Animal models of gastrointestinal and liver diseases. New mouse models for studying dietary prevention of colorectal cancer

James C. Fleet

1Department of Nutrition Science, Purdue University, West Lafayette, Indiana; and 2Purdue University Center for Cancer Research, Purdue University, West Lafayette, Indiana

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Fleet JC. Animal models of gastrointestinal and liver diseases. New mouse models for studying dietary prevention of colorectal cancer. Am J Physiol Gastrointest Liver Physiol 307: G249–G259, 2014. First published May 29, 2014; doi:10.1152/ajpgi.00019.2014.—Colorectal cancer is a heterogeneous disease that is one of the major causes of cancer death in the U.S. There is evidence that lifestyle factors like diet can modulate the course of this disease. Demonstrating the benefit and mechanism of action of dietary interventions against colon cancer will require studies in preclinical models. Many mouse models have been developed to study colon cancer but no single model can reflect all types of colon cancer in terms of molecular etiology. In addition, many models develop only low-grade cancers and are confounded by development of the disease outside of the colon. This review will discuss how mice can be used to model human colon cancer and it will describe a variety of new mouse models that develop colon-restricted cancer as well as more advanced phenotypes for studies of late-stage disease.

Chemoprevention is the term given to efforts that block cancer initiation, slow tumor growth and metastasis, or inhibit cancer recurrence after treatment. The basic premise of chemoprevention is that by extending the natural history of colorectal cancer, we can reduce the number of people who need treatment, expand the window during which highly treatable low-grade cancers are detected and diagnosed, and reduce the chance of cancer recurrence after treatment (113). Many population-based studies suggest that dietary factors are important chemopreventative agents against colorectal cancer (59, 69). However, studies identifying associations between dietary factors and cancer risk cannot prove causation, and this makes the links between diet and colorectal cancer tenuous. Clinical trials carefully designed to test the impact of dietary agents on colorectal cancer are practical for some (e.g., biomarker studies, cancer recurrence) but not other (e.g., lifelong changes, studies of tumor promotion, progression, or metastasis) chemoprevention goals. For example, human tumors develop over a lifetime and are usually diagnosed in middle-aged adults. This makes early intervention studies aimed at regulating cancer initiation and preventing tumor development impractical from a cost and sample size standpoint.

As a result, alternative strategies to test the biological validity of various chemoprevention hypotheses are needed. Preclinical animal models provide the best hope for the careful evaluation of dietary chemoprevention strategies, to study the molecular mechanisms of colorectal carcinogenesis, and to translate mechanistic hypotheses derived from cell models into whole organisms. Many animal models for colorectal cancer have been developed and used to understand colon carcinogenesis or to test dietary prevention strategies (Table 1). Interested readers should refer to earlier reviews for detailed descriptions of long-standing models like the ApcMin mouse or those whose cancer results from chemical induction (48, 91, 108). In this review, I will provide a perspective for how mouse models can be developed to best reflect human colon cancer. In addition, I will discuss how a number of recent models have improved our ability to study colorectal cancer and dietary chemoprevention.

What Makes an Animal Model Useful for Chemoprevention Research?

Although it may seem obvious, the goal for using mouse models to study dietary chemoprevention is to gain insight into the value of specific agents for use against human colon cancer. Because of this, the starting point for choosing an animal model has to be how it relates to the human disease. Historically, colon cancer has been viewed in three broad categories: tumors characterized by chromosomal instability (CIN) and progressing from adenoma to carcinoma, those arising from microsatellite instability (MSI), and those associated with inflammation-induced dysplasia. These cancers are formed by sporadic mutations in specific genes that serve as drivers for the initiation and progression of cancer. However, recent advances in cancer genomics are showing that these categorizations, although useful, are overly simplistic. In this section I will provide a brief overview of the molecular etiology and pathological features of colorectal cancer in humans. This information is summarized in Fig. 1.

Molecular and pathological features of human colorectal cancer. Most sporadic colorectal cancers arise from benign adenomatous polyps over a course of several decades. These tumors are typically found in the descending colon and rectum and are initiated through the mechanism of CIN (37). Our understanding of this type of cancer started with studies on the rare human colorectal cancer susceptibility syndrome, familial adenomatous polyposis (FAP), a disease caused by germline mutations in the tumor suppressor gene APC. APC is a 2,843-
amino acid protein that is part of the canonical WNT signaling pathway. In the cytoplasm it helps form a destruction complex that reduces the cytoplasmic level of β-catenin (93), whereas in the nucleus it sequesters and inhibits β-catenin binding to the TCF/LEF transcriptional complex (100) and mediates β-catenin export to cytoplasm (40, 82, 92). As a part of a complex formed with the TCF/LEF family of transcription factors, β-catenin regulates genes whose protein products stimulate cell proliferation and delay differentiation. The loss of APC function in FAP patients or in animal models leads to the development of pedunculated adenomatous polyps (119). Progression of these polyps to invasive and metastatic cancer (i.e., adenoma-to-carcinoma progression) is driven by the sequential accumulation of mutations in at least three additional pathways: the MAPK pathway (i.e., KRAS-activating mutations), TGF-β-induced differentiation pathway (e.g., deletions that disrupt SMAD genes), and DNA repair pathways (i.e., inactivation of TP53); this is known as the Vogelstein model (29).

