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A GFP–Tcf4 reporter mouse model for monitoring effects of *Apc* mutations during intestinal tumorigenesis

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Abstract

Apc mutations cause intestinal tumorigenesis through Tcf4 activation. However, direct techniques for studying Tcf4 activation *in vivo* are limited. Here, we describe the development of a GFP–Tcf4 reporter mouse model for directly studying Tcf4 activation. We first developed a GFP reporter construct (GFP-Tcf4) and transfected it into SW480 cells that have constitutively activated Tcf4. Reporter activity increased 47-fold. Next, we created transgenic (Tg) mice by transducing the construct into C57BL6 mice. Fluorescence microscopy did not detect GFP in intestinal sections, but flow cytometry showed 5% of crypt cells to be GFP^+. We then established cross-bred mice (Tg X $Apc^{Min/+}$), which have a germline *Apc* mutation and sustained Tcf4-activation. Here, fluorescence microscopy showed GFP^+ cells at or near the base of normal-appearing crypts. In adenomas, in which *Apc* is inactivated, GFP^+ signal was even greater. Immunostaining for the Tcf4 target genes survivin and cyclin D1 showed that their expression also paralleled GFP positivity. We conclude that GFP directly reports Tcf4 activation *in vivo* and tracks the predicted increases in Tcf4 activation that result from *Apc* inactivation, and that *Apc* mutation contributes to survivin and cyclin D1 overexpression through Tcf4 activation. Our Tcf4 mouse should be useful in studying the effects of chemopreventive agents on Wnt signaling and changes in proliferative crypt cell populations — including stem cells — during intestinal tumorigenesis.

Keywords

stem cells; colorectal cancer; Tcf4; adenomatous polyposis coli; survivin; adenoma

INTRODUCTION

Colorectal cancer (CRC) represents the third leading cause of cancer-related deaths in the United States. CRC development is due to the accumulation of mutations of a number of tumor suppressor genes and oncogenes. Patients with familial adenomatous polyposis

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(FAP), a hereditary disease characterized by the presence of multiple adenomas in the colon, carry a germline mutation of the adenomatous polyposis coli (*APC*) tumor suppressor gene. Adenoma development in FAP patients, however, requires at least one additional genetic event, an acquired *Apc* mutation in the second *Apc* allele. This is consistent with the "two-hit" hypothesis for tumorigenesis in which cancer arises from inactivation of tumor suppressor genes. The *APC* gene is also mutated in most cases of sporadic CRC and in some cases of hereditary nonpolyposis colon cancer (HNPCC); in both diseases, two somatic *APC* mutations are acquired. The same mechanism occurs during intestinal tumorigenesis in $Apc^{Min/+}$ mice, an animal model for FAP. Alterations in gene expression and cell signaling caused by *APC* mutations are thought to be key to the pathogenesis of CRC because these mutations represent the earliest molecular changes during carcinogenesis.

Accordingly, intensive research efforts have been made to elucidate *APC*'s molecular roles in cellular signaling pathways in carcinogenesis. One mechanism involves the inability of neoplastic cells with mutant *APC* to downregulate β-catenin[1–4]. The increased β-catenin then binds to and activates Tcf4, a key transcription factor[5,6]. In the nucleus, the resulting β-catenin/Tcf4 complex binds to the DNA regulatory elements of β-catenin/Tcf4 target genes and, along with other transcription factors, modulates expression of target genes[7,8]. For example, Tcf4 activation normally modulates expression of Myc[9], CD44[10], PPARδ[11], survivin[12,13], cyclin D1[14], and other genes known to be involved in the regulation of cell proliferation. In carcinogenesis, in contrast, activation of the β-catenin/Tcf4 pathway is constitutive/uncontrolled, which links *APC* mutations to CRC development[7].

At the cellular level, Tcf4 activation appears to be involved in regulating intestinal crypt stem cells (SC). This is important because SC are almost certainly the cells of origin of intestinal cancers[15]. Korinek et al[16] showed that knocking out Tcf4 leads to SC depletion, indicating that maintenance of the SC compartment in the normal crypt requires Tcf4 activity. Other studies[17] showed that nuclear localization of β-catenin is restricted to enterocytes located at the crypt base, and it is thought that nuclear localization of β-catenin indicates Tcf4 activation[7]. This is consistent with the fact that SC reside at the crypt base[18].

Our earlier work[19] suggested that overpopulation of colonic SC (change at the cellular level) links the initiating *APC* mutation (change at the molecular level) to the known proliferative abnormality in FAP crypts (change at the tissue level). This led to our hypothesis that wild type *APC*, via β-catenin degradation and Tcf4 inactivation, regulates SC population size. In this view, when *APC* is mutant, as it is in most cases of CRC, the β-catenin/Tcf4 pathway becomes activated, leading to SC overproduction. Moreover, intestinal tumor cells that have *APC* mutations have also been shown to have activated Tcf4 in vitro[7] and, as noted above, intestinal tumors are thought to originate from clonal expansion of mutant SC[15]. Thus, Tcf4 activation may contribute to the SC origin of CRC.

However, studying Tcf4 activation is problematic. While it is currently possible to assay for Tcf4 protein and nuclear β-catenin using immunohistochemistry, this does not directly measure Tcf4 activation. The purpose of the current study was to develop a specific

research tool — GFP-Tcf4 transgenic mice — to report Tcf4 activation in vivo directly. To monitor the effect of *Apc* mutation on Tcf4 activation, we established a cross-bred line, GFP-Tcf4 X $Apc^{Min/+}$ mice because both the germline *Apc* mutation in this mouse strain and the acquired *Apc* mutation (second hit) occurring in adenomas[20,21] will progressively increase Tcf4 activation. Comparisons were made to control mice lacking the GFP-Tcf4 reporter (a strain used to test for autofluorescence), and transgenic (Tg) mice having the reporter but lacking *Apc* mutations.

MATERIALS AND METHODS

There were four phases to this study: **1)** to monitor activation of the Tcf4 transcription factor in situ, we constructed a GFP-Tcf4 reporter plasmid; **2)** to validate expression of the GFP-Tcf4 reporter plasmid construct, we transfected the construct into SW480 cells, a colon carcinoma cell line containing mutant *Apc* and activated Tcf4; **3)** to develop an in vivo model that reports activated Tcf4, we generated a transgenic (Tg) mouse strain, containing the GFP- Tcf4 reporter construct; **4)** to develop an in vivo model that evaluates the response of the reporter to different levels of Tcf4 activation, we crossed Tg mice with $Apc^{Min/+}$ mice which have tissues with mutations in one *Apc* allele (in normal appearing colonic mucosa) or in both *Apc* alleles (in advanced adenomatous mucosa)[20,21]. This study was approved by the Animal Studies Committee of Thomas Jefferson University.

Construction of a reporter plasmid to monitor activation of the Tcf4 transcription factor.

A GFP-Tcf4 reporter plasmid was constructed to monitor the status of Tcf4 transcription activity. Six reporter plasmids and six control plasmids (12 constructs total) were designed and constructed as potential reporters of Tcf4 functioning in cells. Differences among these plasmids were in the combination of promoter, enhancer and reporter gene sequences. In one set of reporter constructs, enhancer sequences were synthesized as repeats E3: 3x(ccc ttt gatc tta cc) or E6: 6x(cc ttt gatc) linked to a minimal HIV promoter and inserted into Bgl II/Hind III site of pEGFP-1 vector (Clontech) in front of a GFP gene, or into Bgl II/Hind III site of pGL3 promoter vector (Promega) in front of a luciferase gene. The minimal HIV promoter had the following sequence: c tca gat gct gca tataa gca gct gct ttt tgc ccg tcc tc act ctc ttc. In the other set of reporter constructs, the synthesized enhancers E3: 3x(ccc ttt gatc tta cc) or E6: 6x(cc ttt gatc) were inserted into the Nhe I/Bgl II site of the pGL3 Promoter vector (Promega) in front of an SV40 promoter which was positioned upstream of a luciferase reporter gene. Control plasmids were identical except for a mutation in their enhancers: mE3: 3x(ccc ttt ggcc tta cc), mE6: 6x(cc ttt ggcc).

Reporters expressing the luciferase enzyme were constructed only for in vitro testing of the enhancer/promoter combination functioning in tissue culture cells. Luciferase systems are beneficial for in vitro studies because they offer an easy way to determine the amount of expressed enzyme in cell lysates and to easily provide quantitative measurements to compare samples. GFP is better suited for in vivo studies.

Testing SW480 cells containing a Tcf4-luciferase reporter.

To test constructed promoter / enhancer combinations, and to investigate the ability of constructed plasmids to serve as reporter systems of Tcf4 functioning in mammalian cells, the SW480 human colon cancer cell line, which is known to contain an activated Tcf4 transcription pathway (secondary to mutant *APC*)[7] was transiently transfected with plasmids pC, pD, pG, pH, pGL3 control (contains SV40 promoter, SV40 enhancer and luciferase gene) or pGL3 promoter (contains SV40 promoter, no enhancer and luciferase gene) using lipofectin (Gibco). Luciferase activity was monitored in cell extracts 48 hours after transfection. All transfections were performed as co-transfections with a reporter plasmid pRL-TK (Promega), which contained a *renila* luciferase gene driven by a thymidine kinase promoter/enhancer and served as an internal standard to compare transfection efficiency among samples. Raw data obtained were converted into relative light units and plotted.

Testing of SW480 cells containing a Tcf4-GFP reporter.

Plasmids pI, pJ, pK, and pL were also transfected into SW480 cells. Selection for transfectants was performed in medium with G418. Cells obtained were fixed in paraformaldehyde and analyzed under a fluorescent microscope with a DAPI/FITC filter.

