

Elimination of Colon Cancer in Germ-free Transforming Growth Factor Beta 1-deficient Mice¹

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Abstract

Patients with ulcerative colitis are at risk for colon cancer and frequently have microsatellite instability, which, in turn, is usually associated with inactivation of transforming growth factor (TGF) β signaling. TGF- β 1 deficiency in mice can lead to colon cancer that is preceded by precancerous lesions having submucosal inflammation and hyperplastic crypts. Germ-free TGF- β 1-deficient mice are free of inflammation, hyperplasia, and cancer, but when reintroduced into a *Helicobacter hepaticus*-containing specific pathogen-free room, these lesions reappear. Because adenoma/carcinoma but not inflammation/hyperplasia is dependent on the genetic backgrounds tested, colitis is required, but not sufficient, for carcinogenesis. This animal model should provide insight into the protective role of TGF- β 1 in early stages of ulcerative colitis-associated human colon cancer.

Introduction

TGF- β 1³ is at the apex of a signaling pathway that is one of the more commonly disrupted pathways in human colon cancer (1). Human colon tumor-derived cell lines are frequently resistant to the growth-inhibitory effects of TGF- β 1 (2), and this loss of sensitivity often results from *TGFBR2* inactivating mutations. *TGFBR2* mutations are found in 90% of MSI-positive tumors, and therefore account for ~13% of all human colon tumors (3). MSI is often associated with UC-associated colon cancer (4). Consequently, it is probable that inactivation of TGF- β signaling also correlates with UC-associated colon cancer. The tumor suppressor effect of TGF- β 1 in humans is thought to occur at a late stage of tumorigenesis because microsatellite unstable tumors usually have *TGFBR2* mutations only if the tumors are at the adenoma/carcinoma transition stage or later (5). These studies indicate that causal relationships may exist among UC, TGF- β signaling, genetic instability, and colon adenocarcinoma, but the basis of these relationships is unclear. Because TGF- β 1-deficient mice on an immunodeficient background develop colon cancer that is associated with inflammation (6), they would be a useful model for determining the relationship among UC, TGF- β , and colon cancer if it could be demonstrated that the inflammatory lesions are required for tumorigenesis. To make this determination we have eliminated inflammatory bowel lesions by making germ-free TGF- β 1-deficient mice. Here we show that in the absence of enteric flora there is no inflammation, hyperplasia, or neoplasia, and that recolonization of the mice with enteric flora can result in the reappearance of colon cancer.

Materials and Methods

A breeding pair of *Tgfb1*^{+/-} *Rag2*^{-/-} mice was generously provided by Dr. Robert L. Coffman (DNX Transgenics, Princeton, NJ, presently, Dynavax Technologies, Emeryville, CA). Specific pathogen-free breeding colonies of (a) *Tgfb1*^{+/-} *Rag2*^{-/-} mice with a mixed genetic background of 85–94% 129S2/SvPas (formerly 129/SvPas) and the remainder CF1; and (b) *Tgfb1*^{+/-} *Prkdc*^{scid/scid} mice with a mixed genetic background of 85–94% C3H/HeJ, and the remainder CF1 and 129S2/SvPas were housed in a barrier facility operated by the University of Cincinnati Laboratory Animal Medicine Services. Germ-free *Tgfb1*^{+/-} *Rag2*^{-/-} mouse colonies were established at the University of Wisconsin Gnotobiotic Research Laboratory (Madison, WI) by caesarean derivation. The establishment, maintenance, and health surveys of the barrier-raised and germ-free mice were carried out as reported (7, 8). PCR for *Helicobacter hepaticus* was carried out by the Research Animal Diagnostic and Investigative Laboratory, Columbia, MO.

When germ-free *Tgfb1*^{+/-} *Rag2*^{-/-} mice were reintroduced into our barrier facility for recolonization with the resident enteric flora, they were placed in two separate specific pathogen-free rooms, SPF1 and SPF2. SPF1 is the room where the animals with colon cancer had originally been housed, and SPF2 is a similar breeding room in the same barrier facility. Maintenance and quality control for the two rooms is identical, the only known difference being that SPF2 was *Helicobacter* sp.-free.

