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Enhanced Stem Cell Survival in Familial Adenomatous Polyposis

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Individuals with heterozygous germline adenomatous polyposis coli (*APC*) mutations or familial adenomatous polyposis (FAP) are born with normal appearing colons but later develop hundreds to thousands of polyps. Tumor progression apparently starts after somatic loss of the normal *APC* allele, but germline *APC* mutations may potentially alter niche stem cell survival through dominant-negative interactions or haploinsufficiency. Although morphologically occult, altered stem cell turnover or clonal evolution rates may be detected by measuring the diversity of crypt sequences, with greater diversity expected with longer lived stem cell lineages. Methylation pattern diversity (numbers of unique patterns per crypt) was higher in normal appearing crypts from four of five FAP colons compared to six non-FAP colons and one attenuated FAP colon. Simulations indicate higher FAP crypt diversity is consistent with slower clonal evolution from enhanced stem cell survival, either through increased stem cell numbers or decreased stem cell lineage extinction, which is predicted to increase progression rates to cancer. Enhanced stem cell survival was associated with *APC* mutations that remove some but not all catenin-binding repeats. Therefore, some *APC* mutations may be common in colorectal cancers because they confer occult pretumor "caretaker" and "gatekeeper" defects. FAP crypts accumulate more alterations from slower stem cell clonal evolution rather than increased error rates. In non-FAP crypts, enhanced stem cell survival conferred by somatic heterozygous *APC* mutations would favor fixation through occult clonal niche expansions. Heterozygous *APC* mutations may change stem cell survival during colorectal pretumor progression. *(Am J Pathol 2004, 164:1369–1377)*

Familial adenomatous polyposis (FAP) is characterized by heterozygous germline mutations in the tumor suppressor gene adenomatous polyposis coli (*APC*).[1,2] Most mutations truncate the APC protein, with mutations near 5′ or 3′ ends associated with reduced polyp numbers (attenuated polyposis, AFAP). FAP colons appear normal at birth and hundreds of polyps start to appear during the second decade of life, suggesting that a single *APC* mutation is insufficient to start tumor progression. Somatic inactivation of the normal allele is observed in most FAP tumors and, consistent with Knudson's hypothesis, *APC* mutations are also present in the majority of sporadic colorectal cancers.[1–3] The germline mutation appears to influence the type of somatic mutation. Inactivation of the normal *APC* allele usually occurs through allelic loss when the germline mutation is in a small region of exon 15 called the mutation cluster region (MCR, codons 1286 to 1513[4]). In contrast, somatic mutations often appear within the MCR when the germline mutation is outside the MCR. MCR mutations are common in FAP and sporadic tumors.[2]

This nonrandom MCR mutation pattern is consistent with selection,[2,5–8] which may occur during tumor progression. However, selection is also possible in normal colon because each crypt contains multiple stem cells that compete for survival within a niche.[9–15] Random stem cell loss with replacement occurs in every crypt[9,10] and eventually results in niche succession or a "bottleneck" whenever all except one stem cell lineage becomes extinct (see Figure 1 in Calabrese et al[16]). Niche succession resembles the clonal evolution of tumor progression[17] but occurs during pretumor progression[16] without a change in visible phenotype. Normally a random stem cell drifts to dominance because mutations are rare in normal colon. However, a dominant stem cell could be selected by mutations that confer survival advantages over other stem cells.

Heterozygous *APC* mutations may confer selection through dominant-negative interactions or haploinsufficiency. Most *APC* mutations disrupt β-catenin (CTNNB1) interaction domains but leave N-terminal oligomerization domains intact, allowing for mutant and wild-type heterodimerization.[2,18] *APC* has a central role in WNT signaling pathways that modulate stem cell and niche dy-

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Table 1. Data for FAP and Non-FAP Colons

Patient	Age/sex	Disease*	Polyp number	Family history?	Germline *APC* mutation	Number of examined crypts	Average tags per crypt	Enhanced stem cell survival?
A	14.5/F	FAP	>500	Yes	1462 (AG deletion)	11	3.45	Yes
B	19/F	FAP	>500	No	1356 (TCA to TGA) stop	11	3.55	Yes
C	34/F	FAP	>500	No	1061 (5 bp deletion)	10	4.30	Yes
D	41/F	FAP	>500	Yes	Not in MCR	11	4.55	Yes
E	41/F	FAP	~150	No	213 (CGA to TGA) stop	10	3.50	No
F	58/F	AFAP	~20	No	2564 (TGG to TGGG)	10	3.20	No
G	17/F	Non-FAP		No		6	2.83	No
H	21/F	Non-FAP		No		10	2.91	No
I	25/F	Non-FAP		No		10	3.30	No
J	31/M	Non-FAP		No		10	3.10	No
K	34/M	Non-FAP		No		9	3.44	No
L	40/M	Non-FAP		No		7	2.57	No

*Colectomies were performed in non-FAP patients for cancer (patients G, I, J, and K), an adenoma (patient L), and a colostomy modification secondary to an imperforate anus at birth (patient H).

