Tgfbr1 Haploinsufficiency Is a Potent Modifier of Colorectal Cancer Development

Qinghua Zeng, Sharbani Phukan, Yanfei Xu, Maureen Sadim, Diana S. Rosman, Michael Pennison, Jie Liao, Guang-Yu Yang, Chiang-Ching Huang, Laura Valle, Antonio Di Cristofano, Albert de la Chapelle, and Boris Pasche

Division of Hematology/Oncology, Department of Medicine and Comprehensive Cancer Center, The University of Alabama at Birmingham, Birmingham, Alabama; Cancer Genetics Program, Division of Hematology/Oncology, Department of Medicine; Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine; Department of Pathology; Department of Preventive Medicine, Northwestern University, Chicago, Illinois; Human Cancer Genetics Program, The Ohio State University Comprehensive Cancer Center, Columbus, Ohio; Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York

Abstract

Transforming growth factor-β (TGF-β) signaling is frequently altered in colorectal cancer. Using a novel model of mice heterozygous for a targeted null mutation of Tgfbr1 crossed with ApcMin/+ mice, we show that ApcMin/+;Tgfbr1−/− mice develop twice as many intestinal tumors as ApcMin/+;Tgfbr1+/+ mice, as well as adenocarcinoma of the colon, without loss of heterozygosity at the Tgfbr1 locus. Decreased Smad2 and Smad3 phosphorylation and increased cellular proliferation are observed in the colonic epithelium crypts of ApcMin/+;Tgfbr1−/− mice. Smad-mediated TGF-β signaling is preserved in both ApcMin/+;Tgfbr1−/− and ApcMin/+;Tgfbr1+/− intestinal tumors, but cyclin D1 expression and cellular proliferation are significantly higher in ApcMin/+;Tgfbr1−/− tumors. These results show that constitutively reduced Tgfbr1-mediated TGF-β signaling significantly enhances colorectal cancer development and results in increased tumor cell proliferation. These findings provide a plausible molecular mechanism for colorectal cancer development in individuals with constitutively altered TGFBR1 expression, a recently identified common form of human colorectal cancer. [Cancer Res 2009;69(2):678–86]

Introduction

There is growing evidence that constitutive and somatically acquired alterations in transforming growth factor-β (TGF-β) signaling are associated with colorectal cancer risk and disease progression. Germ-line mutations of the SMAD4 and BMPR1A genes are associated with juvenile polyposis (1), and common and functionally relevant alleles of SMAD7 influence colorectal cancer risk (2). There is also evidence that loss of SMAD signaling in human colorectal cancer is associated with advanced disease and poor prognosis (3). Analysis of 13,023 genes in human colorectal cancers has shown that four of the 69 most frequently mutated genes are constitutive elements of the TGF-β signaling pathway: TGFBR2, SMAD2, SMAD3, and SMAD4 (4).

The central role of impaired TGF-β and bone morphogenic protein (BMP) signaling in colon cancer development and progression was first shown in animal experiments by the use of cis-Apc−/−/− Smad4−/− compound mutant mice (5). In the compound mutant mice, complete loss of Smad4-dependent TGF-β signaling causes intestinal adenomas to develop into adenocarcinomas. Other animal experiments have shown that complete loss of Tgfbr2 in intestinal epithelial cells promotes the invasion and malignant transformation of tumors (6). Complete Smad3 deficiency promotes tumorigenesis in the distal colonic ApcMin/+ mice (7), and complete loss of Smad4-dependent signaling in T cells has been shown to increase spontaneous gastrointestinal tumorigenesis (8). Whereas increased gastrointestinal tumor susceptibility has not yet been reported in Tgfbr1−/−, Tgfbr2−/−, Smad2−/−, or Smad3−/− mice, Smad4−/− mice are predisposed to the development of late-onset polyps in the upper gastrointestinal tract (9–11). Whether haploinsufficiency of any of the TGF-β genes contributes to colorectal cancer development is unknown.

