Stem Cell Reviews

ISSN 1550-8943 / 05 / 1:233-241 / $30.00

Original Article

Gastrointestinal Stem Cells and Cancer—Bridging the Molecular Gap

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Abstract

Cancer is believed to be a disease involving stem cells. The digestive tract has a very high cancer prevalence partly owing to rapid epithelial cell turnover and exposure to dietary toxins. Work on the hereditary cancer syndromes including famalial adenomatous polyposis (FAP) has led to significant advances, including the adenoma-carcinoma sequence. The initial mutation involved in this stepwise progression is in the "gatekeeper" tumor suppressor gene adenomatous polyposis coli (*APC*). In FAP somatic, second hits in this gene are nonrandom events, selected for by the position of the germline mutation. Extensive work in both the mouse and human has shown that crypts are clonal units and mutated stem cells may develop a selective advantage, eventually forming a clonal crypt population by a process called "niche succession." Aberrant crypt foci are then formed by the longitudinal division of crypts into two daughter units—crypt fission. The early growth of adenomas is contentious with two main theories, the "top-down" and "bottom-up" hypotheses, attempting to explain the spread of dysplastic tissue in the bowel. Initial X chromosome inactivation studies suggested that colorectal tumors were monoclonal; however, work on a rare XO/XY human patient with FAP and chimeric Min mice showed that 76% of adenomas were polyclonal. A reduction in tumor multiplicity in the chimeric mouse model has been achieved by the introduction of a homozygous tumor resistance allele. This model has been used to suggest that short-range interaction between adjacent initiated crypts, not random polyp collision, is responsible for tumor polyclonality.

Index Entries: *APC*; stem cells; clonality; niche succession; crypt fission; top down; bottom up.

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Introduction

The cells that line the gastrointestinal tract are among the most rapidly proliferating cells in the body with differentiated cells undergoing continual replacement. They are also exposed to a hostile environment as they come into close contact with numerous toxins and carcinogens contained in digested food. Thus it is of little surprise that cancer of the digestive system is common, with 255,640 new cases in the US alone in 2004 *(1)*. The gastrointestinal epithelium is an important tissue in the understanding of cancer biology partly owing to its rapid cell turnover and high cancer prevalence. Colonic polyposis syndromes were first recognized 200 yr ago, and it has been 100 yr since inflammatory and adenomatous polyps were characterized *(2)*. The observation of familial cancer syndromes led to the establishment of polyposis registries, with one of the largest starting at St. Marks Hospital in London in 1925. Work on the familial colonic cancer syndromes including familial adenomatous polyposis (FAP) has led to a number of advances in the

understanding of intestinal tumor initiation including the recognition that many colonic adenocarcinomas arise from adenomas *(3)*. The adenoma-carcinoma sequence has subsequently become established as a stepwise pattern of mutational activation of oncogenes and inactivation of tumor suppressor genes that result in cancer *(4)*. Malignant cells share a number of characteristics with stem cells, such as the ability to self-replicate and proliferate, and it is widely believed that the gastrointestinal stem cell is the target of the mutational changes. This review will summarize the molecular and cellular events involving the stem cell, which occur at the birth of the adenoma, the spread of dysplastic tissue around the bowel, and the development of malignancy.

Gastrointestinal Stem Cells

The immature, relatively undifferentiated nature of gastrointestinal epithelial stem cells means that they are not directly identifiable and researchers in this field in the past have had to rely on ingenious indirect methods to identify their position and track their progeny *(5)*. Recent work on molecules uniquely involved in the biochemical pathways of the stem cell may provide useful tools for cell identification. One such protein is Mushashi-1, the mammalian equivalent of a Drosophila protein. It is responsible for the upregulation of expression of the transcriptional repressor Hes-1, a protein involved in neural stem cell self-renewal *(6)*. Both these proteins are coexpressed in cells superior to the Paneth cells in the mouse intestine, but Hes-1 alone is only seen in differentiating cells, thus it is hypothesized that the colocalization of these two markers may denote the small bowel stem cell population *(7)*. The putative stem cell compartment position varies according to the location in the digestive tract. In the stomach, the epithelial lining is formed into long tubular glands, each subdivided into foveolus, isthmus, neck, and base regions. The foveolae and mucosal surface is made up of gastric foveolar mucous cells. The acid-secreting parietal (oxyntic) cells and the pepsinogen-secreting peptic/chief (zymogenic) cells are found in the base of the gastric glands, and in the body and the fundus/body of the stomach, respectively *(8)*. Within the gastric glands cell migration is bidirectional, with the differentiating mucous cells migrating upward and the developing parietal and chief cells moving down toward the gland base. The putative stem cell compartment is thus believed to lie in the neck/isthmus region of the gastric gland. Throughout the small and large intestine the luminal surface is composed of a columnar epithelial mucosa, with glandular invaginations called crypts. Several of these crypts contribute epithelium to finger-like projections called villi in the small bowel. The cells of the intestinal epithelium are arranged hierarchically, becoming progressively more differentiated as they age and pass along the crypt-to-villus axis. The stem cell compartment is believed to be at the origin of this axis, the base of the colonic crypt, and at cell position 4–5 in the small bowel (reviewed in ref. *9*). The number of stem cells within this compartment is debated but is generally believed to be between 4 and 6 *(10,11)*. Stem cells themselves divide infrequently and it is the first few generations of stem cell daughters, known as transit amplifying cells, which proliferate in the lower part of the crypt *(12)*. Stem cells reside within a stem cell compartment or "niche." This is a group of epithelial and mesenchymal cells and extracellular substrates, which provide an optimal microenvironment for stem cells to give rise to their differentiated progeny. In the intestinal crypts this is formed by a fenestrated sheath of surrounding mesenchymal cells that regulate stem cell behavior through paracrine secretion of growth factors and cytokines *(13)*. Functionally, a niche is characterized by its persistence on removal of stem cells and the cessation of stem cell potential when cells are removed from this niche *(14)*. The rapid turnover of the gastrointestinal epithelium means that differentiating cells are shed into the lumen and replaced every few days, thus do not have a sufficient life-span to gather the multiple genetic defects required for malignant transformation. Therefore, the perpetual stem cell has long been considered the target of carcinogenic mutations *(12,15,16)*.