namics. For example, alteration of a β-catenin homologue changes stem cell survival in Drosophila gonadal niches,[19] and an activated β-catenin transgene appears to increase stem cell survival during murine brain development.[20]

Despite a potential to alter stem cell survival, heterozygous *APC* mutations appear inert (see Figure 1A) because FAP and non-FAP crypts are morphologically identical,[21,22] although crypt fission is more frequent in FAP.[23] The first *APC* mutation may predispose to tumorigenesis simply because biallelic *APC* mutations are necessary for neoplasia.[1] However, stem cells cannot be visualized, and normal or abnormal survival is usually inferred in model systems that artificially create heterogeneous appearing crypts.[11–15] Such experimental approaches are impractical in human colons, but recent studies[9] illustrate that colon stem cell niche dynamics can be inferred from crypt methylation patterns that arise during aging. An analysis of FAP crypt sequences may potentially detect altered stem cell survival because the diversity of a population reflects the time since a last bottleneck. Older populations have longer-lived lineages and therefore are more genetically diverse. If heterozygous *APC* mutations enhance stem cell survival, FAP niche succession should occur less frequently and crypt methylation patterns should be more diverse. Here we compare methylation patterns between FAP and non-FAP colons and infer that stem cell clonal evolution is slower and stem cell survival is enhanced in some normal appearing FAP colons.

Materials and Methods

Patients

Fresh colons were obtained from five FAP, one AFAP, and six non-FAP patients (Table 1). These colons have not been previously reported except for the non-FAP patient L, which was previously used to characterize normal niches.[9] All FAP colons had more than 100 polyps. Patient D had a previous partial colectomy at age 21 because of polyposis.

APC mutations were found by sequencing germline DNA from the AFAP and four FAP patients. DNA extracted from paraffin-embedded tissue was only available for FAP patient D and no *APC* mutation was found in the MCR. FAP patients B, C, and E apparently acquired new germline *APC* mutations because their family histories were negative for colorectal polyps or cancers.

Crypt Isolation

Crypts were isolated from ~1 to 2 cm^2 polyp-free patches of fresh colons with an ethylenediaminetetraacetic acid solution as previously described.[7] DNA was isolated from individual crypts in 10-μl volumes (100 mmol/L Tris-HCl, 4 mmol/L ethylenediaminetetraacetic acid, pH 8.0, with 200 μg/ml Proteinase K) for 4 hours at 56°C. After boiling for 5 minutes the DNA was bisulfite converted using an agarose bead method.[24]

Methylation Analysis

Bisulfite-treated DNA from individual crypts was amplified with primers for a CpG island present in the *CSX* gene (also called MINT23[25]) as previously described.[9] After 42 polymerase chain reaction cycles, products were cloned (TOPO TA cloning kit; Invitrogen, Carlsbad, CA), and sequences from eight individual clones were analyzed from each crypt. All C's not 5′ to G's were converted to T's, consistent with complete bisulfite conversion.

Methylation patterns are defined as the 5′ to 3′ order of methylation at CpG sites and each polymerase chain reaction product or tag can be summarized with a binary code (0 is unmethylated and 1 is methylated). The *CSX* polymerase chain reaction product has eight CpG sites and therefore 256 combinations or unique tags are possible. The multiple tags sequenced from each crypt can be described by two relevant measurements.[9] A measurement of crypt diversity is the number of unique tags per crypt. This value may range from one to eight because eight tags are sampled from each crypt. Average methylation is defined as the average number of CpG sites methylated in a tag or crypt.