We have previously identified TGFBR1*6A, which encodes a common human TGFBR1 variant (12) and transduces TGF-β signaling less effectively than TGFBR1 (13, 14). Cancer risk is higher for TGFBR1*6A homozygotes than for TGFBR1*6A heterozygotes among patients with hereditary colorectal cancer and no evidence of mismatch repair deficiency, which suggests that constitutively decreased TGF-β signaling modifies cancer risk (15, 16). These findings led us to hypothesize that decreased Tgfbr1-mediated TGF-β signaling may be a modifier of cancer susceptibility (17). Here we report on a novel Tgfbr1−/− mouse model generated to test the hypothesis that constitutively decreased Tgfbr1 signaling is causally involved in colorectal cancer development. When Tgfbr1−/− mice in mixed 129SvIm/C57BL/6 background were crossed with ApcMin/+ mice, a significantly higher number of tumors was observed in Apc−/−;Tgfbr1−/− mice than in Apc−/−;Tgfbr1+/− mice. These findings confirmed our hypothesis and prompted us to investigate the relevance of these findings in humans. We considered TGFBR1 to be a notable candidate for a gene that, when mutated, causes predisposition to colorectal cancer or acts as a modifier of other genes resulting in a predisposition. This led to the discovery that 12% of patients with colorectal cancer and 1.5% of healthy controls have evidence of germ line decreased TGFBR1 expression (18). Thus, this novel phenotype likely accounts for a significant proportion of human colorectal cancers (18). This report describes new mechanistic insights into the role of Tgfbr1 signaling in colorectal cancer development both in mixed 129SvIm/C57BL/6 and pure C57BL/6 backgrounds with significant implications for human colorectal cancer.
Materials and Methods

Generation of a targeted Tgfb1 mouse model. Using mouse genomic DNA as a template, we designed Tgfb1 primers amplifying a 491-bp fragment spanning from position 27 (exon 1) to position 517 (exon 3). Using an isogenic 129SvIm genomic library (Stratagene), we picked several clones, grew them, and excised the insert by NotI cleavage. Two overlapping clones were obtained that spanned this genomic region. We found a NotI site 5 bp downstream of the ATG start codon. The targeting vector has been designed to insert the neomycin resistance cassette (Neo) immediately upstream of this NotI site (Fig. 1A). Following transfection and selection of 129SvIm embryonic stem cells, knockout clones were karyotyped and injected into C57BL/6 blastocysts. Germ-line transmission from the resulting chimeras was obtained and a minimum of eight Tgfb1+/+ knockout clones were obtained that spanned this genomic region. We found a NotI site located immediately after the start codon and removing 1.1 kb mouse genomic sequence immediately upstream of this NotI site (Fig. 1A).

Histopathology of intestinal polyps and polyp scoring. The number and size of polyps were scored by two examiners. Tissue specimens were prepared according to standard protocols. Polyps from seven randomized mice from each group were sectioned and stained with H&E to differentiate tumors from lymphoid aggregates.

Mouse embryonic fibroblasts. Mouse embryonic fibroblasts (MEF) were collected at embryonic day 12.5 according to standard protocol (19) and cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin (20).

Spontaneous cell proliferation assays. MEFs were seeded in normal growth medium at a concentration of 5 x 10³ per well in six-well plates on day 1. Cultures were counted on days 1 and 3 of growth. Colony formation assays were performed using 5 x 10³ MEFs per six-well plate. Colonies were counted after 10 days. The number of colonies per well was calculated and expressed as a percentage of the control media.

Histology. Tissues from the small intestine were fixed in 10% formalin for 24 hours and then embedded in paraffin. Sections were stained with H&E or Masson's trichrome for collagen.

Figure 1. Generation of a novel Tgfb1 exon 1 knockout mouse model. A, strategy for interrupting the Tgfb1 open reading frame by insertion of a Neo cassette. A classic targeting vector was generated by inserting a Neo cassette immediately upstream of the Tgfb1 open reading frame and removing 1.1 kb of mouse genomic sequence immediately upstream of this NotI site (Fig. 1A). B, PCR genotyping of Tgfb1+/− mice using three primers reveals a band at 240 bp, corresponding to the knocked out allele, and the wild-type Tgfb1 band at 314 bp. C, quantitative reverse transcription-PCR assessment of Tgfb1 expression levels in MEFs (M), colon intestinal tissue (C), tail (T), and peripheral lymphocytes (L) of Tgfb1+/− and Tgfb1−/− mice. Tissues were collected from three animals of each genotype. Each experiment was done at least three times in triplicates. Tgfb1 levels in Tgfb1−/− tissues are expressed as ratio of Tgfb1/Gapdh compared to each corresponding Tgfb1+/+ tissue. D, Western blot analysis of Tgfb1 and Tgfb2 expression of two representative pairs of MEFs from Tgfb1−/− and Tgfb1+/− mice. A, B, C, D. Western blot analysis of Tgfb1 and Tgfb2 expression of two representative pairs of MEFs from Tgfb1−/− and Tgfb1+/− mice.
day 0. Cell number was determined by trypsinizing and counting cells on days 1, 2, and 3.

**TGF-β-mediated cell proliferation assays.** TGF-β-mediated cell growth inhibition was assessed by [3H]thymidine incorporation assays as previously described (21).

