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Advances in Brief

Evidence That APC Regulates Survivin Expression: A Possible Mechanism Contributing to the Stem Cell Origin of Colon Cancer

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Abstract

Because colorectal cancers (CRCs) frequently display *APC* mutation, inhibition of apoptosis, and increased expression of the antiapoptotic protein survivin, we hypothesized that *APC* mutation inhibits apoptosis by allowing constitutive survivin expression. Using HT-29 CRC cell lines having inducible wild-type *APC* (*wt-APC*) or transfected dominant-negative *TCF-4*, we show that wt-APC down-regulates survivin expression via APC/β-catenin/TCF-4 signaling. Using normal colonic epithelium, we found survivin by immunostaining/reverse transcription-PCR to be preferentially expressed in the lower crypt (which inversely correlates with wt-APC's expression pattern). Thus, wt-APC, by progressively decreasing survivin and increasing apoptosis from crypt bottom to top, may limit the population size of stem cells and other proliferative cells in the lower crypt; mutant APC may allow expansion of these populations, thereby initiating tumorigenesis.

Introduction

APC[2] mutations are thought to initiate both sporadic and hereditary forms of CRC. In the latter, a germ-line *APC* mutation in familial adenomatous polyposis patients is associated with a proliferative shift in normal-appearing colonic crypts, the earliest tissue change that has been identified. However, the mechanisms linking *APC* mutation to changes at the cell and tissue level are only poorly understood. *APC* mutation leads to TCF-4 activation which modulates the transcription of several genes including *c-MYC* (1), *Cox-2* (2), *cyclin D1* (3), *CD44* (4) and *PPARδ* (5). Nonetheless, how changes in the expression of these or other genes leads to tissue changes in colon tumorigenesis remains unclear. We hypothesized that loss of apoptosis is involved. Specifically, we postulated that wt-APC protein suppresses the expression of the antiapoptotic protein survivin. This hypothesis was based on several lines of evidence. First, *APC* is mutant in the majority of CRCs (6). Second, the gradual transformation of colorectal epithelium to carcinomas is associated with the progressive inhibition of apoptosis (7). Third, expression of *wt-APC* by recombinant means in CRC cell lines that have only inactive APC, induces apoptosis (8, 9). Fourth, survivin is highly expressed in the majority of CRCs (10–13). In this view, if suppression of survivin were lost because *APC* became mutant, it could lead to constitutive survivin expression, inhibition of apoptosis, and possibly development of CRC. In the present study, we tested the hypothesis that APC suppresses survivin expression and that this occurs through APC/β-catenin/TCF-4 signaling. Because an important prediction of this hypothesis is that wt-APC should, in normal colonic epithelial cells, suppress survivin expression, we also ascertained survivin expression in normal human colonic epithelium.

Materials and Methods

Cell Culture and Induction of *APC*. The colon carcinoma cell line HT-29 containing a zinc-inducible *APC* gene (HT-29-*APC*) and a control cell line containing an analogous inducible *lacZ* gene (HT-29-Gal; Ref. 8; kindly supplied by Drs. B. Vogelstein and K. Kinzler of the Howard Hughes Medical Institute and Johns Hopkins Oncology Center, Baltimore, MD) were cultivated in McCoy's 5A (Cellgro, Herndon, Virginia), supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 mg/ml streptomycin, and 0.6 mg/ml hygromycin. *wt-APC* expression was induced with 120 μM $ZnCl_2$ for the times indicated.

Construction of the Expression Plasmid. The fragment of *TCF-4* cDNA containing the β-catenin binding site, but lacking the portion responsible for the binding of the protein to DNA, was obtained using the RT-PCR method from total RNA isolated from the SW480 colon cancer cell line. The oligonucleotide AAATGCCGCAGCTGAACG, derived from the gene translation initiation region, and the reverse primer CCTTTTTGGAGTCCTGATGC, hybridizing to the region located in front of the DNA binding region, were used in a single-tube RT-PCR reaction using a Titan RT-PCR kit (Roche Molecular Biochemicals). The PCR products were cloned into a pCR3.1-Uni mammalian expression vector (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. The plasmids from a few randomly picked colonies were isolated and sequenced, and the clones with correct sequences were used in subsequent experimental procedures.

