Development of a mouse model for sporadic and metastatic colon tumors and its use in assessing drug treatment

Kenneth E. Hung,1, Marco A. Maricevich,1, Larissa Georgeon Richard,2 Wei Y. Chen,3 Michael P. Richardson,1 Alexandra Kunin,4 Roderick T. Bronson,4 Umar Mahmood1, and Raju Kucherlapati5

1Division of Gastroenterology, Tufts Medical Center, Boston, MA 02111; 2Center for Molecular Imaging Research, Massachusetts General Hospital, Boston, MA 02114; 3Department of Genetics, Harvard Medical School and Department of Medicine, Brigham and Women’s Hospital, Boston, MA 02115; and 4Dana Farber/ Harvard Cancer Center, Boston, MA 02115

Edited by Richard D. Kolodner, Ludwig Institute for Cancer Research, La Jolla, CA, and approved December 7, 2009 (received for review August 3, 2009)

Most genetically engineered mouse (GEM) models for colon cancer are based on tissue-wide or germline gene modification, resulting in tumors predominantly of the small intestine. Several of these models involve modification of the adenomatous polyposis coli (Apc) gene and are excellent models for familial cancer predisposition syndromes. We have developed a stochastic somatic mutation model for sporadic colon cancer that presents with isolated primary tumors in the distal colon and recapitulates the entire adenoma–carcinoma–metastasis axis seen in human colon cancer. Using this model, we have analyzed tumors that are either solely mutant in the Apc gene or in combination with another colon cancer-associated mutant gene, the Kras G12D allele. Because of the restricted location in the distal colon, the natural history of the tumors can be analyzed by serial colonoscopy. As the mammalian target of rapamycin (mTOR) pathway is a critical component of the complex signaling network in colon cancer, we used this model to assess the efficacy of mTOR blockade through rapamycin treatment of mice with established tumors. After treatment, Apc mutant tumors were more than 80% smaller than control tumors. However, tumors that possessed both Apc and Kras mutations did not respond to rapamycin treatment. These studies suggest that mTOR inhibitors should be further explored as potential colorectal cancer therapies in patients whose tumors do not have activating mutations in KRAS.

Colon cancer continues to be one of the leading causes of cancer-related deaths. The prognosis for patients with early stage cancers is good, but the majority of cancers are diagnosed at later stages (1). Most drug development strategies use transplantation of human tumor cells into immunocompromised mice. These models are not truly predictive of response in human patients because they are derived from tumor cell lines grown in vitro and are implanted into ectopic sites that bear no resemblance to the colonic microenvironment (2). Furthermore, these xenograft models fail to recapitulate the heterogeneous nature of cancer and the critical endogenous interplay between tumor and supporting stroma. Genetically engineered mouse (GEM) models circumvent these shortcomings, making them an attractive platform for biomarker discovery, study of cancer biology, and preclinical therapeutic trials (2, 3).

Several GEMs that use germline or tissue-wide modification of genes known to be mutated in human colon cancer spontaneously develop intestinal tumors (4). Depending on the gene(s) that is modified, these are reasonable models for inherited cancer predisposition syndromes, such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colon cancer (HNPCC). However, these are not models for sporadic colon cancer, which comprises 75–80% of all cases in humans (5). In these mouse models for FAP and HNPCC, the genetic mutations are present in the germline. Consequently, they are expressed throughout intestinal development. In sporadic colon cancer, the genetic mutations develop somatically. As such, mouse models that somatically modify critical carcinogenic genes in a stochastic fashion are needed to accurately model sporadic colon cancer.

A true sporadic model for colon cancer should have the following features: (i) the model develops one or two tumors in the colon, (ii) the tumors derive from somatic modification of genes known to be involved in human colorectal cancer, (iii) the somatic mutations involve the colonic epithelium, and (iv) the tumors present along the entire adenoma–carcinoma–metastasis axis. Mice with conditional mutations in Apc are an excellent starting point for developing such models.