Histological examination and scoring for lesion classification was done as described previously (6). All of the analyses were performed blinded to prevent bias. A one-tailed Student *t* test was used for statistical analysis. Genotyping the secretory group II phospholipase A₂ wild-type (*Pla2g2a*, C3H strain) and mutant (*Pla2g2a*^{Mom1}, 129 strain) alleles were identified by the PCR genotyping technique described previously (9). DSS treatment was performed on eight each of *Tgfb1*^{+/+} *Prkdc*^{scid/scid} and *Tgfb1*^{-/-} *Prkdc*^{scid/scid} 6–8 week-old mice as described (10). The concentration and duration of treatment were chosen to induce a chronic inflammation similar to that seen in the original *Tgfb1*^{-/-} *Rag2*^{-/-} SPF1 colony without inducing acute symptoms.

Results and Discussion

From the outset it is necessary to make a clear distinction between the autoimmune-like multifocal inflammatory phenotype of *Tgfb1*^{-/-} mice and the inflammatory lesions found in the cecums and colons of *Tgfb1*^{-/-} *Rag2*^{-/-} mice. Immunocompetent *Tgfb1*^{-/-} mice have inflammatory lesions in multiple organs, and their median age of death is 20 days (11). When made germ-free, these mice still die from the autoimmune-like disease (7). However, if *Tgfb1*^{-/-} mice are genetically combined with lymphocyte-deficient mice, such as *Rag2* knockout (6) or *Prkdc*^{scid/scid} (12) mice, they are rescued from the autoimmune phenotype, and they can live as long as 8 months. However, submucosal, primarily granulocytic inflammatory lesions with accompanying hyperplasia now occur in the cecum and colon of these mice. These lesions occur in nearly all of the immunodeficient mice regardless of the presence or absence of TGF- β 1. Consequently, the inflammatory lesions that are associated with cecum and colon in *Tgfb1*^{-/-} *Rag2*^{-/-} mice are unrelated to the autoimmune-like inflammatory lesions of immunocompetent *Tgfb1*^{-/-} mice (6).

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³ The abbreviations used are: TGF, transforming growth factor; *TGFBR2*, transforming growth factor β receptor type II; MSI, microsatellite instability; UC, ulcerative colitis; DSS, dextran sodium sulfate.

Genetic Background Effects on Carcinogenesis in TGF- β -deficient Mice. Immunodeficient *Tgfb1*^{-/-} *Prkdc*^{scid/scid} and *Tgfb1*^{-/-} *Rag2*^{-/-} mice were placed on predominantly C3H or 129 genetic backgrounds, respectively. In both cases the animals were devoid of the autoimmune-like inflammatory disease described above. However, in each colony immunodeficient *Tgfb1*^{+/+} and *Tgfb1*^{-/-} animals developed submucosal inflammatory foci of the large intestine, and hyperplastic crypts were associated with the inflammatory lesions for *Tgfb1*^{+/+} or ^{-/-} *Rag2*^{-/-} mice (6) and for *Tgfb1*^{+/+} or ^{-/-} *Prkdc*^{scid/scid} mice (Fig. 1). However, only in immunocompromised TGF- β -deficient animals on a predominantly 129 background (*Tgfb1*^{-/-} *Rag2*^{-/-} mice) was progression to adenoma and adenocarcinoma observed (see Fig. 1 in Ref. 6). These results suggest that there may be modifier genes present in 129 strains that increase susceptibility for progression from hyperplasia to adenoma, and that inflammation is required but not sufficient for the development of cecum and colon cancer in immunocompromised TGF- β -deficient mice on a 129-strain genetic background.

