Nuclear adenomatous polyposis coli suppresses colitis-associated tumorigenesis in mice

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Mutation of tumor suppressor adenomatous polyposis coli (APC) initiates most colorectal cancers and chronic colitis increases risk. APC is a nucleo-cytoplasmic shuttling protein, best known for antagonizing Wnt signaling by forming a cytoplasmic complex that marks β-catenin for degradation. Using our unique mouse model with compromised nuclear Apc import (ApcmNLS), we show that ApcmNLS/m mice have increased susceptibility to tumorigenesis induced with azoxymethane (AOM) and dextran sodium sulfate (DSS). The AOM–DSS-induced colon adenoma histopathology, proliferation, apoptosis, stem cell number and β-catenin and Kras mutation spectra were similar in ApcmNLS/m and Apc+/+ mice. However, AOM–DSS-treated ApcmNLS/m mice showed more weight loss, more lymphoid follicles and edema, and increased colon shortening than treated Apc+/+ mice, indicating a colitis predisposition. To test this directly, we induced acute colitis with a 7 day DSS treatment followed by 5 days of recovery. Compared with Apc+/+ mice, DSS-treated ApcmNLS/m mice developed more severe colitis based on clinical grade and histopathology. ApcmNLS/m mice also had higher lymphocytic infiltration and reduced expression of stem cell markers, suggesting an increased propensity for chronic inflammation. Moreover, colons from DSS-treated ApcmNLS/m mice showed fewer goblet cells and reduced Muc2 expression. Even in untreated ApcmNLS/m mice, there were significantly fewer goblet cells in jejunum, and a modest decrease in colonicocyte Muc2 expression compared with Apc+/+ mice. Colonocytes from untreated ApcmNLS/m mice also showed increased expression of inflammatory mediators cyclooxygenase-2 (Cox-2) and macrophage inflammatory protein-2 (MIP-2). These findings reveal novel functions for nuclear Apc in goblet cell differentiation and protection against inflammation-induced colon tumorigenesis.

Introduction

As the second leading cause of cancer-related mortality in the USA, colorectal cancer (CRC) is responsible for nearly 50,000 deaths each year. Inflammation is considered a major risk factor for CRC and one in five persons with inflammatory bowel disease develop colon cancer. This colitis-associated cancer is characterized by poor prognosis and a relatively high mortality rate of ~50% (1). Anti-inflammatory drugs such as aspirin and celebrex can reduce the risk of CRC, not only in colorectal cancer patients but also in the general population (2). A better understanding of the contribution of inflammation to the underlying biology of CRC is needed to develop more effective, diagnostic and therapeutic regimens.

Abbreviations: APC, adenomatous polyposis coli; AOM, azoxymethane; Cox, cyclooxygenase; CRC, colorectal cancer; CXCR-2, CXC cytokine receptor-2; DSS, dextran sodium sulfate; GRO-α, growth-related oncoenzyme-α; IL, interleukin; MIP-2, macrophage inflammatory protein-2; mRNA, messenger RNA; NLS, nuclear localization signals; OPN, osteopontin; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Inflammation is a part of the immune response to harmful stimuli including pathogens and tumors. However, extensive and/or prolonged inflammation can result in tissue damage. Tissue repair begins with the start of inflammation and both processes are controlled by several mediators and growth factors (3). Chronic inflammation and repair have been linked to tumor formation in several tissues (4).

The colonic mucosa has several lines of defense against the immunological challenges posed by foreign antigens and microorganisms. Collectively, these lines of defense are called the intestinal epithelial barrier (6). Disruption of this barrier is a hallmark of inflammatory bowel disease and inflammation-associated colon carcinogenesis (7). Experimentally, mice unable to produce mucus develop spontaneous inflammation and are at increased risk of colon tumor formation (8). In addition to the intestinal epithelial barrier, colonocytes are continuously generated from stem cells, replacing damaged cells at the luminal surface (9).

Adenomatous polyposis coli (APC) is the primary CRC suppressor gene, with APC mutations implicated in initiation of >80% of all CRC. The APC gene product is a large multidomain protein, which localizes to both the cytoplasm and nucleus (10). APC has many cellular functions, the most recognized being as a Wnt signaling pathway antagonist (11). In this capacity, APC forms a cytoplasmic complex with axin, glycogen synthase kinase 3β and other proteins that targets the oncoprotein β-catenin for proteasome-mediated degradation (12). APC also shuttles between the cytoplasm and the nucleus using at least two nuclear localization signals (NLS) and five nuclear export signals (13-15).

