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

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Induction and downregulation of *Sox17* and its possible roles during the course of gastrointestinal tumorigenesis

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Conflict of Interest

No conflicts of interest exist.

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Abbreviations: APC, adenomatous polyposis coli; COX-2, cyclooxygenase-2; ES cells, embryonic stem cells; GSK3β, glycogen synthase kinase 3β; HGF, hepatocyte growth factor; Ihh, indian hedgehog; HMG, high mobility group; mPGES-1, microsomal prostaglandin E synthase-1; PDGF, platelet-derived growth factor; PGE_2, prostaglandin E_2; RT-PCR, reverse-transcription polymerase chain reaction; SFRP, secreted frizzled-related proteins; TNF-α, tumor necrosis factor-α.

A list of involvement of each author: Yu-Chen Du performed cell culture and mouse experiments. Hiroko Oshima constructed mouse models. Keisuke Oguma examined Sox17 expression levels. Takanori Kitamura prepared mouse intestine samples. Hiraku Itadani performed microarray analyses. Takashi Fujimura prepared human benign tumor samples. Ying-Shi Piao performed technical assistance. Tanihiro Yoshimoto discussed for manuscript preparation. Toshinari Minamoto prepared tumor samples. Hidehioto Kotani supervised microarray analyses. Makoto M. Taketo discussed the design of the study. Masanobu Oshima designed the study and prepared the manuscript.

Abstract

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 downregulated 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, while strong nuclear staining of Sox17 was found in more than 70% of benign gastric and intestinal tumors. Treatment with a demethylating agent induced Sox17 expression in gastric cancer cells, thus indicating the downregulation 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 downregulated by methylation during malignant progression. It is therefore conceivable that *Sox17* protects benign tumors from malignant progression at early stage of tumorigenesis, and downregulation of Sox17 contributes to malignant progression through promotion of Wnt activity.

Introduction

The binding of the Wnt ligand to a Frizzled receptor destabilizes the β-catenin degradation complex containing APC, AXIN, and 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[4,5] and mice[6,7]. It has also been shown that β-catenin 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]. Growth factors PDGF and HGF, as well as inflammatory cytokine TNF-α, have been shown to promote Wnt/β-catenin signaling activity in tumor cells[9-11]. On the other hand, downregulation of Wnt antagonists like *SFRPs* contribute 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 and/or downregulation 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 HMG box of the sex-determining gene *SRY*[19]. *Sox17*-null mouse embryos exhibit a deficiency of definitive endoderm[20], and overexpression of *Sox17* in ES 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

downregulated in colon cancer cells by promoter methylation[22], and the expression of *Sox17* 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 demonstrated 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 downregulation of *Sox17* causes tumor progression.

Materials and Methods

Mouse Models

Construction of *Apc*$^{\Delta716}$, *cis-Apc*$^{\Delta716}$ *Smad4* (+/-) knockout (*cis-Apc*$^{\Delta716}$ *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*$^{\Delta716}$ mice carry a heterozygous mutation in the *Apc* gene which develop intestinal polyps. *cis-Apc*$^{\Delta716}$ *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, #5 and #9, were established, which showed similar phenotypes. Accordingly, we herein present the results with line #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 using histological sections. C57BL/6 mice (CLEA, 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 using the Vectorstain Elite Kit (Vector Laboratories, Burlingame, CA). The MOM Kit (Vector Laboratories, Burlingame, CA) 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 using the ApopTag Apoptosis Detection Kit (Chemicon). 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 using 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, 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 using public data[30] (NCBI 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 using the pooled RNA samples and Wnt signaling Pathway Oligo GEArray (SABiosciences, Frederick, MD) according to the manufacturer's protocol.

Real-time RT-PCR

Total RNA was extracted from tissues using ISOGEN (Nippon Gene, Tokyo, Japan), reverse transcribed with PrimeScript RT reagent Kit (Takara, Tokyo, Japan), and PCR-amplified by ABI Prism 7900HT (Applied Biosystems) using SYBR Premix Ex Taq II (Takara, Tokyo, Japan). Primers were purchased (Takara) and primer sequences are available upon request.

Cell Culture Experiments

Gastric cancer cell lines, AZ521 (Riken Bioresource Center, Tsukuba, Japan) Kato-III (Cell Resource Center, Tohoku Univ., Japan), and AGS (ATCC) and colon cancer cell line SW-480, and HEK293 cells (ATCC) were cultured in RPMI1640 or DMEM supplemented with 10% FBS. 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 using the Luciferase Assay System

(Promega, Madison, WI). TOPflash results were normalized with FOPflash. For demethylation, cells were treated with 1 μM of 5-aza-2'-deoxycytidine (DAC; Sigma) for 72 h. For the inhibition of GSK3β and protein synthesis, cells were treated with SB-216763 at 10 μM for 24 h and cycloheximide at 0.5 μg/ml (Sigma) for 12 h. 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 using 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 thus calculated.

Immunoblotting Analysis

Tissue samples were homogenized and sonicated in lysis buffer. After centrifugation at 2,000 × g, 10 μg of the protein sample was separated in a 10% SDS-polyacrylamide gel. Antibodies for Sox17 (R&D, Minneapolis, MN), active β-catenin (Millipore, Temecula, CA), total β-catenin (Sigma, St. Louis, MO), 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 ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to detect the specific signals. Band intensities were measured by Image J (NIH).