Transfection of Plasmid Constructs into HT-29 Cells. The dominant-negative plasmid construct was transfected transiently into the HT-29 cell line using a lipofection technique. Briefly, for each transfection, 2 μg of DNA were incubated with 12 μl of Lipofectin (Life Technologies, Inc., Grand Island, New York) in McCoy's growth medium without serum to form complexes according to the manufacturer's recommendations. Subsequently, 1×10^6 cells seeded in 6-well plates were exposed to a DNA/Lipofectin mixture for 5 h. After 5 h, the medium was adjusted to the normal serum concentration, and the incubation of cells continued under regular growth conditions until the cells were harvested. Cells were harvested after 24 h, and total RNA was isolated (Invitrogen; Total RNA Isolation kit) and analyzed using RT-PCR. To generate stable transfections, 2 μg of linearized DNA plasmid constructs were introduced into cells using the above-mentioned procedure. Subsequently (48 h after transfection) cells were trypsinized and plated into the medium containing G418 (Life Technologies, Inc.). Stable transfectants were harvested 10 days after the beginning of transfection.

RNA Extraction and cDNA Synthesis. Total RNA was isolated from each section of the crypt and from cell lines with RNeasy Mini kit (Qiagen, Valencia, California) in accordance with the manufacturer's instructions. The first strand of cDNA was synthesized from RNA molecules using Avian Myeloblastosis Virus reverse transcriptase (Promega, Madison, Wisconsin), as indicated by the manufacturer. One μg of RNA was used as a template for first-strand synthesis in quantitative analysis of mRNA expression in the HT-29-*APC* cell line.

RT-PCR Amplification. The expression of survivin was evaluated with RT-PCR. cDNA was made by reverse transcription with random primers. Survivin was amplified by PCR. The primers used to detect fragments of the

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[2] The abbreviations used are: APC, adenomatous polyposis coli; RT-PCR, reverse transcription-PCR; CRC, colorectal cancer; TCF-4, T-cell factor 4; wt, wild-type.

survivin gene were designed from published human sequences and span exons 1–4. The sequences were: 5′-AGCCCTTTCTCAAGGACCAC-3′ and 5′-GCACTTTCTTCGCAGTTTCC-3′, giving an amplified product of 363 bp. The PCR reaction contained 2 units of Taq polymerase (Roche Molecular Biochemicals); 10× PCR buffer; 0.5 μg of each oligonucleotide primer; 200 μM each dATP, dCTP, dGTP, dTTP; 1 μl of nascent cDNA; and sterile distilled water to bring the volume to 25 μl. The amplification cycle included a denaturation step of 94°C for 2 min, followed by 25 or 28 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and concluded with a final primer extension step of 72°C for 5 min. Positive controls included the human colon cancer cell line HCT116 cDNA. Negative controls included replacing RNA or cDNA with distilled water. Controls were consistently found to be negative for survivin. PCR products were resolved in a 1.5% agarose gel in Tris-acetate/EDTA buffer and visualized by ethidium bromide staining under UV illumination. To confirm the integrity of cDNA, fragments of the housekeeping gene *β-actin* were amplified concurrently. The sequence of the cDNA was compared with that in GenBank, and they were found to be identical.

Tissue Procurement and Preparation of Crypts. Samples of normal colon tissue were obtained from each of 28 individuals undergoing surgery for colon cancer. The tissue procurement was approved by the IRB of Thomas Jefferson University. In each case, only colonic tissue that appeared normal was collected. Crypts were isolated from these specimens using a chelation method. Briefly, tissue samples were washed with PBS and incubated in 3 mM EDTA/0.05 mM DTT in PBS for 90 min at room temperature. The samples were shaken at 300 pulses per min for 3 min at room temperature, at which point isolated mucosal crypts could be seen in suspension. The supernatant was transferred to a new centrifuge tube and centrifuged at 300 rpm and 4°C to pellet crypts. The supernatant was removed, and the pellet was resuspended in PBS. The crypts were diluted and suspended, and a drop was placed in a dish under a dissecting microscope. Using two hypodermic needles and a dissecting microscope, the crypts were microdissected into three compartments containing top, middle, or bottom sections, and isolated sections were collected and pooled.