APC has several proposed nuclear functions. APC and β-catenin can interact in the nucleus leading to transcriptional repression of Wnt target genes and ultimately inhibition of cellular proliferation (12). Nuclear APC interacts with topoisomerase IIs, a critical enzyme required for DNA replication and a target for traditional cancer chemotherapeutics (16). Nuclear APC has also been implicated in DNA repair (17) and synthesis (18). Until recently, analyses of nuclear APC activities relied on purified proteins or cultured cells as there was no animal model to enable characterization of nuclear APC functions in the context of a whole organism.

To better understand the role of nuclear APC in tissue homeostasis and tumor suppression, we generated a mouse model in which the nuclear import of APC was compromised by the introduction of mutations into each NLS (19). We found that these mutant mice (ApcmNLS/m) have higher rates of cellular proliferation than their wild-type counterparts and increased expression of Wnt target genes (19). We also found that ApcmNLS/m mice, the most widely utilized mouse model of intestinal polyposis mediated by truncating germline APC mutation, develop more and larger intestinal polyps when they also harbor the ApcmNLS allele (ApcmNLS/m). Together these data suggest a tumor suppressor function for nuclear APC (19).

In this study, we tested the potential roles for nuclear APC in a mouse model of colitis-mediated colon cancer. In this model, colon cancers are initiated with a single injection of the mutagen, azoxymethane (AOM) and are promoted with repeated oral administration of dextran sodium sulfate (DSS) to induce colonic inflammation (20). Using this model, we examined the requirement of nuclear APC in suppression of colitis-associated CRC. In contrast with the polyposis that...
predominantly develop in the small intestines of Apc<sup>Min/−</sup> mice (21,22), the AOM–DSS-treated mice develop tumors in the colon, the site of tumor formation in humans with mutated APC (23). Furthermore, most mouse models with germline Apc mutations express truncated Apc lacking both β-catenin degradation and nuclear localization domains (22). Apc mutations are not typically found in colon tumors from AOM–DSS-treated mice. Rather, mutations in β-catenin that render it incapable of Apc-mediated destruction are considered the initiating step (20). This feature allows us to distinguish tumor suppressor functions of nuclear Apc from the cytoplasmic role of Apc in targeting β-catenin for destruction.

Here, we show that nuclear Apc suppresses colon tumorigenesis in the AOM–DSS mouse model. Although they differ in relative number, the colonic polyposis themselves show very similar histology, β-catenin mutations, apoptosis, stem cell number and proliferation indices in AOM–DSS-treated Apc<sup>minNLS/minNLS</sup> and Apc<sup>+/+</sup> mice. Apc<sup>minNLS/minNLS</sup> mice are also more sensitive to DSS-induced colitis with fewer stem cells and proportionally less crypt branching relative to ulceration extent, suggesting less ability to repair inflammation-induced tissue damage. These mice also display a goblet cell deficiency, consistent with a role for nuclear Apc in goblet cell differentiation. Based on the cumulative data obtained using the Apc<sup>minNLS</sup> model, we propose that nuclear Apc protects against colitis-associated colon tumorigenesis by suppressing inflammation, inhibiting Wnt signaling, and increasing colonocyte differentiation and mucus production.

**Materials and methods**

**Mouse husbandry**

Mice were maintained at the animal care unit at the University of Kansas according to animal use statement number 137-01. The research complied with all relevant federal guidelines and institutional policies. Mice were fed ad libitum with Purina Lab Diet 5001 and were housed in cages in adjoining animal rooms. All mice are C57BL/6J and Apc<sup>minNLS/minNLS</sup> mice are congenic (>N15).

**Acute inflammation model and colitis severity clinical scoring**

Thirteen Apc<sup>minNLS/minNLS</sup> and 14 Apc<sup>+/+</sup> mice (6–7-week-old) were provided 2.5% DSS (MW 36 000–50 000, MP Cat# 160110) in their drinking water for 7 days followed by 5 days with regular water. DSS used in both test and control groups was from the same batch. Animal weight, stool consistency, and presence or absence of blood in stool were recorded daily. At study’s end, mice were killed and removed from cecum to anus and opened longitudinally. Histopathological examination and scoring

**Histopathological examination and scoring**

Tumors and representative lymphoid follicles were dissected from the surrounding tissues, processed and paraffin embedded. Colons from the cecum to the anus were Swiss-rolled, paraffin embedded, sectioned (8 μm) and stained with hematoxylin and eosin for histopathological analysis. Colon tissue from the acute inflammation study was scored using a modified system from that described previously for the acute inflammation study. Colonic polyps and lymphoid follicles were identified and measured by an investigator blind to the animal’s genotype using a dissecting microscope with 10 magnification and with the aid of an eyepiece graticule calibrated to a 50 mm scale stage micrometer with 0.1 and 0.01 mm graduation (Leica).

