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Polyclonal Tumors in Mammalian Intestine

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Mitotic Arrest and Cell Fate

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CYFIP2, a Direct p53 Target

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Perspective

Polyclonal Tumors in the Mammalian Intestine

Are Interactions Among Multiple Initiated Clones Necessary for Tumor Initiation, Growth, and Progression?

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KEY WORDS

intestinal cancer, crypt purification, polyclonal structure, random collision, short-range interactions, Apc, Cox-2, and EgfR

ABBREVIATIONS

Apc/APC	adenomatous polyposis coli
Cox-2	cyclooxygenase 2 (also known as PTGS2, prostaglandin-endoperoxide synthase 2)
DMBA	7,12-dimethylbenz(a)anthracene
EgfR	epidermal growth factor receptor
FAP	familial adenomatous polyposis
G6PD	glucose-6-phosphate dehydrogenase
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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ABSTRACT

Studies in both man and mouse indicate that the majority of familial intestinal tumors are polyclonal being composed of cells from at least two distinct progenitors. The formation of polyclonal tumors in the mouse can be explained by short-range interactions between multiple initiated clones within one or two crypt diameters of each other. These clonal interactions might be critical, if not necessary, for initiation, growth, progression, or all three stages of tumorigenesis. This view is diametrically opposed to the widely held view that intestinal tumors are monoclonal and progress by clonal expansion. The data supporting the latter are neither extensive nor definitive. In addition, the results from a recent study indicate that earlier studies of tumor clonality were heavily biased because lineage patches in the intestinal epithelium of humans resulting from X-inactivation are relatively large. Consequently, hundreds of tumors from familial and sporadic cases need to be analyzed to accurately assess tumor clonality. Investigators must keep an open mind regarding the clonality of tumors in the mammalian intestine as new experimental approaches are developed which will eventually provide a definitive answer to this fundamental question in the field of cancer biology.

BACKGROUND

Colorectal cancer is a leading cause of cancer death in the United States with half the population developing one or more colonic tumors by 80 years of age.[1] Many investigators believe that each tumor is derived from a single somatic progenitor cell and that a tumor progresses from a benign to malignant form as a series of mutations in oncogenes and tumor suppressor genes accumulate.[2] Studies in which intestinal tumors were analyzed from a XO/XY mosaic man with a hereditary form of colorectal cancer and from a mouse model of this same disease, however, indicate that the majority of early familial intestinal adenomas are not monoclonal but polyclonal being composed of cells from at least two distinct progenitors.[3,4] The goals of this Perspective are to summarize the results from studies that were designed to determine the clonal structure of tumors in the mammalian intestine, to reconcile why investigators have reached different conclusions about tumor clonality, and to discuss how short-range interactions among initiated cells from multiple lineages in a tumor could potentially affect all stages of tumorigenesis.

The epithelium in the small intestine of mammals is arranged into two fundamental structures: crypts of Lieberkuhn, which are invaginations into the mucosa, and villi, which are finger-like projections into the lumen. A cohort of four to 16 stem cells with unlimited capacity for self-renewal lies at the base of each crypt in the mouse.[5] Each stem cell divides asymmetrically and infrequently. One daughter replaces the progenitor, while the other migrates upward and becomes highly proliferative with a doubling time of approximately 12 hours. As cells reach the top of the crypt, they stop dividing and terminally differentiate into one of several different cell types. Absorptive enterocytes, enteroendocrine cells, and goblet cells continue migrating upward and populate the surface of villi. This migration takes three days, after which time cells are exfoliated into the lumen. By contrast, Paneth cells migrate downward to the base of the crypt. After approximately three weeks, they are removed by phagocytosis. The structure of the epithelium in the colon is similar to that of the small intestine, except cells migrating from crypts form epithelial cuffs rather than villi. The clonality of the cohort of stem cells and consequently a crypt depends on the age of the animal[6] (Fig. 1). Crypts are often polyclonal throughout the entire neonatal intestine, whereas crypts are monoclonal throughout the entire adult intestine.

POSITIVE AND NEGATIVE REGULATORS OF INTESTINAL TUMORIGENESIS

The incidence of colorectal cancer is high because spontaneous mutations can occur during any one of the 10^{11} cell divisions that occur per day in this tissue.[7] The molecular changes that correlate with the transformation of the intestinal epithelium into its cancerous counterpart are being elucidated. Genetic alterations in *Adenomatous Polyposis Coli* (*APC*), *RAS* and *p53* are detected in tumors from patients with sporadic cases of colorectal cancer.[8-10] The inactivation of *APC* appears to initiate tumorigenesis in most cases, while the activation of *RAS* and inactivation of *p53* correlate with tumor progression and metastasis, respectively. Additional genes affecting tumorigenesis in the mammalian intestine are continuing to be discovered. Eschrich and his colleagues reported that a molecular fingerprint based on 43 genes, including neuregulin and osteopontin, predicted 36-month survival more accurately than Duke's staging, the standard method of histological grading.[11] Similarly, Kwong and her colleagues demonstrated that changes in expression of 2187 genes as monitored by microarrays can be used to differentiate normal intestinal epithelium, benign adenomas, and malignant carcinomas.[12] Thus, a number of genes clearly affect tumorigenesis in the mammalian intestine.

