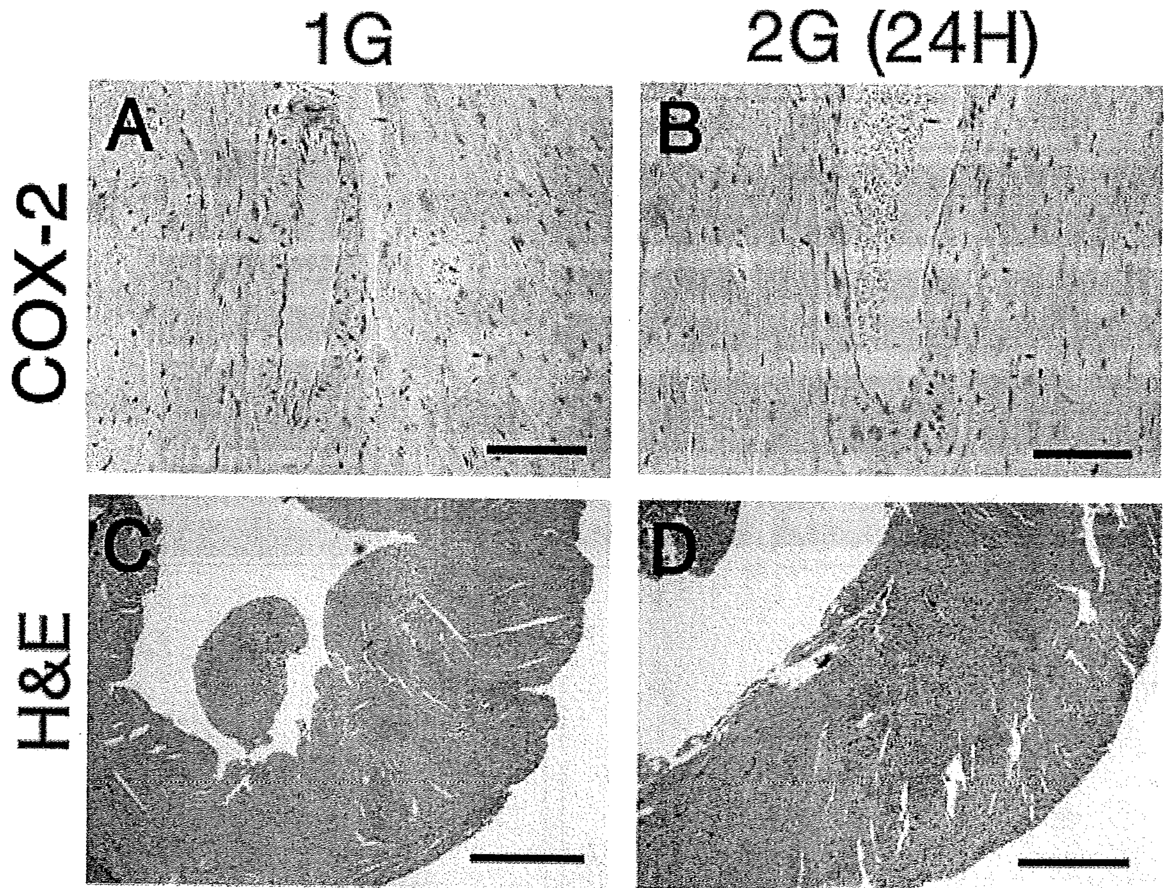

Fig. 2. Immunostaining for COX-2 in the heart of 1G-control (A) and 2G (24H)-exposed (B) mice. COX-2 expression was not detected in the 2G (24H)-exposed mouse heart including vessels nor in the 1G control. Histology of the heart (H&E) of 1G control (C) and 2G (24H)-exposed (D) mice. Note that neither necrosis nor infarction were found in 2G (24H)-exposed mouse heart. Bars in A, B, and C, D indicate 100 μm and 200 μm, respectively.

possible that some of these stimuli were generated temporarily in the heart by hypergravity, which resulted in transient induction of heart COX-2. However, the molecular mechanism underlying COX-2 induction under hypergravity remains to be investigated.

Interestingly, we found that the serum VEGF level was significantly increased in the 2G (24H)-exposed mice compared with the 1G control mice (Fig. 3). Although meloxicam treatment resulted in a slight decrease of the VEGF level, it was not a significant reduction. These results indicate that long-term exposure to hypergravity induces VEGF expression in tissues other than the heart in a COX-2-independent manner. In this study, we did not determine tissue(s) expressing VEGF under hypergravity. However, it has been reported that increased mechanical stretch in the artery causes induction of VEGF in the vessel walls [4]. Therefore, it is possible that hemodynamic changes caused by constitutive gravitational force induced VEGF in blood vessels, increasing the serum VEGF levels. Accordingly, it is possible that increased gravitational stress affects systemic angiogenesis through induction of serum VEGF from blood vessels.

On earth, normal gravity at 1G continuously stimulates our body. Accordingly, 1G gravity may also contribute to the basal level of serum VEGF. It has been demonstrated that induction of immediate early genes in cultured cells is significantly suppressed when experiments were performed in the Space Shuttle [3], indicating that 1G gravitational stress is required for

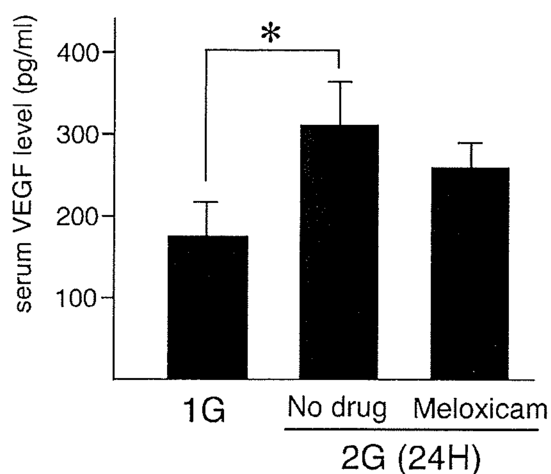

Fig. 3. Serum VEGF levels of 1G control, 2G (24H)-exposed, and meloxicam-treated 2G (24H)-exposed mice (mean ± SD). Asterisk indicates significant difference; P=0.0215. Serum VEGF level was significantly increased by exposure to 2G (24H), and it was not suppressed by treatment with a COX-2 inhibitor, meloxicam.

induction of these genes. Therefore, our results suggest the possibility that the serum VEGF level is decreased by long-term exposure to microgravity, like in a space station. Gene targeting of VEGF in the mouse leads to severe reduction in the size and caliber of the developing blood vessels [1, 2]. Accordingly, further experiments under microgravity are required to investigate the serum VEGF level and vascular development by breeding mice in a space station.

Acknowledgment(s)

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Induction and Down-regulation of *Sox17* and Its Possible Roles During the Course of Gastrointestinal Tumorigenesis

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BACKGROUND & AIMS: The activation of Wnt/β-catenin signaling causes the development of gastric and colon cancers. Sox17 represses Wnt/β-catenin signaling and is down-regulated in colon cancer. This study was designed to elucidate the role of Sox17 during the course of gastrointestinal tumorigenesis. **METHODS:** *Sox17* expression was examined in gastrointestinal tumors of mouse models and humans. The roles of Sox17 in gastric tumorigenesis were examined by cell culture experiments and by construction of *Sox17* transgenic mice. **RESULTS:** *Sox17* was induced in *K19-Wnt1/C2mE* mouse gastric tumors and *K19-Wnt1* preneoplastic lesions, where Wnt/β-catenin signaling was activated. Consistently, Wnt activation induced *Sox17* expression in gastric cancer cells. In contrast, Sox17 was rarely detected by immunohistochemistry in gastric and colon cancers, whereas strong nuclear staining of Sox17 was found in >70% of benign gastric and intestinal tumors. Treatment with a demethylating agent induced *Sox17* expression in gastric cancer cells, thus indicating the down-regulation of *Sox17* by methylation. Moreover, transfection of *Sox17* in gastric cancer cells suppressed both the Wnt activity and colony formation efficiency. Finally, transgenic expression of *Sox17* suppressed dysplastic tumor development in *K19-Wnt1/C2mE* mouse stomach. **CONCLUSIONS: Sox17 plays a tumor suppressor role through suppression of Wnt signaling. However, *Sox17* is induced by Wnt activation in the early stage of gastrointestinal tumorigenesis, and *Sox17* is down-regulated by methylation during malignant progression. It is therefore conceivable that Sox17 protects benign tumors from malignant progression at an early stage of tumorigenesis, and down-regulation of *Sox17* contributes to malignant progression through promotion of Wnt activity.**

The binding of the Wnt ligand to a Frizzled receptor destabilizes the β-catenin degradation complex containing adenomatous polyposis coli (APC), AXIN, and glycogen synthase kinase 3β (GSK3β), allowing the nuclear translocation of β-catenin, followed by the transcriptional activation of the Wnt target genes.[1] This canonical Wnt signaling (Wnt/β-catenin signaling) plays a key role in the maintenance of intestinal stem cells and progenitor cells.[2,3] Moreover, the constitutive activation of Wnt/β-catenin signaling causes gastrointestinal tumorigenesis in both human beings[4,5] and mice.[6,7] It has also been shown that β-catenin nuclear accumulation, a hallmark of Wnt activation, is particularly enhanced in the invasion front and metastasized colon cancer cells, suggesting that promotion of Wnt/β-catenin signaling is important for malignant progression.[8] Platelet-derived growth factor and hepatocyte growth factor, as well as tumor necrosis factor-α, have been shown to promote Wnt/β-catenin signaling activity in tumor cells.[9–11] On the other hand, down-regulation of Wnt antagonists such as secreted frizzled-related proteins contributes to gastric and intestinal tumorigenesis by boosting Wnt/β-catenin signaling activity.[12–14] These results suggest that the enhancement of Wnt activity by the induction of Wnt promoters or down-regulation of Wnt antagonists is important for gastrointestinal carcinogenesis.

Sox17 and other Sox family members, Sox3, Sox7, and Sox9, have been shown to inhibit Wnt/β-catenin signaling.[15–18] The *Sox* gene family was first identified by homology to the high mobility group box of the sex-determining gene *SRY*.[19] *Sox17*-null mouse embryos exhibit a deficiency of definitive endoderm,[20] and overexpression of *Sox17* in embryonic stem cells results in the establishment of stable endoderm progenitors.[21] These results indicate that Sox17 plays a key role in definitive endoderm development. On the other hand, *Sox17* expression is down-regulated in colon cancer cells by promoter methylation,[22] and the expression of *Sox17*

Abbreviations used in this paper: APC, adenomatous polyposis coli; DAC, 5-aza-2′-deoxycytidine; GSK3β, glycogen synthase kinase 3β; HMG, high mobility group; PGE_2, prostaglandin E_2; RT-PCR, reverse-transcription polymerase chain reaction; TCF, T-cell factor.