Genetic Background Affects Susceptibility to Mouse Colon Cancer. One modifier locus that has been implicated in modifying tumor development in the *Apc*^{Min} mouse can be identified by the secretory group II phospholipase A₂ gene allele *Pla2g2a*^{Mom1}. The *Pla2g2a*^{Mom1} allele is a spontaneously occurring mutation propagated in specific strains of mice that results in severely deficient enzyme activity. It is linked to increased tumor numbers in *Apc*^{Min} mice on the 129 background compared with *Apc*^{Min} mice with a wild-type *Pla2g2a* allele, such as C3H mice. However, it is not completely clear whether the *Pla2g2a*^{Mom1} allele is responsible for the increased susceptibility (9). Because TGF- β -deficient mice are maintained on a partially mixed genetic background to circumvent embryonic lethality, some of the predominantly C3H-background *Tgfb1*^{-/-}

Prkdc^{scid/scid} mice should be homozygous for the *Pla2g2a*^{Mom1} susceptibility locus. Four such mice ranging in age from 4–6 months were identified and analyzed. Two had no lesions, one had inflammation and hyperplasia, and one had an adenoma (Fig. 2). One *Tgfb1*^{+/+} wild-type *Prkdc*^{scid/scid} mouse with the *Pla2g2a*^{Mom1} locus also had inflammation and hyperplastic lesions but no adenomas (data not shown). Because all of the *Tgfb1*^{-/-} *Rag2*^{-/-} animals with *H. hepaticus* and the *Pla2g2a*^{Mom1} locus develop adenomas and adenocarcinomas, another modifier locus (loci) must account for most of the susceptibility in that strain. Consequently, *Pla2g2a*^{Mom1} could represent a weak modifier locus conferring a minor degree of susceptibility to colitis-induced colon cancer, but it does not account for the full penetrance of the colon cancer phenotype in predominantly 129 *Tgfb1*^{-/-} *Rag2*^{-/-} mice, because nearly all of the *Tgfb1*^{-/-} *Rag2*^{-/-} mice develop colon cancer (6). Hence, there is at least one other major colon cancer susceptibility locus in strain 129 mice.

Colitis Is Required but Not Sufficient for Carcinogenesis in *Tgfb1*^{-/-} *Rag2*^{-/-} Mice. A previous study demonstrated that barrier-raised *Tgfb1*^{-/-} *Rag2*^{-/-} mice develop colon cancer from 3 to 6 months of age, and that inflammatory foci were always associated with the tumorigenic lesions, whereas the *Tgfb1*^{+/+} and ^{+/-} *Rag2*^{-/-} mice infrequently developed lesions beyond hyperplasia (6). Assuming that some complement of normal and/or pathogenic microbial flora resulted in an inflammatory response that, in turn, induced the tumorigenic lesions, we reasoned that making the mice germ-free would eliminate the inflammation and subsequent cancer. Barrier-raised *Tgfb1*^{+/+} *Rag2*^{-/-} mice were crossed, and the pregnant females were sent to the Gnotobiotic Facility in Madison, WI, where a germ-free standing colony of *Tgfb1*^{+/+} *Rag2*^{-/-} mice was established. Eighteen germ-free *Tgfb1*^{-/-} *Rag2*^{-/-} mice, 7 *Tgfb1*^{+/+} *Rag2*^{-/-} mice, and 6 *Tgfb1*^{+/+} *Rag2*^{-/-} mice ranging from 3 to 6

