Smad3 Deficiency Promotes Tumorigenesis in the Distal Colon of Apc\(^{Min/+}\) Mice

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Abstract

Colorectal cancer, one of the most common human malignancies in the Western world, is often subdivided based on tumor location in either the distal or proximal colon. Several mouse models have been developed to study human colorectal cancer, but few display this clear distinction between the two colonic locations. By crossing Apc\(^{Min/+}\) and Smad3 mutant mice, we showed that combined activation of the Wnt pathway and attenuation of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) pathway causes high multiplicity and rapid onset of invasive tumorigenesis almost exclusively in the distal colon, closely mimicking the familial adenomatous polyposis (FAP) disease and consisting with distinct colorectal cancer etiologies based on tumor location. Transcriptional profiling revealed higher expression of several TGF-\(\beta\) activators in the normal distal mucosa than in proximal mucosa, suggesting a stronger reliance on TGF-\(\beta\)-mediated growth control in the distal than in the proximal colon. Apc\(^{Min/+}\)/smad3\(^{-/-}\) mice provide an alternative model to Apc\(^{Min/+}\) mice to study FAP and distal sporadic colorectal cancer. This model will be useful in dissecting mechanistic and etiologic differences between proximal and distal colonic cancer, whereas the confinement of tumorigenesis to the distal colon offers unique advantages in monitoring tumor progression by \textit{in vivo} imaging. (Cancer Res 2006; 66(17): \textit{8430-8})

Introduction

Colorectal cancer is the second leading cause of death by malignancy in the Western world. Colorectal cancer is a heterogeneous disease with epidemiologic, biological, and clinical differences between cancers of the proximal and the distal colon, reflecting distinct etiologies and molecular pathways of carcinogenesis for proximal and distal disease. These differences may result from the existence of distinct embryologic, morphologic, physiologic, environmental, and biochemical factors between the two colonic locations (1). For example, the proximal and the distal colon have different embryonic origins (midgut and hindgut, respectively) and a different vascular supply (2).

Mutant and genetically engineered mouse models of intestinal neoplasia are broadly divided into five categories: mutations in the adenomatous polyposis coli (Apc) gene and Wnt signaling pathway, alterations in the mismatch repair genes, alterations in the transforming growth factor-\(\beta\) (TGF-\(\beta\)) pathway, immunodeficient mice with colitis, and carcinogen-treated mice (3). Although these models have provided useful information about colorectal cancer, they all have various limitations and do not faithfully mimic the disease in humans. For example, germ-line mutations in the human \(\textit{APC}\) gene cause familial adenomatous polyposis (FAP). Patients with FAP develop, during their late teens and early twenties, hundreds to thousands of adenomatous polyps distributed predominantly in the distal colon; such individuals have a high risk of malignancies before the age of 40 years (4). The multiple intestinal neoplasia (Min) mouse model for FAP has a transversion mutation from T to A in the \(\textit{Apc}\) gene (5, 6). These mice also develop dozens to hundreds of adenomas and have a reduced survival. However, unlike in human FAP cases, these adenomas are mainly located in the small intestine and generally do not progress to malignancy.

The Wnt and the TGF-\(\beta\) signaling pathways participate in multiple developmental events during embryogenesis and are implicated in maintaining adult tissue homeostasis (7–10). The Wnt signaling pathway is mediated by the oncogenic \(\beta\)-catenin protein, which is regulated by a complex containing glycogen synthase kinase-3\(\beta\), \(\textit{APC}\), and axins (11). Signals from the TGF-\(\beta\) pathway are initiated on the binding of TGF-\(\beta\) superfamily ligands to cell surface serine/threonine kinase receptors and are transmitted to the nucleus by the Smad family of intracellular proteins (12).

Alterations in both the Wnt and the TGF-\(\beta\) pathways have been described in human colorectal cancer (13, 14). For example, mutations in \(\beta\)-\textit{CATENIN (CTNNB1)} and \(\textit{APC}\) were detected in sporadic colon cancers and in a large variety of other tumor types, such as melanoma and ovarian carcinoma (14). \(\textit{SMAD4}\) is deleted or mutated in colorectal, pancreatic, and ovarian tumors. Mutations in \(\textit{SMAD2}\) have been found in some colorectal adenocarcinomas (13). Many of the alterations cited above have been tested in mice in an attempt to understand their role in the tumorigenic process. The \textit{Min} model was the first intestinal neoplasia model to be developed, and other models are often compared with it. Mice carrying different \textit{Apc} mutations have been developed (15). The tumors arising in these mice are histologically similar but vary with respect to age of onset, number, and location (3). Mice that harbor targeted disruption of \textit{Smad2}, \textit{Smad3}, or \textit{Smad4} have also been described. \textit{Smad2} heterozygous mice appear normal, but \textit{Smad2} homozygous mutant mice die \textit{in utero} (16, 17). \textit{Smad4} homozygous mutant mice die during embryogenesis (18), and \textit{Smad4} heterozygous mice are predisposed to the development of multiple polyps in the upper gastrointestinal tract (19, 20). Although \textit{Smad3} mutations have not been detected in human cancer, one model of \textit{Smad3}\(^{-/-}\) mice developed colonic lesions, including adenocarcinomas and metastatic carcinomas.
(21). On the 129/Sv background, 100% of Smad3−/− mice had tumors by 6 months of age, whereas on the hybrid background of 129/Sv and C57BL/6, the tumors occurred in only 30% of mice and their onset was delayed to 10 months of age.