Cancers found in the ascending colon account for 20% of all colon cancers and they are distinct from those that develop through the adenoma-to-carcinoma progression. Our initial understanding of these cancers comes from the study of Lynch Syndrome (a.k.a. hereditary nonpolyposis colorectal cancer, or HNPCC). HNPCC is caused by germline mutations in mismatch repair genes (90% by MSH2 and MLH1 gene mutations; 10% by MSH6, PMS2, or MLH3 gene mutations) (35). Inactivation of these DNA mismatch repair (MMR) mechanisms is associated with MSI (51) and dysregulation of MMR increases the potential for mutations in other oncogenes, e.g., a high percentage of HNPCC colon cancers exhibit increased WNT signaling (45) and β-catenin gene (CTNNB1) mutations (75). Sporadic colon cancers with MSI commonly result from hy-

Table 1. A summary of models used to study dietary prevention

<table>
<thead>
<tr>
<th>Model</th>
<th>Examples</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Spontaneous cancer</td>
<td>“Western” diet (high fat, low Ca, vitamin D)</td>
<td>48</td>
</tr>
<tr>
<td>Chemically induced</td>
<td>AOM, NMU, DMH, MNNG</td>
<td>91</td>
</tr>
<tr>
<td>APCnull mouse</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Genetic modification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td>11 APC lines including APC^AT5, APC^AT4; Msh2; Mlh1; PTEN; Smad3</td>
<td>48, 108, 131</td>
</tr>
<tr>
<td>Floxed alleles</td>
<td>LSL-Kras^{G12D}, Msh2 (exon 2), APC (exon 14, exon 15)</td>
<td>48</td>
</tr>
<tr>
<td>Intestine-specific promoter</td>
<td>Villin, Car1, CDX2P9.5, Fabl</td>
<td></td>
</tr>
<tr>
<td>Inducible</td>
<td>Cre-ER^{T2}, rtTA, AhRo/CYP1A1</td>
<td>48</td>
</tr>
<tr>
<td>Xenograft</td>
<td>Cell lines: SW480, SW620, HCT116, KM12SM, LIM1215</td>
<td></td>
</tr>
<tr>
<td>Allograft</td>
<td>CT26 cells</td>
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**Fig. 1.** Molecular changes associated with colorectal cancer development. Colorectal cancer is a heterogeneous disease. Traditional pathways for the molecular etiology of sporadic colon cancer are 1) tumors that develop through an adenoma-to-carcinoma progression that is similar to the heritable cancer familial adenomatous polyposis (FAP), 2) tumors that are characterized by high levels of microsatellite instability (MSI) due to inactivation of mismatch repair enzymes, similar to Lynch Syndrome, and 3) tumors that develop from dysplastic lesions resulting from recurrent bouts of colonic inflammation. CIN, chromosomal instability; MHC, major histocompatibility complex; ROS, reactive oxygen species; RNS, reactive nitrogen species. Pathological features leading to the development of each tumor are shown. Common gene mutations or epigenetic changes leading to initiation and progression are shown on top of arrows leading from one phenotype to the next. Additional biochemical features of specific phenotypes are presented below the phenotype.
permethylation of MMR gene promoters, particularly MLH1 (23), and these tumors have a high frequency of mutations in the BRAF gene and in genes controlling the canonical WNT pathway (109), as well as inactivating mutations of the type II TGF-β receptor (67) and the proapoptotic gene BAX (86). Histologically, MSI-associated tumors exhibit an exophytic or sessile gross phenotype and are characterized microscopically by excessive mucin production, poor epithelial differentiation, and lymphocytic infiltration (51). A unique morphological variant of hyperplastic polypl with a serrated glandular morphology and BRAF mutations is a precursor lesion to MSI cancers.

Recent research demonstrates that a form of MSI exists that is independent of mutations in or hypermethylation of genes for MMR enzymes: elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) or MSI-low (41, 120). Moderately and poorly differentiated colorectal tumors have higher frequencies of EMAST (56.9 and 40%, respectively) than either well-differentiated colorectal tumors (12.5%) or adenomas (33%) (56). Although the importance of EMAST in tumors is not yet known with certainty, patients with stage II or III colorectal cancer and EMAST tumors were found to have a worse prognosis, with a shorter time to tumor recurrence, and were more likely to develop distant metastasis than patients that has microsatellite stable or MSI-high tumors (33). This is consistent with the observation that EMAST may increase the frequency of loss of heterozygosity in humans (125). EMAST is characterized by a change in the cellular distribution of MSH3 from the nucleus to the cytosol (41), and this redistribution is enhanced by oxidative stress in a number of colorectal cancer cell lines (111).