Construction of a transgenic mouse strain containing the Tcf4-GFP reporter construct.

To be able to determine the level of Tcf4 activation in colonic crypt cells *in vivo*, the reporter constructs described above were made, and used to create transgenic mice. Both wild type and mutant *Tcf4-GFP* constructs were prepared for microinjection by first separating the respective inserts from their vector sequences by cleavage with appropriate restriction endonucleases, followed by gel excision and purification of inserts. Both constructs were independently microinjected into fertilized eggs from the C57BL/6J (B6) inbred strain. Construction of transgenic reporter mice was performed on a pure inbred strain background to eliminate the influence of genetic variation present when mixed hybrid embryos are used. The B6 strain was chosen 1) because of its high susceptibility to $Apc^{Min/+}$-induced intestinal neoplasia and 2) because the $Apc^{Min/+}$ mutation is congenic on this inbred strain background. Our transgenic Tcf4-GFP construct is co-isogenic on the pure B6 background.

Testing of mice for the integration of microinjected constructs.

Genotyping of potential Tg mice for the *Tcf4-GFP* reporter construct pK was done by standard PCR, real-time PCR, and/or Southern blots. Three transgenic mice (INSK2: 7258, 7180, 7182) showed the presence of plasmid pK. These three founders were subsequently crossed with B6 mice to expand a population of founders and the resulting pups were also analyzed by PCR and Southern blots. From 51 generated pK-based transgenic pups, 8 were positive (4 pups from founder 7258 and 4 from 7182). Genotyping of 74 potential founder mice for the control *Tcf4-GFP* reporter construct pL was done. Three potential founders were found to test positive for plasmid pL.

Development of a GFP-Tcf4-Apc$^{Min/+}$ transgenic mouse and fluorescent microscopy of its tissues.

We created an *Apc*$^{Min/+}$-GFP-Tcf4 mouse by cross-breeding male *Apc*$^{Min/+}$ mice (starting with 6 males) with female Tg GFP-Tcf4 mice (from our two different Tg mouse strains – 7182 & 7258). This new mouse line was therefore designed to contain both a germline *APC* mutation and a GFP-Tcf4 reporter construct. This line was designed to serve as a reporter of *Tcf4* activation *in vivo* and to determine the effects of the germline *Apc*$^{Min/+}$ mutation on Tcf4 and GFP expression. Specifically, we developed *Apc*$^{Min/+}$ X *Tcf4*-GFP mice (on a C57BL/6J background) that carry a germline *Apc*$^{Min/+}$ mutation and the *GFP* gene downstream of the *Tcf4* activation response element. This resulted in offspring from this cross-breeding which were positive (using PCR analysis) for the desired combined genotype.

Protocols for tissue specimen collection, histopathology, and necropsy.

Mice were observed daily, euthanized at the designated time (or earlier if they appeared moribund), and subjected to detailed necropsy. Intestinal tissue was dissected and immediately processed, sectioned, and slides stained with hematoxylin and eosin. Intestinal epithelial tissue samples were examined as described below. At 100–110 days of age, GFP-Tcf4 mice were sacrificed by CO_2 asphyxiation. The entire small intestine along with the colon was dissected. The small intestine was cut into 3 sections (proximal, middle, and distal) and the colon into 2 sections (proximal and distal). Intestinal sections were gently scraped to remove fecal matter and then cut open longitudinally. Each piece was washed with phosphate buffered saline (pH 7.0) to clear away any residual fecal matter. Vibratome sections (80 micron thickness) were prepared using a Vibratome 1000 classic vibratome (Vibratome Company, St. Louis MO). Vibratome sections of various thicknesses were prepared by a short fixation in paraformaldehyde, followed by embedding in agarose and cutting with the machine. Longitudinal sections of small and large intestinal epithelium were examined by fluorescence microscopy (Nikon) with FITC and GFP filters and histologic images were recorded electronically using a digital camera (Nikon CoolPix).

Protocols for tissue specimen collection and genotyping in Apc$^{Min/+}$ mice.

Samples of intestinal tumors arising from all offspring of GFP-Tcf4 X *Apc*$^{Min/+}$ were saved for molecular and histopathologic analyses. Tumors were dissected along with adjacent normal tissue and immediately processed, sectioned, and slides stained with hematoxylin and eosin. The investigator, blinded to mouse genotype, performed histopathologic analyses on the tissue samples. We used PCR and Southern blot analysis to genotype tumor and normal-appearing samples using probes for the *Apc* gene and the GFP construct. Intestinal epithelial tissue samples were examined as described below. GFP-Tcf4 X *Apc*$^{Min/+}$ offspring from the above crosses were allowed to mature to 100–110 days of age. Histology and fluorescent GFP levels were assessed in sectioned materials.

Analysis of tissues from GFP-Tcf4 transgenic mice by fluorescence microscopy.

Tissues were obtained at necropsy from offspring of GFP-Tcf4 transgenic mice (7258 and 7182) and crossbred *Apc*$^{Min/+}$ X GFP-Tcf4 mice. Mice positive for the combined phenotype

were identified and intestinal tissues of some of them analyzed using fluorescence microscopy. To overcome auto fluorescence of the surrounding tissue, we tried various methods of sample preparation: frozen sections of various thickness and different ways of freezing, differing methods of fixation, and use of a vibratome to cut tissues immobilized in gels. To observe samples, both a regular fluorescent microscope and a confocal microscope were used. The short time-fixed vibratome cut thick sections (70–150 μm) gave the best results. Focusing on different planes of the tissue allowed us to analyze patterns of GFP expression.

Evaluating Tcf Activation Using Tg(Fos-lacZ) Mice

In addition, Tg(Fos-lacZ) mice, commonly known as lacZ or Top-Gal, were obtained from Jackson Laboratory as an alternate method of validating Tcf activation in intestinal epithelia. These transgenic mice express beta-galactosidase in the presence of LEF/TCF (lymphoid enhancer binding factor 1/transcription factor) and beta-catenin[22]. This reporter strain contains the lacZ gene under the control of a regulatory sequence consisting of three consensus LEF/TCF-binding motifs upstream of a minimal c-fos promoter.

Five hemizygote lacZ mice and one wildtype mouse of the same strain were used for breeding hemizygote and homozygote progeny. Mating cages were set up to generate progeny with this desired genotypes including $lacZ^{+/-}$ X $lacZ^{+/-}$, $lacZ^{+/-}$ X $Apc^{Min/+}$, $Apc^{Min/+}$- $lacZ^{+/-}$ X $Apc^{Min/+}$-$lacZ^{+/-}$ and $lacZ^{+/-}$X wildtype. These progeny were tested using the same aforementioned procedure for genotyping. The primers for lacZ were successful in performing PCR for the genotype of these lacZ mice, which include: 5’ caaatgttgcttgtctggtg 3’ (20 bp); 5’ gtcagtcgagtgcacagttt 3’ (20 bp); 5’ atcctctgcatggtcaggtc 3’ (20 bp); 5’ cgtggcctgattcattcc 3’ (18 bp). Two TgR(ROSA26)26Sor mouse was purchased from Jackson Laboratory to be used as a positive control for lacZ immunostaining. The B6 mouse was used as a negative control.

Analysis of tissues by immunohistochemistry for beta-galactosidase and survivin.

Immunohistochemistry was performed on normal-appearing and adenomatous intestinal tissues from C57BL/6, $Apc^{Min/+}$ and transgenic mice as we previously described[23]. Anti-survivin antibody (Biogenex, San Ramon CA) was used at 1:400 to 1:600 dilutions, anti–cyclin D1 antibody (Santa Cruz Biotechnology, Santa Cruz CA) was used at 1:100, anti-Ki67 antibody (Santa Cruz Biotechnology) was used at 1:100, and anti-beta-galactosidase (Novus Biologicals, Littleton CO) at 1:100 to 1:200. The slides were prepared by first deparaffinizing them with xylene and then incubating them for 3 min in 95% alcohol. They were then rehydrated and treated with Citra Plus Target Retrieval buffer (Biogenex), microwaved at 70% power for 10 minutes, and allowed to cool for 20 minutes. They were then rinsed in PBS for 5 minutes, blocked with H_2O_2 for 10 min, treated with a blocking solution (Avidin/Biotin) for 30 minutes. They were then rinsed in PBS followed by a normal serum block for 20 minutes. The primary antibodies were administered at the respective dilutions for 60 minutes and rinsed 3–5 times in PBS. The second antibody was administered for 30 min and rinsed in PBS 5 times. LSAB+ biotinylated link (DAKO Corporation, Carpenteria CA) was used for survivin staining for 20 minutes. After the biotinylated link step, slides were treated with DAKO LSAB+Streptavidin-HRP

for 20 minutes, rinsed in buffer 5 times, transferred to DAB+chromagen solution for 10 minutes, rinsed in PBS once, and rinsed in distilled H_20 3 times. Finally, all slides were counterstained with Harris hematoxylin and coverslipped.

Analysis of isolated enterocytes for GFP positivity using flow cytometry.