**Luciferase assays.** The 3TP-Lux and SBE4-Lux reporter constructs were gifts from Dr. Joan Massagué (Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY) and Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The experiments were done as described before (22).

**Immunoblotting and immunohistochemistry.** Nuclear extracts from MEFs were obtained using a NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Inc.). Cell lysates were collected in lysis buffer [TNT buffer (10 mM Tris pH 8.0, 1% Triton X-100, 1 mM EDTA, 150 mMol/L NaCl) supplemented with phosphatase inhibitor cocktails 1 and 2 and protease inhibitor cocktail (Sigma)] and centrifuged at 14,000 × g for 15 min. All lysates were separated in SDS-PAGE gels (Invitrogen) and transferred onto nitrocellulose membrane (GE Healthcare). Immunoblotting was done using the following antibodies: rabbit anti-Tgfbr1, anti-cyclin D1, anti-TGFBR2, anti-p15, anti–cyclin-dependent kinase (Cdk)-4, mouse anti-Cdk2, anti-Cdk6, anti-p21, anti-p27, and anti–histone 1 (Santa Cruz Biotechnology); rabbit anti–pSmad2 (Cell Signaling Technology); and rabbit anti-pSmad3 (a gift from Dr. Koichi Matsuzaki, Kanzai Medical University, Osaka, Japan). Signal detection was measured by SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific). Films were scanned and densitometry was done using Fujifilm LAS-3000 (Fuji Medical System).

**Immunohistochemistry.** Immunohistochemistry was done with the DAKO EnVision System. Percentage of positively stained cells was determined by assessing the number of strongly positive stained cells of the total number of cells in a field. Five representative fields in three different samples were assessed.

**Loss of heterozygosity analyses.** The SNaPshot method (PE Applied Biosystems) was used to identify each allele and to detect loss of heterozygosity (LOH) in tumor DNA.

**Statistical analysis.** Data were analyzed by Student's t test and are expressed as mean ± SE. P < 0.05 was considered significant. All tests were two-tailed. Data were transformed in logarithm scale when normality assumption was violated. One-way ANOVA was used for the analysis of Tgfbr1 expression in various tissues (Fig. 1C). Chi-square analysis was used to compare the proportion of intestinal tumors in Tgfbr1+/− and Tgfbr1−/− mice and the proportion of colonic tumors in ApcMin+/−:Tgfbr1+−/− and ApcMin−/−:Tgfbr1−/− mice.

**Results**

**Generation of a novel mouse model of targeted Tgfbr1 inactivation.** A knockout mouse model of Tgfbr1 generated by targeted deletion of exon 3 has been previously described (23). There is growing evidence that the signal sequence of human TGFBR1 6A may have intrinsic biological effects, which are caused by mutations within the exon 1 GCC repeat sequence (21, 22). Whereas the exon 3 Tgfbr1 knockout model does not result in the generation of functional Tgfbr1 (23), the generation of a functionally active signal sequence cannot be excluded. To circumvent this potential problem, we designed a classic knockout vector to insert a Neo cassette into a NotI site located immediately after the start codon and removing 1.1 kb of mouse genomic sequence immediately upstream of this NotI site (Fig. 1A). This approach precludes the generation of any signal sequence, which is encoded by part of the removed sequence. The Tgfbr1−/− mice were viable and fertile and appeared normal in their morphology and behavior. A total of 50 pups from the heterozygous intercrosses were genotyped and no Tgfbr1−/−−/− pups were found, with only the wild-types and the heterozygotes at a ratio of 1:2. Dead Tgfbr1−/− embryos were found at a ratio of 1:4 at the time of collection of MEFs. These findings are consistent with the previous report of targeted disruption of Tgfbr1 exon 3 in which mice lacking Tgfbr1 die at midgestation (23). We did not therefore attempt to determine the stage of lethality. At 16 months, follow-up of 10 Tgfbr1−/−/− mice does not suggest increased mortality as compared with 10 wild-type littermates.

Tgfbr1 expression levels in different tissues were first compared by real-time PCR. Tgfbr1 expression in Tgfbr1−/− mice tissues ranged from 54% in embryonic fibroblasts to 62% in colon epithelium, 44% in tail, and 67% in blood lymphocytes when compared with corresponding expression levels in Tgfbr1+/− mice (Fig. 1C). Tissue-specific differences between Tgfbr1−/− and Tgfbr1+/− mice were significant for each corresponding tissue: P = 0.016 for embryonic fibroblasts, P = 0.04 for colon epithelium, P = 0.009 for tail, and P = 0.01 for blood lymphocytes. The differences in Tgfbr1 expression levels between the various Tgfbr1−/− tissues were not statistically significant, P = 0.429. To assess the functional consequences of Tgfbr1 haploinsufficiency, we measured Tgfbr1 and Tgfbr2 protein expression in MEFs. Tgfbr1 expression levels were lower in the Tgfbr1−/− MEFs than in Tgfbr1+/− MEFs (Fig. 1D). As expected, Tgfbr2 levels were similar (Fig. 1D).