**Detection of Cmylb (β-catenin) and Kras mutations**

DNA was extracted using the QIAamp DNA formalin-fixed paraffin-embedded tissue kit (Qiagen) from 3–8 manually microdissected, 8 μm sections of paraffin-embedded tissue for each tumor and amplified using primers spanning β-catenin (Cmylb) exon 3 (forward: 5'-TTCAAGTGAGTTTTCTGTAAGC-3'; reverse: 5'-GTTGACCTTCCTGAAAATGC-3') and Kras exon 1 (forward: 5'-TGAATTCCGTTGCTGAAAATTC-3'; reverse: 5'-GACCCCGAGCTGAGACG-3'). Gel-purified PCR products were sequenced (ACGT Inc.).

**Assessment of proliferation**

Paraffin-embedded tumors were sectioned (8 μm) and stained for proliferation marker Ki-67 as described previously (19). Images from at least three fields per tumor (100 × 100 μm) were analyzed for Ki-67-positive cells at ×40 magnification.

**Assessment of apoptosis**

Apoptosis was detected using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay DeadEnd<sup>TM</sup> Fluorometric TUNEL. System

### Table I. Primers to quantify inflammatory mediators shown previously to be differentially expressed in Apc<sup>Min</sup> polyposis and stem cell markers

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<tr>
<th>Gene</th>
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<th>Reverse primer</th>
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<td>Cxcr1</td>
<td>5'-AAGGAGTCTTCCGCTCCTGG-3'</td>
<td>5'-CTGTTCTTCGGCACGCGATAGT-3'</td>
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<tr>
<td>Cxcr2</td>
<td>5'-CATCCTTGCCGACACTTC-3'</td>
<td>5'-GCGGACATTTAATTTGTCTTG-3'</td>
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<td>Gro-α</td>
<td>5'-TGGTTTGGCCGGAAGAATGTC-3'</td>
<td>5'-TCCAACCTCAACCCTAGCTA-3'</td>
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<tr>
<td>Mip-2</td>
<td>5'-CAGATCCCAGCACCATCCTA-3'</td>
<td>5'-TACAACACACCCCTCCCCACCA-3'</td>
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<tr>
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<td>5'-GACTTACCCGCTCTCATG-3'</td>
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<td>5'-GTCGTAGTGGCTTGCTGCTGCT-3'</td>
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<td>5'-TATGGCAGCGAAGAAAGAG-3'</td>
</tr>
<tr>
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<td>5'-TGTCGAGATGTCGATAGG-3'</td>
<td>5'-TATGTTCCCCCTGGTACCTAG-3'</td>
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Stem cell scoring
Formalin-fixed paraffin-embedded tissues were sectioned (7 μm), deparaffinized in SafeClear (Fisher Scientific) and rehydrated in ethanol. Antigen retrieval was performed by boiling the slides in 10 mM citrate buffer (pH 6.0) for 25 min in a microwave. Anti-DCAMKL1 antibody (1:100, ab31704; Abcam) was used for primary staining with Alexafluor goat anti-rabbit 564 (Molecular Probes) as a secondary and 4',6-diamidino-2-phenylindole as a nuclear stain. Positive cells in whole Swiss-rolled colon sections were counted by an investigator blind to the genotyope.

Statistical analysis
P-values were calculated using Student’s t-test, Mann–Whitney non-parametric tests, Fisher’s exact test and GraphPad Prism software as indicated in figure legends. The distribution of exon 3 mutations in AOM/DSS-treated Apc<sup>mNLS</sup>/mNLS<sup>mNLS</sup> and Apc<sup>+/+</sup> mice was analyzed using a one-way analysis of variance. Statistical analysis of goblet cell scores in small intestines from untreated mice was performed by Dr J.Wick, Department of Biostatistics at The University of Kansas Medical Center. A linear mixed model was used as described previously (27). The mixed model approach utilizes an analysis of variance model with an additional random ‘mouse’ effect to model the inherent correlation that exists between samples from the same mouse. We assumed the random mouse effect is normally distributed with zero mean and unknown variance.

Results

**Apc<sup>mNLS</sup>/mNLS<sup>mNLS</sup> mice have increased susceptibility to colitis-associated tumorigenesis**

We previously reported that the Apc<sup>mNLS</sup> allele increases intestinal tumorigenicity in mice harboring a germline mutation that results in Apc truncation, Apc<sup>Min</sup> (19). In both Apc<sup>Mito</sup> and Apc<sup>mNLS/mNLS</sup> mice, the vast majority of tumors develop in the small intestine (19). To examine the role of nuclear Apc as a suppressor of colon cancer, we initiated colon tumors with the mutagen AOM followed by cycles of DSS to induce chronic inflammation (Figure 1A). Unlike tumors in Apc<sup>Min</sup> mice, AOM–DSS-treated wild-type mice develop colonic tumors, which do not typically have Apc mutations (20).