We used flow cytometry to analyze purified enterocytes for GFP positivity to identify the presence of GFP positive cells in crypts isolated from tg/Tcf4-GFP$^{+/+}$ mice that have insufficient GFP signal levels to be seen by fluorescence microscopy. Briefly, intestinal samples from tg/Tcf4-GFP$^{+/+}$ mice were obtained. Crypts were isolated by incubating the colonic mucosa in 3 mM EDTA at 4^{o}C for 1 hour[24]. The sample was vigorously shaken to release the crypts. Isolated crypts were washed in saline and dissociated using 0.3% pancreatin solution. Purified enterocytes in single cell suspension were fixed in 70% cold ethanol for 30 minutes. Enterocyte samples were run on a flow cytometer (Beckman Coulter XL 4 Color Analyzer). Dissociated mouse crypts from C57BL/6 mice were used as controls. The proportion of GFP positive enterocytes from tg/Tcf4-GFP$^{+/+}$ mice was taken as the proportion of cells above a threshold gate set to eliminate false positive cells due to autofluorescence as determined by the maximum fluorescence level in control cells.

RESULTS

Testing the Tcf4-luciferase reporter and Tcf4-GFP reporter constructs using SW480 cells.

Among the Tcf4 luciferase reporter constructs we made, the one with enhancer E6 linked to a minimal HIV promoter (pC) gave optimal results (Figure 1). Results for this construct showed a 47-fold increase of luciferase activity in extracts of SW480 cells transfected with pC compared with a control plasmid pD that carries mutated enhancer mE6 instead of the functional sequence E6. Thus, the Tcf4 enhancer element in the pC construct was selected for use in subsequent studies.

Results on Tcf4-GFP reporter constructs are shown in Figure 2. There was a significant difference in fluorescence intensity between transfectants with plasmid pK and those with the control plasmid pL, which contains a mutated enhancer. These data demonstrate that the combination of the minimal HIV promoter and E6 enhancer coupled with the GFP reporter can be used to monitor Tcf4 activity in colonic epithelial cells.

GFP^{+} signal for Tcf4 activation in GFP-Tcf4 transgenic mice with wild-type Apc.

Tg mice containing the Tcf4-GFP construct (*Tcf4-GFP*) were generated as outlined in Methods. Intestinal samples from these Tg mice, which have wild type *Apc*, showed a spotty, intracellular signal in a few cells scattered throughout the mucosa (Figure 3, left panel). Closer inspection of such cells showed a non-homogeneous (stellate) fluorescence pattern. There were no positively fluorescing cells that had the morphology of crypt epithelial cells or had a homogeneous staining pattern. Both colon and small intestine showed this pattern.

Given the possibility that fluorescence microscopy had insufficient sensitivity to detect baseline levels of Tcf4 activation in these mice, flow cytometry was also used. Using flow

cytometry to detect GFP fluorescence, we identified GFP positivity in isolated intestinal cells from *Tcf4-GFP* mice (Figure 4). About 5% of intestinal crypt cells from transgenic mice were GFP positive when compared to cells isolated from C57BL/6 control mice which lack the GFP-Tcf4 construct (any signal in the latter must therefore be due to autofluorescence).

GFP$^+$ signal for Tcf4 activation in normal-appearing intestinal epithelium of cross bred (GFP-Tcf4 X Apc$^{Min/+}$) transgenic mice having an Apc germline mutation.

Results from Tg mice crossbred with *Apc*$^{Min/+}$ mice showed numerous intensely bright regions of GFP positive (GFP$^+$) fluorescence in the area where small intestinal crypts are located, but not in small intestinal villi (Figure 3, center panel). This pattern was clearly different from the pattern we saw in transgenic mice that lack *Apc* mutations (Figure 3, left panel). The pattern observed in small intestinal crypts from *Apc*$^{Min/+}$ mice (Figure 3, center panel) was confirmed, by immunohistochemistry, to be due to expression of the GFP reporter in epithelial crypt cells (Figure 3, right panel). Normal-appearing *colonic* epithelium in crossbred mice appeared similar to *small intestinal* epithelium, except that in colonic epithelium we found a unique fluorescence pattern of GFP-positive signals in select, morphologically identifiable cells, at the crypt bottom (Figure 5). In each of these select cells, the cytoplasm was homogeneously stained. This fluorescence pattern in colonic and small intestinal tissues was absent in non-transgenic, control, C57BL6 mice (not shown), indicating that the signal was not caused by tissue autofluorescence.

Using Tg(Fos-lacZ) mice as an independent method to evaluate Tcf activation in normal-appearing intestinal epithelium of cross-bred (Fos-lacZ X Apc$^{Min/+}$) transgenic mice having an Apc germline mutation.

The Tg(Fos-lacZ) mouse strain was used as an independent method to validate findings for the GFP-Tcf4 reporter mouse model. The Tg(Fos-lacZ) strain expresses beta-galactosidase in response to increased β-catenin, which binds to another Tcf transcription factor — intracellular LEF-1 (lymphoid enhancer binding factor 1). Figure 6 shows immunostaining using anti-β-galactosidase antibodies to evaluate LEF/Tcf activity in intestinal tissue in vivo using Tg(Fos-lacZ) mice. These results show that LEF/Tcf activation is highest in the lower region of small intestinal crypts (Figure 6, upper panels) and colonic crypts (Figure 6, lower panels). C57BL6 mice served as negative controls. The staining signal intensity is higher in Fos-lacZ x *Apc*$^{Min/+}$ mice than in Fos-lacZ mice.

GFP$^+$ signal for Tcf4 activation in adenomas from cross-bred (GFP-Tcf4 X Apc$^{Min/+}$) transgenic mice (dysplastic intestinal tissue with inactivation of the second Apc allele).

In adenomatous small intestinal tissue from crossbred mice, which is known to have acquired a second hit at the *Apc* locus[20,21], we found greater signal than in normal-appearing mucosa from the same mice (normal-appearing mucosa contains only the first hit at the *Apc* locus) (Figure 7). These GFP$^+$ cells were found throughout the adenoma and had a homogeneous intracellular fluorescence pattern.

Immunohistochemical staining for Ki67 and for Tcf4 target genes survivin and cyclin D1

Immunostaining using anti-Ki67 antibodies was used to evaluate cell proliferation in crypts (Figure 8) and showed an increase in proliferation from normal small intestine of controls to normal-appearing small intestine of $Apc^{Min/+}$ mice to adenomatous tissue of $Apc^{Min/+}$ mice. Staining of normal large intestine of controls is also shown compared to normal-appearing large intestine of $Apc^{Min/+}$ mice; the latter showed increased proliferation..

We also analyzed intestinal tissues for expression of the Tcf4 target genes survivin and cyclin D1 as an additional method to validate our model. In Figure 9, the left panel shows that survivin-positive staining only occurs in the cell nucleus and only at the crypt base in small intestinal tissue from controls (B6 mice). The center right panel shows that in normal-appearing small intestinal mucosa from $Apc^{Min/+}$ mice, staining intensity is stronger, appears to include areas further up the crypt, and begins to show cytoplasmic localization compared to adenomatous tissue of small intestine of $Apc^{Min/+}$ mice (right panel). The center left panel shows survivin-positive staining of isolated cells near the base of colonic crypts in $Apc^{Min/+}$ mice (arrowheads). The right panel shows that in adenomatous mucosa, survivin staining is even stronger, and is found throughout the crypt, including both nuclear and cytoplasmic regions.

Immunostaining was also done using anti–cyclin D1 antibodies to evaluate expression of the Tcf4 target gene cyclin D1. Figure 10 shows a progressive increase in survivin expression from (i) normal small intestine of controls to (ii) normal-appearing small intestine of $Apc^{Min/+}$ mice to (iii) adenomatous tissue of $Apc^{Min/+}$ mice. Staining of normal-appearing large intestine of $Apc^{Min/+}$ mice reveals positive staining near the crypt base.

DISCUSSION

In this study we first evaluated our GFP construct for its ability to monitor Tcf4 activity, both in vitro and in vivo. This strategy relies on the fact that *Apc* mutations activate Tcf4 by allowing β-catenin levels to rise[20,21]. That SW480 cells (having homozygous mutant *Apc*) showed a highly enhanced signal, validated the use of the GFP reporter construct for monitoring Tcf4 activity in vitro. The intensity of GFP expression in our mice correlated with the degree of *Apc* inactivation: transgenic mice < normal-appearing mucosa from cross-bred mice < adenomatous mucosa of cross-bred mice. Therefore, the GFP-Tcf4 construct appears to report Tcf4 activation in vivo as well. Our findings that results for the Fos-lacZ model were consistent with results for the GFP-Tcf4 model helps validate the latter model. Our finding that survivin and cyclin D1 expression parallels GFP expression further helps validate the model since survivin and cyclin D1 are Tcf4 target genes[12,25].

That there was little if any GFP signal in intestinal tissue sections from Tg mice (only wildtype *Apc*) is consistent with several earlier findings for transgenic mouse strains designed and developed to respond to Tcf4 activity. Two of them[26,27] were designed to study Tcf signaling in intestine and two[22,28] in skin. These other studies also failed to find reporter signal in transgenic mice without first genetically manipulating the system to activate Tcf4 (e.g., by overexpressing β-catenin). In the study by Maretto et al[26] using a different reporter, they crossed reporter mice with $Apc^{Min/+}$ mice, as we did. However, they

reported data on Tcf4 reporter signal only for non-transgenic controls and for adenomatous tissues, not for normal-appearing mucosa from $Apc^{Min/+}$ mice. Thus, while they observed increased reporter signal in adenomas, it was not shown whether or not there was any signal indicating Tcf4 activity in normal-appearing crypts from cross-bred mice with a germline *Apc* mutation.

When we used flow cytometry as yet another independent method to analyze GFP signal, we found that 5% of crypt cells from transgenic mice were GFP positive. First, this suggests that the GFP signal from fluorescence microscopy of tissue sections from transgenic mice is not totally absent, but only below the lower limit of detection of our fluorescence microscopy assay. Second, our finding that 5% of colonic crypt cells have activated Tcf4 is consistent with an earlier estimate that 2–13% of cells in the murine colonic crypt are SC[29,30].