Statistical Analysis

The data were analyzed by the unpaired *t*-test using Microsoft Excel (Microsoft), and presented as the mean ± standard deviation (s.d.). A value of $P < 0.05$ 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 COX-2/PGE_2 pathway[7]. As expected, the Wnt target genes, such as *Vegfa, Cdx1 and Cd44*, were upregulated 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, while those of *Sox6*, *Sox10*, *Sox12* and *Sox14* significantly decreased (Figure 1*B*). Notably, *Sox2*, *Sox7* and *Sox9* were upregulated 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 RT-PCR, while *Sox17* was not induced in *K19-C2mE* mice (Figure 1*C*).

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* 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 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.

Downregulation of Sox17 in human gastric cancer cells

We next examined the expression of all *Sox* family members in human gastric cancer using public microarray databases[30]. *Sox2*, *Sox4* and *Sox*9 were significantly upregulated 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* downregulation 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/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 downregulation 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 using tissue microarrays. Consistent with the microarray results (Figure 3*A*), Sox17 expression was rarely detected in gastric cancer tissues (2.8%), whereas clear 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 downregulated 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*), while strong nuclear Sox17 staining was found in 80% of the colon polyps (Figure 4*D* and *E*).

Downregulation of Sox17 during malignant progression of mouse intestinal tumors

We next examined Sox17 expression in mouse intestinal adenomas and adenocarcinomas developed in $Apc^{\Delta 716}$ and *cis*-$Apc^{\Delta 716}$ *Smad4* mice, respectively. $Apc^{\Delta 716}$ mice develop intestinal polyps caused by activation of Wnt signaling[6,24], while *cis*-$Apc^{\Delta 716}$ *Smad4* mice develop invasive adenocarcinomas by suppression of the TGF-β pathway in addition to Wnt activation[28]. We found β-catenin accumulation in $Apc^{\Delta 716}$ mouse adenoma cells, but staining intensity was not uniform; *i.e.*, the distinct strongly stained area and moderately stained area were mixed (Figure 5*A*). Notably, *Sox17* was induced in the $Apc^{\Delta 716}$ 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 non-invasive polyps of *cis*-$Apc^{\Delta 716}$ *Smad4* mice (Figure 5*B*). Importantly, in the invasive adenocarcinomas of *cis*-$Apc^{\Delta 716}$ *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 dramatically downregulated 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 downregulated 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 ES 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) using 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 when 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 due to 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 (Figure 8*A*), which was consistent with the previous reports[7,32]. 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 shown that the expression of several *Sox* family genes, including *Sox2*, *Sox9* and *Sox17* are induced by Wnt/β-catenin signaling[31,33,34]. 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 early stage of tumorigenesis is strictly regulated to maintain the Wnt/β-catenin activity for dysplastic tumor development.

On the other hand, *Sox17* expression is dramatically suppressed in most human gastric cancer cells possibly through promoter methylation, which may results in increase of Wnt/β-catenin signaling activity in comparison to that in benign tumors. We also confirmed *Sox17* downregulation 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 downregulation 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 $Apc^{\Delta 716}$ and *cis-*$Apc^{\Delta 716}$ *Smad4* mice. The previous genetic studies indicate that mutations in both *Apc* and *Smad4* cause malignant tumor development in the intestine[28,35]. However, due to the fact that *Sox17* downregulation was tightly associated with invasive tumor phenotype in *cis-*$Apc^{\Delta 716}$ *Smad4* mice, it is conceivable that *Sox17* suppression in addition to *Apc* and *Smad4* mutations is required for malignant progression to adenocarcinoma. Because *Sox17* downregulation was found only in the *cis-*$Apc^{\Delta 716}$ *Smad4* mouse tumors but not in $Apc^{\Delta 716}$ polyps, it is possible that disruption of the TGF-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 demonstrate 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 gastrointestinal tract. Dysregulation of morphogen signals, such as Notch, Hh or Wnt, in adult tissues often results in pathological 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 downregulation of *Sox17* expression is important for tumor initiation and malignant progression, respectively, in the course of gastrointestinal tumorigenesis.

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Figure Legends

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 upregulated 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 wild-type (*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 ± s.d) (*bottom*). (*C*) The relative *Sox17* mRNA levels of gastric tissues of indicated genotypes to the wild-type mouse stomach (mean ± s.d.). Asterisks in (*B*, *C*), $P < 0.05$ versus wild type mouse stomach.

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. Arrows in (*A* and *B*) indicate tumor cells and dysplastic cells, respectively, while arrowheads in (*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 ± s.d.). Asterisks, $P < 0.05$.

Figure 3

Downregulation 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 ± s.d.) (*bottom*). (*B*) The relative expression level of *Sox17* in DAC-treated cells examined by real-time RT-PCT (mean ± s.d.). (*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 ± s.d.) (*E*) The number of colonies of AGS cells transfected with empty vector (*EV*) or Sox17 expression vector (*Sox17*) (mean ± s.d.). Asterisks in (*A*, *B*, *D* and *E*) $P < 0.05$.

Figure 4

Immunohistochemistry for Sox17 in malignant cancers and benign tumors. (*A, B*) Representative H&E staining, and immunostaining for Sox17 and β-catenin in two sets of

serial sections from gastric cancer (*A*) and colon cancer (*B*) tissue microarrays. Insets indicate enlarged images. (*C, 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.

