Transforming Growth Factor β Receptor Type II Inactivation Induces the Malignant Transformation of Intestinal Neoplasms Initiated by *Apc* Mutation

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Abstract

The transforming growth factor-β (TGF-β) signaling pathway is a tumor-suppressor pathway that is commonly inactivated in colon cancer. TGF-β is a secreted ligand that mediates its effects through a transmembrane heteromeric receptor complex, which consists of type I (TGFBR1) and type II subunits (TGFBR2). Approximately 30% of colon cancers carry *TGFBR2* mutations, demonstrating that it is a common target for mutational inactivation in this cancer. To assess the functional role of TGFBR2 inactivation in the multistep progression sequence of colon cancer, we generated a mouse model that recapitulates two common genetic events observed in human colon cancer by mating $Apc^{1638N/wt}$ mice with mice that are null for *Tgfbr2* in the intestinal epithelium, *Villin*-Cre;$Tgfbr2^{E2flx/E2flx}$ mice. In this model, we observed a dramatic increase in the number of intestinal adenocarcinomas in the $Apc^{1638N/wt}$;*Villin*-Cre;$Tgfbr2^{E2flx/E2flx}$ mice (called $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$) compared with those mice with intact *Tgfbr2* ($Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$). Additionally, *in vitro* analyses of epithelial tumor cells derived from the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice showed enhanced expression and activity of matrix metalloproteinase MMP-2 and MMP-9, as well as increased TGF-β1 secretion in the conditioned medium. Similarly, primary tumor tissues from the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice also showed elevated amounts of TGF-β1 as well as higher MMP-2 activity in comparison with $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$–derived tumors. Thus, loss of TGFBR2 in intestinal epithelial cells promotes the invasion and malignant transformation of tumors initiated by *Apc* mutation, providing evidence that Wnt signaling deregulation and TGF-β signaling inactivation cooperate to drive the initiation and progression, respectively, of intestinal cancers *in vivo*. (Cancer Res 2006; 66(20): 9837-44)

Introduction

The transforming growth factor-β (TGF-β) superfamily of proteins is a family of dimeric secreted ligands that includes the TGF-βs, bone morphogenetic proteins (BMP), activins, and inhibin, among others. TGF-β regulates tissue homeostasis and development through affecting fundamental biological processes such as cell proliferation, differentiation, motility, and programmed cell death. TGF-β exerts its effects on the cells by binding to the transmembrane TGF-β type II receptor (TGFBR2), which causes the recruitment of the TGF-β type I receptor (TGFBR1) with subsequent activation of the receptor complex. SMAD2 and SMAD3 are direct substrates of the activated TGF-β receptor complex, and once phosphorylated they form a heterotrimeric complex with the common mediator Smad, SMAD4. This trimeric complex translocates into the nucleus where it functions as a member of different transcription factor complexes that regulate the expression of a variety of genes (1, 2). In addition, TGF-β receptor activation can induce a variety of non-SMAD signaling pathways, including phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and RhoA (1).

The role of TGF-β signaling in the process of tumor formation is complex and its effects seem to be context dependent as they vary between tumors arising from different organs and between tumors in different stages of progression (1, 2). Components of the TGF-β signaling pathway, including *TGFBR2, SMAD2*, and *SMAD4*, have been shown to be altered frequently in colon cancer, consistent with the idea that TGF-β signaling prevents tumor development, presumably through the induction of growth arrest, differentiation, or apoptosis (3–6). However, evidence from *in vitro* tissue culture systems shows that, paradoxically, TGF-β signaling may be able to induce tumor progression due to its ability to promote epithelial-to-mesenchymal transition; its capacity to inhibit cell death caused by growth factor deprivation; and its immunosuppressant function (7–9).

To investigate the complex effects of TGF-β signaling deregulation in primary colon cancer, several genetically engineered mouse models have been generated that harbor alterations in components of the TGF-β signaling pathway; specifically *Tgfbr2, Tgfb1, Smad2, Smad3*, and *Smad4* have been altered *in vivo* (reviewed in ref. 10). $Tgfb1^{-/-}$;$Prkdc^{scid/scid}$ mice develop colonic carcinomas (9). In addition, constitutive abrogation of *Smad3* and *Smad4* leads to the development colonic and intestinal neoplasms, respectively (11, 12). Of interest, the $Tgfb1^{-/-}$;$Prkdc^{scid/scid}$ and $Smad3^{-/-}$ mice require intestinal colonization with *Helicobacter* to develop colon neoplasms (13, 14). Furthermore, conditional inactivation of *Tgfbr2* in the colonic epithelium promotes the development and progression of azoxymethane-induced tumors (15). Interestingly, compound heterozygous *Cis-*$Apc^{\Delta 716}$(+/−), *Dpc4*(+/−) mice develop large and invasive tumors not seen in the $Apc^{\Delta 716/+}$ heterozygous

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mice; however, *Smad2*$^{+/-}$ mice do not develop intestinal neoplasms spontaneously and it does not seem that *Smad2* haploinsufficiency has a dramatic effect on the formation of intestinal tumors in the *Apc*$^{\Delta 716}$ or *Apc*^{580D} mice (16, 17). These studies show that deregulation of the Wnt and SMAD pathways can cooperate in the development of intestinal neoplasia. However, because SMADs regulate BMP, activin, and inhibin signaling and because TGF-β activates non-SMAD pathways, these studies do not permit a precise assessment of the effect of deregulation of TGF-β–mediated signaling in intestinal cancer formation *in vivo* (16–18). In addition, except for the *Fabp*$^{4xat-132}$-Cre*; Tgfbr2*$^{flx/flx}$ mouse, these models abrogate TGF-β signaling in all cell types, raising the question of whether the neoplasms observed in these mouse models are a consequence of direct loss of TGF-β signaling in the intestinal epithelial cells or a consequence of loss of TGF-β signaling in the immune or stromal cells (19, 20).

With the aim of understanding the implications of TGF-β receptor inactivation in the intestinal epithelium in the process of tumor development, we generated a mouse model that replicates two common genetic events observed in human colorectal cancer, *APC* mutation, and TGFBR2 inactivation. Thus, we mated *Tgfbr2*$^{E2flx/E2flx}$;*Villin*-Cre mice (called *Tgfbr2*^{IEKO}) with *Apc*$^{1638N/wt}$ mice to elucidate the effect of disrupting TGF-β signaling exclusively in intestinal epithelial cells in the context of Wnt pathway deregulation. We observed that the *Apc*^{1638N};*Tgfbr2*^{IEKO} mice develop intestinal adenocarcinomas at a substantially higher incidence than mice that carry the *Apc*^{1638N} mutation alone. Furthermore, potential cell autonomous and nonautonomous mechanisms through which the inactivation of *Tgfbr2* in intestinal epithelial cells may promote tumor progression were identified.