Delivery of adenovirus expressing cre recombinase (adeno-cre) to conditional knockout mice is an attractive approach, as the spatial and temporal sequence of gene modification(s) can be controlled (6). This approach has been used to locally modify critical carcinogenic genes in lung, liver, ovarian, and other mouse cancer models (7–12). Colon tumorigenesis using rectal adeno-cre enemas in mice carrying floxed Apc alleles has been described, but we and other groups have found that the incidence, multiplicity, and location of the intestinal tumors can be highly variable in this model (13).

In this report, we describe a unique surgical procedure to limit adeno-cre infection to the most distal colon, resulting in highly penetrant tumor formation (14). These tumors present with the full spectrum of adenomas, invasive carcinoma, and metastases. The restricted location of the primary tumors makes this an ideal model for serial endoscopic assessment in preclinical therapeutic trials. With the increasing interest in mTOR blockade as an anticaner therapy, we used this model to examine the efficacy of rapamycin as a therapeutic agent. We observe that tumors in mice with Apc mutation respond well to treatment with rapamycin, but when the Apc mutation is combined with an activating mutation in Kras, the resulting tumors no longer respond.

Results

Adenovirus Infects Basal Colonic Crypt Cells. To generate a mouse model for sporadic colon cancer, we hypothesized that adeno-cre could be delivered to stochastically modify floxed genes within the distal colonic epithelium. Because the murine intestinal epithelium
renews every 5 days, infection of the functional stem cell compartment in the basal crypt is required for these changes to have a biological impact (15). To assess the infection efficiency in the basal crypt, we administered 10^7 pfu of adenovirus expressing β-galactosidase (adeno-LacZ) in 100 μL PBS to the distal colon of C57BL/6 mice. To increase the efficiency of adenoviral infection, we employed an approach in which surgical clips, flanking the site of injection, were placed throughout the entire infection period (Fig. S1A). After 48 h, the distal colons were removed, sectioned along the sagittal plane, and stained for β-galactosidase. Histological examination revealed positive blue staining mainly in the superficial epithelium (Fig. S1B). However, occasional staining was noted in basal crypt cells (Fig. S1C). Although the precise nature of all of the cells infected by the adenovirus cannot be accurately determined, the staining of basal crypt cells and the fact that these mice do develop tumors (see below) suggest that at least a few of the critical cells in the functional stem cell compartment are indeed infected by adenovirus.

Adeno-Cre Treatment of Apc CKO Mice Results in Isolated Distal Colonic Tumors. Apc inactivation is known to be one of the critical initial genetic alterations for entry into the adenoma–cancer sequence (5). In many different mouse models, germline or tissues-wide inactivation of the Apc gene results in predominantly small intestinal tumor formation (4). To assess whether critical genes involved in colon carcinogenesis could be stochastically modified to produce distal colonic tumors in a highly reproducible fashion, 10^7 pfu of adenovirus in 100 μL PBS was introduced into the colons of mice that were homozygous for a floxed exon 14 allele of the Apc gene (Apc CKO) (16). As a control, 10^7 pfu of adenovirus containing an empty expression cassette (adeno-WT) was administered to Apc CKO mice. In one experiment, of the 66 mice that were infused with adenovirus, we were able to detect tumors in 47 (71%) of the mice in as little as 6 weeks after viral administration (Fig. 1A). None of the mice injected with adenovirus WT ever developed tumors (n = 12). The mean tumor multiplicity was 1.3 per animal, and the mean distance from the anus was 22.5 mm. We examined colonic tumors from 60 different Apc CKO mice ranging from 9 to 35 weeks after adenovirus injection. Of these, 56 (93%) exhibited uniform cells with minimal pleomorphism that recapitulated glandular structures in an organized fashion and were classified as adenomas (Fig. 1B). As early as 18 weeks after adenovirus infection, tumor cells in 4 (7%) of the mice showed greater mitotic activity, were more anaplastic, and had very pleomorphic nuclei. The cells were not orderly arranged in glands or crypts, but exhibited finger-like projections of cells, with individual cells breaking off at the deepest parts and some invading the muscularis layer. Furthermore, there was a vigorous stromal reaction to the tumor. On the basis of these findings, these tumors were classified as carcinomas (Fig. 1C).