The importance of negative regulators in maintaining homeostasis in the intestinal epithelium is exemplified by the etiology of familial adenomatous polyposis (FAP).[13] Patients with this syndrome carry apparent loss-of-function mutations in the *APC* gene and develop hundreds to thousands of benign adenomas in the colon by the second or third decade of life.[14,15] The majority of the mutations are in the 5′ half of the *APC* gene and result in the premature truncation of the 2843 amino acid gene product. The APC polypeptide contains several armadillo repeats and binding sites for itself, axin, b-catenin, conductin, glycogen synthase kinase (GSK3B), microtubules, DLG, and EB1.[16] Such interactions with other molecules link APC to a diverse array of cellular processes including cell adhesion, microtubule formation, and transcription.[17,18]

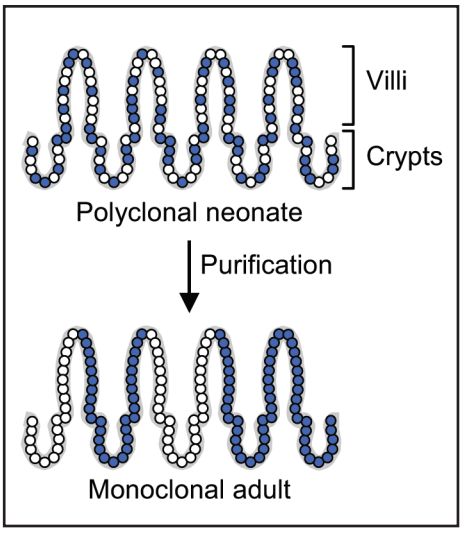

Figure 1. The architecture of the intestinal crypt changes as neonates mature into adults. Crypts are polyclonal in the neonatal intestine but monoclonal by 14 days of age in the mouse. The conversion is called crypt purification and is not well understood. Multiple crypts contribute cells to a single villus.

CLONALITY OF HUMAN COLORECTAL TUMORS

A few studies over the past four decades have tested whether tumors from patients with hereditary or sporadic forms of colorectal cancer are derived from a single somatic progenitor cell and its descendents by analyzing the status of tumors in females that are mosaic for X-linked genes (Table 1). Fearon and his colleagues

Table 1 **Intestinal tumor clonality studies**

Species	Publication	N	Assay	Tumor Type	Heterotypic/Total
Human	Buetler et al. (1967)	1	X-inactivation mosaicism for G6PD isozymes detected by gel electrophoresis	Sporadic colonic carcinoma	1/1
				Sporadic hepatic metastases	7/24
Human	Hsu et al. (1983)	3	X-inactivation mosaicism for G6PD isozymes detected by gel electrophoresis	Familial colonic adenomas	7/7
Human	Fearon et al. (1987)	?[a]	X-inactivation mosaicism for PGK or HPRT detected by RFLP	Sporadic colonic adenomas	0/12
				Familial colonic adenomas	0/18
				Sporadic colonic carcinomas	0/20
Human	Novelli et al. (1996)	1	XO/XY mosaicism detected by nonisotopic in situ hybridization	Familial colorectal adenomas	13/263
Mouse	Griffiths et al. (1989)	12	X-inactivation mosacism for G6PD activity detected by immunohistochemistry	Carcinogen-induced colonic tumors	1/28[b]
Mouse	Merritt et al. (1997)	2	Chimerism for ROSA26 activity detected by X-Gal staining	Familial intestinal adenomas	22/260
Mouse	Thliveris and Halberg et al. (2005)	7	Chimerism for ROSA26 activity detected by X-Gal staining	Familial intestinal adenomas	22/100

[a]The number of patients was not reported in this paper. [b]Loss of G6PD activity might have been due to a carcinogen-induced somatic mutation.

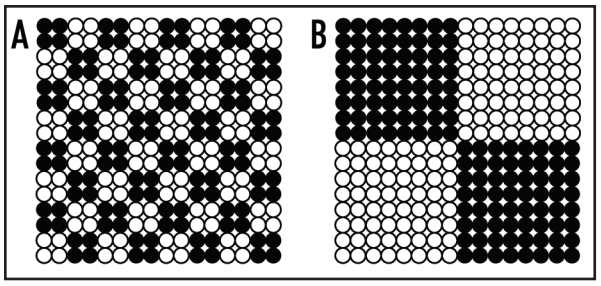

Figure 2. Patch size greatly influences the probability that a tumor will have an overtly polyclonal structure being composed of cells from two or more distinct progenitors, especially if interactions between initiated clones are limited to a short distance. In a region with very small patches, as represented in panel A, all the "crypts" lie on borders between patches of white and black. As the patch size increases, the percentage of "crypts" lying on borders decreases. For example, in a region with larger patches, as represented in panel B, the majority of crypts do not lie on borders. A tumor arising from interactions between initiated clones in a region of intestinal epithelium like panel B would be much more likely to be homotypic than a tumor arising in a region like panel A. Thus, a study that assesses tumor clonality based on the analysis of mosaicism in which patch size is large is heavily biased towards the conclusion that tumors are monoclonal because polyclonal tumors in this situation are likely to be homotypic.