Figure 5

Downregulation of Sox17 in mouse intestinal adenocarcinomas. (*A-C*) Immunostaining for β-catenin and Sox17 in $Apc^{\Delta 716}$ mouse polyps (*A*), non-invasive polyps of *cis-*$Apc^{\Delta 716}$ *Smad4* mice (*B*), and invasive adenocarcinomas of *cis-*$Apc^{\Delta 716}$ *Smad4* mice (*C*). H&E staining (*A-C*, *left*; and *C, center*), immunostaining for β-catenin (*green*) (*A-B, center*) and for Sox17 (*red*) (*A-C*, *right*), and counter staining with DAPI (*blue*) (*B-C, right*). β-Catenin and Sox17 in (*A-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. Arrows in (*A, B*) indicate reciprocal strong stained area of β-catenin (*center*) and Sox17 (*right*). Arrowheads in (*C, right*) indicate remained Sox17-expressing tumor cells. *Bars* indicate 200 μm in (*A-C, left*) and 100 μm in (*A-C, center and right*).

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 two 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*). Asterisks in (*B* and *C*), $P < 0.05$ to the adult mouse level and wild-type level, respectively.

Figure 7

Suppression of gastric tumor development by transgenic expression of *Sox17*. (*A*) Schematic construction of transgenic vector. *Pm*, *PmeI*; *B*, BamHI; *Xb*, *XbaI*. (*B*) Western blotting results of Sox17 in gastric mucosa of wild-type (*WT*), *K19-Sox17* (*Tg*) line #5 and #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*). Asterisks in (*C*, *D*), $P < 0.05$. (*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 ± s.d.). (*F*) Representative photographs for apoptotic cells in *Gan* and *Gan K19-Sox17* mouse tumors, and relative Ki-67 labeling

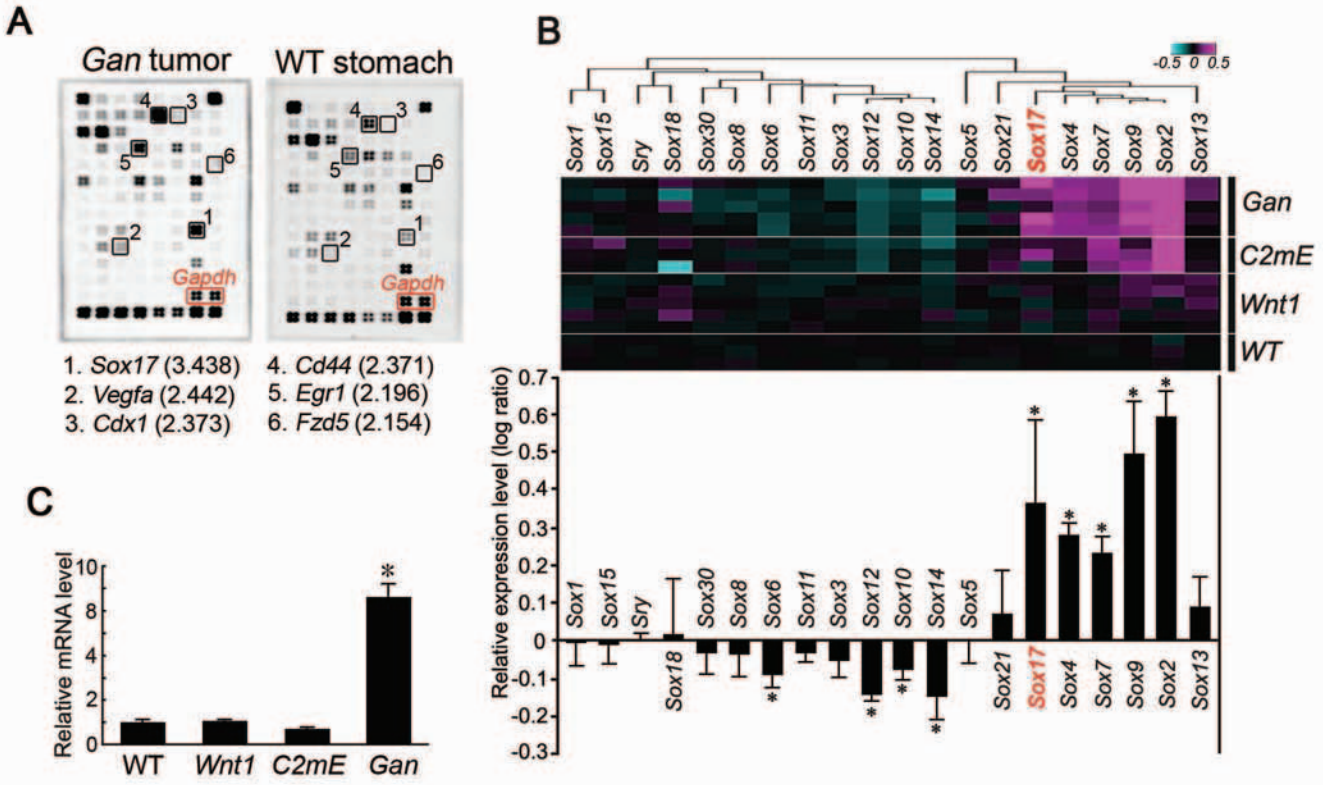
index in *Gan K19-Sox17* tumors (*Gan/Sox17*) to that in *Gan* mouse tumors. Arrowheads indicate apoptotic cells on the tumor surface. Asterisks in (*E*, *F*), $P < 0.05$.

