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Production of Gastrointestinal Tumors in Mice by Modulating Latent Transforming Growth Factor Beta 1 Activation

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Abstract

Transforming growth factor-β (TGF-β) and its signaling pathways are important mediators in the suppression of cancers of the gastrointestinal (GI) tract. TGF-β is released from cells in a latent complex consisting of TGF-β, the TGF-β propeptide (LAP) and a latent TGF-β binding protein (LTBP). We previously generated mice in which the LTBP-binding cysteine residues in LAP TGF-β1 were mutated to serine precluding covalent interactions with LTBP. These *Tgfb1*$^{C33S/C33S}$ mice develop multiorgan inflammation and tumors consistent with reduced TGF-β1 activity. To test whether further reduction in active TGF-β levels would yield additional tumors and a phenotype more similar to *Tgfb1*$^{-/-}$ mice, we generated mice that express TGF-β1^{C33S} and are deficient in either integrin β8 or TSP-1, known activators of latent TGF-β1. In addition we generated mice that have one mutant allele and one null allele at the *Tgfb1* locus, reasoning that these mice should synthesize half the total amount of TGF-β1 as *Tgfb1*$^{C33S/C33S}$ mice and the amount of active TGF-β1 would be correspondingly decreased compared to *Tgfb1*$^{C33S/C33S}$ mice. These compound mutant mice displayed more severe inflammation and higher tumor numbers than the parental *Tgfb1*$^{C33S/C33S}$ animals. The level of active TGF-β1 in compound mutant mice appeared to be decreased compared to *Tgfb1*$^{C33S/C33S}$ mice as determined from analyses of surrogate markers of active TGF-β, such as P-Smad2, C-Myc, KI-67, and markers of cell cycle traverse. We conclude that these mutant mice provide a useful system for modulating TGF-β levels in a manner that determines tumor number and inflammation within the GI tract.

Keywords

TGF-β1; Integrin β8; GI Tumors; Inflammation

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Introduction

Transforming growth factor β (TGF-β) has divergent effects on tumor generation and progression (1, 2). During the early stages of tumorigenesis, TGF-β acts as a tumor suppressor presumably because TGF-β is a powerful inhibitor of the growth of epithelial cells (3, 4). As tumors progress in their evolution, the growth suppressive property of TGF-β can be lost allowing a growth enhancing effect of TGF-β to predominate (5-8). Thus, the participation of TGF-β in tumorigenesis is complicated and dependent upon multiple factors including cell type, location, and the phase of tumor development.

After synthesis and secretion, TGF-β undergoes a series of modifications in order to produce the active growth factor (9). Mature TGF-β is a 25 kDa homodimer derived from a larger precursor by intracellular proteolytic processing (9). The cleaved propeptide, the latency associated protein (LAP), remains non-covalently bound to the mature growth factor, rendering the TGF-β latent, even after secretion. Dissociation from LAP is required for TGF-β receptor binding. LAP is usually covalently bound to a second protein, a latent TGF-β binding protein (LTBP), through cysteine residues at the amino terminal region of the LAP monomers and a pair of cysteines in the LTBP. There are four *Ltbp* genes (*Ltbp1, 2, 3, and 4*), but only LTBP-1, -3 and -4 bind to LAP (10, 11). The complex of TGF-β, LAP, and LTBP is called the large latent complex (LLC), whereas the complex of TGF-β and LAP is called the small latent complex (SLC). There are three TGF-β genes – *Tgfb1, b2* and *b3*, and all three proteins are secreted as latent complexes (12). The conversion of LLC to active TGF-β is mediated by several mechanisms, including proteolytic processing of LTBP and/or LAP to release active cytokine, the interaction of competitive LAP-binding sequences of thrombospondin-1 (TSP-1) or F-spondin with the latent complex, and the binding of integrins to RGD sequences in TGF-β1 and TGF-β3 LAPs (9, 13). Several different β integrins including β3, β5, β6 and β8 bind LAP and may activate the latent complex, but two different mechanisms of activation appear to be employed. β5 and β6 integrin-mediated activation utilizes force supplied by the cell cytoskeleton acting through the integrin upon the LLC tethered to the matrix via the LTBP (14, 15). The application of force distorts the LLC thereby exposing or liberating the TGF-β. SLC binding to LTBP is required for latent TGF-β activation by the integrins αvβ5 and αvβ6 (15, 16). The integrin β8 activates SLC, but activation requires a metalloprotease in addition to the integrin (17). In this activation process, the integrin serves to co-localize the latent complex and the protease, thereby enhancing the rate of protease-mediated activation. TSP-1 also activates both LLC and SLC (18).

Elucidating the role of LTBPs in TGF-β biology has been the focus of a number of investigations (10, 19). Mice and/or people missing LTBP-1, -3 or -4 have phenotypes consistent with alterations in TGF-β levels indicating the importance of LTBPs in TGF-β biology (20-24). However, because binding to LTBP facilitates SLC secretion, the cause of LTBP-related phenotypes is unclear; i.e., are they related to decreased SLC secretion or impaired latent TGF-β activation. To address this question, we generated mice in which the cysteines at position 33 in the TGF-β1 LAP, which normally bind to LTBP, were changed to serines (25). Animals ($Tgfb1^{C33S/C33S}$) with this mutation should produce all of their TGF-β1 as SLC. If LTBP is required for proper TGF-β1 sequestration and activation, these mice should display a TGF-β1-null-like phenotype. If LTBP is not required for TGF-β1 generation, the mice should display a normal phenotype. The mutation of the cysteines to serines also allows the SLC to be readily secreted; therefore mutant phenotypes would not be caused by decreased extracellular latent TGF-β1 (14, 26).

$Tgfb1^{C33S/C33S}$ mice displayed phenotypes, such as shortened life span, lack of epidermal Langerhans cells, and multiorgan inflammation, consistent with decreased active TGF-β1

(25). The mutant mice also developed tumors of the stomach, colon, cecum, rectum and anus. Measurement of active TGF-β1 in serum revealed a decrease in the amount of active TGF-β1, but little change in the amount of total (latent plus active) secreted TGF-β1. These results indicated that LTBP, as part of the LLC, is required for proper latent TGF-β1 activation. We suggested that these animals were hypomorphs rather than nulls for TGF-β1 because the inflammation and shortened life span were not as severe as observed in TGF-β1 null mice. We hypothesized that the *Tgfb1*$^{C33S/C33S}$ animals activated some secreted TGF-β1 SLC, albeit inefficiently.

Here, we report a series of experiments designed to test this contention. We reasoned that if we further lowered active TGF-β1 levels in *Tgfb1*$^{C33S/C33S}$ mice by eliminating activators of the SLC or by deleting one *Tgfb1* allele, the abnormal phenotype would be enhanced to resemble more closely the *Tgfb1*$^{-/-}$ phenotype.

Materials and Methods

Mouse Lines and Reagents

Tgfb1^{+IC33S}, *Tgfb1*$^{+/-}$, *Tsp1*$^{+/-}$, and *Itgb8*$^{+/-}$ mice have been described previously (25, 27-30). All mice were housed in a Specific Pathogen Free facility and routinely checked for infections and parasites. All procedures were conducted according to the regulations of the NYU Langone Medical Center IACUC.