reported that all human colonic tumors have a monoclonal origin,[19] whereas studies by Buetler and his colleagues as well as Hsu and her colleagues reported that the majority of human colorectal tumors have a polyclonal origin[20,21] (Table 1). Fearon and his colleagues suggested in their report that the Buetler study might not be representative because the patient developed cancer at a very young age and that the Hsu study might be misleading because tumor samples of one lineage type were likely contaminated with normal cells from the other lineage type. Thus, the answer to the clonality question is unresolved from these three studies.

Novelli and his colleagues reported that the majority of colorectal adenomas from an XO/XY male patient with FAP were polyclonal.[3] They analyzed 263 colonic adenomas: 246 XY, 4 XO and 13 XO/XY. The homotypic tumors composed of either XO or XY cells might be polyclonal but derived from two somatic lineages with the same genotype, or else these tumors might simply be monoclonal. By contrast, heterotypic XO/XY tumors are polyclonal, so at least 5% of the tumors in this individual have a polyclonal structure. Novelli and his colleagues acknowledged that an XO/XY tumor might arise from an XY tumor that focally lost the Y chromosome. This point is often raised to dismiss this work. Novelli's analysis of normal crypts, however, revealed that only 4 out of 12,614 (0.03%) had lost the Y chromosome, so instability associated with the abnormal Y chromosome seems unlikely to account for the 13 XO/XY adenomas observed in the study unless the instability increases significantly in very early tumors. The Novelli study supports the Hsu and Buetler studies, indicating that tumors from patients with FAP can have a polyclonal origin.

BIAS OF EXPERIMENTS RELYING ON MOSAICISM

Investigators might have reached different conclusions regarding tumor clonality because the fraction of observable polyclonal tumors depends on the size and structure of lineage patches throughout the intestinal epithelium (Fig. 2). If patches are small, the number of crypts lying on borders is high and consequently the probability a polyclonal tumor will have a heterotypic structure is high. By contrast, if patches are large, the number of crypts lying on borders is low and consequently the probability a polyclonal tumor will have a heterotypic structure is low.

Novelli and his colleagues analyzed the size and structure of patches in the intestinal epithelium of humans arising from the random inactivation of X-linked genes.[22] They analyzed glucose-6-phosphate dehydrogenase (G6PD) expression in nine samples of normal intestinal tissue from an 80 year-old woman. Patches of expression were relatively large, with only 8% of the crypts lying on borders. Based on this observation, they estimated that 43 adenomas must be shown to be monoclonal to exclude the possibility that all human colorectal tumors are polyclonal. Note that Fearon and his colleagues tested only 30 adenomas. The number of adenomas that must be tested increases as the hypothesized fraction of polyclonal tumors decreases. A total of 430 adenomas must be shown to be monoclonal to exclude the possibility that 1 out of 10 human colorectal tumors is polyclonal. An accurate estimate of the fraction of polyclonal tumors is clearly important. Thus, studies that assess tumor clonality based on the analysis of mosaicism for X-linked genes are heavily biased towards the conclusion that human colorectal tumors are monoclonal.

CLONALITY OF MURINE INTESTINAL TUMORS

Griffiths and his colleagues determined the clonality of colonic tumors induced by treating mice that were mosaic for G6PD expression with 1,2 dimethylhydrazine.[23] They found 12 tumors had a uniform high G6PD expression, 15 tumors had uniform low G6PD expression, and one tumor had mixed G6PD expression. The heterotypic tumor appeared to have arisen from a carcinogen-induced, somatic mutation in the G6PD gene. Griffiths and his colleagues concluded that colonic tumors in this carcinogen-induced mouse model are monoclonal. They, however, raised the concern that several hundred tumors need to be analyzed to exclude the possibility that 5% are polyclonal because of size and structure of lineage patches.

