American Journal of Pathology, Vol. 165, No. 5, November 2004

Tumorigenesis and Neoplastic Progression

Colonic Crypt Changes during Adenoma Development in Familial Adenomatous Polyposis

Immunohistochemical Evidence for Expansion of the Crypt Base Cell Population

Bruce M. Boman,* Rhonda Walters,† Jeremy Z. Fields,‡ Albert J. Kovatich,† Tao Zhang,* Gerald A. Isenberg,§ Scott D. Goldstein,§ and Juan P. Palazzo†

From the Department of Pathology, Anatomy, and Cell Biology,† and the Divisions of Genetic and Preventive Medicine and of Colorectal Surgery,§ Thomas Jefferson University and Kimmel Cancer Center, Philadelphia; and CA*TX Incorporated,‡ Gladwyne, Pennsylvania*

Familial adenomatous polyposis patients, who have a germline *APC* mutation, develop adenomas in normal-appearing colonic mucosa, and in the process usually acquire a mutation in the other *APC* allele as well. Nonetheless, the cellular mechanisms that link these initiating genetic changes with the earliest tissue changes (upward shift in the labeling index) in colon tumorigenesis are unclear. Based on the tenet that colorectal cancer originates from crypt stem cells (SCs) and on our kinetic modeling, we hypothesized that overpopulation of mutant colonic SCs is the missing link. Directly testing this hypothesis requires measuring changes in the size of the SC population, but specific markers for human colonic SCs are lacking. Hence, we used immunohistochemical mapping to study crypt base cells, of which SCs are a subset. Using colectomy specimens from 16 familial adenomatous polyposis and 11 control cases, we determined the topographic profiles of various cell populations along the crypt axis and the proportions of each cell type. In the formation of adenomatous crypts, the distribution of cells expressing crypt base cell markers (MSH2, Bcl-2, survivin) expanded toward the crypt surface and showed the greatest proportional increase (fivefold to eightfold). Cells expressing a marker for the upper crypt ($p27^{kip1}$) shifted to the crypt bottom and showed the smallest increase. This suggests that: 1) during adenoma development, *APC* mutations cause expansion of the crypt base cell population, including crypt SCs; 2) SC overpopulation can explain the shifts in pattern of proliferative crypt cell populations in early colon tumorigenesis, and 3) mutant crypt SCs clonally expand to form colonic adenomas and carcinomas. *(Am J Pathol 2004, 165:1489–1498)*

Even though the genetic changes (*APC* mutations) associated with tissue changes (upward shift of the crypt proliferative compartment and adenoma formation) have been characterized in familial adenomatous polyposis (FAP) patients, the cellular mechanism linking the changes at these two levels is not fully established. It has been inferred that changes in the crypt stem cell (SC) population are involved in carcinogenesis in general, and in this mechanism in particular.[1] This inference is based on several lines of evidence: 1) SCs are the only cells to reside in the colonic crypt long enough to acquire the multiple mutations required for colon cancer;[2] 2) SCs already have several characteristics of transformed cells—lifetime capacity for self-renewal and proliferation, and anchorage in the crypt; 3) histological evidence from adenomas in $APC^{Min/+}$ mice[3] and colon carcinomas in rodents[4] indicates that multiple differentiated intestinal cell types exist in these tumors, which suggests that they must have arisen from a multipotent cell, such as a crypt SC. Based on our studies that modeled the kinetics of crypt dynamics and the proliferative abnormality in FAP crypts,[5] we hypothesized that SC overpopulation under-

Supported by the National Institutes of Health (1R21 DK063014-01, 1P01 CA72027-01A2, and P30CA56036) and CA*TX, Inc., Gladwyne PA.

Accepted for publication June 29, 2004.

Address reprint requests to Bruce M. Boman, M.D., Ph.D., Director, Division of Genetic and Preventive Medicine, Director, GI Cancer Program, Kimmel Cancer Center, 1100 Walnut St., Suite 400, Thomas Jefferson University, Philadelphia PA 19107. E-mail: bruce.boman@mail.tju.edu.

Table 1. Primary Antibodies Used for Immunohistochemistry

Antigen	Source	Dilution
HMSH2	Clone FE11, mouse IgG1 kappa (Zymed Laboratories, San Francisco, CA)	1:400
Bcl2	Clone 124, mouse IgG1 kappa (DAKO Co., Carpenteria, CA)	1:400
Survivin	Rabbit polyclonal antibody (Alpha Diagnostics, San Antonio, TX)	1:400
Ki67	Clone MIB1, mouse IgG1 (Immunotech, Westbrook, ME)	1:100
Topoisomerase II	Clone KiS 1, mouse IgG2a (DAKO Co.)	1:100
p21	Clone EA 1O, mouse IgG1 (Oncogene Sciences, Cambridge, MA)	1:100
p27	Clone 1B4, mouse IgG2a (Novocastra Laboratories, Newcastle, UK)	1:160
p53	CM1, rabbit polyclonal (Novocastra/Vector, Burlingame, CA)	1:500

lies the upwards proliferative shifts in the crypt in early colorectal cancer (CRC) initiation and adenoma development.

Directly testing this hypothesis, however, is hampered because there are no unambiguous molecular markers for SCs, and because the crypt is a rather complex system. Accordingly, we used immunohistochemistry to study changes in the number of crypt cells expressing markers for cells in the crypt base, the region where SCs reside. Markers were selected based on their known staining patterns in normal colonic epithelium showing that their expression is restricted to specific colonocyte populations residing within different regions of the crypt. MSH2, Bcl-2, and survivin were selected as markers for crypt base cells (which includes SCs);[6–11] Ki-67 and topoisomerase II for proliferative cells in the lower crypt;[6,11] and $p21^{WAF1/C1P1}$ and $p27^{kip1}$ for differentiated cells in the upper crypt.[6,12–14] We performed immunostaining on both normal-appearing and adenomatous crypts from FAP patients because these patients represent a genetically well-defined model in which the *APC* genotype has been correlated with histopathological changes that occur during colon tumorigenesis.

Materials and Methods

Patient Samples

Sixteen patients with FAP were selected from the Pathology Department at Thomas Jefferson University. Inclusion criteria included: 1) surgical treatment involving a total proctocolectomy that was performed at Thomas Jefferson University; 2) availability of the pathology report from the colectomy to verify histopathological evidence of the classical, multiple polyposis phenotype (documenting FAP diagnosis); and 3) availability of formalin-fixed paraffin-embedded tissue blocks containing both normal and adenomatous colonic epithelium. Four of the sixteen patients had CRC present at colectomy. Ages ranged from 16 to 60 years (median, 29 years) and there were 11 males and 5 females. Patients were also registered in Jefferson's Familial Colorectal Cancer Registry (institutional review board approved) and had documentation of nuclear pedigrees. Immunohistochemistry was also done on colectomy specimens from 11 non-FAP patients with no history of CRC or inflammatory bowel disease. The pathology reports and hematoxylin and eosin-stained slides were reviewed by one pathologist (J.P.P.) to determine that each case had both suitable normal-appearing colonic mucosa and adenomatous epithelium for immunohistochemical analysis.