TGF-*β* Assays

The preparation of sera and measurement of TGF-*β*1 with the Quantine (R & D Systems) kit have been described previously (25). Tissue extracts were prepared from wet tissue samples. The samples were homogenized at 4°C in Tissue Protein Extraction Reagent and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientifc, Rockford, USA). The extracts were centrifuged at 8000 × g for 10 min at 4°C. After the protein concentrations in the supernatants were determined, the levels of total and active TGF-β1 were measured using a mouse TGF-β1 duo-set (DY1679) according to the manufacturer's instructions (R&D Systems, Minneapolis, USA). Total TGF-β1 levels were determined by acid activation (final concentration 0.2 M hydrochloric acid, 10 min, room temperature) of the latent TGF-β1 in the homogenate. Untreated and activated samples from the same homogenate were assayed in parallel.

Immunoblotting

Western blotting was performed as described by Yoshinga et al. (25).

Immunohistochemistry

Preparation of tissues, sectioning, and antibody staining were performed as described in Yoshinaga et al. (25).

Quantitative Real-Time PCR (qPCR) Analysis

RNA was extracted from 5 pairs of mice from each genotype using TRIzol (Invitrogen). Reverse transcription was performed using 1 μg of RNA and the SuperScript III Reverse Transcriptase (Invitrogen) (50 °C, 60 min). The resulting cDNA was used for qPCR analysis. qPCR was performed using specific primers and QuantiFast SYBR Green PCR Kit (Qiagen) on an iCycler Thermal Cycler (Bio-Rad). Each target transcript expression was quantified by comparing the threshold cycle (Cq) with that of β-actin by using the comparative Cq method (31). The primers used are described in Supplemental Table 1.

Statistical Analysis

Descriptive statistics were performed with StatView J-4.5 program (SAS Institute). The Kaplan–Meier method was used to estimate all survival curves from mouse studies. The log-rank statistic was used to compare the overall survival distributions.

Pathological Scoring of Inflammation

A scoring system in which three parameters were monitored in individual histological sections was used for quantification of inflammation (25). The three parameters were; Inflammation, which was measured as cell infiltration of the mucosa by mixed populations of inflammatory cells and edema, hyperplasia, which was monitored by the hyperplasia of the mucosal epithelium including lengthening of crypts, increased density of epithelial cells and crypts and thickening of mucosa, and necrosis/ulceration, which was monitored my examining for necrosis of mucosal epithelial cells with attenuation, erosion, or ulceration of the epithelial barrier. A scoring system for each parameter was used in which 0 = within normal limits, 1 = minimal to mild, 2 = moderate and 3 = severe. Two individuals monitored slides in a blinded fashion. Within the stomach tissue, we observed no necrosis and both the wild type and mutants showed hyperplasia. Therefore, only the inflammation parameter was used.

Results

Tumor production in *Tgfb1*$^{C33S/C33S}$;*thrombospondin1*$^{-/-}$ mice

To test our hypothesis that *Tgfb1*$^{C33S/C33S}$ activate TGF-β1 SLC, we first generated *Tgfb1*$^{C33S/C33S}$ mice deficient in either of two known activators of SLC, the matricellular protein thrombospondin-1 (TSP-1) or the integrin β8 (17, 18), and examined these animals for changes in inflammation, tumor number, and markers for TGF-β activity.

Tgfb1$^{C33S/C33S}$;*Tsp1*$^{-/-}$ mice displayed a phenotype that was essentially unchanged compared to *Tgfb1*$^{C33S/C33S}$ mice (Supplemental Table 2). (*Tsp1*$^{+/-}$ mice, used as controls, show no phenotype.) There was a slight increase in the degree of inflammation in the colon and rectum when *Tgfb1*$^{C33S/C33S}$;*Tsp1*$^{+/-}$ and *Tgfb1*$^{C33S/C33S}$;*Tsp1*$^{-/-}$ mice were compared, but inflammation in all other organs was essentially equivalent amongst mice with the two genotypes (Supplemental Table 2A (12 weeks) and Supplemental Fig. 1). There was no change in survival of *Tgfb1*$^{C33S/C33S}$;*Tsp1*$^{-/-}$ mice compared to *Tgfb1*$^{C33S/C33S}$ mice (data not shown). When these mice were examined for presence of tumors, a slight but statistically insignificant increase in tumor incidence was observed upon elimination of TSP-1 (Supplemental Table 2B). The distribution of tumor types was approximately equivalent in *Tgfb1*$^{C33S/C33S}$;*Tsp1*$^{+/-}$ and *Tgfb1*$^{C33S/C33S}$;*Tsp1*$^{-/-}$ mice and similar to results in our previous studies (25). Therefore, Tsp1 appears to make only a minor, if any, contribution to the level of active TGF-β in tumor production in *Tgfb1*$^{C33S/C33S}$ mice.

Tumor production in *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice

We next examined the degree of inflammation and tumor production in *Tgfb1*$^{C33S/C33S}$ versus *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice. The integrin αvβ8 is an important modulator of TGF-β1 levels within the intestine (32, 33). Unlike *Tgfb1*$^{C33S/C33S}$;*Tsp1*$^{-/-}$ mice, *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice presented with significant changes compared to *Tgfb1*$^{C33S/C33S}$ animals. The compound mutant mice had a shortened survival compared to *Tgfb1*$^{C33S/C33S}$ mice but similar to *Itgb8*$^{-/-}$ mice (Fig. 1A). The level of inflammation was increased in the stomach, cecum, colon and rectum (Table 1A). The increase in inflammation was clearly apparent when tissues from the different groups were compared by a scoring method that quantified inflammation, hyperplasia and necrosis/ulceration (Supplemental Fig. 2A). Tumor incidence also was substantially increased from 19% to 53%

when *Tgfb1*$^{C33S/C33S}$ animals were compared to *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ animals (Table 1B). Although only rectal tumors were observed in *Tgfb1*$^{C33S/C33S}$ mice, more than half of the tumor-bearing *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice had multiple tumors and these occurred in several different organs. In Figure 2, images of a rectal adenosquamous cell carcinoma (Fig. 2Aa) and rectal adenocarcinoma (Fig. 2Ac) are illustrated. All of the tumors were invasive, as tumor cells were observed in the submucosal muscle layer.