Figure 8

Suppression of dysplastic tumor phenotype by transgenic expression of *Sox17*.

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

Du YC et al. Figure 1



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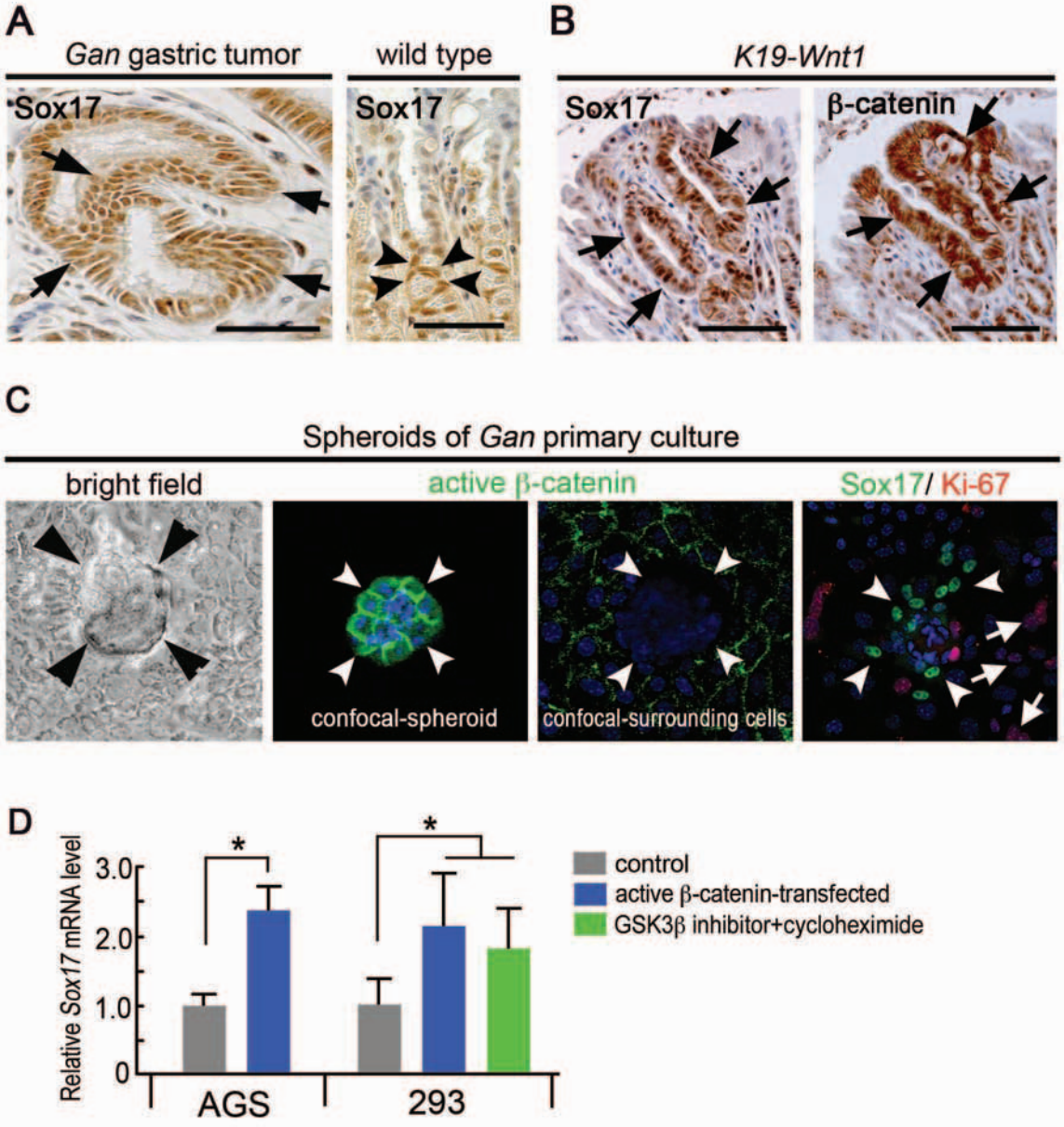