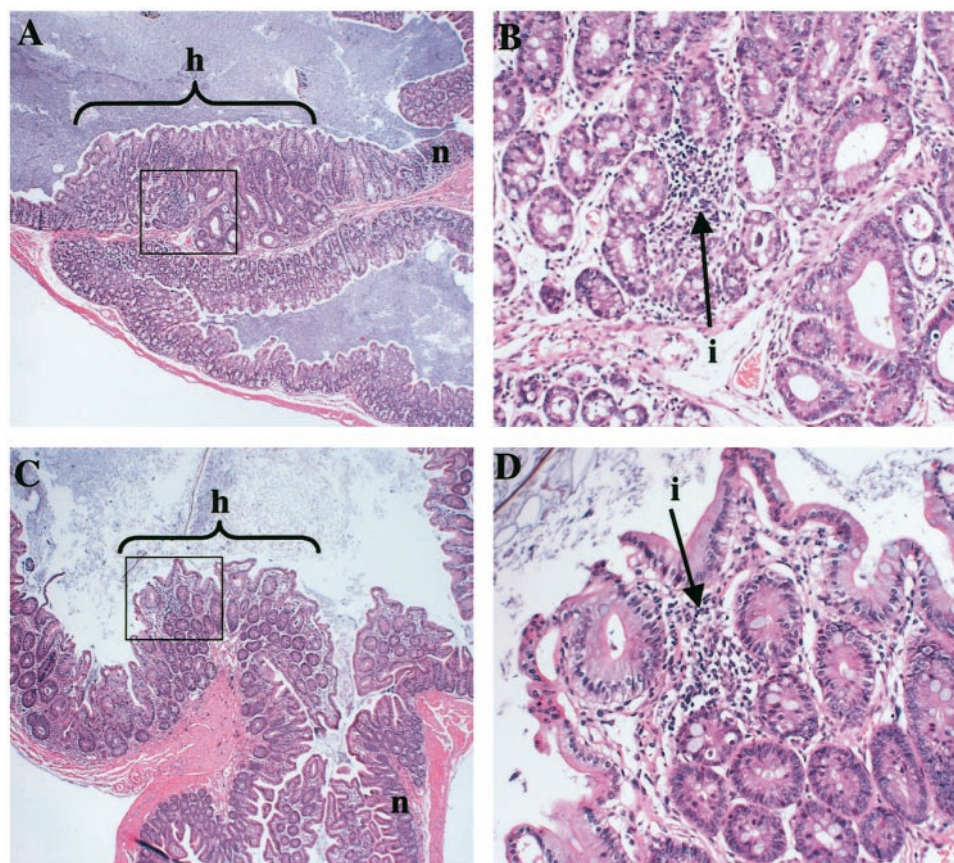


Fig. 1. Inflammation and hyperplasia in *Tgfb1*^{+/+} and ^{-/-} *Prkdc*^{scid/scid} Mice. A, inflammation and hyperplasia in cecum of *Tgfb1*^{+/+} *Prkdc*^{scid/scid} mice ($\times 10$ magnification). B, 5-fold magnification of inset depicted in A. C, inflammation and hyperplasia in cecum of *Tgfb1*^{-/-} *Prkdc*^{scid/scid} mice ($\times 10$ magnification). D, 5-fold magnification of inset depicted in C. h, hyperplasia; n, normal; i, inflammation.

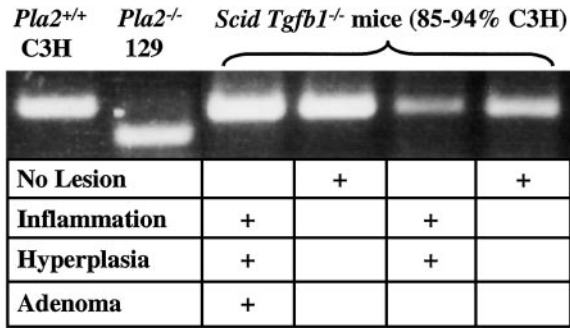


Fig. 2. *Pla2g2a*^{Momi} is not a major colon tumor susceptibility locus in *Tgfb1*^{-/-} *Rag2*^{-/-} mice. The bottom PCR-generated band represents the wild-type *Pla2g2a* allele (*Pla2*^{+/+}), which is present in C3H mice. The top band represents the mutant *Pla2g2a*^{Momi} allele (*Pla2*^{-/-}) that is present in 129 mice. *Tgfb1*^{-/-} *Prkdc*^{scid/scid} mice (predominantly C3H background) are represented in Lanes 3–6 and are all homozygous for the 129 strain *Pla2g2a*^{Momi} susceptibility locus, yet only one of them (Lane 4) progressed beyond hyperplasia.