The risk of colon cancer can be increased by inflammation, especially as seen in conditions like Crohn’s disease or ulcerative colitis in humans or in rodents cycled through multiple rounds of colitis with agents like dextran sodium sulfate (DSS) (49, 90). Like distal colon cancer, inflammation-associated colon cancer is strongly associated with CIN but in contrast to distal colon cancer, the disease follows a dysplasia-to-carcinoma progression that includes accumulation of mutations in some, but not all, of the genes from the Vogelstein model (112) (Fig. 1). For example, p53 gene mutations are a late event in sporadic cancer of the distal colon whereas p53 mutations are commonly seen in dysplastic lesions that precede colitis-associated colon cancer (12). In contrast, it is unclear whether APC mutations are important during colitis-associated cancer. Although several small studies report a 30–50% incidence of APC mutations in colitis-associated colon tumors (39, 50, 87), others found few, if any, APC gene mutations in such tumors (5a, 57, 114). Inflammation can also accelerate the development of colon cancer that has been initiated by deletion of a single APC gene allele or with a chemical carcinogen (49).

Proposed mechanisms for how inflammation initiates or promotes the early stages of colon cancer include: genotoxin-induced mutations resulting from microorganisms that breach the epithelial barrier, DNA damage resulting from reactive oxygen species (ROS), cytokine-induced regulation of microRNAs, and aberrant DNA methylation resulting from mislocalization of DNA methyltransferases (4, 17, 121). Although inflammation is a critical modifer of colon cancer, and mouse models for inflammation-induced colon cancer exist, these have been described extensively elsewhere and will not be discussed further here (107).

There is significant mutational diversity in colorectal cancer. After reading the brief description of colorectal cancer development above, one might get the impression that all colon cancers follow predictable molecular paths. However, significant heterogeneity exists in the mutational profile among (83) and within (36) tumors. The scope of colon tumor diversity was first described by Sjoblom et al. (102), who sequenced the exomes of 13,023 genes in 11 human colon tumors and found that each tumor had an average of 93 mutated genes. More recently, The Cancer Genome Atlas research network conducted exome sequencing on 224 colon tumors and found that distal colon tumors have a median of 58 nonsilent mutations and that 24 genes were mutated in a significant number of cases (14a). Although the genes from the Vogelstein model were commonly mutated (e.g., 81% of tumors contain APC mutations, 60% p53, 43% KRAS, 9% SMAD4), it is clear that they don’t define all cases of distal colon cancer. This is consistent with the “unique tumor principle” suggested by Ogino and Goel (83). This principle states that the intertumor histological heterogeneity observed in clinical samples is due to the fact that each tumor, in each patient, arises through a unique pathway of molecular disruption that is unlikely to be exactly recapitulated in any other tumor (83). More recently, Gerlinger et al. (36) extended this concept by showing extensive intratumoral heterogeneity across multiple tumor biopsies from four patients with metastatic renal cell carcinoma, e.g., >63% of all somatic mutations were not detectable across every tumor region sampled. This suggests the existence of branched evolutionary tumor growth, with some ubiquitous common mutations in all tumor cells but heterogeneity developing at later stages of tumor growth (128). It is likely that similar evolutionary diversity exists during development of tumors in animal models but this has not yet been experimentally demonstrated.

Mutations in tumors are frequently categorized as “drivers” responsible for the primary development of the tumors or “passengers” that occur as a consequence of the driver mutations and have no functional significance in carcinogenesis. Of the somatic mutations Sjoblom et al. (102) observed in a typical tumor, 5–12 were classified as driver mutations that modulate 12–20 core pathways (55). The implication of the driver/passenger mutation model is that preventative and therapeutic approaches could be targeted to driver mutations or pathways influenced by driver mutations. However, the distinction between driver and passenger mutations may be overstated. Recent evidence shows that passenger mutations occur in genes may have a modest individual impact on cells but their cumulative impact alters cancer progression (72). Regardless, both the diversity of driver mutations and the potential importance of passenger mutations complicate the challenge of making mouse models that perfectly recapitulate human colorectal cancer.

Defining the Factors that Make a Good Mouse Model for Dietary Prevention and Colon Cancer Research

The diversity of human colorectal cancer makes it impossible for a single animal model to adequately represent all forms of the disease. Notwithstanding this challenge, I believe that
certain fundamental characteristics are important for maximizing the translational potential of mouse models for dietary chemoprevention.

First, the metabolism of the nutrient or bioactive agent has to be similar between mice and humans. Without this, results from mice are unlikely to apply to humans. For example, our understanding of the role of β-carotene in lung carcinogenesis was delayed because, in contrast to humans, most laboratory animals break down β-carotene in their intestine and thus do not absorb it intact (118). Related to this, the mechanism of action for a dietary agent should be present in the cancer stage represented by the mouse. High serum levels of the vitamin D metabolite 25-hydroxyvitamin D have been proposed to reduce colorectal cancer risk by serving as a substrate for local conversion to the active hormone 1,25-dihydroxyvitamin D, a ligand that activates gene transcription through the vitamin D receptor (VDR). However, Xu et al. (123) showed that VDR mRNA and protein expression were lower in the small intestine and colon tissue of ApcMin mice compared with wild-type mice, thus making it more difficult to study the role of vitamin D status on colon cancer prevention in this model.