Immunohistochemistry

Table 1 summarizes the antibodies used in our study. Immunohistochemistry was performed on colon samples according to methods we previously reported.[6,11] Briefly, 5-μm-thick sections of formalin-fixed paraffin-embedded tissues were cut onto neoprene-coated slides (Aldrich Chemical, Milwaukee, WI). Routine deparaffinization from xylene to 95% alcohol and rehydration before patented microwave antigen recovery were performed on a Leica autostainer (Leica, Inc., Deerfield, IL). The deparaffinization included a 30-minute methanol peroxide block for endogenous peroxidase activity.

The antigen recovery step (Microwave Antigen Retrieval, U.S. Patent no. 5244,787) was performed in a microwave oven (800 W, model no. NN-5602A; Panasonic, Franklin Park, IL). The slides were placed in Tissue Tek slide holders and staining dishes (Miles, Elkhart, IL) and submersed in 200 ml of Citra Plus solution, pH 6.0 (BioGenex, San Ramon, CA). The slide holder with slides was placed in the microwave oven for 5 minutes on the high-energy setting. Fifty ml of dH_2O was added to the vessel to replenish the evaporative loss and the holder was returned to the microwave oven for an additional 5 minutes on the high setting. The holder and slides were then removed from the oven and cooled for 20 minutes before continuing the immunostaining procedure. For one antigen, Bcl-2, ethylenediaminetetraacetic acid solution, pH 8.0 (Zymed Laboratories, San Francisco, CA) was used instead of the citrate buffer in the antigen recovery step that was otherwise the same as described above.

The slides were washed in dH_2O and placed in DAKO Tris-buffered saline (TBS) (TBS containing carrier protein and sodium azide). The immunostaining was performed on a DAKO autostainer (Carpinteria, CA). The slides were incubated for 60 minutes with each predetermined primary antibody dilution (see Table 1). The slides were then washed and incubated for 5 minutes with TBS, which was followed by a 30-minute incubation with peroxidase-labeled polymer (EnVision +, DAKO). The slides were then washed and incubated for 5 minutes in TBS. The DAB/peroxide (DAB+, DAKO) was applied for 5 minutes. Slides were washed in TBS and then tap water.

The slides were removed from the automated immunostainer and placed on the Leica autostainer for coun-

terstaining and dehydration with xylene. The counterstain was Harris hematoxylin (Surgipath, Richmond, IL) followed by bluing in lithium carbonate (3%). The slides were then coverslipped using the Hacker robotic coverslipper (Hacker Instruments, Inc., Fairfield, NJ). Placenta and normal colonic mucosa served as positive controls for p2l and $p27^{kip1}$, a p53-positive known colon cancer for p53; tonsil for Bcl-2, Ki-67, and topoisomerase II; and normal colonic mucosa for hSMH2 and survivin. Absence of primary antibody was used as a negative control.

Analysis of Staining

Immunostained sections from each tissue block—from normal, FAP, or adenomatous colonic mucosa—were scored to determine the percentage of crypt cells that were stained. We evaluated multiple fields, focusing on longitudinally oriented crypts. The proportion of cells in each crypt that showed staining was visually scored. Crypt cells were scored as positive for a marker if any staining (weak, moderate, or strong) was detectable, and negative if absent. We then determined average proportions across each slide, each sample, and each experimental group. We also identified whether staining was nuclear, cytoplasmic, or both. The intracryptal distribution—bottom, middle, top—of positively staining cells was evaluated for each experimental group. A secondary outcome—staining intensity—was rated as negative, weak, moderate, or strong. When staining was not uniform, the comment of heterogeneous was recorded.

Results

Immunohistochemical Staining Patterns

Staining of control colonic mucosa showed nuclear hMSH2 staining of epithelial cells (Figure 1A) in the lower crypt. In normal-appearing (nonadenomatous) FAP mucosa, the MSH2 staining intensity was greater and the population of MSH2-positive cells extended upwards into the middle of the crypt (Figure 1B). There were also some MSH2-positive cells with lower nuclear staining intensity at the luminal surface of the crypt in normal-appearing FAP crypts but not in control crypts. Staining for hMSH2 in adenomatous mucosa showed intense nuclear staining throughout the entire crypt with similar intensity of staining at the crypt surface and at the crypt base (Figure 1C).

Staining for survivin was more intense in the nuclei of cells of normal crypts with some cytoplasmic staining of cells observed in adenomatous crypts. Survivin staining was preferentially located in the lower region of normal control crypts (Figure 1D). Staining showed an expansion of the survivin-positive cell population into the middle of normal-appearing FAP crypts (Figure 1E) compared to control crypts. Staining of adenomatous mucosa (Figure 1F) showed strong survivin-positive staining of colonocytes along the entire crypt axis, with the greatest number of positively staining cells in the upper crypt (Figure 1F).

Bcl-2 staining was seen in the cytoplasm of epithelial cells and did not show significant differences between normal-appearing FAP crypts (Figure 2A) and control crypts (not shown), both showing staining confined to the crypt base. In contrast, adenomatous mucosa, showed positive staining cells diffusely distributed along the entire crypt axis and often displayed a heterogeneous pattern (Figure 2B).

Ki-67 nuclear staining in normal control colonic mucosa was observed mainly in the lower crypt (Figure 1G). Ki-67 staining of cell nuclei was intense and showed an expansion of this cell population into the middle of normal-appearing FAP crypts (Figure 1H) compared to control crypts. In comparison, adenomatous mucosa showed positive nuclear Ki-67 staining throughout the crypt, with increased staining intensity from the mid-crypt region to the lumenal surface (Figure 1I).

Staining for topoisomerase II did not show significant differences between normal-appearing FAP crypts (Figure 2C) and control crypts (not shown). Topoisomerase II staining in both cases showed nuclear staining mainly in the lower portion of the crypt (Figure 2C). Staining of adenomatous mucosa (Figure 2D) showed strong expression throughout the crypts.

Staining for $p21^{WAF1/C1P1}$ did not show significant differences between normal-appearing FAP crypts (Figure 2E) and control crypts (data not shown). Immunohistochemical staining for $p21^{WAF1/C1P1}$ in both cases showed nuclear staining and this was restricted to the surface epithelium (Figure 2E). $p21^{WAF1/C1P1}$ staining of adenomatous mucosa (Figure 2F) also showed nuclear staining in the upper third and lumenal surface of crypts with scattered cells showing nuclear positivity in the crypt bottom.