Immunohistochemistry of Tissue. Sections (5 μm each) cut from formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and rehydrated in graded alcohol. Quenching of endogenous peroxidase activity was attained by treatment with 3% H_2O_2 in methanol. The slides were first boiled in pH 6 citrate buffer (Bio Genex, San Ranmon, California) for 15 min in a microwave oven, then incubated overnight with anti-survivin polyclonal antibody (supplied by Professor Dario Altieri, Department of Pathology, Yale University; dilution of 1:500) at 4°C. After three washes of 5 min each in PBS, slides were incubated with biotin-conjugated antirabbit antibody (Roche Molecular Biochemicals) for 30 min at room temperature, followed by incubation with conjugated streptavidin-horseradish peroxidase for 30 min (Roche Molecular Biochemicals). After three washes of 5 min each in PBS, the slides were stained with 3′,3′-diaminobenzidine for 10 min and then counterstained for 5 min with hematoxylin. In control experiments, endogenous biotin was blocked by applying avidin for 30 min, followed by biotin for 30 min prior to the application of the biotinylated detection reagent. Negative controls obtained by replacing antisurvivin antibody with 10% fetal bovine serum, and positive controls (colonic adenocarcinoma tissue) were also included in the study.

Western Blot Analysis. The cells were lysed by 1× SDS "running" buffer (100 mM Tris chloride, 200 mM DTT, 4% SDS, and 20% glycerol). The amount of protein in cell lysates was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, California). After boiling for 10 min, 50 mg of protein was separated, resolved by 12% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked for 30 min in blocking buffer (Tris-buffered saline with 0.2% Tween 20 and 5% nonfat milk), and incubated with rabbit antihuman survivin polyclonal antibody (1:500) overnight at 4°C. The membrane was then washed in Tris-buffered saline containing 0.2% Tween 20, incubated with phosphatase-conjugated goat antirabbit antibody (1:1500) for 60 min, and developed with a substrate reagent kit (Bio-Rad). As a control, tubulin protein was blotted concurrently. All of the experiments were repeated at least three times.

Analysis of the Survivin Promoter for a TCF-4 Binding Element. We used computer analysis to search for the presence of a basepair sequence corresponding to one of the known Tcf-4 binding elements in the reported promoter sequence of human *survivin* (1, 14, 15).

Results

Regulation of Survivin Expression by APC. The colon carcinoma cell line HT-29 containing a zinc-inducible *APC* gene (HT-29-*APC*) and a control cell line containing an analogous inducible *lacZ* gene (HT-29-Gal) was used to test our hypothesis. Induction of wt-*APC* expression in HT-29 *APC* cells containing mutant *APC* is known to cause apoptosis (8). Using RT-PCR and Western blotting, we assessed the expression of survivin in HT-29-*APC* cells before and after *wt-APC* expression was induced with zinc treatment. Both RT-PCR and Western blot experiments showed that (*a*) HT-29 cells contain high endogenous levels of survivin, and (*b*) induction of *wt-APC* expression causes a decrease in survivin mRNA and protein expression (Fig. 1). Survivin expression was repressed within 8 h after *APC* induction (Fig. 1), whereas no change was detected in control (HT-29-Gal) cells even 8 h after zinc treatment.

To investigate whether the APC/β-catenin/TCF-4 signal transduction pathway regulates survivin expression, a dominant-negative *TCF-4* gene construct was used to block TCF-4-mediated activation of gene transcription. In experiments designed to determine the dose effect of a transiently transfected dominant-negative *TCF-4* construct on survivin expression, we observed, using RT-PCR, that survivin expression decreased within 24 h, and the suppression of survivin expression correlated with the amount of the *TCF-4* construct transfected (Fig. 2*A*). When the dominant-negative *TCF-4* was stably transfected into CRC cell lines, survivin RNA and protein expression remained significantly reduced (Fig. 2, *B* and *C*).

To further evaluate whether APC might regulate survivin expression via APC/β-catenin/TCF-4 signaling, we analyzed the human *survivin* promoter sequence (14) for the presence of one of the known TCF-4 binding elements. We found that a TCF-4 binding element CTTTGAA was located 263 bp upstream of the transcription start site and perfectly matched the consensus for the TCF-4 binding sequence CTTTG(A/T)(A/T) (1, 15).