Although the TGF-β and Wnt signaling pathways do their biological functions by using two separate families of secreted molecules, they cooperate to regulate certain developmental events. For instance, induction of the expression of Spemann’s organizer genes in Xenopus requires both pathways (22). Studies have shown that the R-Smads, Smad2, and Smad3, as well as the Co-Smad Smad4 can physically associate with lymphoid enhancer binding factor 1 (LEF1) on a DNA fragment from the promoter of the Xenopus homeobox gene, twin (Xtin), which is expressed in the Spemann’s organizer. A transcriptional activation complex composed of β-catenin, R-Smads, Co-Smad, and LEF1 is required for the maximal activation of the Xtin gene (23, 24).

Combined alterations in the TGF-β and Wnt signaling pathways have been shown to cooperatively affect tumorigenesis. The introduction of Smad4 mutations accelerated tumor progression from intestinal polyps to malignant cancer in Apc−/− mice (25) and increased tumor multiplicity in Apc+/−N1638 mice (26). Compound inactivation of Smad2 in heterozygous Apc mutant mice accelerated tumor progression in one study (17) but showed no effect on tumor formation in a second study (27). Because the homozygous inactivation of either Smad4 or Smad2 causes early embryonic lethality, the effect of deficiency in the TGF-β signaling pathway on tumor formation could be addressed by homozygous disruption of Smad3 in an Apc mutant mouse model. Traditionally, experiments involving Apc mutant mouse models are done in a C57BL/6 background or in an F1 hybrid of C57BL/6 and 129/Sv, a combination that does not work well for Smad3 mutant mice.

In this study, we hypothesized that mutations in Smad3 would enhance tumorigenesis in the colons of Apc−/− mice. Because of the strain specificity of Smad3 mutant mice with respect to tumor formation, we first backcrossed the Apc+/− allele to the 129/Sv background for 12 generations. We then crossed the 129/Sv Smad3−/− mice with 129/Sv Apc−/− mice to generate 129/Sv Apc−/−Smad3−/− mice to determine whether the combination of high tumor multiplicity of the Min mice with the invasive colorectal location of the Smad3−/− mice would provide a better model system of human colorectal tumor progression than previously reported models.

Materials and Methods

Genotyping of mice. DNA was isolated from tail biopsies of 3-week-old mice as described previously (28). Smad3 mice were genotyped by a multiplex PCR assay. Apc−/− mice were genotyped using a fluorescence-based, allelic discrimination PCR. A detailed description of the experimental procedures and the primers used for PCR analysis is provided in Supplementary Data.

Examination of mice. The 129-Smad3tm1Par−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and then backcrossed onto the 129/Sv strain. For simplicity, the tm1Par allele (targeted mutation 1; Luis F. Parada, UT Southwestern, Dallas, TX) is referred to as the (−) allele. The 129/Sv Smad3−/− mice were then transferred to 129/Sv Apc−/− mice to generate Apc−/−Smad3−/− mice. Apc+/−Smad3−/− males were then crossed to Smad3−/− females to generate 179 offspring, which were monitored on a daily basis for signs of illness or tumor development. These mice were euthanized at 2 months of age or at the first signs of morbidity and then autopsied. The intestinal tract was removed immediately after euthanasia, opened longitudinally, washed with 70% ethanol, fixed overnight in PBS-buffered 10% formalin, and then transferred to 70% ethanol for storage at 4 °C. The entire intestine was examined, and the number of tumors was scored using a dissecting microscope. All animal experiments were planned and conducted in compliance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Southern California (Los Angeles, CA).