$p27^{kip1}$ staining, which was mainly nuclear and to a lesser extent cytoplasmic, was present in the upper portions of normal control crypts, although there were occasional isolated $p27^{kip1}$-positive cells at the crypt base (Figure 1J). Compared to control crypts, normal-appearing FAP crypts showed $p27^{kip1}$-positive cells with greater staining intensity at the lumenal surface and showed an increased number of isolated $p27^{kip1}$-positive cells at the crypt bottom (Figure 1K). $p27^{kip1}$ staining of adenomatous mucosa (Figure 1L) showed both nuclear and cytoplasmic staining of epithelial cells, mainly in the lower crypt (Figure 1L).

Staining for p53 was also done because it is considered by some[15] to be a marker for SCs. This showed solitary p53-positive cells at the base of the normal control crypt (data not shown), but only in half of the samples. In normal-appearing FAP crypts, approximately two-thirds of the samples showed p53 staining of isolated cells at the crypt base. There was an increased number of p53-positive cells per crypt compared to controls. Adenomatous crypts showed staining for p53 in approximately three-fourths of the cases. The staining was heterogeneous with a variable number of positively staining cells (5 to 30%) throughout the crypt. Overall, the pattern of staining for any given marker was similar among all samples in any given experimental group. Table 2 summarizes these staining patterns.

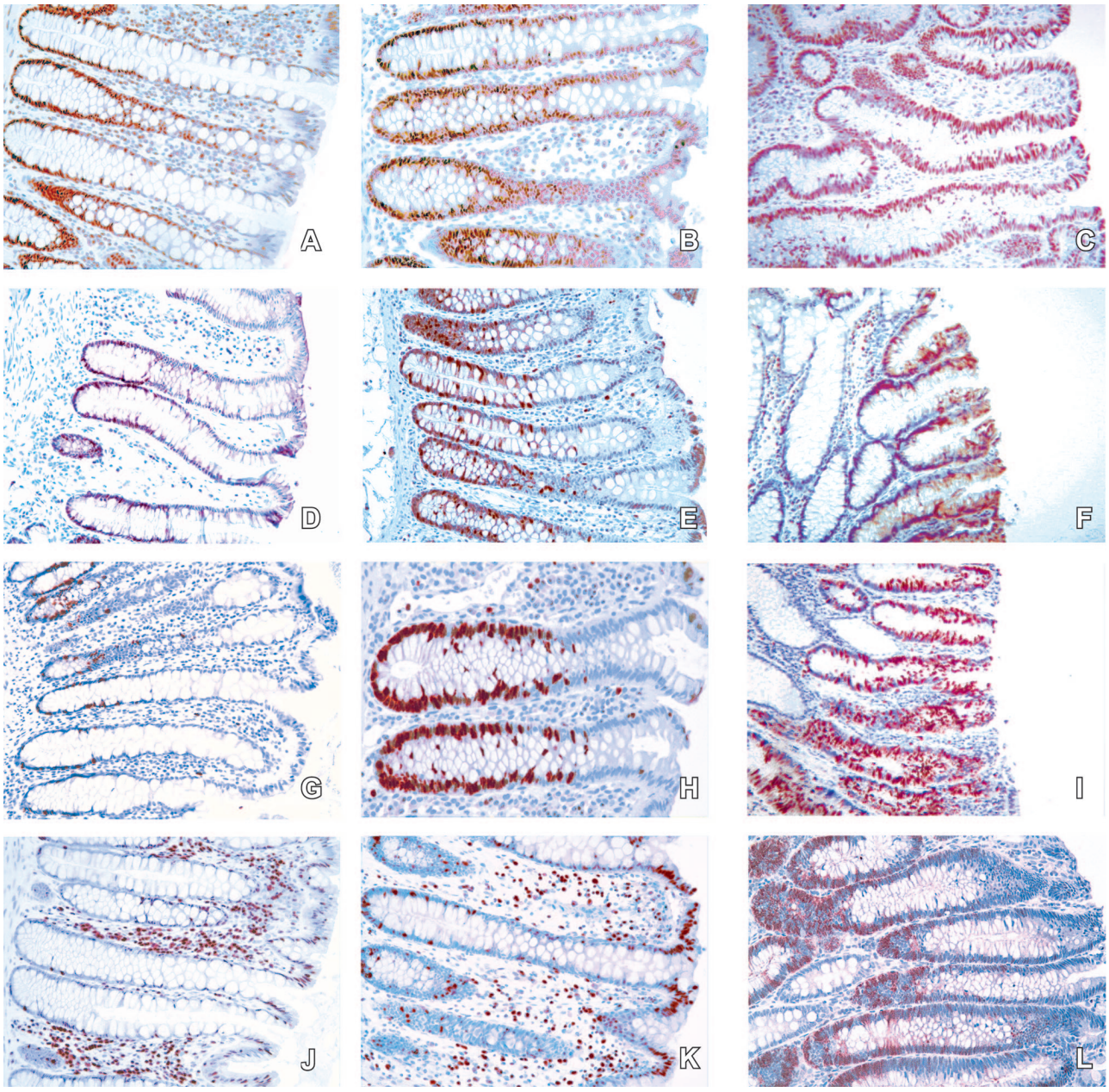

Figure 1. Immunostaining of normal crypts from control (non-FAP) patients (**A**, **D**, **G**, **J**) versus normal-appearing crypts from FAP patients (**B**, **E**, **H**, **K**) versus adenomatous crypts from FAP patients (**C**, **F**, **I**, **L**). Staining patterns are shown for hMSH2 (**A–C**), survivin (**D–F**), Ki67 (**G–I**), and p27 (**J–L**).

Proportion of Positively Staining Cells

Figure 3 shows changes in the proportions of cells staining for different markers. In general, going from normal control crypts, to normal-appearing FAP crypts, to adenomatous FAP crypts, we found an increase in the proportion of cells that stained for each marker relative to the total number of cells in the crypt. The proportion of cells staining for crypt base cell markers changed the most dramatically. That is, in adenomatous crypts, a greater proportion of cells expressed MSH2, bcl-2 and survivin compared to cells expressing markers for other crypt phenotypes. In contrast, in normal control crypts, cells expressing markers for crypt base cells showed the lowest proportions relative to cells with other phenotypes. As noted above, this staining for crypt base cell markers occurred in cells throughout the adenomatous crypt, not just in the crypt base region.