To determine if the increased tumor incidence in *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice correlated with changes in TGF-β levels and/or TGF-β signaling, we analyzed stomach and rectal tissue from wild type, *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ animals for markers of TGF-β1 activity. Because TGF-β1 is an inhibitor of epithelial cell growth, decreases in TGF-β1 signaling should result in enhanced epithelial proliferation. Consistent with potentially diminished levels of active TGF-β1, *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice displayed increased (more than twice that observed in wild type (WT) tissues) cell proliferation of the epithelial cells of the mucosal layer in the rectal epithelium, as monitored by immunostaining for the proliferation marker KI-67 (Fig. 3A). We observed a progressive increase in the number of proliferating cells when WT samples were compared to *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ samples. (Fig. 3A). Similar results were found in stomach tissues from the three genotypes (data not shown). TGF-β signaling is usually marked by increases in the level of P-Smad2, an intracellular mediator of the canonical TGF-β signaling pathway. P-Smad2 immunostaining in the rectum was heterogeneous in *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice with some areas showing decreased intensity, whereas other areas appeared to have normal levels (Fig. 4Aa-d). In general there appeared to be less P-Smad2 in the rectal tissue of each of the two mutant genotypes and significantly less staining in the double mutant. However, in order to survey the entire rectum for P-Smad levels, we performed immunoblotting and scanning after SDS-PAGE on the soluble proteins from rectal tissue (Fig. 4Ae). The results show a clear loss of P-Smad2 reactivity in the double mutant tissue, whereas tissue from animals with only the *Tgfb1*$^{C33S/C33S}$ or *Itgb8*$^{-/-}$ mutations appeared to contain amounts of P-Smad2 close to or slightly more than that of the wild type sample. The level of C-Myc expression, which normally is suppressed by TGF-β, was enhanced in the *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ rectal tissue compared to wild type tissue (Fig. 5A). We also measured the transcript levels for the cell cycle regulators p15, p21, and p27, which are known to be regulated by TGF-β, in the three genotypes (Fig. 5A). There was only a slight decrease, which was not statistically significant, in p21 expression in *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice compared to either wild type or *Tgfb1*$^{C33S/C33S}$ mice. However, there were pronounced decreases in the expression of the negative regulators p15 and p27 compared to wild type in both *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ rectal tissues consistent with a decrease in active TGF-β1.

Next, we measured TGF-β1 in sera from WT, *Itgb8*$^{-/-}$, *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice (Fig. 6A). When total TGF-β1 was quantified after acid activation of mouse sera, the *Tgfb1*$^{C33S/C33S}$, *Itgb8*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ samples were equivalent and slightly lower than the control WT animal samples (Fig. 6A). When active TGF-β1 in serum was quantified, the samples from *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice were approximately half of the control values (Fig. 6B). However, we did not detect a difference between those two samples. This may indicate that the contribution of integrin αvβ8 to the activation of latent TGF-β1 in serum is negligible. We also evaluated the amount of total and active TGF-β1 in rectal tissue extracts (Supplemental Fig. 3A and B). Although there were small differences with respect to total TGF-β1 amongst the four experimental groups, there was no statistical difference in the amount of active TGF-β1 amongst the samples. The reasons for this are not known.

Tumor production in *Tgfb1*$^{-/C33S}$ mice

We reasoned that if enhanced tumorigenesis in *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ versus *Tgfb1*$^{C33S/C33S}$ mice was the result of lower TGF-β1 levels, a second approach to decrease active TGF-β1 levels would be to generate *Tgfb1*$^{-/C33S}$ mice. As these animals would have only one functional TGF-β1 allele and that would be the C33S allele, they should produce approximately half as much TGF-β1 as *Tgfb1*$^{C33S/C33S}$ mice. Therefore, we generated *Tgfb1*$^{-/C33S}$ mice by crossing *Tgfb1*$^{+/C33S}$ and *Tgfb1*$^{-/+}$ animals. *Tgfb1*$^{-/C33S}$ mice were produced at the expected Mendelian frequency (Data not shown) and had a shortened life span compared to wild type or *Tgfb1*$^{C33S/C33S}$ mice (Fig. 1B). *Tgfb1*$^{-/C33S}$ mice displayed more severe inflammation in their lungs, heart, stomach, liver and colon than did *Tgfb1*$^{C33S/C33S}$ mice (Table 1C; Supplemental Fig. 2B). Tumor incidence in *Tgfb1*$^{-/C33S}$ mice was also significantly enhanced from 20% to 73% when *Tgfb1*$^{C33S/C33S}$ mice were compared with *Tgfb1*$^{-/C33S}$ mice (Table 1D). Interestingly, there were only 2 *Tgfb1*$^{-/C33S}$ mice with multiple tumors. The reason for this is unclear. The distribution of tumor types in *Tgfb1*$^{-/C33S}$ mice was also different compared to *Tgfb1*$^{C33S/C33S}$ mice. By 12 weeks of age 53% of the *Tgfb1*$^{-/C33S}$ animals had gastric adenocarcinomas compared to 9% in *Tgfb1*$^{C33S/C33S}$ mice. (The incidence of gastric adenocarcinomas in *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice was 20% (Table 1B)). Two gastric tumors are shown in figure 2B. One tumor is an adenocarcinoma (Fig. 2Ba and b) and one is a squamous cell carcinoma (Fig. 2Bc and d). Both tumors were invasive, as tumor cells were found in the submucosal muscular layer. The rectal tumors in *Tgfb1*$^{-/C33S}$ mice were similar to those observed in animals with the *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ genotype (Data not shown).

We next measured a number of parameters to characterize signaling and TGF-β1 levels in *Tgfb1*$^{-/C33S}$ mice, as we did with the *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice. We focused on the gastric tumors because of their high incidence and relative rarity in other mouse models. As we observed with *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice, stomach tissue from *Tgfb1*$^{-/C33S}$ mice had higher levels of KI-67 positive cells than did wild type or *Tgfb1*$^{C33S/C33S}$ tissues (Fig. 3B). Quantification of the number of KI-67 positive cells revealed that there was a 4-fold increase in *Tgfb1*$^{-/C33S}$ tissue compared to controls and a 2-fold increase compared to *Tgfb1*$^{C33S/C33S}$ tissue (Fig. 3B). P-Smad2 staining in the stomachs of wild type, *Tgfb1*$^{C33S/C33S}$, and *Tgfb1*$^{-/C33S}$ mice revealed that there was progressively less staining, as the expected level of total TGF-β1 decreased (Fig. 4B). We observed that C-Myc expression in the stomach tissue of *Tgfb1*$^{-/C33S}$ mice was elevated compared to wild type mice, but was not increased compared to *Tgfb1*$^{C33S/C33S}$ animals (Fig. 5B). We also measured tumor suppressor gene expression. As observed with the *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ tissues, there was little difference in expression of p21 when WT, *Tgfb1*$^{C33S/C33S}$, and *Tgfb1*$^{-/C33S}$ stomach tissues were compared by qPCR (Fig. 5B). Similar to the samples in figure 5A, there was a decrease in the expression levels of p15 amongst WT, *Tgfb1*$^{C33S/C33S}$, and *Tgfb1*$^{-/C33S}$ tissue (Fig. 5B).

Finally, we measured the level of total and active TGF-β1 in sera from wild type, *Tgfb1*$^{C33S/C33S}$, and *Tgfb1*$^{-/C33S}$ mice (Fig. 6C and D). The total TGF-β1 in sera from *Tgfb1*$^{C33S/C33S}$ mice, as measured after acid activation, was equivalent to wild type as described earlier (25). The *Tgfb1*$^{-/C33S}$ mouse sera had less than half of the total amount of TGF-β1 consistent with the loss of one allele. Both the mutant sera (*Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{-/C33S}$) had less than 30% of the amount of active TGF-β1 than the wild type sample (Fig. 6D). The amount of active TGF-β1 in the *Tgfb1*$^{-/C33S}$ mouse sera was slightly more than half of that found in the *Tgfb1*$^{C33S/C33S}$ mouse sera. When the amount of total and active TGF-β1 was measured in tissue, there were small differences in the total and active concentrations of cytokine between the genotypes and the amount of active was approximately 10% of total (Supplemental Fig. 3C and D). There were no statistically

significant differences between the *Tgfb1*$^{C33S/C33S}$ and Tgfb1$^{-/C33S}$ samples, although both of these samples were lower than the wild type control sample.