Survivin Expression in Normal Colonic Mucosa. Investigation of the expression of survivin in microdissected isolated subsections of the normal colonic crypt, using RT-PCR, showed that specimens from all 28 donors (100%) were strongly positive for survivin in the bottom section, seven (25%) were weakly positive in the middle section, and

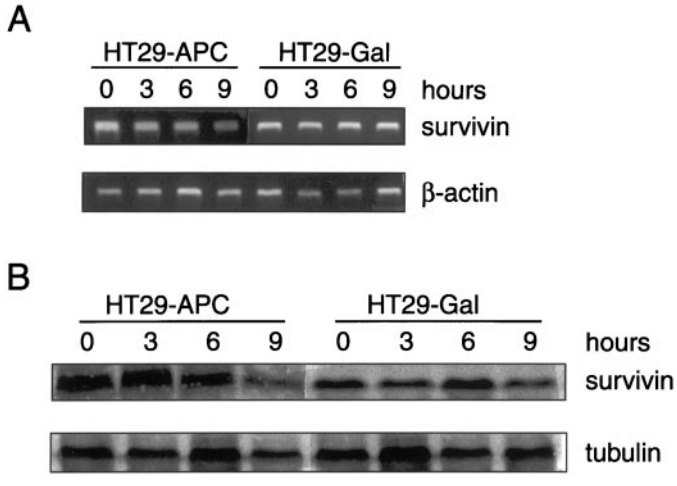

Fig. 1. Changes in survivin expression after induction of wt-APC expression in HT29 cells. The data shows that induction of wt-APC decreases expression of survivin mRNA and protein in recombinant HT-29 cells (HT-29-APC), but not in control cells (HT-29-Gal), in which wt-APC is not induced. *A*, survivin mRNA (363 bp) levels were assessed by RT-PCR in HT-29-APC cells and HT-29-Gal cells at the indicated times after $ZnCl_2$-induced expression of wt-APC. β-actin mRNA (564 bp) levels are shown as an internal control. *B*, survivin protein (M_r 16,500) levels were assessed by Western blots in the same cell lines. Tubulin protein (M_r 55,000) levels are shown as an internal control.

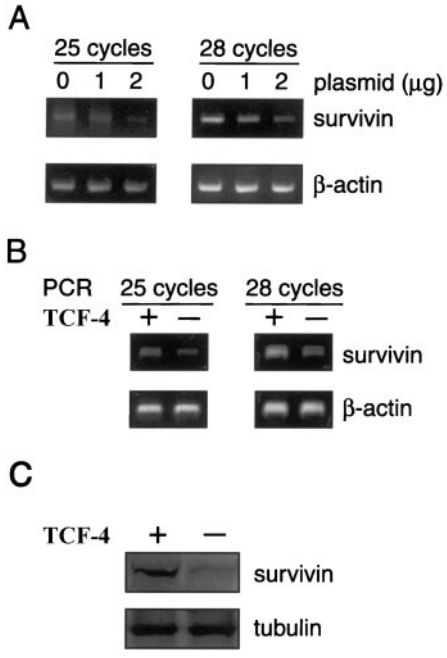

Fig. 2. Changes in survivin expression in HT-29 cells that were transfected with a dominant-negative *TCF-4* gene construct. *A*, HT-29 cells transiently transfected with varying amounts of plasmid DNA (0, 1.0, and 2.0 μg) encoding a dominant-negative *TCF-4* gene construct. These data show that transiently blocking TCF-4 mediated activation of gene transcription decreases survivin expression. Survivin mRNA levels were evaluated by RT-PCR for two different protocols (25 or 28 cycles). β-actin levels are shown as an internal control. *B*, HT-29 cells stably transfected with plasmid DNA encoding a dominant-negative *TCF-4* gene construct (−) or the empty plasmid (+). These data show that stably blocking TCF-4-mediated activation of gene transcription decreases survivin expression. Survivin mRNA levels were evaluated by RT-PCR for two different protocols (25 or 28 cycles). *C*, survivin protein levels in HT-29 cells stably transfected with the dominant-negative *TCF-4* gene construct were evaluated by Western blots. Tubulin levels are shown as an internal control.

six (21%) were weakly positive in the top section of the crypt. Survivin mRNA expression for 10 different samples is shown in Fig. 3*A*. The results indicate that normal colonic epithelium expresses survivin and does so preferentially in the lower crypt.

To confirm that survivin is preferentially expressed in the lower section of normal human crypts, survivin immunoreactivity was studied in parallel samples of normal colon tissue. Consistent with the RT-PCR results, survivin immunostaining in histologically normal colorectal mucosa was observed in epithelial cells at the base of the crypt (Fig. 3, *B* and *C*). A few cells in the middle compartment also showed staining, but none of the cells in the upper crypt were positive for immunostaining.