To confirm that the tumors are indeed the result of adenovirus-mediated recombinant, we assessed the status of the Apc gene in several tumors. In all tumors, we detected recombinant Apc alleles that resulted from the homozygous deletion of exon 14, suggesting that tumor initiation occurred after inactivation of the Apc gene. Immunohistochemistry revealed that unlike normal epithelium, the colonic tumors exhibited strong nuclear β-catenin staining, suggesting that tumor progression occurs through the activation of the canonical Wnt signaling pathway (Fig. ID). These results suggest that adenovirus can be administered to Apc CKO mice to reliably induce isolated tumor formation in the distal colon that results from Apc inactivation and subsequent activation of Wnt signaling.

Apc Tumors Do Not Develop Spontaneous Kras Mutations. Thirty to fifty percent of human colonic adenomas and carcinomas contain activating mutations in one of the Ras genes, primarily KRAS (5). It is also known that such mutations are relatively early events during the development of these tumors. As germline Apc mutant mouse models do not develop spontaneous Kras mutations, we hypothesized that the tumors derived from Apc CKO similarly do not develop spontaneous Kras mutations (17). To test this hypothesis, we examined Kras gene transcripts by direct sequencing of RT-PCR products from 20 different colonic tumors at 17–41 weeks following adenovirus treatment of Apc CKO mice. All of these tumors contained cDNA that was wild type for Kras.

Adeno-Cre Treatment of Apc CKO/LSL-Kras Mice Results in Advanced Primary and Metastatic Colon Tumors. To assess whether the incorporation of an activated Kras gene would alter tumor progression in our mouse model, we generated mice that were homozygous for the Apc CKO allele and heterozygous for a latent activated allele of Kras (Kras<sup>fl</sup>oxed<sup>−/−</sup>) (Apc CKO/LSL-Kras) (7). The distal colons of these mice were infused with 10^7 pfu of adenovirus in 100 μL PBS. As with the Apc CKO mice, the induced Apc CKO/LSL-Kras mice presented 20 weeks after adeno-cre injection. Of the 55 mice that were infused with adenovirus, we were able to detect tumors by colonoscopy (see below), in 53 (96%) of the mice in as little as 3 weeks after viral administration, with an average tumor burden of 3.6 lesions per mouse. Tumor histology was assessed using the same criteria as was used for tumors from the Apc CKO mice. Of the 42 tumors examined, 27 (64%) were adenomas and the remaining 15 (36%) were carcinomas. However, carcinomas first presented 20 weeks after adenovirus injection. Of the 30 tumors that were examined after this time, 15 (50%) were carcinomas (Fig. 2A–C). Furthermore, spontaneous gross liver metastases were noted starting 24 weeks after adenovirus injection (Fig. 2D). Of the 25 mice that were examined after this time, 5 (20%) mice showed these lesions. Upon histological examination, these lesions were classified as adenocarcinomas (Fig. 2E). To confirm that the metastatic tumors were of intestinal origin, both primary and metastatic tumors were stained with the intestinal-specific transcription factor cdx-2 (Fig. 2F and G). Wnt activation was noted in metastatic tumors by nuclear β-catenin staining. (Fig. 2H). These results suggest that the addition of an activated Kras allele can accelerate tumor progression and lead to eventual metastasis.

Endoscopic Assessment of Mouse Sporadic Colon Cancers. The ability to longitudinally follow the development of tumors provides a...
number of opportunities to study progression. Although murine colonoscopy systems have been developed, they have proven to be not useful because the tumors in germline mutant models are predominantly located in the small intestine (18, 19). Because the model described above develops primary tumors only in the distal colon, we were able to use a custom-made colonoscopy system to monitor the natural history of tumor growth. To visualize tumors, the distal colon was cleansed with PBS, and air was carefully insufflated to distend the colon, avoiding perforation. This procedure was well tolerated by all animals. The colonoscope was inserted into the intestinal tract through the anus and white light images were obtained. Representative results from a few such colonoscopies are shown in Fig. 3A. These results suggest that colonoscopy can be used to follow the natural history of tumor formation in this model.