Of the 15 Apc<sup>mNLS/mNLS</sup> mice, five (33%) developed rectal prolapse and were killed at age 20.6, 20.6, 21, 21 and 22.6 weeks, whereas only one of the eight treated Apc<sup>+/+</sup> mice required early termination (at 22.9 weeks) due to rectal bleeding. The remaining mice were killed at 24 weeks. Two untreated control groups of 14 Apc<sup>mNLS/mNLS</sup> and 10 Apc<sup>+/+</sup> mice were also killed at 24 weeks of age, at which time none displayed detectable colon polyps. There were no detectable tumors in the small intestines from AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> mice (data not shown). However, while all of the AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice developed at least one colonic polyp, over one-third of the treated Apc<sup>+/+</sup> mice remained free of colon tumors at the end of the study (Figure 1B–D). Moreover, in AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice, colonic polyp numbers were more than double that of treated Apc<sup>+/+</sup> mice (Figure 1E). Based on these results, we conclude that AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice have higher tumor incidence and multiplicity than treated Apc<sup>+/+</sup> mice.

To determine the mechanism by which Apc<sup>mNLS</sup> increases polyp formation in the AOM–DSS model, we investigated several predicted contributory elements. Histopathological examination of 20 polyps from AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice and 10 tumors from treated Apc<sup>+/+</sup> mice indicated that all were polyposidal, semi-flat or flat adenomas with some degree of atypia (Figure 1F and G). Moderate to marked lymphoplasmacytic infiltration was observed in most polyps with varying degree of inflammatory reactions. Polyp size in treated Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> mice did not significantly differ between groups (Figure 2A). There was also no significant difference in proliferation level as assessed by proliferation marker, Ki-67 (Figure 2B and C) nor in apoptosis as detected by TUNEL assay (Figure 2D and E). Staining for the intestinal stem cell marker DCAMKL1, we found only a few positive cells, mainly at the tumor base, with no significant difference between tumors from both groups (data not shown). Taken together, it appears that adenoma growth is not altered in AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice.

Stabilizing mutations in Cnn1b (b-catenin) exon 3 are characteristic of tumors from mice treated with AOM–DSS, with activating mutations in Kras codon 1 found later in development. We identified mutations in β-catenin exon 3 in each of the nine examined adenomas from AOM–DSS Apc<sup>+/+</sup> mice and in 24 of 25 examined adenomas from treated Apc<sup>mNLS/mNLS</sup> mice (Figure 2F–H). Furthermore, the specific missense mutations in Cnn1b (b-catenin) were similar in both groups (Figure 2G and H, P = 0.22). No mutations were found in exon 1 of kras in tumors from either group, indicating that the tumors were at an early stage of progression (data not shown).

Weight loss and shortening of colon length are considered inflammation parameters in AOM–DSS mouse models (23). We found that AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice lost significantly more weight than treated Apc<sup>+/+</sup> mice relative to the corresponding untreated control groups (Figure 2I). Colon lengths were reduced by 12% in treated Apc<sup>mNLS/mNLS</sup> mice and by only 8% in treated Apc<sup>+/+</sup> mice (Figure 2I). Untreated Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> mice had similar numbers of colonic lymphoid follicles visible with the aid of a dissecting microscope with x10 magnification (Figure 2K) and there were no significant differences in the number of small intestinal lymphoid follicles in AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> mice (data not shown). However, the AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice had significantly more visible colonic lymphoid follicles than the treated Apc<sup>+/+</sup> mice (Figure 2K). Furthermore, three of the five AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice analyzed showed residual mild mucosal edema, whereas only one of five treated Apc<sup>+/+</sup> mice analyzed displayed histopathological signs of edema (data not shown). Together, these data are indicative of more severe inflammation in colons of AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice than in treated Apc<sup>+/+</sup> mice.

**Apc<sup>mNLS/mNLS</sup> mice are more susceptible to DSS-induced colitis**

To more directly examine the role of nuclear Apc in inflammation, Apc<sup>mNLS/mNLS</sup> mice were provided 2.5% DSS in their drinking water for 7 days to induce acute inflammation and then analyzed after five additional days of recovery. Starting at day 7, DSS-treated Apc<sup>mNLS/mNLS</sup> mice lost significantly more weight than treated Apc<sup>+/+</sup> mice (Figure 3A). Once returned to regular water, DSS-treated Apc<sup>+/+</sup> mice showed first signs of weight gain one day prior to treated Apc<sup>mNLS/mNLS</sup> mice (days 10 and 11, respectively), indicating delayed recovery in the DSS-treated Apc<sup>mNLS/mNLS</sup> mice. At the end of the 12 day study, DSS-treated Apc<sup>mNLS/mNLS</sup> mice showed clinical signs indicating more severe colitis than treated Apc<sup>+/+</sup> mice (Figure 3B). Histopathological assessment of colon tissue revealed more extensive ulceration and more severe inflammation in DSS-treated Apc<sup>mNLS/mNLS</sup> mice compared with treated Apc<sup>+/+</sup> mice (Figure 3K–N). Scoring the extent of ulceration and inflammation confirmed that these differences were significant (Figure 3C and D).

