Suppressing TGFβ Signaling in Regenerating Epithelia in an Inflammatory Microenvironment Is Sufficient to Cause Invasive Intestinal Cancer

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Abstract

Genetic alterations in the TGFβ signaling pathway in combination with oncogenic alterations lead to cancer development in the intestines. However, the mechanisms of TGFβ signaling suppression in malignant progression of intestinal tumors have not yet been fully understood. We have examined Apc716/Tgfbr2−/− compound mutant mice that carry mutations in Apc and Tgfbr2 genes in the intestinal epithelial cells. We found inflammatory microenvironment only in the invasive intestinal adenocarcinomas but not in noninvasive benign polyps of the same mice. We thus treated simple Tgfbr2−/− mice with dextran sodium sulfate (DSS) that causes ulcerative colitis. Importantly, these Tgfbr2−/− mice developed invasive colon cancer associated with chronic inflammation. We also found that TGFβ signaling is suppressed in human colitis–associated colon cancer cells. In the mouse invasive tumors, macrophages infiltrated and expressed MT1-MMP, causing MMP2 activation. These results suggest that inflammatory microenvironment contributes to submucosal invasion of TGFβ signaling–repressed epithelial cells through activation of MMP2. We further found that regeneration was impaired in Tgfbr2−/− mice for intestinal mucosa damaged by DSS treatment or X-ray irradiation, resulting in the expansion of undifferentiated epithelial cell population. Moreover, organoids of intestinal epithelial cells cultured from irradiated Tgfbr2−/− mice formed ‘long crypts’ in Matrigel, suggesting acquisition of an invasive phenotype into the extracellular matrix. These results, taken together, indicate that a simple genetic alteration in the TGFβ signaling pathway in the inflamed and regenerating intestinal mucosa can cause invasive intestinal tumors. Such a mechanism may play a role in the colon carcinogenesis associated with inflammatory bowel disease in humans.

Introduction

Accumulating evidence indicates that inflammatory responses play an important role in cancer development (1, 2). The disruption of genes encoding cyclooxygenase 2 (COX-2) or prostaglandin E2 (PGE2) receptor, EP2, in Apc716−/− knockout mice results in significant suppression of intestinal polyposis (3, 4). Moreover, blocking transcription factors NF-κB and Stat3 in a chemically induced colitis-associated colon tumor model in mice causes suppression of colon tumor development (5–7). These results indicate that activation of inflammatory pathways through PGE2, NF-κB, and Stat3 is required for intestinal tumorigenesis. However, it has not yet been elucidated what role the inflammatory responses play in the progression of benign intestinal tumors to invasive adenocarcinomas.

Most intestinal adenomas are induced by APC mutations, resulting in the Wnt signaling activation, and tumors progress to adenocarcinomas by additional mutations such as those encoding RAS or transforming growth factor β (TGFβ) type II receptor (TGFβRII; ref. 8). Mouse genetic studies have indicated that suppression of TGFβ signaling accelerates development of malignant intestinal tumors in combination with mutations in Kras or Pten, although TGFβ suppression alone does not cause tumorous changes (9, 10). These results indicate that suppression of the TGFβ signaling is a key process involved in the malignant progression.

The TGFβ ligand binds TGFβRII, followed by the activation of TGFβRI. Activated TGFβRI then phosphorylates Smad2/3, which causes their binding with Smad4, and the Smad complex induces transcription of TGFβ target genes (11). We have previously shown that immature myeloid cells (iMC) are recruited and express matrix metalloproteinase 2 (MMP2) at the invasion front of compound Apc716−/− Smad4−/− knockout mouse intestinal adenocarcinomas, which contribute to their submucosal invasion (12, 13). Moreover, disruption of Tgfbr2, encoding TGFβRII, in mouse mammary tumor cells results in the recruitment of...
myeloid cells into tumor tissues, which promotes tumor metastasis through a process involving metalloproteinase activation (14). These results indicate that suppression of TGFβ signaling generates a microenvironment that is critical for progression of intestinal tumors. However, it is not understood how the inflammatory responses affect the tumor progression induced by TGFβ suppression. Moreover, it has not been elucidated whether TGFβ-suppressed epithelial cells acquire an invasive phenotype in tumor tissues.