Our finding that survivin expression, like GFP expression, parallels the degree of *Apc* inactivation, indicates that mutant *Apc* leads to survivin overexpression through activation of Tcf4. This is consistent with our current model of *Apc*:survivin signaling[12,25]. Specifically, in normal intestinal epithelium of control mice, there is a gradient for *Apc* expression along the murine intestinal crypt axis from minimal at the bottom to maximal at the villus tip[31,32]. Our current data show that the gradient for survivin expression has an inverse orientation — high at the bottom and low at the top.

In $Apc^{Min/+}$ mice, survivin staining in normal-appearing crypts showed greater intensity and extended further up the crypt compared to crypts in control animals; intestinal adenomas in $Apc^{Min/+}$ mice showed even more intense survivin staining. We previously reported similar findings for human colonic crypts in normals and in FAP patients[25]. These findings, along with our previous report[12] and findings by others[13] that wild type *Apc* downregulates survivin, establishes that *Apc* is a critical regulator of survivin expression. This suggests a mechanism by which mutant *Apc* allows survivin levels to rise, which inhibits apoptosis and promotes mitosis and contributes to the development of colon tumors.

Since Tcf4 knockout mice show depletion of intestinal SC[16], we hypothesize that Tcf4 activation is an index of SC population size. This is consistent with our finding that in normal appearing intestine the highest levels of GFP^+ signal are found in the crypt base, the region where SC reside[18]. This finding is also consistent with our observation that survivin expression was highest at the crypt base in the present mouse study and in our previous studies on human crypts[12,25].

We conclude that GFP expression in our model parallels the degree of *Apc* inactivation, that GFP reports Tcf4 activation, and that mutant *Apc* leads to survivin overexpression through Tcf4 activation. To our knowledge, ours is the first study to show that normal-appearing mucosa in cross-bred mice with a germline *Apc* mutation exhibits Tcf4 activation. Accordingly, identification and characterization of GFP^+ cells should now allow us to determine, using functional assays[18,33], whether some or all of them are indeed SC. Because specific genes, which are controlled by Wnt/Tcf pathway, are expressed at the position of the crypt stem cells[34], the expression of these target genes can be examined

using our GFP-Tcf4 reporter mouse model to demonstrate that changes in their expression parallel changes in Tcf4 activation. Moreover, since our Tcf4 reporter mouse should allow direct study of Tcf4 activation, it will be useful in studying the effects of chemopreventive agents on Wnt signaling and changes in proliferative crypt cell populations — including stem cells — during intestinal tumorigenesis.

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REFERENCES.

1. Rubinfeld B, Souza B, Albert I et al. Association of the APC gene product with beta-catenin. Science 1993;262(5140):1731–1734. [PubMed: 8259518]
2. Su LK, Vogelstein B, Kinzler KW. Association of the APC tumor suppressor protein with catenins. Science 1993;262(5140):1734–1737. [PubMed: 8259519]
3. Munemitsu S, Albert I, Souza B, Rubinfeld B, Polakis P. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc Natl Acad Sci U S A 1995;92(7):3046–3050. [PubMed: 7708772]
4. Hao X, Tomlinson I, Ilyas M, Palazzo JP, Talbot IC. Reciprocity between membranous and nuclear expression of beta-catenin in colorectal tumours. Virchows Arch 1997;431(3):167–172. [PubMed: 9334837]
5. Morin PJ, Sparks AB, Korinek V et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 1997;275(5307):1787–1790. [PubMed: 9065402]
6. Travis A, Amsterdam A, Belanger C, Grosschedl R. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function [corrected]. Genes Dev 1991;5(5):880–894. [PubMed: 1827423]
7. Korinek V, Barker N, Morin PJ et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science 1997;275(5307):1784–1787. [PubMed: 9065401]
8. Hsu SC, Galceran J, Grosschedl R. Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol Cell Biol 1998;18(8):4807–4818. [PubMed: 9671490]
9. He TC, Sparks AB, Rago C et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281(5382):1509–1512. [PubMed: 9727977]
10. Wielenga VJ, Smits R, Korinek V et al. Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol 1999;154(2):515–523. [PubMed: 10027409]
11. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell 1999;99(3):335–345. [PubMed: 10555149]
12. Zhang T, Otevrel T, Gao Z, Ehrlich SM, Fields JZ, Boman BM. Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res 2001;61(24):8664–8667. [PubMed: 11751382]
13. Kim PJ, Plescia J, Clevers H, Fearon ER, Altieri DC. Survivin and molecular pathogenesis of colorectal cancer. Lancet 2003;362(9379):205–209. [PubMed: 12885482]
14. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398(6726):422–426. [PubMed: 10201372]
15. Brittan M, Wright NA. Stem cell in gastrointestinal structure and neoplastic development. Gut 2004;53(6):899–910. [PubMed: 15138220]
16. Korinek V, Barker N, Moerer P et al. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet 1998;19(4):379–383. [PubMed: 9697701]
17. van de Wetering MSE, Verweij C, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241–250. [PubMed: 12408868]

18. Booth C, Potten CS. Gut instincts: thoughts on intestinal epithelial stem cells. J Clin Invest 2000;105(11):1493–1499. [PubMed: 10841502]
19. Boman BM, Fields JZ, Bonham-Carter O, Runquist OA. Computer modeling implicates stem cell overproduction in colon cancer initiation. Cancer Res 2001;61(23):8408–8411. [PubMed: 11731419]
20. Levy DB, Smith KJ, Beazer-Barclay Y, Hamilton SR, Vogelstein B, Kinzler KW. Inactivation of both APC alleles in human and mouse tumors. Cancer Res 1994;54(22):5953–5958. [PubMed: 7954428]
21. Luongo C, Moser AR, Gledhill S, Dove WF. Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res 1994;54(22):5947–5952. [PubMed: 7954427]
22. DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development 1999;126(20):4557–4568. [PubMed: 10498690]
23. Boman BM, Walters R, Fields JZ et al. Colonic crypt changes during adenoma development in familial adenomatous polyposis: immunohistochemical evidence for expansion of the crypt base cell population. Am J Pathol 2004;165(5):1489–1498. [PubMed: 15509520]
24. Wildrick DM, Roll R, Lointier P, Quintanilla B, Nichols DH, Boman BM. Comparative study of cultured human colonic epithelium isolated by two different methods. In Vitro Dev Cell Biol 1997;33:18–27.
25. Boman BM ZT, Fields JZ. Correspondence: Evidence that APC Regulates Survivin Expression: A Possible Mechanism Contributing to the Stem Cell Origin of Colon Cancer. Cancer Res 2004;64:776–779. [PubMed: 14744799]
26. Maretto S, Cordenonsi M, Dupont S et al. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci U S A 2003;100(6):3299–3304. [PubMed: 12626757]
27. Cheon SS, Cheah AY, Turley S et al. beta-Catenin stabilization dysregulates mesenchymal cell proliferation, motility, and invasiveness and causes aggressive fibromatosis and hyperplastic cutaneous wounds. Proc Natl Acad Sci U S A 2002;99(10):6973–6978. [PubMed: 11983872]
28. Van Mater D, Kolligs FT, Dlugosz AA, Fearon ER. Transient activation of beta -catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice. Genes Dev 2003;17(10):1219–1224. [PubMed: 12756226]
29. Potten CS, Booth C, Tudor GL et al. Identification of a putative intestinal stem cell and early lineage marker; musashi-1. Differentiation 2003;71(1):28–41. [PubMed: 12558601]
30. Potten CS, Rezvani M, Hendry JH, Moore JV, Major D. The correction of intestinal microcolony counts for variation in size. Int J Radiat Biol Relat Stud Phys Chem Med 1981;40(3):321–326. [PubMed: 7026475]
31. Miyashiro I, Senda T, Matsumine A et al. Subcellular localization of the APC protein: immunoelectron microscopic study of the association of the APC protein with catenin. Oncogene 1995;11(1):89–96. [PubMed: 7624136]
32. Smith KJ, Johnson KA, Bryan TM et al. The APC gene product in normal and tumor cells. Proc Natl Acad Sci U S A 1993;90(7):2846–2850. [PubMed: 8385345]
33. Whitehead RH, Demmler K, Rockman SP, Watson NK. Clonogenic growth of epithelial cells from normal colonic mucosa from both mice and humans. Gastroenterology 1999;117(4):858–865. [PubMed: 10500068]
34. Van der Flier LG, Sabates-Bellver J, Oving I et al. The Intestinal Wnt/TCF Signature. Gastroenterology 2007;132(2):628–632. [PubMed: 17320548]

