Colon Tumors with the Simultaneous Induction of Driver Mutations in APC, KRAS, and PIK3CA Still Progress through the Adenoma-to-carcinoma Sequence

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Abstract

Human colorectal cancers often possess multiple mutations, including three to six driver mutations per tumor. The timing of when these mutations occur during tumor development and progression continues to be debated. More advanced lesions carry a greater number of driver mutations, indicating that colon tumors might progress from adenomas to carcinomas through the stepwise accumulation of mutations following tumor initiation. However, mutations that have been implicated in tumor progression have been identified in normal-appearing epithelial cells of the colon, leaving the possibility that these mutations might be present before the initiation of tumorigenesis. We utilized mouse models of colon cancer to investigate whether tumorigenesis still occurs through the adenoma-to-carcinoma sequence when multiple mutations are present at the time of tumor initiation. To create a model in which tumors could concomitantly possess mutations in Apc, Kras, and Pik3ca, we developed a novel minimally invasive technique to administer an adenovirus expressing Cre recombinase to a focal region of the colon. Here, we demonstrate that the presence of these additional driver mutations at the time of tumor initiation results in increased tumor multiplicity and an increased rate of progression to invasive adenocarcinomas. These cancers can even metastasize to retroperitoneal lymph nodes or the liver. However, despite having as many as three concomitant driver mutations at the time of initiation, these tumors still proceed through the adenoma-to-carcinoma sequence. Cancer Prev Res; 8(10); 952–61. ©2015 AACR.

Introduction

Colorectal cancer is the second-leading cause of cancer-related mortality in the United States (1). An improved understanding of the processes by which tumorigenesis occurs will allow for the rational development of chemopreventive and therapeutic agents. The canonical adenoma-to-carcinoma sequence has been proposed to describe the processes by which mutations in driver genes accumulate over time causing the progression of adenomas to invasive adenocarcinomas in the colon (2, 3).

An important early step in tumorigenesis is the acquisition of alterations in the Adenomatous Polyposis Coli (APC) tumor suppressor gene. Loss of this gatekeeper gene is thought to be the initiating event in the majority of sporadic human colorectal cancers with approximately 80% to 90% of human colon cancers harboring somatic mutations in APC (4–6). In the canonical sequence, tumor initiation caused by loss of APC is followed by mutations in other genes including KRAS, TP53, PIK3CA, and BRAF (7). Some studies have questioned whether the accumulation of mutations over time is necessary for colon cancer development (8–11). For example, KRAS mutations have been detected in non-neoplastic tissue of the colon (8, 12). This observation indicates that somatic mutations occurring before tumor initiation might subsequently influence tumor development and progression (13). In addition, it could be a potential explanation for interval cancers that form between routine screening colonoscopies.

Because of the prevalence of APC loss in human colorectal cancer, mice carrying mutations in Apc, the murine homolog, have been generated to more fully understand intestinal tumorigenesis and investigate pharmacologic strategies for chemoprevention (14–16). Here, we utilized genetically engineered mouse models of colon cancer to examine the adenoma-to-carcinoma sequence in the setting of the concurrent induction of common driver mutations. We developed a minimally invasive procedure for inoculating an adenovirus expressing the Cre recombinase (Adeno-Cre) to induce multiple mutations at a desired location. An important early step in tumorigenesis is the acquisition of alterations in the Adenomatous Polyposis Coli (APC) tumor suppressor gene. Loss of this gatekeeper gene is thought to be the initiating event in the majority of sporadic human colorectal cancers with approximately 80% to 90% of human colon cancers harboring somatic mutations in APC (4–6). In the canonical sequence, tumor initiation caused by loss of APC is followed by mutations in other genes including KRAS, TP53, PIK3CA, and BRAF (7). Some studies have questioned whether the accumulation of mutations over time is necessary for colon cancer development (8–11). For example, KRAS mutations have been detected in non-neoplastic tissue of the colon (8, 12). This observation indicates that somatic mutations occurring before tumor initiation might subsequently influence tumor development and progression (13). In addition, it could be a potential explanation for interval cancers that form between routine screening colonoscopies.

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in the colon. This method allows for tumors to be initiated with a predetermined mutation profile at a specific time and location desired by the investigator.