**Tgfbr1 haploinsufficiency enhances tumor formation.** Because the gastrointestinal tract is a common site of cancer in humans with constitutively altered TGF-β signaling (1, 16), we tested the effect of Tgfbr1 haploinsufficiency on ApcMin−/−-mediated intestinal tumorigenesis. ApcMin−/− mice harbor a premature stop codon in one allele of the Apc tumor suppressor gene (ApcMin−/−). These mice develop multiple intestinal adenomas and mimic human familial adenomatosis polyposis coli (24, 25). These mice were crossed into the C57BL/6 using speed congenics markers (Fig. 2A). F2 Tgfbr1+/− females were crossed with ApcMin−/− male mice (C57BL/6). Mice were sacrificed at 12 weeks and examined for intestinal tumors. The tumors counted were verified by histology. We did not observe any tumors in the small and large bowels of eight Tgfbr1+/− and nine Tgfbr1−/− mice in wild-type Apc background. A total of nine ApcMin−/−:Tgfbr1+/− mice developed an average of 5.4 ± 1.7 tumors (mean ± SE), whereas the number of tumors observed in 10 ApcMin−/−:Tgfbr1−/− mice was almost three times higher, 14.5 ± 1.1 tumors (Fig. 2A). The difference in the number of tumors between the two groups was highly significant, 9.8 tumors (95% confidence interval, 4.8–13.4; P = 0.0004). The majority of tumors was small (<3 mm) and predominantly scattered in the small intestine. Five ApcMin−/−:Tgfbr1+/− mice (50%) had an average of 2.4 ± 0.2 colonic tumors whereas only two ApcMin−/−:Tgfbr1−/− mice (22%) had one colonic tumor each, a nonsignificant difference, P = 0.437. The identity of each lesion as tumor rather than lymphoid aggregates was confirmed in seven mice from each group by histopathology.

To determine the reproducibility of our initial findings obtained in a mixed 129SvIm × C57BL/6 background in 2006, we repeated these experiments with Tgfbr1+/− mice, which were fully backcrossed into the C57BL/6 using speed congenics markers (Supplementary Table S1). As seen in Fig. 2B, there was an average of 30.2 ± 0.9 tumors in 12 ApcMin−/−:Tgfbr1+/− mice and 61.4 ± 3.4 tumors in 7 ApcMin−/−:Tgfbr1+/− mice (mean ± SE). The difference in the number of tumors between the two groups was highly significant, 31.2 tumors (95% confidence interval, 25.3–37.2; P = 4.8 × 10−5). Importantly, the number of colonic tumors was higher among ApcMin−/−:Tgfbr1+/− mice (49.0 ± 0.3) than among ApcMin−/−:Tgfbr1−/− mice (30.4 ± 0.3), P = 0.0005.
Six Apc<sup>Min</sup>/Tgfbr1<sup>+/+</sup> mice (three in the mixed background and three in the pure C57BL/6 background) exhibited large colonic tumors with a maximal diameter >7 mm (Fig. 2C). Histologic analysis of these polyoid and ulcerated colonic tumors revealed the presence of carcinoma (Fig. 2D; Supplementary Fig. S3) as evidenced by the presence of distinct cytologic and nuclear atypia. The largest tumors in the Apc<sup>Min</sup>/Tgfbr1<sup>+/+</sup> mice with colonic tumors >7 mm (35.3%) harboring carcinoma. Among all mice examined at 12 weeks, the proportion of Apc<sup>Min</sup>/Tgfbr1<sup>+/+</sup> mice with colonic tumors ≥3 mm in size, and none of them harbored carcinoma. Among all mice examined at 12 weeks, the proportion of Apc<sup>Min</sup>/Tgfbr1<sup>+/+</sup> mice with colonic tumors ≥7 mm (35.3%) harboring carcinoma.