months of age were analyzed for hyperplastic, adenomatous, and neoplastic lesions, as well as for submucosal inflammatory foci. Fig. 3, A and B, compare histological sections of colon samples from the progenitor barrier-raised and germ-free colonies, respectively, demonstrating that the inflammatory bowel lesions had been eliminated by the germ-free conditions. None of the mice in this colony developed hyperplasia, adenoma, or adenocarcinoma, strongly suggesting that colitis is required for the development of colon cancer in TGF- β 1-deficient mice.

Recolonization of Germ-free Mice with Enteric Flora. To confirm that the presence of enteric microbial content can lead to tumorigenic cecum and colon lesions associated with inflammatory bowel foci, germ-free *Tgfb1*^{+/+} *Rag2*^{-/-} mice from the Wisconsin germ-free colony were reintroduced into two independent SPF rooms in the University of Cincinnati barrier facility. Within 1 year the incidence of colon cancer had reappeared in the SPF1 room where mice had been housed for the original colon cancer study (6). In contrast, no colon cancer has reappeared in 3 years in SPF2. Consistent with our previous results, colitis was always present in mice developing colon cancer in SPF1 (Fig. 3C) and was never present in SPF2 where mice were free of tumorigenic lesions (Fig. 3D). These results demonstrate that gut microbial content is integral to the development of colitis in *Rag2*^{-/-} mice and to the development of colon cancer in *Tgfb1*^{-/-} *Rag2*^{-/-} mice.

To determine whether pathogenic microflora may be involved we tested the SPF1 and SPF2 colonies for *Helicobacter* species. *H. hepaticus* was identified as the only *Helicobacter* species in the SPF1 colony where colitis and colon cancer had been reestablished in *Tgfb1*^{-/-} *Rag2*^{-/-} mice. In the *Helicobacter*-free SPF2 colony, no colitis, hyperplasia, adenoma, or adenocarcinoma reappeared. These results suggest that *H. hepaticus* may be a causative factor for colitis in the *Rag2*^{-/-} colonies, and that in the absence of TGF- β 1 the resulting hyperplasia can progress to adenoma and adenocarcinoma. This is consistent with the observation that *Smad3* knockout mice develop colon cancer when on a 129 genetic background (100% incidence) or a mixed 129 \times C57BL/6 background (30% incidence; Ref. 13). However, it is not known whether the colon cancer in these mice is associated with *H. hepaticus*. Another 129-strain *Smad3*^{-/-} colony has been maintained free of *H. hepaticus*, and no colitis or adenocarcinoma has been observed.⁴ In another study, immunodeficient C57BL/6J *Rag1*^{-/-} mice were infected with *H. hepaticus* and *bilis* (14). In approximately one-third of the *Rag1*^{-/-} mice inflam-