Genetic Pathways Leading to Tumorigenesis

Numerous steps are involved in the progression of normal tissue from dysplasia to malignancy and it is estimated that a typical colorectal tumor contains at least 11,000 genomic alterations *(17)*. Some tumor suppressor genes such as *p53* and *DCC* are predominantly mutated in carcinomas rather than early adenomas, suggesting a late role in the transition from adenoma to carcinoma (reviewed in ref. *18*). Other genes such as *SMAD2* and *SMAD4* are seen in 50% of adenomas as well, and their role in tumorigenesis is unclear *(19)*. Mutations also occur in proto-oncogenes, such as *K-RAS*, which are seen in 50% of large adenomas and tumors; however, similar changes are also seen in nondysplastic lesions such as hyperplastic polyps *(20)*. Based on the observation that the accumulation of molecular alterations seemed to parallel the clinical progression of tumors, Vogelstein et al. *(4)* proposed a stepwise model of colorectal tumorigenesis. The molecular pathogenesis of FAP has shed much light on the initial mutations required in this step, like progression. FAP results in the formation of multiple bowel adenomas in the second and third decades of life. Colonic cancer is inevitable in these patients who therefore require prophylactic colectomy. The heritable nature of FAP was first recognized at the end of the nineteenth century, however, not until 1986 was observed an interstitial deletion of chromosome 5q in an FAP patient *(21)*. This prompted linkage analysis studies which codemonstrated tight linkage of the condition to markers on chromosome 5q21 *(22,23)*. The gene responsible was adenomatous polyposis coli (*APC*) *(24,25)* which encodes a large (approx 2800 amino acids) multifunctional cytoplasmic protein *(26)*. This important protein binds and downregulates β-catenin and is vital in the regulation of Wnt signaling, as well as maintenance of apoptosis, cell-cycle progression, and chromosomal stability (reviewed in refs. *27–30*). Subsequent work revealed that mutations in *APC* are also found in 63% of sporadic adenomas *(31)* and up to 80% of sporadic colorectal cancers *(32)*. This led Kinzler and Vogelstein to propose that *APC* acts as a "gatekeeper" gene—a gene involved in the control of normal epithelial cell proliferation required for cellular homeostasis. Mutation of a gatekeeper gene results in an imbalance of cell division over death, thus FAP is a disease of accelerated tumor initiation *(33)*. Other hereditary bowel cancer syndromes have been used in the identification of alternative pathogenetic mechanisms. Hereditary nonpolyposis coli is a condition that predisposes to cancers of the colon, endometrium, and several other extracolonic sites, notably

without prior polyp formation (reviewed in ref. *34*). The use of microsatellite markers to try to identify allelic losses in this syndrome led to the discovery of multiple new di- and trinucleotide repeats throughout the genome. This was termed microsatellite instability and is explained by defects in DNA mismatch repair (MMR) genes, which normally recognize and repair single base and larger strand slippage mismatches in DNA replication. The mutated MMR genes in the majority of HNPCC are *hMSH2*, *hMLH1*, and *hPMS2* (reviewed in ref. *33*). This led to the proposal of MMR genes as "caretaker" genes and that HNPCC is a disease of tumor progression, with impaired DNA repair accelerating the process. The observation that the median age of cancer development in both HNPCC and FAP is 42 demonstrates the importance of both gatekeeper and caretaker gene functions *(33)*. Gatekeeper gene mutations, such as *APC*, are present in very early adenomas *(31)* and are sufficient to promote small adenoma growth in the absence of microsatellite instability, K-ras, or β-catenin mutation or allelic loss of 1p *(35)*. Thus, *APC* inactivation provides a stem cell with a selective growth advantage by allowing unregulated activation of Wnt signaling. Mutations in β-catenin, preventing its breakdown, can also promote adenoma initiation, however, small adenomas with β-catenin mutations alone do not progress to larger adenomas or carcinomas as frequently as adenomas with *APC* mutations *(36)*. Therefore, although *APC*'s role in the regulation of Wnt signaling is the most important in prevention of tumor initiation, its involvement in apoptosis and chromosomal stability also has an effect on the progression of the adenoma growth (reviewed in ref. *19*).