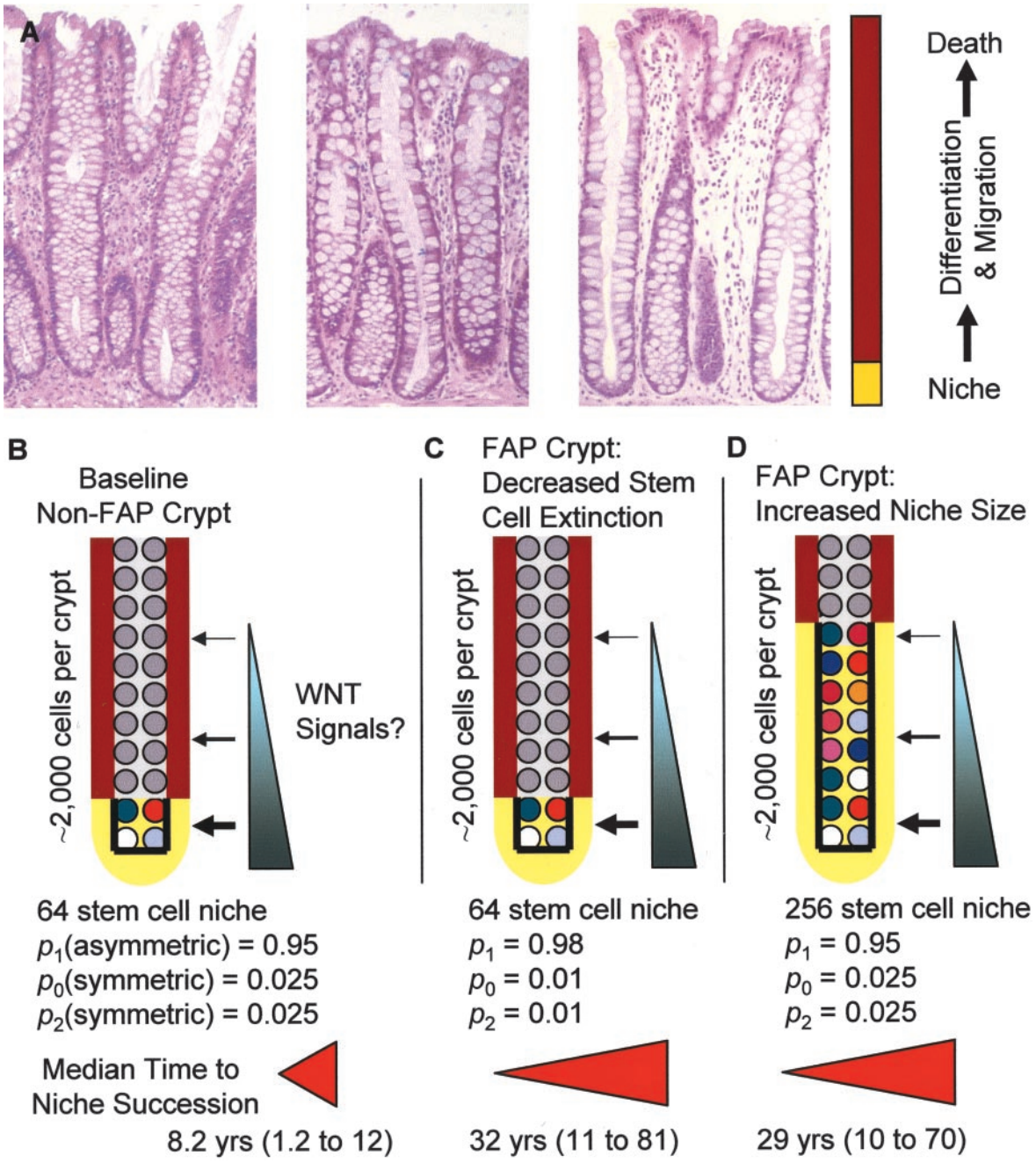

Figure 1. A: Microscopic sections of three colons. Although morphologically similar, they are (from **left** to **right**) FAP with enhanced stem cell survival (patient A), FAP without enhanced stem cell survival (patient E), and non-FAP (patient I) colons. The static images fail to reveal ongoing niche stem cell clonal evolution, cell migration/differentiation, and death. **B:** Normal niche scenario. Stem cells (**circles**) are located within a niche (yellow), possibly defined by WNT signals. In our non-FAP model a niche contains 64 stem cells, but only four are illustrated. After stem cell division, half the daughter cells leave the niche and differentiate. Most stem cell divisions are asymmetric (p_1 = 0.95), producing one differentiated and one stem cell daughter, but some divisions are symmetric, producing either two stem cell (lineage expansion) or two differentiated daughters (lineage extinction). Lineage extinction is balanced by lineage expansion ($p_0 = p_2 = 0.025$) such that niche stem cell numbers remain constant. Eventually all lineages except one are lost, resulting in niche succession (see also Figure 1 in Calabrese et al[16]). **C:** Enhanced stem cell survival in some FAP crypts may be because of decreased stem cell lineage extinction (p_0 changing from 0.025 to 0.01), possibly secondary to increased adhesion resulting in better retention of stem cell daughters. **D:** Enhanced stem cell survival may be because of increased sensitivity to WNT signals, which effectively expands niche size from 64 to 256 cells, or from 3 to 12% of all crypt cells. The crypt is visibly unchanged because there are no morphological features that distinguish stem cells from nonstem cells. Both FAP crypt scenarios slow stem cell clonal evolution, with niche succession intervals increased from ~8 to 30 years, which would increase intracrypt diversity because stem cells and their alterations persist longer.

Crypt Niche Simulations

Stem cell niches were simulated as previously described.[9] This model starts at birth with unmethylated CpG islands and predicts how percent methylation and numbers of unique tags per crypt (crypt diversity) increase with age. Parameters (Figure 1B) and their values for a baseline non-FAP niche scenario are numbers of stem cells (n = 64), probabilities of a stem cell division