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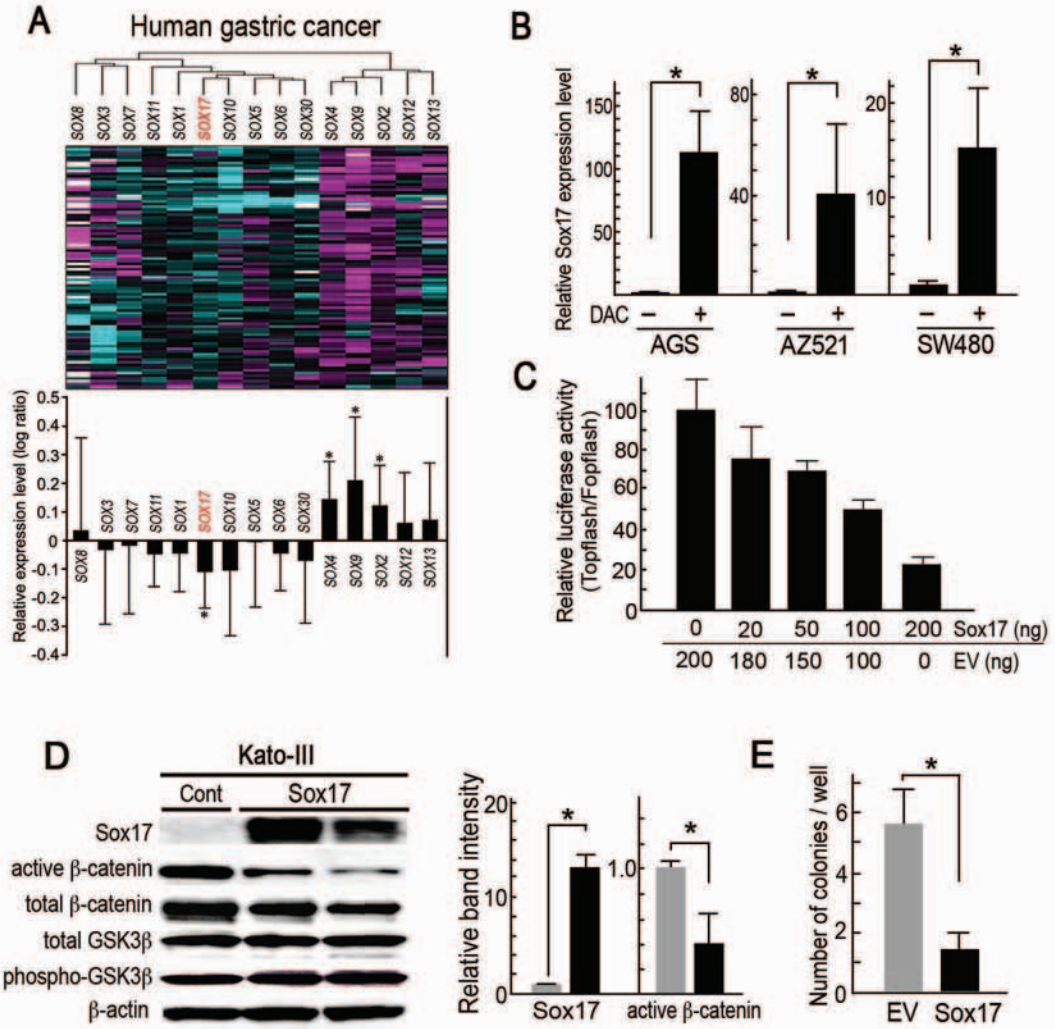