Discussion

The association of TGF-β with cancers of the GI tract is well established. Extensive work has shown that elimination of the growth factor or interference with its signaling either by TGF-β receptor mutations or mutations in the intracellular signaling pathway yield carcinomas (34). Our previous work showing that blocking the formation of the disulfide bond between the TGF-β1 propeptide and its matrix localizing proteins, the LTBPs, resulted in the production of GI cancers, was consistent with the hypothesis that binding to LTBP was required for proper TGF-β1 generation from its latent complex (25). Interference with active TGF-β1 production yields results similar to ablation of TGF-β1 signaling. Because the inflammatory response observed in *Tgfb1*$^{C33S/C33S}$ mice was not as severe as that observed in *Tgfb1*$^{-/-}$ mice (28, 29), we presumed that some latent TGF-β1 was activated in our mutant animals. Indeed, by further impairing active TGF-β1 production either by eliminating an activator of latent TGF-β or by simply decreasing the amount of total TGF-β1 produced, we enhanced both the tumor and inflammatory phenotypes. Therefore, these mutant animals provide a hypomorphic series in which the relationship of different TGF-β1 levels, inflammation, and relatively rapid tumor production can be explored. In addition, the results re-enforce our earlier conclusions of the importance of TGF-β LLC in TGF-β biology as well as highlighting the role of the integrin αvβ8 in activation of TGF-β1 SLC.

Both integrins αvβ6 and αvβ8 are important for active TGF-β1 formation (27, 33). Because Tgfb1$^{C33S/C33S}$ animals produce only the SLC of TGF-β1 and this complex is not activated by αvβ6 (14), we focused on the effects of αvβ8 loss. Mice that were deficient for integrin β8 and that produced TGF-β1^{C33S} displayed a higher degree of inflammation and considerably more tumors than *Tgfb1*$^{C33S/C33S}$ mice. The enhanced inflammation is consistent with published reports that αvβ8 is crucial for latent TGF-β1 activation by dendritic cells (33) and supports the observed association of inflammation and tumorigenesis (35, 36). Similar results were observed on the degree of inflammation with *Tgfb1*$^{C33S/C33S}$ mice that were housed in helicobacter-free conditions (Data not shown). Yet, *Itgb8*$^{-/-}$ mice do not get tumors, indicating that αvβ8 is not the exclusive latent TGF-β1 activator in the GI tract. The other potential activator of TGF-β1 SLC, TSP-1, appeared to make no contribution to the level of TGF-β in *Tgfb1*$^{C33S/C33S}$ mice as determined by our assays. Perhaps analysis of a larger cadre of animals might reveal a minor contribution. Our second approach to diminish TGF-β1 levels, using mice with one null allele and one *Tgfb1*^{C33S} allele, also enhanced both inflammation and tumor frequency. Together, our results with the different mutant animals indicate that tumor production is dependent upon the level of TGF-β1 signaling.

A limitation of our study is that we do not know the precise level of active TGF-β1 in the specific tissues examined. The TGF-β1 levels in serum reflect the expected changes, the secondary markers for TGF-β1 signaling in the tissues reflect decreased TGF-β1, and the level of active TGF-β1 from stomach is decreased when *Tgfb1*$^{C33S/C33S}$ mice are compared to wild type mice. However, we were unable to detect statistically significant differences in active TGF-β1 when tissue extracts from *Tgfb1*$^{C33S/C33S}$ animals were compared to either *Tgfb1*$^{-/C33S}$ or *Tgfb1*$^{C33S/C33S}$*;Itgb8*$^{-/-}$ tissue extracts. The reason for this is not apparent but might reflect the fact that the amount of active TGF-β1 in the tissue is close to the limit of sensitivity of the assay. The availability of an assay that directly monitored TGF-β1 signaling within the tissue would resolve the question of tissue levels of active cytokine.

An interesting question is why the tumor types and distribution appeared to be different between *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ and *Tgfb1*$^{-/C33S}$ mice, as each mutation should lower the amount of active TGF-β1. This may reflect the fact that in one case, *Tgfb1*$^{-/C33S}$ mice, the total amount of TGF-β1 was diminished, whereas in the second case, *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$, the total TGF-β was not decreased; only one of the potential activators was decreased compared to *Tgfb1*$^{C33S/C33S}$ animals. The contribution of integrin β8 to active TGF-β1 levels also may vary throughout the GI tract, thereby yielding a unique pattern of tumorigenesis. Alternatively, the differences in tumor incidence may reflect the different mouse strain backgrounds of the two sets of mutant and control animals generated for our studies. The *Itgb8* null mutation is an embryonic lethal when placed in the C57BL genetic background (27). Only when the mutation is placed into mixed background of C57BL plus ICR, do pups with the null mutation survive for up to 8 months; this precludes using inbred animals. Therefore, differences in genetic backgrounds might account for differences in results observed between the two sets of animals. Finally, the fact that the microenvironment in the stomach and rectum may be different and may differentially affect tumorogenesis.

It is interesting to speculate on the actual cause of tumors in these mutant mice. Tumor onset is relatively rapid but the frequency is not particularly high. In this respect, our model differs from other models where tumor frequency and rate are quite robust. Usually these other models involve the alteration of tumor suppressor genes and/or oncogenes. TGF-β1$^{-/C33S}$-mediated gastric tumor production, therefore, is in some ways closer to that seen in humans. Since all of the epithelial cells have the same genotype, why are there not more tumors or what is unique about the cells comprising the tumors that occur? Thus far, we have not explored what additional genetic changes may have occurred within these tumors because at the time of sacrifice (12 weeks), the tumors are all microscopic. Additional experiments need to be done using macroscopic tumors from older animals and analyzing the involved tissue for genetic alterations.