**Activated Kras Accelerates Growth of Early Lesions.** We next assessed the feasibility of quantitating the rate of tumor growth by colonoscopy. To accomplish this, endoscopic images obtained in real time were saved for later offline analysis. Assessing the absolute tumor size from these images is difficult because tumor size in these images is a function of the distance between the endoscopic probe and the tumor itself. Therefore, we hypothesized that if the colon were fully insufflated, the relative lumen sizes could be used as a normalization factor. We used ImageJ software to determine the ratio of the tumor and lumen cross-sectional areas. To validate this ratio as a surrogate metric for tumor size, mice underwent colonoscopy followed by immediate necropsy. The dissected tumor size was then assessed by standard caliper measurements. Comparison of the ratio of the tumor/lumen cross-sectional area and the absolute ex vivo tumor size showed good correlation (r = 0.87, n = 12). On the basis of this approach, we generated growth curves for tumors in the induced Apc CKO and Apc CKO/LSL-Kras mice (Fig. 3B). For the Apc CKO mice, tumors were seen in as little as 6 weeks. However, for the Apc CKO/LSL-Kras mice initial lesions were seen in as little as 3 weeks. Nonetheless, the difference between the overall composite growth curves was not significant (P = 0.41).

A significant advantage of the colonoscopy approach is the ability to assess tumor multiplicity at the earliest stages of tumor formation. If tumor multiplicity were only assessed at necropsy, multiple initial tumors may have coalesced and appear as a single tumor. However, initial tumor multiplicities can be assessed at the time of earliest tumor formation by colonoscopy. We compared the tumor multiplicity of Apc CKO and Apc CKO/LSL-Kras at each endoscopic time of assessment. When the Kras mutation is activated, the overall tumor multiplicity increased from 1.3 to 3.6 in a statistically significant manner (P = 0.002, Fig. 3C). Taken together, these results suggest that the presence of K-Ras mutations may provide an added advantage for the tumor clone to survive and develop into a tumor rather than providing a specific advantage in the rate of established tumor growth.

**Assessment of Colonoscopy Adjuvants.** In the clinical arena, optical colonoscopy is the gold standard for colon cancer screening, but a significant number of polyps are still missed (20). There is growing interest in the use of molecular beacon protease-activated synthetic probes for improved detection of early stage cancers including flat adenomas. Prosense 680 is a long circulating graft copolymer that can be selectively enzymatic cleavage of lysine-lysine bonds within it, primarily by cathepsin B in vivo. Our previous mass spectrometry-based study of the plasma proteome of intestinal tumor-bearing Apc ∆580 mice revealed high levels of plasma cathepsin B (21). Immunohistochemical analysis indicated that cathepsin B was preferentially expressed in tumor compared with normal mucosa. Ex vivo imaging after injection with Prosense 680 demonstrated highly specific staining for intestinal tumors. To assess whether Prosense 680 could be used as an endoscopic adjuvant, the same agent was i.v. injected into adeno-cre-induced Apc CKO mice with established tumors and imaged with near-infrared colonoscopy. Representative results are shown in Fig. 3D and E. Near-infrared endoscopic images revealed that Prosense 680 signal correlates well with tumor lesions and that it can be a useful adjunct to white light colonoscopy. These results demonstrate the utility of this model in assessment of colonoscopic adjuvant strategies.

**mTOR Pathway Is Activated in Tumors from Mouse Sporadic Colon Cancer Models.** Recent studies have revealed the importance of the mTOR growth pathway in a mouse model for FAP (22). To assess whether this pathway was activated in our mouse sporadic colon cancer model, we examined the levels of phosphorylated S6 ribosomal protein (S6-P) as a metric for mTOR activation. Comparison of tumor and normal epithelium by Western blot analysis showed similarly increased levels of S6-P in both Apc CKO and Apc CKO/LSL-Kras mice (Fig. 4A). These results were confirmed...
by tumor immunohistochemistry in these mice (Fig. 4B). Taken together, it appears that the mTOR pathway is activated in tumors derived from Apc CKO and Apc CKO/LSL-Kras mice.