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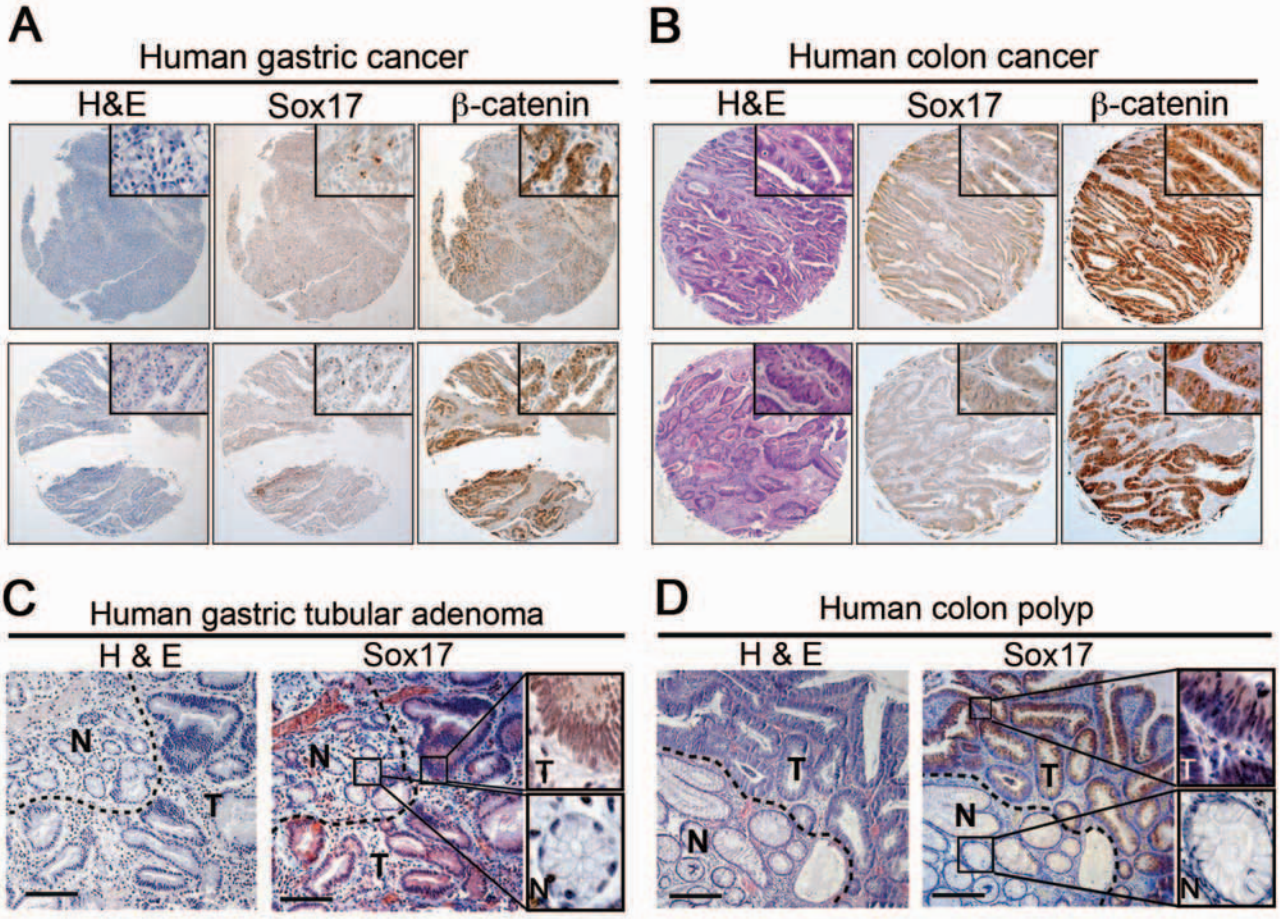
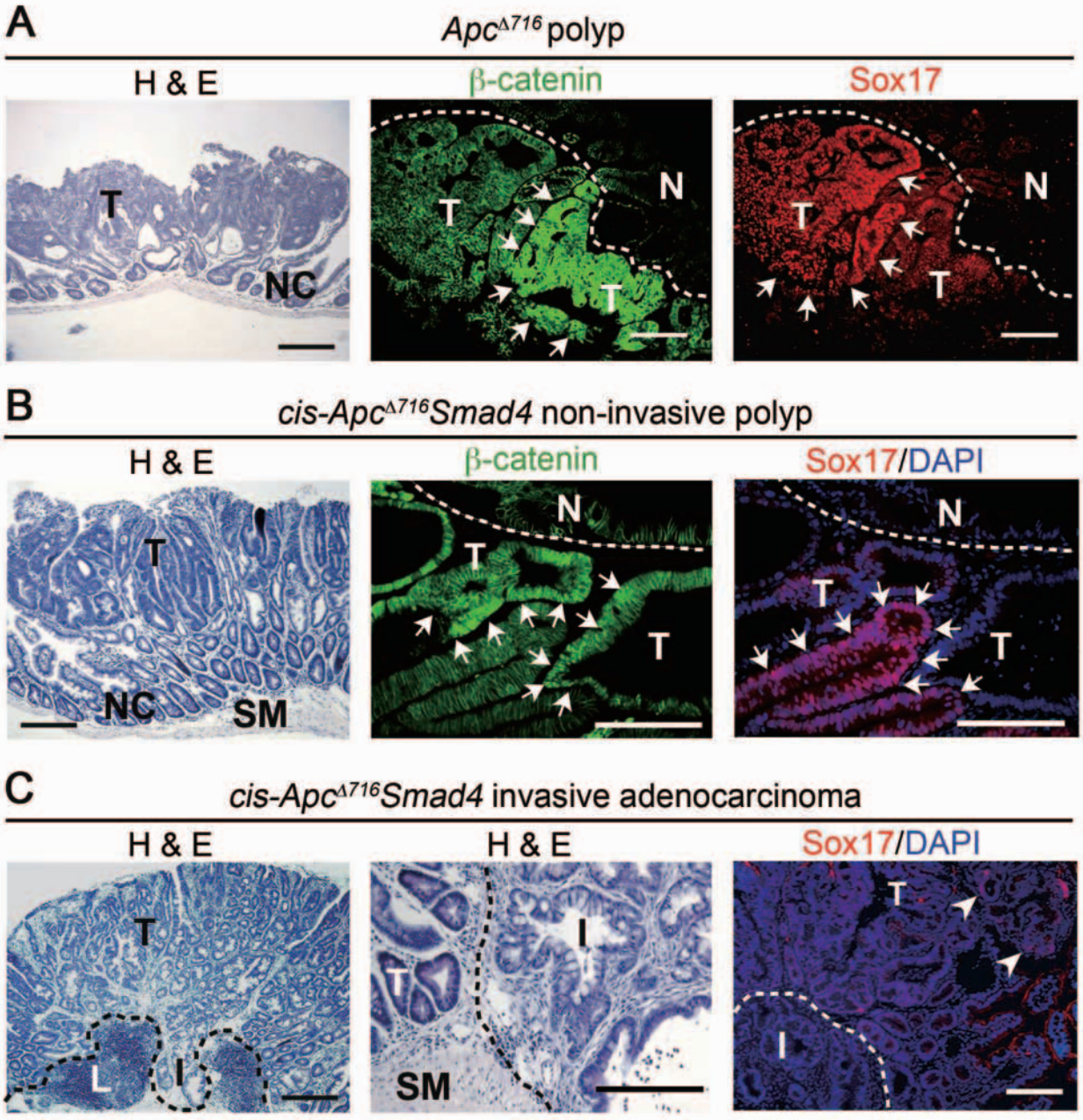


E

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)