APC Mutations

Knudson's two-hit hypothesis postulates that inactivation of a tumor suppressor gene occurs only after two independent mutation events, and certainly biallelic mutation of *APC* can be detected in early intestinal tumors *(31)*. In FAP, initial germ-line mutations are frequently small-scale truncating mutations. Second-hit, somatic mutations may be point mutations, which commonly cluster in a region between codons 1286 and 1513—termed the mutation cluster region (MCR) *(32)*, or large-scale genetic changes that are collectively termed allelic loss or loss of heterozygosity *(37)*. Careful mutation analysis of multiple polyps from FAP patients showed that the position and type of the somatic hit in the *APC* gene depended on the position of the germ-line mutation. Lamlum et al. studied 210 early polyps from 35 different patients from 26 FAP families. They found loss of heterozygosity as the cause of the somatic mutation in 20% of adenomas and this was strongly associated with cases whose germ-line mutations were around codon 1300. Patients with germ-line mutations away from this region tended to acquire their second hits by point mutation within the MCR. *(38)*. A strong genotype-phenotype correlation was noted with more severe polyposis associated with patients whose germ-line mutations were between codons 450 and 1600, especially those with mutations around codon 1300, who had thousands of colonic polyps. Patients with mutations at the 3′or 5′ end of the gene had less severe diseases with tens of polyps and later age of onset *(39,40)*. It appears that different *APC* mutations provide a cell with different selective advantages, and the somatic mutations are nonrandom events that are selected for on the growth advantage they confer to the tumor cell. The greatest growth advantage is conferred by germ-line mutation around codon 1300, which then obtains a second hit by allelic loss, an event that occurs at a high spontaneous frequency. Patients with germ-line mutations that provide less growth advantage to the cell need to acquire second or even third hits, often in the MCR to compensate for the weaker selective advantage provided by the first mutation *(38)*. Based on this work Albuquerque et al. and Crabtree et al. were able to define the "just-right" and "loose-fit" models to explain these findings. These models suggest that for a cell to acquire a selective advantage the function of *APC* must be impaired to a specific degree to allow sufficient nuclear accumulation of β-catenin promoting excess proliferation, but not so much as to cause cell apoptosis. The regulation of β-catenin by APC is achieved by three 15 amino acid and seven 20 amino acid repeats that respectively bind and downregulate β-catenin. The number of amino acid repeats left in an abnormal *APC* protein depends on the position of the gene mutation, with an early mutation leaving fewer repeats. As an optimal just-right level of β-catenin activity is required for tumor cell growth advantage, the somatic mutation is selected for by the number of amino acid repeats it will code for usually resulting in one or two residual β-catenin-binding amino acid repeats (*see* Fig. 1) *(41)*. The loose-fit model adapts this slightly to show that some variation in *APC* is tolerable *(42)*.

Crypts as Clonal Populations

The importance of the APC protein in a number of different cell-regulatory functions means that mutations in the *APC* gene alone are sufficient to provide a cell with a selective growth advantage *(35)*. To determine the next steps in the pathway from stem cell genetic mutations to the smallest recognizable adenomas, it is important to understand the organization and cell dynamics of the basic functional unit of the gut—the crypt. The clonality of the gastrointestinal crypt has been extensively studied in both the mouse and the human.

Mouse

In the mouse, clonality experiments frequently involve the formation of aggregation chimeras and/or the induction of somatic mutations in crypt stem cells. Studying the contribution to the crypt structure of the different cell populations in the chimera, or the cells bearing a new induced somatic mutation helps determine the cell lineages of the crypt occupants. The chimeric B6/SWR mouse is an aggregation chimera where the two populations of cells can be simply distinguished. The lectin dolichos biflorus agglutinin (DBA) binds to sites on the B6-derived but not the SWR-derived cells in B6/SWR chimeras. Staining for DBA binding thus segregates cells from the different strains. In neonatal B6/SWR chimeras, the intestinal crypts exhibit mixed staining patterns for the first 2 wk of life *(43)*. After this time and in the adult mouse, the crypts are either positive or negative for DBA staining, with no mixed crypts in the many thousands studied *(44)*. This suggests that during development multiple stem cells are present in the crypt, but as neonatal development progresses the crypts undergo a process of monoclonal conversion, resulting in a clonal population of cells where all cell lineages are derived from a single parental strain. Chemical mutagenesis has been used in other chimeric mice to demonstrate the dynamics of

