Mouse Model of Colonic Adenoma-Carcinoma Progression Based on Somatic Apc Inactivation

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Abstract

Mutations in the adenomatous polyposis coli (APC) gene are pivotal in colorectal tumorigenesis. Existing mouse intestinal tumor models display mainly small intestinal lesions and carcinomas are rare. We defined human CDX2 sequences conferring colon epithelium-preferential transgene expression in the adult mouse. Mice carrying a CDX2-NLS Cre recombinase transgene and a loxP-targeted Apc allele developed mainly colorectal tumors, with carcinomas seen in 6 of 36 (17%) of mice followed for 300 days. Like human colorectal lesions, the mouse tumors showed biallelic Apc inactivation, β-catenin dysregulation, global DNA hypomethylation, and aneuploidy. The predominantly distal colon and rectal distribution of tumors seen in mice where one Apc allele was inactivated in epithelial cells from distal ileum to rectum suggests that regional differences in the intestinal tract in the frequency and nature of secondary genetic and epigenetic events associated with adenoma outgrowth have a contributing role in determining where adenomas develop. The presence of large numbers of small intestine tumors seemed to inhibit colo-
rectal tumor development in the mouse, and gender-specific effects on tumor multiplicity in the distal mouse colon and rectum mimic the situation in humans where males have a larger number of advanced adenomas and carcinomas in the distal colon and rectum than females. The mouse model of colon-preferential gene targeting described here should facilitate efforts to define novel factors and mechanisms contributing to human colon tumor pathogenesis, as well as work on tumor-promoting environmental factors and agents and strategies for cancer prevention and treatment. [Cancer Res 2007;67(20):9721–30]

Introduction

Colorectal cancer is a leading cause of cancer-related death in much of the industrialized world (1, 2). Despite much progress, we lack a full accounting of the molecular pathogenesis of colorectal cancer or key dietary and environmental factors in colorectal cancer development. A current view is that most colorectal cancers arise from adenomatous precursors, and accumulated gain-of-function mutations in proto-oncogenes and loss-of-function mutations in tumor suppressor genes underlie initiation of adenomatous lesions and progression to carcinoma (2–4). The identification of germline mutations predisposing to tumors in man and/or the mouse has highlighted genes with roles in sporadic tumor development and has clarified mechanisms via which cancers arise. For instance, whereas familial adenomatous polyposis (FAP) syndromes account for <1% of colorectal cancer cases, the identification of heterozygous germline mutations in the adenomatous polyposis coli (APC) gene in FAP patients led to efforts showing that somatic APC defects are present in ~75% of sporadic colorectal adenomas and carcinomas (2, 4). Work on the APC gene also spurred studies that showed that a nonsense mutation at codon 850 of the murine Apc gene was responsible for predisposition to a multitude of small intestinal tumors in a mouse genetic model of multiple intestinal neoplasia (Min; refs. 4, 5). Other studies established that the APC protein regulates β-catenin, a key downstream mediator of Wnt signals, with oncogenic effects as a transcriptional coactivator of T-cell factor proteins (reviewed in ref. 6).

C57BL6/J mice carrying the ApcMin mutation develop ~50 adenomas and infrequent carcinomas in the small intestine by 120 to 140 days of age, along with rare colon adenomas and carcinomas (7, 8). The ApcMin mouse has been of keen interest for studying intestinal tumorigenesis mechanisms and potential cancer prevention and treatment strategies (8). Nevertheless, the ApcMin mouse has notable limitations as a model for human colorectal tumorigenesis. The ApcMin mouse manifests chiefly small intestinal lesions, but the vast majority of gastrointestinal tumors in FAP patients are found in the colon and rectum. Overall, small intestine carcinomas are rare in humans, with a prevalence that is about one thirtieth of that of colon carcinomas (9) Perhaps because ApcMin mice usually die by 140 days of age due to anemia and/or intestinal obstruction, few adenomas progress to carcinoma in ApcMin mice (8). Mice with other Apc germ-line mutations (10–14) and mice in which Apc has been targeted for somatic inactivation by Cre recombinase (15) have been described. However, whereas some of these mice develop more colonic lesions than ApcMin mice, mice with constitutional Apc mutations develop predominantly small intestinal tumors. As such, they offer modest value over the ApcMin mouse for modeling adenoma-carcinoma progression in the colon. In fact, most genetically engineered mouse models of intestinal tumorigenesis manifest largely or exclusively small intestinal lesions (13), and adenoviral delivery of Cre to target Apc in distal colon tissues is a technically challenging approach described in only a single article (15).