Du YC et al. Figure 5



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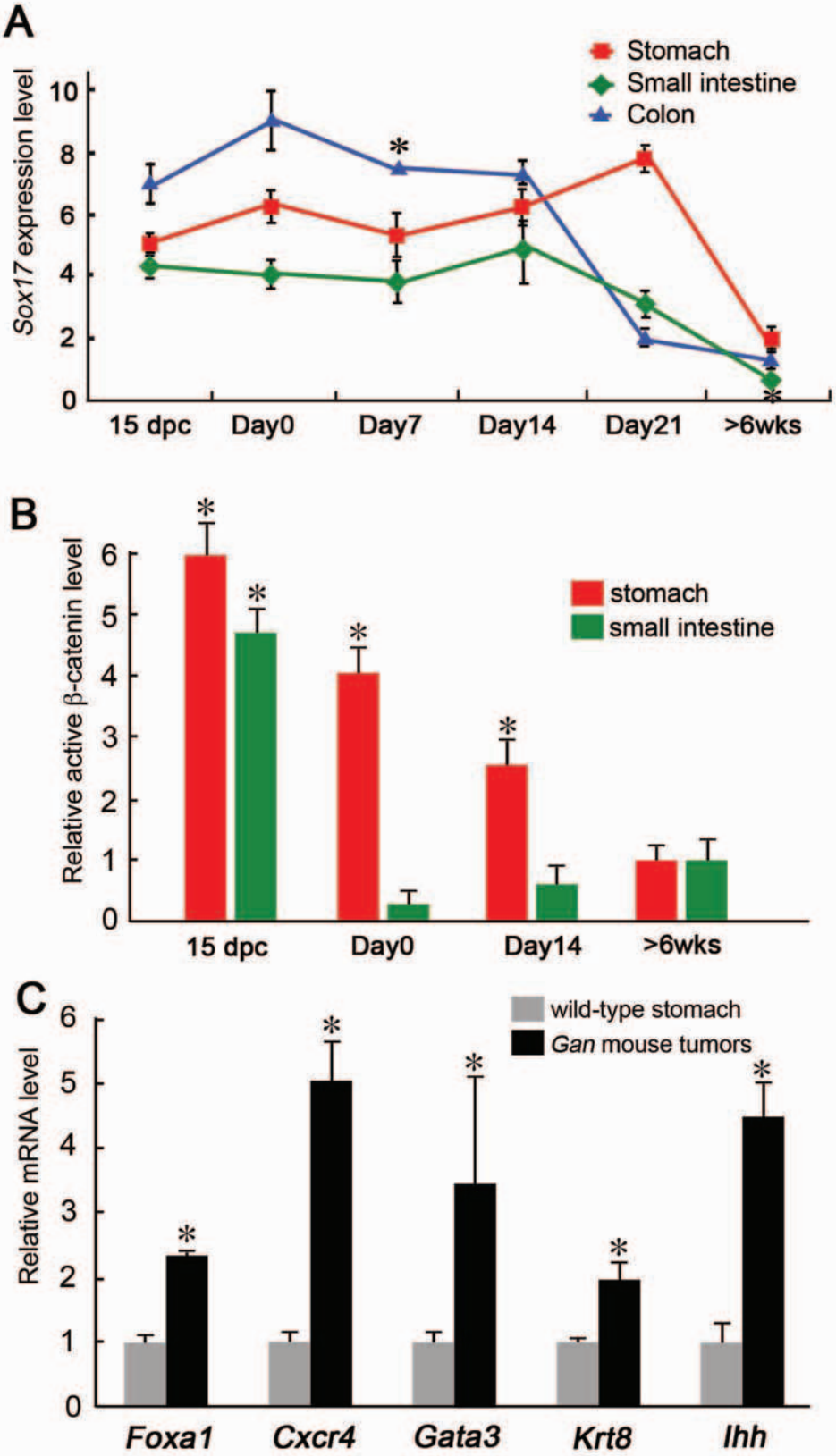