Discussion

It is well known that when colonic crypts become adenomatous, as they do in FAP, there is an increase in the total number of colonocytes, an increase in the number of mitotic figures, and an apparent reversal in the distribution of proliferating cells from the crypt bottom toward the upper crypt and luminal surface.[16–19] The present results

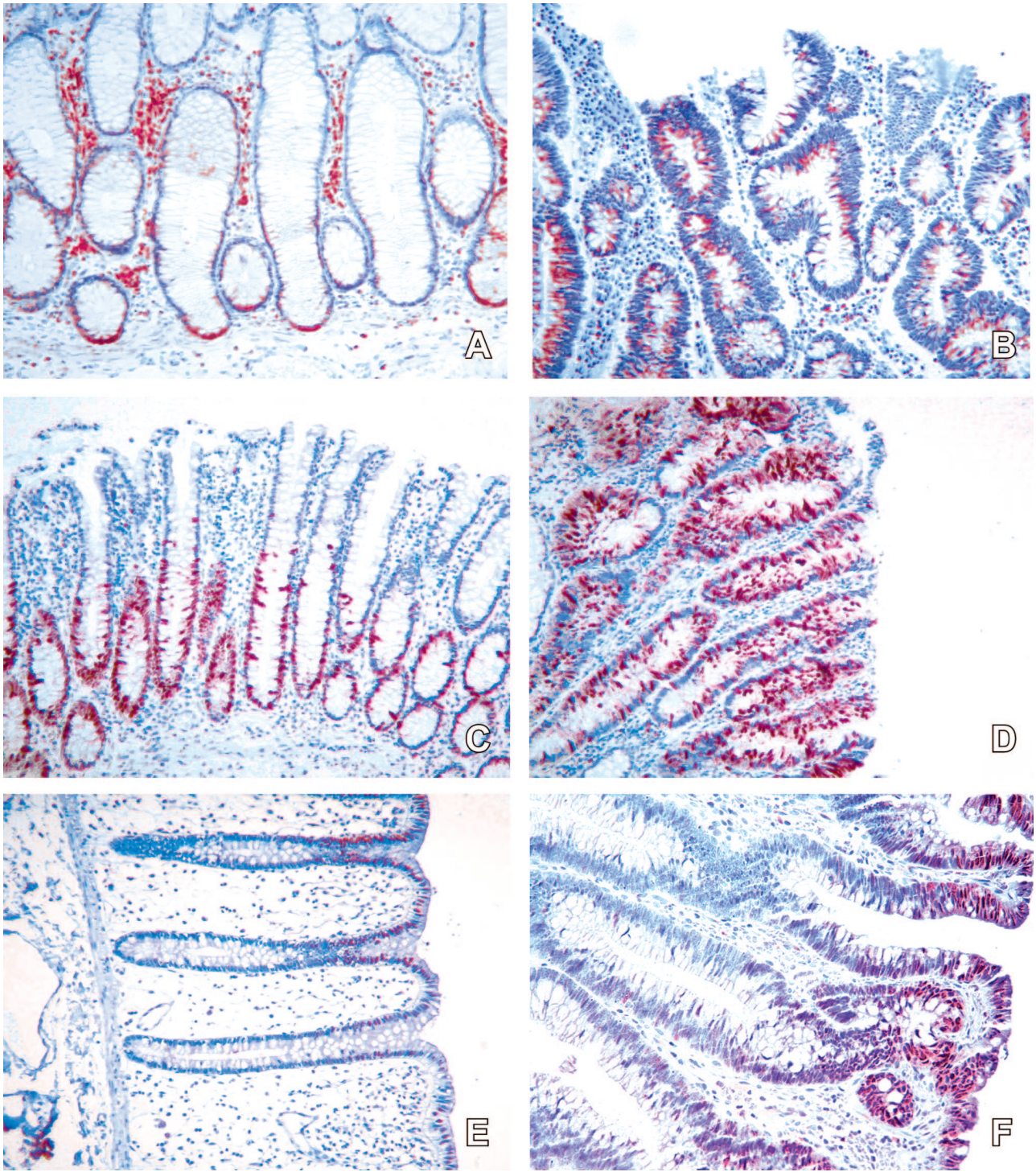

Figure 2. Immunostaining of normal colonic crypts (**A**, **C**, **E**) versus adenomatous crypts (**B**, **D**, **F**) from FAP patients for Bcl-2 (**A**, **B**); topoisomerase II (**C**, **D**), and $p21^{WAF1/C1P1}$ (**E**, **F**).

using markers for different cell phenotypes indicate that there is not only a change in the distribution of proliferative cells, but also, there is an increase in the proportion of certain types of crypt cells, which was greatest for cells having a crypt base cell phenotype. In this view, during adenoma development, the colonic epithelial cell population changes in number of cells (increased), location of cell types (different patterns of distribution), and composition (different proportions).

We compared the proportion of cells staining positively for any given marker within adenomatous glands to that within normal-appearing FAP crypts and to normal control crypts. We found a step-wise increase, from control to FAP to adenomatous crypts, in the proportion of cells

Table 2. Immunostaining Patterns in Normal Control Crypts, Normal-Appearing FAP Crypts, and Adenomatous FAP Crypts

Antigen	Associated function	Pattern in normal control crypts	Pattern in normal-appearing FAP crypts	Pattern in adenomatous crypts
HMSH2	Mismatch repair protein	Lower crypt	Lower and middle crypt	Diffuse staining throughout crypts with greatest number of positive cells in the upper crypt
Bcl2	Anti-apoptotic protein	Crypt base	Lower crypt	Diffuse staining throughout crypts
Survivin	Anti-apoptotic protein	Lower crypt	Lower and middle crypt	Staining throughout crypts with greatest number of positive cells in the upper crypt
Ki-67	Proliferation antigen	Lower crypt	Lower and middle crypt	Staining throughout crypts with greatest number of positive cells in the upper crypt
Topo II	Prevents DNA tangling	Lower crypt	Lower crypt	Diffuse staining throughout crypts
p21	Cell cycle suppressor	Crypt top and mucosal surface	Crypt top and mucosal surface	Upper crypt and mucosal surface
p27	Cell cycle suppressor	Crypt top and mucosal surface with rare positive cells in lower crypt	Crypt top and mucosal surface with isolated positive cells in lower crypt	Lower crypt

expressing MSH2, Bcl-2, survivin, and Ki-67. Increases for topoisomerase II, $p21^{WAF1/C1P1}$, and $p27^{kip1}$, were smaller. We conclude that during progression to adenoma there is a substantially increased proportion of cells having the crypt base cell phenotype. Because SCs are a subset of base cells in normal crypts, these data are consistent with the idea that the population of mutant SCs, or SC-like cells, in FAP and adenomatous FAP crypts has also increased in size. Our data do not exclude some other possibilities including the possibility that differentiated crypt cells in mutated crypts might recapitulate a dedifferentiated stem-cell phenotype. In such a scenario, the SCs would not be the origin of mutant cells that produce adenomas; rather it would be a differentiated cell. It has been argued that this latter possibility is an unlikely mechanism in adenoma morphogenesis.[2]