**Materials and Methods**

**Mouse husbandry**

All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison (Madison, WI), following the guidelines of the American Association for the Assessment and Accreditation of Laboratory Animal Care. FC mice (FVB/N-Tg [Fabp1-Cre]1Htg; NCI Mouse Repository, strain number 01XDA), Kras^{G12D/+}, mice (C57BL/6J Gt(Rosa)26Sortm7(Cg krej39)/E125Mr1), Pik3cap110^{+/−} mice (C57BL/6Gt(Rosa)26Sortm7(Pik3cap110^{−/−}; E125Mr1); The Jackson Laboratory, stock number 019104), Pik3cap110^{+/−} mice (C57BL/6Gt(Rosa)26Sortm7(Pik3cap110^{−/−}; E125Mr1); The Jackson Laboratory, stock number 012343), Apc^{+/−} mice (C57BL/6. Cg-Apc^{−/−}; Nci; NCI Mouse Repository, strain number 01XAA), and mT/mG mice (B6.129(Cg-Gt[Rosa]26Sortm1(CAG-tdTomato, -EGFP)Luo/J; The Jackson Laboratory, stock number 007676) were maintained and genotyped as previously described (17–21). For FC and mT/mG, a ± denotes carrier and a − noncarrier. Pik3cap110^{+/−} and Kras^{G12D/+} mice were also crossed to Apc^{+/−} mice to generate Apc^{+/−} Kras^{G12D/+}, Apc^{+/−} Pik3cap110^{+/−}, and Apc^{+/−} Kras^{G12D/+} Pik3cap110^{+/−} mice on a homozygous B6 genetic background for Adeno-Cre delivery. In addition, mT/mG mice were crossed to generate B6 mT/mG^{−/−} Kras^{G12D/+} Pik3cap110^{+/−} mice. All Pik3cap110^{+/−} mice were hemizygous for this allele.

**Nonsurgical exposure of the colon to Adeno-Cre**

Polyethylene tubing (I.D. 1.4 mm, O.D. 1.90 mm; Becton Dickinson, Sparks, MD) was cut to sizes appropriate for mice (7–12 cm). A 1-cm window was notched into the tubing and the end of the tubing was closed with edges being rounded to avoid perforation of the bowel (Fig. 1A). Marks corresponding to 1 cm intervals were made on the tubing. A longitudinal stripe was also applied corresponding to the orientation of the window. Similarly sized polyethylene tubing was cut to size without a slot to be used as a sheath for a 2.2-mm caliber soft bristle brush (DenTek Oral Care).

Mice were anesthetized using 2% isoflurane. The colon was irrigated with PBS. A narrow ribbon of GelFoam (Pharmacia and Upjohn) was inserted into the window cut into the polyethylene tube. Of note, 200 μL of 0.05% trypsin (Hyclone) was injected into the tubing and inserted into the mouse colon at the desired depth and radial orientation. After 10 minutes, the slotted tube was removed and the sheath with the small soft bristle brush was introduced at the same intraluminal location. The brush was then used to abrade the epithelium for up to 3 minutes. After PBS irrigation, a slotted tubing containing GelFoam was then filled with 200 μL PBS containing 10^5 PFU of Adeno-Cre (Ad5CMVCre and Ad5CMVEmpty, University of Iowa Gene Transfer Vector Core, IA). After 30 minutes of incubation, the tubing was removed. Owing to the anatomical limitations of the mouse, only the most distal half of the colon (~4 cm) could be inoculated in this way. Mice recovered quickly after the procedure and did not exhibit overt signs of pain or distress following the procedure, as they quickly became active. Note that nonsteroidal anti-inflammatory drugs were not used for post-procedure analgesia as these agents have been shown to suppress intestinal tumorigenesis in both humans and laboratory mice.

**Figure 1.**

A minimally invasive technique for inoculation of the colon with Adeno-Cre initiates tumorigenesis in Apc^{−/−} mice. We have developed a novel technique that can be utilized to sequester Adeno-Cre within the colon without laparotomy, as was required with prior methodologies. A, in this technique, a slotted tube housing GelFoam allows for the treatment of the colon with trypsin. Abrasion of the colon is then performed before incubation with Adeno-Cre-soaked GelFoam at the desired location. B, colonic tumors are visualized by endoscopy in 3 to 7 weeks following treatment with Adeno-Cre and monitored over time with serial endoscopy. C, interestingly, these tumors have varying growth patterns over 14 weeks of observation, despite a similar initiation. D, histologic evaluation demonstrated that the majority (83%) of these lesions were adenomas with nuclear CTNNB1 consistent with activation of the WNT pathway. The area indicated by the rectangle is shown enlarged at the right; at the far right the same area in an adjacent slide stained for CTNNB1 is shown. Size bar = 1 mm.
Murine colonoscopy/PET imaging
Mice were anesthetized using 2% isoflurane and the colons were flushed with PBS. The Coloview System was used to monitor tumor formation and growth in the distal half of the colon as previously described (Karl Storz; ref. 22). Image analysis was utilized to measure the percent lumen occlusion as previously described (23).