Histology of tumors and immunohistochemistry. Tumor specimens dissected from 10% formalin-fixed intestinal tissues were embedded in paraffin and then sectioned to 4-μm slices. Paraffin sections were stained with H&E for histologic examination. For immunohistochemistry, paraffin sections were deparaffinized, rehydrated, and subjected to high temperature antigen retrieval in 10 mmol/L citrate buffer (pH 6.0). Sections were blocked in 1.5% normal goat serum and then incubated with either rabbit anti-Apc COOH-terminal polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-β-catenin COOH-terminal polyclonal antibody (Neomarkers, Fremont, CA). The detection of the primary antibodies was achieved using biotin-avidin-horseradish peroxidase–complexed anti-rabbit antibodies (Immunoperoxidase Secondary Detection kit, Chemicon, Temecula, CA).

Apc loss of heterozygosity analysis. DNA was extracted from tumors of 6-week-old mice as described previously (28). The loss of Apc was analyzed by fluorescence-based real-time PCR. PCRs specific for Apc and the Min Apc allele (Apc+/11) were designed. In each DNA sample, the fraction of cells, in which loss of heterozygosity (LOH) occurs, was evaluated by dividing the initial template quantity derived from the Apc allele amplification to that derived from Apc. Apc universal reactions located downstream from the LOH reaction were set to amplify both Apc−/− and Apc−/+ alleles. Apc universal reactions were quantified by real-time PCR and normalized to proliferating cell nuclear antigen (PcnA) or Mlh1 control reactions. A detailed description of the Apc LOH assay and the Apc universal assay in addition to primer sequences are provided in Supplementary Data.

RNA extraction and microarray analysis. Total RNA was isolated from the colonic mucosa of 129/Sv 6-week-old wild-type (WT) mice via the RNeasy Protect Mini kit as described by the manufacturer (Qiagen, Valencia, CA). Total RNA was processed for microarray hybridization using Affymetrix protocols (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Mouse Genome GeneChip 430 2.0 arrays used in the analysis were purchased from Affymetrix (Santa Clara, CA) and analyzed at the Children’s Hospital of Los Angeles biotechnology core facility (Los Angeles, CA). A detailed description of the experimental procedures is provided in Supplementary Data.

RNA expression analysis. Total RNA was isolated as described above. Contaminating DNA was removed by DNase treatment (DNA-free, Ambion, Austin, TX). Total RNA purified from each section was then reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers (Amersham Pharmacia, Piscataway, NJ). Real-time reverse transcription-PCR (RT-PCR) was used to measure gene expression that was normalized to PcnA control reaction. The primer and probe sequences are listed in Table 1 as Supplementary Materials.

Mouse bowel preparation and MicroCAT scanning. Mice were given fresh green leafy vegetables and water ad libitum for 2 days followed by a fluid diet of GoLYTELY solution (Braintree Scientific, Braintree, MA) for 16 hours before MicroCAT imaging. Mice were anesthetized with 2% isoflurane (Isoflurane, Abbott Laboratories, North Chicago, IL) in oxygen and scanned after rectal administration of 60% w/v barium sulfate solution (E-Z-Em Canada, Inc., Anjou, Quebec, Canada). Images were acquired with a MicroCAT scanner (Imetreck, Inc., Knoxville, TN) and then viewed and analyzed with the Metamorph 6.26 program. A detailed description of the experimental procedure is provided in Supplementary Data.

Results

Generation of Apc−/−Smad3 mutant mice. To generate 129/Sv Apc−/−Smad3−/− mice, we first backcrossed C57BL/6 Apc−/− mice to the 129/Sv background for 12 generations (n = 12) and then crossed 129/Sv Smad3−/− females with 129/Sv Apc−/−Smad3−/− males. We monitored the mice with the various genotypes on a
regular basis and found that the majority of the Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice exhibited signs of illness and were moribund by 2 months of age. We did not observe decreased survival in any of the single mutant mice (Apc\(^{Min/+}\) or Smad3\(^{-/-}\)) at this young age. We therefore set up our experiment so that all the mice were euthanized by 60 days or at first sign of morbidity. Our cross produced 179 offspring, of which 20% were Apc\(^{Min/+}\)Smad3\(^{-/-}\) (n = 35), 23% Apc\(^{Min/+}\)Smad3\(^{+/+}\) (n = 41), 10% Apc\(^{Min/+}\)Smad3\(^{+-}\) (n = 18), 13% Apc\(^{Min/+}\)Smad3\(^{+/-}\) (n = 23), 23% Apc\(^{Min/+}\)Smad3\(^{+/+}\) (n = 42), and 11% Apc\(^{Min/+}\)Smad3\(^{-/+}\) (n = 20). The numbers of mice with the six different combinations of Apc and Smad3 genotypes were consistent with the expected Mendelian ratios for independent segregation of two genes, indicating that Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice proceeded normally through embryonic development.