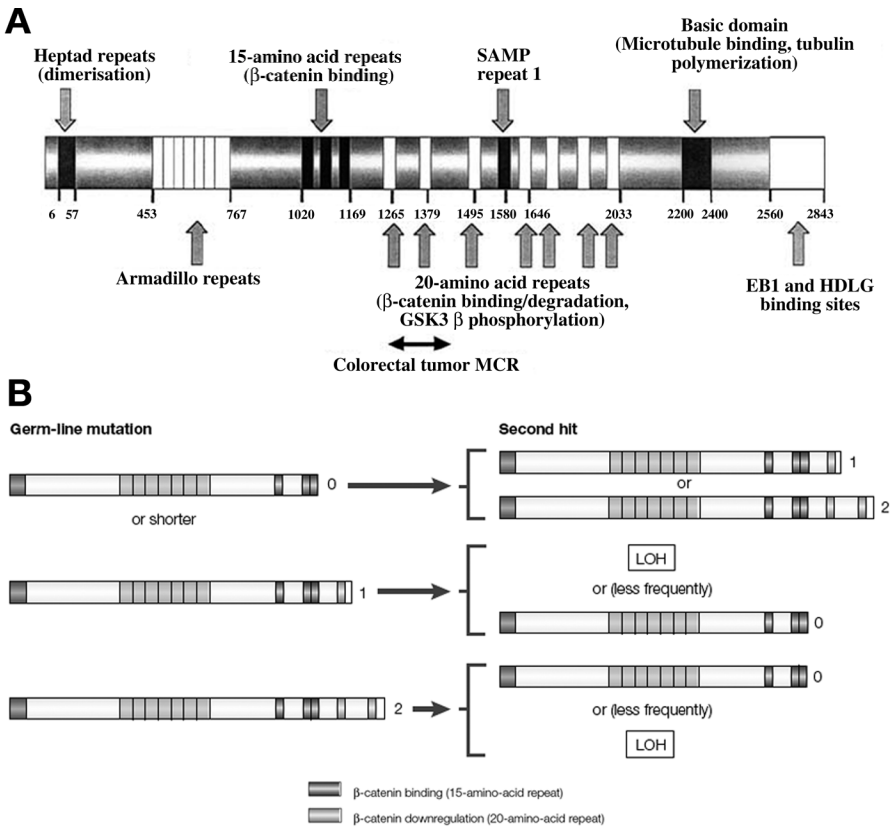

Fig.1. **(A)** The adenomatous polyposis protein showing major functional domains by amino acid position. (Reprinted with permission from ref. *42*.) **(B)** Interdependence of germ-line and somatic APC mutations according to the just-right hypothesis. The germ-line and somatic mutations are represented by the truncated protein they produce. Numbers to the right of the protein are the remaining number of 20 amino-acid repeats after protein truncation. Germ-line mutations that result in a protein lacking all amino acid repeats tend to acquire somatic mutations that result in a protein that retains 1 or 2 repeats. When the germ-line mutation results in a protein containing 1 amino acid repeat, the second hit tends to be allelic loss, or more rarely, point mutations resulting in short proteins. When the germ-line hit leaves a protein with two residual repeats then somatic mutations causing allelic loss or short proteins are selected for. In all cases the combination of germ-line and somatic mutations results in a truncated protein that retains one or two amino acid repeats. LOH:loss of heterozygosity.(Reprinted with permission from ref.19.)

adult crypt populations. C57BL/6J/SWR mice show heterozygous expression at the DBA binding site so that in the nonmutated state crypt cells stain positive for bound agglutinin. Spontaneous or ethyl nitrosurea (ENU)-induced mutation at the Dlb-1 locus on chromosome 11 abolishes the DBA binding site, and mutated cells are then distinguished by the loss of ability to bind DBA. After ENU treatment, crypts emerge, which are partially and then completely negative for DBA staining, suggesting mutation of a stem cell, which forms a clone that expands stochastically. Eventually, the mutated progeny populate the entire crypt *(45)*. Bjerknes et al. used a knock-in model to study cell dynamics and fate. SWR mice, normally negative for DBA binding, can be induced to bind the agglutinin by ENU treatment. By observing the morphology, location, and longevity of the mutant clones in the mouse small intestine they suggest that stem cell division gives rise to committed epithelial progenitor cells—the columnar cell progenitor, the mucus cell progenitor, and to a lesser extent, the mixed progenitor. These cells undergo further transit-amplifying divisions but are committed to differentiation down their cell line *(11)*.

In the stomach the situation is similar. The demonstration of either Y chromosome-positive or -negative gastric glands in XX-XY chimeras *(43,46)* and the CH3 or Balb/c origin of glands in CH3:BALBc chimeras *(47)* demonstrates the clonality of gastric crypts. In the developing mouse stomach, glands are initially polyclonal, but in a fashion similar to the intestine they undergo purification to form clonal crypts. However, unlike the intestine, a small population of gastric glands (5–10%) remains polyclonal into adulthood *(48)*.

Human

Studies in the human rely on natural mutations and polymorphisms. One such mutation is in the gene coding for the enzyme O-acetyl transferase (OAT). This enzyme is responsible for the O-acetylation of sialic acid in goblet cell mucus. Non-O-acetylated mucus will stain positively with mild periodic acid–Schiff reagent (mPAS). Approximately 9% of the Caucasian population has a homozygous mutation ($OAT^{-/-}$), resulting in mPAS staining of goblet cells carrying the mutation, allowing their identification; 42% of the population are heterozygotes ($OAT^{+/-}$) and will not stain with mPAS unless