Materials and Methods

Generation and characterization of *Villin*-Cre, *Tgfbr2*$^{E2flx/E2flx}$, and *Apc*$^{1638N/wt}$ mice. The generation of transgenic *Tgfbr2*$^{E2flx/E2flx}$, *Villin*-Cre, and *Apc*^{1638N} mice has been previously described (21–24). The animals were mated to generate the following compound genotypes: *Villin*-Cre;*Tgfbr2*$^{E2flx/E2flx}$ (called *Tgfbr2*^{IEKO}), *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$, or *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO}, and were fed *ad libitum* with a standard rodent diet and water. The mice were genotyped using PCR-based assays with visualization of the PCR products by UV transillumination after staining with ethidium bromide. The following primer sequences were used for genotyping: *Tgfbr2* primer 1: 5′-GCAGGCATCAGGACCTCAGTTTGATCC-3′ and primer 2: 5′-AGAGTGAAGCCGTGGTAGGTGAGCTTG-3′, which generate a 556 bp amplicon from the wild-type *Tgfbr2* allele and a 711 bp amplicon from the *Tgfbr2*^{E2flx} allele; *Villin*-Cre primer 1: 5′-GTGTGGGACAGAGAACAAACCG-3′ and primer 2: 5′-TGCGAACCTCATCACTCGTTGC-3′, which generate a 1,000 bp product from Cre-positive DNA (22); and *Apc*^{1638N} primer 1: 5′-TGCCAGCACAGAATAGGCTG-3′, primer 2: 5′-TGGAAGGATTGGAGCTACGG-3′, primer 3: 5′-GTTGTCATCCAGGTCTGGTG-3′ that generate a 300 bp amplicon from the wild-type *Apc* allele and 400 bp amplicon from the *Apc*^{1638N} mutant allele (25). Animals with the genotypes *Tgfbr2*$^{E2flx/E2flx}$ or *Tgfbr2*^{IEKO} were harvested at 3.5, 6, 10, 17, and 24 months of age to evaluate the morphology of the intestinal mucosa and for the development of spontaneous tumors. Similarly, mice with the genotypes *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$ and *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} were harvested at 9 and 12 months of age.

Tissue harvesting. Two hours before sacrifice, the mice were injected i.p. with bromodeoxyuridine (BrdUrd, Sigma-Aldrich, St. Louis, MO) at a dose of 100 mg/kg of weight. Immediately after sacrifice, the small intestine and colon were dissected, flushed with PBS, opened flat, and assessed for macroscopically visible lesions. Tissue samples were fixed in 10% neutral buffered formalin overnight at 4°C, and subjected to standard histologic processing and H&E staining. The histologic evaluation of all lesions was done by a gastrointestinal pathologist (M.U.), who was unaware of the genotype of the mice.

Immunostaining. For BrdUrd immunostaining, tissue sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked with H_2O_2 treatment. Antigen unmasking was achieved by incubation with Target Retrieval Solution (DakoCytomation, Carpinteria, CA) for 20 minutes. Nonspecific binding was blocked by treating the sections with 1.5% rabbit normal serum for 10 minutes, and the immunostaining was done with the rat monoclonal antibody anti-BrdUrd (clone BU1/75, Accurate Scientific, Westbury, NY) diluted 1:2,000. For β-catenin immunostaining, the sections were treated similarly; but the antigen was retrieved using boiling sodium citrate buffer (pH 6.0) for 15 minutes. A mouse monoclonal antibody anti-β-catenin (clone 14, BD Transduction Laboratories, San Diego, CA) was used in a 1:200 dilution with a DakoCytomation ARK system (DAKO, Carpinteria, CA).

Assessment of Cre-mediated recombination. Genomic DNA was extracted from tissues and cell cultures using a standard phenol-chloroform extraction protocol as previously described (26). Cre-mediated recombination of *Tgfbr2*^{E2flx} allele was assessed using a PCR-based assay that only generates an amplicon if the *Tgfbr2*^{E2flx} allele has undergone Cre-mediated recombination. The primer sequences are as follows: primer 1, 5′-AGGGATGAATGGGCTTGCTT-3′, and primer 2, 5′-CTCACCTCAGAGCCTGATTA-3′.

Tumor-derived cells isolation and long-term culture. Tumor cells were isolated following a modification of a protocol previously described (27). In summary, pieces of the tumors of interest were rinsed several times in PBS with 10 μg/mL gentamicin (10 μg/mL), penicillin (20 units/mL), and streptomycin (20 μg/mL; called PBS-A). Subsequently, the samples were incubated for 20 minutes in a 0.04% sodium hypochlorite solution (Sigma-Aldrich) at room temperature and handled as sterile specimens afterward. The tissues were rinsed with PBS-A and minced in small pieces, which were then centrifuged at 100 × *g* for 5 minutes in PBS-A. The pellet was resuspended in liver digest medium (Life Technologies/Invitrogen, Grand Island, NY) with the antibiotics mentioned above, and incubated for 90 minutes at 37°C with occasional shaking. The samples were centrifuged at 100 × *g* for 5 minutes; the pellet was resuspended in liver digest medium with antibiotics, and then incubated overnight at 4°C. Subsequently, the samples were centrifuged and the pellet washed twice with tissue culture medium [RPMI 1640, 10% fetal bovine serum (FBS), 10 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 1 mL/L ITS-X (Life Technologies/Invitrogen), 0.5 μg/mL Fungizone (Life Technologies/Invitrogen), and the same antibiotics used previously (see above)]. The cell suspension was plated in six-well plates coated with Matrigel (10 μg/mL; BD Biosciences, Bedford, MA). When the cells reached confluence, they were disaggregated with 0.25% Trypsin/EDTA (Life Technologies/Invitrogen) and subcultured using standard technique. To remove tumor-associated fibroblasts, the cultures were subjected to several rounds of differential trypsinization and the medium was changed to keratinocyte SFM supplemented with bovine pituitary extract and recombinant EGF (Life Technologies/Invitrogen) until no further fibroblasts were observed. After cultures of epithelial cells showed stable growth patterns, they were grown in CellBind (Corning, Corning, NY) culture vessels.