Tumor incidence and multiplicity of Apc\(^{Min/+}\)Smad3 mutant mice. To assess the cause of early morbidity in Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice, we examined the intestines for tumor formation. We found that Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice (Fig. 1A) developed multiple large colonic tumors (majority, >1 mm) by 60 days of age. This phenotype was not observed in Apc\(^{Min/+}\)Smad3\(^{+/+}\), Apc\(^{Min/+}\)Smad3\(^{+/-}\), Apc\(^{Min/+}\)Smad3\(^{+/-}\), or Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice (Fig. 1A). The distribution of the tumors in Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice was surprisingly distinctive. The majority of the tumors were localized to the distal colon, with very few in the proximal colon or cecum (Fig. 1A and B). The boundary between the colonic segments with and without tumors was quite sharp. Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice had a mean tumor multiplicity of 15 in the distal colon compared with 1 in Apc\(^{Min/+}\)Smad3\(^{-/-}\), 0.4 in Apc\(^{Min/+}\)Smad3\(^{+/-}\) mice and Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice, 0.1 in Apc\(^{Min/+}\)Smad3\(^{-/-}\), and 0 in Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice (Fig. 1B). These observations indicate that mutations in Apc and Smad3 cooperate to enhance tumorigenesis in the distal colon in a synergistic manner. The Apc\(^{Min/+}\)Smad3\(^{-/-}\) mouse model appears to mimic human FAP disease with respect to the high multiplicity of the tumors and their predominant distribution in the distal colon. In addition, the data suggest that the major cause of morbidity in Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice may be intestinal obstruction and rectal prolapse (found in 40% of these mice) resulting from large tumors in the distal colon or, more specifically, in the rectal region.

Tumor characteristics in Apc\(^{Min/+}\)Smad3 mutant mice. To examine the colonic tumors in Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice, sections from the distal colon were embedded in paraffin for histologic evaluation. H&E staining of histologic sections of colonic tumors revealed a mixture of benign adenomas, carcinomas in situ, and invasive carcinomas (Fig. 2A and B). Because FAP patients have a significantly increased risk of developing metastatic liver cancer (29), we analyzed H&E-stained and keratin-stained sections of paraffin-embedded liver tissues from six Apc\(^{Min/+}\)Smad3\(^{-/-}\) mice and found no signs of liver micrometastasis (data not shown). In addition, we did not observe any macroscopic or microscopic evidence of tumor invasion to regional lymph nodes (data not shown).

The adenomas that develop in Apc\(^{Min/+}\) mice are characterized by loss of the Apc allele (30), mainly through homologous somatic recombination (31). Because of the cooperativity between the Apc mutation and Smad3 homozygous mutation in our model system, we investigated whether the loss of Apc was still required for the emergence of colonic tumors or whether homozygous mutation of Smad3 could substitute for the loss of Apc in the neoplastic process. To detect the presence or the absence of Apc expression in colonic tumor, we did immunohistochemistry analysis using an NH\(_2\)-terminal mouse Apc-specific antibody. We found low expression of Apc in the tumor compared with the normal surrounding tissues (Fig. 2D). In addition, we assessed the level of β-catenin protein in the tumors previously examined for Apc expression. Mutation of Apc leads to β-catenin stabilization and accumulation in the nucleus (11). We found a strong nuclear staining of β-catenin in the tumors compared with normal surrounding tissues, consistent with loss of Apc function in these tumors (Fig. 2C). This suggests that loss of WT Apc expression is required for tumor formation irrespective of the presence of Smad3 genetic alterations.

To investigate whether LOH is the molecular mechanism underlying the loss of Apc expression in our model, we developed a novel Taqman-based real-time PCR assay using MGB probes to specifically detect LOH at the Apc locus. In this assay, we designed FAM-labeled MGB probes to distinguish between Apc\(^{+}\) and Apc\(^{Min/+}\) mice.
alleles (Fig. 3A). In a situation of heterozygosity, both Apc<sup>+</sup> and Apc<sup>Min</sup> alleles are present, and the ratio of Apc<sup>+</sup> allele to Apc<sup>Min</sup> allele is 1:1. LOH, whether followed by duplication of the Apc<sup>Min</sup> allele, is characterized by the absence of the Apc<sup>+</sup> allele and a ratio of Apc<sup>+</sup> allele to Apc<sup>Min</sup> allele approaching zero, provided there is little stromal contamination (Fig. 3A). In our assay, 30 tumors from Apc<sup>Min/+</sup>/Smad3<sup>−/−</sup> mice were analyzed for the presence of the Apc<sup>+</sup> allele and compared with control tail DNA from Apc<sup>Min/+</sup> (n = 13) and Apc<sup>Min−</sup>/Smad3<sup>−/−</sup> (n = 7) mice (Fig. 3A). In tail DNA, the ratios of Apc<sup>+</sup>/Apc<sup>Min</sup> have a median of 0.99 with a minimum value equal to 0.55. In tumor DNA, the median is 0.33 and the minimum value is 0.16. A ratio of Apc<sup>+</sup>/Apc<sup>Min</sup> equal to zero was not observed in tumor DNA likely because of stromal contamination. We set a threshold for LOH at 0.55, the lowest ratio observed in tail DNA, below which we categorized tumors as having sustained LOH. Using this criterion, 77% (23 of 30) of the tumors showed loss of the Apc<sup>+</sup> allele (Fig. 3A). The difference in mean Apc<sup>+</sup>/Apc<sup>Min</sup> ratio between the tumor and the tail DNA was highly significant (P < 0.0001). The six tumors that did not qualify as having undergone LOH may have had excessive stromal contamination, preventing detection of LOH, or the tumors may have lost Apc function through some other mechanism, such as point mutation or transcriptional silencing. These alternatives were not investigated.