Mouse models of FAP also permit the clonality of intestinal tumors to be assessed knowing patch size and structure because aggregation chimeras can be generated by fusing together embryos with different genotypes (Fig. 3). Merritt and her colleagues analyzed intestinal tumors from *Apc*$^{Min/+}$ mice chimeric for ROSA26 expression.[4] They found that 22 out of 260 tumors from these mice were heterotypic, composed of ROSA26$^-$ (white) and ROSA26$^+$ (blue) neoplastic cells, while 238 were composed solely of ROSA26$^-$ neoplastic cells and 6 were composed solely of ROSA26$^+$ neoplastic cells. Thus, a significant number of tumors were clearly polyclonal. An analysis of Apc expression by immunohistochemistry revealed that the protein was absent in both ROSA26$^-$ and ROSA26$^+$ cells contributing to polyclonal tumors. Merritt and her colleagues proposed four hypotheses to explain the formation of heterotypic tumors.

Hypothesis 1: A heterotypic tumor forms because the ROSA26 cell lineage marker is lost focally within a ROSA26$^+$ adenoma. This explanation is implausible because ROSA26 shows no mosaicism in *Apc*$^{Min/+}$ *ROSA26*$^+$ mice.[4] Thus, a major concern of the Novelli study i.e., stability of the markers, was not an issue with this mouse model.

Hypothesis 2: A heterotypic tumor forms because the ROSA26 marker is silenced epigenetically. Again, this explanation has been ruled out. Regions of heterotypic tumors that were white did not carry the ROSA26 marker.

Hypothesis 3: A heterotypic tumor is polyclonal and forms because of clonal interactions between multiple initiated clones which are neoplastic.

Hypothesis 4: A heterotypic tumor is polyclonal and forms because of random collision between two or more distinct tumors.

Merritt and her colleagues favored Hypothesis 3, but could not rule out Hypothesis 4. The multiplicity of intestinal tumors was very high in the two *Apc*$^{Min/+}$ ↔ *Apc*$^{Min/+}$ *ROSA26*$^{+}$ aggregation chimeras in their study and the XO/XY mosaic FAP individual in the Novelli study.[3,4]

SHORT-RANGE INTERACTIONS

With our biostatistical colleagues, we have tested the random collision hypothesis by reducing tumor multiplicity.[24] We analyzed tumors from *Apc*$^{Min/+}$ ROSA26 chimeras homozygous for the tumor-resistance allele of *Mom1*. Tumors often had a polyclonal structure (Fig. 4), despite the fact that the mice had a markedly reduced tumor multiplicity. Statistical analyses ruled out random collision and indicated that the formation of polyclonal tumors could be explained by short-range interactions between multiple initiated clones within one or two crypt diameters of each other.[24,25] If interactions are limited to close neighbors, the ability to detect the interactions requires mosaicim or chimerism to be very fine grained. This point emphasizes the limitation of X-inactivation mosaics in detecting polyclonality. Fortunately, 17% of the crypts were on borders in the chimeras that we analyzed in our study.[24]

We estimated that 50 to 100% of the tumors in our study were polyclonal using a different statistical approach than described previously.[24,26] The range is large because the clonal structure of a homotypic tumor is unclear: a solid blue tumor could be either monoclonal derived from a single ROSA26^{+} progenitor or else polyclonal derived from two or more ROSA26^{+} progenitors. This problem can be overcome only when mosaics or chimeras are developed in which cells within a single crypt carry a unique marker making them distinguishable from cells in neighboring crypts. Kim and Shibata demonstrated that neighboring crypts in the human colon have distinct patterns of methylation at the *BGN* locus.[27] They concluded that crypts are long-lived structures that become mosaic because of random epigenetic changes that occur during aging. Unfortunately, the methylation pattern at a specific locus is unlikely to be useful in uniquely identifying crypts within the intestinal epithelium of a mouse because the lifespan of the mouse is so short relative to that of humans, and consequently very few random epigenetic changes occur that could be used to distinguish neighboring crypts.

Greaves and her colleagues demonstrated that the intestinal epithelium in humans is often mosaic with respect to cytochrome c oxidase expression.[28] They analyzed small blocks of normal tissue from 14 individuals and found that some intestinal crypts lacked cytochrome c oxidase expression because of spontaneous mutations in this mitochondrial gene. Interestingly, the number of crypts with the same mutation within a patch increased with age, indicating that crypt fission can give rise to a distinct field of such crypts. Cytochrome c oxidase activity in the intestinal epithelium of the laboratory mouse has not been analyzed, so it is unclear whether this experimental approach would yield fine-grained mosaics that would be beneficial in the assessment of tumor clonality in the mammalian intestine.