Next, we studied the effects of Tgfbr1<sup>+/+</sup> and Tgfbr1<sup>−/−</sup> MEFs from Apc<sup>Min</sup>/Tgfbr1<sup>+/+</sup> mice on cell proliferation using MEFs from Tgfbr1<sup>−/−</sup> and Tgfbr1<sup>+/+</sup> mice. In the absence of TGF-β, the growth of Tgfbr1<sup>−/−</sup> and Tgfbr1<sup>+/+</sup> MEFs was identical (Fig. 3A). In the presence of exogenously added TGF-β, the proliferation of Tgfbr1<sup>−/−</sup> MEFs decreased by 58.24 ± 3.44% whereas that of Tgfbr1<sup>+/+</sup> MEFs decreased by 58.24 ± 5.74% (Fig. 3B), P = 0.0005. To directly analyze the signaling activity of Tgfbr1<sup>−/−</sup> and Tgfbr1<sup>+/+</sup> MEFs, we used as readouts the TGF-β reporter 3TP-lux (26) and the TGF-β reporter SBE4-Lux (27). As seen in Fig. 4A, following addition of TGF-β to the cell culture medium, induction of TGF-β signaling was significantly higher for Tgfbr1<sup>−/−</sup> than Tgfbr1<sup>+/+</sup> MEFs for 3TP-Lux (3.62-fold versus 2.73-fold; P = 0.02) and SBE4-Lux (5.76-fold versus 4.47-fold; P = 0.04).

The differences between Tgfbr1<sup>+/+</sup> and Tgfbr1<sup>−/−</sup> with respect to the induction of SBE4-Lux and 3TP-Lux on exposure to TGF-β were almost similar, 24.6% and 22.4%, respectively.

Because the TGF-β signaling pathway is a potent regulator of hematopoietic differentiation (28) and because alterations in lymphocyte TGF-β signaling have been implicated in colorectal tumor progression in mice (8, 29), we sought to determine whether Tgfbr1 haploinsufficiency had any measurable effects on the hematopoietic compartment. Complete blood counts of five Tgfbr1<sup>−/−</sup> and five Tgfbr1<sup>+/+</sup> mice obtained at 12 weeks did not reveal any difference in the average RBC, WBC, or platelet numbers, thus indicating that Tgfbr1 haploinsufficiency alone does not significantly alter hematopoiesis. The average lymphocyte count was 13.11 ± 0.31 and 12.73 ± 0.55 (mean ± SD) for Tgfbr1<sup>−/−</sup> and Tgfbr1<sup>+/+</sup> mice, respectively, a nonsignificant difference (P = 0.181).

Tgfbr1 haploinsufficiency impairs Smad2 and Smad3 signaling. We first assessed the levels of TGF-β-mediated generation of pSmad2 in Tgfbr1<sup>−/−</sup> and Tgfbr1<sup>+/+</sup> MEFs over 24 hours. Whereas pSmad2 levels were almost identical at 1 and 4 hours, pSmad2 levels decreased by ~50% at 8 hours and by 80% at 24 hours in Tgfbr1<sup>−/−</sup> MEFs, whereas they decreased only slightly in Tgfbr1<sup>+/+</sup> MEFs (Fig. 4A). It has been previously shown that phosphorylation of Smad3 is an essential step in signal transduction by TGF-β for inhibition of cell proliferation (30), and Smad3-deficient mice are prone to colon cancer development (7, 31). To assess the effect of Tgfbr1 haploinsufficiency on the phosphorylation of Smad3, we used an antibody targeting the Ser<sup>127/129</sup> site on Smad3 (32, 33). As seen in Fig. 4B, following exposure to TGF-β,
pSmad3 levels were higher at 1 and 16 hours in Tgfbr1+/+ MEFs than in Tgfbr1−/− MEFs. Hence, Tgfbr1 haploinsufficiency was associated with a small but significant decrease in TGF-β signaling mediated by decreased phosphorylation of both Smad2 and Smad3.

**Downstream effects of decreased Tgfbr1-mediated signaling in vitro.** To dissect the downstream effects of decreased TGF-β signaling, we assessed the expression levels of selected mediators of the cell cycle and downstream effectors of TGF-β signaling. As seen in Fig. 4C, there was no difference in the levels of these mediators in the absence of TGF-β with the exception of mildly decreased baseline levels of Ccn1 in Tgfbr1−/− MEFs when compared with Tgfbr1+/+ MEFs. This differential expression pattern was significantly enhanced following exposure to TGF-β as exemplified by reduced Ccn1 expression in Tgfbr1−/− MEFs after 4 hours, whereas Ccn1 levels initially increased and remained elevated at 16 hours in Tgfbr1+/+ MEFs (Fig. 4C). Levels of Cdkn2b remained unchanged on exposure to TGF-β in Tgfbr1+/+ MEFs, whereas we observed a small decrease in Cdkn2b levels in Tgfbr1−/− MEFs. The emergence of differential expression of pSmad2 (Fig. 4A), pSmad3 (Fig. 4B), and Ccn1 (Fig. 4C) levels occurred in parallel, which suggests that decreased Smad signaling results in persistently high Ccn1 levels in Tgfbr1−/− MEFs.