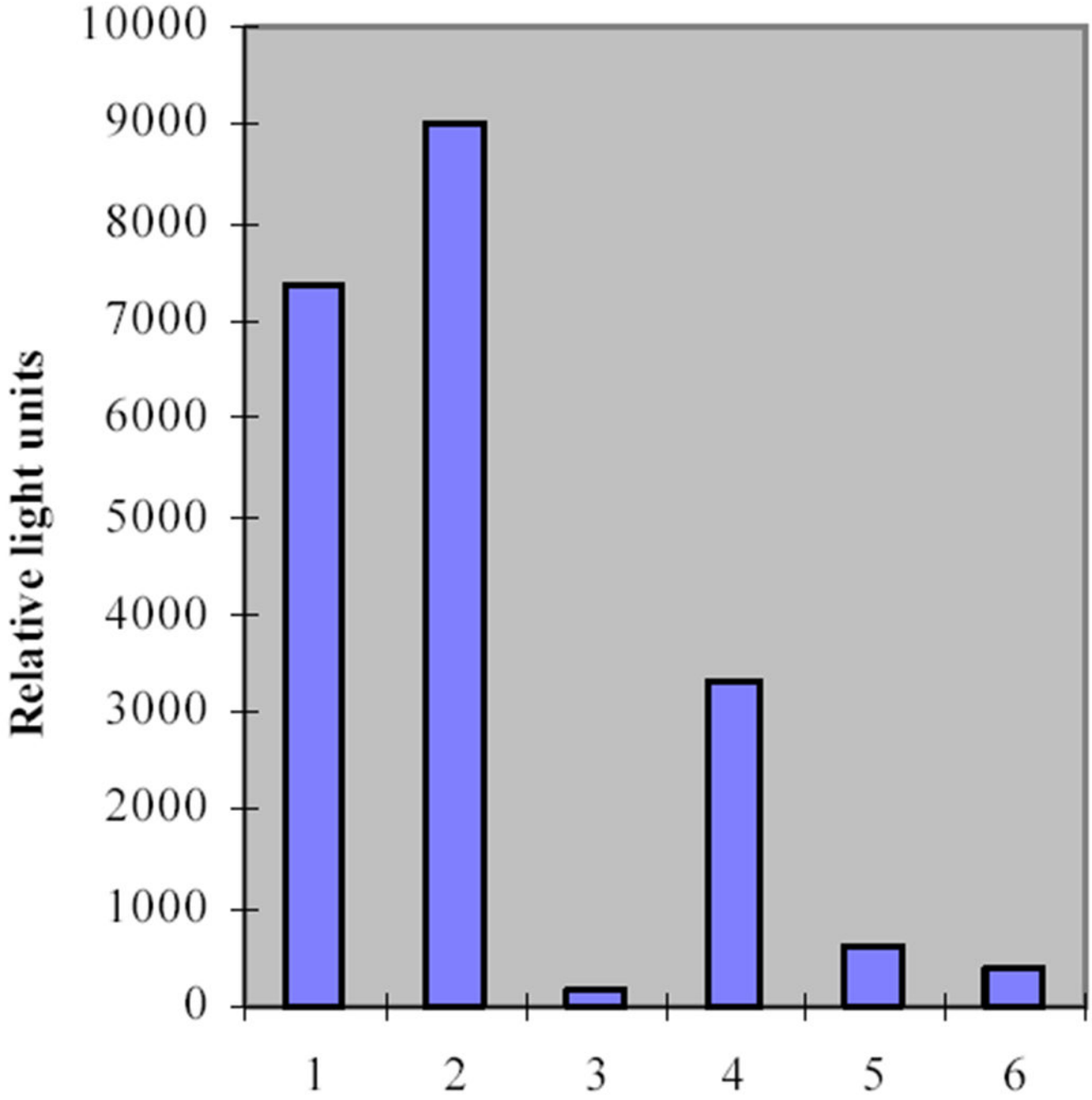

Figure 1.

A representative experiment showing comparison of signals produced by different luciferase constructs transfected into SW480 cells. Transfected constructs for the different bars are: 1 = pGL3 positive control; 2 = pC; 3 = pD (negative control); 4 = pG; 5 = pH; 6 = pGL3 promoter.

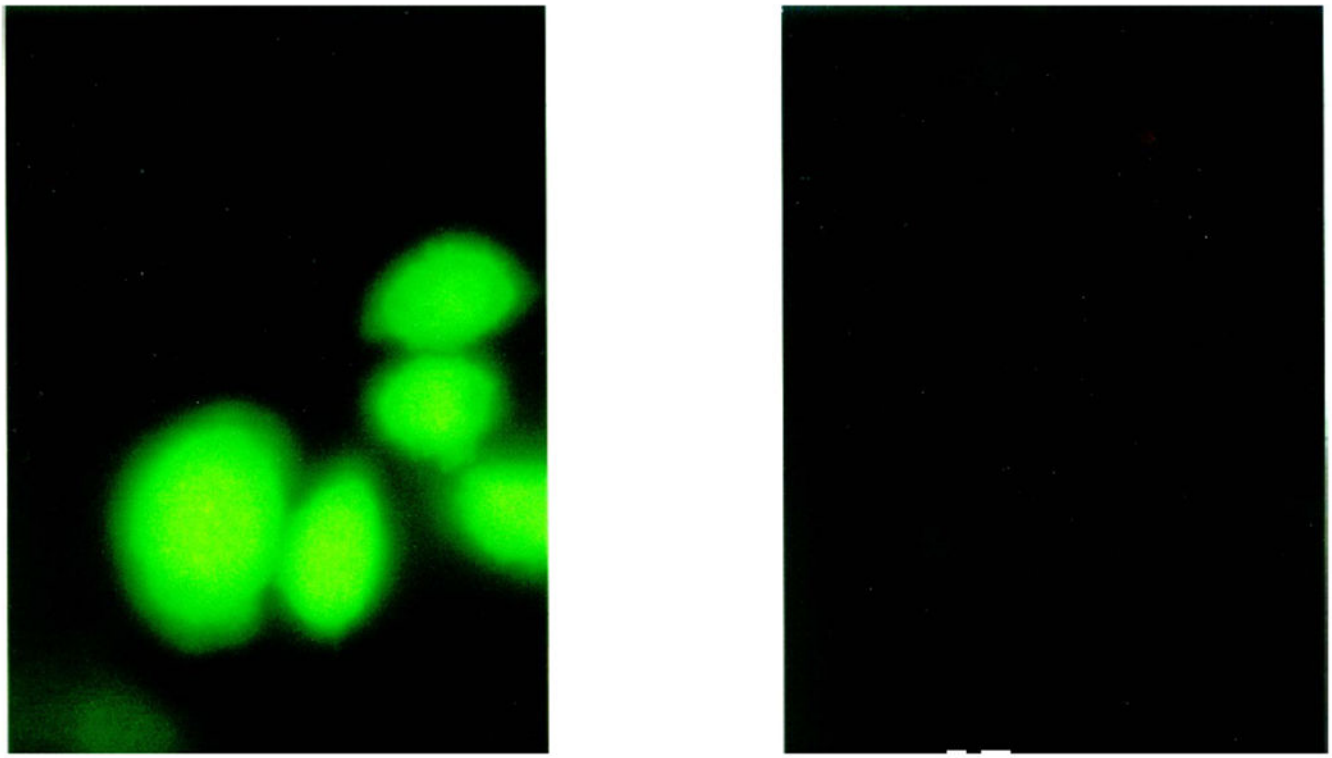

Figure 2.
GFP fluorescence signal observed in SW480 cells transfected with the GFP-Tcf4 pK plasmid (left panel) versus cells transfected with the control pL plasmid (right panel) containing a mutated enhancer.

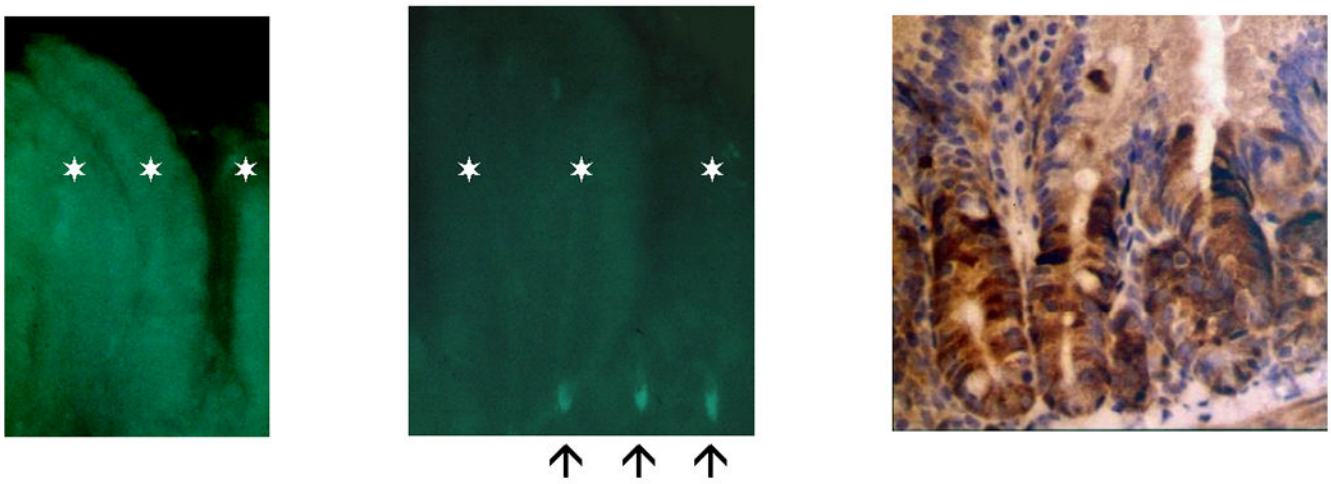

Figure 3.

Fluorescence microscopic analysis of small intestinal samples from transgenic (Tg) mice lacking *Apc* mutations (left panel) and from crossbred mice (*Tcf4-GFP*$^{+/+}$ X *Apc*$^{Min/+}$) with a germline *Apc* mutation (center panel). The center panel shows several bright regions of GFP positive (GFP^{+}) fluorescence in the area where small intestinal crypts are located (arrows), but not in small intestinal villi (asterisks). This pattern was clearly different from the pattern in transgenic mice that lack *Apc* mutations (left panel). The right panel shows immunostaining of small intestinal crypts from *Apc*$^{Min/+}$ mice using GFP antibodies to show that expression of the GFP reporter for Tcf4 activity is limited to epithelial crypt cells. This also showed that Paneth cells at the crypt bottom have lower levels of GFP expression.