Crypts within the mouse intestinal epithelium might be distinguishable based on the position of a transposable element that randomly integrates into the genome. Collier and her colleagues have developed a mouse model in which the Sleeping Beauty element transposes in all somatic tissues tested, including the intestine.[29] Mice carrying Sleeping Beauty, the transposase, and *Apc*^{Min} might permit us to estimate more accurately the percentage of polyclonal tumors if the mosacism created by the hopping of the transposon generates a finer grain of patches than does chimerism for the ROSA26 lineage marker. Interestingly, Collier and her colleagues found that sarcomas from ARF-deficient mice carrying Sleeping Beauty and the transposase had multiple transposon insertions.[29] This observation does not necessarily indicate that sarcomas from these mice are polyclonal. A detailed analysis of the transpositions is required to determine whether multiple transpositions occurred in a lineage derived from a single progenitor, or else occurred in cells derived from multiple progenitors. Thus, new experimental systems might allow us to determine more accurately the extent of polyclonality in mouse

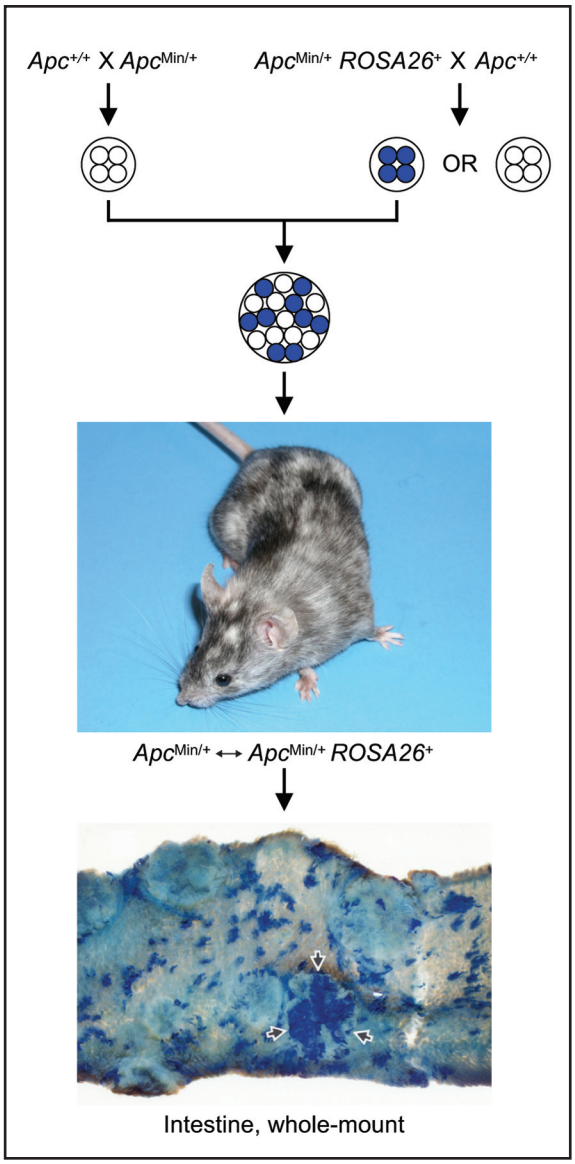

Figure 3. *Apc*$^{Min/+}$↔*Apc*$^{Min/+}$ *ROSA26*$^{+}$ aggregation chimeras develop polyclonal intestinal tumors. Embryos from *Apc*$^{+/+}$ females in matings with *Apc*$^{Min/+}$ males were fused to embryos from *Apc*$^{+/+}$ females in matings with *Apc*$^{Min/+}$ *ROSA26*$^{+}$ males. One out of eight aggregation chimeras will be composed of *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ *ROSA26*$^{+}$ components. The intestines of these mice were patchworks because cells carrying the *ROSA26*$^{+}$ transgene express LacZ and are blue following staining with 5-bromo-4-chloro-indolyl-D-galactopyranoside (X-Gal). Tumors from these *Apc*$^{Min/+}$↔*Apc*$^{Min/+}$ *ROSA26*$^{+}$ aggregation chimeras are often heterotypic being composed of ROSA26^{-} (white) and ROSA26^{+} (blue) neoplastic cells (arrows).