**Rapamycin Treatment Causes Tumor Regression Only in Apc CKO Mice.** Because the mTOR pathway is activated in both Apc CKO and Apc CKO/LSL-Kras mice, we hypothesized that these tumors might be “oncogenically addicted” to mTOR signaling. If so, mTOR blockade might be expected to result in growth arrest or even regression of established tumors. To assess this feature, we treated adeno-cre-induced Apc CKO and Apc CKO/LSL-Kras mice with established tumors as determined by colonoscopy (n = 8) with the mTOR inhibitor rapamycin. Mice were treated with daily i.p. injection of rapamycin (4 mg/kg) and subsequent tumor growth was followed by twice weekly colonoscopy. After 24 days of rapamycin treatment, tumors from Apc CKO mice showed an 82% decrease in tumor size as compared to control animals (Fig. 5A, P = 0.04). Interestingly, the growth curves between treated case and control Apc CKO/LSL-Kras mice (n = 8) was not significantly different (Fig. 5B, P = 0.26). To assess whether the treatment failure was simply due to increased drug requirements secondary to mutated Kras, Western blot and immunohistochemistry were used to demonstrate that S6-P was equivalently decreased in both the treated Apc CKO and Apc CKO/LSL-Kras mice (Fig. 4A and B). These results suggest that rapamycin has powerful anticolonic tumor effects that are abrogated in the presence of activated Kras alleles.

**Mutant Kras Activates the MAPK Pathway.** We reasoned that the additional mutant Kras allele might provide an alternate signaling pathway that allows these tumors to escape inhibition of “oncogenic addiction” to mTOR signaling. As Kras is known to activate the MAPK pathway, we assessed its activation using levels of phosphorylated ERK (p-ERK) as a metric. Western blot analysis revealed that p-ERK was increased in tumors from Apc CKO/LSL-Kras mice as compared to those from Apc CKO (Fig. 6A). These results were also confirmed by immunohistochemistry analysis (Fig. 6B). These results suggest that the activation of MAPK pathway might serve as a parallel signaling pathway that permits tumor growth in the face of mTOR blockade.

**Discussion**

Several GEM models for human colon cancer have been described that show an intestinal tumor predisposition phenotype. Many of these incorporate genetic modification in the Apc gene, which is mutated in a majority of human colorectal cancers, whereas others have mutations in DNA mismatch repair genes (4). Although these are excellent models for hereditary gastrointestinal cancer, none of them are appropriate models for sporadic colorectal cancer. Because the majority of human colon cancers are sporadic in nature, having a robust mouse model for such cancers would prove to be an important adjunct for our understanding of tumor initiation, progression, and treatment.

A faithful sporadic GEM model of colon cancer must have the following features: (i) the mice should develop one or a very few colonic adenomas that have the capacity to progress to adenocarcinoma and metastasis, (ii) the tumor(s) must be restricted to colon, (iii) the tumors should involve loss of function of the Kras gene leading to activation of Wnt signaling, (iv) the tumor must develop in the context of normal colonic epithelium and stroma, and (v) the model should have a short and predictable timeline for tumor progression. The mouse we have described in this report meets all of these expectations. We began with a mouse that is homozygous for a floxed allele of the Apc locus (13). We developed a reliable and reproducible strategy to inactivate the Apc locus in a...
small subset of colonic epithelial cells, leading to the development of one to two tumors only in the distal colon. These tumors develop very rapidly in the setting of normal colonic epithelium and stroma with the earliest macroscopically detectable tumors visualized in as little as 6 weeks after the genetic modification.

Because loss of germline Apc gene function results in embryonic lethality, only mice that are heterozygous for an Apc mutation can be used in most mouse models. In models carrying different Apc mutations, the latency period required before detection of tumors can be quite variable. In the sporadic model described here, we have initiated tumor formation through simultaneous modification of both floxed Apc alleles, thereby reducing the time of latency required for tumor development. The short latency period and high tumor penetrance are ideal for preclinical drug development studies.