Animals were fasted for at least 6 hours before injection of 18F-FDG (160 μCi; IBA Molecular). After injection, the animals were kept under anesthesia for 60 minutes and then prepared for microPET colonography as described previously (24).

Histology and IHC
Mice were euthanized and the colons were excised and opened longitudinally. Tumor tissue was fixed in 10% buffered formalin. Fixed tumors were embedded in paraffin and cut into 5 μm sections. Every tenth section was stained with hematoxylin and eosin (H&E) for histologic review. IHC was carried out as described previously (25) with the exception that tissues were blocked with Background Sniper (Biocare Medical) for 10 minutes. The primary antibodies included rabbit anti-phospho-AKT (Ser473, 1:1000, Cell Signaling Technology #4060), rabbit anti-phospho-S6 Ribosomal Protein (Ser235/236, 1:50, Cell Signaling Technology #4858), rabbit anti-CTNNB1 (1:100–1:200, Cell Signaling Technology #8480), rabbit anti-Ki67 (1:400, Cell Signaling Technology #12202), and rabbit anti-phospho-ERK1/2 (1:400, Cell Signaling Technology #4370). The Ki-67 proliferation index was measured as the percent of nuclei staining positive for Ki-67 per tumor using ImmunoRatio, an ImageJ plugin (http://jmscimicroscope.uta.uthscsa.edu/sites/default/files/software/immunoratio-plugin/index.html).

Recombination testing
To validate that all alleles were recombined within tumors, neoplastic tissue was scraped from FFPE sections with a sterile size 10 surgical blade with normal epithelium left behind. DNA was isolated from the scraped tissue using the Maxwell 16 FFPE Tissue LEV DNA Purification Kit (Promega). Samples with a sufficient amount of DNA were analyzed for Cre-mediated recombination between loxP sites.

Recombination of the loxP sites within the ApcΔ allele and upstream of the KRASG12D allele was confirmed using previously described PCR primers and protocols (http://jacks-lab.mit.edu/protocols/genotyping/kras_cond; ref. 20). Recombination of the loxP sites upstream of the Pik3cdΔ allele was confirmed using the forward primer 5′-GGACTGTCGAGGA-CATTCTCCT-3′ and the reverse primer 5′-AACCATAATCCAC-CAACCT-3′. Cycling conditions were one cycle of 94°C for 3 minutes, followed by 35 cycles of 94°C for 20 seconds, 61°C for 60 seconds, and 72°C for 60 seconds, with one final extension cycle at 72°C for 2 minutes. Recombined DNA resulted in amplification of a 561-bp product.

Results
Colon tumors can be initiated using a noninvasive inoculation of the Adeno-Cre virus
KRAS and PIK3CA mutations have been identified as occurring concomitantly with the loss of APC in human adenomas and carcinomas (26, 27). Multiple investigations have examined murine models with combinations of loss of APC and activating mutations in either KRAS or PIK3CA (28–31). These studies had limitations. C57BL/6 (B6) mice carrying the Min allele of Apc (ApcMin/−) have a ubiquitous spatial and temporal expression of a mutant germline allele resulting in alterations of homeostasis along the entire intestinal tract (14). The life expectancy of these mice is limited due to their high tumor burden, especially in the small intestine, resulting in anemia or intestinal obstruction. B6 ApcMin/− mice do not develop advanced colon cancers as a consequence of this shortened lifespan. Finally, triggering multiple mutations in the colon concurrently by expressing Cre recombinase from the FABP-1 promoter is lethal (Supplementary Fig. S1). To determine whether additional concomitant mutations would alter the progression of colon tumors through the adenoma-to-carcinoma sequence alternative methodologies were required. A model allowing for spatial control of tumor initiation that can be used to express a number of mutations was needed to further explore their effects on tumorigenesis.