To investigate whether the loss of Apc<sup>+</sup> allele in the tumor samples was accompanied by reduplication of the Apc<sup>Min</sup> locus, we designed an Apc universal reaction, in which the PCR primers and probe can hybridize and amplify both Apc<sup>+</sup> and Apc<sup>Min</sup> alleles. In addition, we designed primers and probe for a control gene on a different chromosome, Pcna, to score for Apc duplication. For example, a ratio of 1:2 of Apc universal to a control gene, such as Pcna indicates LOH without duplication of the Apc<sup>Min</sup> locus, whereas a ratio of 2:2 (1:1) suggests duplication of the Min locus (Fig. 3B). In this assay, we analyzed 23 tumors from Apc<sup>Min/+</sup>/Smad3<sup>−/−</sup> mice that had undergone LOH at the Apc locus and compared them with control tail DNA from WT (n = 7), Apc<sup>Min−/−</sup> (n = 13), and Apc<sup>Min/+</sup>/Smad3<sup>−/−</sup> (n = 7) mice (Fig. 3B). The ratios of Apc<sup>Min</sup>/Pcna have a median value of 1.02 in tail DNA and 0.93 in tumor DNA (Fig. 3B). Apc<sup>Min−/−</sup>/Pcna ratios of tumor DNA from Apc<sup>Min−/−</sup>/Smad3<sup>−/−</sup> mice were not significantly different from those of tail DNA from WT, Apc<sup>Min−</sup>, and Apc<sup>Min−/−</sup>/Smad3<sup>−/−</sup> mice (P = 0.60). These results suggest the presence of two copies and thus duplication of the Apc<sup>Min</sup> allele in the tumors of Apc<sup>Min−/−</sup>/Smad3<sup>−/−</sup> mice.

Transcriptional profiling of normal mucosa. In human colorectal cancer, different risk factors are associated with proximal and distal colon tumor formation. In our mouse model, the inappropriate activation of the Wnt pathway via Apc mutation and the alterations in the TGF-β pathway via Smad3 inactivation have rendered the distal colon considerably more susceptible to tumor formation than the proximal colon. This suggests that the mechanisms and biological pathways associated with tumor initiation and/or progression may differ between the distal and proximal colon. The distinctive tumor distribution also suggests that, in a normal precancerous colon, a different balance of protumorigenic and antitumorigenic factors may exist in the two parts of the colon. On inactivation of Smad3 gene, factors present in the proximal colon could compensate for the lack of Smad3 and thus protect the proximal colon from tumor formation. To investigate this hypothesis, we used transcriptional profiling to analyze gene expression differences between the proximal and distal epithelial colonic mucosa of histologically normal colon from WT mice.

Our primary goal was to investigate expression differences between the proximal and the distal colon focusing on genes directly involved in the TGF-β and Wnt signaling pathways because genes in the somewhat redundant TGF-β pathway in particular would most likely be able to compensate for lack of Smad3 in the proximal colon. For each of these two pathways, we divided the genes into three groups: activators for the pathway...
[Wnt (116 probes for 54 genes) and TGF-β (114 probes for 41 genes)], inhibitors of the pathway [Wnt (56 probes for 21 genes) and TGF-β (64 probes for 26 genes)], and undefined [Wnt (41 probes for 13 genes) and TGF-β (77 probes for 27 genes)]. Information about the Wnt genes were collected based on the description of genes in the "Mouse Genome GeneChip 430 2.0" array provided by Affymetrix and with the help of "The Wnt Homepage" (http://www.stanford.edu/~rnusse/wntwindow.html). The list for TGF-β genes was made based on information from Affymetrix and from recent research and review articles, especially from a review article by Liu in Frontiers in Bioscience (32).