Our studies provide an explanation as to why the development of a robust sporadic colon cancer model using a simple adenoviral enema strategy has been problematic. X-gal staining after adeno-lacZ administration revealed that the majority of infection occurs in the superficial intestinal epithelium, which would result in gene modification of already well-differentiated cells that would not have the capacity to develop into a tumor. Our surgically based infection strategy physically confines the adenovirus to the distal colon for an adequate period to increase the effective local concentration and to allow for infection of the cells. With this surgical approach, we have determined that simple adenoviral enemas result in leakage of the solution containing the virus to either proximally into the right colon, cecum, or small intestine, or distally out through the anus, resulting in suboptimal adenoviral infection. In addition, our surgical strategy allows direct visualization of the desired region to confirm the absence of stool that might decrease the overall infection efficiency. Most importantly, this approach physically constrains tumor formation to the distal colonic region that is flanked by the surgical clips, thereby preventing the formation of extracolonic tumors and allowing for subsequent endoscopic tumor assessment.

Traditional mouse models, such as ApcMin, can present with greater than 100 intestinal tumors, show a high level of morbidity, and die by 4–5 months of age secondary to anemia (4). In contrast, our adenoviral induction strategy mimics human sporadic colon cancer in its very low colonic tumor burden. As a result, these mice remain healthy for extended periods past initial tumor formation. We have determined that simple adenoviral enemas result in leakage of the solution containing the virus to either proximally into the right colon, cecum, or small intestine, or distally out through the anus, resulting in suboptimal adenoviral infection. In addition, our surgical strategy allows direct visualization of the desired region to confirm the absence of stool that might decrease the overall infection efficiency. Most importantly, this approach physically constrains tumor formation to the distal colonic region that is flanked by the surgical clips, thereby preventing the formation of extracolonic tumors and allowing for subsequent endoscopic tumor assessment.

Traditional mouse models, such as ApcMin, can present with greater than 100 intestinal tumors, show a high level of morbidity, and die by 4–5 months of age secondary to anemia (4). In contrast, our adenoviral induction strategy mimics human sporadic colon cancer in its very low colonic tumor burden. As a result, these mice remain healthy for extended periods past initial tumor formation. We have determined that simple adenoviral enemas result in leakage of the solution containing the virus to either proximally into the right colon, cecum, or small intestine, or distally out through the anus, resulting in suboptimal adenoviral infection. In addition, our surgical strategy allows direct visualization of the desired region to confirm the absence of stool that might decrease the overall infection efficiency. Most importantly, this approach physically constrains tumor formation to the distal colonic region that is flanked by the surgical clips, thereby preventing the formation of extracolonic tumors and allowing for subsequent endoscopic tumor assessment.

Traditional mouse models, such as ApcMin, can present with greater than 100 intestinal tumors, show a high level of morbidity, and die by 4–5 months of age secondary to anemia (4). In contrast, our adenoviral induction strategy mimics human sporadic colon cancer in its very low colonic tumor burden. As a result, these mice remain healthy for extended periods past initial tumor formation. We have determined that simple adenoviral enemas result in leakage of the solution containing the virus to either proximally into the right colon, cecum, or small intestine, or distally out through the anus, resulting in suboptimal adenoviral infection. In addition, our surgical strategy allows direct visualization of the desired region to confirm the absence of stool that might decrease the overall infection efficiency. Most importantly, this approach physically constrains tumor formation to the distal colonic region that is flanked by the surgical clips, thereby preventing the formation of extracolonic tumors and allowing for subsequent endoscopic tumor assessment.

Traditional mouse models, such as ApcMin, can present with greater than 100 intestinal tumors, show a high level of morbidity, and die by 4–5 months of age secondary to anemia (4). In contrast, our adenoviral induction strategy mimics human sporadic colon cancer in its very low colonic tumor burden. As a result, these mice remain healthy for extended periods past initial tumor formation. We have determined that simple adenoviral enemas result in leakage of the solution containing the virus to either proximally into the right colon, cecum, or small intestine, or distally out through the anus, resulting in suboptimal adenoviral infection. In addition, our surgical strategy allows direct visualization of the desired region to confirm the absence of stool that might decrease the overall infection efficiency. Most importantly, this approach physically constrains tumor formation to the distal colonic region that is flanked by the surgical clips, thereby preventing the formation of extracolonic tumors and allowing for subsequent endoscopic tumor assessment.