Although there are multiple methods of expressing Cre in the mouse, including organ-specific promoter-based systems, the delivery of an adenovirus carrying Cre recombinase allows for the greatest spatio-temporal control of initiation of colon tumors (18). To sequester the virus in the colon, an invasive laparotomy procedure has been described (23). Surgical clips were directly placed both proximally and distally around the colon after exposure of the peritoneal cavity. Adeno-Cre was then injected into the area between the clips. This surgical Adeno-Cre instillation technique allows for the development of colon tumors without the comorbidities related to Apc mutations occurring outside of the area of interest. However, this surgical intervention is no trivial task; preparation, surgery, and recovery may all contribute detrimental effects to the overall health of the animal. Unintended consequences from surgical wounds may alter various cellular mechanisms, including inflammatory and wound-healing responses, involved in tumorigenesis (32).

To overcome limitations of the surgical approach, we developed a device to nonsurgically inoculate Adeno-Cre into the colon in homozygous Apc fiz mice (Fig. 1A; ref. 20). Upon expression of Cre recombinase in these mice, exon 14 of Apc is excised, resulting in the expression of a truncated, non-functional APC protein. Cre expression controls the location and time at which tumors form, in turn alleviating many of the limitations associated with models carrying germline Apc mutations. This nonsurgical approach induced tumorigenesis in a physiologically relevant setting, while minimizing the systemic effects of an operative procedure.

Biocompatible materials have been used to enhance the delivery of viruses in a variety of research settings (33). One such biomaterial, GelFoam (Pharmacia and Upjohn), is a collagen-based sponge. It has been used to deliver viruses in gene therapy research due to its ability to localize and protect virus (34). GelFoam saturated in adenovirus solution has been implanted in mice and rats to facilitate gene expression for the study of wound healing and to elicit immune responses in tumors (35, 36). These studies have demonstrated that GelFoam can be used to improve the sustained delivery of adenovirus in vivo. Therefore, we found it an attractive alternative to surgery for use in localizing the virus in specific areas of the mouse.

A slotted tube housing GelFoam soaked with Adeno-Cre was placed 1.5 to 3.5 cm into the colon of each prepared Apc fiz mouse (see Materials and Methods). After the procedure, tumor formation and growth were monitored by endoscopy. Tumors were visible as early as 3 weeks post-treatment and these tumors were
monitored over 14 weeks post-treatment. Fifty-eight percent (18/31) of treated Apc\(^{fl/fl}\) mice developed tumors. Tumors originated at the desired location, both in radial orientation and depth in the colon (Fig. 1B). No tumors developed in mice infected with adenovirus carrying an empty vector.

**Differential growth patterns occur in tumors after the simultaneous loss of both normal APC alleles**

Small animal endoscopy was performed every other week over a 14-week period to follow tumor growth in homozygous Apc\(^{fl/fl}\) mice treated with Adeno-Cre. Tumors visible one month after adenoviral treatment were relatively small, occluding less than 30% of the lumen. However, in the following weeks, tumors exhibited many different patterns of growth (Fig. 1C). Some tumors grew rapidly to eventually occlude the entire lumen, while others grew more incrementally, or even remained stable in size over this time. One small tumor ultimately regressed. These dynamic growth patterns, observed even in the earliest adenomas, were similar to tumor growth patterns in other murine colon tumor models with loss of APC (25).

After 14 weeks of monitoring, necropsy was performed. None of the mice became moribund before this time point. Histologic evaluation was completed on six tumors which revealed that 83% were adenomas, and 17% were invasive adenocarcinomas with invasion into the muscularis mucosa (Fig. 1D). No evidence of regional nodal disease or metastatic disease was observed.

**Epithelial tumors arise secondary to inoculation of the colon with Adeno-Cre**

When the Adeno-Cre virus is instilled into the colon, it is possible that the virus is infecting cells other than the epithelial cells lining the intestine. To make certain that only epithelial cells were being infected with the Adeno-Cre virus, mT/mG\(^{fl/fl}\) Apc\(^{fl/fl}\), Kras\(^{G12D}\), Pik3cap110\(^{++}\) mice were treated with Adeno-Cre. mT/mG is a reporter from which red (tdTomato) fluorescent protein is ubiquitously expressed preceding Cre recombinase expression and GFP is expressed following Cre recombinase expression. Green fluorescence was detected only within the colonic epithelium confirming that the intended cell type was being infected with the Adeno-Cre (Fig. 2). Nuclear localization of CTNNB1 (β-catenin) and activation of the anticipated downstream signaling cascades were observed within the transformed epithelial cells indicating that recombination following Cre expression was occurring as anticipated (Fig. 2).