We herein show that ulcerative colitis causes submucosal invasion of Tgfbr2-disrupted intestinal epithelia, leading to the development of invasive colon cancer. Moreover, TGFβ signaling suppression in regenerating epithelia caused long crypt formation in Matrigel, which may reflect an increased capacity for invasion. We also found that TGFβ signaling is suppressed in human colitis-associated colon cancer cells. These results provide a novel mechanism for the development of invasive colon cancer where TGFβ signaling suppression, chronic inflammation, and the regeneration of epithelial cells are compounded.

Materials and Methods

Animal models

Wild-type C57BL/6 mice were purchased from CLEA. Ap716 mice, Tgfr2fl/fl mice, and villin-CreER mice have been described previously (15–17). Tgfr2fl/fl mice were obtained from Mouse Repository (NCI-Frederick, Strain Number: 01255, Frederick, MD). All animal experiments were carried out according to the protocol approved by the Committee on Animal Experimentation of Kanazawa University, Japan.

Animal experiments

Ap716 Tgfr2fl/fl and villin-CreER mice were treated with tamoxifen at 4 mg/mouse once a week, from 5 weeks of age to generate Ap716 Tgfr2fl/fl mice. Intestinal tumors of Ap716 fl and Ap716 Tgfr2fl/fl mice were examined at 15 weeks of age (n = 4). For the dextran sodium sulfate (DSS) treatment experiments, Tgfr2fl/fl villin-CreER mice were treated with tamoxifen at 4 mg/mouse for 3 consecutive days to generate Tgfr2fl mice, and mice were treated with 2% DSS in drinking water (MP Biomedicals) for 5 days. After DSS treatment, Tgfr2fl mice were treated with tamoxifen for 2 days, and were examined at 3 days, 4 weeks, 6 to 10 weeks (n = 10 for each) or 40 weeks (n = 2) after DSS treatment. For the X-ray irradiation experiments, C57BL/6 mice and Tgfr2fl mice were irradiated with X-ray at 9 Gy (n = 15 for each), and were examined for the intestine phenotype chronologically from days 0 to 6. For the azoxymethane/DSS colitis-associated colon tumor model, wild-type mice (n = 10) were intraperitoneally injected with 10 mg/kg azoxymethane (Sigma), followed by treatment with 2% DSS (MP Biomedicals) in drinking water for 5 days (week 1). This cycle was repeated twice during weeks 4 and 7, and mice were euthanized at week 15. Tumor tissues were used for immunoblotting analysis and gelatin zymography.

Histology and immunohistochemistry

The tissue sections were stained with H&E or Masson’s trichrome stain, or were processed for immunohistochemistry. Staining signals of immunohistochemistry were visualized using the Vectorstain Elite Kit (Vector Laboratories). Antibodies against E-cadherin (R&D Systems), α-SMA (Sigma), F4/80 (Serotec), MT1-MMP (GeneTex), K67 (Life Technologies), GFP (Molecular Probes), Collagen type IV (Nichirei Biosciences), CD44 (Millipore), SOX7 (R&D Systems), phosphorylated Smad2 (PSmad2) at Ser465/467 (Millipore) and β-catenin (Sigma) were used for immunohistochemistry. For fluorescence immunohistochemistry, Alexa Fluor 594 or Alexa Fluor 488 antibodies (Molecular Probes) were used as the secondary antibody. Approval for the projects using human tissue sections was obtained from the Tokyo Medical and Dental University Ethics Committee and Keio University Ethics Committee.

Bone marrow transplantation

Bone marrow (BM) cells were prepared from the femurs and tibias of green fluorescent protein (GFP) gene transgenic mice. Recipient mice were irradiated with 9 Gy of X-rays, followed by intravenous injection of 2 × 10⁶ BM cells.

Real-time RT-PCR

Size-classified intestinal polyps and normal intestines of Ap716 mice (n = 5 for each) were used for RNA extraction. For DSS-treated mouse samples, normal colon tissues or invasive colon tumors at 3 days, 4 weeks or 6 to 10 weeks (n = 10 for each) after DSS treatment were used for RNA extraction. The total RNAs were reverse-transcribed using the PrimeScript RT reagent kit (TaKaRa) and were PCR-amplified using the SYBR Premix ExTaqII (TaKaRa). The primers used for the real-time RT-PCR were purchased from TaKaRa.