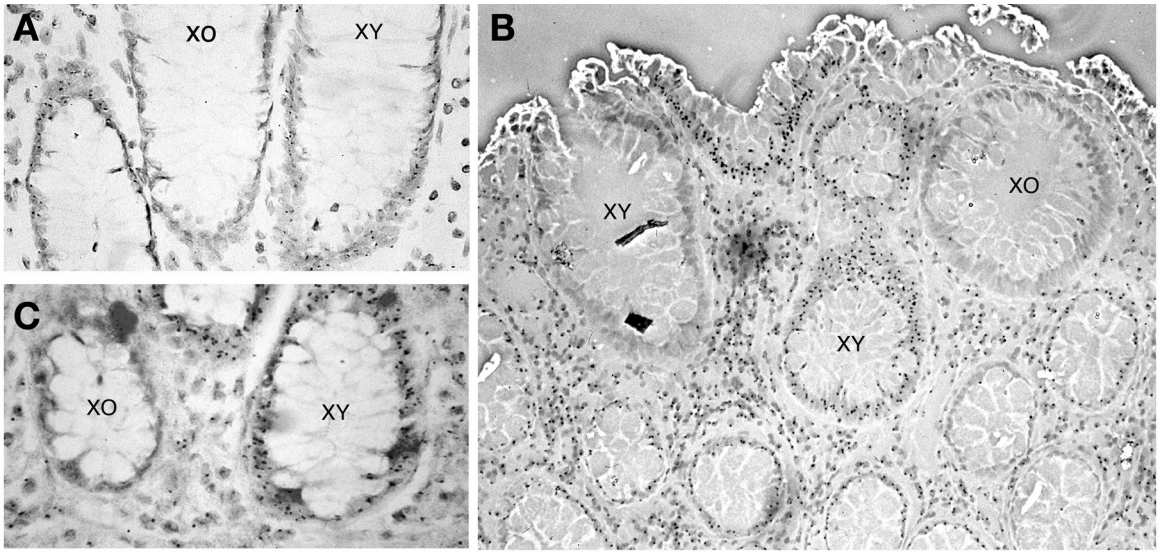

Fig.2. **(A,B)** Monoclonal origins of human colonic crypts. Normal colonic mucosa from a rare XO/XY patient with FAP. Crypts stained by *in situ* hybridization with a Y chromosome-specific probe either stain positively or negatively for the Y chromosome (small black dot within XY cells). **(C)** Polyclonal adenoma in the same chimeric patient, with a mixture of XO and XY crypts. (Reprinted with permission from ref.78.)

further mutation causes loss of the remaining allele. Second hits in the heterozygote increase with age and this results in randomly located positive mPAS-stained crypts with all intracryptal goblet cells affected from base to lumen *(49)*. The frequency of positive crypts was increased after irradiation and initially crypts showed a partial crypt-staining pattern, which then became uniform. The time taken for partially trans-formed crypts to become clonal after radiation damage was about 1 yr and was called the "clonal stabilization time" *(50)*.The best evidence for the clonality of human colonic crypts came from the fortuitous discovery of an XO/XY chimeric patient who required a colectomy for FAP. Nonisotopic in situ hybridization for the Y chromosome revealed that all except4 of 12,614 colonic crypts studied, were composed of Y chromosome-positive or -negative cells (see Fig. 2A,B). The four mixed clonality crypts were explained by nondisjunction with loss of the Y chromosome in a stem cell.

More recently, Taylor et al. have shown that the colonic crypt cells accumulate sufficient mitochondrial DNA(mtDNA) mutations with age to cause a biochemical defect in the mtDNA-coded subunits of cytochrome-coxidase (COX). This defect can also be stained for with immunohistochemistry. Normal colonic tissue shows numerous completely COX-deficient crypts, but also a few partially stained crypts. Serial sections of these partial crypts allowed them to reconstruct three-dimensional images of the crypt, revealing a ribbon of COX-negative cells extending from the base of the crypt to the top. The ribbon of mutated, COX-negative cells appears to be the progeny of one of the small number of stem cells in the niche, and that the partially negative crypts are likely to be intermediate steps in the expansion of the mutated clone with eventual formation of a completely clonal COX-deficient crypt *(51)*. Although Taylor et al. have demonstrated what appears to be multiple stem cells within the niche, there is equally compelling evidence for clonality in the crypt, with all cells arising from a parent stem cell.

Clonal Expansion of Mutated Cells—Niche Succession and Crypt Fission

There are three possibilities that can result from a stem cell division *(52)*.

1. The production of one stem cell and one daughter cell—asymmetric division.
2. Symmetric division with self-replication, in which two stem cells are produced.
3. Symmetric division with stem cell loss, in which both daughter cells go on to differentiate.

The majority of divisions are thought to be asymmetric and there is some evidence supporting the retention of the template DNA strand within the stem cell located in the niche—the so-called "immortal strand" hypothesis *(53)*. This allows any DNA replication errors to pass into the differentiating, short-lived daughter cell affording a mechanism of stem cell genome protection *(54)*. Park et al. used ENU to induce mutations in the X-linked gene for glucose-6-phosphate dehydrogenase (G6PD) to demonstrate the expansion of a mutated clone within the crypt. G6PD gene mutation resulted in loss of staining in affected cells. After ENU treatment they initially observed crypts that were only partially stained for G6PD, which eventually disappeared with the contemporaneous emergence of fully mutated crypts (monoclonal conversion or crypt purification). These eventually gave rise to patches of crypts that failed to stain with G6PD *(55)*. More recently, Yatabe et al. used CpG methylation patterns in three non-expressed genes to study the dynamics of the stem cells within the niche, and proposed the niche succession model. They showed that the differences in methylation tag sequence among cells in adjacent crypts (intercrypt variation) were more pronounced

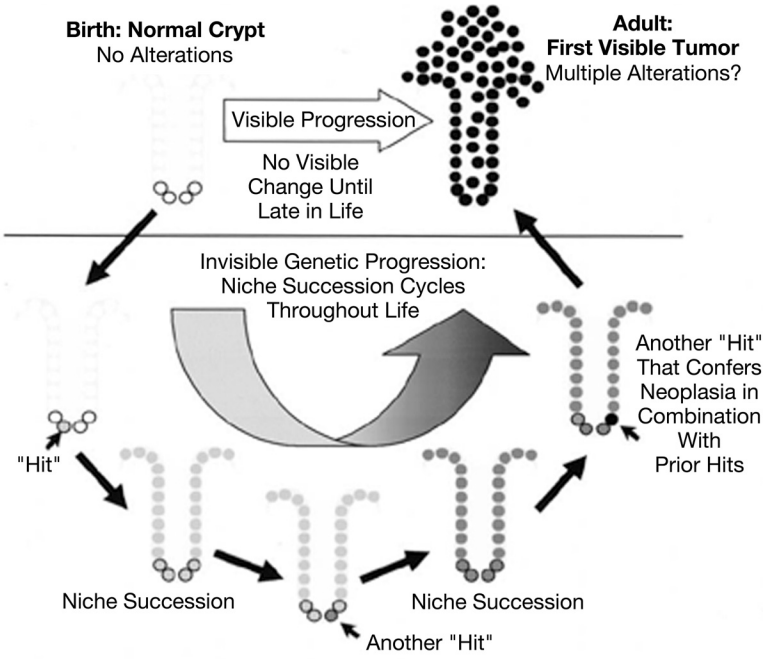

Fig. 3. A niche succession sequence. Although most mutations will be lost by stochastic extinction of stem cell lines, random genetic mutations in a stem cell may be passively fixed as a consequence of niche succession. Rarely, combinations of mutations in the same cell can hitchhike their way to clonal dominance, through successive niche succession cycles (small arrows) and can then confer a tumor phenotype on the crypt. (Reprinted with permission from ref. *58*.)