Western blotting. For evaluation of TGF-β–induced Smad-2 phosphorylation, tumor-derived cell cultures at 70% confluence were treated with 10 ng/mL TGF-β1 (R&D Systems, Minneapolis, MN) for 24 hours in keratinocyte SFM. Protein lysates were prepared in radioimmunoprecipitation assay buffer supplemented with a complete protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 1 and 2 (Sigma, St. Louis, MO), and then used for SDS-PAGE. The mouse monoclonal antibodies anti-phospho-Smad-2 (Ser$^{465/467}$, Cell Signaling, Beverly, MA) and Smad-2 (Cell Signaling) were used for Western blot analyses.

Luciferase reporter assays. To evaluate TGF-β–mediated transcription, tumor-derived cells were transiently transfected with the p3TP-lux or CAGA reporters (provided by Joan Massague, Memorial Sloan-Kettering Cancer Center, New York, NY and Bert Vogelstein, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, respectively) concomitantly with the pRL-TK reporter construct (Promega, Madison, WI). Subsequently, the cells were treated with 10 ng/mL TGF-β1 and luciferase activity was

evaluated 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega) with a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). Similarly, for the analysis of β-catenin–mediated transcription, the cells were transfected with the superTOPFLASH (28) and pRL-TK reporters, and examined 48 hours later with the Dual Luciferase System.

Gelatin zymography. The activity of MMP-2 and MMP-9 produced by tumor-derived cells *in vitro* was assessed through gelatin zymography as previously reported (29). Briefly, 70% confluent cultures were switched from FBS-containing medium to keratinocyte SFM, and the supernatant collected 24, 48, and 72 hours after serum deprivation started. The supernatants were concentrated with Microcon YM-10 Centrifugal Devices (Millipore, Bedford, MA) and equivalent amounts of all samples were loaded into the gel. To evaluate metalloprotease activity in the tumors, proteins were extracted from fragments of frozen tissue (30-80 mg) using lysis buffer [0.5 mol/L Tris (pH 7.5), 0.2 mol/L NaCl, 10 mmol/L $CaCl_2$, and 1% Triton X-100] supplemented with a complete protease inhibitor cocktail (Roche). The protein lysates were homogenized by sonication, clarified by centrifugation, and quantified with the BCA Protein Assay kit (Pierce, Rockford, IL). Fifty micrograms of protein per sample were loaded in 10% Zymogram gels (Bio-Rad) and processed as described above (29).

***In situ* gelatin zymography.** *In situ* gelatin zymography was done as previously published (30). Briefly, OCT-embedded tissues were sectioned (10 μm) and incubated at 37°C for 20 hours with reaction buffer [50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 2 mmol/L NaN_3] containing 40 μg/mL FITC-labeled DQ-gelatin (Molecular Probes, Eugene, OR) and 0.02 μg/mL 4′,6-diamidino-2-phenylindole. At the end of the incubation period, the samples were evaluated by fluorescence microscopy without prior washes or fixation processes.

Quantitative reverse transcription-PCR. TaqMan gene expression assays (Assays-on-Demand from Applied Biosystems) for Serpine1 or PAI-1, TGFB1, MMP2, and 18S were used for quantitative RT-PCR. mRNA was extracted from 70% confluent tumor-derived cultures that were serum deprived for 24 hours, or from snap-frozen tumor tissue samples using TRIzol (Invitrogen) and following the instructions of the manufacturer. cDNA was obtained through standard techniques. The assays were done using an ABI 7900 real-time PCR system and 18S values were used for normalization.

TGF-β1 ELISA. The amount of TGF-β1 present in the conditioned medium of tumor-derived epithelial cells was evaluated using the TGF-β1 Quantikine kit (R&D Systems). The supernatants were obtained similarly to those used for gelatin zymography, after 48, 72, and 96 hours after serum removal from the medium. Alternatively, fragments of frozen tissue (80-100 mg) were lysed using 20 mmol/L Tris (pH 7.5), 140 mmol/L NaCl, 1 mmol/L EDTA (pH 8.0), 1 mmol/L EGTA (pH 8.0), 1% Triton X-100, supplemented with a complete protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktails 1 and 2 (Sigma). These protein lysates were homogenized by sonication, clarified by centrifugation, and quantified with the BCA Protein Assay kit (Pierce). One hundred twenty-five micrograms of protein per tumor were used in the assay. Latent TGF-β1 present in the samples was acid-activated before developing the ELISA assay according to the indications of the manufacturer.

Results

***Villin*-Cre;*Tgfbr2*$^{E2flx/E2flx}$ (*Tgfbr2*^{IEKO}) mice show *Tgfbr2* recombination in the intestinal mucosa.** It has been previously shown that Cre expression in *Villin*-Cre transgenic mice occurs exclusively in the epithelial cells of the small intestine and colon, and that Cre-mediated DNA recombination is continuous in the crypt-villus and longitudinal axes of these organs (22). To confirm that the *Tgfbr2*^{E2flx} allele was undergoing Cre-mediated recombination in the intestinal epithelium, genomic DNA from intestinal mucosa obtained through a blunt dissection was assessed using a PCR assay that specifically amplifies the recombined *Tgfbr2*^{E2flx} allele. The recombined *Tgfbr2*^{E2flx} allele could only be detected in the intestinal epithelium from the mice carrying the *Villin*-Cre transgene, providing evidence that the *Tgfbr2*^{E2flx} allele is being deleted in the *Tgfbr2*^{IEKO} mice (Fig. 1).