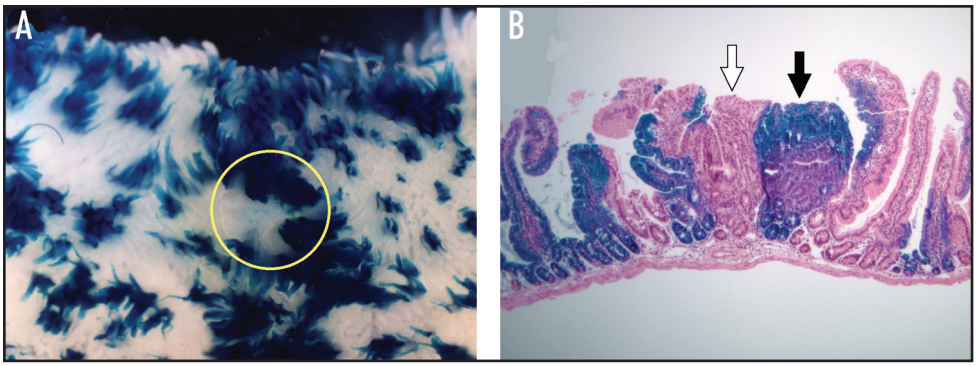

Figure 4. $Apc^{Min/+}$ $Mom1^{R/R}$↔$Apc^{Min/+}$ $Mom1^{R/R}$ *ROSA26*$^{+}$ aggregation chimeras develop polyclonal intestinal tumors. These chimeras were generated, and then sacrificed at 60-72 days of age. The intestinal tract was removed, stained, and scored for tumor number. Tumor multiplicity was low because the tumor-resistance allele of *Mom1* suppresses tumorigenesis, but tumors with a polyclonal structure were still evident. An example is shown in a wholemount image (panel A; yellow circle). The tumor was removed, embedded in paraffin, and sectioned. Every 14th section was stained with hematoxylin and eosin to determine whether the white (white-filled arrow) and blue (black arrow) components were composed of neoplastic cells (panel B). Three pathologists confirmed that this tumor was polyclonal. A total of 22 out of 100 tumors from six different aggregation chimeras had similar structures.

models of human colorectal cancer. An accurate assessment is critical because polyclonal tumors might respond differently to drug intervention than monoclonal tumors and mouse models are frequently used to test drug efficacy.

TRANSFORMING THE NEIGHBORHOOD

In an earlier discussion of the polyclonality of early familial adenomas, we have drawn attention to several classical observations:

- "the community effect" in developmental transitions[30,31]
- "the polyclone" in transdetermination in *Drosophila*;[32] and
- "tumor promoters" that synergize with mutagenic "tumor initiators" by stimulating cell proliferation.[33]

A recent study of the transformed phenotype in culture has indicated that a neighborhood of untransformed 3T3 fibroblasts can suppress the neoplastic phenotype of SV40-transformed derivatives.[34] This observation is consistent with the central hypothesis of this Perspective that the neoplastic transformation is stimulated by clonal interaction.

The clonal interactions invoked in the community effect and the polyclone do not presuppose that each interacting partner has incurred a genetic event. The interactions that are suggested to explain the action of tumor promoters and the 3T3 suppression experiments would involve partners that are clonal descendents of a single initiated cell. By contrast, it remains puzzling that the interactions between epithelial clones in intestinal neoplasia[3,4,24] and between epithelial and mesenchymal clones in prostatic intraepithelial neoplasia[35] involve cooperation between partners that are each expected to be rare somatic variants. For intestinal cancer, we suggest below possible solutions to this mystery.

How do polyclonal tumors form in the mammalian intestine? Apc is the gatekeeper in the intestinal epithelium that is absolutely essential for maintaining homeostasis.[36] Clarke recently reported that loss of Apc activity drastically alters the intestinal epithelium, causing cells to proliferate rapidly, migrate improperly, and fail to differentiate.[37] If loss of Apc activity is necessary to transform a normal progenitor into its neoplastic counterpart, the development of a polyclonal intestinal tumor in patients with FAP or mouse models of this disease would require only two somatic mutations in *APC/Apc* because every cell in the body already carries a germline mutation in this gene. Kuraguchi and her colleagues demonstrated that the majority of tumors from mice carrying a germline mutation in *Apc* and lacking mismatch repair activity often carried two further somatic mutations in *Apc*.[38,39] They found that 29 out of 44 tumors from $Apc^{1638N/+}$ $Mlh1^{-/-}$ mice carried two distinct somatic mutations in *Apc*.[38] Similar results were obtained when analyzing tumors from $Apc^{1638N/+}$ $Msh6^{-/-}$ and $Apc^{1638N/+}$ $Msh3^{-/-}$ $Msh6^{-/-}$ mice.[39] Thus, polyclonal tumors in the intestine might be limited to individuals carrying a germline mutation that predisposes them to the cancer. However, short-range interactions between distinct initiated clones may be essential for all cases of tumor initiation. Interestingly, FAP patients present with hundreds to thousands of colonic tumors during the second and third decades of life. This lag in the onset is consistent with more than one further mutation being required for a tumor to form.

Does the loss of Apc activity in one crypt affect the loss of Apc activity in a neighboring crypt? Neoplastic cells within a dysplastic crypt might release mitogenic factors that affect cellular proliferation in neighboring normal crypts. Indeed, the normal intestinal epithelium adjacent to tumors is often hyperplastic.[40] This change could increase the chance that Apc activity is lost in a neighboring crypt. A mutation might occur in *Apc* because errors during replication are not repaired in the context of high rates of proliferation, or the wildtype allele might be lost by somatic recombination. Haigis and his colleagues demonstrated that the wildtype allele of *Apc* is lost by somatic recombination during intestinal tumorigenesis in C57BL/6 $Apc^{Min/+}$ mice.[41] Alternatively, the proliferative change could thwart cellular checkpoints that eliminate transformed cells lacking Apc activity. Meniel and her colleagues demonstrated that cells lacking Apc and p53 persist in the mammary gland of females beyond parturition, in contrast to cells lacking Apc but expressing p53 which do not persist.[42] They proposed that the persistence of such double mutant cells is either a contributing factor or a driving mechanism to neoplasia in the mammary gland. We have demonstrated that a lack of p53 activity affects the development of intestinal tumors in C57BL/6 $Apc^{Min/+}$ mice.[43] Thus, formation of polyclonal tumors in the mammalian intestine might involve an active recruitment process in which a dysplastic crypt causes one or more neighboring normal crypts to be transformed. Mitogenic factors causing proliferative changes might be targets for chemoprevention.