Given this background, we sought to define sequence elements that might confer colon epithelium-preferential transgene expression in the mouse. Our previous studies revealed that the human CDX2 homeobox gene, which is expressed in epithelial cells throughout adult small intestine and colon, contained key cis elements for regulating transcription in cultured colon cancer cells
We show here that the CDX2 elements confer colon epithelium-preferential transgene expression in the adult mouse. Mice carrying a CDX2P-NLS Cre recombinase transgene and a floxed Apc allele developed colorectal adenomas, and carcinomas arose in ~15% to 20% of mice. Morphologic and molecular studies of the mouse tumors revealed their similarity to human colorectal tumors, suggesting that mice in which the CDX2P-NLS Cre transgene is used to target Apc and other genes of interest will be valuable for studies in colorectal cancer prevention, diagnosis, and therapy.

Materials and Methods

Plasmid and transgene construction. Plasmid vectors for the CDX2P 9.5-NLS lacZ and CDX2P 0.5-NLS lacZ transgenes were constructed using the pMlacF vector (R. Palmiter, University of Washington), which provided the β-gal (lacZ) expression cassette with nuclear localization signal (NLS) sequences and a polyadenylation addition site from mouse protamine gene (mP1pA). The 9.5- and 0.5-kb fragments, containing various 5′-flanking sequences from the human CDX2 promoter region, have previously been described (16). These fragments were subcloned into the pMlacF vector. The CDX2P-NLS Cre transgene was generated by subcloning the 9.5-kb CDX2 promoter fragment along with a polyadenylation cassette from the bovine growth hormone gene (BGHpA) into an Cre-1 plasmid (S. Camper, University of Michigan, Ann Arbor, MI), which provides the NLS Cre recombinase cassette.

Mouse colony. We cared for mice and carried out experimental procedures under approval from the University Committee on Use and Care of Animals, University of Michigan and according to the Michigan state and U.S. federal regulations. Transgenic founders on a mixed background [C57BL6/J X SJL/J] were backcrossed with C57BL6/J mice. CDX2P 9.5-NLS Cre mice or Villin-Cre mice [B6SJ-Tg(Vill-cre)997Gam/J] were intercrossed with mice harboring the Apc homozygously (ApcAPFPlas, 580S; ref. 15) or the loxP-Stop-loxP lacZ [Gt(Rosa)26Sor, R26R] reporter allele (17). Progeny from the cross between CDX2P 9.5-NLS Cre and ApcAPFPlas were intercrossed to obtain an outbred colony, segregating for the C57BL6/J and SJL genomes at a ratio of 93.75% and 6.25%, respectively. All the mice were housed in specific pathogen-free conditions. Teklad Mouse Breeder Diet 8626 (Harland-Teklad) and automatically supplied water were provided ad libitum to mice for tumorigenesis experiments. R26R reporter mice were kindly provided by S. Camper (University of Michigan, Ann Arbor, MI).

Tissue fixation and quantification for β-galactosidase. Dissected gastrointestinal tissues were opened and washed with PBS containing 0.01% Triton X-100 at 4°C with agitation. After brief fixation with 4% paraformaldehyde containing 1.25 mmol/L EGTA, 2 mmol/L MgCl2 in PBS, tissues were placed in 4% 1:3:3:3 (v/v/v/v) 0.1 M of 3′-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining solution [1 mg/mL X-gal in N,N-dimethylformamide, 5 mmol/L K4Fe(CN)6, 5 mmol/L K3Fe(CN)6, 2 mmol/L MgCl2, 125 mmol/L MgCl2 in PBS] for 4 to 12 h at 37°C (18). The tissue was fixed with 0.2% glutaraldehyde for 10 min and with 4% paraformaldehyde for 4 h at 4°C. Embryos and tissues were then stored in 70% ethanol. For quantitative analysis of β-gal expression, 30 to 50 μg of total protein from homogenate were incubated in β-gal buffer (60 mmol/L Na2HPO4, 40 mmol/L NaH2PO4, 1 mmol/L MgCl2, 50 mmol/L β-mercaptoethanol, 2.2 mmol/L o-nitrophenyl-β-D-galactopyranoside) at 37°C for 3 h, and absorbance at 405 nm was measured.

Immunohistochemical staining. We carried out immunohistochemical staining as described (19). The antibodies against β-catenin (BD Transduction Laboratories), CDX2 (19), p53 (Novocastra Laboratories, NCL-p53-CM5p), and 5-methylcytochrome C (Calbiochem, NA81) were used at dilutions of 1:200, 1:1,000, 1:1,000, and 1:200 (final concentration, 5 μg/mL, respectively).