Second, since the research goal is to study sporadic colorectal cancer, the cancer that develops in the mouse model should be limited to the large intestine. Primary tumors that develop in other sites have the potential to disrupt the health of the animal in ways that confound interpretation of the study results. ApcMin mice classically used for dietary chemoprevention studies develop many more small intestinal tumors than colon tumors (79). The small intestinal tumors frequently hemorrhage, causing anemia that negatively affects the general health of the mouse. In addition, the oxidized heme in the intestine after hemorrhage can promote tumorigenesis but this mechanism reflects only a subgroup of human sporadic colorectal cancers (38). Mlh1+/− mice used to model colon cancer originating from defects in DNA mismatch repair and microsatellite instability develop cancer in both lymphoid tissue and in the intestine (27). Similarly, whereas 70% of MutS homologue 2 (Msh2) knockout mice developed intestinal tumors after 6 mo of age, these mice succumb primarily to lymphomas (89). Because of the extracolonic effects, global gene deletion or transgenic mouse models more closely reflect rare heritable cancers like FAP and HNPCC in which the heritable mutations are found in all cells and where extracolonic manifestations are present in two-thirds of affected individuals.

Third, the pathological and molecular features of colorectal tumors in the mouse model, as well as the timing and location of those features, should be similar to those observed in a subtype of human colorectal cancer. This will increase the potential of a mouse study to translate into the complexity of human cancer. Because modifying the mouse genome is now commonplace, deleting or overexpressing any gene or microRNA is achievable. Many such models are available and have been reviewed elsewhere (48, 108). In addition, large scale gene knockout mouse projects are making libraries of embryonic stem (ES) cells with single gene deletions; as of 2011 almost 17,000 genes had been deleted in ES cells by various teams around the world (25). This effort is dramatically reducing the barriers to study the role of specific genes in colorectal cancer. The pathological features of rodent models of intestinal cancer was recently published; interested readers should also refer to this report (119). In particular, this report indicates that newer mouse models with multiple genetic alterations develop intestinal tumors that closely resemble the morphology of human tumors. The report also identifies a website for sharing information about the histological features of published models (mmgint.org). However, there is a significant limitation to the use of global gene knockouts and transgenic mice for dietary chemoprevention studies; the defect is present in embryos so dietary interventions can’t be implemented before the genetic modification has had an influence on cell biology. This can be overcome with inducible transgenes or gene knockouts so that dietary interventions can be introduced prior to the mutation event.

Finally, the models should capture the microenvironmental features relevant to human colon cancer. For colitis-associated colon cancer this will include cellular interactions and factors produced by infiltrating immune cells that induce DNA damage (e.g., oxidative stress), regulate cancer-relevant cell growth, or promote angiogenesis (17). In addition, there is a growing appreciation that genotoxins or metabolites from the gut microbiome can influence carcinogenesis or tumor growth (5, 21). Unfortunately these issues are often ignored by researchers.

New Mouse Models for Colon Cancer Research

The great advantage of the mouse as a research model is the relative ease with which it can be genetically modified. Genetically modified mice offer the potential to precisely recapitulate specific molecular changes relevant to human colorectal cancer. As I noted earlier, there are many mouse models for colon cancer research (Table 1; Refs. 48, 108). This section will discuss new mouse models that have been developed over the last 5 years.

Tissue-specific and inducible models. Controlled induction of genetic mutations is possible in mice and the most common system for doing this is by genomic recombination using Cre recombinase (Cre), a bacteriophage enzyme that deletes sequences between two LoxP DNA sites (11, 30). This approach requires a transgenic mouse expressing Cre and a mouse with LoxP sites flanking a biologically important DNA sequence within a gene. In addition, a modified version of the Cre protein has been designed that allows tamoxifen-inducible control of Cre activity (31). Another system for tissue-specific inducible transgene activation is the reverse tetracycline activator system (32, 52). This approach requires development of mice that express the reverse tetracycline-regulated transactivator (rtTA), mice that have a transgene whose promoter is controlled by the rtTA, and, if the transgene encodes for Cre, mice with a gene containing LoxP sites. The complications of this approach make it less common.

Most genetically modified mouse models were generated to have a single gene defect in all cells in the body so they are often confounded by the existence of precancerous or cancerous lesions in other tissues. This can be limited with transgenes driven by tissue-specific promoters such as 1) the Villin promoter, which limits intestinal transgene expression to the epithelial cells along the crypt–villus axis and from the duodenum to the colon (10, 28, 64); 2) the intestinal fatty acid binding protein promoter (Fabp4kum−135), which limits intestinal transgene expression to the proximal small intestine, cecum, and colon (95); and 3) a 6.3-kb fragment of the cytoker-
atin 19 (CK19) promoter that is expressed throughout the intestinal epithelium (74). Unfortunately, each of these promoter systems has expression that extends beyond the colon (i.e., villin in small intestine and kidney; Fabpl in ileum, bladder, and testes; CK19 in small intestine, stomach, pancreatic ducts, and hepatic ducts) (48). Because of this, over the last several years researchers have developed mouse models that use promoters from genes with a more restricted cell or tissue distribution. However, even with these advances we do not yet have mouse models where transgene expression is limited to the epithelium of the just proximal colon, just the distal colon, or just the rectum.