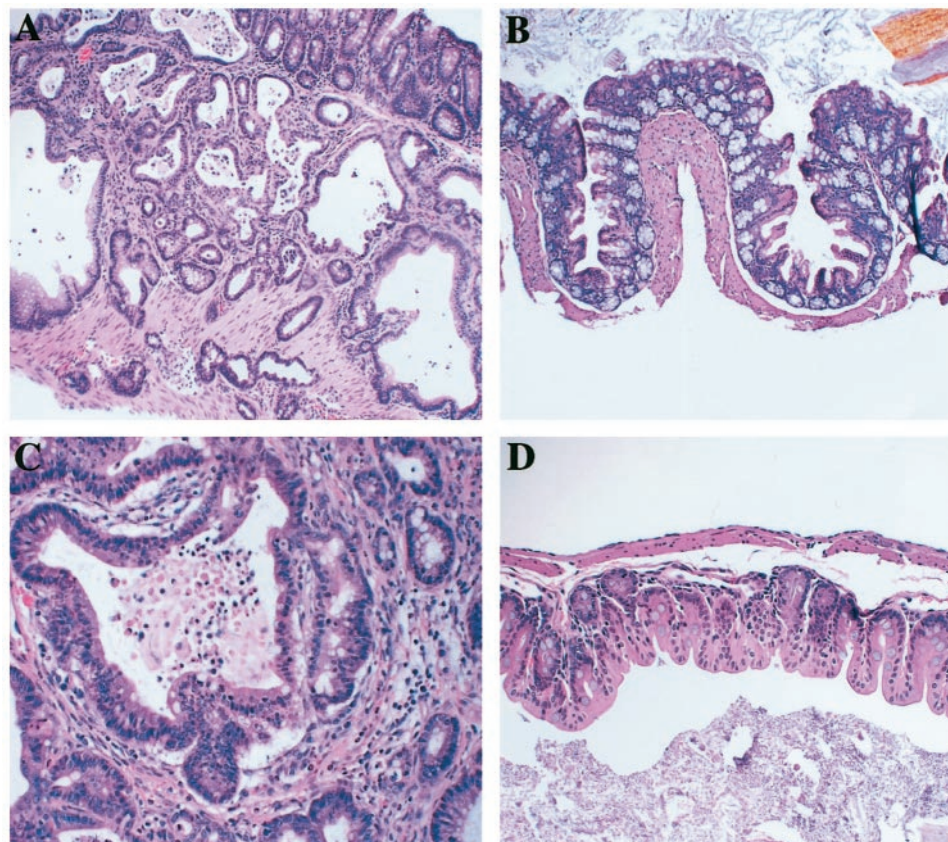
matory lesions with occasional hyperplasia were found, but no progression to adenoma or carcinoma was reported. None of the *Rag1*^{+/+} mice had lesions. This study is consistent with our findings that adenoma/carcinoma but not inflammation/hyperplasia are dependent on genetic background, that *H. hepaticus* could be causative, and that colitis is not sufficient for progression to adenoma/carcinoma in this mouse model.

DSS-induced Inflammatory Stress in TGF- β 1-deficient Mice. Our experiments strongly suggest that inflammatory stress, while required, is not sufficient for the development of colon cancer in TGF- β 1-deficient mice. To test this hypothesis, *Tgfb1*^{-/-} *Prkdc*^{scid/scid} mice, which normally have inflammatory/hyperplastic lesions but which do not progress to colon cancer, were subjected to a significant increase in large intestinal inflammatory stress. DSS treatments (1.25% DSS, 60 days) were used to determine whether increased inflammatory stress might be sufficient to induce a transition from hyperplasia to adenoma/carcinoma in 6–8-week-old *Tgfb1*^{-/-} *Prkdc*^{scid/scid} mice. Subsets of animals were analyzed for cecum and colon lesions at 22, 29, and 32 days of treatment and at 2, 22, and 33 days after treatment. No adenomas or adenocarcinomas developed in DSS-treated mice (Fig. 4), suggesting that increasing inflammatory stress in the large intestine does not induce progression to adenoma and adenocarcinoma. As expected, both *Tgfb1*^{+/+} and ^{-/-} *Prkdc*^{scid/scid} mice develop inflammation and moderate mucosal hyperplasia during treatment. However, the *Tgfb1*^{+/+} *Prkdc*^{scid/scid} mice are able to rapidly repair the mucosal damage, whereas inflammation and mucosal hyperplasia are still present in *Tgfb1*^{-/-} *Prkdc*^{scid/scid} mice 22 and 33 days after treatment. This supports previous evidence (6) suggesting that TGF- β 1-deficient mice may be less able to repair and maintain tissue damage than wild-type mice. These results confirm that inflammatory lesions are required for the development of colon cancer in TGF- β 1-deficient mice. However, because TGF- β 1-deficient mice on a *Prkdc*^{scid/scid} (predominantly C3H) background all develop inflammatory lesions but rarely colon cancer, it is clear that inflammatory lesions are not sufficient for progression from hyperplasia to adenoma and adenocarcinoma.