Traditional mouse models, such as ApcMin, can present with greater than 100 intestinal tumors, show a high level of morbidity, and die by 4–5 months of age secondary to anemia (4). In contrast, our adenoviral induction strategy mimics human sporadic colon cancer in its very low colonic tumor burden. As a result, these mice remain healthy for extended periods past initial tumor formation. We have determined that simple adenoviral enemas result in leakage of the solution containing the virus to either proximally into the right colon, cecum, or small intestine, or distally out through the anus, resulting in suboptimal adenoviral infection. In addition, our surgical strategy allows direct visualization of the desired region to confirm the absence of stool that might decrease the overall infection efficiency. Most importantly, this approach physically constrains tumor formation to the distal colonic region that is flanked by the surgical clips, thereby preventing the formation of extracolonic tumors and allowing for subsequent endoscopic tumor assessment.
macologic mTOR blockade. Taken together, examination of the Kras mutational status and activation of the MAPK pathway may be critical before the administration of mTOR inhibitors. Furthermore, colon cancer with activating KRAS mutations might be amenable to combination therapy with rapamycin and a MEK inhibitor, as has recently been demonstrated in a mouse lung cancer model using combination treatment with a PI3K and a MEK inhibitor (31).

In summary, we present the results of our efforts to develop a robust GEM model for sporadic colon cancer. This model mimics human disease in that it presents along the entire spectrum from adenomas to invasive cancer to metastatic cancer. The restricted location in the colon will allow more specific biomarker studies and preclinical therapeutic trials in both primary and metastatic tumors. Our preclinical studies suggest that the use of mTOR inhibitors warrants further investigation in human colorectal cancer. However, these also suggest that such agents might fail in the setting of patients that possess activating KRAS mutations. This phenomenon might explain why the efficacy of mTOR inhibitors against colon cancer has been underappreciated. As such, future clinical trials should perhaps focus on wild-type KRAS patient populations. Taken together, this treatment paradigm may add to the personalized medicine arsenal against colon cancer.

Methods

Animals. Apc conditional knockout (Apc CKO) mice were used as has been previously described (16). To generate compound mutants in Apc and Kras, the above mice were crossed to Kras mice bearing a latent mutant Kras allele (7).

Adenoviral Infection of Colonic Epithelium. Mice were fasted overnight and anesthetized using 2% isoflurane. A midline incision was performed and the distal colon was clamped 3 cm from the anus. After washing with PBS, 100 μL trypan blue was injected into the colon for 10 min. The lining of the distal colon was then mechanically abraded using a small caliber brush. After washing with PBS, 10^7 pfu of adenovirus in 100 μL PBS was injected into the colon for 30 min.

For all incubations, a second clamp was placed 1 cm from the anus to ensure localization during the entire incubation period. After the infection period, both clamps were removed and the abdominal wall was closed in two layers. The procedures were well tolerated by all animals. All of the above procedures were approved by the Harvard Medical Area Standing Committee on Animals. Ad5CMVcre, Ad5CMVEmipt and Ad5CMVkras2 adenoviruses were obtained from the Gene Transfer Vector Core, University of Iowa.

Mouse Colonoscopy. Mice were fasted overnight and anesthetized using 2% isoflurane. The colon was washed with PBS to cleanse the bowel. A custom-made colonoscopy system was used as previously described (32). Air was carefully insufflated into the colon to allow full visualization, but avoid perforation. Endoscopic images and movies were saved for later offline analysis by ImageJ software to calculate the ratio of the tumor area relative to that of the lumen (33). For cathespin imaging, Prosense 680 (VisEn Medical) was injected IV at a dose of 2 nmol/mouse 24 h before colonoscopy. White light colonoscopy and simultaneously acquired near-infrared imaging using a filter set optimized for CysS was performed on a custom-made colonoscopy system as previously described (21, 32).