The concept of SC overpopulation is consistent with the results of our kinetic modeling studies.[5] In that modeling study, we investigated how a germline *APC* mutation, the earliest molecular alteration in colon tumorigenesis, might be linked to the proliferative shift in normal-appearing FAP crypts, the earliest known tissue change. To address this, we used kinetic modeling to investigate the premalignant crypt phenotype in FAP patients. Our modeling showed that only an increase in crypt SC number, not changes in the rates of cell cycle proliferation, differentiation, or apoptosis of the non-SC population, simulated the biological data (the labeling index) for the change from normal to FAP crypts, which exhibit a proliferative abnormality. Our results suggested that the protein product of the *APC* gene regulates the number of colonic crypt SCs, and that when *APC* is mutant it causes expansion of the crypt SC population. This kinetic modeling study led to our hypothesis that colon tumor initiation results from crypt SC overpopulation.

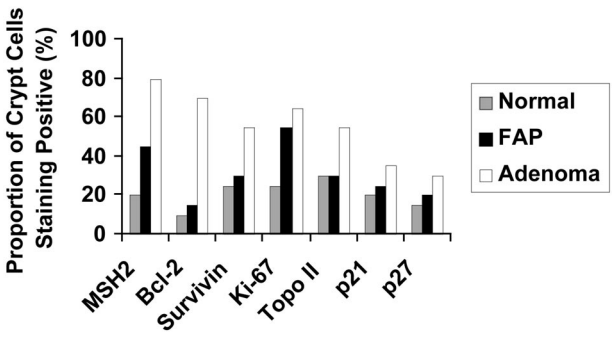

Figure 3. Changes in proportions of different kinds of cells based on differential staining between normal control crypts (**gray bars**), normal-appearing FAP crypts (**black bars**), and adenomatous mucosa (**white bars**) from FAP patients. The proportion of cells expressing markers for the crypt base (where SCs reside) shows the greatest increase in adenomatous crypts relative to normal-appearing crypts.

Because the SC phenotype includes immortality, and the ability to avoid apoptosis, the fact that more cells in adenomatous crypts express Bcl-2 and survivin, proteins that prevent apoptosis, is also consistent with SC overpopulation. Survivin has previously been reported to be overexpressed in adenomas.[20] Because SCs are immortal and drive cell renewal in the crypt throughout the life of the individual, MSH2 may also be crucial to the SCs by maintaining DNA fidelity through an intact mismatch repair mechanism. Indeed, it was recently reported[21] that MSH2 appears to be a marker for SCs in at least some tissues. Our data on MSH2 staining are therefore consistent with there being more SCs.

If the size of the SC population increases, as our current and previous reports suggest, then the SC population will produce more offspring. Our results showing that adenomas contain an increased number of cells expressing markers for proliferative cells (Ki-67 and topoisomerase II) are consistent with this concept. These changes should cause an overall increase in the size of the proliferative cell population, which expands into the upper crypt and toward the luminal surface. Indeed, the presence in adenomas of proliferative cells throughout the crypt, including the upper crypt regions and superficial

mucosa, has been well-documented by several studies using Ki-67 staining.[19,20,22–24]

A crucial point, in our view, is that proliferative cells (which are labeled by Ki-67[25,26]) and proliferating cells (which are labeled by BrdU or [^{3}H]thymidine uptake) need to be distinguished. The proliferating cells in adenomas are at the crypt top (the reverse of the labeling index pattern in controls).[16–18] However, our data, as well as data of others,[19,24] show that Ki-67 staining for proliferative cells occurs throughout the adenomatous crypt with greater staining intensity in the upper crypt being reported by some studies.[20,22,23] We submit that the distinction between proliferating and proliferative cells can account for the observed differences between the labeling index distribution and the Ki-67 staining profile in normal and abnormal colonic crypts. The proliferative cell population thus includes both cells that are proliferating and cells that can proliferate but are not actively proliferating, which includes SCs. The SC population consists of cells that are proliferative in that they have the capacity to proliferate, but they are only very slowly proliferating and are not readily labeled by BrdU or [^{3}H]thymidine uptake.[1,27–30]

Consistent with this distinction is our observed reversal of P27^{kip1} staining, from the upper crypt in normals to the lower crypt in adenomas. That is, the fact that P27^{kip1} is a cell-cycle inhibitor is consonant with the fact that SCs cycle very slowly.[1,27–30] P27^{kip1} is a component of the signaling pathway of another growth inhibitor, transforming growth factor (TGF)-β, and TGF-β signaling, via p27^{kip1}, inhibits progression from G_1 to S by inhibiting cyclin-dependent kinase activity.[31] The TGF-β staining pattern in adenomatous crypts is similarly reversed from crypt top to bottom compared to normal crypts.[22] These authors also found that the distribution of apoptotic cells was changed—from the upper crypt in normal mucosa to the lower crypt in adenomatous crypts. These results[22] coupled with reversal of the labeling index in adenomatous crypts,[18,32] suggested the possibility that the direction of cell migration is inward toward the crypt base, in contrast to normal crypts where it is outward toward the lumen. However, the possibility that adenomas grow inward is counterintuitive to the paradigm that, in normal crypts, SCs reside at the crypt base and offspring cells migrate toward the crypt lumen.[1] Moreover, the findings that support this possibility must be viewed in the context that the epithelium at the base of adenomatous crypts resembles the germinative epithelium at the base of normal crypts[33,34] and, while adenomatous crypts have more apoptotic cells at the crypt base, a few apoptotic cells are also found in the bottom of normal crypts.[35,36] If there truly were inward growth, the hierarchy of differentiation should also be reversed, and one would expect histological evidence of differentiated or at least partially differentiated cell populations at the crypt base (contrary to the germinative phenotype). Other studies provide evidence against an inward growth mechanism.[24,37–39]

We found overlap between Ki-67 staining and P27^{kip1} staining in the lower third of the adenomatous crypt. We relate our observed overlap to a specific mechanism, SC overpopulation. That is, the overlap is consistent with the cells in this region being SCs. SCs, as stated above, are proliferative (consistent with Ki-67 staining) yet relatively quiescent (consistent with P27^{kip1} staining). A cell that stains for p27 but not Ki-67 would be consistent with a terminally differentiated cell.