Lgr5 has been identified as a marker of one type of intestinal stem cell (9). Barker et al. (9) used homologous recombination of the Lgr5 gene locus to create a gene that marks cells expressing Lgr5 with EGFP and that permits tamoxifen-inducible Cre-mediated deletion of floxed alleles from intestinal stem cells (Lgr5-EGFP-IRESCreERT2 mice). Inducing deletion of both APC alleles with this model led to formation of colon adenomas while inducing APC allele deletion in more mature intestinal epithelial cells did not (8). This demonstrates that stem cells can be the cells of origin for colon cancer. However, there are two weaknesses with this model. First, Lgr5 is expressed in the stem cells throughout the gastrointestinal tract, as well as in a number of other tissues (e.g., hair follicle) (7). Second, the expression of the Lgr5-EGFP-IRESCreERT2 transgene is not uniform across the intestine; based on EGFP expression only 10–15% of crypts express the transgene. As a result, although this mouse may be useful to study early carcinogenic transformation in vivo, it may also lead to confounding phenotypes in other tissues and more mice may be needed if tumor incidence is low.

Hinoi et al. (42) developed a mouse model using a 9.5-kb fragment of the CDX2 gene promoter to drive Cre expression (CDX2P9.5-NLS-Cre). After birth, this transgene has expression that is limited to the ileum, cecum, and colon. However, the CDX2P9.5-NLS promoter is transiently expressed in the posterior of the mouse within multiple tissues during embryonic development. As a result, when the promoter is used to drive Cre for the recombination of floxed alleles, the gene is knocked out in the proximal intestine as well as in the muscle, skin, and bones of the rear legs, and in essential organs like the kidney and spleen. This explains why CDX2P9.5-NLS;Cre; Apc580D/580D embryos were not viable. Depending on the gene being studied, it is also possible that CDX2P9.5-NLS;Cre mice may develop additional cancers outside the colon. Although the adverse effects of fetal and extraintestinal expression could be overcome by coupling the CDX2P9.5-NLS promoter to Cre-ERT2, allowing recombination of floxed alleles after birth, this model has not yet been reported.

The CDX2P9.5-NLS promoter construct has also been used to construct the CDX2P9.5-G22-Cre mouse (2). The “G22” refers to the addition of 22 guanine repeats in the transgene coding region before the normal translation start site for Cre. The repeats are out of phase with the Cre start site; this means that Cre can be transcribed and translated only after a somatic frameshift mutation occurs, thus making embryonic expression unlikely. When spontaneous, single-base deletions in the G22 repeat occur, it puts the Cre coding sequence in phase, which allows translation of a functional Cre enzyme and deletion of floxed alleles. β-Galactosidase expression from a CDX2P9.5-G22-LacZ transgene was limited to the ileum and proximal colon. This makes the CDX2P9.5-G22-LacZ mouse an interesting model to study the effects of dietary agents on spontaneous mutations in the colon.

A unique approach to restrict the temporal and spatial activity of Cre was reported by Hung et al. (43). To get recombination of floxed alleles limited to the colon of adult mice, they introduced an adenovirus vector expressing Cre through the rectum to infect colonic epithelial cells. This approach provides precise control over when and where Cre will be expressed. However, the technique requires anesthetizing the mice, applying surgical clamps to the distal colon, and creating abrasive injury to the colonic mucosa before a 30-min adenoviral infection period. Without the injury to stimulate cell proliferation, floxed alleles in the self-renewing stem cells of the colonic crypt are not infected by the adenovirus and deletion of floxed alleles does not occur (99).

A final recent model is one developed in my laboratory. We used a bioinformatics approach to identify Carboxylic anhydrase-1 (CA) as a gene that is highly expressed in the colon of mice. We modified the CA promoter/enhancer to drive Cre expression (CAC) (124). CAC transgene expression affects ~10% of large intestinal epithelial cells. In the distal colon transgene expression occurs from the crypt base to the luminal surface in groups of three to six crypts whereas in the proximal colon expression is mainly in the surface epithelium. When the CAC transgene is used to remove a lox-stop-lox signal in ROSA26R mice, β-galactosidase expression is seen at 14.5 days postcoitum and a small amount of staining is also seen in the liver. When we used the CAC mouse to drive recombination of the APC580D locus and delete one APC allele, recombination caused 15% of the mice to develop at least one distal colon tumor by 20 wk of age. The penetrance of colon cancer can be increased several ways in CAC mice. First, deletion of two APC alleles results in 100% penetrance of colon cancers, with florid tumor growth in the distal colon by 6 wk of age (124). We also used the CAC mouse to delete both a single APC580D allele and a lox-stop-lox allele controlling expression of a constitutively active form of KRAS (G12D) (13). By 15 wk of age 100% of the double-mutant mice develop an average of 4.6 tumors in the distal colon. In none of these cases (single or double APC deletion, APC/KRAS double mutant) did tumors develop in the small intestine or liver; this large-intestine-specific expression makes the CAC model unique among genetically mouse models. In addition, we have recently learned that a 5-day course of DSS leads to activation of the CAC transgene in the epithelium of both the proximal and distal colon (unpublished data). This likely explains our published observation that tumor incidence and multiplicity are dramatically increased in CAC mice with one floxed APC allele following DSS treatment (124). Thus using the CAC model to delete a floxed APC allele may be particularly useful for studies on the role of dietary agents in inflammation-induced colon cancer.