Discussion

In this study, we investigated the hypothesis that APC suppresses survivin expression, a mechanism that might regulate apoptosis in normal human colonic epithelial cells. Our results support the hypothesis that APC suppresses survivin expression and does so via TCF-4/β-catenin signaling. This conclusion was based on the convergence of several independent lines of evidence: (*a*) zinc induction of wt-APC expression in cells that otherwise lack wt-APC leads to decreased expression of survivin; (*b*) a dominant-negative *TCF-4* gene construct that blocks TCF-4-mediated activation of transcription led to decreased survivin expression; and (*c*) a TCF-4 binding site was identified within the *survivin* promoter sequence.

Because, as noted above, an important prediction of our hypothesis is that wt-APC should, in normal colonic epithelial cells, suppress survivin expression, we also ascertained survivin expression in normal human colonic epithelium. Despite this prediction, we found that survivin is expressed in normal colonic epithelium. In comparison, results from previous studies have been controversial as to whether survivin is expressed in colonic mucosa. In two studies (10, 11), survivin expression was found to be absent in normal adult colonic epithelium. In another study (12), survivin expression was found in 29% of biopsies of normal colonic epithelium taken from mucosa adjacent to colorectal tumors, although this finding was considered to represent an "intermediate biological change identifying histologically normal mucosa at risk of neoplastic transformation." A recent study by Gianini *et al.* (13) reported survivin expression (seen by immunostaining) to be present in all normal, adult colonic mucosa specimens examined. In this study, survivin expression localized to the crypt base, which was similar to our current findings. The apparent discrepancy between both Gianini's and our present study on the one hand, and earlier studies on the other hand, could conceivably result from differences in the sensitivity of the methods. In one of the two studies with negative findings, Ambrosini *et al.* (11) used a different method, *in situ* hybridization of survivin mRNA. The other negative study from the research of Kawasaki *et al.* (10) involved immunohistochemistry as did ours and Gianini's (13). Although the study by Sarela *et al.* (12) used RT-PCR to analyze biopsies of normal colonic mucosa, these specimens are likely to have a significant component of nonepithelial tissue that could hinder the detection of survivin. In our study, we used RT-PCR and isolated, microdissected normal colonic crypts because we consider the combination of these two techniques to provide the most sensitive method.

Nonetheless, our finding that survivin is expressed in normal crypts needs to be reconciled with our prediction that wt-APC would sup-

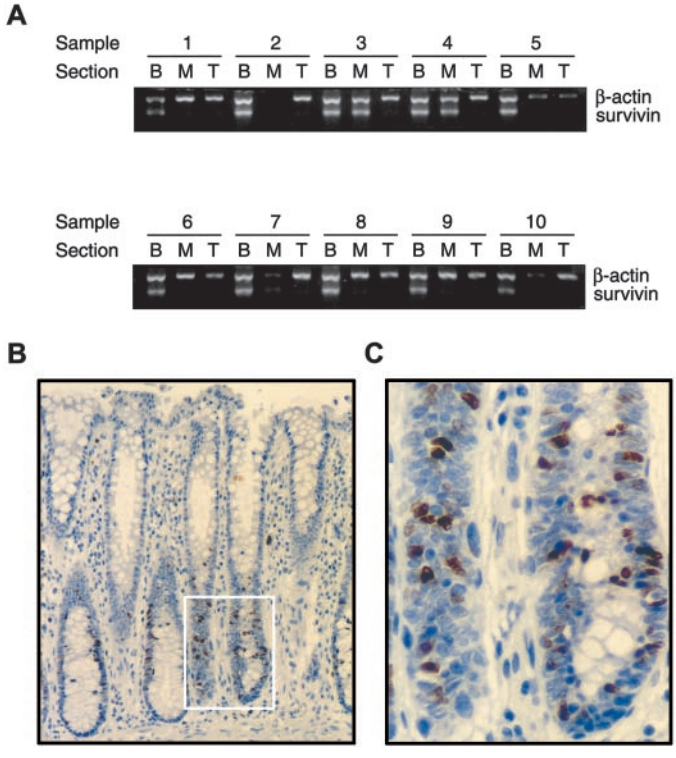

Fig. 3. Survivin expression in normal human colonic mucosa. *A*, survivin mRNA expression in different sections of microdissected normal crypts isolated from 10 patients. Using RT-PCR, we showed that survivin is preferentially expressed in the bottom (*B*) crypt section as compared with the middle (*M*) and top (*T*) sections of normal human crypts. β-actin levels are shown as an internal control. *B* and *C*, survivin immunoreactivity in normal human colonic mucosa. Staining is most prominent at the base of the crypt, considerably lower in the middle region, and not present at the crypt top. ×100 (*B*). *Outlined area in B* is shown at higher magnification (×400) in *C*.