Immunoblotting analysis

Tissues were homogenized in lysis buffer, and protein sample was separated in a 10% SDS-polyacrylamide gel. Antibodies against active form of β-catenin (dephosphorylated at Ser37 and Thr41; Millipore) and total β-catenin (Sigma), Stat3 (Cell Signaling Technology), and phosphorylated Stat3 at Tyr705 (Cell Signaling Technology) were used. An anti-β-actin antibody (Sigma) was used as the internal control. The ECL detection system (GE Healthcare) was used to detect the signals.

Gelatin zymography

Tissue sample was lysed in SDS sample buffer, incubated for 20 minutes at 37°C and separated in a 10% polyacrylamide gel containing 0.005% gelatin labeled with Alexa Fluor 670 (Abcam). After electrophoresis, gels were soaked in 2.5% Triton X-100 for 1 hour, then gelatinolysis was carried out by incubation at 37°C for 24 hours. The gel was monitored by an Odyssey infrared imaging system (LI-COR).

Organoid culture

The organoid culture using small intestinal epithelial cells was performed as described previously (18). Briefly, organoids were cultured in Matrigel with Advanced DMEM/F12 medium (Invitrogen) supplemented with 50 ng/mL EGF (Invitrogen), R-Spondin1 conditioned medium (a kind gift from Dr. Marc Leushacke, A*STAR, Institute of Medical Biology, Singapore), and 100 ng/mL Noggin (Peprotech). The cultures were passaged once, and the crypt length was measured under a dissecting microscope. Organoid cell proliferation was detected using the Click-iT EdU Imaging System (Invitrogen). After EdU staining, organoids were
incubated with anti-CD44 antibody (Chemicon) followed by secondary antibody, Alexa Fluor 488 (Molecular Probes). The stained organoids were analyzed using a Zeiss 510 META laser-scanning microscope (Zeiss).

Statistical analyses
The data were analyzed using an unpaired t test, and are presented as the means ± standard deviation (SD). A value of \( P < 0.05 \) was considered as statistically significant.

Results
Size-dependent submucosal invasion of intestinal tumors
It has been demonstrated that the disruption of Tgfbr2, Smad4, or Smad3 in Apc-mutant mice causes development of invasive adenocarcinomas in the intestine, whereas simple Apc-mutant mice develop only benign adenomas (12, 19–21). Accordingly, the combination of Wnt signaling activation and TGFβ signaling suppression is implicated in the malignant invasion of intestinal tumors. To further investigate the mechanism underlying the submucosal invasion of intestinal tumors, we constructed compound mutant mice carrying both Apc<sup>Δ716</sup> and Tgfbr2 mutation in intestinal epithelial cells (Apc<sup>Δ716</sup>Tgfbr2<sup>ΔIEC</sup>). We confirmed that Apc<sup>Δ716</sup>Tgfbr2<sup>ΔIEC</sup> mice developed adenocarcinomas with submucosal invasion, whereas simple Apc<sup>Δ716</sup> mice had only noninvasive adenomas (Fig. 1A and Supplementary Fig. S1). Notably, only the polyps >1 mm in diameter showed submucosal invasion in the Apc<sup>Δ716</sup>Tgfbr2<sup>ΔIEC</sup> mice (Fig. 1B and Supplementary Fig. S1B). Histologically, large invasive polyps in Apc<sup>Δ716</sup>Tgfbr2<sup>ΔIEC</sup> mice were associated with increased stroma and macrophage infiltration, which was not found in the small noninvasive polyps (Fig. 1C).