Diversity in the colon cancer phenotypes of mice with APC gene mutations. The most commonly modified gene in mice used for dietary chemoprevention research is APC. Although APC mutant models have been available for a long time, there are many such models and it is confusing which of them should be used for dietary prevention studies. This section will give an overview of the mutant APC models.
APC truncation mutations occur in >90% of FAP patients and in >75% of sporadic colon cancers. Because of the critical role Wnt signaling plays in development, homozygosity for most germline APC truncation mutations is embryonically lethal. As a result, FAP patients and genetically modified mice with APC gene modifications are heterozygotes and loss of heterozygosity of the remaining wild-type ApC allele is required for adenoma formation (63). In terms of dietary prevention, the protection from acquisition of a “second hit” that leads mice heterozygous for APC mutations to form aberrant crypt foci or adenomas is a critical end point.

Over 90% of germline APC mutations in FAP patients (76) and somatic APC mutations in colorectal tumors (77) occur within exon 15 in a cluster between codons 1286 and 1513 and this results in truncation of the APC protein to between 426 and 504 amino acids long (vs. 2,843 for the wild-type protein). The most common mutant APC mouse model is the ApcMin mouse that was generated in an N-ethyl-N-nitrosourea-induced mutation screen (79). The ApcMin mouse expresses a truncated APC protein 850 amino acids long, resulting from a mutation at codon 2549 (105). In addition to the ApcMin mouse, there are 11 genetically engineered APC mutant mice with germline modifications and 2 mutant lines with LoxP sites flanking either exon 14 or exon 15 (48-131). There is significant phenotypic heterogeneity in these APC mutant mice in terms of tumor number and location. All global APC mutants form more tumors in the small intestine compared with the colon and several form tumors at extraintestinal sites (131). Several of the APC mouse models have phenotypes similar to ApcMin mice (i.e., Apc1572N, Apc1309, Apc1309), in several the phenotype is more severe (Apc1322T, ApcΔSAMP, ApcΔ15, ApcΔ580S or Δ580S), and a very high intestinal tumor burden is seen in ApcΔ15 mice. Recently, the Apc1572T mouse has been shown to have hammy cancer but not intestinal cancer (34). This suggests that the last SAMP repeat in APC accounts for the mammalian phenotype of APC gene truncation. This phenotypic variability reflects the importance of structural features necessary for protein-protein interactions affecting APC function. Important motifs within APC confer the ability to bind to β-catenin [three 15-amino-acid repeats between residues 1014 and 1210 (106), seven 20-amino-acid repeats between amino acids 1034 and 2130 (93)] or to the destruction complex through Axin (104) (three serine-alanine-methionine-proline repeats between amino acids 1034 and 2130).

In addition to the phenotypic variation that exists among APC mutants, classical genetic mapping strategies have revealed 13 loci that modify the ApcΔmin phenotype when it is present on different genetic backgrounds (71); similar genetic modifiers of cancer phenotypes are likely to exist in humans as well as to affect cancers with other molecular etiologies. Thus it is critical that chemoprevention researchers control the genetic background of their mouse models to limit this diversity (especially when crossing lines).

Variation in the gut microbiome can influence the development of colon cancer. It has long been recognized that the microbes within the colon can ferment dietary starch and fiber and that the resultant fermentation products can influence colon carcinogenesis (22, 97). In addition, fewer adenomas develop in APC mutant mice raised in germ-free (GF) conditions (19, 26, 58) and in Smad3 knockout mice grown in specific pathogen-free housing (65) whereas colonic levels of specific microbes are associated with increased colon cancer in animals and humans [e.g., F. nucleatum (15, 53)]. These examples reveal a role for the gut microbiota in the etiology of colon cancer, and recent evidence suggests that this is due to specific microbial metabolites or genotoxins that directly influence the integrity of DNA (44). Consistent with this hypothesis, Conlon et al. (20) found that feeding resistant starches to rats increased gut short-chain fatty acid (SCFA) and reduced gut ammonia levels and that these changes were inversely (SCFA) and positively (ammonia) associated with DNA strand breaks in colonocytes. Also, Arthur et al. (4) used IL-10 knockout mice with monoclonization to show that only mice whose colons had been colonized with microbes expressing the bacterial genotoxin colibactin developed colon tumors in response to azoxymethane (AOM).

The impact of gut microbes on colon cancer is clearly seen in mice lacking the mucin 2 (Muc2) gene. Mucin 2 is a large gel-forming protein that makes up the bulk of the mucus layer coating colonic epithelial cells. Mucins are secreted by goblet cells and provide a barrier between the gut microbiome and the epithelial barrier (47). In contrast to normal mice, gut bacteria are in direct contact with the intestinal epithelium of Muc2 knockout mice and they extend deep into the crypt where the normal intestinal stem cell resides. Muc2 knockout mice also have more intestinal epithelial cell proliferation than normal mice and 68% of Muc2 knockout mice develop intestinal cancer within 1 year (115). In addition, deletion of Muc2 from Apc1638N/ and ApcMin mice increased the incidence of tumors, increased the number of tumors per mouse, and shifted tumor burden toward the large intestine, where bacterial loads are greater (127). Thus the Muc2 knockout mouse is an excellent model for researchers who want to learn whether a dietary agent can either reduce the presence of genotoxins from the gut microbiome or stimulate epithelial cell systems for that prevent or repair genotoxin-mediated DNA damage.