Tissue inflammation and regeneration are physiologically linked processes aimed at healing injury and repairing the damage (3). One key component of regeneration is the ability of stem cells to repopulate damaged tissues. Intestinal stem cell markers Hopx and Bmi1 messenger RNA (mRNA) were significantly reduced in colons from DSS-treated Apc<sup>mNLS/mNLS</sup> mice relative to those from treated Apc<sup>+/+</sup> mice (Figure 3E and F). We also found significant reduction in the number of cells expressing the intestinal stem cell marker DCAMKL1 in colons from DSS-treated Apc<sup>mNLS/mNLS</sup> mice compared with those from Apc<sup>+/+</sup> mice (Figure 3H–J). Collectively, these data indicate stem cell deficiency in the DSS-treated Apc<sup>mNLS/mNLS</sup> mouse colon. The expression of Bmi1 and Hopx and the number of DCAMKL1-positive cells did not significantly differ in colons from untreated Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> mice (data not shown).

Intestinal crypts can increase in number by crypt branching and fission (28, 29). In DSS-treated Apc<sup>+/+</sup> mice, there was a strong positive correlation between ulceration and crypt branching, whereas in treated Apc<sup>mNLS/mNLS</sup> mice, this correlation was not seen (Figure 3G). Other parameters for modified colonic epithelial architecture including crypt separation, crypt shortening and enlarged and elongated
Fig. 1. Apc^mNLS^ allele increases tumor incidence and multiplicity in AOM–DSS mouse model. AOM–DSS treatment protocol (A). Representative samples of large intestines from treated Apc^+/+ (n = 8) mice (B) and Apc^mNLS/mNLS (n = 15) mice (C). Scale bars = 1 cm. (D) Polyp incidence calculated following polyp detected using a dissecting microscope with x10 magnification. Significant differences are indicated with * (P < 0.05, Fisher’s exact test). (E) Average polyp number in AOM-DSS treated mice (P < 0.05, Student’s t-test). Histopathology of representative polypoidal (F) and semi-flat (G) colonic adenomas from AOM–DSS-treated Apc^mNLS/mNLS mice. Higher magnifications of boxed areas are presented. Scale bars 200 µm (left panels), 100 µm (middle) and 50 µm (right). Error bars represent standard error of the mean throughout.
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Fig. 2. Polyps from AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> mice do not differ in size, proliferation, apoptosis or β-catenin mutations, but treated Apc<sup>mNLS/mNLS</sup> mice are more prone to chronic inflammation. (A) Colon polyp diameters from AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> mice presented as box and whiskers plots. (B) Representative images of Ki-67 staining, scale bar = 100 µm. (C) Box and whiskers plots of proliferation marker Ki-67-positive cells per 100×100 µm high-power field (hpf) from AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> mice. (D) Representative images of TUNEL assay showing apoptotic cells in green and 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei in blue. (E) The average number of apoptotic cells per high-power field (×40) in tumors from AOM–DSS-treated Apc<sup>+/+</sup> (upper panel) and Apc<sup>mNLS/mNLS</sup> (lower panel) mice. (F) Representative sequencing chromatographs showing a G→A (middle) or a C→T (lower) mutation (arrows) or the normal control β-catenin coding sequence (top). (G) Predicted amino acid alterations resulting from the missense mutations in exon 3 of β-catenin are presented for polyps from AOM–DSS-treated Apc<sup>mNLS/mNLS</sup> (top) and Apc<sup>+/+</sup> (bottom) mice. (H) The distribution of β-catenin exon 3 mutations found in tumors from Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> mice is displayed as the relative frequency at each codon. (I) The average weight change of AOM–DSS-treated mice is shown relative to the untreated mice from both Apc<sup>mNLS/mNLS</sup> and Apc<sup>+/+</sup> groups (*P < 0.01, Student’s t-test). (J) The average reduction of colon length in AOM–DSS-treated mice as a percentage to the untreated mice (*P < 0.05, Student’s t-test). (K) The average number of visible colonic lymphoid follicles in untreated and AOM–DSS-treated mice (*P < 0.05, Student’s t-test). Error bars represent standard error of the mean throughout.
Fig. 3. DSS induces more severe tissue damage and less effective tissue repair in Apc\textsuperscript{NLS/nLS} mice. (A) Weight changes in 2.5% DSS-treated Apc\textsuperscript{NLS/nLS} and Apc\textsuperscript{+/+} mice (treatment schematic shown below graph). * indicates $P < 0.05$, using Student’s $t$-test. (B) The average colitis severity clinical score in DSS-treated Apc\textsuperscript{NLS/nLS} and Apc\textsuperscript{+/+} mice ($P < 0.05$, using Mann–Whitney test). (C and D) Histopathological scoring for ulceration (C) and inflammation (D) in DSS-treated Apc\textsuperscript{NLS/nLS} and Apc\textsuperscript{+/+} mice ($P < 0.05$, using Mann–Whitney test). (E and F) Relative expression of the intestinal stem cell markers; Hopx (E) and Bmi1 (F) in DSS-treated Apc\textsuperscript{+/+} and Apc\textsuperscript{NLS/nLS} mice ($P < 0.05$, using Mann–Whitney test). (G) Correlation between ulceration score and crypt branching score in 14 DSS-treated Apc\textsuperscript{+/+} (black squares) and 13 treated Apc\textsuperscript{NLS/nLS} mice (blue triangles). The number of mice at each point are presented in black (Apc\textsuperscript{+/+}) and blue (Apc\textsuperscript{NLS/nLS}). (H–J) Intestinal stem cell marker DCAMKL (red) in colons from DSS-treated Apc\textsuperscript{+/+} (H) and Apc\textsuperscript{NLS/nLS} (I) mice. The average number of positive cells/cm of colon from DSS-treated mice is presented in (J) ($P < 0.05$, Student’s $t$-test). (K–N) Representative pictures of colonic lesions from DSS-treated mice is presented in (J) ($P < 0.05$, Student’s $t$-test). (K and M) and treated Apc\textsuperscript{NLS/nLS} mice (L and N) showing inflammation with no ulceration and lymphocytic infiltration (K), ulceration with lymphocytic infiltrations (L), crypt branching (M) and ulceration (N). Scale bars 200 µm (left panels) and 100 µm (right panels).
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crypts did not show significant differences between colons from DSS-treated Apc<sup>+/+</sup> and treated Apc<sup>mNLS/mNLS</sup> mice (data not shown). Tumors were not seen in any of the acutely DSS-treated mice and only one mouse exhibited epithelial dysplasia that was not associated with inflammation. Together, these data suggest that regeneration in DSS-treated Apc<sup>mNLS/mNLS</sup> mice is lagging that of treated Apc<sup>+/+</sup> mice and that Apc<sup>mNLS/mNLS</sup> mice are more susceptible to DSS-induced acute inflammation than their wild-type counterparts.