Figure 1
Size-dependent submucosal invasion of intestinal tumors of Apc<sup>Δ716</sup> mouse benign adenomas (left) and Apc<sup>Δ716</sup>Tgfbr2<sup>ΔIEC</sup> mouse invasive adenocarcinomas (right). HE (top) and enlarged images of the boxed areas (middle), and fluorescence immunohistochemistry for E-cadherin (red) and αSMA (green; bottom). T, tumor; SM, submucosa. Arrowheads, submucosal invasion of tumor epithelial cells. Bars, 400 μm (top) and 200 μm (middle and bottom). B, size classification of intestinal tumors of Apc<sup>Δ716</sup> mice (top) and Apc<sup>Δ716</sup>Tgfbr2<sup>ΔIEC</sup> mice (bottom) scored using “Swiss roll” histology sections. Each dot indicates an individual polyp. Different colors indicate independent mice. C, representative photographs of noninvasive (top) and invasive polyps (bottom) of Apc<sup>Δ716</sup>Tgfbr2<sup>ΔIEC</sup> mice. H&E staining, immunohistochemistry for F4/80 and MT1-MMP, and Masson trichrome (MT) staining (left to right). Insets, high-powered magnification. Bars, 200 μm (top) and 400 μm (bottom). D, expression levels of the indicated factors relative to the mean level of normal mucosa in size-classified small intestinal and colon tumors of Apc<sup>Δ716</sup> mice (mean ± SD). * \( P < 0.05 \) compared with the normal mucosal level.
Membrane-type 1 matrix metalloproteinase (MT1-MP) expression was induced in the stroma of the large invasive tumors, and collagen fiber deposition was also found in the stroma by Masson trichrome staining. Moreover, expression of IL1β, IL6, and COX-2 was induced when polyp size increased beyond >1 mm in diameter in ApcΔ716 mouse small intestine, and expression levels of MT1-MMP, Adam10, and epiregulin were also increased in the large polyps (Fig. 1D). The induction of these factors was also found in the colon polyps >2 mm in diameter.

Figure 2.
Submucosal invasion of TGFβ signaling-suppressed cells by ulcerative colitis. A, schedule for tamoxifen and DSS treatment and examination of mice. B, representative photographs (H&E) of DSS-treated wild-type (left) and DSS-treated Tgfbr2ΔIEC (right) mouse colons 3 days (top) and 4 weeks (down) after treatment. Arrows (top), ulcer lesions. Arrowheads (bottom right), submucosal invasion of epithelial cells. *, impaired regeneration of the normal gland structure. Bars, 200 μm. C, representative photographs of invasive tumors of a DSS-treated Tgfbr2ΔIEC mouse 10 weeks after treatment. H&E (top), enlarged images of the boxed areas (bottom left), and Masson trichrome (MT) staining of serial section (bottom right). Arrows, location of muscularis mucosae. *, impaired regeneration of the normal gland structure. Bars, 500 μm (top and middle) and 200 μm (bottom). D, representative macroscopic photographs of invasive tumors developed in a DSS-treated Tgfbr2ΔIEC mouse at 40 weeks after treatment (arrows, top left), and the H&E (top right) and immunohistochemistry for Ki67 (bottom). Arrows (bottom), the location of muscularis mucosae; and inset, enlarged image of the boxed area. Ce, cecum; Ic, ileum; Co, colon. Bars, 2 mm (top) and 400 μm (bottom). E, immunoblotting for active β-catenin and total β-catenin in the intestinal mucosa and polyps in ApcΔ716 and AOM/DSS-treated mice (left) and DSS-treated Tgfbr2ΔIEC mice (right) at the indicated time points. NM, normal mucosa; and inv, invasive tumors. β-Actin was used as an internal control. F, representative immunohistochemical findings of p-Smad2 in ulcerative colitis–associated human colon cancer tissues (left, center), and the β-catenin in sporadic colon cancer (top right) and ulcerative colitis–associated colon cancer (UC; right bottom). Arrows (center), surface epithelial cells and invading tumor cells. Bars, 500 μm (left) and 50 μm (center and right).
together, these results indicate that the inflammatory microenvironment is generated by a tumor size-dependent mechanism in both the small intestinal and colon polyps. It is therefore possible that such microenvironment is required for submucosal invasion.