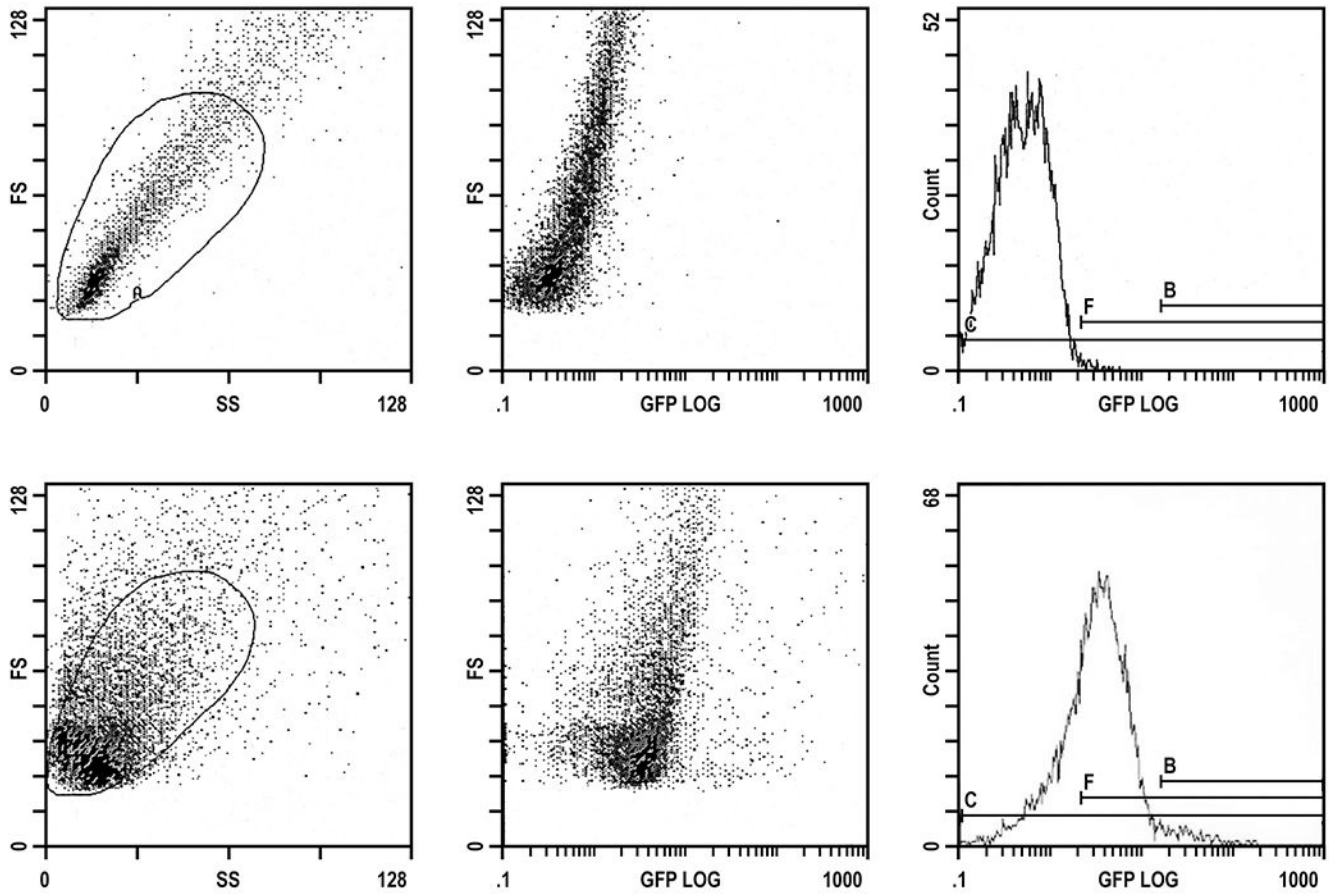

Figure 4.

Analysis of GFP-positive enterocytes by flow cytometry. Cells from C57BL/6 control mice were used as a negative control for GFP autofluorescence signal (top row). The top left panel shows the scatter of the cells with the gated region indicating live (non-apoptotic) cells. The top center panel gives the side-scatter (y-axis) vs. log GFP intensity (x-axis). The top right panel gives a plot of the results in the center panel. Results for homozygous tg/Tcf4-GFP+/+ mice are shown at bottom. Scatter analysis shows that enterocytes from transgenic mice have greater side scatter (bottom left panel) compared to control cells (top left panel). Enterocytes from transgenic mice also show increased GFP intensity (bottom middle panel versus top middle panel), as indicated by a curve shift to the right (bottom right panel) compared to control cells (top right panel). The top right panel also shows a subpopulation (3.65%) of cells (in gated region B) with strong GFP signal intensity (>42.3), as indicated by the shoulder of the curve that extends to the right of the peak.

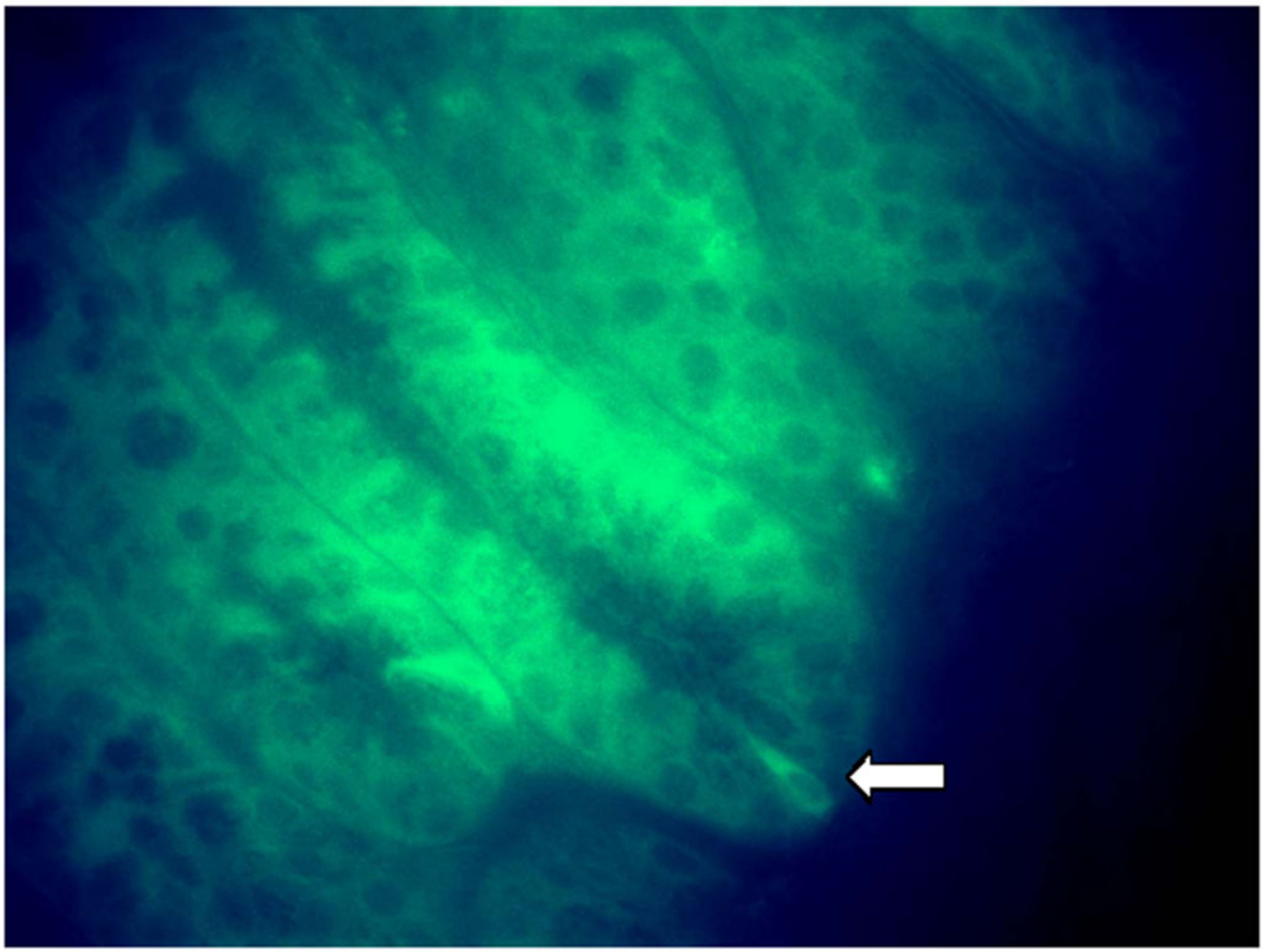

Figure 5.

Fluorescence microscopy analysis of normal-appearing colonic epithelium from crossbred mice that are homozygous for the transgene (*Tcf4-GFP*$^{+/+}$ X *Apc*$^{Min/+}$). Morphologically identifiable cells at the crypt bottoms (arrow) had homogeneously staining GFP-positive signal.