Apc<sup>mNLS/mNLS</sup> mice have fewer goblet cells

While examining hematoxylin- and eosin-stained tissue from DSS-treated mice, we found that colons from treated Apc<sup>mNLS/mNLS</sup> mice appeared to have fewer goblet cells than those from treated Apc<sup>+/+</sup> mice. This observation was confirmed using Alcian Blue, which stains mucopolysaccharides and thus marks goblet cells. Colons from DSS-treated Apc<sup>mNLS/mNLS</sup> mice had fewer and lighter-staining goblet cells than those from DSS-treated Apc<sup>+/+</sup> mice (Figure 4A–D). Moreover, a significantly lower level of Muc2 mRNA (the major colonic mucin) was found in colon epithelia from DSS-treated Apc<sup>mNLS/mNLS</sup> mice than in treated Apc<sup>+/+</sup> mice, further validating the goblet cell phenotype originally noted in colon tissue sections (Figure 4E).

Goblet cells secrete mucus that serves as a protective barrier from microbes and foreign antigens, which could otherwise induce inflammation (5). Reduction of goblet cell number is associated with both ulcerative colitis and colorectal carcinoma; Apc and reduced Wnt signaling have been implicated in goblet cell differentiation (30). Therefore, we predicted that alterations in goblet cell abundance might even be detected in tissue from untreated Apc<sup>mNLS/mNLS</sup> mice, which showed elevated expression of Wnt targets (19). There were significantly fewer goblet cells in jejunum from untreated Apc<sup>mNLS/mNLS</sup> mice than in Apc<sup>+/+</sup> mice (Figure 4G). Although not statistically significant, the same trend was also seen in the ileum (data not shown). Colon crypts from untreated mice contain such a high proportion of goblet cells that their accurate quantification is challenging. Using Muc2 mRNA levels as an indirect assessment of goblet cell abundance, we found a reduction in Muc2 mRNA in colonocytes from untreated Apc<sup>mNLS/mNLS</sup> mice compared with those from untreated Apc<sup>+/+</sup> mice, albeit not of statistical significance (Figure 4F, P = 0.073). We conclude that Apc<sup>mNLS/mNLS</sup> mice have fewer goblet cells than Apc<sup>+/+</sup> mice.
Increased expression of inflammatory mediators in colon epithelial cells from untreated Apc<sup>αNLS/mNLS</sup> mice