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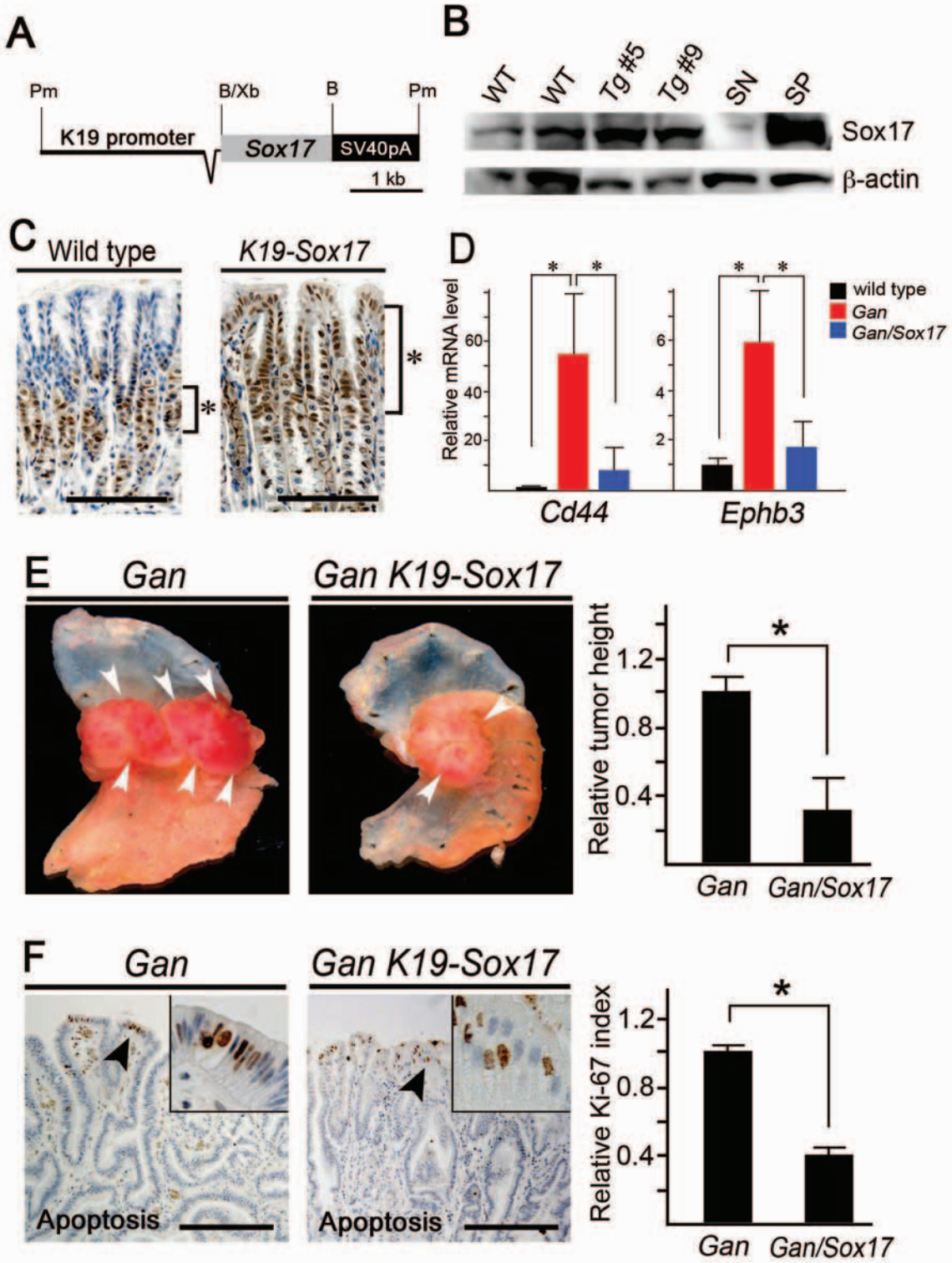
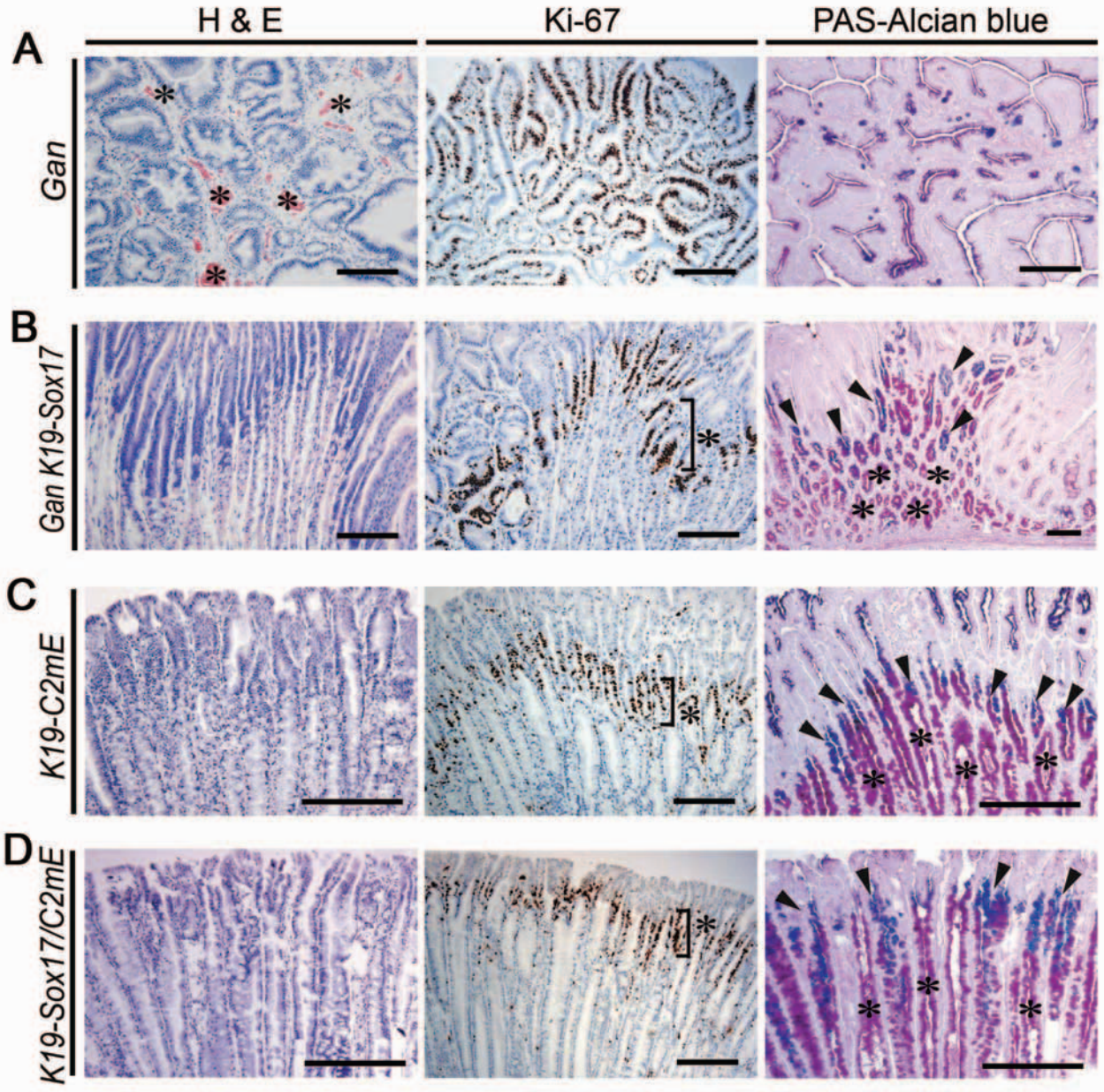
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