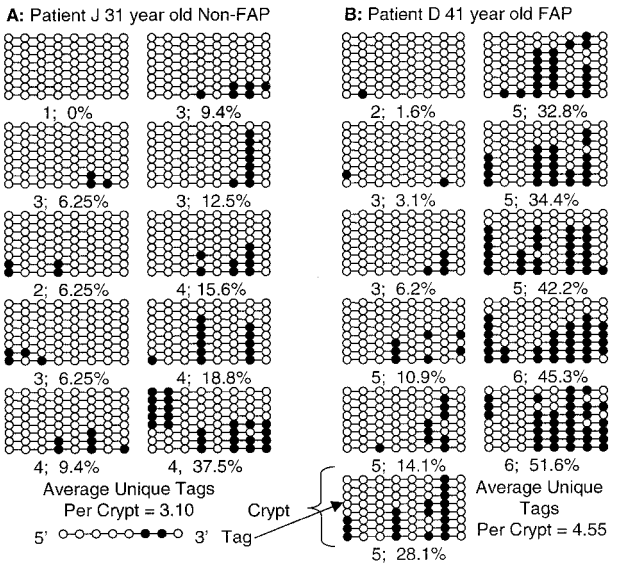

Figure 2. Crypt methylation patterns sampled from FAP and non-FAP patients. CSX tags contain eight CpG sites (**circles**), illustrated horizontally in a 5′ to 3′ direction. Methylated sites are **filled**. Each crypt is represented by eight individual CSX tags, with numbers of unique tags per crypt and percent methylation summarized below each crypt. Crypt diversity is greater in the FAP patient, with an average of 4.55 unique tags per crypt.

asymmetrically producing one differentiated and one stem cell daughter (p_1 = 0.95), or symmetrically two differentiated or two stem cell daughters (p_0, p_2, with $p_0 = p_2 = 0.025$), methylation error rates (2×10^{-5} per division per CpG site, with both methylation and demethylation possible), and stem cell division rates (once per day). At least 1000 simulations were performed for each scenario.

Results

Crypt methylation patterns represent complex interactions between random methylation errors and random stem cell loss with replacement. With aging, methylation patterns drift and eventually may differ between and within individual crypts. Consistent with underlying stochastic processes, methylation patterns sampled from five FAP, one AFAP, and six non-FAP patients (Table 1) were complex and different between crypts (Figure 2).

Percent crypt methylation was similar (P = 0.58, two-tailed t-test, df = 104) between the FAP (18.9%) and non-FAP (17.4%) patients (Figure 3). Methylation increases with aging at our locus,[9] and ages were similar (P = 0.78) between the FAP (average, 29.9 years) and non-FAP (average, 28.0 years) patients. However, numbers of unique tags per crypt were significantly greater (P = 0.0024, two-tailed t-test, df = 95) for FAP (average of 3.9 per crypt) compared to non-FAP (average of 3.1 per crypt) colons (Figure 3).

Although tag differences are numerically small between the FAP and non-FAP crypts, such small but significant differences may represent biologically important changes. Seemingly random crypt methylation patterns (Figure 2) encode stem cell histories because only a limited number of stochastic outcomes are consistent with specific niche scenarios. A previous analysis of three different CpG islands developed a model of normal human crypts maintained by niches containing multiple stem cells.[9] Changes in stem cell numbers, methylation error or division rates, or probabilities of symmetric *versus* asymmetric divisions may potentially explain differences between the FAP and non-FAP crypts.

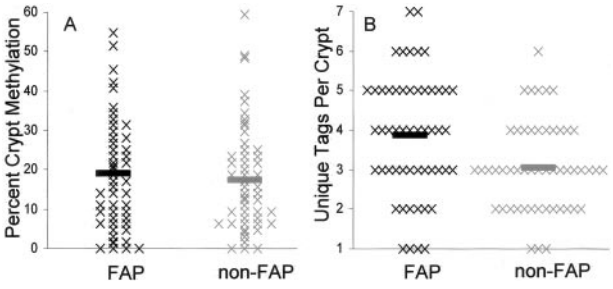

Figure 3. A: Methylation levels were similar between the five FAP and six non-FAP patients. **B:** Numbers of unique tags per crypt were significantly greater (P = 0.0024) in FAP crypts compared to non-FAP crypts. **X**, Individual crypt values; **bars**, average crypt values.

Individual crypt values may vary because of stochastic methylation errors and stem cell turnover. Therefore, to determine whether measured crypt values are consistent or inconsistent with various crypt model parameters, simulations were performed based on the ages and numbers of crypts sampled from each colon. These simulations produce varying outcomes, and intervals that include 95% of their outcomes were plotted on Figure 4. Average numbers of unique tags per crypt measured from the AFAP and the six non-FAP colons were consistent with the baseline crypt scenario (Figure 4). Only one FAP colon (patient E) was consistent with the baseline crypt scenario (Figure 4).