It has been proposed that both p27^{kip1} as well as TGF-β are involved in at least two different physiological processes that are related to their ability to inhibit cell proliferation. First, they both have been reported to arrest cells in the G_1/S phase before the cells undergo cellular differentiation.[1,22,31,40–42] This is in agreement with our finding that p27^{kip1}-positive cells are located at the normal crypt top and lumenal surface where differentiated cells reside. Second, both p27^{kip1} and TGF-β are involved in other processes in which cell growth is downregulated in the absence of cellular differentiation.[43,44] This is consonant with the presence of p27^{kip1} and TGF-β at the adenomatous crypt bottom where these proteins may maintain these cells in a quiescent state, which is consistent with our hypothesis that the adenomatous crypt is overpopulated with mutant SCs and the known SC property of extremely slow proliferation. Indeed, it has been reported that TGF-β slows the rate of proliferation of cells in the lower crypt, including SCs.[45] Protracted *in vivo* administration of TGF-β has been shown to reduce crypt cell proliferation and possibly alter SC cycling.[45] It has been hypothesized that TGF-β may regulate the output of offspring cells from SCs in the crypt bottom.[46] This role for TGF-β has also been proposed for hematopoietic SCs.[47] Thus, TGF-β-mediated signal transduction may slow the rate of proliferation of cells at the crypt bottom, including SCs, via inhibition by p27^{kip1} of the cell cycle.

Our findings are also consistent with previous data on staining for these markers in sporadic carcinomas. Because mutation in the *APC* gene not only promotes development of adenomas in FAP patients, but also does so for most cases of sporadic CRC, we compared our results to results reported for similar immunohistochemical marker studies on sporadic human colorectal carcinomas. Like FAP adenomas, in the development of sporadic colon cancers, a mutation in the second *APC* allele usually occurs by the adenomatous polyp stage, and complete inactivation of wild-type *APC* occurs in the majority of colorectal carcinomas.[48] In sporadic CRC[6,7,9] as in our FAP adenomatous crypts, there was an increase in the proportion of total neoplastic epithelial cells staining for Bcl-2, survivin, and Ki-67 compared to normal colonic mucosa. Colon carcinomas are reported to contain fewer cells expressing p21$^{WAF1/C1P1}$ and p27^{kip1} than normal epithelium,[13,14,49] and we observed only a slight increase in the proportion of p21$^{WAF1/C1P1}$- and p27^{kip1}-stained cells in FAP adenomas. Overall, our interpretation of the immunohistochemical studies on sporadic colon carcinomas is that they suggest that during colon carcinogenesis there is an increase in the number of cells that express the crypt base cell phenotype. These data for sporadic CRC are consistent with our findings for FAP adenomas, and further support our hypothesis that SC overproduction underlies CRC initiation and promotion including the upward proliferative shift in early colon tumorigenesis and adenoma formation.

If SC overpopulation is important to understanding colon tumorigenesis, then it would be valuable to investigate SC mechanisms that are involved, because such mechanisms might provide a basis for new approaches to colon cancer treatment and chemoprevention. For example, we and others recently reported that APC down-regulates survivin expression via the β-catenin/Tcf4 pathway.[10,50] In our study,[10] in the current study, and in another study,[9] it was shown that survivin is preferentially expressed in the lower crypt in normals, which means that survivin expression (higher toward the crypt bottom) inversely correlates with the expression pattern (intracryptal gradient) of wild-type APC (higher toward the crypt top).[51,52] Thus, the wild-type APC gradient, by progressively decreasing survivin and increasing apoptosis from crypt bottom to top, would limit the population size of SCs and other proliferative cells and restrict them to the lower crypt. This may be a critical mechanism through which *APC* mutation leads to SC overpopulation and contributes to colon tumorigenesis.

Survivin also enhances the activity of aurora B kinase, a protein that catalyzes chromosome segregation and cytokinesis during mitosis.[53,54] Hence, intracellular survivin not only prevents apoptosis, but also promotes cell division in proportion to the amount of survivin that binds to and activates aurora B kinase. Thus, as the APC gradient increases upwards along the crypt axis, survivin will be increasingly down-regulated, which will increasingly induce apoptosis, and inhibit mitosis, and which will progressively decrease the likelihood that the cell is a proliferative cell. Consequently, it would be predicted that the high concentrations of survivin at the crypt bottom would help maintain cells there in a proliferative state. We hypothesized that up-regulation of survivin expression occurs when *APC* is mutant, and that this mechanism helps promote colon cancer development.[55] Our current staining data do indeed indicate that the number of survivin-positive cells correlates with the predicted number of *APC* mutations in human colon: none in controls, one in normal-appearing FAP, two in adenomatous FAP mucosa. This data, coupled with survivin's known role in activating aurora B kinase, is also consistent with the fact that normal-appearing FAP and adenomatous crypts have a greater number of mitotic figures.[37,56]

Our data provide an alternative explanation to the unresolved issue in colon carcinogenesis as to whether the morphogenesis of colorectal adenomas proceeds from the top of the crypt down (top down; inward growth)[17,18,22,23,57] or from the bottom of the crypt up (bottom up; outward growth).[24,34,37–39] We believe that our data on the staining of normal, FAP, and adenomatous FAP crypts are most readily explained by another mechanism, namely, SC overpopulation. This view takes into account the above-noted distinction between proliferating and proliferative cells. The expansion of the population of proliferative cells (those staining for Ki-67 and topoisomerase II) both in number and in area (into the upper crypt in adenomatous FAP crypts) can be most simply viewed as a sequel to the shift in cell number and distribution found in normal-appearing FAP crypts in which the population of proliferative cells has, compared to normal control crypts, expanded in both cell number and in distribution from the lower crypt toward the crypt middle.[58] In both cases, the driving force is, in our view, SC overpopulation.

During the conduct of the work presented herein, the Musashi protein was reported to be a putative marker for colonic SCs.[59] We therefore did immunohistochemistry on normal colonic epithelium using commercially available polyclonal antibodies (Chemicon Int., Temecula, CA) to the Musashi protein. We observed immunostaining of cells at the crypt base, but staining of an equal number of cells was also present in the upper crypt and luminal surface (not shown). Based on these results, and the evidence[60] that intestinal SCs in normal epithelium are located in the crypt base, we were unable to conclude that Musashi staining (at least with the available polyclonal antibody) is a specific marker for SCs in the human colonic crypt. It has also been reported that Musashi staining of human colonic crypts shows weaker and less reproducible staining than do mouse crypts.[61]

In summary, our view is that adenoma morphogenesis in patients with FAP depends on increasing SC population size. Studying changes in molecular and cellular processes involved in regulating crypt SC population size may be an important prerequisite to the understanding of histological changes during adenoma progression.

Acknowledgments

We thank Gretchen Matika, MS; Debra Rose, MS; Emin G. Kandemir, MD; Robert Fry, MD; Burt Cajir, MD; and Jan Rakinic, MD, for their assistance on this project.