Microdissection. Apc allele analysis, and KRAS analysis. Formalin-fixed, paraffin-embedded tissues were sectioned at 5 μm and mounted on glass slides, then weakly stained with hematoxylin. Specific regions (neoplastic versus non-neoplastic tissue) were carefully laser-capture microdissected using PixCell IIe apparatus (Arcturus Engineering). The number of cells that were captured ranged from 100 to 500 cells. DNAs were extracted in 30 to 50 μL of proteinase K buffer using PicoPure DNA extraction kit (Arcturus). Analysis for loss of heterozygosity of Apc alleles was done by multiple PCR using Apc-P3 primer 5′-GTTCTGTATCATG-GAAAGATAAGGTTGCT-3′, Apc-P4 primer 5′-CAGCTAAACGCTTTTGA-GTTGAATTC-3′, Apc-P5 primer 5′-GAATGGCGGCTTCCTGCTCAGTA-3′, targeted allele (580S), deleted allele (580D), and wild-type allele amplified as 314-bp (P5 and P3), 258-bp (P3 and P5), and 226-bp (P3 and P4) PCR products, respectively (15). For p53 mutational analysis, Trp53E5-S (5′-TC-CAATGTGCTGAGCACATGTG-3′), Trp53E5-AS (5′-CCTAACAGAGCAAA-TAAGTCAG-3′), Trp53E6-S (5′-CTGCTCAGGTAGTATGGAA-3′), Trp53E6-AS (5′-CTAAAGCTAGTAGCAGC-3′), Trp53E7-S (5′-AAC-TTATTAGGCTATAGCAG-3′), Trp53E7-AS (5′-ACTTAGAGTAGGATTGAG-3′), Trp53E8-S (5′-CTTGGCGCTAGATATGCAAG-3′), and Trp53E8-AS (5′-TGTGAGAGAGAGACAGGTT-3′) were used. For KRAS codon 12 or 13 mutational analysis, KRAS 1S (5′-TTATTGTA-AGGCCTGCTGAA-3′) and KRAS 1AS (5′-GACCTGTTACCTCTATCGTA-3′) were used.

Microsatellite stability analysis. Microsatellite analysis of mouse tumors was done as described using six microsatellite repeat markers previously shown to be informative in tumors from DNA mismatch repair-deficient mice (20–22): A33, G429, D7Mit91, D17Mit123, mBat-26, and mBat-37.

Primary culture of tumors. Primary culture of colon tumor cells was largely done as described (23, 24). Freshly isolated tumor specimens were minced with sterile razor blades, digested in HBSS containing 60 units/mL collagenase, 0.02 mg/mL dispase I, 0.2 mg/mL soybean trypsin inhibitor, and 2% bovine serum albumin for 1 h at 37°C. Tumor cells were separated and collected as pellet after sedimentation in DMEM containing 10% sorbitol and 5% fetal bovine serum. The pellets were washed with DMEM containing 2% sorbitol, twice, and cells were cultured in 60-mm plates with NIH 3T3 feeder cells grown on Matrigel-coated surface and treated with 10 μg/mL mitomycin C for 2 h using DMEM/Ham’s F-12 (1:1) with 1-glutamate and 15 mmol/L HEPES containing 5 μmol/L dexamethasone, 10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 mg/mL selenium, 2 μg/mL ethanolamine, 5 μmol/L triiodothyronine, 10 ng/mL epidermal growth factor, 0.2% α-glucose, 50 μg/mL gentamicin, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2% fetal bovine serum for 48 h.

Spectral karyotyping analysis. Spectral karyotyping analysis was done by treating the cells with 80 mmol/L 4-calycullin A in Ham’s F-10 medium for 1 h at 37°C to induce premature chromosome condensation (25) instead of inducing mitotic arrest. Chromosomal aberrations were quantified with an Olympus BX-61 microscope equipped with an Applied Spectral Imaging interferometer and 40× and 63× objectives. Chromosome counts were determined by analysis of 10, 8, 19, 10, and 13 independent chromosome spreads from one short-term cultured cell line from each of five independent adenomas.