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in colon cancer cells reduces the efficiency of the colony formation.[22,23] These results suggest that Sox17 is a tumor suppressor for colorectal cancer development.

Activation of Wnt/β-catenin signaling has been shown to cause the development of gastric tumors as well as intestinal tumors.[6,7,24,25] However, *Sox17* expression during the course of gastrointestinal tumorigenesis has not been fully investigated yet. We herein show the *Sox17* expression to be unexpectedly induced at the early stage of gastric and intestinal tumors both in human and mouse models. Sox17 and β-catenin have been shown to cooperate to function as a transcription factor in definitive endoderm development.[26,27] Interestingly, we found that Sox17 target genes were induced in mouse gastric tumors, thus suggesting that Sox17 plays a role in early tumorigenesis in cooperation with β-catenin. Moreover, the transgenic expression of *Sox17* in mouse stomach suppressed dysplastic tumor development in *K19-Wnt1/C2mE* mice.[7] These results suggest that Sox17 prevents malignant progression as a tumor suppressor at an early stage of tumorigenesis, and down-regulation of *Sox17* causes tumor progression.

Materials and Methods

Mouse Models

Construction of $Apc^{\Delta 716}$, *cis-*$Apc^{\Delta 716}$ *Smad4* (+/−) knockout (*cis-*$Apc^{\Delta 716}$ *Smad4*), *K19-Wnt1*, *K19-C2mE*, and *K19-Wnt1/C2mE* (*Gan* for Gastric neoplasia) mouse models has been described previously.[6,7,28,29] Briefly, $Apc^{\Delta 716}$ mice carry a heterozygous mutation in the *Apc* gene, which develop intestinal polyps. *cis-*$Apc^{\Delta 716}$ *Smad4* mice are compound heterozygotes of *Apc* and *Smad4* which develop invasive intestinal adenocarcinoma. *K19-Wnt1* and *K19-C2mE* mice express *Wnt1* and a combination of *Ptgs2* and *Ptges*, respectively, in gastric epithelial cells. *K19-Wnt1* mice develop gastric preneoplastic lesions, whereas *K19-C2mE* mice show inflammation-associated metaplastic hyperplasia. *Gan* mice expressing *Wnt1*, *Ptgs2*, and *Ptges* are compound transgenic mice of *K19-Wnt1* and *K19-C2mE*, which develop dysplastic gastric tumors. To construct *K19-Sox17* mice, mouse *Sox17* cDNA fragment was subcloned into pBluescript (Stratagene, La Jolla, CA) with cytokeratin 19 (K19) gene promoter and SV40 poly(A) cassette. The expression vector was microinjected into the fertilized eggs of F1 (C3H and C57BL/6) mice to obtain *K19-Sox17* mice. Two *K19-Sox17* lines, no. 5 and

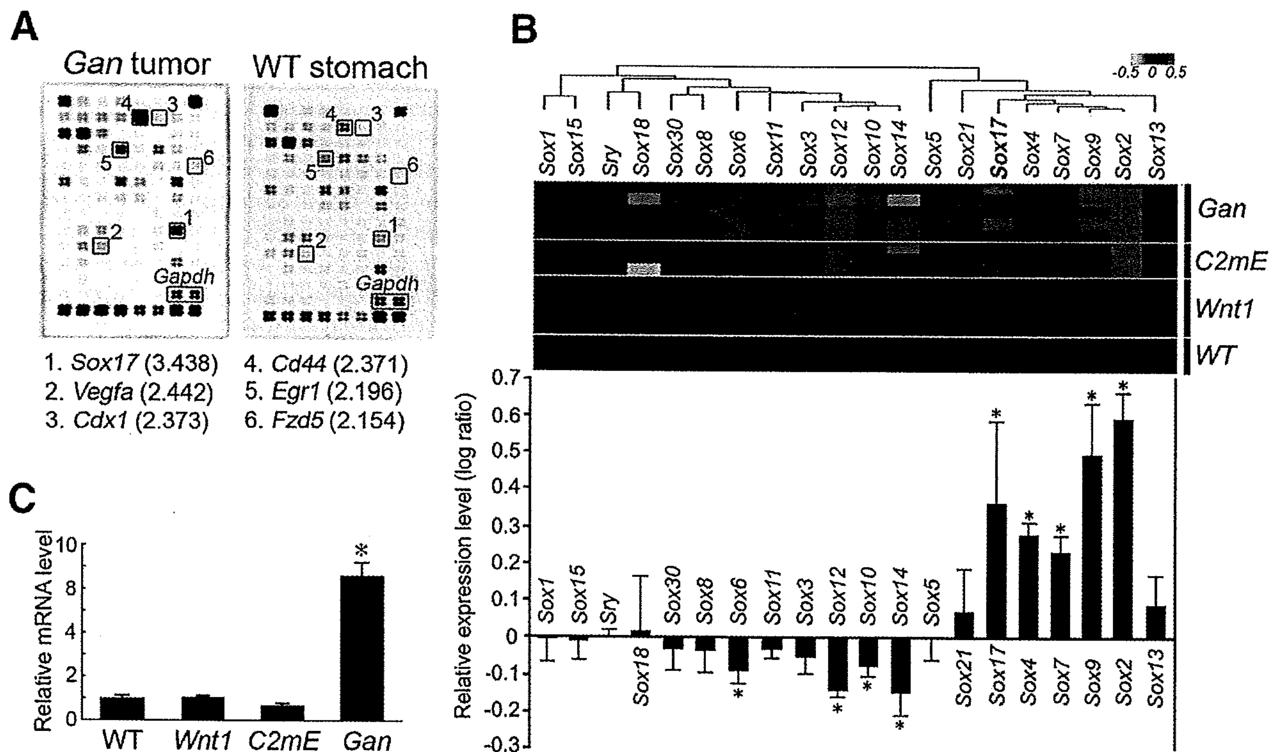


Figure 1. Induction of *Sox17* in *Gan* mouse gastric tumors. (*A*) Results of filter array analysis of Wnt pathway in *Gan* mouse tumors and wild-type mouse (WT) stomach. *Squares* indicate up-regulated genes in *Gan* tumors, and fold increases are indicated in *parentheses*. (*B*) Expression profiles of *Sox* family genes in *Gan*, *K19-C2mE* (*C2mE*), *K19-Wnt1* (*Wnt1*), and WT mice. The gene expression levels are shown in log10 ratios to wild-type as shown in the *cyan-magenta color bars* (*top*) and the mean log10 ratios in *Gan* tumors to the wild-type are shown in bar graph (mean ± SD) (*bottom*). (*C*) The relative *Sox17* mRNA levels of gastric tissues of indicated genotypes to the wild-type mouse stomach (mean ± SD). (*B* and *C*) $^{*}P < .05$ versus wild-type mouse stomach.

no. 9, were established, which showed similar phenotypes. Accordingly, we herein present the results with line no. 5. *K19-Sox17* mice were crossed with *Gan* mice to obtain *Gan K19-Sox17* and *K19-Sox17/C2mE* compound mice. The gastric tumors of *Gan* (n = 4), *Gan K19-Sox17* (n = 3), and *K19-Sox17/C2mE* mice (n = 3) were examined at 30 weeks of age. The tumor height was measured with the use of histologic sections. C57BL/6 mice (CLEA, Osaka, Japan) were used for RNA and protein preparation from stomach and intestines of embryos, pups, and adult mice. All animal experiments were carried out according to the protocol approved by the Ethics Committees on Animal Experimentation of Kanazawa University.

Histology and Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, embedded and sectioned at 4-μm thickness. The sections were stained with H&E, PAS-Alcian blue, or processed for immunostaining. Human tissue microarrays of gastric cancer and colon cancer (Biomax US, Rockville, MD) were used for immunostaining. Antibodies for Sox17 (R&D Systems, Minneapolis, MN), β-catenin (Sigma, St Louis, MO), active β-catenin (Millipore, Temecula, CA), and Ki-67 (DakoCytomation, Carpinteria, CA) were used as the primary antibody. Staining signals were visualized with the use of the Vectorstain Elite Kit (Vector Laboratories, Burlingame, CA). The MOM Kit (Vector Laboratories) was used to minimize the background staining signals. For fluorescence immunostaining, Alexa Fluor 594 or Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR) was used as the secondary antibody. Apoptosis was examined with the ApopTag Apoptosis Detection Kit (Chemicon, Temecula, CA). The mean Ki-67 labeling index was calculated by counting Ki-67–labeled cells per microscopic field (×200) in 5 fields.

Microarray Analyses

Total RNA samples were prepared from either tumors or the normal stomach of *Gan* (n = 5), *K19-C2mE* (n = 3), *K19-Wnt1* (n = 5), or wild-type (n = 3) mice at 30 weeks of age with the use of RNeasy Mini Kit (QIAGEN, Valencia, CA). The expression profiles of *Sox* family genes were examined with the Affymetrix GeneChip system and Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara,