References

1. Bach SP, Renehan AG, Potten CS: Stem cells: the intestinal stem cell as a paradigm. Carcinogenesis 2000, 21:469–476
2. Potter JD: Colorectal cancer: molecules and populations. J Natl Cancer Inst 1999, 91:916–932
3. Moser AR, Dove WF, Roth KA, Gordon JI: The Min (multiple intestinal neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. J Cell Biol 1992, 116:1517–1526
4. Pierce GB, Stevens LC, Nakane PK: Ultrastructural comparison of differentiation of stem cells of adenocarcinomas of colon and breast with their normal counterparts. J Natl Cancer Inst 1967, 39:755–773
5. Boman BM, Fields JZ, Bonham-Carter O, Runquist OA: Computer modeling implicates stem cell overproduction in colon cancer initiation. Cancer Res 2001, 61:8408–8411
6. Palazzo JP, Kafka NJ, Grasso L, Chakrani F, Hanau C, Cuesta KH, Mercer WE: The role of p53, p21WAF1/C1P1, and bcl-2 in radioresistant colorectal carcinoma. Hum Pathol 1997, 28:1189–1195
7. Sinicrope FA, Hart J, Michelassi F, Lee JJ: Prognostic value of bcl-2 oncoprotein expression in stage II colon carcinoma. Clin Cancer Res 1995, 1:1103–1110
8. Thibodeau SN, French AJ, Roche PC, Cunningham JM, Tester DJ, Lindor NM, Moslein G, Baker SM, Liskay RM, Burgart LJ, Honchel R, Halling KC: Altered expression of hMSH2 and hMLH1 in tumors with microsatellite instability and gene alterations in mismatch repair genes. Cancer Res 1996, 56:4836–4840
9. Gianani R, Jarboe E, Frost M, Bobak J, Lehner R, Shroyer KR: Expression of survivin in normal, hyperplastic, and neoplastic colonic mucosa. Hum Pathol 2001, 32:119–125
10. Zhang T, Otevrel T, Gao ZQ, Gao ZP, 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, 61:8664–8667
11. Edmonston TB, Cuesta KH, Burkholder S, Barusevicius A, Rose D, Kovatich AJ, Boman B, Fry R, Fishel R, Palazzo JP: Colorectal carcinomas with high microsatellite instability: defining a distinct immunologic and molecular entity with respect to prognostic markers. Hum Pathol 2000, 31:1506–1514
12. Fredersdorf S, Burns J, Milne AM, Packham G, Fallis L, Gillett CE, Royds JA, Peston D, Hall PA, Hanby AM, Barnes DM, Shousha S, O'Hare MJ, Lu X: High levels of p27kip1 and cyclin D1 in some breast cancer cells: inverse correlation between the expression of p27kip1 and degree of malignancy in human breast and colorectal cancers. Proc Natl Acad Sci USA 1997, 94:6380–6385
13. Sinicrope FA, Roddey G, Lemoine M, Ruan S, Stephens LC, Frazier ML, Shen Y, Zhang W: Loss of $p21^{WAF1/Cip1}$ protein expression accompanies progression of sporadic colorectal neoplasms but not hereditary nonpolyposis colorectal cancers. Clin Cancer Res 1998, 4:1251–1261
14. Viale G, Pellegrini C, Mazzarol G, Maisonneuve P, Silverman ML, Bosari S: $p21^{WAF/CIP!}$ expression in colorectal carcinoma correlates with advanced disease stage and p53 mutations. J Pathol 1999, 187:302–307
15. Sherley JL: Asymmetric cell kinetics genes: the key to expansion of adult stem cells in culture. Stem Cells 2002, 20:561–572
16. Deschner E, Lewis CM, Lipkin M: In vitro study of human rectal epithelial cells. 1. Atypical zone of H^3 thymidine incorporation in mucosa of multiple polyposis. J Clin Invest 1963, 42:1922–1928
17. Cole JW, McKalen A: Studies on the morphogenesis of adenomatous polyps in the human colon. Cancer 1963, 16:998–1002
18. Maskens AP: Histogenesis of adenomatous polyps in the human large intestine. Gastroenterology 1979, 77:1245–1251
19. Polyak K, Hamilton SR, Vogelstein B, Kinzler KW: Early alteration of cell-cycle-regulated gene expression in colorectal neoplasia. Am J Pathol 1996, 149:381–387
20. Fogt F, Poremba C, Shibao K, Itoh H, Kohno K, Zimmerman RL, Gortz HG, Dockhorn-Dworniczak B, Urbanski SJ, Alsaigh N, Heinz D, Noffsinger AE, Shroyer KR: Expression of survivin, YB-1, and Ki-67 in sporadic adenomas and dysplasia-associated lesions or masses in ulcerative colitis. Appl Immunohistochem Mol Morphol 2001, 9:143–149
21. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA: "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 2002, 298:597–600
22. Moss SF, Liu TC, Petrotos A, Hsu TM, Gold LI, Holt PR: Inward growth of colonic adenomatous polyps. Gastroenterology 1996, 111:1425–1432
23. Shih I-M, Wang T-L, Traverso G, Romans K, Hamilton SR, Ben-Sasson S, Kinzler KW, Vogelstein B: Top-down morphogenesis of colorectal tumors. Proc Natl Acad Sci USA 2001, 98:2640–2645
24. Preston SL, Wong WM, Chan AOO, Poulsom R, Jeffery R, Goodlad RA, Mandir N, Elia G, Novelli M, Bodmer WF, Tomlinson IP, Wright NA: Bottom-up histogenesis of colorectal adenomas: origin in the monocryptal adenoma and initial expansion by crypt fission. Cancer Res 2003, 63:3819–3825
25. Scholzen T, Gerdes J: The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000, 182:311–322
26. Riso M: Cell proliferation in colorectal tumor progression: an immunohistochemical approach to intermediate biomarkers. J Cell Biochem Suppl 1992, 16G:79–87
27. Potten CS, Owen G, Booth D: Intestinal stem cells protect their genome by selective segregation of template DNA strands. J Cell Sci 2002, 115:2381–2388
28. Cheshier SH, Morrison SJ, Liao X, Weissman IL: In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci USA 1999, 96:3120–3125
29. D'Urso G, Datta S. Cell cycle control, checkpoints and stem cell biology. Stem Cell Biology. Edited by DR Marshak, RL Gardner, D Gottlieb. Cold Spring Harbor, Cold Spring Harbor Press, 2001, pp 61–94
30. Kim SJ, Cheung S, Hellerstein MK: Isolation of nuclei from label-retaining cells and measurement of their turnover rates in rat colon. Am J Physiol 2004, 286:C1464–C1473
31. Ciarallo S, Subramaniam V, Hung W, Lee JH, Kotchetkov R, Sandhu C, Milic A, Slingerland JM: Altered $p27^{Kip1}$ phosphorylation, localization and function in human epithelial cells resistant to transforming growth factor β-mediated G_1 arrest. Mol Cell Biol 2002, 22:2993–3002
32. Lightdale C, Lipkin M, Deschner E: In vivo measurements in familial polyposis: kinetics and location of proliferating cells in colonic adenomas. Cancer Res 1982, 42:4280–4283
33. Fenoglio-Presier CM, Noffsinger AE, Stemmermann GN, Lantz PE, Listrom MB, Rilke FO: Gastrointestinal Pathology. Lippincott-Raven, Philadelphia, 1999, pp 918–921
34. Lane N, Lev R: Observations on the origin of adenomatous epithelium of the colon. Cancer 1963, 16:751–764
35. Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, Zehnbauer BA, Hamilton SR, Jones RJ: Inhibition of apoptosis during development of colorectal cancer. Cancer Res 1995, 55:1811–1816
36. Strater J, Koretz K, Gunthert AR, Moller P: In situ detection of enterocyte apoptosis in normal colonic mucosa, and in familial adenomatous polyposis. Gut 1995, 37:819–825
37. Wong W-M, Mandir N, Goodlad RA, Wong BCY, Garcia SB, Lam S-K, Wright NA: Histogenesis of human colorectal adenomas and hyperplastic polyps: the role of cell proliferation and crypt fission. Gut 2002, 50:212–217
38. Wiebecke B, Brandts A, Eder M: Epithelial proliferation and morphogenesis of hyperplastic adenomatous and villous polyps of the human colon. Virchows Arch A Pathol Anat Histol 1974, 364:35–49
39. Nakamura S-I, Kino I: Morphogenesis of minute adenomas in familial polyposis coli. J Natl Cancer Inst 1984, 73:41–49
40. Howe PH, Draetta G, Loef EB: Transforming growth factor β1 inhibition of p34/CDC2 phosphorylation and histone H1 activity is associated with G_1/S-phase growth arrest. Mol Cell Biol 1991, 11:1185–1194
41. Quaroni A, Tian JQ, Seth P, Ap Rhys C: p27(Kip1) is an inducer of intestinal epithelial differentiation. Am J Physiol 2000, 279:C1045–C1057
42. Kurokowa M, Lynch K, Podolsky DK: Effects of growth factors on an intestinal epithelial cell line: transforming growth factor beta inhibits proliferation and stimulates differentiation. Biochem Biophys Res Commun 1987, 142:775–782
43. Hu X, Zuckerman KS: Transforming growth factor: signal transduction pathways, cell cycle mediation and effects on hematopoiesis. J Hematother Stem Cell Res 2001, 10:67–74
44. Tamir A, Petrocelli T, Stetler K, Chu W, Howard J, Croix BS, Slingerland J, Ben-David Y: Stem cell factor inhibits erythroid differentiation by modulating the activity of G_1-cyclin-dependent kinase complexes: a role for p27 in erythroid differentiation coupled G1 arrest. Cell Growth Differ 2000, 11:269–277
45. Potten CS, Owen G, Hewitt D, Chadwick CA, Hendry H, Lord BI, Woolford LB: Stimulation and inhibition of proliferation in the small intestine crypts of the mouse after in vivo administration of growth factors. Gut 1995, 36:864–873
46. Koyama SY, Podolsky DK: Differential expression of transforming growth factors alpha and beta in rat intestinal epithelial cells. J Clin Invest 1989, 83:1768–1773
47. Strife A, Lambek C, Perez A, Darzynkiewicz Z, Skierski J, Gulati S, Haley JD, ten Dijke P, Iwata KK, Clarkson BD: The effects of transforming growth factor beta 3 on the growth of highly enriched hematopoietic progenitor cells derived from normal human bone marrow and peripheral blood. Cancer Res 1991, 51:4828–4836
48. 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, 54:5953–5958
49. Palmqvist R, Stenling R, Oberg A, Landberg G: Prognostic significance of $p27^{Kip1}$ expression in colorectal cancer: a clinico-pathological characterization. J Pathol 1995, 1888:18–23
50. Kim PJ, Plescia J, Clevers H, Fearon ER, Altieri DC: Survivin and molecular pathogenesis of colorectal cancer. Lancet 2003, 362:205–209
51. Miyashiro I, Senda T, Matsumine A, Baeg G, Kuroda T, Shimano T, Miura S, Noda T, Kobayashi S, Monden V, Toyoshima K, Akiyama T: Subcellular localization of the APC protein: immunoelectron microscopic study of the association of the APC protein with catenin. Oncogene 1995, 11:89–96
52. Smith KJ, Johnson KA, Bryan TM, Hill DE, Markowitz S, Willson JKV, Paraskeva C, Petersen GM, Hamilton S, Vogelstein B, Kinzler KW: The APC gene product in normal and tumor cells. Proc Natl Acad Sci USA 1993, 90:2846–2850