Comparison of Genetically Engineered Mouse Models of Colon Cancer. Besides *Tgfb1 Rag2* double knockout mice, there are several other mouse models for human colon cancer: *Smad3*, *Il2*, *Il10*, *G α ₁₂*, and *Muc2* knockout mice, and *Cdx2* and *Apc*^{Min} heterozygous mice. SMAD3 is in the TGF- β signaling pathway, and in its absence mice can develop metastatic colon cancer with associated inflammation (13). Similarly, *Il10*, *Muc2*, and *G α ₁₂* knockout mice, as well as *Il2* and *β 2 microglobulin* (*β 2m*) double-knockout mice, all develop inflammatory bowel-associated colon cancer (Refs. 15, 16; reviewed in Ref. 17). *Cdx2* heterozygous mice develop primarily large intestinal and occasionally small intestinal tumors without loss of heterozygosity at the *Cdx2* locus, and there is no associated colitis, suggesting that mild disruption of normal topographical tissue relationships could alone initiate tumorigenesis (18). *Apc*^{Min} mice have predominantly small intestinal adenomas with a few large intestinal tumors. Inhibition or elimination of inflammatory activity reduces the penetrance and multiplicity, but does not eliminate the tumor phenotype, so there may be a mild association of inflammation with the tumors, but it is not required (19). In most of the models in which a gene was knocked out that had no previous association with colon cancer, no evidence for other mutations in any genes normally associated with colon cancer were found. Similarly, in the *Tgfb1*^{-/-}, *Smad3*^{-/-}, and *Apc*^{Min} models, no mutations were found in colon cancer genes other than the gene of the original mutation. An exception is the *Il2 β 2m* double-knockout mouse in which mutations in *Apc* and *p53* were found (16). In summary, colitis seems to be required for tumorigenesis in most large intestinal cancer models in which *Apc* is not mutated. *Cdx2*

⁴ J. Letterio, personal communication.

Fig. 3. Reconstitution of natural gut flora in germ-free *Tgfb1*^{-/-} *Rag2*^{-/-} mice. Representative samples are from previously germ-free *Tgfb1*^{-/-} *Rag2*^{-/-} mice that were reintroduced into barrier rooms SPF1 in which *H. hepaticus* was present (A and C) or SPF2 in which *H. hepaticus* was not present (B and D). A, adenocarcinoma from cecum of a 3-month-old mouse ($\times 25$ magnification). B, no significant lesions from colon of a 4-month-old mouse ($\times 25$ magnification). C, adenocarcinoma from cecum of a 4-month-old mouse ($\times 50$ magnification). D, no significant lesions from cecum of a 6-month-old mouse ($\times 20$ magnification).



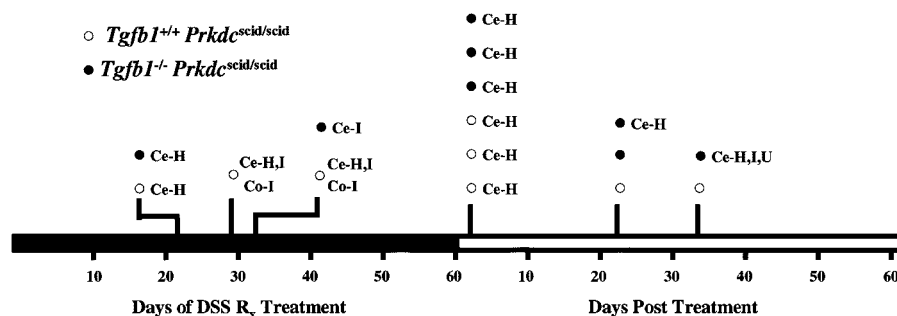
heterozygous mice are an exception because they develop grossly altered crypt architecture without colitis, and *Apc*^{+/-min} mice are an exception because they can be made germ-free without major loss of the tumor phenotype (19). Progression to tumors could result either directly from deficiency in a tissue architecture gene, as in the *Cdx2* heterozygous mice, or through an inability of the epithelium to maintain tissue integrity in the presence of inflammatory stress. Altered inflammatory response and a deficiency in maintenance of tissue architecture may combine to induce large intestinal cancer in *Smad3*, *Tgfb1* *Rag2*, *Il10*, and *G α ₁₂* knockout mice.