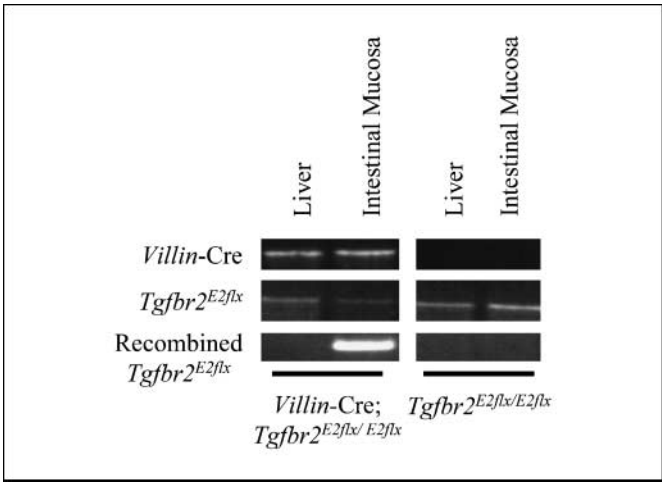

Figure 1. Status of *Tgfbr2* in the intestines of *Villin*-Cre;*Tgfbr2*$^{E2flx/E2flx}$ and *Tgfbr2*$^{E2flx/E2flx}$ mice. Results of PCR-based assays showing evidence of recombination of the *Tgfbr2*^{E2flx} allele in the intestinal epithelial layer of the *Villin*-Cre;*Tgfbr2*$^{E2flx/E2flx}$ but not in the *Tgfbr2*$^{E2flx/E2flx}$ mice. The *Tgfbr2*^{E2flx} product generated from the intestinal mucosa of the *Villin*-Cre;*Tgfbr2*$^{E2flx/E2flx}$ mouse is presumably derived from contaminating submucosa. The liver was used as a negative control and has no detectable recombined *Tgfbr2*^{E2Flx} allele.

Inactivation of *Tgfbr2* in the intestinal epithelium neither increases proliferation of the epithelial cells nor induces the development of spontaneous tumors. TGF-β has been shown to be a potent inhibitor of intestinal epithelial cell proliferation *in vitro* (31, 32). To evaluate the effect of TGF-β signaling abrogation on the basal proliferation rate of the intestinal epithelium, BrdUrd incorporation in the intestines of *Tgfbr2*^{IEKO} and *Tgfbr2*$^{E2flx/E2flx}$ mice was analyzed at 3.5, 6, and 10 months of age (n = 5-14 mice per time point). The organs of the animals from both genotypic groups were grossly and histologically normal at each time point. In addition, evaluation of the proliferation indices of the intestinal epithelial cells using BrdUrd incorporation by the intestinal mucosa revealed similar distribution of the indices in the *Tgfbr2*^{IEKO} and *Tgfbr2*$^{E2flx/E2flx}$ mice at all the time points considered (P > 0.05 as determined by the nonparametric Wilcoxon's rank sum test).

With regard to spontaneous neoplasm formation, elderly *Tgfbr2*^{IEKO} and *Tgfbr2*$^{E2flx/E2flx}$ mice (17 and 24 months of age) developed small duodenal adenomas. Specifically, at 17 months of age 71% (n = 10 of 14) *Tgfbr2*^{IEKO} and 78% (n = 7 of 9) *Tgfbr2*$^{E2flx/E2flx}$ mice developed duodenal adenomas. Similarly, at 24 months of age, 57% (n = 8 of 14) *Tgfbr2*^{IEKO} and 62% (n = 5 of 8) *Tgfbr2*$^{E2flx/E2flx}$ animals had this type of lesion. Furthermore, we observed invasive intestinal adenocarcinomas in 24-month-old *Tgfbr2*^{IEKO} and *Tgfbr2*$^{E2flx/E2flx}$ mice (n = 2 of 14 and n = 1 of 8, respectively). Of note, the three colonic adenocarcinomas observed in the *Tgfbr2*^{IEKO} animals did not show evidence of β-catenin nuclear localization, whereas the only invasive neoplasm observed in the *Tgfbr2*$^{E2flx/E2flx}$ mouse displayed nuclear localization of β-catenin. Thus, our findings indicate that deletion of *Tgfbr2* in the epithelial cells does not affect basal cell proliferation in the intestinal epithelium and that suppression of TGF-β signaling in intestinal epithelial cells alone is not sufficient to promote the formation of intestinal tumors. This observation is consistent with those of previous

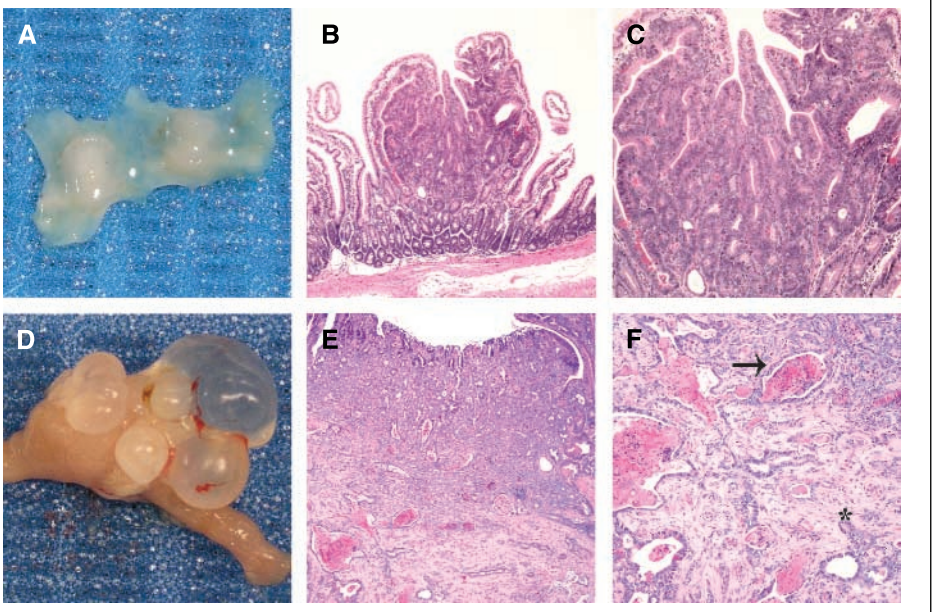

Figure 2. Gross and histologic features of the intestinal neoplasms initiated by the *Apc*^{1638N} mutation. Gross appearance of representative intestinal adenomas (*A*) and adenocarcinoma (*D*) observed in *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$ and *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} mice, respectively. Photomicrographs of H&E–stained sections of a representative adenoma [magnifications, ×40 (*B*) and ×100 (*C*)] and an adenocarcinoma [magnifications, ×40 (*E*) and ×100 (*F*)]. Cystic structures filled with mucin (*arrow*) were frequently observed in the invasive neoplasms, as well as a dramatic desmoplastic response (*) in the invasive component of such tumors (*F*).

studies of *Fabp*$^{4xat-132}$-Cre;*Tgfbr2*$^{E2flx/E2flx}$ mice, which lack *Tgfbr2* in the colonic epithelium (15).