Submucosal invasion by TGFβ signaling suppression and ulcerative colitis

We thus examined the role of inflammatory responses in TGFβ signaling suppression-associated submucosal invasion using a colitis mouse model by treating Tgfb2<sup>AIEC</sup> mice with DSS (Fig. 2A). The wild-type mice treated with DSS showed ulcerative colitis beginning 3 days after DSS treatment, and the colonic mucosa was repaired in 4 weeks after treatment (Fig. 2B). Although mucosal ulcers were repaired in the Tgfb2<sup>AIEC</sup> mice in 4 weeks after DSS treatment, regeneration of the normal gland structure was significantly impaired (Fig. 2B, asterisk). Importantly, colonic epithelial cells invaded to the submucosa in Tgfb2<sup>AIEC</sup> mice by the end of fourth week after treatment. These invading epithelial cells continued proliferation, and formed invasive tumors associated with deposition of collagen fibers by the 10 weeks after DSS treatment (Fig. 2C). Furthermore, large solid tumors were visible from outside of the colon and cecum of Tgfb2<sup>AIEC</sup> mice to the 10 weeks after DSS treatment (Fig. 2D). Epithelial cells of tumors were positive for Ki67, indicating that the tumor cells continued proliferation even at 40 weeks after DSS treatment. We confirmed that disruption of Tgfb2<sup>AIEC</sup> in normal intestinal mucosa did not cause any morphologic changes (Supplementary Fig. S3), which is consistent with previous reports (20, 22). The level of active β-catenin was significantly increased both in the Apc<sup>Min<sup>−/−</sup></sup> mouse intestinal tumors and colitis-associated colon tumors that were chemically induced by treatment with azoxymethane and DSS, however, active β-catenin level was not increased in the invasive tumors of DSS-treated Tgfb2<sup>AIEC</sup> mice (Fig. 2E). These results indicate that ulcerative colitis induces invasive tumors in the TGFβ-suppressed mucosa without activation of the Wnt signaling.

We next examined human inflammatory bowel disease (IBD)—related colon cancer. Nuclear localized P-Smad2 was found in the
surface epithelial cells of ulcerative colitis, however, it was not detected in the invaded tumor cells, indicating that there was suppression of TGFβ signaling (Fig. 2F). A loss of P-Smad2 staining in tumor cells was found in five out of eight cases (62.5%) of ulcerative colitis–associated colon cancer. Moreover, β-catenin accumulation was not detected in seven out of eight ulcerative colitis–associated tumors (87.5%). Accordingly, it is possible that suppression of TGFβ signaling causes colon cancer invasion in human patients with IBD without Wnt signaling activation.

**Chronic inflammatory responses in the invasive tumors**

We found that BM-derived cells and macrophages infiltrated in the stroma of invasive colon tumors, but not in the noninvasive mucosa of DSS-treated Tgfbr2<sup>fl<sup>ex</sup></sup> mice (Fig. 3A). Consistently, expression of monocyte-trophic chemokines (CCL2, CCL3, CCL4, CCL7, and CCL8), was increased significantly in the invasive tumors of DSS-treated Tgfbr2<sup>fl<sup>ex</sup></sup> mice, whereas its level was decreased in the repaired mucosa of DSS-treated wild-type mice (Fig. 3C). These results indicate that inflammatory responses are chronically maintained in the invasive tumor tissues, even after the repair of DSS-induced ulcers. We thus determined the levels of inflammatory cytokines in both the invasive tumors and DSS-induced ulcerative colitis. Expression was significantly upregulated for TNFα, IL1β, IL6, CXCL1, CXCL2, and CXCL5 by DSS-induced ulcerative colitis in both the wild-type and Tgfbr2<sup>fl<sup>ex</sup></sup> mice (Fig. 3D).
Expression of MT1-MMP and activation of MMP2 in invasive tumors

To investigate the mechanism underlying chronic inflammation-associated invasion, we examined expression of MMPs that are important for submucosal invasion (24). It has been shown that MT1-MMP plays a key role in activation of MMP2 (25) and MT1-MMP is expressed in macrophages, regulating inflammatory responses (26, 27). The expression levels of MT1-MMP, MMP2, and MMP9 were significantly increased in the invasive tumors of DSS-treated Tgfbr2<sup>AEC</sup> mice (Fig. 4A). Fluorescence immunohistochemistry showed that the stromal cells of invasive tumors expressed MT1-MMP, and most MT1-MMP-expressing cells in tumor stroma were F4/80-positive macrophages (Fig. 4B). Moreover, gelatin zymography analyses revealed that MMP2 was activated in the invasive tumor tissues, but not in the normal colonic mucosa of the same DSS-treated Tgfbr2<sup>AEC</sup> mice (Fig. 4C). Consistently, the immunostaining signal for collagen type IV in the basement membrane was significantly decreased in the invading epithelial cells (Fig. 4D). These results suggest that chronic inflammation contributes to the submucosal invasion through macrophage-expressed MT1-MMP, which leads to MMP2 activation, resulting in degradation of basement membrane.

