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Advances in Brief

Transforming Growth Factor β Receptor Type II Inactivation Promotes the Establishment and Progression of Colon Cancer

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Abstract

Deregulation of members of the transforming growth factor (TGF)-β signaling pathway occurs often in colon cancers and is believed to affect the formation of primary colon cancer. Mutational inactivation of *TGFBR2* is the most common genetic event affecting the TGF-β signaling pathway and occurs in ~20–30% of all colon cancers. By mating *Fabpl$^{4xat-132}$* Cre mice with *Tgfbr2$^{flx/flx}$* mice, we have generated a mouse model that is null for *Tgfbr2* in the colonic epithelium, and in this model system, we have assessed the effect of loss of TGF-β signaling *in vivo* on colon cancer formation induced by azoxymethane (AOM). We have observed a significant increase in the number of AOM-induced adenomas and adenocarcinomas in the *Fabpl$^{4xat-132}$* Cre *Tgfbr2$^{flx/flx}$* mice compared with *Tgfbr2$^{flx/flx}$* mice, which have intact TGF-β receptor type II (TGFBR2) in the colon epithelium, and we have found increased proliferation in the neoplasms occurring in the *Fabpl$^{4xat-132}$* Cre *Tgfbr2$^{flx/flx}$* mice. These results implicate the loss of TGF-β-mediated growth inhibition as one of the *in vivo* mechanisms through which TGFBR2 inactivation contributes to colon cancer formation. Thus, we have demonstrated that loss of TGFBR2 in colon epithelial cells promotes the establishment and progression of AOM-induced colon neoplasms, providing evidence from an *in vivo* model system that *TGFBR2* is a tumor suppressor gene in the colon.

Introduction

Members of the transforming growth factor (TGF)-β signaling pathway are common targets for mutation in colon cancers. Deregulation of this pathway appears to play an important role in colon carcinogenesis by affecting TGF-β-mediated growth inhibition, apoptosis, and differentiation as well as other TGF-β-regulated processes (1, 2). The TGF-β ligands mediate their effects on cells through a heteromeric receptor complex that consists of type I and type II [TGF-β receptor type II (TGFBR2)] components. The most common mechanism for deregulating TGF-β signaling in colon cancers is the mutational inactivation of *TGFBR2*. Functionally significant mutations of *TGFBR2* have been identified in ~90% of microsatellite unstable colon cancers and in up to 30% of all colon cancers (1). However, despite genetic evidence and *in vitro* experimental data implicating *TGFBR2* as a tumor suppressor gene in colon cancer, recent *in vivo* experimental data suggest that the TGF-β signaling pathway has a contextual effect in carcinogenesis and may be both tumor-suppressing and tumor-promoting (3–7). Consequently, we crossed a *Tgfbr2$^{flx/flx}$* line with the *Fabpl$^{4xat-132}$* Cre driver line to generate an *in vivo* model system to determine the effect of TGFBR2 inactivation on colon carcinogenesis (8, 9). We observed that *Fabpl$^{4xat-132}$* Cre *Tgfbr2$^{flx/flx}$* mice, which were followed until the age of 9 months, do not frequently develop spontaneous colon neoplasms. Consequently, azoxymethane (AOM), a rodent colon carcinogen, was used to induce colon neoplasms in the *Fabpl$^{4xat-132}$* Cre *Tgfbr2$^{flx/flx}$* mice, which lack TGFBR2 expression in ~70–90% of the colonic epithelial cells, and *Tgfbr2$^{flx/flx}$* mice, which have intact TGFBR2 in the colon epithelium, to investigate the effect of *Tgfbr2* inactivation on tumor formation and progression. We observed an increase in the number and pathological stage of colon neoplasms in the *Fabpl$^{4xat-132}$* Cre *Tgfbr2$^{flx/flx}$* mice compared with the *Tgfbr2$^{flx/flx}$* mice. These findings are consistent with *TGFBR2* functioning as a tumor suppressor gene that has its predominant effect on impeding the progression of colon neoplasms toward malignancy.

Materials and Methods

Generation and Characterization of *Tgfbr2$^{flx/flx}$*/*Fabpl$^{4xat-132}$* Cre Mice. The generation of the *Tgfbr2$^{flx/flx}$* mice has been described previously (8). These mice were mated with *Fabpl$^{4xat-132}$* Cre mice (kindly provided by J. Gordon; Washington University School of Medicine) to generate *Tgfbr2$^{flx/flx}$* *Fabpl$^{4xat-132}$* Cre mice (9, 10). Both lines of mice were maintained on the FVB genetic background (11). The mice were maintained in a specific pathogen-free facility in accordance with standard animal use protocols and fed either a standard chow diet or, if receiving AOM, an AIN-76A diet.