***Tgfbr2* inactivation promotes the malignant transformation of intestinal adenomas initiated by *Apc* mutation.** In light of the infrequent formation of spontaneous tumors in *Tgfbr2*^{IEKO} mice, we reasoned that *Tgfbr2* inactivation may have its primary effect on tumorigenesis in the background of other gene mutations and deregulated signaling pathways. Consequently, we evaluated the effect of *Tgfbr2* inactivation in the context of mutations in *Apc*, the most common initiating genetic event observed in colon neoplasms (33). Hence, we mated *Tgfbr2*^{IEKO} mice with *Apc*$^{1638N/wt}$ mice, which develop three to four intestinal adenomas during their first year of life (23, 24), and evaluated the mice for tumor development at 9 and 12 months of age (Fig. 2). As shown in Table 1, the total number of neoplastic lesions in the *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} and *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$ mice at 9 and 12 months of age was similar; however, the number of advanced tumors (i.e., adenomas with high-grade dysplasia and adenocarcinomas) was significantly greater in the *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} mice than in the *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$ mice. Accordingly, the odds ratio of probabilities for developing high-grade lesions for the *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} mice is 10.2 compared with *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$ mice (95% confidence interval, 4.9-21.3; logistic regression with age adjusted). The increase in the number of advanced neoplastic lesions in the *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} mice suggests that suppression of TGF-β signaling, in the context of *Apc* mutation and aberrant activation of the Wnt pathway, promotes the transformation of benign neoplasms into malignant neoplasms. Our observations are similar to those made in the Cis-*Apc*$^{\Delta 716}$ (+/−), *Dpc4* (+/−) mouse model previously described (18). In these mice, deregulation of both the Wnt and Smad pathways leads to a rapid progression of intestinal adenomas to invasive adenocarcinomas. Further, the invasive lesions of both the *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} and Cis-*Apc*$^{\Delta 716}$ (+/−), *Dpc4* (+/−) animals show significant desmoplasia, and in neither of the two models are metastatic lesions observed. However, it is important to note

Table 1. Comparative tumor incidence in *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$ and *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} mice

Genotype	No. mice with tumors	Average no. tumors per mouse	Total no. adenomas	Total no. adenomas w/HGD*	Total no. adenocarcinomas*	Total no. tumors
9 mo						
Apc$^{1638N/wt}$, *Tgfbr2*$^{E2flx/E2flx}$ (n = 10)	8	2.62	21	0	0	21
Apc$^{1638N/wt}$, *Tgfbr2*^{IEKO} (n = 11)	10	2.70	9	4	14	27
12 mo						
Apc$^{1638N/wt}$, *Tgfbr2*$^{E2flx/E2flx}$ (n = 12)	12	4.92	44	11	4	59
Apc$^{1638N/wt}$, *Tgfbr2*^{IEKO} (n = 12)	12	5.17	19	17	26	62

Abbreviation: HGD, high grade dysplasia.
*Odds ratio of developing high-grade lesions for the *Apc*$^{1638N/wt}$, *Tgfbr2*^{IEKO} mice is 10.2 compared with *Apc*$^{1638N/wt}$, *Tgfbr2*$^{E2flx/E2flx}$ mice (95% confidence interval, 4.9-21.3).

that deregulation of the Smad pathway in the Cis-$Apc^{\Delta 716}$ (+/−), *Dpc4* (+/−) mice is constitutive, and it affects both TGF-β and BMP signaling in several cellular compartments that clearly have an effect on tumor formation. Recent studies have shown that impairment of TGF-β-Smad signaling in lymphocytes can affect intestinal epithelial cell neoplasm formation (19, 34). Therefore, the results obtained with the $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ mouse model more specifically identify the role of TGF-β signaling deregulation in the epithelial cells during the process of intestinal tumor formation, which we propose is the most relevant model for assessing the role of TGF-β signaling deregulation in human intestinal cancer.

To investigate if loss of the growth inhibitory effect of TGF-β on the epithelial cells plays a role in the progression of the intestinal neoplasms of the $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ animals, we determined the proliferation index of representative adenomas arising in the $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ and $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ mice. Interestingly, we observed no difference between the proliferation indices of tumors from $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ and $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ mice (data not shown; $P = 0.24$ as determined by the nonparametric Wilcoxon's rank sum test).

Effects of *Tgfbr2* inactivation on production of factors implicated in tumor invasion in neoplasms arising in the $Apc^{1638N/wt}$ mice. To assess the mechanism(s) affecting tumor progression that result from the cooperation of *Apc* inactivation with *Tgfbr2* deletion in the tumors arising in the $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ mice, we derived cultures of epithelial cells from neoplasms of each genotype. We first characterized the status of *Apc* and *Tgfbr2* in these lines by assessing the cell lines for allelic imbalance of the *Apc* locus and for deletion of the $Tgfbr2^{E2flx}$ allele. A tumor cell line derived from $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ mice, named AVcTT, displayed *Apc* loss of heterozygosity (LOH) and loss of *Tgfbr2*, whereas a cell line derived from a tumor from the $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ mice, named ATT, displayed only *Apc* LOH (Fig. 3*A*). Both cell lines showed increased Wnt pathway activity by the superTOPFLASH reporter assay; however, only the tumor cell line from the $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ mice showed loss of TGF-β–induced phosphorylation of Smad2 and TGF-β–mediated transcription (Fig. 3*B-D*). As anticipated, the p3TP-lux luciferase reporter assay showed no basal or TGF-β inducible activity in the AVcTT cell line, but p3TP-lux reporter activity could be induced by reconstitution of the AVcTT cell line with wild-type *Tgfbr2*.

We then assessed the effect of TGFBR2 inactivation on the secretion of proteins that promote tumor invasion. We determined the concentration of secreted TGF-β1 in the conditioned media from the AVcTT cells and found it to be higher than that in media from the ATT cell line (Fig. 4*A*). Moreover, we found that the concentration of TGF-β1 in tumor tissues was also higher in the tumors from the $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ mice compared with those from the Apc^{1638N};$Tgfbr2^{E2flx/E2flx}$ mice (Fig. 4*B*). These results suggest that TGF-β1 secretion is elevated in the tumors from the $Apc^{1638N/wt}$; $Tgfbr2^{\text{IEKO}}$ mice and could have oncogenic effects by inducing angiogenesis, extracellular matrix remodeling, immune suppression, etc.