Other factors could alter the microenvironment such that tumor initiation is favored. The short-range interactions between cells within dysplastic crypts might be mediated by epidermal growth factor receptor (EgfR) and cyclooxygenase 2 (Cox-2). These molecules are coupled through a positive feedback loop.[44] An elevated level of EgfR in one initiated clone could conceivably affect the loss of Apc activity in neighboring clones and affect the expression of Cox-2 in surrounding stromal cells.[45-47] Cox-2 catalyzes the synthesis of PGE_2, a prostaglandin that stimulates cell proliferation and angiogenesis while blocking apoptosis and the immune response of

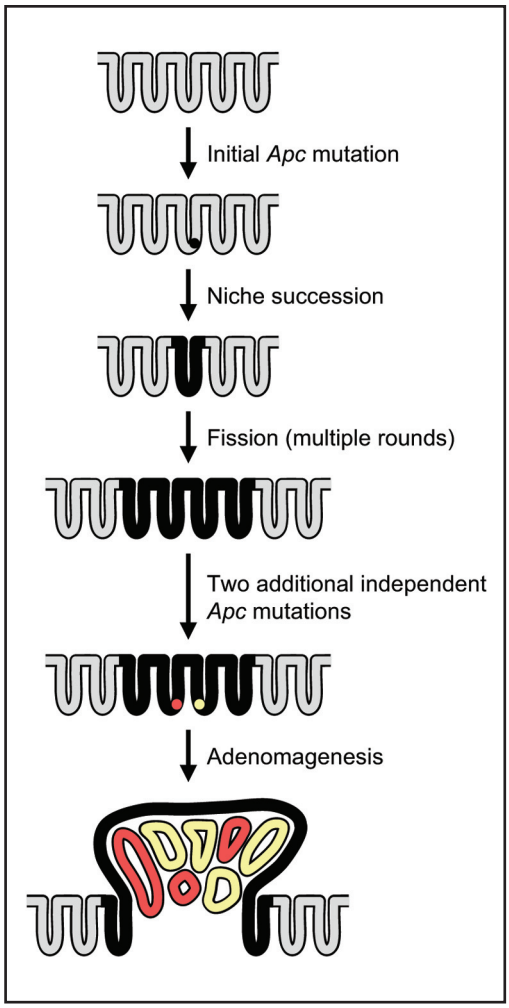

Figure 5. The development of a polyclonal tumor in the intestine of a normal individual might require only three mutations in *APC/Apc*. A spontaneous mutation in a single stem cell (black dot) could generate a field of crypts composed entirely of cells carrying this mutation. The exposure of this field to a dietary carcinogen could then generate two additional independent mutations (red and yellow dots) and consequently lead to the development of a polyclonal tumor.

the host.[48-58] Thus, the EgfR/Cox-2 system could establish a microenvironment that favors tumor initiation. Both molecules are prime pharmacologic targets for the chemoprevention of colorectal cancer. Torrance and his colleagues demonstrated recently the combination of EKI-569, an inhibitor of EgfR, and sulindac, a specific inhibitor of Cox-2, often blocked completely the development of intestinal tumors in C57BL/6 $Apc^{Min/+}$ mice.[59]

Are colorectal tumors from patients with sporadic disease polyclonal? This question has gone unanswered because most researchers have focused on tumors from patients with FAP except for Fearon and his colleagues (Table 1). The development of a polyclonal tumor in the intestine of a normal individual would require a total of four mutations in *Apc*. Armitage and Doll estimated as many as seven mutations are necessary to transform the normal intestinal epithelium into its cancerous counterpart based on mathematical models that account for the sharp increase in the incidence of colorectal cancer after the sixth decade of life.[60] But an underlying assumption was that intestinal tumorigenesis is a monoclonal process in which successive mutations in cells derived from a single progenitor provide a selective growth advantage rather than a polycolonal process in which neighboring cells acquire mutations in the same gene.