Results

CDX2 elements regulate gene expression in colon epithelium of adult mice. Previously, we found that a 9.5-kb fragment from the CDX2 proximal promoter region (CDX2P 9.5) strongly activated reporter gene expression in cultured colon cancer cells that expressed CDX2 transcripts (16). The CDX2P 9.5 reporter construct had weak activity in cells with reduced or absent CDX2 endogenous expression. Based on these data, we generated transgenic mice in which the CDX2P 9.5 fragment or a much shorter CDX2 promoter fragment (CDX2P 0.5) was present upstream of sequences encoding β-galactosidase (β-gal; Fig. 1A, CDX2P 9.5-NLS lacZ and CDX2P 0.5-NLS lacZ). Analysis of CDX2P 0.5-NLS lacZ transgenic mice revealed no specific and robust β-gal expression in the gastrointestinal tract, and they were not studied further. In adult mice from three independent transgenic lines carrying the CDX2P 9.5-NLS lacZ transgene, strong homogeneous β-gal expression was seen in...
epithelium of the cecum and proximal colon, with more variable expression in epithelium from the terminal ileum and distal colon in low-copy-number transgenic lines (Fig. 1B). Of the three lines, the highest copy line showed β-gal expression through a larger region of the distal ileum and in all colon epithelium (Fig. 1B). Restricted expression of the transgene in terminal ileum, cecum, and colon in adult high-copy transgenic CDX2P 9.5-NLS lacZ mice was confirmed with a quantitative assay (Fig. 1C).

We analyzed β-gal expression in CDX2P 9.5-NLS lacZ transgenic embryos at 8.5, 10.5, and 12.5 days postconception (dpc; Fig. 1D). Consistent with prior data on endogenous Cdx2 expression during development (26), β-gal expression was seen in the tail bud and caudal part of the neural tube at 8.5 and 10.5 dpc. At 12.5 dpc, when Cdx2 expression in gut endoderm has been reported, β-gal expression was seen in the presumptive colon region of CDX2P 9.5-NLS lacZ transgenic embryos (Fig. 1D). We further documented β-gal expression in colon, but not small intestine, of embryos by analyzing endogenous CDX2 protein expression simultaneously via immunohistochemistry (Fig. 1E). The findings indicate that the CDX2P 9.5 fragment regulates gene expression in the caudal region...
of the embryo during early development, but, later in development and in the adult, the fragment is active predominantly in colon epithelium.

**CDX2P 9.5-NLS Cre transgene-mediated targeting of loxP sites in vivo.** We generated transgenic mice carrying a Cre recombinase gene controlled by the CDX2P 9.5 fragment (Fig. 1A; CDX2P-NLS Cre transgene) to assess the utility of the mice for somatic targeting of loxP-containing genes. We used a line with ~50 copies of the transgene for subsequent studies. By crossing the CDX2P-NLS Cre mice with R26R reporter mice (17), we found that Cre recombinase had been active in epithelium from the distal ileum to the distal colon and from the crypt base to the luminal surface (Fig. 2A). Consistent with our findings from analysis of the embryonic expression pattern of the CDX2P 9.5-NLS lacZ transgene, the CDX2P-NLS Cre transgene mediated recombination that led to R26R reporter expression in extraintestinal cell populations of adult mice (e.g., tissues of the hind legs; Fig. 2B). The patterns of extraintestinal β-gal expression in adult mice were predictable based on CDX2P-NLS Cre transgene activity in the caudal region of the embryo (Fig. 2C).