AOM-Induced Tumorigenesis. Beginning at 6 weeks of age, the mice received s.c. injection twice a week with AOM at a dose of 10 mg/kg body weight (total dose, 120 mg/kg). The mice were sacrificed at 10 or 17 weeks after the end of the treatment. These studies were carried out under Institutional Animal Care and Use Committee-approved protocols.

Tissue Harvesting. After sacrifice, the colon was flushed with normal saline, fixed in 10% neutral buffer formalin overnight at 4°C, and then inspected for polypoid lesions. The tissues were then subjected to standard histological preparation and H&E staining, followed by assessment by a pathologist (K. W.) who was unaware of the genotype of the mice. The lesions were characterized using criteria established by the Consensus Report of the Pathology of Mouse Models of Intestinal Cancer (12). The tissue sections were analyzed using bright-field microscopy with a Nikon E800 compound microscope mounted with a Micropublisher digital camera (Q Imaging) using Q-capture software (Q Imaging).

Immunostaining. Tissue sections were deparaffinized, rehydrated, and then treated with either 0.05% saponin in water for 30 min at room temperature for TGFBR2 immunostaining, 10 mM citric acid (pH 6.0) at 98°C for 30 min for cyclooxygenase (COX)-2 immunostaining, or casein-based protein block (DakoCytomation) for Ki-67 immunostaining. TGFBR2 immunostaining was performed as described previously (13). COX-2 immunostaining was performed with anti-PGHS-2 polyclonal antisera (PG 27B; Oxford Biomedical Research, Oxford, MI) diluted 1:100 in PBS. Tissue sections were then incubated overnight at 4°C, followed by incubation with biotinylated rabbit antisera (Vector Laboratories, Burlingame, CA) diluted 1:500 in PBS with 1% BSA for 60 min at room temperature, and incubation with streptavidin following the manufacturer's protocol (DAKO). Ki-67 immunostaining was

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performed by incubating the tissue sections with rabbit antihuman Ki-67 (ki67p; NovaCastra Laboratories Ltd.) diluted 1:1500 for 60 min, followed by the Dako Envision+ system, 3,3′-diaminobenzidine/peroxidase (DakoCytomation). The stained sections were scored to generate a proliferation index [mean number of Ki-67-positive nuclei/total number of nuclei per high-power field (×400); 5 fields assessed/tumor]. For terminal deoxynucleotidyl transferase-mediated nick end labeling staining, the deadEnd colorimetric terminal deoxynucleotidyl transferase-mediated nick end labeling system (Promega, Madison, WI) was used following the manufacturer's protocol. An apoptotic index was determined by counting five ×400 fields per lesion, which was done independently by two individuals. The slides were assessed with the same microscope system used to assess the H&E-stained tissue sections.

***Kras2* Mutation Analysis in Exons 1 and 2.** Briefly, DNA samples extracted from paraffin-embedded, formalin-fixed microdissected tumors were subjected to PCR amplification, and then the amplicons were sequenced to assess for point mutations. For exon 1 PCR amplification, the following primer sequences were used: sense primer, 5′-AAACTTGTGGTGGTTGGAGC-3′; and antisense primer, 5′-TGAGCGGCGTTACCTCTATC-3′. For exon 2, previously published primers were used (14). The reaction mix consisted of 125 nM deoxynucleoside triphosphates (Sigma), 300 nM sense and antisense primer (Sigma-Genosys), PCR buffer (Perkin-Elmer, Foster City, CA), and 1 unit of Amplitaq DNA polymerase (Perkin-Elmer). The PCR reactions were carried out in a Tetrad thermocycler (MJ Research) using the following conditions for exon 1: 94°C for 5 minutes; 94°C for 1 minute, 54.3°C for 1 minute, 72°C for 3 minutes for 40 cycles. The same conditions were used for exon 2, except that the annealing temperature was 60°C. The PCR products were gel purified using the Qiaquick PCR purification kit (Qiagen) following the manufacturer's protocol and then subcloned using the pGEM-T Easy vector system (Promega). Pools of 6–10 clones from each sample were then sequenced in the Vanderbilt Ingram Cancer Center DNA sequencing facility using an ABI 3730X1 automated sequencer and the same primers used for PCR amplification.