**Characterization of Tgfbr1 haploinsufficiency effects on the intestinal epithelium.** To characterize the in vivo consequences of constitutively decreased TGF-β signaling, we performed pSmad2 immunostaining of normal-appearing intestinal tissue and tumor sections. Whereas pSmad2 staining was homogeneous throughout the intestinal mucosa of ApcMin−/+;Tgfbr1+/+ mice (Fig. 5A), we observed reduced pSmad2 staining in the crypts but not in the villi of ApcMin−/+;Tgfbr1−/− mice (Fig. 5B). To comprehensively assess the effect of Tgfbr1 haploinsufficiency on Smad-mediated TGF-β signaling, we also performed pSmad3 immunostaining of the same tissues. As seen in Fig. 5C, we observed homogeneous pSmad3 staining in the crypts of ApcMin−/+;Tgfbr1+/+ mice, whereas pSmad3 staining was markedly reduced in the crypts of ApcMin−/+;Tgfbr1−/− mice (Fig. 5D), mirroring the pSmad2 findings and showing that Tgfbr1 haploinsufficiency results in decreased phosphorylation of both receptor Smads within the intestinal epithelial crypts, thus resulting in overall decreased Smad-mediated TGF-β signaling in vivo. To determine whether the differential expression of Smads within the intestinal crypts modifies cellular proliferation in vitro, we assessed the levels of proliferating cell nuclear antigen (PCNA) in the normal intestinal epithelium of ApcMin+/ApcMin−/+;Tgfbr1+/+ and ApcMin−/+;Tgfbr1−/− mice. PCNA staining was significantly more intense in ApcMin−/+;Tgfbr1−/− mice (62.2 ± 2.2% positive staining; Supplementary Fig. S4B) than in their wild-type counterpart (44.4 ± 2.8% positive staining; Supplementary Fig. S4A; P = 0.008), thus confirming in vivo the observed in vitro increased cellular proliferation of Tgfbr1−/− on exposure to TGF-β.

**Characterization of Tgfbr1 haploinsufficiency effects on intestinal tumors.** Tumors arising from both ApcMin−/+;Tgfbr1+/+ and ApcMin−/+;Tgfbr1−/− mice had uniform pSmad staining, reflecting preserved in vivo Smad signaling. However, we found focal areas of decreased pSmad2 staining among ApcMin−/+;Tgfbr1+/+ mice (Fig. 6A) and ApcMin−/+;Tgfbr1−/− mice (Fig. 6B) but not in their wild-type counterparts (Fig. 6A). Consistent with the findings of preserved TGF-β signaling activity in the tumors of both ApcMin−/+;Tgfbr1+/+ and ApcMin−/+;Tgfbr1−/− mice, we found no evidence of Tgfbr1 LOH in six microdissected colonic tumors from three different ApcMin−/+;Tgfbr1−/− mice (Supplementary Table S2). The combined evidence from pSmad2 immunohistochemistry and LOH analysis of intestinal tumors shows that reduced dosage, rather than abrogation of Tgfbr1-mediated Smad signaling, is sufficient to enhance the TGF-β-mediated development of intestinal tumors and adenocarcinoma at 12 weeks.
The role of Ccnd1 as a mediator of colon cancer development and progression is reflected by the fact that decreased Ccnd1 expression reduces tumor formation in ApcMin/+ mice (34). Conversely, the role of the Wnt pathway in promoting intestinal stem cell proliferation has been previously documented (35). Located in the intestinal crypts, stem cells constantly generate progeny that differentiate as they flow upward to the tip of the villi, where they die within days. T-cell factor–mediated induction of Ccnd1 expression reduces tumor formation in ApcMin/+ mice (34).

To assess the downstream effects of decreased Tgfbr1-mediated TGF-β signaling on Ccnd1, we measured the levels of Ccnd1 by immunohistochemistry and found that Ccnd1 staining was significantly more intense in the tumors of ApcMin/+/Tgfbr1+/+ mice (50.7 ± 4.1% positive staining; Fig. 5D) than in those of ApcMin/+/Tgfbr1+/− mice (20.1 ± 5.7% positive staining; Fig. 5C; P = 0.002). To determine whether Tgfbr1 haploinsufficiency modifies tumor proliferation in vivo, we measured the levels of Ccnd1 by immunohistochemistry and found that Ccnd1 staining was significantly higher in the tumors of ApcMin/+/Tgfbr1+/− mice (34).