To find new crypt scenarios consistent with the FAP data, niche parameters were independently varied. An increased stem cell division rate could not further in-

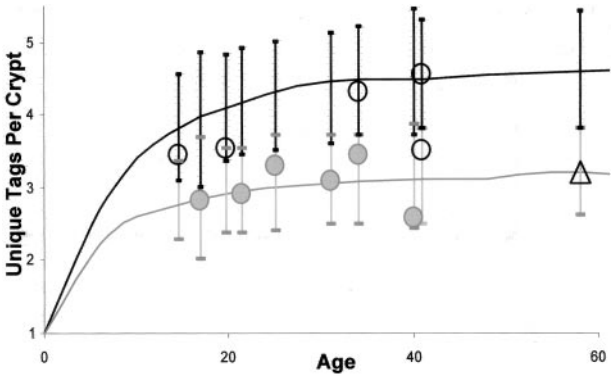

Figure 4. Plotted are average numbers of unique tags per crypt for FAP (**black circles**), non-FAP (**gray circles**), and AFAP (**black triangle**) colons with respect to age at removal. Simulations were performed based on the numbers of sampled crypts for each colon. The AFAP and all of the non-FAP colons were consistent with baseline niche parameters (**gray line** gives average values, **gray bars** indicate 95% of simulated outcomes). Only one FAP colon (from 41-year-old patient E) fell within the baseline niche simulation intervals. Simulations better fitting the other FAP colons (**black lines**) either increased stem cell numbers from 64 to 256 or decreased stem cell extinction (p_0 changing from 0.025 to 0.01).

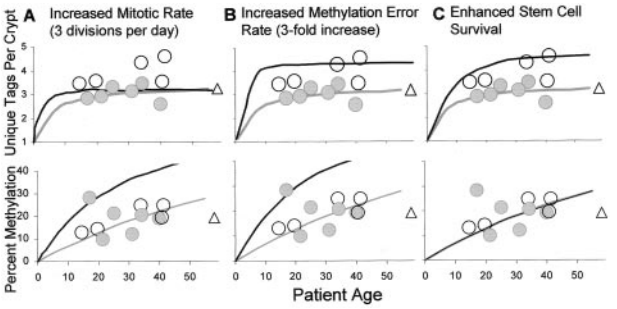

Figure 5. Alternative niche scenarios. Symbols are as in Figure 4, with **gray lines** representing average values of the non-FAP baseline scenario, and **black lines** representing crypts with different niche parameters. **A:** Increasing the stem cell mitotic rate (to three per day) does not increase niche diversity as a limit of ~3.2 unique tags is reached. **B:** Increasing the methylation rate (threefold to 6×10^{-5} per CpG site per division) can increase crypt diversity to FAP levels, but also increases crypt percent methylation to levels higher than experimentally observed. **C:** Enhanced stem cell survival (**black lines**) because of decreased stem cell extinction (p_o changing from 0.025 to 0.01) or an increased niche size (**green lines**, from 64 to 256 stem cells) matched increased FAP crypt diversity and percent methylation. Both enhanced stem cell survival scenarios gave equivalent outcomes (**black lines**).

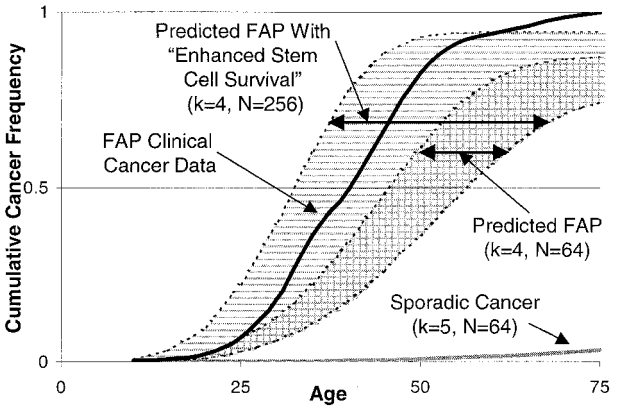

Figure 6. Cumulative cancer frequency *versus* age as a function of model parameters for our neutral pretumor progression model.[16] The **solid black line** is the data for FAP patients.[26] For parameters inferred from the SEER data set, our model predicts the **bottom gray line**. When we decrease the number of required mutations from five to four but otherwise keep the parameters the same, our model predicts the range in the **checkered region** between the two **bottom dotted lines**. When we also increase the number of stem cells per crypt, our model predicts the range in the region with **horizontal stripes** (including the **checkered region**). For all these model predictions the mutation rate is that inferred from the SEER data set.

crease diversity because a limit of ~3.2 average unique tags per crypt was observed even when rates were increased to three divisions per day (Figure 5A). A threefold increase in methylation error rates increased diversity to FAP levels, but predicted crypt percent methylation levels were higher than experimentally observed (Figure 5B). Therefore, increased FAP crypt diversity does not appear consistent with changes in methylation error or stem cell division rates.

In contrast, changes in niche size or stem cell survival increased diversity and still matched observed crypt methylation levels (Figures 4 and 5C). One FAP scenario decreased stem cell extinction with only 1% (instead of 2.5% with normal crypts) of divisions producing two differentiated daughters (Figure 1C). Another FAP scenario increased stem cell numbers fourfold from 64 to 256 per FAP crypt (Figure 1D). Both FAP crypt scenarios produced similar simulation outcomes (Figure 5C) that were consistent with FAP patients A to D (Figure 4). The FAP colon from patient E, and the AFAP and non-FAP colons were not consistent with these FAP models (Figure 4). The exact changes in FAP stem cell number or extinction probabilities are uncertain because larger or smaller changes could also produce simulation intervals that include the FAP data. The above values were chosen because their simulation intervals are consistent with the FAP data, but primarily distinct from the baseline niche model.