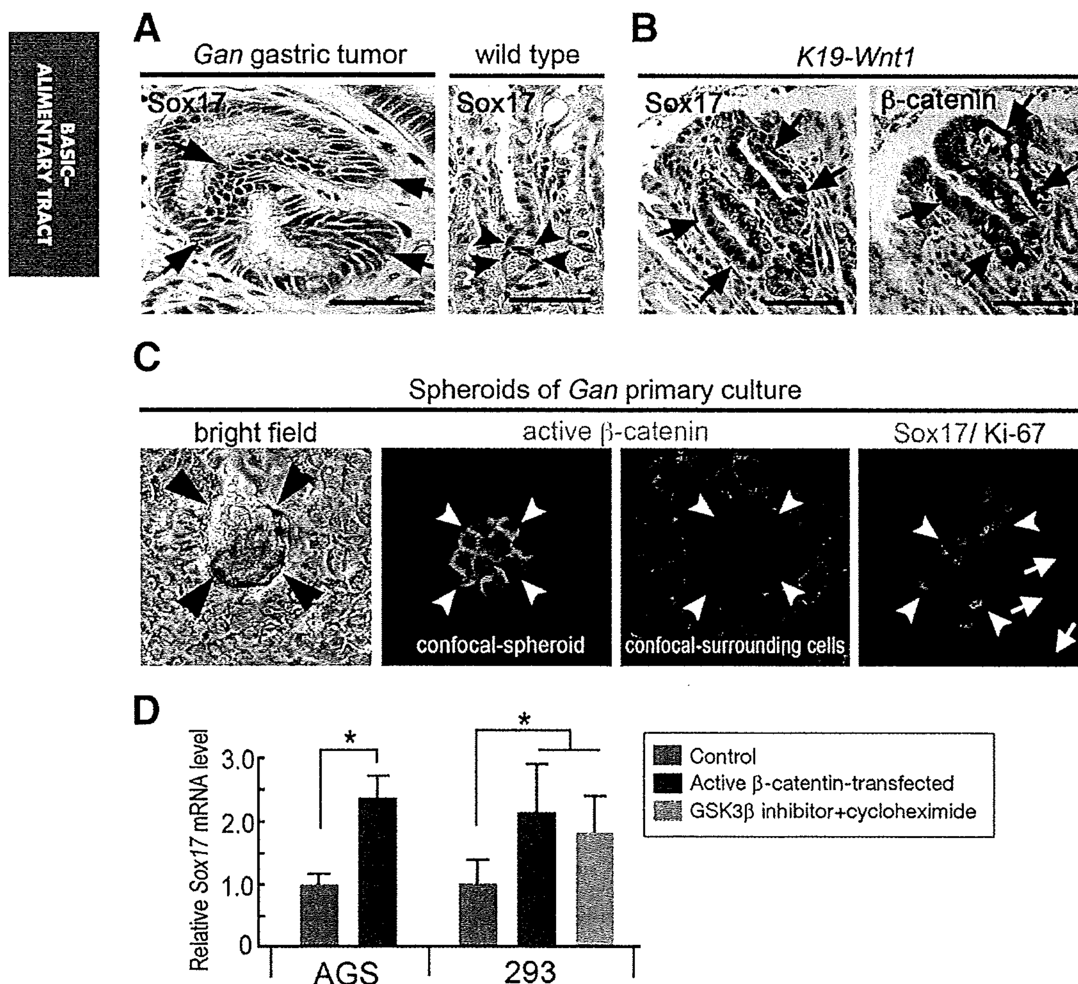


Figure 2. Induction of *Sox17* in Wnt-activated gastric epithelial cells. (*A*) Immunostaining for Sox17 in *Gan* mouse gastric tumor and wild-type gastric gland. (*B*) Immunostaining for Sox17 and β-catenin in serial sections of *K19-Wnt1* mouse preneoplastic lesion. (*A* and *B*) *Arrows* indicate tumor cells and dysplastic cells, respectively, whereas *arrowheads* (*A*) indicate normal undifferentiated epithelial cells. *Bars* indicate 100 μm. (*C*) Photographs of the primary cultured *Gan* mouse tumor epithelial cells. *Arrowheads* indicate the place of spheroid. Bright field photograph (*left*) and confocal microscopy photographs of spheroid (*second from left*) and surrounding monolayer cells (*third from left*) immunostained with active β-catenin antibody (*green*). Double immunostaining for Sox17 (*green*) and Ki-67 (*red*) (*right*). *Arrows* indicate Ki-67–positive cells. (*D*) The relative *Sox17* mRNA levels in active β-catenin-transfected cells (*blue*) and GSK3β inhibitor/cycloheximide-treated cells (*green*) to that in untreated control cells (*gray*) (mean ± SD); $^{*}P < .05$.

CA). Wild-type sample data were combined in silico in Rosetta Resolver (Rosetta Biosoftware, Seattle, WA) and used as a reference for all samples. The expression profile of *Sox* family genes in human gastric cancer was examined with public data[30] (National Center for Biotechnology Information GEO, GSE4007). These expression data were transformed to log10 ratios to the average of all normal samples.

Filter Array Analyses

Total RNA was prepared from wild-type mouse stomach (n = 3) and *Gan* mouse gastric tumors (n = 3). The gene expression profiles were examined with the pooled RNA samples and Wnt signaling Pathway Oligo GEArray (SABiosciences, Frederick, MD) according to the manufacturer's protocol.

Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from tissues with the use of ISOGEN (Nippon Gene, Tokyo, Japan), reverse transcribed with PrimeScript RT reagent Kit (Takara, Tokyo, Japan), and polymerase chain reaction (PCR)-amplified by ABI Prism 7900HT (Applied Biosystems) with the use of SYBR Premix Ex Taq II (Takara, Tokyo,

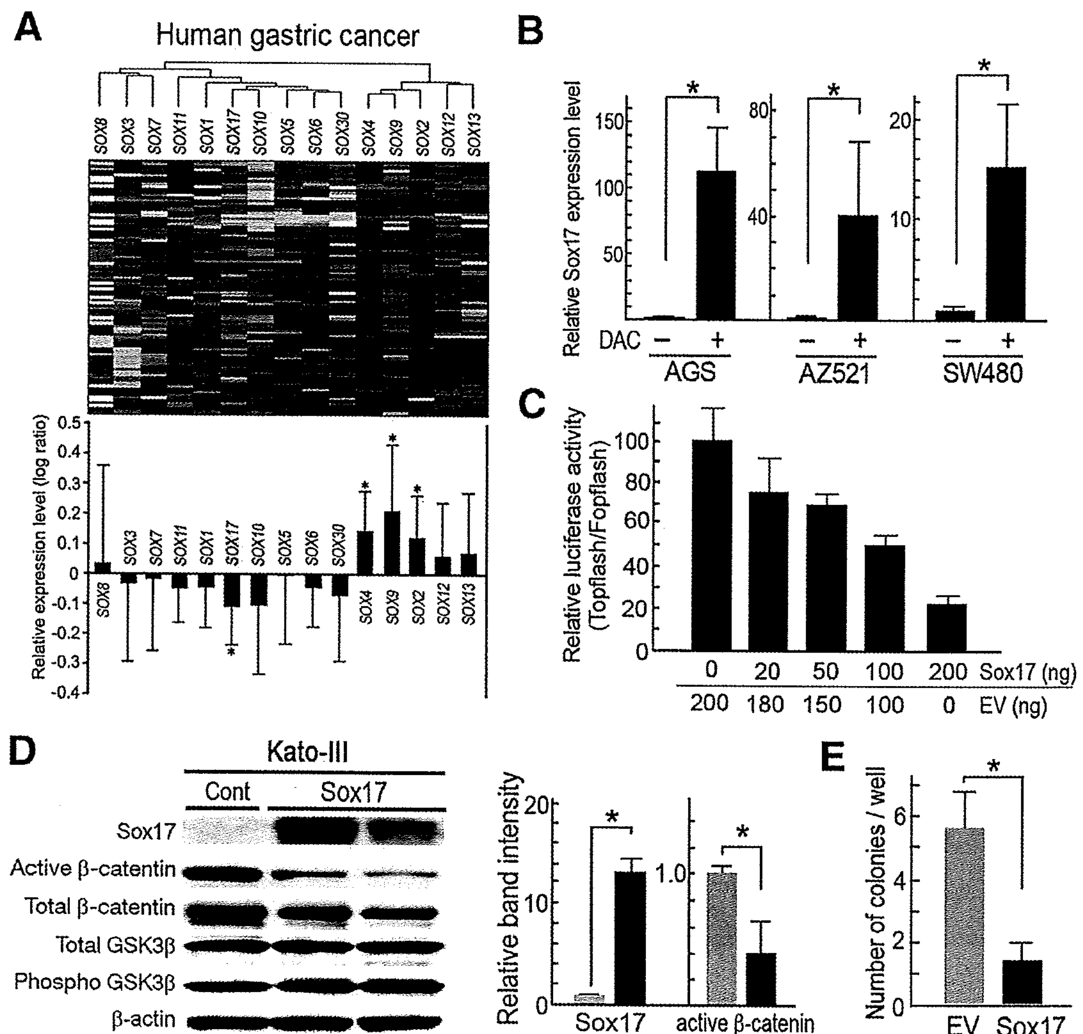


Figure 3. Down-regulation of *Sox17* in human gastric cancer, and suppression of Wnt/β-catenin signaling by *Sox17* expression. (*A*) The expression profiles of *Sox* family genes in human gastric caner. Gene expression levels are shown in log10 ratios as shown in *cyan-magenta color bars* (*top*) and in *bar graph* (mean ± SD) (*bottom*). (*B*) The relative expression level of *Sox17* in DAC-treated cells examined by real-time RT-PCT (mean ± SD). (*C*) The relative TCF/β-catenin transcription activity examined by TOPflash/FOPflash assay in *Sox17*-transfected AZ521 cells. The DNA amounts of transfected *Sox17* expression vector (*Sox17*) and empty vector (EV) are indicated. (*D*) Representative Western blotting results in control and *Sox17*-transfected Kato-III cells. β-Actin was used as an internal control. The relative band intensities of Sox17 and active β-catenin in the *Sox17*-transfected cells (*closed bars*) to the control levels (*gray bars*) are shown in bar graph. (mean ± SD) (*E*) The number of colonies of AGS cells transfected with empty vector (EV) or *Sox17* expression vector (*Sox17*) (mean ± SD). (*A*, *B*, *D*, and *E*) $^*P < .05$.

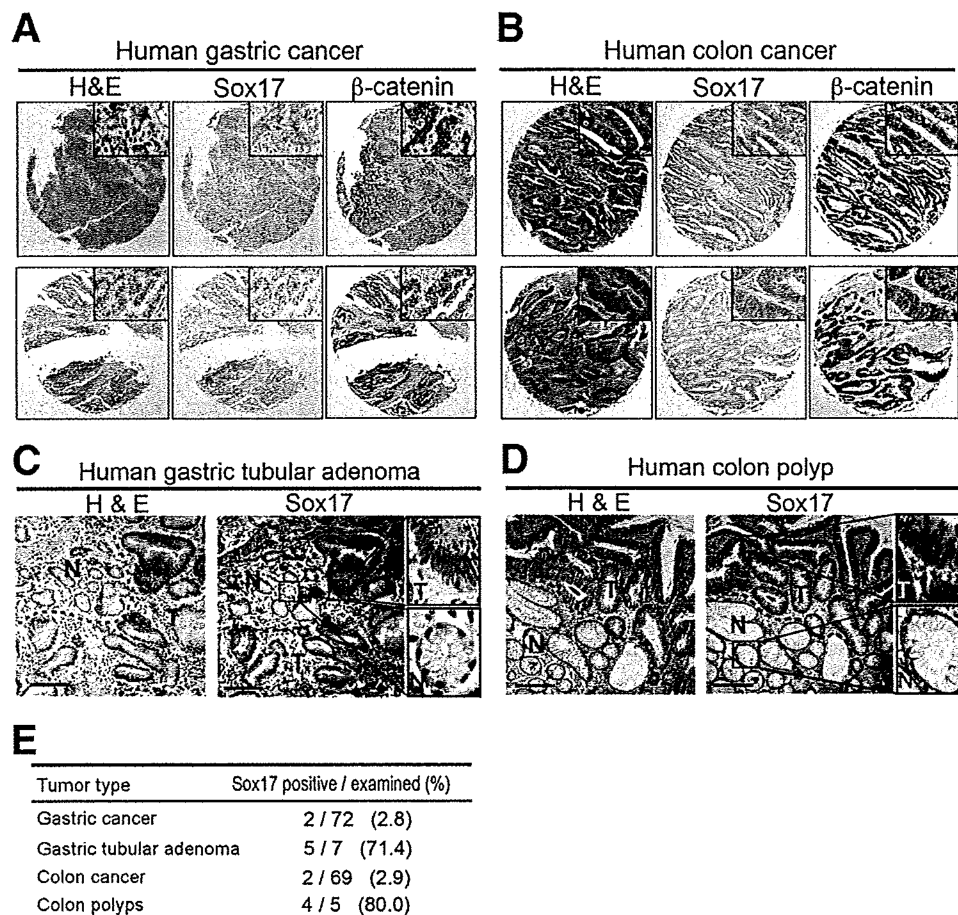
Japan). Primers were purchased (Takara), and primer sequences are available on request.