An alternative hypothesis is that the development of a polyclonal tumor in the intestine of a normal individual requires only three mutations in *Apc* because a single mutation gives rise to a field of aberrant crypts. The concept of field cancerization was first introduced by Slaughter and his colleagues in 1953 and predicts that multiple independent tumors arise from a field of epithelial cells carrying a common genetic alteration following exposure to a carcinogen.[61] This mechanism could explain the development of polyclonal tumors in the mammalian intestine (Fig. 5). In this view, one of the four to 16 stem cells residing at the base of a crypt would acquire spontaneously a somatic mutation in *Apc* and eventually become the sole progenitor of all cells within the crypt because of niche succession. This crypt would then undergo fission to produce a patch. The size of the patch would increase and become a field within the normal epithelium following additional rounds of crypt fission. As mentioned above, Greaves and her colleagues recently demonstrated that crypts carrying a common genetic alteration form patches in the intestinal epithelium of humans through niche succession and crypt fission.[28] The exposure of crypts composed entirely of cells carrying a mutation in *Apc* to a dietary carcinogen could then result in the loss of Apc activity in two or more neighboring crypts. The dysplastic crypts that form then coalesce because of their close proximity. The persistence of the polyclonal structure would depend on whether one population of neoplastic cells has a growth advantage over the other or whether the structure itself is advantageous for tumorigenesis. Thus, the formation of polyclonal tumors in the mammalian intestine might require only three somatic mutations in *Apc* because a field of aberrant crypts can result from single somatic mutation. Reddy and Fialkow found that papillomas induced by multiple paintings of 7,12-dimethylbenz(a)anthracene (DMBA) were polyclonal.[62] They concluded that polyclonal tumors form because multiple paintings increased the likelihood that neighboring cells would be transformed to create a focus. By contrast, these authors found that papillomas induced by a single painting of DMBA followed by promotion with 12,0-tetradecanoylphorbol-13-acetate were monoclonal.[62] A possible interpretation of this observation is that promotion eliminates the need for clonal interactions between transformed neighbors because it dramatically increases the number of transformed cells in early neoplasms and thereby permits intraclonal cooperation.

Different mechanisms can be envisioned that explain why a rare event, like the loss of Apc activity, occurs in neighboring crypts. Regardless of the exact mechanism, the probability that a tumor has a polyclonal structure is still lower than the probability that a tumor has a monoclonal structure because the former requires two progenitors to be transformed, whereas the latter requires only one progenitor to be transformed. Why are tumors polyclonal unless this structure provides a selective advantage? Polyclonal tumors might become established at a higher frequency than monoclonal tumors because interactions between initiated clones alter the microenvironment. Molecules mediating interactions within a polyclonal tumor might also be critical for tumor progression. This view of tumorigenesis in the mammalian intestine is diametrically opposed to the widely held view that intestinal tumors are monoclonal and progress by clonal expansion.

POLYCLONALITY AND TUMOR PROGRESSION

Polyclonality might not be limited to short-range interactions among epithelial clones. Transformation of the normal intestinal epithelium into its cancerous counterpart appears to affect the activity of tumor suppressor genes in surrounding mesenchymal tissue. Wernert and his colleagues demonstrated that fibroblasts associated with human colorectal tumors often carry mutations in the *p53* gene.[63] This genetic change might occur because of selective pressure. Hill and his colleagues demonstrated that the formation of an epithelial tumor in the prostate gland elicits a paracrine response.[35] This response suppresses proliferation in the surrounding mesenchyme, but the oncogenic stress eventually leads to the loss of p53 activity in tumor-associated fibroblasts. Thus, these prostate tumors are polyclonal being composed of epithelial and mesenchymal components. Hill and his colleagues suggest the selective changes in the stroma, in turn, might lead to additional genetic changes in the epithelial tumor. In this case, short-range interactions between transformed epithelial and mesenchymal cells might be critical for tumor progression.

Do the molecules mediating short-range interactions in a polyclonal tumor dictate the site of metastasis? Colorectal tumors metastasize primarily to the liver and lung.[1] Both organs have normally a very high level of Egf signaling.[64] Neoplastic cells within a polyclonal tumor might acquire changes and migrate away from the favorable microenvironment created by short-range interactions that are mediated by EgfR and Cox-2 to other sites that provide the same signals. Interestingly, Buetler and his colleagues found that many hepatic metastases expressed only the A or B variant of GPD even though the primary tumor expressed both (Table 1). Several inhibitors of EgfR are currently being tested to determine whether they are effective against advanced stages of colorectal cancer.[65]

CONCLUSIONS

The clonality question with respect to human colorectal tumors remains unresolved and provocative. Some investigators believe these tumors have a monoclonal origin, while others believe these tumors have a polyclonal origin. Data supporting these different beliefs are neither extensive nor definitive. Our minds must remain open to both possibilities as new experimental approaches are developed to address this fundamental question in cancer biology. By contrast, the clonality question with respect to adenomas in mouse models of FAP is resolved. These early tumors commonly have a polyclonal structure, which is amazing given that at least two additional copies of *Apc* are inactivated somatically in these tumors. Additional experiments are necessary to understand the mechanism by which polyclonal tumors form in this model and to determine the relevancy of this model to other mouse models that develop advanced cancer as well as hereditary and sporadic forms of human disease. The results could fundamentally change our understanding of tumorigenesis in the intestine and consequently impact chemoprevention and treatment.

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