Because MT1-MMP expression was also induced in the Apc<sup>D716</sup> benign adenomas (Fig. 1C and D), we further examined MMP2 activation in other mouse tumor models. As anticipated, MMP2 was activated in the invasive tumors of Apc<sup>A716</sup> Tgfbr2<sup>AEC</sup> mice (Fig. 4E). Notably, MMP2 activation was also found in the noninvasive benign tumors of Apc<sup>A716</sup> and AOM/DSS-treated mice, although the band intensities were lower compared with those of the Apc<sup>A716</sup> Tgfbr2<sup>AEC</sup> mouse invasive tumors (Fig. 4E). These results suggest that MMP2 activation is already induced in benign intestinal tumors, and its activation level increases with the progression of the tumor. Accordingly, it is also possible that
Impaired mucosal regeneration by suppression of TGFβ signaling

Impaired mucosal regeneration from ulcer in Tgfbr2ΔMEC mice (Fig. 2B and C) suggested a role of TGFβ signaling in regeneration of injured intestinal mucosa. To test this possibility, mice were irradiated with X-ray at 9 Gy. In the irradiated wild-type mice, the number of proliferating cells in the crypt decreased on days 1 to 3 after irradiation, followed by destruction of the mucosal structure by day 4 (Supplementary Fig. S4). At the same time, the undifferentiated cell population expanded, and normal crypt-villous structures were regenerated by day 6, which was consistent with a previous report (28). X-ray irradiation in Tgfbr2ΔMEC mice also showed a decrease in the proliferating crypt cell population, followed by increased numbers of proliferating undifferentiated cells (Supplementary Fig. S4). However, the extent of the injury was more severe in the Tgfbr2ΔMEC mice, and the regeneration of the normal mucosal structure was impaired in both the small intestine and colon (Fig. 5A and Supplementary Fig. S5). Because of such severe phenotypes, most irradiated Tgfbr2ΔMEC mice died by day 7 (Fig. 5B). Moreover, markers of undifferentiated epithelial cells, CD44 and SOX7, were expressed in the entire intestinal mucosa of the X-ray–irradiated Tgfbr2ΔMEC mice, whereas their expression was limited to the crypt bottom in the wild-type mice (Fig. 5A and Supplementary Fig. S5B). Consistently, an intestinal progenitor cell marker, SOX9 (29), was upregulated in the intestine by irradiation, and the SOX9 level was significantly higher in Tgfbr2ΔMEC mice than in wild-type mice (Fig. 5C). Moreover, the nuclear accumulation and stabilization of β-catenin was not found in the irradiated Tgfbr2ΔMEC mouse intestinal epithelia (Fig. 5D and E). Taken together, these results indicate that TGFβ signaling is required for differentiation of regenerating epithelial cells, and that blocking the TGFβ pathway causes expansion of the proliferating and undifferentiated epithelial cell population without activation of Wnt signaling.

Acquisition of invasive phenotype by blocking TGFβ signaling in regenerating epithelial cells

We next studied the effect of TGFβ signaling suppression in regenerating mucosa on invasive phenotype. Organoid culture of the irradiated wild-type mouse-derived intestinal epithelial cells showed budding from cysts, forming mini-crypt structures (Fig. 6A), which was consistent with the original report (18). Importantly, intestinal epithelial cells derived from irradiated Tgfbr2ΔMEC mice formed gland-like long crypt structures in the Matrigel (Fig. 6A). We further examined the epithelial cell proliferation in the organoids by evaluating the EdU incorporation to nuclei, and examined the undifferentiated status by determining the CD44 expression. Notably, the expression of EdU and CD44 was found in the epithelial cells along the long crypts of Tgfbr2ΔMEC mouse-derived organoids, although that was detected only in budding crypts of wild-type organoids. These results suggest that the long crypts are comprised of proliferating undifferentiated epithelial cells. The proportion of long crypts (>200 μm) was significantly higher in the irradiated Tgfbr2ΔMEC mouse-derived intestinal epithelial cells (42% ± 13% of all crypts) compared with epithelial cells derived from irradiated Tgfbr2ΔMEC mouse control mice (3.9 ± 4.5%; Fig. 6B). Such long crypt formation was not found also in organoids of nonirradiated Tgfbr2ΔMEC mouse-derived intestinal epithelial cells. It is possible that long crypt formation in Matrigel reflects "collective cell migration" in the extracellular matrix, which is one of strategies used by cancer cells for invasion (30). Therefore, it is conceivable that suppression of TGFβ signaling in regenerating mucosa results in the acquisition of invasive phenotype, which leads to collective migration in the inflammatory microenvironment.