Cell Culture Experiments

Gastric cancer cell lines, AZ521 (Riken Bioresource Center, Tsukuba, Japan), Kato-III (Cell Resource Center, Tohoku University, Sendai, Japan), and AGS (ATCC, Manassas, VA) and colon cancer cell line SW480, and HEK293 cells (ATCC) were cultured in RPMI1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To examine the Wnt/β-catenin signaling activity, cells were transfected with TOPflash or FOPflash vector (Upstate Biology, Temecula, CA), and the luciferase activities were measured with the Luciferase Assay System (Promega, Madison, WI). TOPflash results were normalized with FOPflash. For demethylation, cells were treated with 1 μmol/L of 5-aza-2′-deoxycytidine (DAC; Sigma) for 72 hours. For the inhibition of GSK3β and protein synthesis, cells were treated with SB-216763 at 10 μmol/L for 24 hours and cycloheximide at 0.5 μg/mL (Sigma) for 12 hours. For activation of Wnt/β-catenin signaling, pcDNA3-S33A-β-catenin, kindly provided by Dr Peter Vogt at the Scripps Research Institute, was transiently transfected as described.[11] The primary gastric epithelial cells were cultured as described.[29] Immunostaining of the primary spheroid cultures were examined with confocal microscopy. For the colony formation assay, AGS cells were transfected with the same amount of *Sox17* expression vector or empty vector (pcDNA3.1-Hygro; Invitrogen, Carlsbad, CA), cultured in hygromycin containing medium at 50 μg/mL for 10 days, and then the mean colony numbers from 6 independent experiments were calculated.

Immunoblotting Analysis

Tissue samples were homogenized and sonicated in lysis buffer. After centrifugation at 2000 × *g*, 10 μg of the protein sample was separated in a 10% sodium dodecyl sulfate-polyacrylamide gel. Antibodies for Sox17 (R&D, Minneapolis, MN), active β-catenin (Millipore, Temecula, CA), total β-catenin (Sigma), total GSK3β (BD Biosciences, San Jose, CA), and phosphorylated GSK3 on Ser9 and 21 (Cell Signaling, Danvers, MA) were used as the primary antibody. The electrochemiluminescence detection system (GE Healthcare, Buckinghamshire, United Kingdom) was used to detect the specific signals. Band intensities were measured by Image J (National Institutes of Health, Bethesda, MD).



Tumor type	Sox17 positive / examined (%)
Gastric cancer	2 / 72 (2.8)
Gastric tubular adenoma	5 / 7 (71.4)
Colon cancer	2 / 69 (2.9)
Colon polyps	4 / 5 (80.0)

Figure 4. Immunohistochemistry for Sox17 in malignant cancers and benign tumors. (*A* and *B*) Representative H&E staining and immunostaining for Sox17 and β-catenin in 2 sets of serial sections from gastric cancer (*A*) and colon cancer (*B*) tissue microarrays. *Insets* indicate enlarged images. (*C* and *D*) Representative H&E staining and Sox17 immunostaining in serial sections of human gastric tubular adenoma (*C*) and colon polyp (*D*). N, normal mucosa; T, tumor region. Enlarged images of normal and tumor regions (*squares*) of Sox17 immunostaining are shown (*right*). *Bars* indicate 100 μm. (*E*) Ratio of Sox17-positive specimens in gastric and colon cancers and benign tumors.

Statistical Analysis

The data were analyzed by the unpaired *t* test with the use of Microsoft Excel (Microsoft, Redman, WA), and presented as the mean ± standard deviation (SD). A value of $P < .05$ was considered to be statistically significant.

Results

Induction of Sox17 in Gan Mouse Gastric Tumors

We first examined the gene expression profile in gastric tumors developed in *K19-Wnt1/C2mE* (*Gan*) mice by filter array analysis. *Gan* mice develop gastric tumors caused by simultaneous activation of the Wnt/β-catenin signaling and cyclooxygenase-2/prostaglandin E_2 (PGE_2) pathway.[7] As expected, the Wnt target genes, such as *Vegfa*, *Cdx1*, and *Cd44*, were up-regulated in the *Gan* mouse tumors (Figure 1*A*). Interestingly, the expression of *Sox17* was also elevated in *Gan* mouse tumors. We thus examined expression of all *Sox* family members by microarray analysis (Figure 1*B*). The expression levels of *Sox2*, *Sox4*, *Sox7*, *Sox9*, and *Sox17* increased significantly in the *Gan* mouse tumors, whereas those of *Sox6*, *Sox10*, *Sox12*, and *Sox14* significantly decreased (Figure 1*B*). Notably, *Sox2*, *Sox7*, and *Sox9* were up-regulated also in the inflamed gastric hyperplasia of *K19-C2mE* mice that express *Ptgs2* and *Ptges* in gastric mucosa,[29] suggesting that these *Sox* genes were induced by PGE_2 or PGE_2-dependent inflammation. We confirmed significant induction of *Sox17* in *Gan* mouse tumors by real-time reverse transcription (RT)-PCR, whereas *Sox17* was not induced in *K19-C2mE* mice (Figure 1C).

Induction of Sox17 *in Wnt-Activated Gastric Epithelial Cells*

We next determined the *Sox17*-expressing cell types by immunostaining. Notably, strong nuclear staining of Sox17 was found in *Gan* mouse gastric tumor cells (Figure 2*A*). In the normal gastric mucosa, Sox17 was detected only in undifferentiated epithelial cells in the gland neck. Notably, nuclear Sox17 staining was also detected in the dysplastic epithelial cells of *K19-Wnt1*

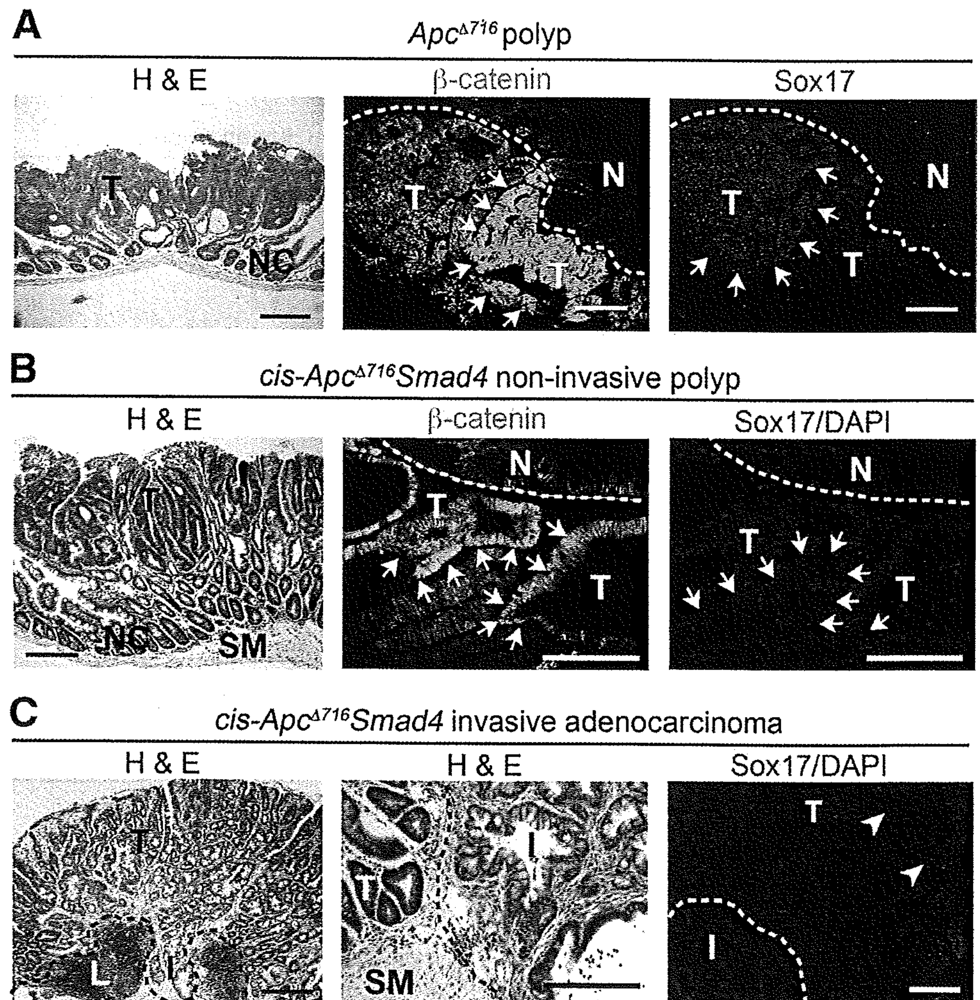


Figure 5. Down-regulation of *Sox17* in mouse intestinal adenocarcinomas. (*A–C*) Immunostaining for β-catenin and Sox17 in $Apc^{\Delta716}$ mouse polyps (*A*), noninvasive polyps of *cis-*$Apc^{\Delta716}$ *Smad4* mice (*B*), and invasive adenocarcinomas of *cis-*$Apc^{\Delta716}$ *Smad4* mice (*C*). H&E staining (*A–C left* and *C center*), immunostaining for β-catenin (*green*) (*A* and *B center*) and for Sox17 (*red*) (*A–C right*) and counterstaining with DAPI (*blue*) (*B* and *C right*). β-Catenin and Sox17 in (*A* and *B center* and *right*) are double immunostaining using the same section. T, tumor area; N, normal mucosa; L, lymphocyte-accumulated area; NC, normal crypt; SM, smooth muscle layer; and I, invasion front. (*A* and *B*) *Arrows* indicate reciprocal strong stained area of β-catenin (*center*) and Sox17 (*right*). (*C right*) *Arrowheads* indicate remained Sox17-expressing tumor cells. *Bars* indicate 200 μm (*A–C left*) and 100 μm (*A–C center and right*).

gastric preneoplastic lesions where Wnt/β-catenin signaling was activated (Figure 2*B*). We next examined *Sox17* expression in the primary cultured gastric tumor cells. Tumor epithelial cells from *Gan* mouse tumors formed dome-shaped spheroid structures on the culture dish, consisting of small epithelial cells (Figure 2*C*). The spheroid cells showed strong accumulation of β-catenin and negative staining of Ki-67, suggesting that they were slow-cycling undifferentiated cells. In contrast, monolayer cells surrounding the spheroids showed weak β-catenin staining and were Ki-67 positive. Notably, a strong Sox17 expression was found only in the spheroids. These results, taken together, suggest that *Sox17* is induced in the Wnt-activated undifferentiated epithelial cells.