Rapamycin Therapy. Rapamycin (LC Laboratories) working stock was prepared at 50 μg/mL in 100% ETFoH. For injection, a fresh working solution of rapamycin was prepared with a final concentration of 4% ethanol, 5% PEG400, and 5% Tween 80. Mice were treated with daily i.p. 250-μL injections of 4 mg/kg rapamycin for 3 weeks. Control animals were treated with diluents vehicle alone.

For further details on β-galactosidase staining, Kras sequencing, Western blotting, and immunohistochemistry, please see SI Text.

ACKNOWLEDGMENTS. We thank Eric Martin and Alain Charest for critical review of the manuscript. The work reported here is supported by grants from the American Gastroenterological Association (Research Scholar Award) and from the National Institute of Diabetes and Digestive and Kidney Diseases, Grant 5R01DK087083 (to K.H.); from the National Institute of Biomedical Imaging and Bioengineering, Grant 5R01EB001872 (to U.M.); and from the National Cancer Institute, Grant U01CA084301 (to R.K.) and Grant S50CA127003 (to U.M. and R.K.).


Supporting Information

Hung et al. 10.1073/pnas.0908682107

β-Galactosidase Staining. The intestinal tract was removed, opened longitudinally, and washed with PBS. Samples were fixed in 4% paraformaldehyde for 1 h at 4°C and washed three times with PBS. Intestines were stained in X-gal solution overnight at 37°C and fixed in 10% formalin overnight at 4°C. Fixed samples were embedded in paraffin, sectioned, and stained with eosin. Slides were examined with a BX51 System Microscope (Olympus).

Kras Sequencing. Total RNA was extracted from tumors using TRIZOL (Invitrogen) and the PureLink total RNA purification system (Invitrogen). cDNA was reverse transcribed and amplified using the SuperScript one-step RT-PCR with platinum Taq high fidelity (Invitrogen) with the following primers F-gcaattaaccctcactaaagggCCTGCTGAAAATGACTGAGTAT and R-aagc-taatagactcactatagggTTAATTTGTTCTCTATAATGGTGAA. Samples were sequenced at the Harvard Biopolymers Facility using the following primers T3-AAT TAA CCC TCA CTA AAG GG and T7-TAA TAC GAC TCA CTA TAG GG.

Western Blot. Whole tumors were processed in lysis buffer containing complete protease inhibitor mixture (Roche) using a TH tissue homogenizer (Omni International). Twenty-five micrograms of protein was separated on 10% SDS/PAGE, transferred to a nitrocellulose membrane, and incubated with primary antibody overnight at 4°C, and then secondary antibody for 1 h at room temperature. Detection was performed using the ECL Western blot detection kit (GE Healthcare). Antibodies for p-ERK, total ERK, p-S6, and total S6 were purchased from Cell Signaling Technologies.

Immunohistochemistry. Five-micrometer paraffin-embedded tissue sections were deparaffinized in xylene, followed by alcohol rehydration. After quenching endogenous peroxidases in 3% H2O2 in methanol, the slides were rinsed in distilled water, and an antigen retrieval step was carried out in a microwave oven for a total of 10 min in preheated citrate buffer (pH 6.0). The slides were then incubated with primary antibody at room temperature overnight. After PBS wash, slides were incubated with secondary antibody for 1 h at room temperature. The Vectastain Elite ABC kit (Vector Laboratories) was used for detection per manufacturer’s instructions. The slides were stained with DAB and counterstained with Mayer’s hematoxylin. Antibodies for p-ERK, total ERK, p-S6, and total S6 were purchased from Cell Signaling Technologies. β-catenin antibody was purchased from BD Biosciences.

![Fig. S1. Adenovirus infects basal colonic crypt. (A) Surgical procedure using clips to limit adenoviral infection to distal colon. Red arrow denotes infection site for adenovirus during incubation period. (B) X-gal staining after adeno-lacZ demonstrates infection predominates in superficial epithelium. (C) X-gal staining after adeno-lacZ demonstrates infection in basal colonic crypt marked by red arrow.](image-url)