**Adeno-Cre tumor initiation rate depends upon the mutation profile**

Our minimally invasive technique allows for temporal and spatial control of the initiation of colon tumors with multiple simultaneous mutations. Mice with Apc\(^{fl/fl}\), Kras\(^{G12D}\), and/or Pik3cap110\(^{++}\) were generated and treated with Adeno-Cre (Fig. 3A). After 3 to 4 weeks, tumors were identified on endoscopy. When additional driver mutations were present in addition to the loss of APC, an increase in the rate of tumor development was observed. Tumors developed in 79% of Apc\(^{fl/fl}\) mice carrying Kras\(^{G12D}\) and/or Pik3cap110\(^{++}\) compared with 58% of Apc\(^{fl/fl}\) mice without these additional driver mutations [Fig. 3A, \(P\) (one-sided) = 0.031, Barnard exact test]. All mice were treated according to the same Adeno-Cre protocol and on the same homogeneous genetic background. In the 14 samples that were successfully tested, recombination was confirmed in nearly all cases (12/14, Supplementary Fig. S2). Exceptions
were found within two of three samples from a single Apc\(^{fl/fl}\) Kras\(^{G12D/+}\) Pik3cap110\(^{+/-}\) mouse: only two of the three Cre-dependent alleles appeared to recombine in two tumors, but all three Cre-dependent alleles recombined in the third tumor.

In the setting of multiple mutations, Adeno-Cre induction resulted in adenoma formation.

Mice with Apc\(^{fl/fl}\) plus Kras\(^{G12D/+}\) and/or Pik3cap110\(^{+/-}\) were generated and treated with Adeno-Cre to determine how the simultaneous induction of these mutations would alter tumor biology. Tumors were identified 3 to 8 weeks after viral treatment (Fig. 3B). Histologic sectioning of tumors revealed that all tumors from these mice carrying mutations in two to three genes at this early time point were small adenomas. An example of a small adenoma from an Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) mouse is presented in Fig. 3B. The expected nuclear localization of CTNNB1 and activation of the PI3K cascade with abundant phosphorylation of RPS6 were observed (Fig. 3B).

The addition of Kras and Pik3ca mutations does not significantly alter the rate of proliferation in colon tumors compared with loss of APC alone.

To estimate the rate at which the tumors with different mutation profiles were growing, Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) and Apc\(^{fl/fl}\) Kras\(^{G12D/+}\) Pik3cap110\(^{+/-}\) mice were monitored with serial endoscopy every 2 weeks following treatment with Adeno-Cre (Fig. 4A). Lumen occlusion by the colonic tumor was used as a marker of cellular proliferation within tumors. Occlusion of 75% of the colonic lumen was observed in 65% of tumors in Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) and Apc\(^{fl/fl}\) Kras\(^{G12D/+}\) Pik3cap110\(^{+/-}\) mice. For the tumors that eventually occurred at least 75% of the lumen, the mean time required to reach 75% occlusion was 15 weeks. No statistically significant difference in the mean time to 75% lumen occlusion was observed between tumors with APC loss and the addition of 1 or 2 oncocenes (Fig. 4B). Interestingly, in one instance, a tumor from an Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) mouse regressed (Supplementary Fig. S3). Histologic analysis of this regressed area revealed a predominant lymphocytic infiltrate, indicating a potential immune-mediated mechanism for the regulation of some colon tumors.

To confirm that mean time to lumen occlusion as is a useful marker for cellular proliferation within tumors, histologic sectioning of tumors from Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) and Apc\(^{fl/fl}\) Kras\(^{G12D/+}\) Pik3cap110\(^{+/-}\) mice was performed and these sections were stained for Ki-67, a standard marker of cell proliferation. Occlusion of 75% of the colonic lumen was observed in 65% of tumors in Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) and Apc\(^{fl/fl}\) Kras\(^{G12D/+}\) Pik3cap110\(^{+/-}\) mice. The mean Ki-67 PI of tumors from these strains was pooled (31/39) for comparison with Apc\(^{fl/fl}\) Kras\(^{G12D/+}\) (18/31); \(P\) (one-sided) = 0.031, Barnard exact test. B, tumors were identified by endoscopy in the colon as early as 3 to 4 weeks after viral inoculation. Histologic sectioning was performed on tumors shortly after identification on endoscopy revealing that these lesions were small adenomas without evidence of invasion into the muscularis mucosa. An adenoma from an Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) mouse is shown here. Nuclear localization of CTNNB1 and phosphorylation of RPS6 were observed without phosphorylation of ERK1/2 indicating that recombination was occurring as expected. The area indicated by each rectangle is shown enlarged at the right. Size bar = 500 μm.