We examined the results from the microarray analysis on a scatterplot to visualize the relationship between Wnt and TGF-β pathway gene expression versus the remaining 34,000 genes on the array. In this plot (Fig. 4), each gene was displayed as an individual point defined by two independent variables, the log2 scale-based proximal colon expression (vertical axis) and the log2 scale-based distal colon expression (horizontal axis). We found that the overall distribution of the TGF-β and Wnt signaling pathways on the plot was not qualitatively different from that of the rest of the gene markers on the array. Nevertheless, this analysis provided us with a subset of Wnt and TGF-β pathway genes that lie off the diagonal on the scatterplot and thus are either overexpressed or underexpressed in the proximal versus the distal colon. Interestingly, we observed several TGF-β activators (Fos, Bmp2, Smad4, and Smad2) with higher expression in the distal colon and several TGF-β inhibitors (Stat1, Nr3c1, and Cav1) with higher expression in the proximal colon. These data suggest that the TGF-β pathway is more active in the distal colonic mucosa. This result is in contrast to a model in which high expression of TGF-β pathway genes would protect the proximal colon from tumorigenesis in the absence of Smad3. Rather, our data suggest that perhaps the distal colon may rely more heavily on the TGF-β pathway for normal growth and differentiation control than does the proximal colon.

**Validation of microarray data.** We used real-time RT-PCR on independent sets of proximal and distal normal mucosal samples from 12 new WT mice to validate the results of the microarray data for a limited number of genes that are involved in the TGF-β or Wnt signaling pathways and that showed a statistically significant difference in mean expression levels between the distal and the proximal colon based on a paired t test analysis.

![Graphical representation of the analysis](image-url)
amounted to 19 genes. Included were Wnt activators (Ccn2, Cdc44, Mmp7, and Pitx2), Wnt inhibitors (Sfrp1, Wnt5a, and Wif1), Wnt undefined (Tcf4 and Tcf3), TGF-β activators (Bmp2, Smad4, Smad2, Fos, and Hipk2), TGF-β inhibitors (Nrc31, Stat1, and Cav1), and TGF-β undefined (Miz1 and Tbrg4). The RT-PCR data were consistent with the microarray data for all of the genes analyzed with the exception of Tcf3, which in the RT-PCR showed a higher expression in the proximal colon versus the distal colon but vice versa in the microarray analysis (Fig. 5A). We have also examined by RT-PCR the expression of the genes in the normal surrounding colonic mucosa from two ApcMin/+ Smad3/C0/C0 mice and found that the disruption in Smad3 and Apc did not alter the expression level of these genes (Fig. 5B).

In vivo imaging of colonic tumors in ApcMin−/− Smad3 mutant mice. The ApcMin−/− Smad3−/− mouse model faithfully mimics FAP cases with respect to the severity of the disease, the presence of numerous tumors, including adenocarcinomas, and the unique distribution of tumors to the distal colon and the rectum. This model seems to be superior to others for investigating the molecular mechanisms of human colorectal carcinogenesis. We were next motivated to determine whether we could apply in vivo imaging tools to noninvasively visualize tumor development in our model and thus to document its usefulness as a system for pharmacologic trials and as a direct preclinical bridge to human trials. Imaging the mouse intestine to detect tumors presents special challenges. Whereas the distal colon is relatively fixed in vivo due to the anal connection, the rest of the intestine is mobile and has a complex geometry, which makes it difficult to define the exact location of tumors. The high multiplicity of the tumors, their large size, the rapid onset, and most importantly, their distribution in the distal colon may make ApcMin−/− Smad3−/− mice an appropriate model for in vivo imaging and a potentially useful system to monitor and evaluate in vivo tumor development in response to therapeutic and preventive agents.

We used small-animal X-ray computed tomography (CT) scanning for the in vivo detection of distal colonic tumors in this mouse model. We focused on noninvasively visualizing and measuring tumors by CT using barium sulfate as a positive-contrast agent. The scans visualized multiple tumors in the distal colon of a 2-month-old ApcMin/+ Smad3/C0/C0 mouse (Fig. 6B) and zero tumors in a WT mouse with the same age and gender (Fig. 6A). CT scanning allows for computer-assisted three-dimensional reconstruction and calculation of tumor volume. Tumor volumes in the ApcMin−/− Smad3−/− mouse varied from 5.1 to 17.8 mm³ (Fig. 6B).