Tumorigenesis within the GI tract due to loss of TGF-β1 is interesting to consider with respect to the cell type responsible for initiation of the lesion. The loss of TGF-β1 signaling within the epithelium removes a potent inhibitor of cell growth and potentially allows for the early growth of initiated cells. Additionally, the presence of inflammatory cells of several types in *Tgfb1*$^{-/-}$ mice due to increased inflammation may supply mediators that promote tumorogenesis. However, removal of T and B cells by crossing with *Rag2*$^{-/-}$ mice appears not to delay colon tumor appearance in *Tgfb1*$^{-/-}$ mice by a significant degree (37). Rather, the absence of TGF-β1 yields disorganized crypt architecture that may predispose the tissue for malignant transformation. Alternatively, two other reports describing tumor production using cells deficient in TGF-β signaling describe contributions of either mutant stroma or T cells to GI tumor production (38, 39). It is unclear in the mutant mice we have examined if the decreases in TGF-β have a direct effect on the epithelium thereby promoting carcinoma formation or whether the lack of TGF-β produced by or signaling through other cell types promotes tumor formation. This question can be further explored with respect to hematopoietic cell contribution to tumor development using bone marrow from *Tgfb1*$^{-/C33S}$ mice transferred to WT animals. Such experiments are planned.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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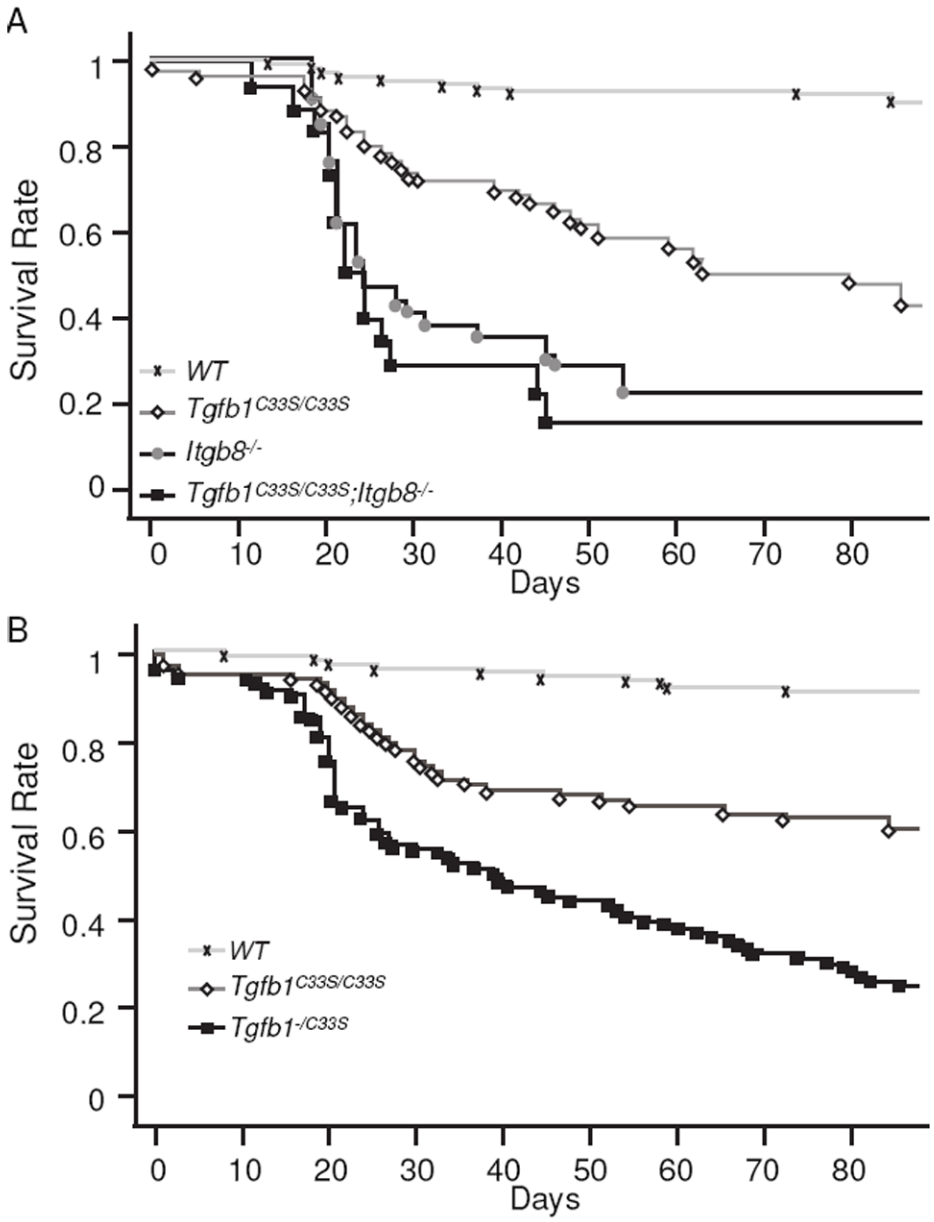

Figure 1.

Survival of *Tgfb1* Mutant Mice. Survival curves were constructed according to the Kaplan-Meier method. A. WT, 129 mice; $Tgfb1^{C33S/C33S}$, 60 mice; $Itgb8^{-/-}$, 33 mice; $Tgfb1^{C33S/C33S}$; $Itgb8^{-/-}$, 18 mice. B. WT, 168 mice; $Tgfb1^{C33S/C33S}$, 112 mice, $Tgfb1^{-/C33S}$, 138 mice.

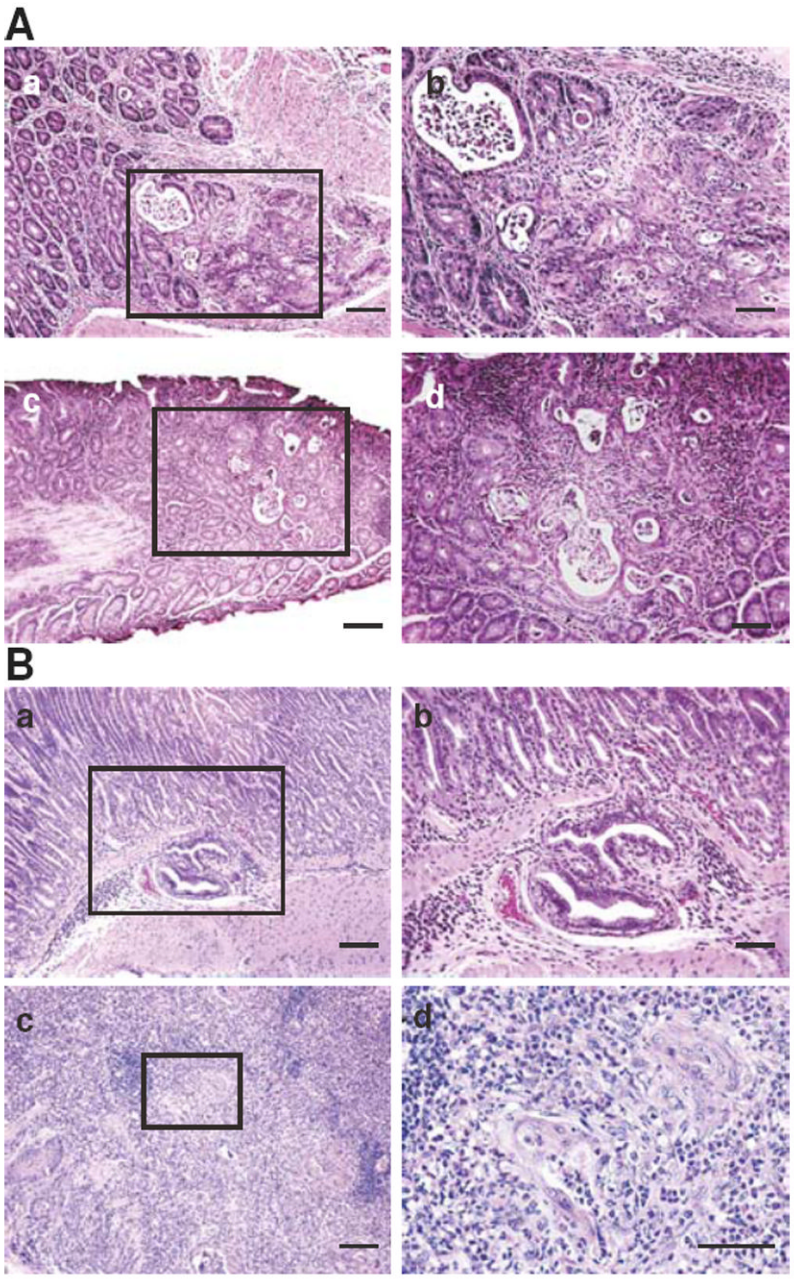

Figure 2.