For people conducting dietary prevention studies that are not explicitly examining the gut microbiome, there is a cautionary note that must be sound ed. It is clear that dietary changes can modify the gut microbiome. For example, David et al. (24) found that, just days after feeding humans subjects a low-fiber, animal protein-based diet, the diversity of the gut microbiome significantly increased to reflect species that were more tolerant of bile. In mice, even the simple act of switching between two commercial chow diets increased microbial diversity in the gut and this increased the sensitivity of mice to DSS-induced colitis (80). In terms of cancer, Mai et al. (66) found that a cancer-protective diet high in olive oil and supplemented with a dried fruit and vegetable extract increased levels of a bacteria from family Lachnospiraceae (order Clostridiales). This suggests that targeting specific bacterial groups by diet could be an effective colon cancer prevention strategy. Unfortunately, we have not identified all of the protective or harmful gut microbes yet nor have we reached the point where we can reliably and predictably alter the gut microbial population by diet. As a result, it is critical for cancer researchers to control their diets and limit unintended variability in diet composition. This would argue strongly against the use of chow-type diets formulated on a least-cost ingredient basis where there is significant batch-to-batch variability in the ingredients used.

Models of late stage colorectal cancer. Mice with single gene modifications generally have mild phenotypes that do not
develop past adenoma. This feature makes them useful for early carcinogenesis studies or for examining the function of specific pathways. Although many dietary cancer prevention studies focus on modifying the early stages of cancer, dietary factors may also have an impact at later stages that could make them valuable adjuvant therapies to complement traditional treatment regimes. The following section will focus on mouse models of advanced colon cancer.

Combining Gene Mutants to Make Mice with More Advanced Cancer Phenotypes. Breeding mice to combine genetic modifications into a single mouse can cause more advanced phenotypes that allow researchers to examine the role that dietary factors can play in later stages of cancer. First, a number of studies have used mice with K-RAS activating mutations to modify tumor development in other genetically modified mouse models. This approach uses mice where the endogenous Kras allele has been replaced with an allele containing a floxed translational stop site (Lox-STOP-Lox, LSL) in front of a gene encoding either a constitutively active allele with either the ApcMin genotype or AOM chemical induction increased the number of colonic lesions (61). Only minor effects were observed in the intestine of the LSL-KrasG12D allele after Cre-mediated recombination (61), but this mutant accelerated tumorigenesis in ApcMin mice (61) as well as the cancer caused by DMH treatment (62). Similarly, although Cre-mediated recombination of floxed TGF-β receptor 2 (TGFb2Rlox/loxp) alleles caused formation of intestinal neoplasms, in LSL-KrasG12D/+;TGFb2Rlox/loxp transgenic mice recombination results in the formation of tumors that metastasize through a β-catenin-independent mechanism (110).

Disruption of enzymes controlling DNA mismatch repair enhances accumulation of mutations in other oncogenes, e.g., tumors from Msh2−/− mice acquire Apc gene mutations (88). When either Mlh1 or Msh2 knockout mice are crossed to mice heterozygous for a mutated Apc allele, intestinal tumorigenesis is markedly increased (27). Similarly, crossing Msh3 knockout mice to ApcMin/+ mice increased the number of frameshift mutations in the wild-type Apc allele and this caused higher incidence and more tumors per mouse compared with the ApcMin/+ mice (16). Finally, combining Msh2-null mice with LSL-KrasG12V mice increased the number of adenomas per mouse fivefold after Cre-mediated recombination (60).

Pten−/− mice do not develop intestinal neoplasia, but, when they are crossed with ApcMin mice, larger, more invasive tumors develop than the adenomas and adenocarcinomas normally seen in the ApcMin mouse (98). Marsh et al. (68) later confirmed this observation by demonstrating that mice with conditional deletion of one Apc580S and one floxed Pten allele in intestinal epithelial cells had more advanced adenocarcinomas than mice with just one deleted Apc allele. Also, although deletion of the TGFβ2 gene alone has a minimal impact on intestinal tumorigenesis, when TGFβ2 deletion is combined with Pten knockout in the intestinal epithelium, 86% of the mice develop malignant tumors in both the small intestine and colon and 8% of the tumor-bearing mice have metastases (129).

The protein mitochondrial transcription factor A (TFAM) is required for expression and maintenance of mitochondrial DNA (mtDNA) (54). Woo et al. (122) found that mice heterozygous for the TFAM knockout (Tfam+/−) have mild mtDNA depletion but no other overt phenotypes. However, they also showed that when these mice are crossed with ApcMin mice, their offspring have increased tumor number and faster tumor growth. This was proposed to be due to increased generation of ROS from mitochondria, suggesting these double-mutant mice might be particularly useful to study the impact of dietary agents on antioxidant systems or on DNA repair mechanisms.

A final model worth considering could be useful to evaluate the impact of dietary agents on CIN, a central feature of tumors developing in the distal colon. Bub1 is a serine/threonine kinase critical for the mitotic checkpoint, and Bub1 hypomorphic mice are prone to aneuploidy and cancer (96). When Bub1 hypomorphic mice are crossed with ApcMin mice, colon tumor incidence increased from 25 to 90% owing to loss of heterozygosity of the remaining wild-type Apc allele and/or duplication of a copy of chromosome 18 with the ApcMin allele (6).