We also evaluated the production of the matrix metalloproteases MMP-2 and MMP-9 in the cell cultures derived from Apc^{1638N};$Tgfbr2^{\text{IEKO}}$ and Apc^{1638N};$Tgfbr2^{E2flx/E2flx}$ tumors. We observed increased *MMP2* mRNA expression and gelatinolytic activity in the AVcTT cell line compared with the ATT cell line, and found that this effect could be inhibited by reconstitution of the AVcTT line with *Tgfbr2* (Fig. 5*A-B*). Assessment of these metalloproteases in tumor tissues showed that MMP-2 has a tendency to a higher enzymatic activity in the tumors derived from Apc^{1638N};$Tgfbr2^{\text{IEKO}}$ compared with those from Apc^{1638N}; $Tgfbr2^{E2flx/E2flx}$ mice; however, we did not find any significant difference in MMP-9 activity in these samples (Fig. 5*C*). We used *in situ* zymography to better identify the cell type involved in

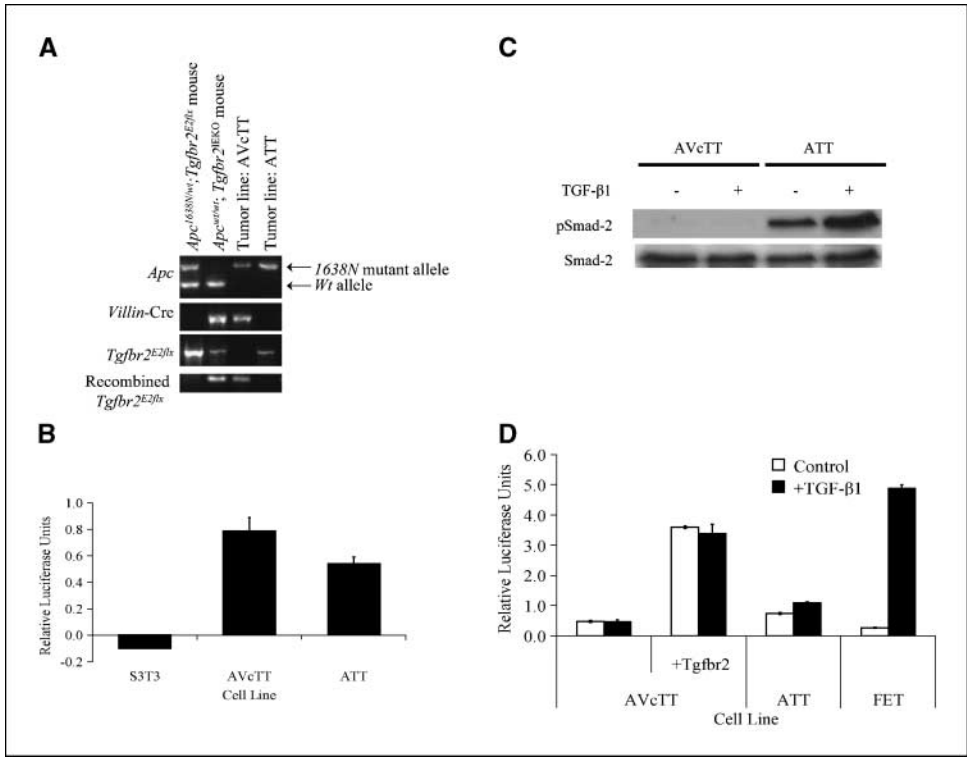

Figure 3. Status of Wnt and TGF-β signaling in tumor-derived epithelial cells. *A,* results of PCR-based analysis indicating *Apc* LOH in tumor lines derived from $Apc^{1638N/wt}$;$Tgfbr2^{\text{IEKO}}$ (AVcTT) and $Apc^{1638N/wt}$, $Tgfbr2^{E2flx/E2flx}$ (ATT) neoplasms, and recombination of the $Tgfbr2^{E2flx}$ allele in the AVcTT but not in the ATT line. Genomic DNA obtained from normal intestinal tissue was used as the reference DNA in the assays. *B,* β-catenin–mediated transcription assessed with the superTOPFLASH reporter showing an increase in both the AVcTT and ATT tumor lines. S3T3 cells have an intact Wnt signaling pathway that is not constitutively activated and were used as negative control (44). *C,* analysis of Smad-2 phosphorylation by western blot, indicating loss of TGF-β responsiveness in the AVcTT but not in the ATT line. *D,* TGF-β–induced transcription evaluated with the p3TP-lux reporter indicates that TGF-β resistance can be restored in the AVcTT line by cotransfection with *Tgfbr2*. Of note, we observed similar results with the CAGA luciferase reporter (data not shown). The human colorectal cancer cell line FET was used as positive control for TGF-β–induced transcription.

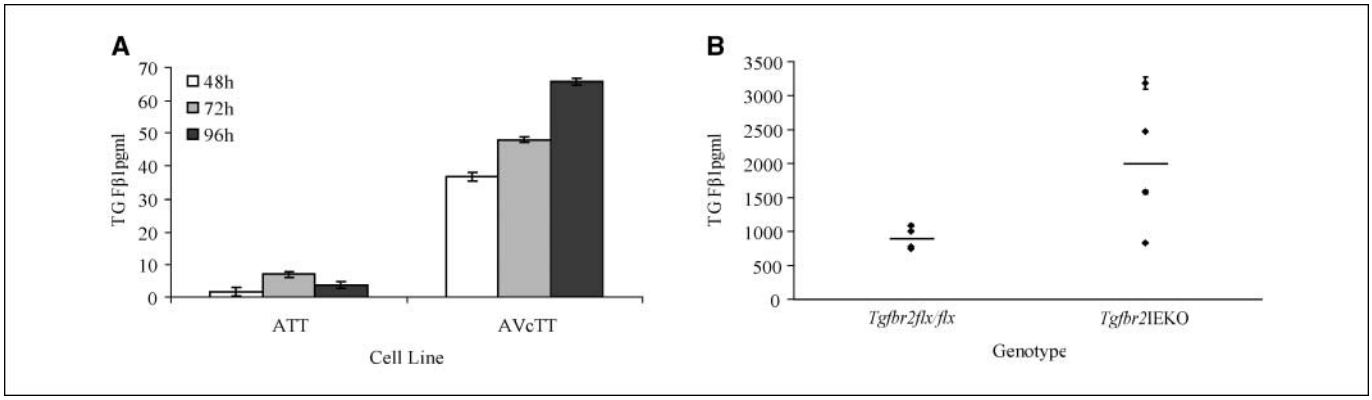

Figure 4. Effect of *Tgbfr2* inactivation on the secretion of TGF-β1. *A,* the concentration of TGF-β1 in the conditioned medium from AVcTT is greater than that from the ATT tumor line. *B,* the amount of TGF-β1 is higher in tumors from $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice than in lesions from $Apc^{1638N/wt}$, $Tgfbr2^{E2flx/E2flx}$ mice.