**Figure 2.** Expression of β-gal reporter gene in CDX2P-NLS Cre;R26R mice. A, left, expression of β-gal from terminal ileum to rectum. Bar, 10 mm. Right, expression of β-gal in colon epithelium from crypt to surface. Bar, 1 mm. B, quantitative analysis of β-gal expression in tissues/organs from CDX2P-NLS Cre;R26R mice. C, β-gal gene expression during embryogenesis in CDX2P-NLS Cre;R26R mice (12.5 dpc).
**CDX2P-NLS Cre;Apc**<sup>+/loxP</sup>** mice develop colorectal adenomas and carcinomas.** Mice carrying an Apc allele in which loxP sites flank exon 14 were crossed with CDX2P-NLS Cre<sup>+/loxP</sup> (CPC;Apc) mice. Cre targeting of the Apc<sup>loxP</sup> allele deletes exon 14, leading to a frameshift mutation at codon 580 and a truncated APC protein (15). We also targeted Apc for conditional inactivation using another transgenic strain expressing Cre in the gastrointestinal tract (i.e., Villin-Cre; Apc<sup>−/−</sup> mice; ref. 18). Using this strain, Cre targeting of loxP-containing genes has been reported in epithelium throughout the small intestine and colon. As shown in Table 1, 36 CPC;Apc mice, 6 Villin-Cre;Apc mice, and 28 Apc<sup>+/loxP</sup> (control) mice were studied for up to 300 days. Thirteen (36%) CPC;Apc mice survived up to the study end point. Three (8.3%) CPC;Apc mice died, likely due to intestinal obstruction by tumor, with the remaining 20 CPC;Apc mice euthanized on signs of distress. The average observation period for CPC;Apc mice was ~200 days (Table 1), and the Villin-Cre;Apc and Apc<sup>+/loxP</sup> mice were euthanized at similar ages to compare phenotypes. The CPC;Apc mice manifested only mild anemia, with hematocrit values in the range of 33% to 36% (Table 1), in spite of rectal tumors, male CPC;Apc mice had similar numbers of small intestinal and cecal tumors, male CPC;Apc mice had ~60% more colon tumors (Table 1; Fig. 3A). In Villin-Cre;Apc mice, we found ~36 tumors per mouse, the vast majority of which were located in the small intestine (Table 1; Fig. 3C and D). The cecal and colon tumors observed in Villin-Cre;Apc mice were smaller than those in similarly aged CPC;Apc mice (Table 1; Fig. 3C and D). Because few Villin-Cre;Apc mice were studied, the significance of the apparent gender differences in tumor multiplicity and distribution in Villin-Cre;Apc mice is uncertain. No tumors were observed in similarly aged control Apc<sup>+/loxP</sup> mice (Table 1) or in CDX2P-NLS Cre or Villin-Cre;Apc mice (data not shown). Embryonic lethality was seen in CDX2P-NLS Cre and Villin-Cre transgenic mice homozygous for the Apc<sup>loxP</sup> allele.

**Colon tumors in CPC;Apc mice manifest features akin to those in human tumors.** Microscopic examination of the 231 colorectal tumors that arose in the 36 CPC;Apc mice revealed that 90% of the lesions resembled pedunculated-type tumors in man, in which a relatively well-differentiated, epithelial-rich lesion with tubular-type glands is attached to the mucosa by a stroma-rich stalk (Fig. 4A). In contrast, 10% of colorectal lesions and 54% of the 40 cecal lesions identified, the tumors displayed a sessile appearance, where the neoplastic glands were minimally, if at all, raised above the mucosal surface (Fig. 4B). Of the 109 small intestine lesions from CPC;Apc mice studied, 71% had a sessile appearance and 29% were pedunculated. In the cohort of 36 CPC;Apc

### Table 1. Phenotypic characteristics of CPC;Apc, Villin-Cre;Apc, and Apc<sup>+/loxP</sup> mice

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<th>Age (d)</th>
<th>Body weight (g)</th>
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<th>Small intestine</th>
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<td>CPC;Apc</td>
<td>36</td>
<td>203 ± 26</td>
<td>26.2 ± 3.4</td>
<td>34.8 ± 9.7</td>
<td>380 (10.6)</td>
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<td>18</td>
<td>196 ± 22</td>
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<td>18</td>
<td>207 ± 29</td>
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<td>Villin-Cre;Apc</td>
<td>6</td>
<td>203 ± 2</td>
<td>28.4 ± 4.6</td>
<td>24.2 ± 7.7</td>
<td>217 (36.2)</td>
<td>185 (30.8)</td>
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<td>Apc&lt;sup&gt;+/loxP&lt;/sup&gt;</td>
<td>28</td>
<td>202 ± 24</td>
<td>31.2 ± 7.8</td>
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*Colon and rectal lesions were scored collectively as colon.
mice, we defined the most advanced lesion histologically, with 22 (66.1%) mice harboring at least one tumor with intramucosal carcinoma features. Six of the 36 (16%) CPC;Apc mice had a total of eight lesions defined as invasive carcinoma into submucosa, with associated desmoplasia and multiple invading irregular glands seen (Fig. 4C). Two of the eight carcinomas were in the cecum and three were in the colon. Intramucosal carcinoma was far more common in tumors >5 mm, but there was no clear relationship between tumor size and the presence of invasive carcinoma into submucosa (data not shown). None of the six CPC;Apc mice with invasive carcinoma had detectable lymph node or distant metastases. Even in the case of a 20-month-old CPC;Apc mouse with a large, deeply invasive distal colorectal carcinoma (Fig. 4D), lymph node or distant metastases were not found. Mammary gland tumors, with histologic features like those reported in Apc\textsuperscript{Min} mice (27), were seen in 2 of 36 CPC;Apc mice. In one Villin-Cre;Apc mouse, a hepatic adenoma was found. No other extracolonic tumors were observed in the CPC;Apc or Villin-Cre;Apc mice studied.