Bromodeoxyuridine Immunostaining. Briefly, the mice were injected with 100 mg/kg body weight bromodeoxyuridine (Sigma) 2 h before sacrifice. After sacrifice, the colon tissue was fixed in 10% neutral buffer formalin and then subjected to standard paraffin embedding and sectioning. The sections were heated in Target Retrieval Solution (DakoCytomation) for 20 min following the manufacturer's protocol and then incubated with a rat monoclonal antibody (H2724; Accurate Labs) for 60 minutes at room temperature. This incubation was followed by staining with the Vectastain ABC Elite system (Vector Laboratories) and 3,3′-diaminobenzidine/peroxidase+ (DakoCytomation). The stained sections were analyzed as described above.

Laser Capture Microdissection and DNA Extraction. Laser capture microdissection was performed using the PixCell laser capture microscope (Arcturus Engineering, Santa Clara, CA) following the manufacturer's protocol. DNA was extracted from paraffin-embedded formalin-fixed tissues as described previously (15).

Statistical Analysis. Statistical analysis of the results was performed using either Student's t test or Fisher's exact test, as appropriate, and with the statistical package SAS version 8.02 (SAS Inc.). $P \leq 0.05$ was considered statistically significant.

Results

***Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* Mice Lack Expression of TGFBR2 in the Colon Epithelium.** Genomic DNA from the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* and *Tgfbr2$^{flx/flx}$* mice was obtained from both the entire colonic mucosal layer using blunt dissection of freshly harvested colons and the separate basal and luminal portions of the crypts using laser capture microdissection of colon tissue sections. A recombined *Tgfbr2^{flx}* allele was detected only in the intestinal mucosa from the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice. Only the intact *Tgfbr2^{flx}* allele was detected in colon epithelium of the *Tgfbr2$^{flx/flx}$* mice. Analysis of the genomic DNA extracted from the basal and luminal portions of the colon crypts from *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice demonstrated the recombined *Tgfbr2^{flx}* allele in both portions of the crypt, which indicates Cre activity in the proliferative compartment of the crypt. (Fig. 1*A*).

The expression of TGFBR2 was then assessed by immunostaining colons resected from both lines of mice with anti-TGFBR2 polyclonal antisera (sc-220; Santa Cruz Biotechnology). As demonstrated in previous studies, TGFBR2 immunoreactivity was present primarily at the luminal surface of the colonic epithelium of the *Tgfbr2$^{flx/flx}$* mice (16). In contrast, the colon epithelium from the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice displayed no TGFBR2 immunoreactivity, except in occasional crypts that comprised ~10–30% of the entire epithelial

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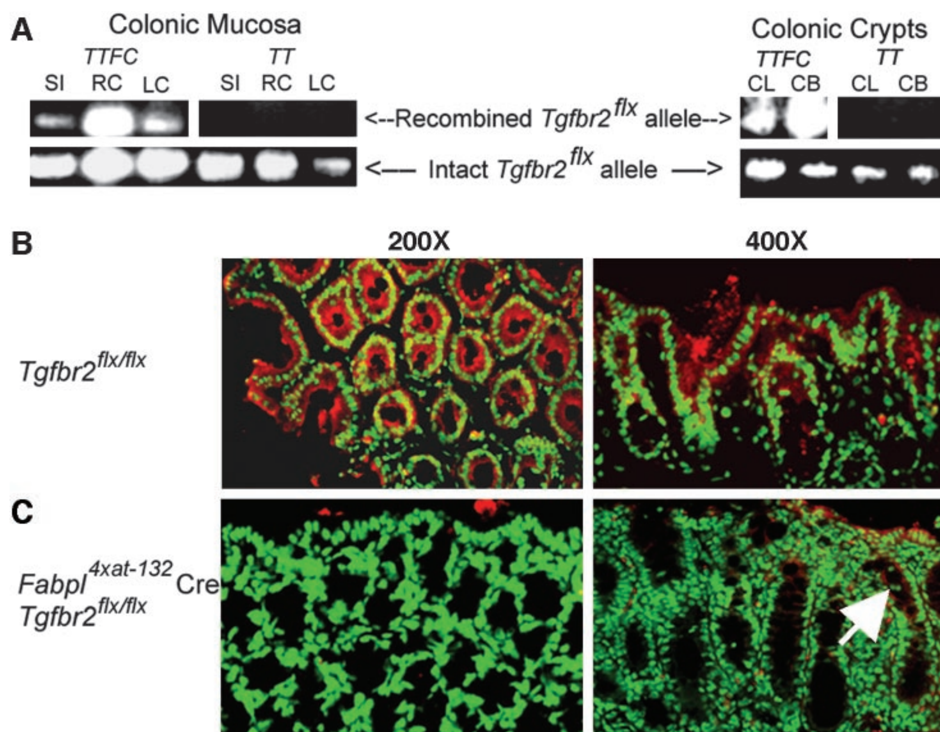