MMP production/activity in tumor tissues. As shown in Fig. 5*D*, minimal MMP activity was detected in the tumors from Apc^{1638N}; $Tgfbr2^{E2flx/E2flx}$ mice. Conversely, gelatinolytic activity was clearly observed in the Apc^{1638N};$Tgfbr2^{IEKO}$ mice and it was found predominantly in the epithelial component of the tumors, thus confirming our observations on the *in vitro* models. Of note, evaluation of *MMP2* mRNA expression by quantitative RT-PCR in the primary tumors from the $Apc^{1638N/wt}$; $Tgfbr2^{IEKO}$ and the $Apc^{1638N/wt}$; $Tgfbr2^{E2flx/E2flx}$ mice did not show a consistent difference between the two groups of mice, possibly because the heterogeneity of the tumor tissue compared with the cultures of epithelial cells, or because of the effect of differences in the proportion of stromal and epithelial cells in the tumors that were analyzed.

Discussion

Our results from the *Villin*-Cre;$Tgfbr2^{E2flx/E2flx}$ ($Tgfbr2^{IEKO}$) mice show that *Tgfbr2* null intestinal epithelium *in vivo* is not highly susceptible to spontaneous tumor formation. Nevertheless, in the context of an *Apc* mutation, *Tgfbr2* inactivation promotes the progression of adenomas to adenocarcinomas. These results are consistent with *Tgfbr2* acting as a tumor-suppressor gene in the intestines that primarily has effects on cells previously initiated into the polyp-carcinoma progression sequence. Furthermore, and importantly, we can conclude from this mouse model that this effect is cell autonomous and not a consequence of impaired TGF-β signaling in T cells or stromal cells, which has been shown to affect tumor formation in mouse models (19, 20).

Interestingly, although TGF-β has been shown to have potent inhibitory effects on epithelial cells *in vitro*, we do not observe any detectable difference between the $Tgfbr2^{IEKO}$ and the $Tgfbr2^{E2flx/E2flx}$ mice with regard to proliferation within the small intestine or colon. We also found no alterations in the histologic appearance of the intestinal epithelium of the $Tgfbr2^{IEKO}$ mice, which is noteworthy because TGF-β has been implicated in regulating apoptosis and differentiation as well as proliferation in the intestinal epithelium (4, 35, 36). These results show that TGF-β signaling is dispensable for the homeostasis of the intestinal epithelium and may only be physiologically required under specific circumstances, such as in response to exposure to specific inflammatory stimuli (9, 14, 37). The absence of an effect of TGF-β inactivation in the intestinal epithelium is in contrast to the result of BMP signaling disruption in the same tissue, which has been shown to lead to ectopic crypt formation and increased cell proliferation secondary to deregulated sonic hedgehog signaling (38–40). It is not clear whether the lack of a phenotypic effect is the consequence of compensation by other TGF-β ligand family members, such as activin, or whether it is because TGF-β signaling is not required for the *in vivo* regulation of intestinal epithelial proliferation, differentiation, and induction of programmed cell death.

In light of the unremarkable effect of *Tgfbr2* deletion on the basal status of the intestinal epithelium, it is perhaps not surprising that we did not observe the development of spontaneous intestinal neoplasms beyond those observed in the control mice. Our findings provide further insight into the role of TGF-β signaling on intestinal tumor formation that has been derived from other mouse models with disrupted elements in the TGF-β signaling pathway, including the $Tgfb1^{-/-}$;$Prkdc^{scid/scid}$, ITF-dnRII (dominant negative *Tgfbr2* transgene in the intestinal epithelium), and $Smad3^{-/-}$ mouse models. None of these models show spontaneous intestinal tumor formation without the presence of a concurrent factor, either intestinal *Helicobacter* infection or azoxymethane treatment (9, 13, 14, 37). Of interest, however, these models do not permit the precise assessment of TGF-β signaling disruption on cell autonomous events that affect intestinal tumor formation either because they are constitutional knockout models (e.g., $Smad3^{-/-}$ or $Tgfb1^{-/-}$;$Prkdc^{scid/scid}$) or because they rely on dominant negative *Tgfbr2* transgenes. The $Tgfb1^{-/-}$; $Prkdc^{scid/scid}$ and $Smad3^{-/-}$ require *Helicobacter* colonization to develop tumors, and it is not clear whether the bacterial infection induces tumor formation through direct effects on the intestinal epithelium or through indirect paracrine effects mediated by lymphocytes that are TGF-β resistant (34). Furthermore, effects of azoxymethane in the ITR-dnRII construct may result from attenuation of TGF-β signaling or blockade of BMP signaling rather than loss of TGF-β receptor function as occurs with the majority of *TGFBR2* mutations in human colon cancer (41).

Our prior assessment of the $Fabp^{4xat-132}$-Cre;$Tgfbr2^{E2flx/E2flx}$ mouse, the only model to date that permits the assessment of cell autonomous effects of TGF-β signaling on intestinal neoplasm formation, showed that colon neoplasms would form after exposure of the mice to azoxymethane, which can induce neoplasms through β-catenin–dependent and β-catenin–independent pathways (15, 42, 43). Thus, to determine the effect