**Somatic gene defects in CPC;Apc mouse colon tumors.** Prior studies in mice carrying constitutional, heterozygous inactivating mutations in the Apc gene (Apc\textsuperscript{Min}, Apc\textsuperscript{-\textDelta14}, Apc\textsuperscript{-\textDelta1638N} and Apc\textsuperscript{-\textDelta124}) have shown that the wild-type Apc allele is inactivated in small intestine and colon adenomas, most often by allelic loss, at least in the case of C57BL6/J mice (11, 12, 14, 28). From a total of 15 CPC;Apc mice, we isolated DNA from neoplastic cells of 129 tumors, along with DNA from adjacent normal epithelium. In 109 of 129 (84.5%) tumors analyzed, the intensity of the wild-type Apc allele was greatly reduced, relative to the pattern for the wild-type and Cre-targeted (580D) Apc alleles in normal mucosa (Fig. 5A).

Based on inefficiencies in eliminating nonneoplastic elements, even in laser-capture microdissection approaches, and alternative mechanisms of inactivating Apc besides allele loss, our analysis likely underestimated the frequency of wild-type Apc allele inactivation in tumors. Indeed, consistent with the view that loss of the APC protein function in regulating \(\beta\)-catenin levels and localization contributed to the development of nearly all tumors, immunohistochemical analysis of 97 tumors from 19 different CPC;Apc mice revealed strong \(\beta\)-catenin accumulation in the nucleus and cytosol of neoplastic cells in all 97 tumors, as compared with the membrane-associated \(\beta\)-catenin staining seen in adjacent normal...
epithelium (Fig. 5B). Alterations in the murine Cdx2 gene have been suggested to cooperate with Apc defects in intestinal tumorigenesis (29, 30). We found reduced or absent Cdx2 protein expression in the neoplastic cells of 88 of the 97 (91%) tumors analyzed (Fig. 5B).

In the case of human colon tumors, missense mutations of codon 12 of the KRAS gene are present in 40% to 45% of sporadic adenomas and carcinomas and KRAS codon 13 missense mutations are found in a fraction of tumors (2, 3). In our sequence-based studies of 97 tumors from the CPC;Apc mice, we did not identify any mutations of KRAS codon 12 or 13. Missense p53 mutations are found in 50% to 60% of human colorectal carcinomas, but such mutations are seen in <10% of colorectal adenomas (2, 3). To address the potential role of p53 defects in tumors in CPC;Apc mice, we first studied p53 expression by immunohistochemistry. We found that 93 of the 97 tumors studied showed moderately elevated p53 expression in the nucleus of neoplastic cells relative to adjacent normal mucosa (Fig. 5B), with the remaining 4 tumors showing p53 expression equivalent to levels in normal mucosa. Consistent with the immunohistochemistry results suggesting that endogenous wild-type p53 expression might be elevated in tumors perhaps due to cellular stresses (e.g., hypoxia), sequence-based analysis of exons 5 to 8 of the Trp53 gene were carried out in 86 of the 93 tumors with moderate p53 expression, and no missense

Figure 4. Histologic analysis of tumors in CPC;Apc mice. H&E staining of a distal colon tumor of pedunculated type (A) and a cecal lesion of sessile type (B). Bar, 1 mm. C, H&E staining (left) and immunohistochemical staining with anti-β-catenin antibody in a tumor showing invasive adenocarcinoma. Bar, 1 mm. Insets, invasion of multiple malignant glands into the submucosa (bar, 100 μm). D, example of a large distal colon carcinoma in a 20-month-old female CPC;Apc mouse. Dissection microscopic image (bar, 5 mm) and H&E staining (bar, 100 μm; m, mucosae; sm, submucosal mp, muscularis propria) reveal sessile appearance with invasive carcinoma, along with desmoplasia and inflammatory cells, in the muscular wall. Arrowheads, invasive front of the neoplastic cells. Immunostaining for β-catenin and p53 in serial sections of the tumor indicates nuclear and cytosol accumulation of β-catenin and strong nuclear accumulation of p53 in the neoplastic elements.
substitutions were seen. The only lesion with robust p53 nuclear staining was the large deeply invasive distal colon carcinoma found in a 20-month-old CPC;Apc mouse (Fig. 4D). This case harbored a p53 missense mutation, resulting in substitution of valine for alanine at mouse p53 codon 156 (corresponding to human codon 159).