The transfection of active β-catenin expression vector caused a significant increase in the *Sox17* mRNA levels in AGS gastric cancer cells and 293 cells (Figure 2*D*). Moreover, the treatment of 293 cells with GSK3β inhibitor and cycloheximide induced *Sox17* expression. These results indicate that unphosphorylated (active) β-catenin directly induces *Sox17* without protein biosynthesis.

Down-regulation of Sox17 in Human Gastric Cancer Cells

We next examined the expression of all *Sox* family members in human gastric cancer with the use of public microarray databases.[30] *Sox2, Sox4,* and *Sox9* were significantly up-regulated in human gastric cancer (Figure 3*A*), which was consistent with the results of *Gan* mice (Figure 1*B*). In contrast, *Sox17* expression was significantly suppressed in human gastric cancer. It has been reported that *Sox17* expression is suppressed in colon cancer by promoter methylation.[22] We found that treatment with a demethylating agent DAC induced *Sox17* expression in AGS and AZ521 gastric cancer cells as well as SW480 cells (Figure 3*B*). Accordingly, it is possible that promoter methylation is one of the major causes for *Sox17* down-regulation in gastric cancer cells.

Suppression of Wnt Signaling and Tumorigenesis by Sox17 Expression

Sox17 represses Wnt/β-catenin signaling in colon cancer cells.[22,23] We found that transfection of *Sox17* expression vector significantly suppressed the β-catenin/T-cell factor (TCF) transcriptional activity in AZ521 cells (Figure 3*C*). We confirmed by Western blotting that *Sox17* transfection resulted in a significant decrease of the active β-catenin level in different gastric cancer cells Kato-III (Figure 3*D*). Because phosphorylated GSK3β stayed at the similar level after *Sox17* transfection, it is possible that Sox17 represses Wnt signaling through a GSK3β-independent mechanism. Importantly, the transfection of *Sox17* in AGS cells significantly suppressed the colony formation efficiency (Figure 3*E*), thus suggesting that down-regulation of *Sox17* enhances tumorigenicity of gastric cancer cells.

Reciprocal Expression Pattern of Sox17 in Malignant Cancers and Benign Tumors

We next examined the Sox17 expression in gastric cancer tissue specimens by immunohistochemistry with the use of tissue microarrays. Consistent with the microarray results (Figure 3*A*), Sox17 expression was rarely detected in gastric cancer tissues (2.8%), whereas clear

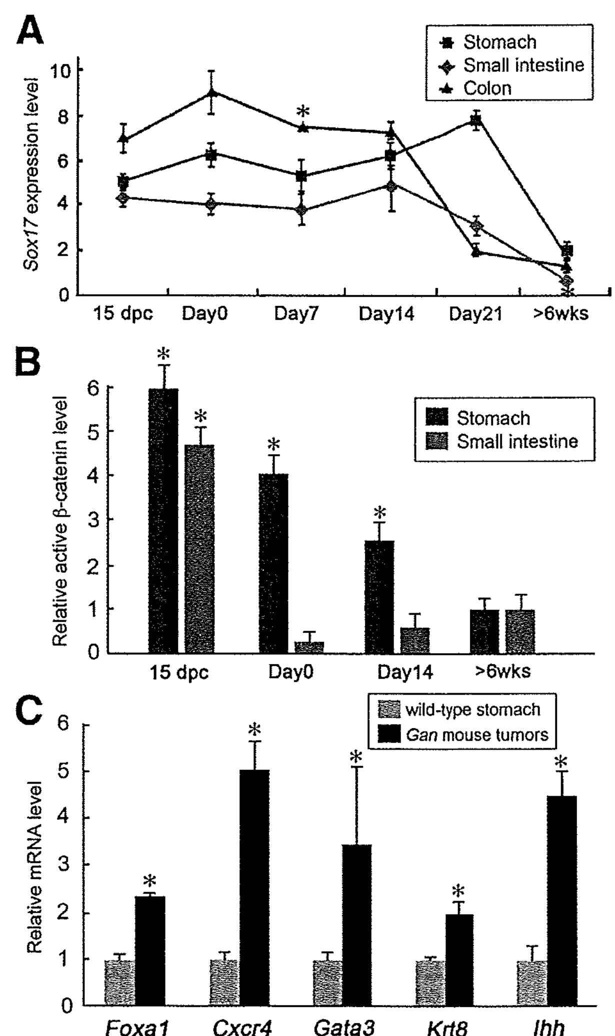


Figure 6. *Sox17* induction and Wnt activation during gastrointestinal development. (*A*) Expression of *Sox17* in the stomach, small intestine, and colon at the indicated ages examined by real-time RT-PCR. *Asterisks* indicate N/A for standard error bars because of 2 samples. (*B*) The relative level of active β-catenin in the stomach and small intestine to the adult mouse level at the indicated ages measured from band intensities of Western blotting. (*C*) The relative mRNA levels of *Foxa1, Cxcr4, Gata3, Krt8,* and *Ihh* examined by real-time RT-PCR in *Gan* tumor tissues (*closed bars*) to those in wild-type mouse stomach (*gray bars*). (*B* and *C*) *$P < .05$ to the adult mouse level and wild-type level, respectively.

β-catenin accumulation was detected in the serial sections (Figure 4*A* and *E*). *Gan* mouse tumors are in the early stage of tumorigenesis because tumor cells do not form soft agar colonies and are not transplantable to immunodeficient mice (data not shown). We thus examined whether *Sox17* is induced in the benign human gastric tumors. Importantly, strong nuclear Sox17 staining was found in 71.4% of gastric tubular adenomas (Figure 4*C* and *E*). These results suggest that *Sox17* expression is induced at the early stage of gastric tumorigenesis and then down-regulated during malignant progression. A similar pattern of *Sox17* expression was found in colon tumors. Namely, Sox17 was rarely detected in colon cancer (2.9%) (Figure 4*B* and *E*), whereas strong nuclear Sox17 staining was found in 80% of the colon polyps (Figure 4*D* and *E*).

Down-regulation of Sox17 During Malignant Progression of Mouse Intestinal Tumors

We next examined Sox17 expression in mouse intestinal adenomas and adenocarcinomas developed in $Apc^{\Delta716}$ and *cis*-$Apc^{\Delta716}$ *Smad4* mice, respectively. $Apc^{\Delta716}$ mice develop intestinal polyps caused by activation of Wnt signaling,[6,24] whereas *cis*-$Apc^{\Delta716}$ *Smad4* mice develop invasive adenocarcinomas by suppression of the transforming growth factor-β pathway in addition to Wnt activation.[28] We found β-catenin accumulation in $Apc^{\Delta716}$ mouse adenoma cells, but staining intensity was not uniform; ie, the distinct strongly stained area and moderately stained area were mixed (Figure 5*A*). Notably, *Sox17* was induced in the $Apc^{\Delta716}$ polyps, including nascent adenomas (Supplementary Figure 1). Interestingly, Sox17 staining intensity in polyps was reciprocal to the β-catenin staining pattern, suggesting that Sox17 negatively regulates the β-catenin accumulation level in polyps (Figure 5*A*). The similar reciprocal staining intensities of β-catenin and Sox17 was found in the noninvasive polyps of *cis*-$Apc^{\Delta716}$ *Smad4* mice (Figure 5*B*). Importantly, in the invasive adenocarcinomas of *cis*-$Apc^{\Delta716}$ *Smad4* mice, *Sox17* expression was dramatically suppressed, and only a limited number of tumor cells expressed *Sox17* (Figure 5*C*). Such suppression of the *Sox17* expression was found in 100% (11/11) of invasive adenocarcinomas. These genetic results clearly indicate that *Sox17* is induced at the initiation stage of intestinal tumorigenesis and is dramatically down-regulated when tumors progress to adenocarcinoma.