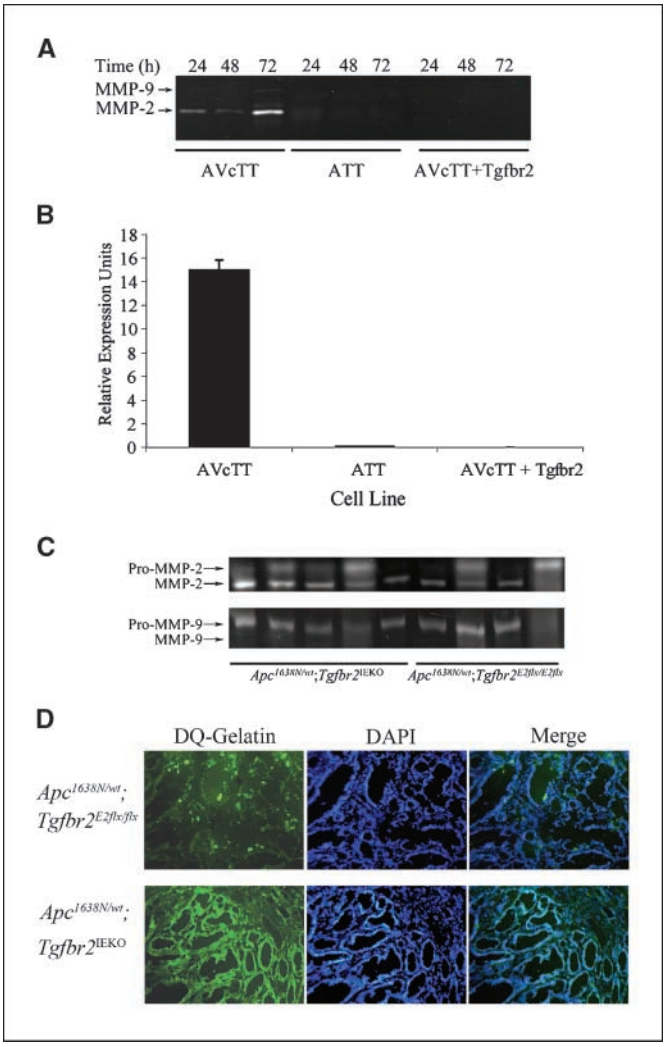

Figure 5. Effect of *Tgfbr2* inactivation on the expression and activity of MMP-2 and MMP-9. *A,* MMP-2 and MMP-9 activities evaluated through zymography reveal that their enzymatic activities are higher in the AVcTT than in the ATT line. The gelatinolytic activities of these metalloproteases are significantly reduced in the AVcTT line after stable reconstitution of *Tgfbr2. B,* quantitative RT-PCR analysis shows that MMP-2 expression is higher in the AVcTT than in the ATT line, and that it is reduced in the AVcTT line after stable reconstitution of *Tgfbr2. C,* the enzymatic activity of MMP-2 is elevated in lesions arisen in $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice. Inverted images were analyzed with Scion Image software, and the average density for MMP-2 in $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ tissues was 1,368.8 $\pm$ 739.5; and for $Apc^{1638N/wt}$, $Tgfbr2^{E2flx/E2flx}$ samples was 702.0 $\pm$ 149. *D, in situ* gelatin zymography of representative tumors from the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ and $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ mice demonstrating increased MMP activity in the tumors from the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice (n = 3) compared with the $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ mice (n = 2). Of note, sections incubated with 10 μmol/L 1,10-phenanthroline (a broad-spectrum MMP inhibitor) did not show any proteolytic activity (data not shown), strongly suggesting that MMPs are indeed responsible for the degradation of DQ-gelatin.

of *Tgfbr2* inactivation in an *in vivo* model that recapitulates the genetic alterations observed in human colon cancer, we generated the $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ +/− *Villin*-Cre mouse model. Our results with this model provide compelling evidence that TGFBR2 acts to suppress the progression of initiated neoplasms and that inactivation of TGF-β signaling in the context of *Apc* mutations strongly promotes the malignant transformation of adenomas to adenocarcinomas. Furthermore, in light of the increased formation of intestinal adenocarcinomas observed in the *Cis-*$Apc^{\Delta 716}$(+/−), *Dpc4*(+/−) mice, our results in the $Apc^{1638N/}$;$Tgfbr2^{E2flx/E2flx}$ +/− *Villin*-Cre suggest that inactivation of Smad signaling is a central effect of TGFBR2 inactivation (18).

With regard to the relevant biological consequences that contribute to colon cancer formation as a result of *Tgfbr2* inactivation, we assessed the effects of loss of *Tgfbr2* on TGF-β1 secretion and on MMP-2 and MMP-9 activity, which are candidate mechanisms that could influence tumor invasion. We assessed these mechanisms through the use of tumor epithelial cell line systems and primary tumors from the mice. We characterized the cell lines for TGF-β responsiveness and activation of the Wnt signaling pathway to accurately determine the status of these pathways in relationship to the effects on TGF-β1 secretion and MMP activity. It is of note that the cell line established from a neoplasm from an $Apc^{1638N/wt}$;$Tgfbr2^{flx/flx}$ mouse (ATT) showed a modest level of 3TP-Lux induction with TGF-β that was substantially lower than the TGF-β–responsive colon cancer cell line, FET. We believe that this is secondary to saturation of the TGF-β receptor with autocrine TGF-β, but it is also possible that the TGF-β signaling pathway is attenuated in this cell line. Our studies of the cell lines and tumor tissues show that loss of TGF-β signaling leads to invasive tumors and that these neoplasms exhibit profound desmoplasia and mucinous features. These histologic characteristics implicate factors that regulate extracellular matrix remodeling, and our *in vitro* studies and studies of the primary tumors have provided evidence suggesting that $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ tumors produce considerably higher levels of TGF-β1 and MMP-2, which could account for their increased invasiveness. Furthermore, we have presented evidence from our *in vitro* models that TGF-β may regulate MMP-2 in part through affecting MMP-2 expression at the level of mRNA synthesis. We have not investigated the role of loss of specific Smad and non-Smad TGF-β–mediated signaling pathways in tumor formation in the $Apc^{1638N/wt}$; $Tgfbr2^{IEKO}$ mice, but based on studies of the *Cis-*$Apc^{\Delta 716}$(+/−), *Dpc4*(+/−) mice and $Smad3^{-/-}$ mice, we predict that impaired Smad signaling has a prominent role in the genesis of these tumors (11, 18).

In summary, we have shown in an *in vivo* model system that loss of *Tgfbr2* in the intestinal epithelium contributes to intestinal cancer formation by promoting the progression of adenomas initiated by *Apc* mutations. The results of these studies using the *Villin-Cre*;$Tgfbr2^{E2flx/E2flx}$ mice provide evidence from an *in vivo* model system that inactivation of *TGFBR2* has a pathogenic role in the formation of human colon cancers by promoting the progression of colon adenomas to invasive adenocarcinomas. These findings have significant implications regarding therapeutic strategies that target the TGF-β signaling pathway for the treatment of cancer.

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