Discussion

We have developed a novel model for human colorectal cancer, the ApcMin−/− Smad3−/− mouse model, resulting from the combined activation of the Wnt pathway and attenuation of the TGF-β pathway. Because Smad3 mutation exhibited a reduced penetrance in the 129/Sv × C57BL/6 hybrid background compared with the 129/Sv background (21) and to minimize the effect of polymorphic modifiers (33) that may impede any cooperation between Smad3 and Apc mutations in intestinal neoplasia, we backcrossed our C57 × 129/Sv background through 10 generations.
Apc<sup>Min</sup>/<sup>+</sup> mice for 12 generations to the 129/Sv background so that both mutations are carried in a homogeneous genetic background. Our mouse model shows a strong enhancement of neoplasia and suggests but does not prove a synergistic cooperation between these two pathways in the distal region of the colon (Fig. 1). Our mouse model mimics the FAP disease, one of the familial forms of colorectal cancer, particularly with respect to the high multiplicity of tumors and their predominant distribution in the distal colon.

Histologic analysis of the distal colonic tumors in Apc<sup>Min</sup>/<sup>+</sup> Smad3<sup>−/−</sup> mice at 2 months of age showed a mixture of adenomas, carcinomas in situ, and invasive carcinomas. We did not detect liver micrometastases in H&E-stained and keratin-stained sections of paraffin-embedded liver tissues from six Apc<sup>Min</sup>/<sup>+</sup> Smad3<sup>−/−</sup> mice, which differs from FAP patients, who have an increased risk of liver metastatic disease (29). This may be the result of the rapid, early tumorigenesis in these mice, providing insufficient time for establishment of metastases before the onset of morbidity.

The mechanism of loss of the Apc<sup>+</sup> allele in adenomas from F1 hybrid Min mice between C57BL/6 and AKR genetic backgrounds has been shown to involve the entire chromosome 18 through mitotic nondisjunction without reduplication (30). In our study, the two Apc universal reactions designed downstream from the Min locus indicate reduplication of the Min allele, a phenomenon also observed in Dpc4 Apc<sup>Min</sup> mice (25).

Functionally significant mutations in SMAD2 have been detected in some human sporadic colorectal cancers (34). Despite the close sequence similarity between SMAD2 and SMAD3, mutations in the SMAD3 gene have not been reported in human colorectal cancer (35). However, the expression of SMAD3 was shown to be suppressed in a subset of human gastric cancers and human gastric cell lines, most likely due to aberrant expression of SMAD3 inhibitors (36). The transfection of SMAD3 in SMAD3-deficient gastric cancer cells restored TGF-β signaling and induced a tumor suppressive activity (36). Smad2 and Smad3 differ in their effect on mouse tumorigenesis. Homozygous Smad3-deficient mice are embryonic lethal, whereas heterozygous Smad2 mice show no sign of tumor development (16, 17). On the other hand, homozygous Smad3-deficient mice in the 129/Sv background develop a low multiplicity of invasive colonic adenocarcinomas. Apc<sup>Min</sup>/<sup>+</sup> mice with WT Smad3 develop adenomas mainly in the small intestine. The introduction of Smad3 mutations in the Min model leads to a high tumor multiplicity in the distal colon, suggesting that Smad3 expression in the Min mouse protects against the type of distal colonic tumors seen in human FAP. The pattern of tumor development in Apc<sup>Min</sup>/<sup>+</sup> Smad3<sup>−/−</sup> mice shows that the distal colon is more prone to tumor initiation and progression when both of these two pathways are altered.

Our model supports evidence from human sporadic colorectal cancer that proximal and distal disease of the colon have different etiologies and that they broadly follow distinct molecular pathways of carcinogenesis (2, 37). The difference between proximal and distal tumors could be due in part to the fact that the two regions of the colon have different embryonic origin (midgut and hindgut, respectively) and vascular supplies (superior and inferior mesenteric artery, respectively; ref. 2). Two forms of genetic instability have been described in colorectal cancer: chromosomal instability (CIN), and microsatellite instability (MIN; ref. 38). Sporadic colonic tumors that show MIN are primarily located in the proximal colon, whereas tumors with CIN are distributed mostly in the distal colon (39, 40). Mutations in the APC gene, the KRAS gene and the P53 gene are primarily involved in CIN tumors but to a lesser extent in MIN tumors. A frequent event in CIN tumors is the loss of material from distal chromosome 18 (18q deletions) that includes the SMAD genes in the TGF-β pathway (39). The progression of tumors in FAP and sporadic CIN cancer is likely to implicate the same genes (39).