Tumor Histology in *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ and *Tgfb1*$^{-/C33S}$ mice. **A.** Tumors in *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ animals. Illustrated are an H&E stained rectal adenosquamous cell carcinoma (**a** and **b**) and a rectal adenocarcinoma (**c** and **d**).

B. Tumors in *Tgfb1*$^{-/C33S}$ mice. Illustrated are an H&E stained gastric adenocarcinoma (**a** and **b**), as well as a squamous cell carcinoma (**c** and **d**). Higher magnifications of the boxed area in each tumor are illustrated in **b** and **d**. Bars; **a** and **c**, 100 μm, **b** and **d**, 50 μm.

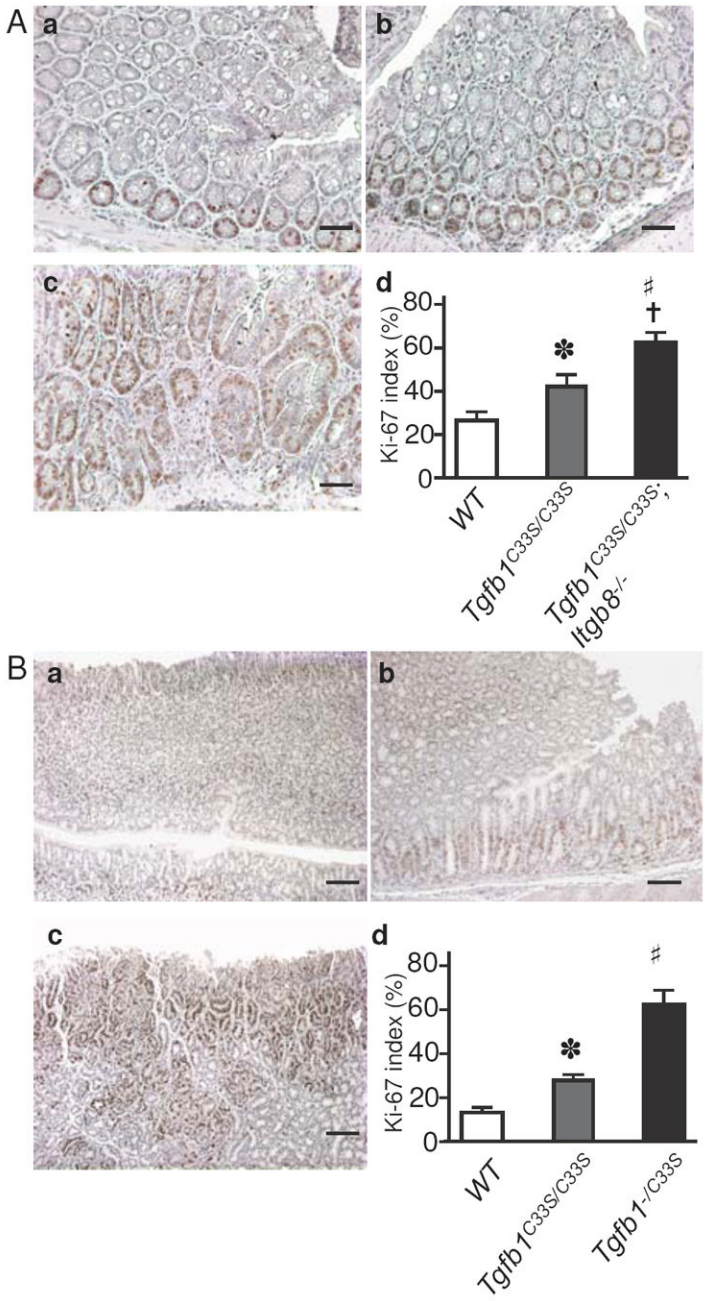

Figure 3.

KI-67 staining increases in mutant tumor tissue compared to WT. **A.** KI-67 staining of *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ tissues. Sections from WT (**a**), *Tgfb1*$^{C33S/C33S}$ (**b**), and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ (**c**) rectal tissues are illustrated after staining for KI-67 to detect proliferating cells. Bars, 100 μm. **d.** The percentage of positive cells in each of the three genotypes was computed by counting the number of positive cells versus total cells in three random fields of sections from the appropriate tissues. The tissue from *Tgfb1*$^{C33S/C33S}$ had significantly more positive cells than did WT tissue (*; $P<0.05$) and tissue from *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ had more than 2 times the number of positive cells than did WT tissue (#; $P<0.0001$) or *Tgfb1*$^{C33S/C33S}$ (†; $P<0.001$). N = 3 animals per group.

B. KI-67 staining of *Tgfb1*$^{-/C33S}$ tissues. Sections from WT (**a**), *Tgfb1*$^{C33S/C33S}$ (**b**), and *Tgfb1*$^{-/C33S}$ (**c**) stomach tissues are illustrated after staining for KI-67 to detect proliferating cells. Bars, 100 μm. **d.** The percentage of positive cells was computed as described above. The *Tgfb1*$^{C33S/C33S}$ tissue had significantly more positive cells than did WT (*; $P<0.0001$) and the *Tgfb1*$^{-/C33S}$ tissue displayed more positive cells than *Tgfb1*$^{C33S/C33S}$ tissue (#; $P<0.0001$). N = 3 animals per group.

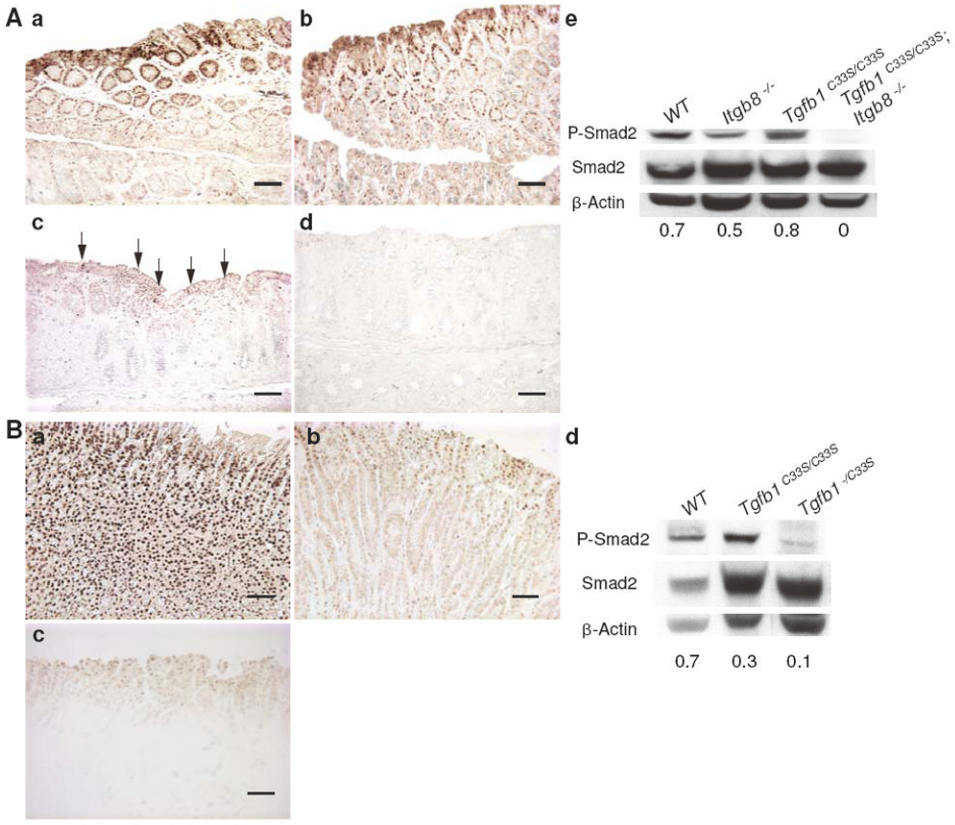

Figure 4.