Figure 3. Apc\(^{fl/fl}\) Kras\(^{G12D/+}\) (A\(^{K2P}\)), Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) (A\(^{K2P}\)), and Apc\(^{fl/fl}\) Kras\(^{G12D/+}\)-Pik3cap110\(^{+/-}\) mice were treated with Adeno-Cre at 40 to 50 days of age. A, incidence of tumors increases with the addition of Kras\(^{G12D/+}\) and/or Pik3cap110\(^{+/-}\) to the mutation profile (due to the relatively small sample sizes and the similar tumor incidence rates among A\(^{K2P}\), A\(^{K2P}\), and A\(^{K2P}\) mice, the tumor incidence data from these strains were pooled (31/39) for comparison with A\(^{K2P}\) (18/31); \(P\) (one-sided) = 0.031, Barnard exact test). B, tumors were identified by endoscopy in the colon as early as 3 to 4 weeks after viral inoculation. Histologic sectioning was performed on tumors shortly after identification on endoscopy revealing that these lesions were small adenomas without evidence of invasion into the muscularis mucosa. An adenoma from an Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) mouse is shown here. Nuclear localization of CTNNB1 and phosphorylation of RPS6 were observed without phosphorylation of ERK1/2 indicating that recombination was occurring as expected. The area indicated by each rectangle is shown enlarged at the right. Size bar = 500 μm.

Tumors with simultaneous loss of Apc and oncogenic mutations in Kras and Pik3ca progress from adenomas to adenocarcinomas to metastatic disease.

Additional Apc\(^{fl/fl}\) Pik3cap110\(^{+/-}\) and Apc\(^{fl/fl}\) Kras\(^{G12D/+}\) Pik3cap110\(^{+/-}\) mice were monitored with serial endoscopies (Supplementary Table S1). After 14 weeks, subsets of these mice underwent necropsy. At this time-point, approximately 50% of tumors from each these genetic profiles were invasive adenocarcinomas. The
The timing of driver mutations versus tumor progression

Timing of Driver Mutations versus Tumor Progression

Figure 4.
The addition of Kras and Pik3ca mutations to the loss of APC has a modest effect on tumor proliferation. A, Apcfl/fl KrasG12D/+ and Apcfl/fl Pik3cap110+/+ mice were aged until moribund. No difference in survival was observed between the two groups with a median survival of approximately 200 days (Fig. 5A). The tumors progressed from small polyloid lesions to invasive adenocarcinomas that filled the majority of the colonic lumen (Fig. 5B). These tumors even progressed to “apple-core” lesions often seen in advanced colon cancers in humans (Fig. 5B, far right; ref. 37). When these mice were moribund, only 20% of their tumors were preinvasive lesions, all of which were associated with high-grade dysplasia. The remaining 80% of tumors in these mice were invasive adenocarcinomas, many of which had a significant proportion of the tumor extending beyond the muscularis propria to involve the serosa (Fig. 5C). Metastatic disease was identified in two of these mice, including metastases to a retroperitoneal para-aortic lymph node (Fig. 5A) and metastatic cancer within the liver (Fig. 5B). These are common locations for human colorectal cancer to metastasize. No lung metastases were identified.

Discussion

The progression from an adenoma to an invasive carcinoma is a histologic determination defined by spread of tumor into the submucosa. Invasion into the submucosa has been associated with the presence of additional driver mutations beyond the loss of APC, but does not necessitate molecular progression beyond mutations that have been observed in adenomas. An important consideration, often ignored, about colon cancer tumorigenesis is the time necessary for the acquired mutations to alter the phenotype of the tumor. This is important when investigating the potential timing of acquired mutations.

Activation of the WNT signaling cascade following the loss of APC or mutations in CTNNB1 results in tumorigenesis in the majority of human colorectal cancers (38, 39). Although activation of the WNT pathway is important for tumor initiation, it is not sufficient for progression to an invasive cancer (40). In fact, the vast majority of polyps that form due to loss of APC will not become invasive cancers and some will actually regress (41). Additional genetic alterations including KRAS, BRAF, PIK3CA, and TP53 mutations are important for adenomas to develop high-grade dysplasia and progress to invasive adenocarcinomas (42). The timing of when these additional genetic changes occur has been debated (43), though these additional genetic changes likely occur very early in tumorigenesis.