A number of prior studies in the literature have established that whereas focal hypermethylation of certain CpG-rich sequence elements (e.g., the promoters of selected genes) can often be seen in colorectal tumors (reviewed in refs. 3, 31), global DNA hypomethylation is a common feature of human colorectal adenomas and carcinomas (31–33). Immunohistochemistry studies with an anti-5-methylcytosine antibody in each of 17 tumors studied yielded data indicating that decreased global DNA methylation in neoplastic cells relative to adjacent normal epithelium was a common feature of CPC;Apc tumors (Fig. 5B).

Genomic instability has been suggested to play a critical role in the initiation and/or progression of the majority of colorectal tumors in man (34). To assess microsatellite instability in the CPC;Apc tumors, we studied three mononucleotide repeat and three dinucleotide repeat markers that have previously been shown to manifest instability in a subset of tissues and tumors from mice defective for mismatch repair (20–22). Of 66 CPC;Apc tumors analyzed with these six markers, one tumor showed variant alleles at one mononucleotide repeat marker (data not shown). The results imply that the vast majority of tumors had a microsatellite stable phenotype. To investigate potential chromosomal instability of CPC;Apc tumors, we carried out spectral karyotyping analysis on one short-term primary culture line of epithelial cells from each of five independent large colorectal adenomas. Spectral karyotyping analysis and 4',6-diamidino-2-phenylindole (DAPI) staining revealed that the primary cultured cells had aneuploidy, with 58.56 ± 17.68 chromosomes seen (Fig. 5C and data not shown), although no specific structural rearrangements were noted. The findings suggest that chromosomal instability has a contributing role in tumor progression in the CPC;Apc mouse model.
Discussion

Data from clinical, pathologic, and molecular studies indicate that most human colorectal cancers arise from adenomatous precursors (1–4). However, only a fraction of adenomas progress to cancer. Adenomas >1 cm in size have a roughly 10% chance of progressing to carcinoma over a 10-year period (35). APC defects seem to have a preeminent role in the pathogenesis of colorectal adenomas and carcinomas in man: (a) biallelic inactivating mutations in the APC gene are found in ~75% of sporadic adenomas and carcinomas, and (b) APC inactivation appears to play a critical initiating role in adenoma development in the inherited setting of FAP and in sporadic cases (2–4). Mouse strains carrying germline Apc mutations, particularly the Apc<sup Min</sup> mouse, have been of keen interest for insights they can offer into intestinal tumor pathogenesis, for their potential value in defining environmental factors affecting tumor development, and for identifying novel agents and strategies for cancer prevention and treatment (8). Nonetheless, Apc<sup Min</sup> mice have limitations for modeling human colorectal cancer, particularly because they manifest predominantly small intestinal lesions (7, 8, 13), whereas sporadic and inherited intestinal tumors in man arise overwhelmingly in the colon and rectum. In addition, perhaps due to their death from severe anemia by 140 days of age, few Apc<sup Min</sup> mice develop carcinomas. Thus, mouse models that better recapitulate features of human colorectal tumorigenesis are needed. We have described a mouse model of colorectal adenoma-carcinoma progression in which a CDXP2-NLS Cre transgene is used to target Apc for somatic inactivation preferentially in the colon.

Whereas mice carrying CDXP2 9.5-regulated transgenes expressed the transgenes throughout the caudal region during early development, transgene expression was restricted to distal small intestine, cecum, and colon and rectum later in development and in adult tissues. Consistent with this expression pattern, the CPC\_Apc mice reproducibly developed multiple colorectal tumors, with few small intestine lesions. The majority of lesions were adenomas, but carcinomas were seen in 17% of CPC\_Apc mice during a 300-day follow-up period. In a 20-month-old CPC\_Apc mouse, we found a large carcinoma in the distal colon. More carcinomas and perhaps even metastatic lesions might have been seen in an older cohort of CPC\_Apc mice because the cohort of 36 CPC\_Apc mice studied in depth here were followed only to 300 days of age and 36% of the CPC\_Apc mice were alive at this time point.

Besides displaying morphologic features similar to human colorectal tumors, tumors arising in CPC\_Apc mice had molecular features resembling those in human lesions. The vast majority of the mouse lesions had biallelic Apc inactivation and β-catenin dysregulation. We also found that CPC\_Apc tumors showed global DNA hypomethylation relative to adjacent normal epithelium. Spectral karyotyping analysis of a short-term primary cell line from each of five independent adenomas revealed aneuploidy in all lines without evidence of specific structural rearrangements. We found no evidence of somatic p53 or KRAS mutations in the vast majority of tumors obtained from CPC\_Apc mice. Based on studies of human colorectal tumors, our results are perhaps not unexpected. Only 10% of human adenomas <1 cm in size have KRAS mutations (3, 36), and only 3 of 380 lesions in our cohort of 36 mice were >1 cm. Additionally, because the overwhelming majority of the CPC\_Apc tumors studied were adenomas, and p53 mutations are rare in human adenomas (2–4), it is not surprising that p53 mutations were not found in the vast majority of tumors studied.