53. Bolton MA, Lan W, Powers SE, McCleland ML, Kuang J, Stukenberg PT: Aurora B kinase exists in a complex with survivin and INCENP and its kinase activity is stimulated by survivin binding and phosphorylation. Mol Biol Cell 2002, 13:3064–3077
54. Chen J, Jin S, Tahir SK, Zhang H, Liu X, Sarthy AV, McGonigal TP, Liu Z, Rosenberg SH, Ng SC: Survivin enhances aurora B kinase activity and localizes aurora B in human cells. J Biol Chem 2003, 278:486–490
55. Boman BM, Zhang T, Fields JZ: Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res 2004, 64:776–779
56. Mills SJ, Mathers JC, Chapman PD, Burn J, Gunn A: Colonic crypt cell proliferation state assessed by whole crypt microdissection in sporadic neoplasia and familial adenomatous polyposis. Gut 2001, 48: 41–46
57. Shiff SJ, Rigas B: Colon adenomatous polyps—do they grow inward? Lancet 1997, 349:1853–1854
58. Potten CS, Kellett M, Rew DA, Roberts SA: Proliferation in human gastrointestinal epithelium using bromodeoxyuridine in vivo: data for different sites, proximity to a tumor, and polyposis. Gut 1992, 33:524–529
59. Nishimura S, Wakabayashi N, Toyoda K, Kashima K, Mitsufuji S: Expression of Musashi-1 in human normal colon crypt cells. Dig Dis Sci 2003, 48:1523–1529
60. Potten CS: Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Phil Trans R Soc Lond B 1998, 353:821–830
61. Potten CS, Booth C, Tudor GL, Booth D, Brady G, Hurley P, Ashton G, Clarke R, Sakakibara S, Okamo H: Identification of a putative intestinal stem cell and early lineage marker; Musashi-1. Differentiation 2003, 71:28–41