press survivin expression in normal colonic crypts. This conflict may be resolved by considering crypt patterns of APC and survivin expression. Our finding is that survivin is preferentially expressed in the lower crypt. In contrast, APC staining in epithelial cells of colonic crypts displays a marked increase from the base of the crypt to the lumenal surface (16, 17). Survivin's pattern thus correlates inversely with wt-APC's expression pattern. Hence, wt-APC would be expected to progressively decrease survivin expression and increase apoptosis from crypt bottom to top. This expectation correlates with the patterns of both: (*a*) survivin expression that we observed in crypts and (*b*) apoptosis in crypt colonocytes that increases as these cells migrate along the crypt axis toward the top (7), where they expire and are extruded or undergo phagocytosis. Therefore, our findings on survivin expression do, after all, support our hypothesis that APC regulates survivin expression.

The findings in this study raise an interesting question: Is APC regulation of survivin expression normally involved in maintenance of crypt cell renewal? The above-discussed patterns of APC and survivin expression suggest a model that provides a possible answer.[3] Our observation that survivin is preferentially expressed in the lower portion of the normal human colonic crypt, in which proliferating cells, including stem cells, reside, suggests that survivin may be integral to preventing apoptosis in these cell populations in the crypt base. Indeed, survivin may confer prolonged survival to basal crypt colonocytes, especially stem cells. In contrast, in the middle portion of the crypt, survivin expression is diminished, and this correlates with the region in which colonocytes stop proliferating and start to undergo differentiation and maturation. In the upper portion of the crypt, APC levels are high and survivin levels are minimal or nonexistent. This correlates with the region in which colonocytes undergo terminal differentiation and apoptosis, and are extruded. Together, these findings suggest that survivin may maintain conditions allowing survival of stem cell populations, and it is these populations that drive the renewal process.

This model is consistent with previous studies. A study of recombinant mice lacking Tcf-4 (which mimics wt-APC signaling) showed that this leads to depletion of epithelial stem cell compartments in the small intestine (18). Two other adult cell renewal type tissues including normal human skin and endometrium have also been found to express survivin (19, 20). Like colon, these tissues are characterized by a constant turnover of cells that are regenerated from stem cells.

Finally, our findings suggest a novel cellular/tissue mechanism for initiation of colon tumorigenesis, a mechanism that suggests how *APC* mutation, the initiating molecular event in CRC, leads to abnormalities at cellular and tissue levels. In normal crypts, in this mechanism, APC-induced suppression of survivin expression in the crypt's middle region causes stem cell progeny to begin to lose, via the initiation of apoptosis, their natural phenotype (*e.g.*, immortality, proliferation) as they migrate up the crypt. In contrast, in crypts containing mutant *APC*, survivin expression may become constitutive, thereby inhibiting apoptosis. In this case, mutant stem cell progeny would tend to maintain their natural, stem-cell-like phenotype as they migrate up the crypt. Such cells would be more likely to proliferate, which is exactly what is found: familial adenomatous polyposis patients show a proliferative shift wherein the proliferative zone extends more and more toward the crypt top. Thus, constitutive expression of survivin prevents apoptosis, contributes to cellular immortality, and may be a key contributing mechanism in early colonic tumorigenesis.

[3] The model that the crypt APC gradient governs crypt stem cell number was presented in preliminary form by Dr. B.M. Boman at the Third Joint Meeting of the Leeds Castle Polyposis Group and the International Collaborative Group for Hereditary Nonpolyposis Colorectal Cancer, Venice, Italy, April 27, 2001.

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Note Added in Proof

Support for our novel cellular/tissue mechanism for initiation of colon tumorigenesis was recently reported in Boman *et al*. Cancer Res., *61:* 8408–8411, 2001.

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