Of interest, the most advanced CPC\_Apc carcinoma studied had acquired a p53 missense mutation. The virtual absence of microsatellite instability in the mouse CPC\_Apc tumors studied is also perhaps not surprising when the situation in man is considered. High-frequency instability of microsatellite repeat tracts is rarely seen in sporadic adenomas or in adenomas arising in FAP patients (2, 4). Microsatellite instability is most common in carcinomas arising in the proximal colon (2), and the vast majority of the CPC\_Apc mouse lesions were adenomas in the distal colon.

Our studies with the CPC\_Apc model yielded some observations that will require further work to understand better the biological significance and underlying mechanisms. First, the distribution of tumors in the intestinal tract of the CPC\_Apc mice was not uniform. On average, one tumor was found in the cecum and five to eight tumors were found in the colon and rectum, with most located distally. These findings on the nonuniform distribution of tumors in affected mice, at least relative to the pattern of Cdx2 9.5 promoter activity and Apc gene targeting in the mice, imply that additional somatic defects promoting the clonal outgrowth of epithelial cells with a single Apc-mutant allele may not occur in a stochastic fashion in the intestinal tract. Further studies are needed to determine whether the nonuniform distribution of tumors is largely due to regional differences in the frequency of wild-type Apc allele inactivation, regional differences in the frequency and types of additional somatic events required for progressive adenoma growth, or perhaps both possibilities.

Second, male and female CPC\_Apc mice had equivalent numbers of small intestinal and cecal tumors, but male mice had a 50% increase in distal colon and rectal tumors relative to females. This tendency toward a 50% increase in distal tumors was also seen in male Villin-Cre\_Apc mice, but the number of Villin-Cre\_Apc mice studied was limited. In humans, males have been reported to have a larger number of colon adenomas of clinically significant size (i.e., >9 mm) in the distal half of the colon, compared with females (37). Data also indicate that hormone replacement therapy in women conferred protection against colorectal adenomas (38), and a recent study reported that estrogen receptors α and β are inhibitory modifiers of Apc-dependent tumorigenesis in the proximal colon of Apc<sup Min</sup> mice (39).

Third, our data suggest that small intestinal tumor burden may inhibit via an unknown mechanism the development and/or progression of colorectal tumors in the mouse. We reported here that CPC\_Apc mice developed, on average, three small intestine tumors and seven to eight colorectal and cecal tumors, whereas Villin-Cre\_Apc mice of similar age manifest ~31 small intestine tumors and five cecal and colorectal tumors, with the colorectal tumors in the Villin-Cre\_Apc mice smaller on average than those in CPC\_Apc mice. Perhaps even more remarkably, although Apc<sup Min</sup> mice carry one inactive Apc allele in all somatic cells, a typical Apc<sup Min</sup> mouse in our colony develops 50 or so small intestinal adenomas and only one or no colon adenomas (data not shown). The early death of Apc<sup Min</sup> mice by 120 to 140 days of age is not sufficient to account for the difference in colon tumor multiplicity because readily detectable distal adenomas can be found in CPC\_Apc mice at 120 days. Insights into mechanisms by which the presence of a large number of small intestine adenomas exerts apparent inhibitory effects on colorectal tumor growth in mice may be of interest for the situation in man.

In closing, whereas we have shown here the utility of the CDXP2-NLS Cre transgene for targeting the ROSA26 reporter and the Apc gene, the CDXP2-NLS Cre transgene should prove valuable for...
studying other genes in an in vivo model of colon tumorigenesis, including tumor suppressor genes that can be conditionally inactivated or proto-oncogenes that can be conditionally activated by lacP-mediated deletions. Furthermore, assuming that biallelic conditional inactivation of a given gene does not result in a developmental phenotype due to inactivation in the caudal region during embryogenesis, the CDX2P-NLS Cre transgene may also be useful for studying other genes in cell fate specification and the physiology of colonic epithelium.

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References

Mouse Model of Colonic Adenoma-Carcinoma Progression Based on Somatic *Apc* Inactivation

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