Clonal evolution is slower with enhanced stem cell survival because intervals between bottlenecks are increased. Calculated median times for niche succession were ~8.2 years (95% intervals of 2.7 to 19.2 years) for non-FAP crypts *versus* ~30 years (95% intervals of 10 to 81 years) for FAP crypts (Figure 1; B to D). The net effect is an increase in crypt diversity without an increase in error or mitotic rates.

Stem cell survival appeared dependent on mutation location, with enhanced survival associated with mutations that remove some but not all catenin-binding repeats (Table 1). The FAP colon with normal stem cell niche dynamics (patient E) had a truncating mutation near the N-terminus (codon 213), and its mutant protein would lack armadillo- and catenin-binding repeats. The AFAP patient had a distal 3′ mutation at codon 2564 that would retain all catenin repeats. Mutations associated with increased stem cell survival retained at least one catenin-binding motif. Mutations in patients A and B were within the MCR (codons 1462 and 1356) and remove some 20-amino acid catenin repeats. The mutation in patient C was within the 15-amino acid repeat region (codon 1061) and would delete the 20-amino acid catenin repeat region.

To determine how changes in stem cell survival may influence progression, we consider an independent data set from Ashley[26] that provides ages at cancer for FAP patients. Compared to sporadic cancers, FAP cancers may require one less somatic oncogenic mutation because an *APC* mutation is inherited. However, others[26,27] have found that in log-log plots of cancer incidence *versus* age, the slope for FAP patients decreases by more than one from the slope for the general colon cancer population. FAP cancers therefore appear earlier than expected if germline *APC* mutations simply reduced the total number of oncogenic mutations by one compared to sporadic cancers. For our pretumor progression model, when we compare the predicted cumulative cancer frequency with parameters inferred from the SEER data set[16] to the FAP data set there is little agreement (Figure 6). However, when we reduce the number of mutations from five to four, but keep the other parameters the same, the model's prediction is similar to the clinical FAP data (Figure 6). Further, when we also increase the number of stem cells per crypt consistent with the simulation described above, the model agrees even more with the FAP data (Figure 6). These calculations indicate that enhanced stem cell survival facilitates progression to cancer, and also

illustrate how our pretumor progression model can link stem cell survival with cancer epidemiology.

Discussion

Colorectal cancer progression is conceptualized as a visible adenoma-cancer sequence,[1] but earlier mutations may accumulate in normal appearing crypts during pretumor progression.[16] Stem cells are likely the earliest cancer progenitors because errors in nonstem cells will not accumulate.[28] Many errors may accumulate in crypt stem cells because many mutations are compatible with normal phenotypes.[10,29] Unfortunately, stem cell evolution would be occult to direct observations because stem cells cannot be visually identified.

Recent studies illustrate colon crypt stem cell survival can be reconstructed using methylation patterns as cell fate markers.[9] Human colon crypts are maintained by multiple stem cells that reside in niches. Methylation patterns are faithfully copied from generation to generation, but sometimes errors occur. Some errors will be propagated but most stem cells and their errors are lost during normal niche turnover.[16] Therefore, crypt methylation patterns reflect a balance between stochastic error loss and error creation. The higher methylation pattern diversity observed in some FAP colons is consistent with enhanced stem cell survival and slower niche stem cell clonal evolution.

Heterozygous APC Mutations Enhanced Stem Cell Survival

Niches are defined by inductive interactions between stem cells and surrounding mesenchyme.[14,15] Stem cell extinction results from a physical loss of inductive interactions by both daughter cells whereas clonal expansion is the retention of both daughter cells within the niche. Stem cell loss with replacement normally results in the random extinction of all niche stem cell lineages except one.[16] However, a dominant stem cell may also be selected if it acquires a mutation that increases its ability to remain within a niche.

WNT signaling pathways influence stem cell niche survival in Drosophila gonads,[19] hair follicles,[30] and murine intestinal crypts.[31] *APC* has a regulatory role in WNT pathways, binding to β-catenin and marking it for degradation.[2] Although *APC* protein levels appear greatest at crypt lumens,[32] low expression in stem cells and their daughters may help determine their fates. In the absence of *APC*, β-catenin enters the nucleus and with TCF factors activates transcription. TCF4/β-catenin-mediated transcription is essential for crypt stem cell survival because Tcf4-deficient mice lack stem cells.[31] Up-regulation of β-catenin/TCF4 transcription by dominant-negative *APC* mutations has been observed *in vitro*,[33] allowing for altered WNT signaling in normal FAP crypts. β-Catenin also interacts with cadherins to modulate cell adhesion. Elevated β-catenin levels in *APC*-deficient cells result in a somewhat paradoxical increase in cell adhesion,[34,35] which may favor niche stem cell retention.