Sox17 Induction and Wnt Activation During Development of Gastrointestinal Tract

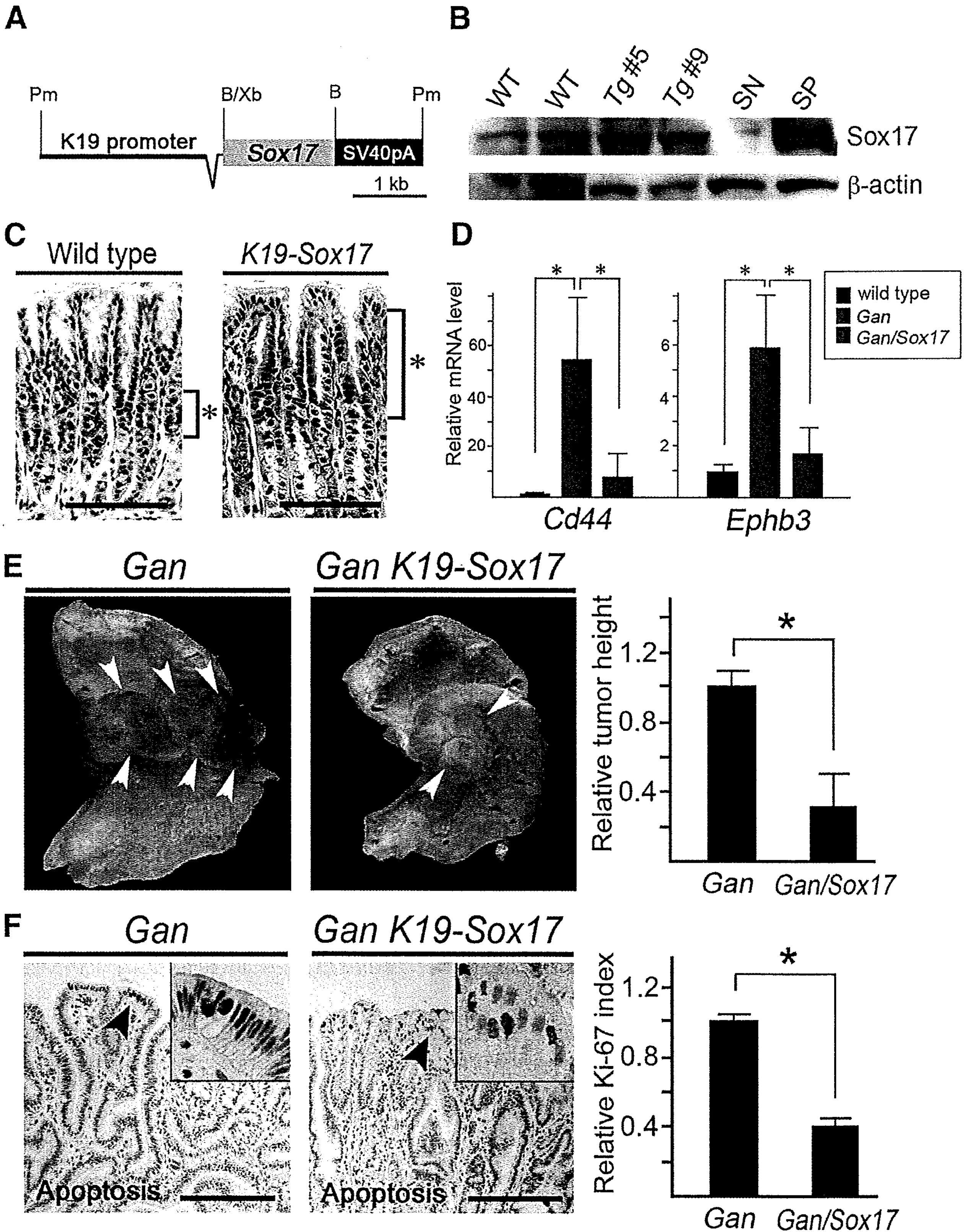
Sox17 plays a key role in the definitive endoderm development in cooperation with β-catenin,[20,26,27] which gives rise to gut formation. Interestingly, *Sox17* expression was also found in the gastrointestinal tract of embryos and pups until 3 weeks of age and then down-regulated in adult mice (Figure 6*A*). Notably, the active β-catenin levels in the stomach and intestine were significantly higher during the developmental stages compared with those in adult mice (Figure 6*B*). These results suggest a cooperative role of Wnt and Sox17 during the development of the gastrointestinal tract. We next examined the expression of Sox17-target endoderm markers in *Gan* mouse gastric tumors by real-time RT-PCR. Notably, expression of *Foxa1*, *Cxcr4*, *Gata3*, *Krt8*, and *Ihh* were increased significantly in *Gan* mouse tumor tissues compared with that in the wild-type mouse stomach (Figure 6*C*). The expression of these genes are induced in *Sox17*-transfected human embryonic stem cells or suppressed in *Sox17*-null mouse embryos.[20,21] Accordingly, it is possible that cooperation of Sox17 and Wnt/β-catenin signaling plays a role also in tumor development through induction of target molecules that function in gastrointestinal development.

Suppression of Gastric Tumor Development by Transgenic Expression of Sox17

To investigate the role of Sox17 in gastric tumorigenesis, we constructed *Sox17* transgenic mice (*K19-Sox17* mice) with the use of a K19 promoter (Figure 7*A*). We confirmed an increased Sox17 level in *K19-Sox17* mouse gastric mucosa by Western blotting and immunohistochemistry (Figure 7*B* and *C*). Although Sox17-expressing gastric epithelial cells increased, histology of the *K19-Sox17* mouse stomach was normal (Figure 7*C*). We thus crossed *K19-Sox17* mice with *Gan* mice to construct *Gan K19-Sox17* compound transgenic mice. We confirmed the transgenic expression of *Ptgs2* and *Ptges* in both *Gan* and *Gan K19-Sox17* mouse stomach at similar levels (Supplementary Figure 2), which ruled out the possibility of the promoter interference of multiple transgenes. Importantly, the expression level of the Wnt target genes, *Cd44* and *Ephb3*, in the *Gan K19-Sox17* mice decreased significantly compared with that in the *Gan* mice (Figure 7*D*), indicating suppression of Wnt/β-catenin signaling by *Sox17* expression. Moreover, the mean height of the gastric tumors in *Gan K19-Sox17* mice decreased significantly to approximately 30% of that in the age-matched *Gan* mice (Figure 7*E*). Apoptotic cells were found on the tumor surface of both *Gan* and *Gan K19-Sox17* tumors at similar levels (Figure 7*F*). However, apoptosis was not detected in the intratumoral tissue specimens of both genotypes. On the other hand, the Ki-67–labeling index decreased significantly in the *Gan K19-Sox17* tumors (Figure 7*F*). These results suggest that *Sox17* suppressed tumor development through the inhibition of cell proliferation rather than because of the induction of apoptosis.

Suppression of Dysplastic Tumor Phenotype by Transgenic Expression of Sox17

Histologically, *Gan* mouse gastric tumors consisted of irregularly branching glands lined with dysplastic tumor cells, and increased angiogenesis was evident


A
Pm
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K19 promoter
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SV40pA
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Tg #5
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SP
Sox17
β-actin
C
Wild type
K19-Sox17
D
Relative mRNA level
wild type
Gan
Gan/Sox17
Cd44
Ephb3
E
Gan
Gan K19-Sox17
Relative tumor height
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Apoptosis
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Relative Ki-67 index
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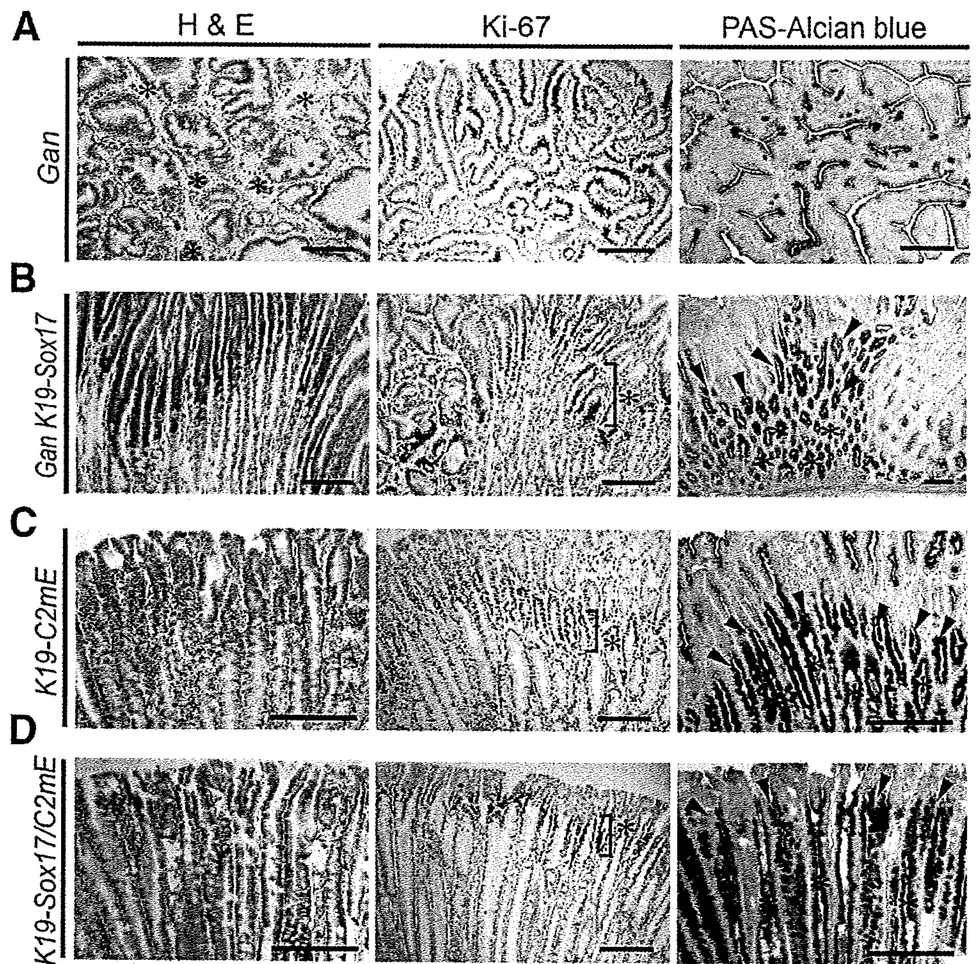

Figure 8. Suppression of dysplastic tumor phenotype by transgenic expression of *Sox17*. Histologic examination of gastric tumors in *Gan* (*A*), *Gan K19-Sox17* (*B*), *K19-C2mE* (*C*), and *K19-Sox17/C2mE* (*D*) mice. H&E (*left*), Ki-67 immunostaining (*center*), and PAS-Alcian blue staining (*right*). (*A left*) *Asterisks* indicate capillary vessels. (*B–D center*) *Asterisks* indicate Ki-67–positive proliferating cells that aligned in gland neck. (*B–D right*) *Asterisks* and *arrowheads* indicate PAS-positive and Alcian blue-positive mucous cells, respectively. Bars indicate 100 μm.

(Figure 8*A*), which was consistent with previous reports.[7,31] In gastric tumors of *Gan K19-Sox17* mice, Ki-67–positive proliferating cells aligned at the gland neck of hyperplastic tumors, and the number of PAS-Alcian blue–positive mucous cells increased (Figure 8*B*), which were similar to the characteristics of metaplastic hyperplasia of *K19-C2mE* mice[29] (Figure 8*C*). *Gan* mice develop dysplastic tumors caused by simultaneous activation of Wnt and PGE_2 pathways. Accordingly, it is possible that *Sox17* expression suppressed Wnt activity to the level insufficient for dysplastic tumor development in *Gan K19-Sox17* mice, thus resulting in development of a PGE_2-dependent gastric phenotype. Moreover, *K19-Sox17/C2mE* mice developed metaplastic hyperplasia but not dysplastic tumors (Figure 8*D*), thus indicating that the induction of *Sox17* and PGE_2 pathways without the activation of Wnt/β-catenin signaling is not sufficient for gastric tumor development.