Fig. 1. Status of *Tgfbr2^{flx}* in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* and the *Tgfbr2$^{flx/flx}$* mouse colons. *A,* results of PCR-based assays that amplify either the recombined or intact *Tgfbr2^{flx}* allele. DNA was extracted from either intestinal mucosa [distal small intestine (*SI*), right colon (*RC*), and left colon (*LC*)] or, using laser capture microdissection, from the luminal (*CL*) and basal (*CB*) portions of colonic crypts from the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* (*TTFC*) mice and *Tgfbr2$^{flx/flx}$* (*TT*) mice. The recombined *Tgfbr2^{flx}* allele is only detected in the intestinal mucosa from the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mouse. An intact *Tgfbr2^{flx}* allele is present in the intestinal mucosa from both genotypes. The intact *Tgfbr2^{flx}* allele in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mouse tissue is presumably from the 10–30% of the colonic mucosa that does not express Cre recombinase and from nonepithelial cells in the mucosal layer. *B* and *C,* TGFBR2 immunostaining of colonic mucosa from the *Tgfbr2$^{flx/flx}$* and *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice, respectively. TGFBR2 has been detected using rabbit polyclonal antisera and Cy3-labeled avidin-biotin complex, which can be seen as *red immunofluorescence*. The *white arrow* indicates a colon crypt in a *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mouse colon that has intact receptor expression, whereas the remainder of the colonic epithelia does not express TGFBR2.

layer. The mosaic expression of TGFBR2 we observed (Fig. 1, *B* and *C*) is consistent with the mosaic expression of Cre-recombinase from the *Fabpl$^{4xat\text{-}132}$* Cre transgene reported by Wong *et al.* (9).

Loss of TGFBR2 in the Colon Epithelium Does Not Have a Significant Effect on the Histological Appearance of the Colon. After observing absent TGFBR2 expression in the colonic epithelium of the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice, we assessed the effect of loss of TGFBR2 on the histological features of the colonic mucosa. Evaluation of randomly selected mice did not reveal any significant difference between *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice and *Tgfbr2$^{flx/flx}$* littermates. The overall status of the mice, including body weight, activity level, and coat quality, as well as the gross and histological appearance of the mucosa, was similar in the two lines. Representative mice (n = 1–4 mice/time point) were sacrificed at 1 month, 6.5 months, and 9 months of age and assessed by gross and microscopic examination. A subtle increase in the lamina propria was noted in a subset of the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice compared with *Tgfbr2$^{flx/flx}$* littermates, but no inflammation or hyperplasia was found in any of the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice. The proliferation rate in the *Tgfbr2*-null colon epithelium was also studied by assessing bromodeoxyuridine uptake in the normal colonic epithelium of the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice and *Tgfbr2$^{flx/flx}$* mice (n = 2–3 mice/group) at 6–7 months of age. No significant difference in the average nuclear labeling index (number of bromodeoxyuridine-positive cells/total number of cells per crypt) was seen between the two groups (0.19 *versus* 0.18, respectively; $P = 0.87$).

AOM-Induced Adenomas and Carcinomas Develop More Frequently in *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* Mice Compared with *Tgfbr2$^{flx/flx}$* Mice. In light of the lack of frequent spontaneous neoplasms in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice, we decided to determine whether loss of TGFBR2 affected colon cancer formation after the initiation phase of the adenoma−carcinoma sequence. Consequently, we treated the mice with AOM, a rodent colon carcinogen that has been shown to induce neoplasms that recapitulate the histological steps of the adenoma−carcinoma sequence. After AOM treatment, we observed a significant increase in colon neoplasm formation in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice at both 10 and 17 weeks after treatment. At 10 weeks after treatment, we observed that 82% (9 of 11) of the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice had colon neoplasms compared with 11% (1 of 9) of the *Tgfbr2$^{flx/flx}$* mice. (Table 1) The tumors in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice included gastrointestinal intraepithelial neoplasia (GIN), adenomas, and adenocarcinomas (Ref. 12; Fig. 2, *A*−*C*). Furthermore, at 10 weeks, the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice had more tumors per mouse (mean, 3.45 ± 3.47 tumors/mouse) than the *Tgfbr2$^{flx/flx}$* mice, in which there was only one mouse with two GIN lesions. (Table 1) This difference in average tumor number was also present at 17 weeks after treatment with an average of 4.88 ± 2.75 tumors/mouse in *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice compared with 2.44 ± 1.94 tumors/mouse in *Tgfbr2$^{flx/flx}$* mice ($P = 0.05$), although the total number of mice in each group that had tumors was not statistically different. The total number of tumors in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice at 17 weeks was 39, compared with 22 in the *Tgfbr2$^{flx/flx}$* mice ($P = 0.11$). In both the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* and *Tgfbr2$^{flx/flx}$* mice, the majority of the neoplasms were located in the left colon (93% and 96%, respectively).