Figure 5. Scatterplot analysis of the RT-PCR data of the few Wnt and TGF-β genes that do not lie on the diagonal of the microarray data scatterplot (Fig. 4). A, gene expression in colonic mucosa of twelve 129/Sv 6-week-old WT mice. B, gene expression in normal surrounding colonic mucosa of two Apc<sup>Min</sup>/<sup>+</sup> Smad3<sup>−/−</sup> mice. RT-PCR data were normalized to PcnA gene expression. Each gene is represented as an individual dot defined by two independent variables, the log<sub>2</sub> scale-based proximal colon expression (Y axis) and the log<sub>2</sub> scale-based distal colon expression (X axis). Gene color-coding is as described in Fig. 4. The gene expression data in the scatter plot were 200 times their initial value.
The molecular mechanism behind the distinct susceptibilities of the proximal and the distal colon to tumor formation in this murine context and in sporadic human colorectal carcinogenesis is unknown (2). The molecular basis for these differing predispositions to cancer could be reflected by distinct gene expression patterns between the two organs (41). Using microarray technology, we examined the transcriptional profile of the distal and the proximal colonic epithelium in WT mice and focused on the expression of genes that are involved in either the Wnt or the TGF-β pathways, the two key signaling pathways required for gut development and homeostasis (42, 43). The overall distribution of the Wnt or the TGF-β pathway genes was not significantly different from the rest of the genes in the array. However, we found a few genes, such as Smad4, Smad2, Fos, and Bmp2, which are activators of the TGF-β pathway, to be overexpressed in the distal colon relative to the proximal colon. Components of the TGF-β signaling pathway are usually localized in differentiated epithelia and have growth-inhibitory effects (44). More specifically, they are associated with growth suppression in the gut, thus counteracting the Wnt signaling pathway (43). The TGF-β-mediated cell growth arrest results in inactivation of cyclin-dependent kinases and down-regulation of c-myc (42, 45). TGF-β is also documented to induce apoptosis in various cell types, including colonic epithelial cells (45, 46). The biochemical mechanism underlying the death process is poorly understood but it is thought to involve two independent pathways, the Daxx adaptor pathway and the Smad pathway (45).

Compared with the proximal colon, the epithelial cells of the human distal colon have a higher apoptotic index that is associated with the expression of the proapoptotic Bcl-2 family member Bak, indicating that the colonocytes in the proximal colon may be lost by other routes than apoptosis (47). In addition, the average crypt length is greater in the distal colon than in the proximal colon (48). The existence of a proliferation gradient among the different regions of the colon is still controversial (2). In rats, the location of the proliferative zone in the crypt varies between the proximal and the distal colon (49). In our system, we propose that the distal colon relies more heavily on the TGF-β signaling pathway for growth control than does the proximal colon and that alterations in this pathway secondary to Smad3 knockout predispose the distal colon toward neoplasia by creating a state of imbalance between epithelial cell proliferation and cell death by apoptosis and other routes. When examining the normal surrounding colonic mucosa in ApcMin/+Smad3−/− mice, we found no change in the expression level of the TGF-β and Wnt genes that were informative by microarray. Whether the disruption in Smad3 and Apc altered the activity and the stability of these genes at the protein level is worthy of further investigation. A recent study has shown that colon cancer in Smad3−/− mice only occurs in the presence of Helicobacter, such as Helicobacter bilis and Helicobacter hepaticus; on the other hand, infection with these Helicobacter species had no effect on ApcMin/+ mice (50). H. bilis is endemic in our mouse colony, as it is the case in most research mouse facilities (51), and is not considered pathogenic in healthy WT mice. Tumorigenesis in our model may depend on the presence of such commensals.

Because of its similarity to human polyposis, the ApcMin/+ Smad3−/− mouse model provides an opportunity to advance the

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**Figure 6.** In vivo CT scans of an Apc+/+Smad3+/+ mouse (A) and an ApcMin/+Smad3−/− mouse (B), both at 2 months of age. In both mice, the colon was filled with barium-contrast material before imaging. A and B, two-dimensional, average maximum plus minimum, projections through the colon. a and c, left and right sides of each mouse, respectively; b, anterior views. Arrows, distal colonic tumors in the ApcMin/+Smad3−/− mouse. Tumor volumes were obtained by summing the areas of regions of interest drawn to outline the tumors on transaxial images using the original two-dimensional slices.
understanding of human colorectal cancer. This model may be useful in generating preclinical data for human pharmacologic trials. It may also enhance our understanding of the mechanisms of genesis and progression of colorectal cancer. The use of small-animal imaging technology to noninvasively monitor tumor development in our model will help us to study the genetics and kinetics of colorectal cancer, which is conceptualized as an adenoma-cancer sequence. We used small-animal CT with our model and found that this technique is effective for detecting and measuring the tumors in vivo. This and other anatomic as well as functional small-animal imaging modalities could be used for noninvasive monitoring of tumors in our model during preclinical pharmacologic studies.

Conversely, the model could be useful for preclinical testing of novel imaging probes for colon cancer.

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