Heterozygous *APC* mutations affecting stem cell survival appear to follow the same general pattern as polyposis,[2] but germline mutations outside the MCR still conferred enhanced stem cell survival. How heterozygous *APC* mutations affect stem cell survival is uncertain, but enhanced survival was associated with mutations (codons 1061, 1356, and 1462) that remove some but not all catenin-binding repeats. The FAP patient without enhanced stem cell survival had a proximal truncating mutation (codon 213) that removed all catenin-binding repeats. A 3′ AFAP mutation (codon 2564) would retain all catenin repeats and also did not exhibit evidence of enhanced stem cell survival.

The absolute number of stem cells per human colon crypt is unknown. A niche size of 64 was chosen for a baseline non-FAP crypt model[9] because its parameters follow the scale of smaller (400 *versus* 2000 cells per colon crypt) but better characterized murine crypts in which 5% of stem cell divisions are thought to be symmetrical.[12] Regardless of absolute niche size, the current data are consistent with enhanced stem cell survival in some FAP crypts, although our analysis cannot distinguish between reduced stem cell loss (Figure 1C), increased numbers of stem cells (Figure 1D), or some combination of these changes. Heterozygous *APC* mutations may increase epithelial cell sensitivity to niche WNT signals, effectively increasing niche size and stem cell number. Heterozygous *APC* mutations may also increase stem cell adhesion to surrounding niche mesenchyme, resulting in decreased stem cell extinction.

Consistent with morphological similarities between normal FAP and non-FAP crypts,[21,22] the enhanced stem cell survival inferred by our analysis would be occult (Figure 1). Crypt morphology, cell numbers, division, migration, and death rates would be unchanged. For example, with or without decreased extinction, every day 64 stem cells divide and 64 stem cell daughters leave the niche and differentiate. The only change is how niche positions are inherited among visually identical stem cell daughters. In the absence of stem cell markers, niche expansion or decreased stem cell lineage extinction would be unrecognizable.

Other FAP studies are consistent with enhanced stem cell survival. Somatic mutation frequencies measured by visible losses of O-acetyltransferase crypt activity are increased threefold in nonneoplastic FAP colons.[36] Enhanced stem cell survival would increase mutation frequencies because more stem cells would be at risk, or such neutral mutations would persist for greater amounts of time. A quantitative study of a labeling index shift after bromodeoxyuridine injection was also consistent with a numerical stem cell increase in FAP crypts.[37]

Pathways to Cancer: APC and Clonal Evolution

Our pretumor progression model may explain why certain pathways to cancer are more common than others. In

non-FAP colons, *APC* alleles are wild-type and niche stem cell survival is random. Mutations could occur anywhere within the *APC* locus, but mutations that do not enhance stem cell survival will be frequently lost during normal stem cell turnover. An *APC* mutation near its MCR appears to enhance stem cell survival, perhaps by increasing niche adhesion or its sensitivity to WNT signals. Although this enhanced stem cell survival was inferred at equilibrium in FAP crypts when all stem cells harbor the same mutation, it is easy to envision that the survival advantage acquired somatically would allow for a rapid selective sweep of a wild-type niche. A selective sweep would increase the number of *APC* mutant cells by division rather than mutation and essentially convert a wild-type crypt into a FAP crypt. Therefore, *APC* mutations around its MCR would be fixed more frequently and appear more frequently in sporadic cancers.

Despite enhanced stem cell survival, FAP crypts were still defined by niches, suggesting immortal stem cells are incompatible with normal colon morphology or pretumor progression. Another *APC* mutation could confer additional selective advantages and displace other less fit niche stem cells. In this way, niches could continuously select for second or even third *APC* mutations[2,38] during pretumor progression. Niche selection could also continue during tumor progression because adenomas appear to maintain a crypt hierarchy, with adenoma stem cells and more differentiated adenoma cells.[39,40]

FAP niches not only allow for additional selective mutations, but also facilitate the accumulation of initially neutral mutations by slowing stem cell clonal evolution. Intervals before extinction are longer in FAP colons, with niche succession occurring on average approximately every 30 years *versus* approximately every 8.2 years in non-FAP crypts (compare Figure 5B with 5A in Calabrese et al[16]). Therefore, initially neutral mutations will persist longer in FAP crypts, increasing the probability of acquiring other mutations that eventually and collectively confer a tumor phenotype. This effect (a stem cell acquiring other mutations before fixation) is similar to the stochastic tunneling progression shortcut described by Nowak and colleagues.[41] *APC* mutations appear to be common in cancers because they confer both niche selection and slow down subsequent clonal evolution, a combination predicted to be among the fastest pathways to cancer if most pretumor mutations are initially neutral.[16] FAP patient E without enhanced stem cell survival illustrates the potential importance of stem cell persistence and subsequent crypt diversity to progression. She had few polyps (~150) and became symptomatic from intestinal blood loss relatively late in life (at 41 years of age), suggesting delayed development of her polyposis.