Xenografts, Allografts, and Models of Metastasis. After tumors have developed, the goal of dietary chemoprevention is to limit tumor cell growth, block neovascularization and angiogenesis, and enhance antitumor immunity. When researchers want to study the impact of a treatment on established tumors they use xenografts of human primary tumor cells and cell lines [e.g., HCT116, KM12SM, LIM1215, SW480 (126), SW620 (73)] implanted in immunodeficient mice, or allografts of mouse tumor cells implanted into genetically matched hosts. Some evidence suggests that human cell xenografts have more clinical predictive value than mice cell allografts (116). However, human xenografts in immunodeficient mice eliminate the ability to study tumor growth and angiogenesis whereas mouse cell allografts can be used to study this biology (94, 117). Unfortunately, both mouse and human cell lines suffer from mutational drift with extended culture (70) and no single cell line models the diversity of human cancer (14a). As a result, patient-derived xenografts (PDX) are being utilized to capture the mutational diversity of human colorectal cancer and to develop personalized treatment regimens (101). Although the challenge of acquiring tumors suggests use of PDX will be limited to researchers in hospital environments, The Jackson Laboratories now has a PDX resource available with many colorectal cancer tumors (http://jaxservices.jax.org/invivo/pdx.html). Given the promise of this approach, similar repositories are likely to be developed elsewhere in the future. In addition, the available tumor samples will likely be fully sequenced so that researchers can choose samples to test based on classification of their mutational spectrum.

Colon cancer generally metastasizes to two tissues: liver and lung. A general weakness of animal models of colorectal cancer is that they rarely develop advanced tumors that metas-
tasize to other tissues. Because of this, researchers interested in the impact of dietary agents on metastasis have been limited to transplantation of cancer cell lines into mice. Morikawa et al. (78) first reported that intrasplenic injection of primary human colon tumor cells into nude mice (Balb/c) leads to lung and liver metastases. Later it was shown that both intravenous and intrasplenic injection of the mouse colon tumor cell line CT26 cells (from Balb/c mice) cause lung and liver metastases in genetically matched hosts (130). Zhang et al. (132) recently reported a modification of this method that significantly improved the efficiency of metastasis. They passed CT26 cells through three rounds of in vivo selection to identify the CT26-FL3 cell line. Injecting these cells directly into the cecum resulted in 90% of the mice developing multiple liver tumors whereas just 8% of mice with cecal injection of standard CT26 cells developed single metastatic liver tumors. Recently, Chowdhury et al. (18) developed a metastasis model whereby HCT116 cells are first grown as xenografts in nude mice then removed and reimplanted orthotopically into the colon where 68% of mice develop either lung or liver metastases. Finally, PDXs also metastasize to lung and liver when implanted into the cecum of nude mice (85).

Several genetically modified mouse models develop metastasis. Smad3 knockout mice on the 129/Sv background developed aggressive colorectal adenocarcinomas within 4–6 mo and this was accompanied by frequent metastasis to regional lymph nodes (133). Hung et al. (43) used surgical introduction of a Cre-expressing adenovirus to induce recombination of floxed alleles in the distal colon of Apc<sup>Min</sup>;LSL-Kras<sup>G12D/+</sup> mice. Carcinomas appeared in these mice within 20 wk of the adenovirus injection and spontaneous gross liver metastases were observed by 24 wk after the injection. Finally, although neither Villin-Cre;LSL-Kras<sup>G12D/+</sup> nor Villin-Cre;TGFb2<sup>−/−</sup> double transgenic mice form intestinal neoplasms, 15% of Villin-Cre;LSL-Kras<sup>G12D/+</sup>;TGFb2<sup>−/−</sup> triple transgenic mice developed lymph node and lung metastases by 22 wk of age through a β-catenin-independent mechanism (110).

Conclusion

The field of dietary cancer prevention holds great promise for reducing cancer incidence, slowing cancer progression, improving cancer outcomes, and limiting cancer recurrence. However, the challenge facing the field is to conduct studies that demonstrate a direct role for a dietary pattern or agent on the course of cancer, i.e., to move past the associations identified in population studies. This is extremely challenging to do with clinical trials, especially for lifelong prevention strategies. As a result, animal models will be a critical component in the process of validating hypothesis regarding dietary chemoprevention. However, to realize this promise of dietary cancer prevention, researchers need to carefully choose the preclinical models they use in their studies. This selection should be based on a clear understanding of human colorectal cancer, recognition of how animal models relate to the human disease, an appreciation for the strengths and weakness of the model they choose, and a clear hypothesis for how the dietary intervention might influence carcinogenesis or cancer cells in the model. Our emerging understanding of the molecular diversity of human colorectal cancer is going to make this challenge greater, and it is clear that neither common models like the Apc<sup>Min</sup> mouse nor any other single model is sufficient for testing all dietary cancer prevention strategies for all subtypes of colorectal cancer. Fortunately, there are now a variety of excellent models that should vastly improve the quality of research resulting from preclinical studies on dietary prevention of colorectal cancer.

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