In light of the known mosaic expression pattern of Cre in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice, which results in a proportion of colonic epithelial cells that have intact *Tgfbr2*, we assessed the recombination status of the *Tgfbr2^{flx}* allele in a representative subset of colon neoplasms arising in these mice to determine whether the tumors arose from *Tgfbr2*-null or *Tgfbr2*-intact cells. We observed that 10 of 10 tumors from four different mice were composed of cells that carried recombined *Tgfbr2* alleles (Fig. 2*D*).

The Colon Neoplasms in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* Mice Are More Histologically Progressed than the Neoplasms in the *Tgfbr2$^{flx/flx}$* Mice. At 10 weeks, the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice had a total of 38 neoplastic lesions (n = 11 mice) compared with 2 neoplastic lesions in the *Tgfbr2$^{flx/flx}$* mice (n = 9 mice). The two lesions in the *Tgfbr2$^{flx/flx}$* mice were both GIN lesions, which are histologically apparent areas of dysplasia that are not visible grossly (12). In contrast, the neoplasms in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice included adenomas and adenocarcinomas as well as GIN lesions. In the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice, 55% (21 of 38) of the neoplasms were adenomas, and 24% were adenocarcinomas (9 of 38). In fact, 45% (5 of 11) of the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice had adenocarcinomas, and 2 of these mice had three synchronous adenocarcinomas. Finally, of the adenomas occurring in this line, 29% (6 of 21) had areas of high-grade dysplasia, further demonstrating the advanced histological progression of the neoplasms occurring in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice compared with the neoplasms occurring in the *Tgfbr2$^{flx/flx}$* mice.

Despite using a high dose of AOM, at the 17 week time point, the proportion of advanced neoplasms was still greater in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice compared with the *Tgfbr2$^{flx/flx}$* mice, although the *Tgfbr2$^{flx/flx}$* mice had developed a substantial number of adenomas by this time. The *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice had a total of 39 neoplasms, of which 56% (22 of 39) were adenomas, and 21% (8 of 39) were carcinomas. The *Tgfbr2$^{flx/flx}$* mice had a total of 22 neoplasms at 17 weeks, of which 86% (19 of 22) were adenomas, and 9% (2 of 22) were carcinomas. Similar to the observation at 10 weeks, synchronous colon adenocarcinomas developed in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice (25%, 2 of 8) but did not occur in the *Tgfbr2$^{flx/flx}$* mice. Of interest, at the 17 week time point, the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice also had significantly more GIN lesions than the *Tgfbr2$^{flx/flx}$* mice, consistent with the continued formation of new neoplastic lesions at a higher rate in the colon epithelium lacking functional TGFBR2 compared with the epithelium in the mice with intact TGFBR2 (Table 1).

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Table 1 *Comparative tumor incidence in Tgfbr2$^{flx/flx}$ and Fabpl$^{4xat\text{-}132}$ Cre Tgfbr2$^{flx/flx}$ mice*

Genotype	No. of mice with tumors[a]	No. of tumors/mouse (average)[b]	No. of mice with GIN[c]	No. of mice with adenomas[d]	No. of mice with carcinoma[e]
10 weeks					
Tgfbr2$^{flx/flx}$ (n = 9)	1	0.22	1	0	0
Fabpl$^{4xat\text{-}132}$ Cre *Tgfbr2$^{flx/flx}$* (n = 11)	9	3.45	5	8	5
17 weeks					
Tgfbr2$^{flx/flx}$ (n = 9)	8	2.44	1	9	2
Fabpl$^{4xat\text{-}132}$ Cre *Tgfbr2$^{flx/flx}$* (n = 8)	8	4.88	5	8	5

[a] $P = 0.006$ (10 weeks); $P = 1.0$ (17 weeks).
[b] $P = 0.05$ (17 weeks).
[c] GIN, gastrointestinal intraepithelial neoplasia. $P = 0.16$ (10 weeks); $P = 0.05$ (17 weeks).
[d] $P = 0.001$ (10 weeks).
[e] $P = 0.04$ (10 weeks); $P = 0.15$ (17 weeks).