Discussion

Genome-wide analyses have indicated that accumulation of genetic alterations in oncogenic and tumor-suppressor pathways
is responsible for development of colon cancer (31). On the other hand, the nature and significance of the individual genetic alterations are not yet understood (32). In addition, relatively few mutations have been identified that are responsible for invasion or metastasis (8, 33), suggesting that microenvironment can promote malignant progression. We have herein demonstrated that simple genetic alterations in the TGFβ pathway can lead to the development of invasive gastrointestinal cancers without additional genetic alterations when the mucosa is inflamed and regenerating from injury (Fig. 7A).

It has been shown that suppression of TGFβ signaling in the intestinal and mammary gland tumor cells induces chemokine expression, which recruits myeloid cells to the tumor microenvironment (12, 14). These myeloid cells express metalloproteinases, such as MT1-MMP, MMP2, and MMP9 that contribute to the invasion or metastasis of tumor cells. These results suggest that elaboration of an inflammatory microenvironment (12, 14) is responsible for development of colon cancer (31). On the other hand, compound mutant mice carrying mutations in the TGFβ signaling pathway may cause the development of invasive tumors under IBD condition (Fig. 7A).

Accordingly, it is required for the development of invasive tumors that both the inflammatory microenvironment where MT1-MMP is expressed and the regenerating epithelial cells with increased invasiveness by inhibition of TGFβ signaling (Fig. 7A). Such a mechanism is possibly important for cancer development associated with IBD. In IBD lesions, the mucosa is continuously regenerating in a chronic inflammatory microenvironment, and the expression of MT1-MMP, together with inflammatory chemokines, is upregulated similar to that observed in Tgfb2−/− mouse tumors (Supplementary Fig. S6). Furthermore, we herein demonstrated that TGFβ signaling is suppressed and Wnt signaling is not activated in more than 60% of ulcerative colitis–related colon cancer cells. Consistently, it has also been reported that human colitis–associated colon cancer does not follow the adenoma–carcinoma sequence, and mutations in β-catenin or APC are not common either (34). Accordingly, simple genetic alterations in the TGFβ signaling pathway may cause the development of invasive tumors under IBD condition (Fig. 7A).

On the other hand, compound mutant mice carrying mutations in Apc and TGFβ pathway genes showed progression of invasive adenocarcinomas from Wnt-activated adenomas (19–21), indicating that the combination of Wnt activation and TGFβ signaling suppression is sufficient for malignant progression. In the regenerating mucosa, stem cell population is expanded, with the signaling in the Wnt and Notch pathways activated (35). It is therefore possible that the activation of Wnt signaling is necessary for malignant progression in sporadic tumors where TGFβ signaling is blocked without mucosa regeneration. Accordingly, combination of Wnt activation and TGFβ suppression in the
MT1-MMP—expressing inflammatory microenvironment is sufficient for the induction of invasive adenocarcinoma in normal intestine (Fig. 7B).

In conclusion, we have demonstrated that suppression of TGFβ signaling in the regenerating epithelial cells results in suppression of epithelial differentiation and acquisition of invasive phenotype of epithelial cells. Chronic inflammation induces development of an MMP2-activating microenvironment. The cooperation between TGFβ signaling suppression in the regenerating epithelia and the inflammatory microenvironment can cause invasive colon cancer development, which may explain mechanism of IBD-associated colon tumorigenesis. Therefore, controlling the inflammatory microenvironment may help an effective preventive or therapeutic strategy against the malignant progression of colon cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Manami Watanabe and Ayako Tsuda for technical assistance.

Grant Support
This work was supported by CREST, the Japan Science and Technology Agency, Japan (M. Oshima). Grants-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan #22114005 (M. Oshima), #24300325 (M. Oshima), and Takeda Science Foundation, Japan (H. Oshima and M. Oshima).

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Received July 9, 2014; revised November 25, 2014; accepted December 1, 2014; published online February 16, 2015.

Invasion by TGFβ Blocking in Regenerating Inflamed Mucosa

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