Interplay between Microbial Flora and Host Response Establishes Conditions for Colon Cancer. Together, these results demonstrate that neither enteric microbial agents nor inflammation alone cause colon cancer in *Tgfb1*^{-/-} *Rag2*^{-/-} mice. Because genetic background also plays an important role in the process, it is clear that the response to the microbial-induced inflammation is critical to the development of colon cancer. This is consistent with evidence that a single microbial agent can have important modulatory effects on the host expression profile, especially in areas of nutrient absorption,

mucosal barrier fortification, xenobiotic metabolism, and angiogenesis (20). Consequently, to fully understand the conditions that lead to colitis-associated colon cancer in mice, the causative microbial agent(s), and the differential responses of the host inflammatory system and colon epithelium will need to be systematically investigated. The existence of *Tgfb1*^{+/-} *Rag2*^{-/-} and *Tgfb1*^{-/-} *Rag2*^{-/-} mice, which have different susceptibilities to colitis-associated colon cancer, will be critical to these investigations.

Relevance of *Tgfb1*^{-/-} *Rag2*^{-/-} Mice to Human Colon Cancer. Having established that colitis can cause colon cancer in mice with mutations in the TGF- β signaling pathway and that the causative factors involve an interplay between host tissue and inflammatory responses to specific component(s) of microbial flora, it is worthwhile to compare this mouse model with UC-associated colon cancer in humans. The original studies on mutations in the human *TGFBR2* gene found mutations in 90% of the 15–20% of all human tumors displaying MSI, and the loss was found to occur at a late stage of tumorigenesis (5). More recent studies have indicated that as much as 70% of microsatellite-stable colon cancer can also exhibit a blockade

Fig. 4. DSS treatment of *Tgfb1*^{+/-} and ^{-/-} *Prkdc*^{scid/scid} mice. Mice were treated for 60 days with DSS, and examined for type of lesion at various days during treatment and after treatment. O, *Tgfb1*^{+/-} *Prkdc*^{scid/scid} mice; \circ , without type of lesion given indicates normal cecum and colon in *Tgfb1*^{+/-} *Prkdc*^{scid/scid} mice; \bullet , *Tgfb1*^{-/-} *Prkdc*^{scid/scid} mice; Ce, cecum; Co, colon; H, hyperplasia; I, inflammation; U, ulceration.



of TGF- β signaling (21), and in these cases the stage at which the tumor-suppressive activity of TGF- β occurs is not clear. In one study of colon cancer in patients with UC, chromosomal instability preceded dysplasia (22). Consequently, it now seems plausible that human patients with UC can develop a genetic instability-induced disruption in TGF- β signaling before or during early stages of tumor formation. This would be consistent with our studies on TGF- β 1-deficient mice.

It is not unreasonable to suggest that in humans years of inflammatory stress could eventually lead to mutations resulting in MSI and mutations in *TGFBR2* or downstream signaling genes. With a blockade in TGF- β signaling the inflammatory stress could result in an altered inflammatory response and/or a disruption in tissue architecture, leading to an accelerated progression from hyperplasia to adenoma and adenocarcinoma. In *Tgfb1*^{-/-} *Rag2*^{-/-} mice the short 1–5-month period of inflammatory stress may be insufficient to generate genetic instability and subsequent mutations in *TGFBR2* or downstream signaling genes, but that would not be necessary because they already have a TGF- β signaling defect.

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