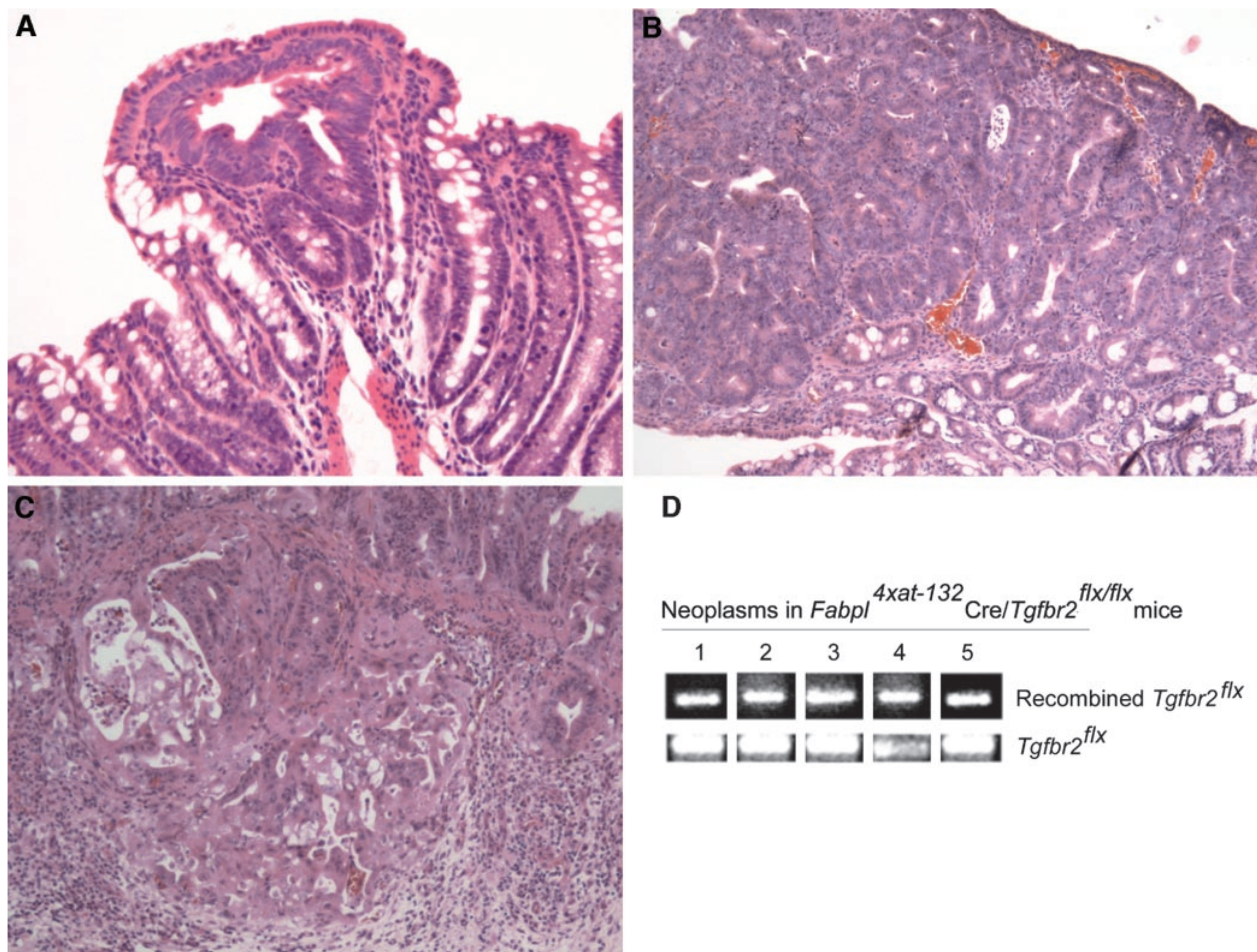

Fig. 2. Colon neoplasms in the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ mice. *A−C,* representative photomicrographs of H&E-stained sections of a gastrointestinal intraepithelial neoplasm (×400), adenoma (×100), and adenocarcinoma (×100), respectively, from *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ mouse colons after azoxymethane treatment. *D,* results of PCR-based assays that detect the intact and recombined *Tgfbr2*^{flx} alleles. A recombined *Tgfbr2*^{flx} allele is present in five representative neoplasms from *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ mice (*Lanes 1–5*), consistent with these tumors arising from *Tgfbr2*-null cells. An intact *Tgfbr2*^{flx} allele is present as well and is most likely from contaminating nonneoplastic cells in the tumor.