than the tag variation seen among cells in the same crypt (intracrypt variation). They proposed that intracrypt variation was a consequence of multiple, yet related, stem cells within each crypt. Stochastic extinction or amplification of one stem cell line by occasional symmetrical division results in a "bottleneck" effect, wherein all cells in the crypt are descended from an original stem cell. Mathematical modeling suggested that this bottleneck occurs once every 8.2 yr in the normal human colon *(56)*. In normal appearing crypts from FAP patients, however, there was a greater intracrypt variation in methylation tags, suggesting slower niche succession, probably as a consequence of enhanced stem cell survival. The longevity of $APC^{+/-}$ stem cells in FAP patients increases the chances of receiving or selecting for a second hit in the gene *(57)*. Once APC protein function is impaired, a growth advantage is bestowed on the cell and clonal expansion would then occur much more quickly (*see* Fig. 3)

Niche succession is a way by which a single stem cell line can "hitchhike" its way to clonal dominance of a single crypt (Fig. 3) *(58)*, but then how does a stem cell line expand into adjacent tissue? Clonality experiments in both mice and humans have shown clustering of mutated, phenotypically similar crypts together in patches *(51,55)*. It is thought that a process called crypt fission, in which crypts undergo basal bifurcation followed by longitudinal division, with the ultimate formation of two daughter crypts, is responsible for the clustering of apparently related crypts. This process is central in the massive increase in crypt number (in both the small and large intestine) in the postnatal period *(59)* and in the regenerative phase following radiation *(60)*. The crypt cycle—crypts born by crypt fission gradually increasing in size until they, themselves, divide by crypt fission—takes approx 108 d in the mouse jejunum and 9–18 yr in the human large intestine *(61,62)*. Studies on the methylation patterns of adjacent crypts showed significant intercrypt variation, both in adjacent crypts and those up to 15 cm apart. It was proposed that this was a consequence of the time taken for crypts to divide, allowing neighboring crypts to develop different methylation patterns during the process *(63)*. It was originally suggested that a crypt would be prompted to go into fission once it had reached a threshold size; however, attention has now focused on the stem cell number being the important factor. The rate of crypt fission is increased in pathological conditions such as ulcerative colitis and Crohn's disease *(64)*, as well as in the flat mucosa of FAP *(65,66)* and sporadic and hyperplastic adenomas *(67)*. In adenoma growth, fission is asymmetrical with atypical branching and budding *(65)*. Clusters of dysplastic crypts are termed microadenomas or dysplastic aberrant crypt foci (ACF), and are thought to be morphologically and genetically distinct lesions that are the precursors of adenomas and cancers *(20,68)*. Studies have shown that dysplastic ACFs are clonal populations *(69)* and expand by crypt fission *(70)*; however, the expansion of a mutated clone from a single cell to form small adenomas is contentious, with two main theories—the top-down and bottom-up models.

Top Down or Bottom up?

The top-down model is based on the frequent observation of dysplastic cells solely at the luminal surface of the crypts *(71–73)*, along with apparent retrograde migration of adenomatous cells from the surface to the base of the crypt *(73)*. Shih et al. examined the morphology and molecular characteristics of small (1–3 mm), well-orientated specimens from sporadic adenomas. Using digital single-nucleotide

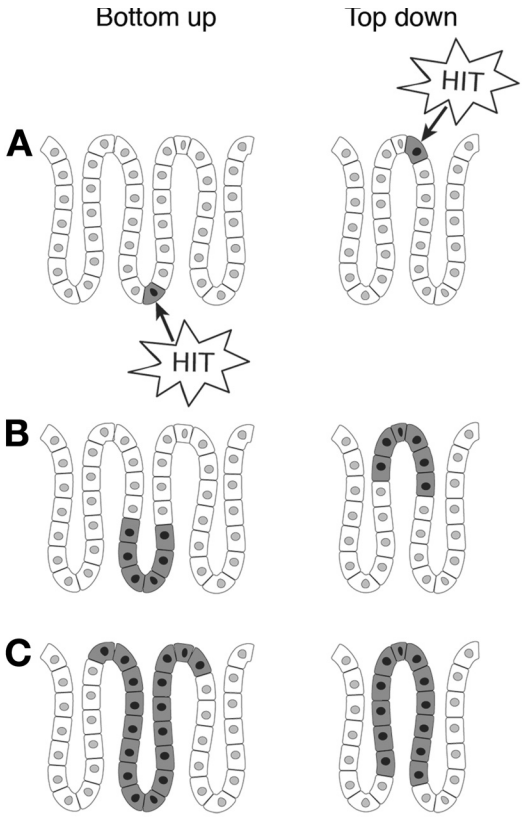

Fig. 4. Top-down, bottom-up growth of colorectal adenomas. Bottom down—the stem cell, located in the crypt base undergoes *APC* mutation **(A)**. The mutated cell proliferates **(B)** and spreads to the top of a crypt to form a monocryptal adenoma **(C)**. Initial further expansion is by crypt fission (based on ref. 78). Top down—the initial transformation event occurs in a cell in the intracryptal zone **(A)** and then spreads laterally and downwards **(B),** eventually filling the whole crypt **(C).** (Adapted from ref. 71.)