Discussion

Accumulating evidence indicates that activation of Wnt/β-catenin signaling is one of the direct causes of gastric and intestinal tumor development.[4–7] It has been

←

Figure 7. Suppression of gastric tumor development by transgenic expression of *Sox17*. (*A*) Schematic construction of transgenic vector. Pm, *Pme*I; B, *Bam*HI; Xb, *Xba*I. (*B*) Western blotting results of Sox17 in gastric mucosa of wild-type (WT), *K19-Sox17* (Tg) line no. 5 and no. 9, normal small intestine (SN) as a negative control, and $Apc^{\Delta 716}$ mouse polyps (SP) as a positive control. β-Actin was used for internal control. (*C*) Immunostaining for Sox17 in gastric mucosa of wild-type and *K19-Sox17* mice. *Asterisks* indicate localization of Sox17-expressing epithelial cells. *Bars* indicate 100 μm. (*D*) The relative mRNA levels of *Cd44* and *Ephb3* in *Gan* (*red*) and *Gan K19-Sox17* mice (*blue*) to the wild-type level (*black*). *$P < .05$. (*E*) Representative photographs of stomach of *Gan* mouse and *Gan K19-Sox17* mouse, and the relative tumor height in *Gan K19-Sox17* (*Gan/Sox17*) to that in *Gan* mice (mean ± SD). (*F*) Representative photographs for apoptotic cells in *Gan* and *Gan K19-Sox17* mouse tumors, and relative Ki-67 labeling index in *Gan K19-Sox17* tumors (*Gan/Sox17*) to that in *Gan* mouse tumors. *Arrowheads* indicate apoptotic cells on the tumor surface. (*E* and *F*) *$P < .05$.

shown that the expression of several *Sox* family genes, including *Sox2*, *Sox9*, and *Sox17* are induced by Wnt/β-catenin signaling.[32–34] In the present study, we found that activation of Wnt signaling causes *Sox17* expression in gastric cancer cells and that *Sox17* is induced in benign gastric tumors. Because Sox17 antagonizes Wnt/β-catenin signaling, it is thus conceivable that the Wnt activation level in the early stage of tumorigenesis is partially suppressed by Sox17 but is sufficient for benign tumor development. Importantly, transgenic expression of *Sox17* in the *Gan* mouse stomach suppressed dysplastic tumor formation. Suppression of the dysplastic phenotype of *Gan K19-Sox17* mouse tumors is possibly caused by repression of Wnt/β-catenin signaling. Namely, overexpression of *Sox17* suppresses Wnt/β-catenin signaling activity to the level insufficient for tumor formation. Therefore, it is possible that *Sox17* induction level at the early stage of tumorigenesis is strictly regulated to maintain the Wnt/β-catenin activity for dysplastic tumor development.

However, *Sox17* expression is dramatically suppressed in most human gastric cancer cells possibly through promoter methylation, which may result in an increase of Wnt/β-catenin signaling activity in comparison to that in benign tumors. We also confirmed *Sox17* down-regulation in human colon cancer tissues. Recently, it has been suggested that promotion of Wnt/β-catenin signaling is required for malignant progression of colon cancer.[8] Consistently, we found that *Sox17* transfection reduced the colony formation efficiency in gastric cancer cells. Accordingly, it is possible that down-regulation of *Sox17* contributes to malignant behavior through promotion of Wnt/β-catenin signaling both in the stomach and colon. Therefore, induction of tumor suppressor *Sox17* at the early stage of tumorigenesis can be considered as a self-protection system against malignant tumor development.

In the present study, we found drastic changes of *Sox17* expression during the course of intestinal tumorigenesis in $Apc^{\Delta716}$ and *cis*-$Apc^{\Delta716}$ *Smad4* mice. The previous genetic studies indicate that mutations in both *Apc* and *Smad4* cause malignant tumor development in the intestine.[28,35] However, because *Sox17* down-regulation was tightly associated with invasive tumor phenotype in *cis*-$Apc^{\Delta716}$ *Smad4* mice, it is conceivable that *Sox17* suppression in addition to *Apc* and *Smad4* mutations is required for malignant progression to adenocarcinoma. Because *Sox17* down-regulation was found only in the *cis*-$Apc^{\Delta716}$ *Smad4* mouse tumors but not in $Apc^{\Delta716}$ polyps, it is possible that disruption of the transforming growth factor-β pathway contributes to suppression of *Sox17* expression.

Sox17 is required for definitive endoderm development, which gives rise to gut formation.[20,21,26] Moreover, it has been shown that β-catenin is also essential for definitive endoderm formation.[36] We have shown here that *Sox17* continuously expresses in the stomach and intestine during organogenesis. Importantly, Wnt signaling is also activated in the gastrointestinal tract of embryonic and neonatal stages. These results suggest that cooperation of Sox17 and Wnt/β-catenin pathway plays a role also in the morphogenesis of the gastrointestinal tract. Dysregulation of morphogen signals, such as Notch, Hh, or Wnt, in adult tissues often results in pathologic conditions, such as tumor development.[37] We herein show that the Sox17 target genes, which encode molecules that play a role in the definitive endoderm development, are induced in *Gan* mouse tumors. Although *Sox17* induction is not sufficient for tumorigenesis, as found in *K19-Sox17* or *K19-Sox17/C2mE* mice, it is possible that the activation of both Wnt- and Sox17-target molecules cooperatively causes gastric tumor development.

In conclusion, *Sox17* is induced at the early stage of tumorigenesis caused by Wnt/β-catenin activation, and Sox17 together with Wnt/β-catenin signaling may play a role in tumor development through induction of target genes. At the same time, Sox17 may play a preventive role against malignant progression through repression of Wnt activity. Therefore, it is conceivable that induction and down-regulation of *Sox17* expression is important for tumor initiation and malignant progression, respectively, in the course of gastrointestinal tumorigenesis.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.06.041.

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BASIC-ALIMENTARY TRACT

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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The Interleukin-6 Family Cytokine Interleukin-11 Regulates Homeostatic Epithelial Cell Turnover and Promotes Gastric Tumor Development

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Background & Aims: Gastric cancer is the second most common cause of cancer-related mortality worldwide, mainly as a result of late-stage detection. Interleukin (IL)-11 is a multifunctional cytokine reported to be up-regulated in human gastric cancer. **Methods:** We investigated the importance of IL-11 in gastric cancer progression by examining its role in a variety of mouse gastric tumor models, as well as in nonneoplastic and tumor tissues taken from gastric cancer patients. We then determined the transcriptional and translational outcomes of IL-11 overexpression in normal gastric mucosa and identified a novel gene signature important early in the progression toward gastric tumorigenesis. **Results:** IL-11 was up-regulated significantly in 4 diverse mouse models of gastric pathology as well as in human biopsy specimens adjacent to and within gastric cancer. Removal of IL-11 co-receptor α significantly reduced HKβ-/- mouse fundic hyperplasia and ablated gp130$^{757F/F}$ mouse tumorigenesis. Exogenous IL-11 but not IL-6 activated oncogenic signal transducer and activator of transcription-3, and altered expression of novel proliferative and cytoprotective genes RegIII-β, RegIII-γ, gremlin-1, clusterin, and growth arrest specific-1 in wild-type gastric mucosa, a gene signature common in gp130$^{757F/F}$ and HKβ-/- tumors as well as nonneoplastic mucosa of gastric cancer patients. One week of chronic IL-11 administration in wild-type mice sustained the gene signature, causing pretumorigenic changes in both antrum and fundus. **Conclusions:** Increased gastric IL-11 alters expression of proliferative and cytoprotective genes and promotes pretumorigenic cellular changes.

Correa's[1] model of gastric cancer development describes a sequence of events beginning with *Helicobacter pylori*–associated gastritis and ending with cancer. A pivotal step involves disruption of cellular proliferation and commitment programs of the gastric epithelium.[1] Factors that influence epithelial cell homeostasis, especially those up-regulated early in disease, could prove crucial as potential therapeutic targets.

Interleukin (IL)-11 belongs to the IL-6 cytokine family, which signals through gp130.[2] We recently showed that IL-11 is increased in human gastric cancer and is mitogenic in vitro.[3] Expression of IL-11 and its co-receptor, IL-11Rα, required for signal transduction,[2] correlates with invasion and proliferation in gastric and colorectal cancer,[4,5] suggesting IL-11 is important in cancer progression. Furthermore, IL-11 can influence epithelial homeostasis by prolonging stem cell survival, decreasing apoptosis, and increasing mitosis,[6–8] as shown in various models of gut injury. In the stomach, IL-11 accelerates ulcer healing by increasing proliferation and angiogenesis,[9,10] both important factors early in gastric cancer.

Signal transducer and activator of transcription-3 (STAT3) and extracellular signal-regulated kinase (ERK 1/2) are the dominant transcription factors induced by gp130 stimulation; they activate target genes involved in survival, differentiation, apoptosis, and proliferation.[2] Active/phosphorylated STAT3 (p-STAT3), is greatly up-regulated in *H pylori* infection, gastric cancer,[3] and correlates with decreased survival.[11] Alterations in phosphorylated ERK1/2 (p-ERK1/2) expression also occur in *H pylori* infection and progression to gastric cancer.[3,12]

We previously developed a mouse model of gastric tumorigenesis similar to intestinal-type human gastric cancer.[13,14] The gp130$^{757F/F}$ mouse develops gastric antral tumors owing to hyper–p-STAT3[15] resulting from a knock-in mutation on gp130 and subsequent disruption of Ras/

BASIC–ALIMENTARY TRACT

Abbreviations used in this paper: AP-1, activator protein-1; BMP, bone morphogenic protein; ERK, extracellular signal-regulated kinase; GAS-1, growth arrest specific-1; IL, interleukin; IL-11Rα, interleukin-11 co-receptor α; p-ERK, phosphorylated extracellular signal-regulated kinase; p-STAT3, phosphorylated signal transducer and activator of transcription-3; PCR, polymerase chain reaction; STAT3, signal transducer and activator of transcription-3; WT, wild-type; WTcIL-11, wild-type treated chronically with IL-11.

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ERK1/2/activator protein-1 (AP-1) output.[13] These tumors fully encompass the antrum by 12 weeks.[14] HKβ-/- mice have a knock-out mutation for the parietal cell enzyme H+/K+ adenosine triphosphatase (ATPase)-β subunit and develop gastrin-dependent, gross fundic hyperplasia by 12 weeks.[16] Transgenic K19-C2mE mice, engineered to overexpress cyclooxygenase-2 and microsomal prostaglandin E synthase-1, develop hyperplastic fundic tumors by 48 weeks,[17] and when crossed with K19-Wnt-1 transgenic mice (K19-Wnt1/C2mE) develop large, dysplastic fundic tumors by 30 weeks.[18] Because these are diverse models of pathology in which epithelial dynamics are affected differentially in the antrum and fundus, they represent excellent models to study early stages of gastric cancer and the effects of IL-11 on this.