Because of the proliferative nature of the intestine, mutations are known to accumulate over time in the normal epithelium (13). In colon cancers, an average of approximately 90 mutations are seen per tumor (44). This number changes significantly depending upon the age at which the patient develops cancer. Most of these mutations are considered to be passenger mutations and are not thought to engender a selective growth advantage (45). It has been estimated that half, if not more, of these mutations occur before tumor initiation (13). To investigate whether driver mutations can occur before tumor initiation, investigations into the presence of KRAS mutations in the normal epithelium have been performed. KRAS mutations have been identified in colon cancers, adenomas, tumor-associated epithelial tissue, and completely normal appearing mucosa (46).
indicates that cells with mutations that are important for tumor progression, such as KRAS, might be present before tumor initiation. If driver mutations are present within intestinal epithelial cells before or at least at the time of tumor initiation, tumor biology may be dramatically affected. Here, we show that the presence of KRAS and PIK3CA mutations at the time of initiation owing to loss of APC results in increased tumor multiplicity and an increased rate of progression to invasive adenocarcinomas. The tumors in the models described here even metastasize to
stromal cell signaling might also limit the proliferation rate be detected in the described experiments when additional driver statistically signifi likely developing very early. This possibility might explain why a containing less than 10^9 cells (\textsuperscript{47}). Because driver mutations are being identifi ed within tumors containing less than 10^5 cells (~1 cm in diameter), clones carrying these mutations are likely developing very early. This possibility might explain why a statistically significant change in the proliferation rate could not be detected in the described experiments when additional driver mutations were added. Other factors including angiogenesis and stromal cell signaling might also limit the proliferation rate \textit{in vivo}. Interestingly, because driver mutations are identified in tumors containing less than 10^5 cells (~1 cm in diameter), clones carrying these mutations are likely developing very early (\textsuperscript{50}). For these clones to outcompete others in the same tumor to a detectable extent, these additional driver mutations are likely occurring when the tumor consists of 10^5 cells or less. The small probability of proliferation causes dramatic changes as the number of cells in these tumors enlarges. These driver mutations \textit{in vivo} augment tumor progression by more than just increasing cell proliferation, but also by suppressing apoptosis, stimulating angiogenesis, recruiting stroma, and evading immune surveillance, among others (\textsuperscript{50}).

The presence of KRAS and PIK3CA mutations at the time of tumor initiation in the setting of loss of APC does not eliminate the formation of the premalignant intermediary. This is important because there is still the possibility to prevent invasion of these tumors if the polyps can be resected at colonoscopy. In addition, the malignant potential of these lesions might not be able to be predicted purely on tumor growth rate and morphology as it appears based on the experiments presented here that loss of APC may be the key mediator of early tumor growth. The increased rate of tumor progression to invasive disease in the setting of concomitant driver mutations indicates that it is likely beneficial to understand the biology of the polyps that are removed from patients. Prognostic stratification of premalignant lesions might be beneficial for determining the likelihood of these tumors harboring foci of invasive disease and potentially the risk of future lesions developing. Further investigation is needed, but it might be necessary to screen patients more frequently if they develop adenomas with high-risk molecular features.

The combinatorial accumulation of driver mutations, opposed to the timing of potential sequential mutations, has the greatest impact on tumor development and progression (\textsuperscript{49}). The timing of when these mutations occur is very important, however, as this will impact the amount of time necessary for these lesions to become invasive cancers. In many instances, the canonical mechanism of tumorigenesis with mutations slowly accumulating over time does likely occur. This study and others demonstrate, though, that there are likely some instances when the canonical pathway is not followed. This could explain the development of interval cancers between colonoscopies. We have characterized an effective mouse model for further investigations into the mechanisms of tumorigenesis that can ultimately be applied to the better understanding of the molecular progression of colon tumors. These studies will allow for improved risk stratification to dictate screening intervals and the development of better chemopreventive strategies.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Colonic Tumors with the Simultaneous Induction of Driver Mutations in \textit{APC}, \textit{KRAS}, and \textit{PIK3CA} Still Progress through the Adenoma-to-carcinoma Sequence

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