FAP cancers appear clinically earlier than expected if germline *APC* mutations merely reduce total numbers of oncogenic mutations by one relative to sporadic cancers.[26,27] Our analysis and others[26,27,42,43] illustrate that small changes in survival during early progression can influence subsequent cancer rates. Our model demonstrates how a single mutation can increase subsequent progression rates by more than a single step, without changing visible phenotype, mutation, or division rates

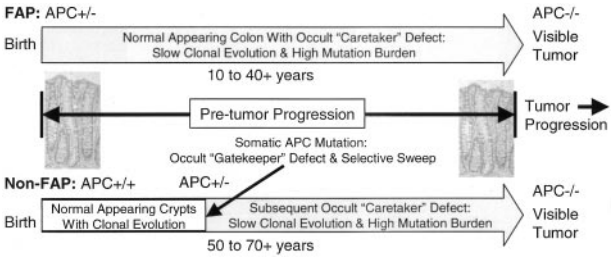

Figure 7. Pretumor progression precedes tumor progression. FAP and non-FAP colons are normal at birth and tumors appear decades later. In FAP, enhanced stem cell survival conferred by some germline *APC* mutations results in slower stem cell clonal evolution or a caretaker defect from birth, leading to a higher mutation burden within each crypt. In non-FAP colons, a somatic *APC* mutation initiates a limited gatekeeper defect that allows a selective niche sweep but not neoplasia. After fixation, the non-FAP *APC*+/− crypt would similarly accumulate a higher mutation burden because of enhanced stem cell survival. Loss of both *APC* alleles exposes a gatekeeper defect, which ends pretumor progression and starts tumor progression.

(Figure 6). AFAP may be associated with inherited *APC* mutations that reduce the total number of somatic oncogenic mutations required for cancer, but do not enhance stem cell survival (like patients E and F), resulting in cancers 10 to 15 years later than FAP.[44] Many factors likely influence progression rates or stem cell survival because identical germline *APC* mutations may yield different FAP phenotypes.[45]

Summary

The roles of *APC* mutations may differ during progression. Specific heterozygous *APC* mutations appear to promote pretumor progression by combining "gatekeeper" and "caretaker" niche defects (Figure 7). Higher FAP crypt diversity is similar to the increased mutation burden expected after caretaker mutations,[46] except diversity results from slower clonal evolution rather than increased mutation rates. In FAP, this "caretaker" defect would start from birth, conserving initially neutral mutations in normal colon until tumors appear decades later. A sporadic *APC* mutation could initiate a limited "gatekeeper" defect allowing a visibly occult selective sweep of its niche, followed by a "caretaker" defect comparable to FAP crypts (Figure 7). Mutation of the second *APC* allele may be a "gatekeeper defect" that causes a permanent imbalance of cell division over cell death, or neoplasia.[1]

The relative importance of selection and survival during stem cell clonal evolution depends on the extent of pretumor progression. If most progression occurs in tumors, enhanced stem cell survival would be of little consequence because a start involves any of millions of essentially identical nonmutant stem cells. However, if a number of mutations accumulate in normal appearing colon, mutations that enhance stem cell survival would be selected for because extinction avoidance and the accumulation of mutations are prerequisites for further pretumor progression. Biallelic *APC* mutations are found in the majority of colorectal cancers,[2] and enhanced stem cell survival may provide a historical explanation for why certain *APC* mutations are so common in colorectal cancers.

Prospectus

A pretumor progression model proposes most oncogenic cancer mutations first occur in normal appearing colon.[16] It is unlikely our model captures all relevant parameters, but it illustrates the type of data and analysis required to reconstruct events before tumorigenesis. Central to our model is the clonal evolution of stem cell populations, which is inherent to niches. Multiple niche stem cells allow for selection, limited clonal expansions, and progression without elevated mutation rates or tumorigenesis. Evidence for our model is scant compared to the adenoma-cancer sequence because in the absence of visual progression clues, pretumor progression must be inferred. Literally none of its changes would ever be seen (Figure 1). Nevertheless, we demonstrate theoretical feasibility[16] and provide experimental data that support major features of pretumor progression. Ages at DNA mismatch repair loss suggest most oncogenic cancer mutations accumulate before tumorigenesis or instability.[47] Enhanced stem cell survival associated with certain heterozygous *APC* mutations is consistent with selection and niche clonal evolution during pretumor progression. In the absence of tumorigenesis or elevated mutation rates, subtle early changes in stem cell niche dynamics acting over many years may favor progression because *APC* mutations are found in most colorectal cancers. Progression is likely to be heterogeneous and a pretumor progression model may best fit cancers that appear shortly after negative clinical examinations. Pretumor progression may be important for a variety of cancers because most mammalian tissues appear to be maintained by stem cell niches[15] and common tumors visibly appear late in life. Cancers and their mutations may reflect a lifetime of competition, first between stem cells during pretumor progression, and later between tumor cells during tumor progression.

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