Early genetic changes in colon tumorigenesis have recently been implicated in altered mucus production and inflammatory response (31). To more directly connect the goblet cell reduction in Apc<sup>αNLS/mNLS</sup> mice with inflammation, we quantified mRNA of presumed inflammatory mediators in colon epithelial cells isolated from untreated Apc<sup>αNLS/mNLS</sup> mice and untreated Apc<sup>α+/+</sup> control mice. We analyzed inflammation-associated genes that were reported to be differentially expressed in colon adenomas that develop in Apc<sup>α+/+</sup> mice (32): osteopontin (OPN), macrophage inflammatory protein-2 (MIP-2), growth-related oncogene-α (Gro-α), CXC cytokine receptor-2 (CXCR-2), and cyclooxygenase-1 and -2 (Cox-1 and Cox-2). There were no significant differences in the expression levels of Cox-1, CXCR-2, Gro-α and Opn in Apc<sup>αNLS/mNLS</sup> samples relative to Apc<sup>α+/+</sup> samples (data not shown). However, Cox-2 and MIP-2 mRNA levels were each significantly higher in colonocytes from untreated Apc<sup>αNLS/mNLS</sup> mice compared with those from Apc<sup>α+/+</sup> mice (Figure 5). The finding that both Cox-2 and MIP-2 are upregulated in colonocytes from untreated Apc<sup>αNLS/mNLS</sup> mice supports a role for nuclear Apc in suppressing colitis.

Discussion

Promiscuous Wnt signaling is a hallmark of CRC in both sporadic and colitis-associated cases, but this promiscuity is the result of different genetic events. Although Apc mutation is the initiating event in most sporadic cases, Apc-independent β-catenin activation is often an early event in colitis-associated CRC. In the latter case, mutation of Apc still occurs, however at a later stage of tumor formation (1). This tendency is recapitulated in a mouse model of colitis-associated CRC, in which a single dose injection of the mutagen AOM leads to ‘stabilizing’ mutations in β-catenin, followed by cycles of DSS to induce colon inflammation (20). A single dose of AOM without subsequent DSS treatment results in only a very low tumor incidence (33), emphasizing that aberrant regulation of Wnt signaling by β-catenin stabilization is not sufficient for tumor formation and inflammation is also required. In contrast, truncation of Apc can initiate tumor formation in both humans and rodent models. Furthermore, most human colon cancers display Apc mutations, whereas tumors in other organs often show mutations in other Wnt pathway components, indicating a Wnt-independent, colon-specific, tumor suppressor function of Apc (11). Together, this evidence supports two distinct tumor suppressor roles for Apc that must be eliminated during CRC progression: Wnt signaling regulation and inflammation suppression. Mutations of Apc early in sporadic cases of CRC might suffice to fulfill both requirements of Wnt signaling activation and induction of inflammation as seen in colitis-associated CRC.

Data supporting a role for APC/Apc in suppressing colitis already exist (31,34). Many inflammatory mediators are upregulated in Apc<sup>α+/+</sup> polyps including Cox-1, Cox-2, MIP-2, OPN, CXCR-2 and Gro-α (32). Although Cox-2 is generally considered a Wnt target (35–37), the other genes are not. Moreover, inflammatory cytokines, interleukin (IL)-23 and IL-17, were elevated in polyps from human CRC and in colons from mice with a conditional Apc mutation (31). Furthermore, reducing inflammation decreases tumor incidence in both humans and mouse models with germline Apc mutations (2,38).

Here, we report that Apc<sup>αNLS/mNLS</sup> mice have increased colitis-associated tumor susceptibility as indicated by an increase in both polyp incidence and multiplicity after treatment with AOM–DSS (Figure 1). We found no change in polyp size, apoptosis or cell proliferation in the polyps from AOM–DSS-treated Apc<sup>αNLS/mNLS</sup> mice. In addition, there was no difference in histopathology or in the number of stem cells in polyps from AOM–DSS-treated Apc<sup>α+/+</sup> and Apc<sup>αNLS/mNLS</sup> mice and they possessed the same spectrum of exon 3 Ctnn1b (β-catenin) mutations and no Kras mutations (Figure 2). Together, these findings suggest loss of nuclear Apc does not change molecular and morphological characteristics of colitis-induced colon tumors. One possible explanation for these findings is that reduction in nuclear Apc enhances colitis. In support of this idea, we found that Apc<sup>αNLS/mNLS</sup> mice have higher susceptibility to DSS-induced colitis (Figure 3) with a greater tendency to progress into chronic inflammation (Figures 2 and 3). This progression was manifested by weight loss, increased ulceration and inflammation score, and more lymphocytic infiltration and lymphoid nodules in colons from DSS-treated Apc<sup>αNLS/mNLS</sup> mice relative to those from treated Apc<sup>α+/+</sup> controls. Of note, we detected signs of inflammation as shown by upregulation of the inflammatory mediators Cox-2 and MIP-2 even in untreated Apc<sup>α+/+</sup> mice. As inflammation is a major risk factor for CRC, our findings support another tumor suppressor role of Apc to protect against inflammation.