Status of *Kras2* Mutations and COX-2 Expression in Tumors from the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ Mice and *Tgfbr2*$^{flx/flx}$ Mice. Tumors arising in AOM models share some of the same genetic events seen in primary human colon neoplasms, such as *CTNNB1* and *KRAS2* mutations and increased COX-2 expression (17–19). In light of the increased adenoma and adenocarcinoma frequency we observed in the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ mice, we assessed *Kras2* mutation status at the mutation hot spots in exon 1 (codons 12 and 13) and exon 2 (codon 61) in a representative group of tumors from the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ and *Tgfbr2*$^{flx/flx}$ mice. No *Kras2* mutations in neoplasms from the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ *mice* ($n = 6$ for exon 1 and exon 2) or from the *Tgfbr2*$^{flx/flx}$ mice ($n = 7$ for exon 1 and 4 for exon 2) were observed. COX-2 expression was assessed by immunostaining a subset of tumors occurring in the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ and *Tgfbr2*$^{flx/flx}$ mice. COX-2 expression was increased in 13% (3 of 23) of neoplasms in the *Tgfbr2*$^{flx/flx}$ mice *versus* 5.2% (1 of 19) of the tumors in the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ mice. This difference was not significantly different ($P = 0.41$; data not shown).

The Tumors Occurring in the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ Mice Show Increased Proliferation but no Difference in Apoptosis when Compared with Tumors Occurring in the *Tgfbr2*$^{flx/flx}$ Mice. In light of the known effects of TGF-β on cell growth and apoptosis, we assessed the proliferation index of tumors arising in the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ and *Tgfbr2*$^{flx/flx}$ mice using Ki-67 immunostaining (2, 20). The tumors occurring in the *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ mice had a mean proliferation index that was 1.4-fold higher than the mean index of the tumors arising in the *Tgfbr2*$^{flx/flx}$ mice ($P = 0.037$, Student's t test; Table 2). In contrast, analysis of apoptosis in the tumors arising in both groups of mice by terminal deoxynucleotidyl transferase-mediated nick end labeling staining showed no significant difference between the two groups (Table 2).

Discussion

Our results demonstrate that *Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$ mice do not express TGFBR2 in the majority of the colonic epithelium and that this *Tgfbr2*-null colon epithelium is highly susceptible to devel-

Table 2 *Proliferation and apoptosis indices in tumors from Tgfbr2*$^{flx/flx}$ *and Fabpl*$^{4xat\text{-}132}$ *Cre Tgfbr2*$^{flx/flx}$ *mice*

For each column, values represent means ± SE of an average index from five fields from each neoplasm. The number of tumors analyzed per group is indicated in parentheses.

	Tgfbr2$^{flx/flx}$	*Fabpl*$^{4xat\text{-}132}$ Cre *Tgfbr2*$^{flx/flx}$
Proliferation index[a]	0.39 ± 0.05 (9)	0.52 ± 0.04 (9)
Apoptotic index[b]	0.02 ± 0.01 (5)	0.02 ± 0.01 (4)

[a] Number of Ki-67-positive nuclei/total number of nuclei in five ×400 fields per lesion. $P = 0.037$.

[b] Number of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive nuclei/total number of nuclei in five ×400 fields per lesion. $P = 0.8$.

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oping AOM-induced colon neoplasms. These results are consistent with *Tgfbr2* acting as a tumor suppressor gene in the colon and acting in a cell autonomous fashion to inhibit neoplasm formation.

Our initial findings of loss of TGFBR2 expression throughout the crypt axis and in the majority of the colon epithelium is consistent with the pattern of Cre-induced DNA recombination demonstrated in the *Fabp-Cre$^{4xat\text{-}132}$ Gt(ROSA)26Sor$^{tm1Sor/tm1Sor}$* (R26R) mice (9). Previous characterization of the *Fabp-Cre$^{4xat\text{-}132}$* R26R mouse has shown that Cre is expressed in 80% of the colonic and ileal mucosa in a mosaic pattern and that Cre-induced recombination in the intestinal endoderm is present as early as embryonic day 14.5 (9). Thus, the *Tgfbr2^{flx}* locus appears to be at least as susceptible to Cre-mediated DNA recombination as the R26R locus.

Interestingly, although the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice lack TGFBR2 expression in most of their colon epithelial cells, they do not frequently form colon neoplasms spontaneously. Thus, our observation of increased colon cancer formation after AOM treatment suggests that TGF-β signaling inactivation can promote tumorigenesis in the colon *in vivo*, but only after a concurrent initiating event. These results are consistent with studies of primary human adenomas and colon adenoma cell lines that have shown that TGF-β signaling deregulation occurs after the establishment of colon adenomas (15, 21). In addition, our results suggest that TGF-β signaling is dispensable for the homeostasis of the colonic epithelium and may only be physiologically required under specific circumstances, such as in response to exposure to specific inflammatory stimuli (22–24).