polymorphism (SNP) analysis of four SNPs within the *APC* gene, they assessed for LOH of APC in cells in the upper portion of the crypts, most of which had truncating *APC* mutations on nucleotide sequence analysis. These were not seen in the histologically normal crypt bases. Only these upper crypt cells showed prominent proliferative activity and nuclear localization of β-catenin. These observations were not easily reconciled with the conventional view of the stem cell origin of cancer, and the authors proposed two possible explanations to explain their findings. First, they considered a relocation of the stem cell area to the intracryptal zone, and second, they suggested that a mutated stem cell migrates from the base of the crypt to the luminal surface before expanding laterally and downwards. Lamprecht et al. adjusted the latter model slightly to suggest that *APC* mutations occur within a transit-amplifying cell, preventing it from terminally differentiating and altering the cell's migration dynamics, allowing it to remain in the mucosa as an incipient aberrant clone *(74)*. The bottom-up model involves the recognition of the earliest lesion in tumor development, the monocryptal adenoma, in which the dysplastic cells occupy an entire single crypt. These lesions are common in FAP *(75)*, and although rare in non-FAP cases, have been described *(76)*. Clonality studies in the XO/XY FAP patient have shown that monocryptal adenomas are clonal populations *(77)*. Analysis of tiny (<3 mm) adenomas in FAP patients showed increased proliferative activity and nuclear β-catenin translocation in morphologically dysplastic cells from the crypt base to the luminal surface. Additionally, there was a sharp cutoff between the dysplastic surface epithelium with nuclear β-catenin activity, and the normal mucosa in a neighboring unaffected crypt. The observation of an increased, asymmetrical crypt fission index in adenomatous tissue led the researchers to propose the bottom-up model—an abnormal stem cell clone with a growth advantage expands from the stem cell niche at the crypt base, to fill an entire crypt. Thereafter, initial spread is by crypt fission to form ACF, with top-down spread undoubtedly occurring in slightly larger lesions (*see* Fig. 4) *(78)*.

The Clonality of Tumors

It is widely accepted that tumors arise from mutations in a single cell, possibly a stem cell, which then clonally expands, with resultant common ancestry of all neoplastic cells. Evidence suggests that in the very early stages of tumor progression, from monocryptal adenoma to ACF, the dysplastic cells do comprise a clonal population *(69,77)*, however, the clonality of larger lesions and tumors is more widely debated. If indeed stem cells, because of their longevity and capacity for self-renewal, are the original targets for the mutation(s) required to initiate a neoplasm, then whether such cells act alone or in cooperation with other mutated stem cells becomes important. In this respect Fearon et al., studying restriction fragment length polymorphisms of inactivation of the X chromosome-linked phosphoglycerate kinase (PGK) gene in colorectal adenomas and carcinomas from 50 female patients, found that in adjacent normal epithelia X chromosome inactivation was random and therefore polyclonal, but in the adenomas and carcinomas, a monoclonal pattern of X chromosome inactivation was seen *(79)*. Conversely, in female patients with Gardner's syndrome, a precancerous bowel condition in which patients develop multiple adenomas of the gastrointestinal mucosa, the adenomas have a multiclonal origin, expressing both forms of G6PD *(80)*. A CAG trinucleotide repeat polymorphism adjacent to methylation sites in the X chromosome-linked human androgen receptor gene is present in approx 90% of females, and provides a method of looking at tumor clonality by polymerase chain reaction amplification of a 600-base pair DNA fragment encompassing the polymorphic *BstXI* site and methylation-sensitive *HpaII* sites. In 15 female gastrointestinal tumors, the majority revealed an unequivocal monoclonal origin with random pattern of X-inactivation in the normal surrounding mucosa, consistent with Southern blot analysis for PGK and the M27(probe (DXS255) which detects X chromosome tandem repeat polymorphisms in about 90% of females *(81)*. However, it is important to note that patch size was overlooked in these studies, and it is possible that tumors were covertly polyclonal but appeared monotypic as they arose within a large X linked patch. In this respect, Novelli et al. studied Sardinian females heterozygous for the G6PD. Mediterranean mutation (563 C→T) and heat deactivated the defective gene product by preheating at 53°C for 5 min followed by enzyme histochemistry to demonstrate G6PD activity. Crypts were arranged in hexagonal arrays in large patches, with irregular patch borders,

containing up to 450 individual crypts *(82)*. These direct observations suggest that, because of the large patch size in the colon, X-inactivation studies are heavily biased toward showing that tumors are monoclonal. To exclude the possibility that all adenomas are polyclonal in origin, every crypt in at least 43 adenomas would need to be shown to be monophenotypic (95% confidence interval). To exclude the possibility that 10% of adenomas are polyclonal, 430 adenomas would need to be examined. Thus patch size confounds measurements of tumor clonality in the colon, and in many other tissues *(82)*.