P-Smad levels are decreased in tumor tissue compared to WT. **A.** P-Smad2 staining in rectal tissue. Staining of WT and *Itgb8*$^{-/-}$ tissues (**a** and **b**) indicated higher levels of P-Smad2 in the epithelium than observed in tissues from *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice (**c** and **d**). **e.** Data from scans of immunoblots are presented under each lane as the ratio of P-Smad2 and Smad2. The results indicate no difference between WT and *Tgfb1*$^{C33S/C33S}$ samples, but there is almost a complete loss of signaling in the *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ tissue. Bars, 100 μm. Arrows indicate P-Smad2-positive areas in panel **c. B.** P-Smad staining in stomach tissue. **a.** WT, **b.** *Tgfb1*$^{C33S/C33S}$, and **c.** *Tgfb1*$^{-/C33S}$. **d.** To measure TGF-β signaling, the amount of P-Smad2 was quantified in gastric tissue after extraction, immunoblotting after SDS-PAGE, and densitometry. The ratio of P-Smad2 to Smad2 is indicated at the bottom of each gel lane. The amount of P-Smad2 was less in *Tgfb1*$^{-/C33S}$ tissue than in *Tgfb1*$^{C33S/C33S}$. Bars, 100 μm.

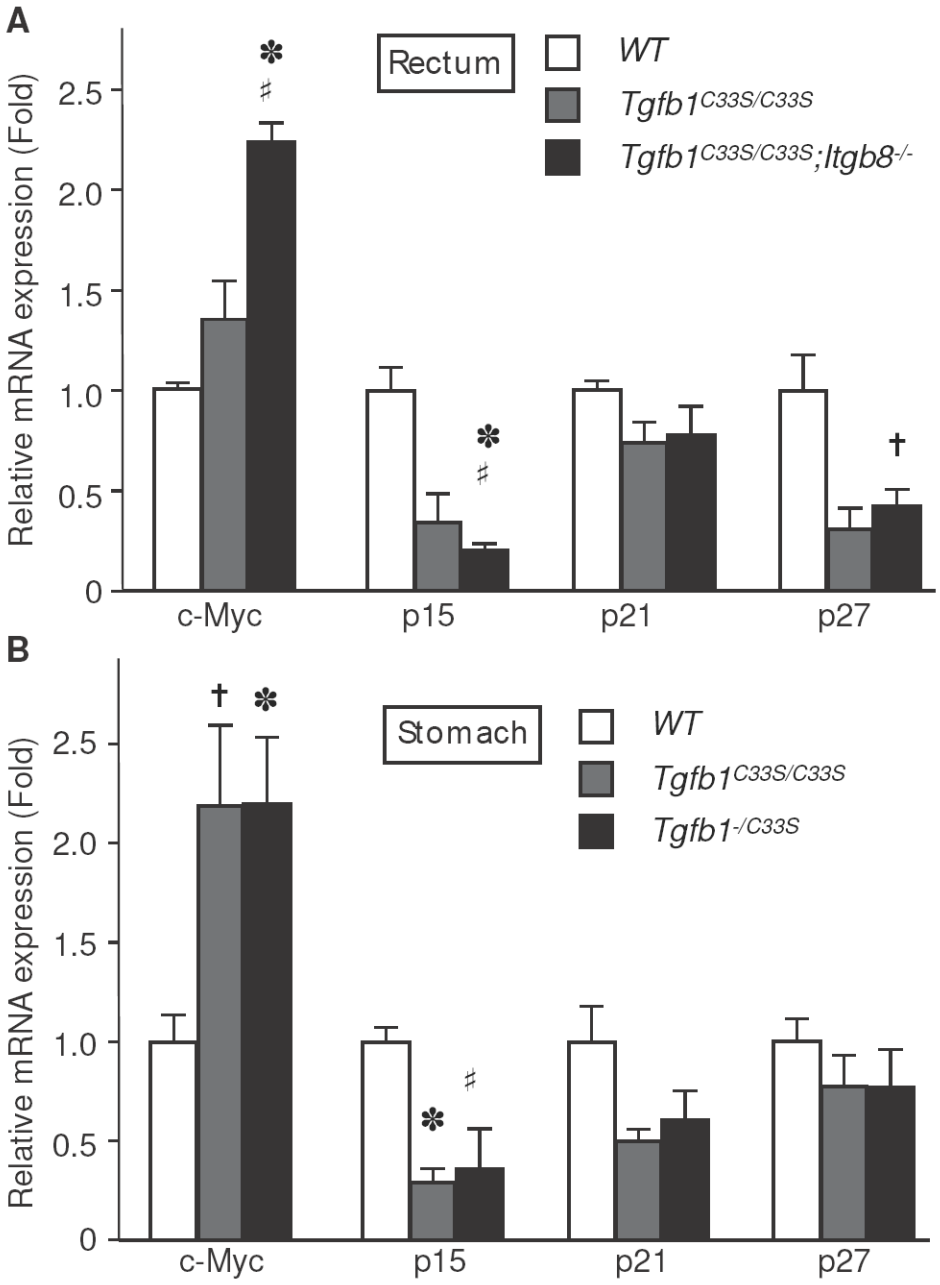

Figure 5.

Transcript levels of cell growth regulators are altered in tumor tissue compared to WT. Rectal (**A**) or gastric (**B**) tissue from WT and mutant animals was extracted and expression levels of the indicated markers were measured by qPCR as described in Methods. **A.** WT, *Tgfb1*$^{C33S/C33S}$, and *Tgfb1*$^{C33S/C33S}$*;Itgb8*$^{-/-}$. *; P<0.01 vs. WT; †; P<0.05 vs. WT; #; P<0.05 vs. *Tgfb1*$^{C33S/C33S}$. **B.** WT, *Tgfb1*$^{C33S/C33S}$, and *Tgfb1*$^{-/C33S}$. *; P<0.01 vs. WT, †; P<0.05 vs. WT, #; P=0.07 vs. WT.