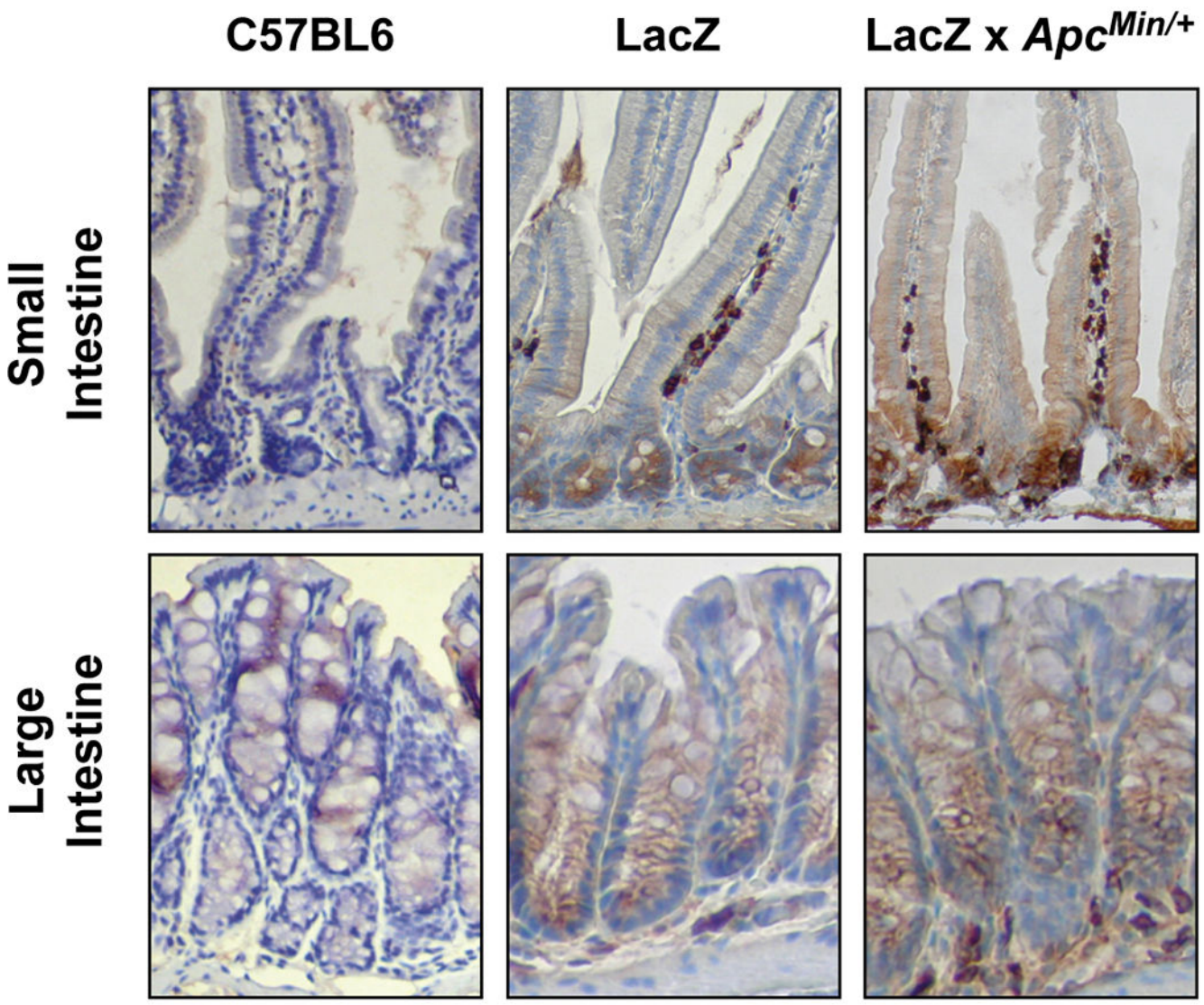

Figure 6.

Immunostaining using anti-β-galactosidase antibodies to evaluate Tcf activity in intestinal tissue *in vivo* using Tg(Fos-lacZ) mice. Tcf activation was highest in the lower region of small intestinal crypts (upper panels) and colonic crypts (lower panels). C57BL6 mice served as negative controls. The signal intensity was higher in LacZ x Apc^{Min+} mice compared to LacZ mice.

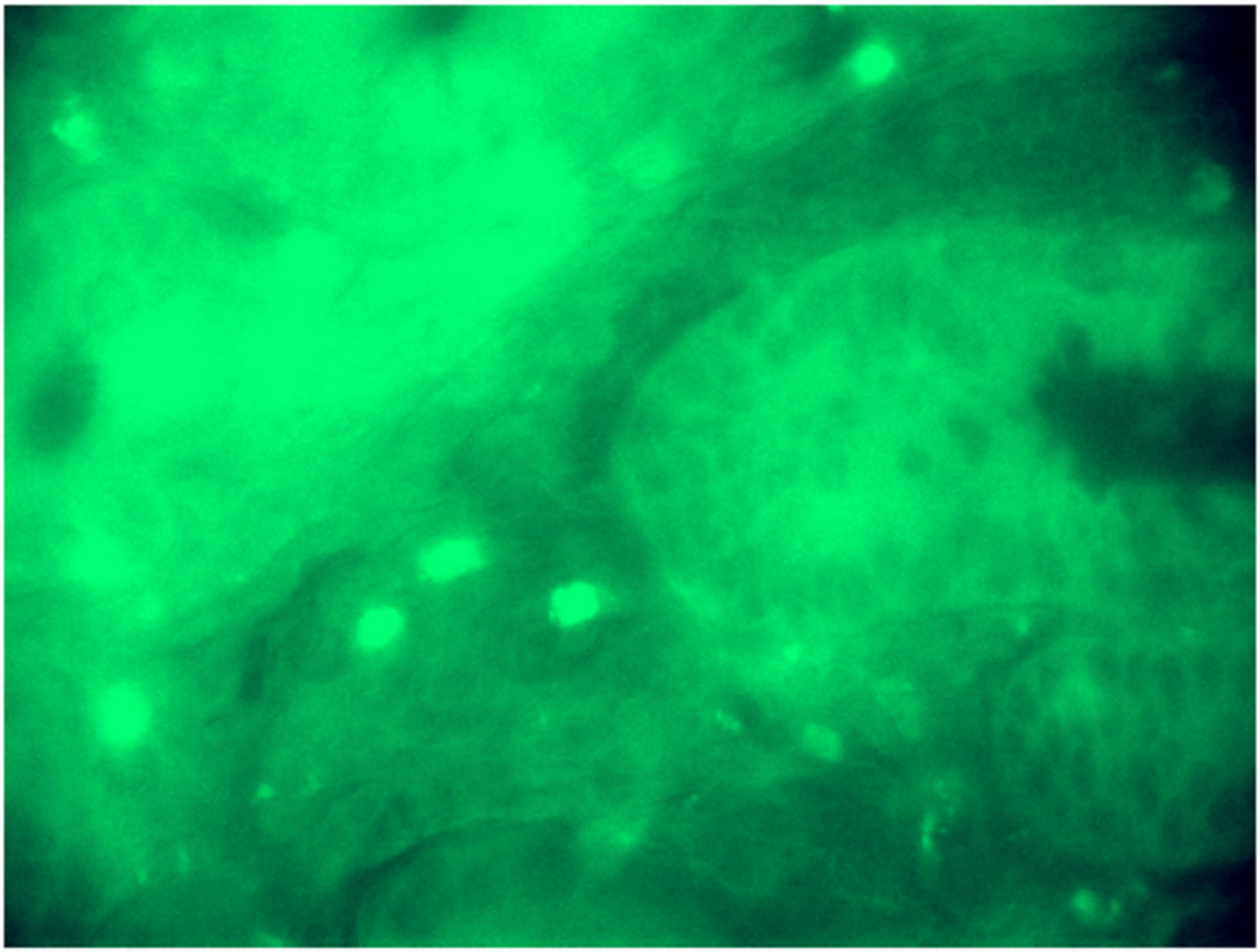

Figure 7.
Fluorescence microscopy of adenomas from crossbred mice. In adenomatous mucosa, GFP-positive cells with high fluorescence intensity were scattered throughout the adenoma.