Here, we investigate the role of IL-11 in gastric cancer initiation and development in vivo. We show increased IL-11 in various mouse models of gastric tumorigenesis as well as in human gastric cancer, not only within the cancer itself, but in adjacent tissue where there is a high risk for cancer development. In the mouse models we identify a gene signature induced by IL-11 in the stomach, which also is evident in human gastric tissue adjacent to a cancer. Further, we show that prolonged exposure to IL-11 in normal mucosa can lead to disruptions in epithelial cell cycle in a similar pattern to that seen in pretumorigenic mouse stomach.

Materials and Methods

Mice

gp130$^{757F/F}$[13], IL-11Rα-/-[19], HKβ-/-[16], K19-C2mE[17], and K19-Wnt/C2mE[18] were developed as previously described. gp130$^{757F/F}$ and IL-11Rα-/- mice were on a mixed 129X1(Sv-J)/C57BL/6 background, HKβ-/- mice on a BALB/cCrSlc background, and K19-C2mE on a C57BL/6 background. K19-C2mE mice were 20–30 weeks of age and all others were 12 weeks of age. Controls were age-matched littermates. Mice lacking mutations were designated wild type (WT). gp130$^{757F/F}$[13], HKβ-/-[16], and IL-11Rα-/- (supplementary Table 1; see supplementary material online at www.gastrojournal.org) mice were genotyped by multiplex polymerase chain reaction (PCR) and housed under conventional conditions, free of *H pylori*. Approval from appropriate animal ethics committees was granted.

Human Gastric Biopsy Specimens

Selection and processing of gastric biopsy specimens from disease-free, CagA-positive, *H pylori*–infected individuals, and nonneoplastic[3] and tumor tissues from gastric cancer patients[20] was undertaken as previously described. Briefly, the presence of *H pylori* was detected by biopsy urease tests and independently by histologic analysis. CagA status was measured by immunoblotting.[3] Specimens of tumor and adjacent nonneoplastic mucosa were collected from patients with gastric cancer requiring curative or palliative resection, each specimen was attributed a diagnosis, and demographic data were obtained from clinical records with patient permission. Supplementary Table 2 (see supplementary material online at www.gastrojournal.org) summarizes the sample characteristics. Written informed consent was obtained before specimen collection and studies were approved by the appropriate ethics committees.

Macroscopic and Histologic Assessment

Paraffin sections (4 μm) were stained with H&E or Alcian blue/periodic acid–Schiff. Area, linear, and point counting measurements were performed using ImageJ software for Windows v1.38 (available from: http://rsb.info.nih.gov/ij/index.html) (n > 5 per group).

Immunoblotting

Proteins were prepared with TRIzol (Life Technologies, Carlsbad, CA) and 20 μg were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Membranes were incubated with STAT3, p-STAT3, ERK1/2, p-ERK1/2, AKT, or p-AKT (Cell Signalling, Danvers, MA), peroxide-conjugated secondary antibody, and visualized by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK). Quantification was performed by using ImageMASTER software (GE Healthcare), and phosphorylated: total protein ratios were determined from duplicate membranes (n ≥ 7, representative bands shown).

Recombinant Cytokine Treatment

WT mice were injected intraperitoneally (intraperitoneally [IP]) with recombinant-human IL-11, mouse IL-6 (5 μg) (Millipore, Billerica, MA), or saline (sham), and euthanized either 3 hours after injection (acute) or 6 hours after 7 days of injections at 6 hourly time points (chronic). The latter group is referred to as WT mice treated chronically with IL-11 (WTcIL-11).

RNA Preparation, Microarray Hybridization, and Computational Methods

RNA was extracted using TRIzol. Amino-allyl RNA was synthesized from RNA (5 ug) using Superscript Indirect amplification (Invitrogen, Carlsbad, CA). Amino-allyl RNA was coupled to fluorescent dye (Alexa Fluor 555 or 647; Invitrogen), fragmented (Ambion Applied Biosystems, Austin, TX), and competitively hybridized with 30% formamide, 2% standard saline citrate, and 1% sodium dodecyl sulfate to Compugen 22K mouse oligonucleotide slides (Adelaide Microarray Centre, Adelaide, Australia). Two of 5 hybridizations were performed with reverse dye configuration.

An Axon 4000B microarray scanner (Molecular Devices, Sunnyvale, CA) with photomultiplicator (PMT) channel settings balancing total Cy3 and Cy5 fluorescence was used. Foreground and background median pixel intensities were extracted using Spot v3 plug-in for R (CSIRO, Clayton South, Australia). After background subtraction, foreground intensities were log_2 transformed and a single ratio (Cy5/Cy3) was obtained. Probe ratio values were normalized

with Limma plug-in[21] for R, using the print-tip-loess normalization option. The 5 replicates were normalized to each other and a linear model was fitted to determine the final value. Statistics were generated using an empiric Bayes method to rank messenger RNA (mRNA) from most to least likely differentially expressed between WT IL-11 and WT sham antrum. The 100 most differentially expressed genes were chosen for evaluation (supplementary Table 3; see supplementary material online at www.gastrojournal.org).

Quantitative PCR

RNA (3 μg) was reverse-transcribed into complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) primed with oligo(dT). Quantitative PCR primers were designed using Primer Express (Applied Biosystems, Austin, TX) (supplementary Table 1; see supplementary material online at www.gastrojournal.org). SYBR green chemistry was used with L32 as the normalizer. PCR conditions were 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 15 seconds (AB7500; Applied Biosystems). Results were analyzed using sequence detector software, relative fold differences were determined using the ΔΔCt method.

Immunohistochemistry

Immunohistochemistry was with antibodies for gastrin (a gift from Dr Arthur Shulkes, University of Melbourne, Melbourne, Australia), Ki-67 (Pharmingen, BD Biosciences, San Diego, CA), and cleaved-caspase 3 (Cell Signalling). Antigen retrieval was in 10 mmol/L citric acid for 30 minutes, 100°C, and staining was completed with biotinylated secondary antibodies, avidin, and biotinylated horseradish-peroxidase complex (Vector Laboratories, Burlingame, MA), 3,3′-diaminobenzidine and hematoxylin counterstained.

Results

IL-11 Is Increased in Four Mouse Models of Gastric Pathology as Well as Human Gastric Cancer

IL-11 was quantified in 4 mouse models of gastric pathology: gp130$^{757F/F}$ antral tumors, HK-β-/- hyperplastic fundus, K19-C2mE hyperplastic fundic tumors, and K19-Wnt1/C2mE dysplastic fundic tumors. In all cases, IL-11 expression was increased consistently in comparison with WT littermates (Figure 1*A*–*C*). IL-11 expression then was determined in gastric cancer tissue, either from within cancer or in nonneoplastic adjacent mucosa, and compared with disease-free tissues. IL-11 was increased significantly in all samples from gastric cancer patients compared with disease-free individuals, including those from adjacent nonneoplastic mucosa (Figure 1*D*). This suggests that IL-11 plays an important role in gastric cancer development and progression.

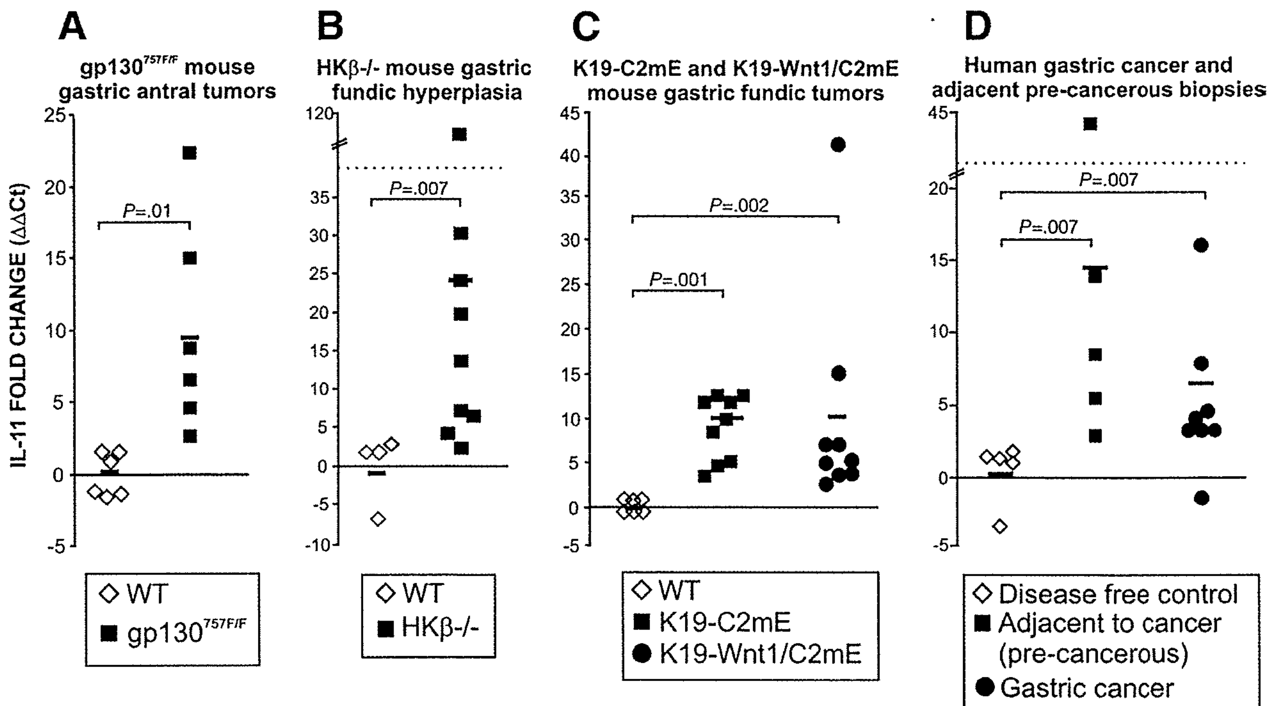

Figure 1. IL-11 fold-change was measured by quantitative PCR in 4 mouse models of gastric pathology: (*A*) gp130$^{757F/F}$, (*B*) HKβ-/-, (*C*) K19-C2mE and K19-Wnt1/C2mE compared with WT littermates, and (*D*) human gastric tissue taken adjacent to and within a cancer compared with disease-free controls.