Our findings are consistent with results from studies of other mouse models that have disrupted the TGF-β signaling pathway. These models include the *Tgfb1$^{-/-}$ Rag2$^{-/-}$* mouse model and a transgenic mouse line, ITF-dnRII, that expresses a dominant negative *Tgfbr2* driven by the mouse intestinal trefoil peptide ITF/TFF3 promoter. Both of these models show an increased predisposition to develop colon neoplasms in the setting of concurrent factors, either *Helicobacter hepaticus* or AOM, respectively (22–24). Of interest, however, there are notable differences between the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* and *Tgfb1$^{-/-}$ Rag2$^{-/-}$* mice. Engle *et al.* (22) observed increased colonic inflammation and hyperplasia in *Tgfb1$^{-/-}$ Rag2$^{-/-}$* mice compared with *Rag2$^{-/-}$* mice and that the *Tgfb1$^{-/-}$ Rag2$^{-/-}$* mice spontaneously developed colon tumors as early as 2 months of age. In contrast, we did not observe spontaneous colon cancer in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice, even at up to 9 months of age. Our results also differ from those seen in the ITF-dnRII transgenic mouse. Similar to the *Tgfb1$^{-/-}$ Rag2$^{-/-}$* mice, the ITF-dnRII mice spontaneously develop colitis, although they have not been reported to develop spontaneous colon neoplasms (24). These differences between the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$*, *Tgfb1$^{-/-}$ Rag2$^{-/-}$*, and ITF-dnRII mice may be related to the degree of TGF-β signaling blockade, to effects caused by modulation of the ligand as opposed to the receptor, to differences in the strains of mice used (FVB *versus* 129/SvPas), or to differences in enteric flora at the respective facilities where the mice were housed. It is also possible that the dnRII construct may impede signaling of other TGF-β-related ligands, which may be causing some of the effects seen in the ITF-dnRII line (25).

Importantly, when our results with the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mouse model are taken into consideration with the results from the mouse models discussed above, it appears that the common mechanism leading to increased colon cancer formation in these mouse models is inactivation of TGF-β signaling in the colonic epithelial cells. Furthermore, our results suggest that loss of TGFBR2 promotes colon neoplasm formation after initiation with AOM by enhancing the formation of the early GIN lesions and by enhancing the progression of these lesions to invasive adenocarcinomas. Our studies of COX-2 expression and *Kras2* mutation status do not support a role for either COX-2 overexpression or *Kras2* mutation in the increased propensity for colon tumor formation in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice. The lack of *Kras2* mutations we observed is likely related to their low prevalence in AOM-induced tumors (19, 26). Finally, one notable difference in our model system compared with human colon cancer is that at least half of colon cancers that have mutationally inactivated *TGFBR2* in humans occur in mutation mismatch repair-deficient tumors and are in the right side of the colon. In contrast, the majority of the neoplasms we observed were in the left side of the colon. This difference is likely related to the use of AOM to induce tumors in these mice, which has been shown to cause predominantly left-sided colon cancer in mice and rats (27, 28).

With regard to the relevant biological consequences that contribute to colon cancer formation as a result of *Tgfbr2* inactivation, our studies implicate effects on cell proliferation but not on apoptosis. Our findings suggest that regulation of cell cycle control is one mechanism *in vivo* through which TGF-β signaling acts as a tumor suppressor. In light of the increased numbers of adenocarcinomas in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice we observed, we also anticipate that loss of TGF-β signaling affects processes implicated in tumor invasion as well. We have not investigated the role of loss of specific TGF-β-mediated signaling pathways in tumor formation in the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice, but based on studies of the *Apc$^{\Delta 716}$ Smad4$^{+/-}$* mice and *Smad3$^{-/-}$* mice, we predict that impaired Smad signaling may have a central role in the genesis of these tumors (29, 30).

In summary, we have demonstrated in an *in vivo* model system that loss of *Tgfbr2* in the colon epithelium contributes to colon cancer formation by promoting the establishment and progression of AOM-induced colon neoplasms. This effect is cell autonomous and may be at least partly secondary to increased cell proliferation in the tumor cells arising from the *Tgfbr2*-null colon epithelial cells. The results of these studies using the *Fabpl$^{4xat\text{-}132}$* Cre *Tgfbr2$^{flx/flx}$* mice provide evidence from an *in vivo* model system that inactivation of *TGFBR2* may have a pathogenic role in the formation of human colon cancers and furthermore suggest that this role is in the progression of colon neoplasms to invasive adenocarcinomas.

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