Discussion
The significant difference in the number of intestinal tumors observed in both mixed 129SvIm × C57BL/6 and pure C57BL/6 backgrounds provides strong support for the novel concept that decreased Tgfbr1-mediated signaling results in the enhanced cell proliferation of normal-appearing intestinal epithelial cells within the crypts as well as tumor cells in the presence of preserved TGF-β signaling. Similarly to what was originally observed with the cis-Apc+/Δ716 Smad4−/− mice in which TGF-β signaling is completely abrogated (5), we found essentially the same results with the F3 (C57BL/6) backcross generation and the fully backcrossed (C57BL/6) generation, except for higher intestinal polyp numbers. It has been previously hypothesized that the reduced polyp numbers in mice with a mixed 129SvIm × C57BL/6 background is presumably due to the background gene(s) brought in from the 129SvIm strain (5). Immunohistochemistry analysis shows that PCNA levels were inversely correlated with pSmad2 and pSmad3 levels in the intestinal crypts, providing strong support for the notion that increased cellular proliferation is a direct consequence of decreased pSmad2/pSmad3-mediated signaling.

Existing mouse intestinal tumor models based on somatic Apc inactivation display mainly small intestinal lesions, and carcinomas are rare (36, 37). Inactivation of one copy of the Smad4 gene accelerated tumor progression from intestinal polyps to adenocarcinoma in compound heterozygous cis-Apc+/Δ716 Smad4−/− mice, whereas control cis-Apc+/Δ716 mice developed adenomas but not adenocarcinomas (5). However, tumor epithelial cells in cis-Apc+/Δ716 Smad4−/− mice carry homozygous mutations in both Apc and Smad4, and there is no evidence of Smad4 protein expression in the colorectal tumor cells (38). This results in complete abrogation of Smad-mediated TGF-β signaling within intestinal tumors. Similar results have been reported in mice in which the Tgfbr2 allele was knocked out in the intestinal epithelium (6). In both models, complete abrogation of TGF-β signaling was required to induce malignant transformation of...
intestinal neoplasms initiated by Apc mutation. Our findings constitute the first report of decreased but not abrogated TGF-β signaling, resulting in adenocarcinoma formation at 3 months. It is also the first report of constitutively altered but not abrogated TGF-β signaling upstream of Smad4 associated with increased colorectal tumor development. These results provide strong evidence that constitutively altered Tgfbr1-mediated TGF-β signaling is a potent modifier of colorectal carcinogenesis. Our initial results with mice bred in a mixed background prompted us to investigate the relevance of this novel concept in human colorectal carcinogenesis. This led to the discovery that germ-line decreased expression of TGFBR1 is a quantitative trait that occurs in 10% to 20% of patients with microsatellite instability-negative colorectal cancer and in 1% to 3% of healthy controls (18). This trait is dominantly inherited, segregates in families, and confers a substantially increased risk of colorectal cancer (18).

Decreased Tgfbr1 signaling leads to decreased levels of phosphorylated Smad2 and Smad3 in MEFs, in in vitro experiments, and in vivo in the normal-appearing colonic epithelium, thus resulting in a global decrease of Smad-mediated signaling. This was observed in vitro on addition of exogenous TGF-β but was only observed in the intestinal crypts and in patches within tumors in vivo. Interestingly, the same pattern of decreased SMAD signaling, as well as evidence of constitutively decreased expression of TGFBR1, was observed in the lymphocytes of patients with colorectal cancer (18). This highlights the critical role of Tgfbr1 as a potentially limiting factor with respect to the activation of the Smad signaling cascade at sites of high TGF-β secretion and/or high cellular proliferation. The absence of effective down-regulation of CcnD1 in Tgfbr1−/− MEFs and the observed increased CcnD1 levels within the tumors of ApcMin+/−;Tgfbr1−/− mice provide the first evidence of the downstream effects of decreased Smad-mediated TGF-β signaling. The TGF-β responses in epithelial cells involve the induction of Cdkn2b by means of the Smads (39). Increased Cdkn2b levels are an important aspect of the TGF-β cytostatic program leading to decreased CcnD1 expression (40). The decreased Cdkn2b levels observed in Tgfbr1+/− MEFs provide a plausible link between decreased Smad-mediated signaling and increased CcnD1 expression. The absence of any obvious phenotype in Tgfbr1−/− mice as well as the absence of phenotypic traits in human beings with constitutionally reduced TGFBR1 expression (18) suggests that decreased Tgfbr1-mediated TGF-β signaling does not affect normal development. One potential explanation is that decreased Tgfbr1 signaling only becomes a limiting factor when persistently decreased phosphorylation of Smad2 and Smad3 leads to decreased TGF-β signaling, which in turn results in higher cell proliferation. As mutations of the APC gene are among the most commonly encountered genetic hallmarks of human colorectal cancer (4, 41), altered Tgfbr1 signaling is emerging as a potent modifier of colorectal cancer development. The effect of decreased Tgfbr1-mediated signaling leading to decreased Smad2 and Smad3 signaling is further highlighted by the recent discovery that both SMAD2 and SMAD3 are among the most commonly mutated genes in human colorectal cancer (4), acting as crucial mediators of colonic carcinogenesis.