However, direct observation, rather than the indirect methods described above tell a different story. Novelli and co-workers *(77)* studied a highly unusual individual who not only had FAP but was also a sex chromosome mixoploid chimera, presumably as a consequence of a dicentric Y chromosome. Thus his tissues were a mosaic with the majority—some 80% of cells being XY and the remaining 20% being XO. Therefore, the detection of the Y chromosome in the excised colonic tissues provides an excellent binary marker for lineage analysis. Of the 263 adenomas analyzed, 246 were wholly XY, four were entirely XO, whereas 13 were of mixed XO/XY genotype. Simplistically, therefore, and considering only the tumors containing the XO lineage, 13/(13 + 4) or 76% of all adenomas would appear to be polyclonal. Subsequent studies in mice heterozygous for the Min germ-line mutation and chimeric for the lineage reporter gene ROSA26 used the same ratio, now called the *Novelli ratio (83)*, rather startlingly produced the same value—76% were polyclonal. However, there are several problems with the Novelli ratio, not the least of which is that the proportion of XO/XY and XO tumors that are polyclonal may not reflect the whole population of tumors leading to an overestimate *(83)*, and this appears confounded when the minority component—here the XO and XY tumors, is small, as in this individual. Making reasonable assumptions about the mechanism of polyclonality, the proportion of polyclonal adenomas becomes closer to 50–60% *(83)*. Three possible explanations were considered for tumor polyclonality in the Novelli paper. First, true polyclonality with adenomas inducing dysplastic growth in adjacent crypts. Second, the XO/XY adenomas may have been clonal XY lesions that had focally lost their Y chromosome. Third, owing to the large numbers of polyps in FAP, mixed tumors may have arisen through random collision of two different lesions. Novelli et al. *(77)* considered the first explanation the most plausible. Chromosomal loss after tumor initiation is usually a late phenomenon in colorectal adenoma formation, and no Y chromosome loss was seen in any larger adenomas from control FAP patients. Additionally, chromosomal loss did not explain the findings in the chimeric mouse analysis of Merritt et al. *(84)*, so this explanation for tumor polyclonality seems unlikely. However, there remains the problem of random collision of adjacent tumors which looms large in a patient with many hundreds of adenomas or the Min, mouse, in which tumors are also very numerous. Although Novelli et al. *(77)* discounted this possibility through statistical inference, an important advance has come from Thliveris et al. *(85)*, in which tumor multiplicity in the chimeric mouse model described above was reduced by introducing homozygosity for the tumor resistance allele of the Mom1 locus (of genotype B6 $Apc^{Min/+}$ $Mom1^{R/R}$ ↔ B6 $Ap^{Min/+}$ ROSA26/+ $Mom^{R/R}$). The percentage of mixed tumors ranged from 8% to 63%, with a mean of 22%. This is much higher than would be expected if heterotypic tumors were formed by random collision. Consequently, the mechanism of this polyclonality becomes pivotal. We have seen above that crypts are clonal populations. So how could tumors arise, which require the interaction of at least two clones? Such considerations are of course anathema to some, reared on the concept of the monoclonality of tumors as a modern shibboleth. It could be of course that the colon is heterogeneous in respect of its tumor susceptibility, but in the Thliveris et al. *(85)* study, since mixed tumors were more frequent in the proximal small bowel where tumor multiplicity is lower than the distal small intestine. Thliveris et al. *(85)* analyzed the chimeric architecture of the intestine in relationship to the appearance of the mixed tumors and considered the spatial extent over which clonal interactions could occur. They concluded that very short-range interactions were important, among clones as close as 1–2 crypts apart. However, is such interaction required for the formation of *all* adenomas? Their analysis was consistent with a proportion of polyclonal adenomas reaching as high as 100%, although higher resolution in terms of the binary marker is required for firm conclusions. In addition, what might be the mechanism of such clonal interactions? Possibilities include microheterogeneity of tumor susceptibility, in which local stroma promotes loss of heterozygosity in adjacent crypts, or induction of elevated proliferative rates in the stem cells of adjacent crypts by a mutated clone, increasing the chance of further mutation in an adjacent stem cell *(85)*. These considerations introduce the concept of stem cell interactions, as yet an unexplored territory in mucosal regeneration and carcinogenesis in the gut.

Conclusion and Future Work

Recently, cancer biology investigators, mostly working in the hematological malignancy field, have focused on the similarities between normal stem cells and malignant cells and have developed the term "cancer stem cell" to describe the ability of certain human cancer cells to regenerate tumor clones in immunocompromised animals, an ability not possessed by the vast majority of the abnormal cells within a neoplasm (reviewed in ref. *86*). Kirkland showed the multilineage differentiation potential of the human rectal adenocarcinoma cell line HRA19a, after injection of the progeny of a single cell into an immunocompromised mouse, caused neoplastic growth containing all three epithelial cell lineages *(87)*. This work has profound implications for the biology of metastasis and for chemotherapy of tumors, since direct targeting of cancer stem cells would prevent recurrence of the tumor rather than simple destruction of the nontumorigenic daughter cells. As yet, the identification of a cancer stem cell in gastrointestinal malignancy has not been achieved, and further work in this area is of great importance.

The adenoma-carcinoma sequence is established as a road map of tumor progression and has altered clinical practice. Intestinal polyps are now routinely removed during endoscopic procedures and surveillance programs established to follow up these patients long term. The molecular changes that occur in a single cell at the very beginning of this process have been elucidated over the past 20 yr of careful experimentation and future further discoveries of important tumor suppressors

and oncogenes involved in carcinogenesis are likely. The events that occur during the growth of the intestinal adenoma remain unresolved but future work in this field may help to clarify the process, and would have profound implications for the understanding of tumor biology in this field.

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