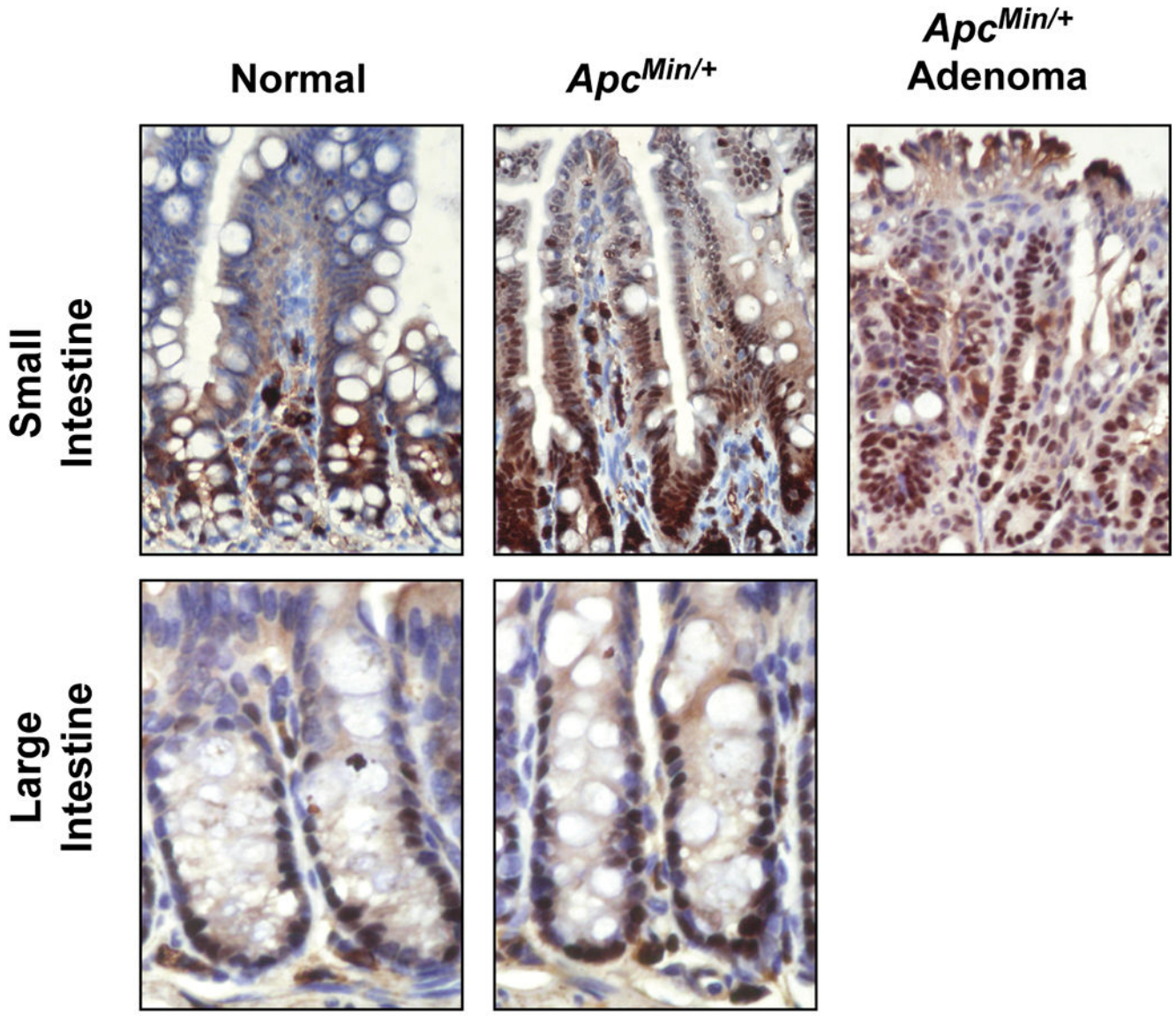

Figure 8.

Immunostaining using anti-Ki67 antibodies to evaluate cell proliferation in crypts. There was a progressive increase in proliferation from normal small intestine of controls (top left panel) to normal-appearing small intestine of $Apc^{Min/+}$ mice (top center panel) to adenomatous tissue of $Apc^{Min/+}$ mice (top right panel). Also shown is staining for normal large intestine of controls (bottom right panel) and normal-appearing large intestine of $Apc^{Min/+}$ mice (bottom right panel); the latter showed increased proliferation.

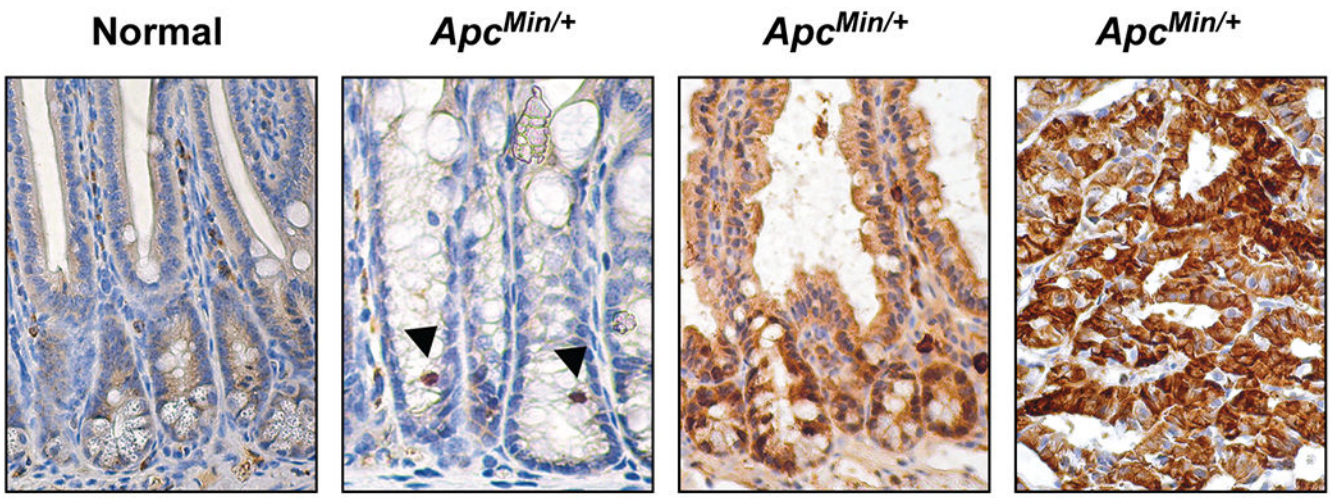

Figure 9.

Immunostaining using anti-survivin antibodies to evaluate expression of the Tcf4 target gene, survivin. There was a progressive increase in survivin expression from normal small intestine of controls (left panel) to normal-appearing small intestine of $Apc^{Min/+}$ mice (center right panel) to adenomatous tissue of $Apc^{Min/+}$ mice (right panel). Also shown is the staining of normal appearing large intestine of $Apc^{Min/+}$ mice (center left panel) that reveals isolated positive cells near the crypt base (arrowheads).

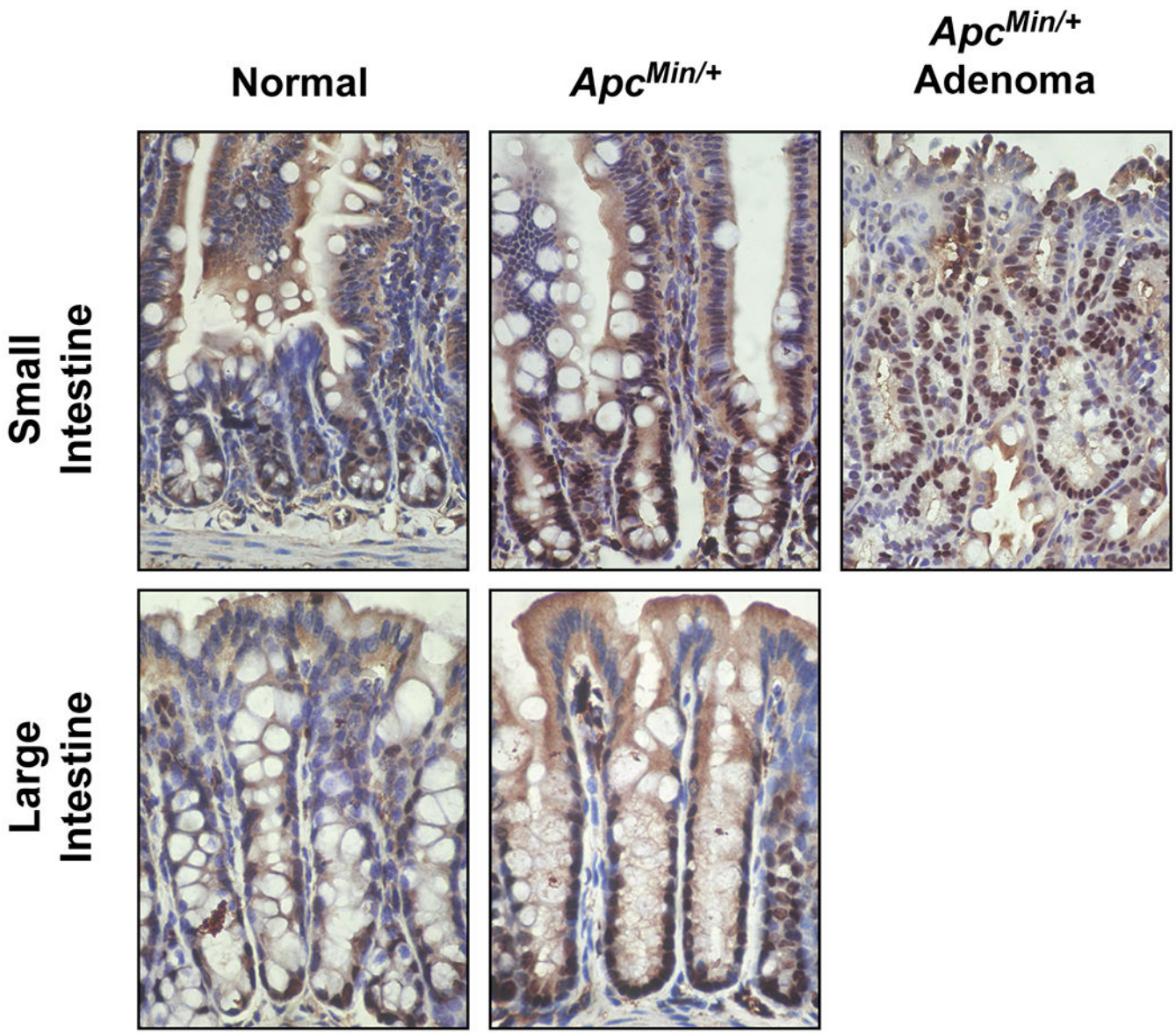

Figure 10.

Immunostaining using anti–cyclin D1 antibodies to evaluate expression of the Tcf4 target gene, cyclin D1. There was a progressive increase in cyclin D1 expression from normal small intestine of controls (top left panel) to normal-appearing small intestine of $Apc^{Min/+}$ mice (top center panel) to adenomatous tissue of small intestine of $Apc^{Min/+}$ mice (top right panel). Cyclin D1 expression was also increased in normal-appearing large intestines of $Apc^{Min/+}$ mice (lower right panel) compared to normal large intestines of control mice (lower left panel).