Thorough histologic review of the normal-appearing colorectal epithelium and tumor tissues did not reveal any difference in the numbers of inflammatory cells in either mouse strain. Together with the findings of comparable lymphocyte counts in ApcMin+/−;Tgfbr1−/− and ApcMin+/−;Tgfbr1+/− mice at 12 weeks, this argues against a major role of inflammation as a contributor to the tumor phenotype observed in ApcMin+/−;Tgfbr1−/− mice. Nonetheless, TGF-β in tumor infiltrating lymphocytes has been shown to control the growth of dysplastic epithelial cells in experimental...
colon cancer (29). Furthermore, abrogation of TGF-β signaling within T cells by means of Smad4 inactivation leads to gastrointestinal cancer development (8). These findings suggest that alterations in lymphocyte-mediated TGF-β signaling may contribute to colorectal cancer development in ApcMin/+;Tgfbr1+/+ mice through a "landscaping" effect (42). Additional studies will be needed to clarify the role of decreased Tgfbr1-mediated signaling and assess potential qualitative differences between Tgfbr1+/+ and Tgfbr1−/− lymphocytes and stromal cells.

In summary, our data provide a strong rationale and a plausible mechanism for the novel concept that Tgfbr1 haploinsufficiency has a causative role in intestinal carcinogenesis. ApcMin/+;Tgfbr1−/− mice may therefore emerge as a valuable human-based mouse model for studying colorectal cancer development and progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 10/15/2008; accepted 10/15/2008.

Grant support: Walter S. Mander Foundation (Chicago, IL); the Jeannik M. Littlefield-AACR Grant in Metastatic Colon Cancer Research; and National Cancer Institute grants CA112520, CA108741, CA67941 and CA16058.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

5. Takaku K, Oshima M, Miyoshi H, Matsui M, Seldin ME. Taketo MM. Intestinal tumorigenesis in compound haploinsufficiency on molecular signaling within tumors. A and B, pSmad2 staining is patchy within tumors arising from ApcMin−/−, Tgfbr1−/− mice, which reflects focally decreased Smad-mediated TGF-β signaling (B), whereas tumors arising from ApcMin−/−, Tgfbr1−/− mice have uniform pSmad2 staining showing preserved Smad-mediated TGF-β signaling (A). C and D, Ccnd1 (cyclin D1) expression is significantly higher within T cells by means of Smad4 inactivation in ApcMin−/−, Tgfbr1−/− mice (F, 50.7 ± 4.1% positive staining) than in those of ApcMin−/−,Tgfbr1−/− mice (E, 20.1 ± 5.7% positive staining), P = 0.002.

Figure 6. Characterization of Tgfbr1 haploinsufficiency on molecular signaling within tumors. A and B, pSmad2 staining is patchy within tumors arising from ApcMin−/−, Tgfbr1−/− mice, which reflects focally decreased Smad-mediated TGF-β signaling (B), whereas tumors arising from ApcMin−/−, Tgfbr1−/− mice have uniform pSmad2 staining showing preserved Smad-mediated TGF-β signaling (A). C and D, Ccnd1 (cyclin D1) expression is significantly higher within T cells by means of Smad4 inactivation in ApcMin−/−, Tgfbr1−/− mice (F, 50.7 ± 4.1% positive staining) than in those of ApcMin−/−,Tgfbr1−/− mice (E, 20.1 ± 5.7% positive staining), P = 0.002.


**Tgfbr1 Haploinsufficiency Is a Potent Modifier of Colorectal Cancer Development**

Qinghua Zeng, Sharbani Phukan, Yanfei Xu, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://cancerres.aacrjournals.org/content/69/2/678">http://cancerres.aacrjournals.org/content/69/2/678</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2009/01/13/69.2.678.DC1">http://cancerres.aacrjournals.org/content/suppl/2009/01/13/69.2.678.DC1</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 41 articles, 20 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/69/2/678.full.html#ref-list-1">http://cancerres.aacrjournals.org/content/69/2/678.full.html#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 11 HighWire-hosted articles. Access the articles at: <a href="http://cancerres.aacrjournals.org/content/69/2/678.full.html#related-urls">http://cancerres.aacrjournals.org/content/69/2/678.full.html#related-urls</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</td>
</tr>
</tbody>
</table>