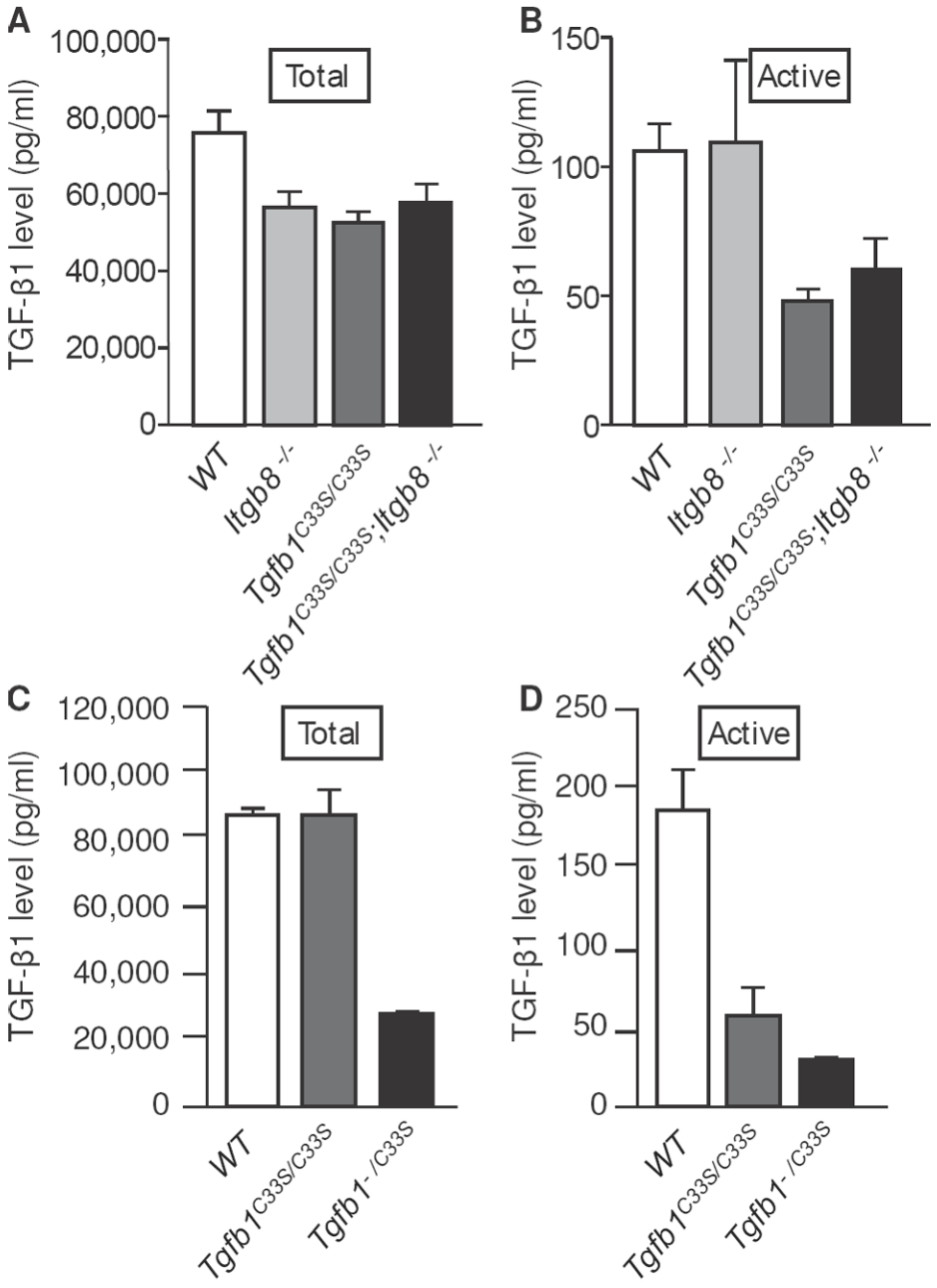

Figure 6.

Active TGF-β1 levels are decreased in sera from mutant versus WT mice. TGF-β1 levels from WT, *Itgb8*$^{-/-}$, *Tgfb1*$^{C33S/C33S}$, and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mouse sera measured by ELISA (**A** and **B**). Total TGF-β1 was measured after acid treatment of serum samples to activate all of the latent TGF-β1 present (**A**), whereas active TGF-β1 was measured in sera without acid treatment (**B**). There was little difference amongst the four genotypes with respect to total TGF-β1. However, *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ sera had significantly less active TGF-β1 than did *WT* or *Itgb8*$^{-/-}$ sera. There was no statistical difference in levels of TGF-β1 in the *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ sera. TGF-β1 levels in WT, *Tgfb1*$^{C33S/C33S}$, and *Tgfb1*$^{-/C33S}$ mice were also measured by ELISA (**C** and **D**). WT and *Tgfb1*$^{C33S/C33S}$ sera had approximately equivalent amounts of total TGF-

β1, while *Tgfb1*$^{-/C33S}$ sera had less than half the amount of total TGF-β1 as did WT sera. When serum active TGF-β1 levels were quantified, *Tgfb1*$^{C33S/C33S}$ sera had slightly less than a third the amount found in WT, whereas *Tgfb1*$^{-/C33S}$ sera had approximately 20% of WT.

Table 1

Inflammation and Tumor Production in Tgfb1 Mutant Mice

A. Inflammation in *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice[a]							
Genotype	N	Lung	Heart	Stomach	Cecum	Colon	Rectum
WT	16	-	-	-	-	-	-
Itgb8$^{-/-}$	6	-	-	-	-	-	-
Tgfb1$^{C33S/C33S}$	16	-~++	-~++	+~++	+~++	-~++	+~++
Tgfb1$^{C33S/C33S}$; *Itgb8*$^{-/-}$	15	+~++	-~++	++	++	++	++
B. Tumors in *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{C33S/C33S}$;*Itgb8*$^{-/-}$ mice[a]							
Genotype	N	Stomach	Cecum	Colon	Rectum	Total	(%)
WT	16	0	0	0	0	0	(0)
Itgb8$^{-/-}$	6	0	0	0	0	0	(0)
Tgfb1$^{C33S/C33S}$	16	0	0	0	3	3	(19)
Tgfb1$^{C33S/C33S}$; *Itgb8*$^{-/-}$	15	3	2	2	7	8	(53) *b, c*
C. Inflammation in *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{-/C33S}$ mice[a]							
Genotype	Lung	Heart	Stomach	Cecum	Colon	Rectum	Liver
WT	-	-	-	-	-	-	-
Tgfb1$^{C33S/C33S}$	-~+	-~+	-~+	++	-~+	++	-
Tgfb1$^{-/C33S}$	+~++	+~++	++	++	+~++	++	+>++
D. Tumors in *Tgfb1*$^{C33S/C33S}$ and *Tgfb1*$^{-/C33S}$ mice[a]							
Genotype	N	Stomach	Cecum	Colon	Rectum	Total	(%)
WT	22	0	0	0	0	0	(0)
Tgfb1$^{C33S/C33S}$	56	5	1	0	7	11	(20) *b*
Tgfb1$^{-/C33S}$	15	8	1	0	4	11	(73) *c, d*

[a] 8-12 weeks of age

- = no inflammation,

+ = mild inflammation,

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$^{++}$= moderate inflammation,

WT = Control *Tgfb1*$^{+/+}$ or *Tgfb1*$^{+/-}$ animals

$^{\sim}$indicates an intermediate value,

^{a}12 weeks of age

Total refers to total number of animals with tumors and includes animals with multiple tumors.

^{b}P<0.05 vs. *Tgfb1*$^{C33S/C33S}$.

^{c}5 mice had multiple tumors.

*12 weeks of age

$^{>}$indicates greater prevalence

Total refers to total number of mice with tumors and includes mice with multiple tumors.

^{b}2 mice had multiple tumors.

^{c}P = 0.0004 vs. *Tgfb1*$^{C33S/C33S}$

^{d}2 mice had multiple tumors.