The finding that there were no proliferation or size differences in tumors from AOM–DSS-treated Apc<sup>αNLS/mNLS</sup> mice relative to those from Apc<sup>α+/+</sup> mice may appear contradictory to our previous report of increased proliferation in colonic crypts from Apc<sup>αNLS/mNLS</sup> mice compared with Apc<sup>α+/+</sup> mice. However, a likely explanation for this difference is that proliferation in tumors from AOM–DSS-treated mice is driven by mutations in Ctnn1b (β-catenin) thus over-riding any proliferation differences seen in the original tissue. These results also suggest that the role of nuclear Apc in protecting against colitis-induced tumorigenesis is at the initiation stage.

The mechanism by which Apc mutations can induce inflammation is not completely known. Reduced intestinal epithelial barrier function, recently observed at an early stage of intestinal tumorigenesis driven by Apc mutations, would be expected to give microbial products access to the stroma, thus inducing an inflammatory response (34). The Apc-mutant KAD rat, which has a germline mutation resulting in truncation of the Apc C-terminus, displays a disruption of vascular endothelial cells and aberrant distribution of the junctional protein Dlg5. These rats also showed delayed colon tissue repair after induction of acute inflammation by DSS (39). The DSS-treated Apc<sup>αNLS/mNLS</sup> mice in this study also showed evidence of delayed tissue repair. However, Apc<sup>αNLS/mNLS</sup> mice express full-length Apc including the C-terminus; therefore, we do not expect the same underlying mechanism for defective tissue repair in both rodent models. In addition, because tissue repair and healing are linked to tissue damage and ulceration, our data of lagging tissue repair in DSS-treated Apc<sup>αNLS/mNLS</sup> mice could not differentiate between a role for nuclear Apc in preventing inflammation and a role in repair. Reduction in stem cell population and the absence of association between ulceration scores and crypt branching in Apc<sup>αNLS/mNLS</sup> mice may represent the severity of DSS-induced ulceration in these mice rather than a compromised ability to repair the damage. Another proposed mechanism by which mutation of Apc compromises intestinal epithelial barrier functions is by affecting mucin production and goblet cells. Mice with an inducible Apc truncating mutation in their distal ileum and colon have increased barrier permeability and defective mucin production (31). A different mouse model expressing only a short Apc also showed more bacterial invasion in intestinal polyps, where both Apc

![Fig. 5. Differential expression of inflammatory mediators in untreated Apc<sup>αNLS/mNLS</sup> mice. Relative expression of Cox-2 and MIP-2 in colon epithelial cells from untreated Apc<sup>αNLS/mNLS</sup> and Apc<sup>α+/+</sup> mice (*P < 0.05 using Mann–Whitney non-parametric test). Error bars represent standard error of the mean.](image-url)
alleles are typically mutated (34). In this study, we found fewer goblet cells and a significant reduction in Muc2 expression in DSS-treated Apc<sup>fl/fl;NLS</sup> mice relative to treated Apc<sup>fl/fl</sup> mice. DSS treatment has previously been associated with loss of goblet cells in general (6). However, we found that even untreated Apc<sup>fl/fl;NLS</sup> mice had fewer goblet cells in their small intestines and reduced Muc2 expression in their colons compared with untreated Apc<sup>fl/fl</sup> mice (Figure 4F and G). Collectively, these data suggest that Apc<sup>fl/fl;NLS</sup> mice have defective mucin production, resulting in exaggerated DSS-induced inflammation and colitis-associated tumorigenesis.

Nuclear APC can repress Wnt target gene expression both in vivo and in cultured cells (10,13,15,40). Loss of nuclear APC increases Wnt signaling and cellular proliferation in Apc<sup>fl/fl</sup> intestinal epithelial cells (19). Increased Wnt signaling by Apc mutation in other mouse models correlates with increased cellular proliferation and reduced differentiation to certain lineages, including goblet cells (30). Considering these findings, higher Wnt target expression might contribute to the increased DSS-induced inflammation and colitis-associated tumorigenicy by reducing differentiated goblet cells. One possible mechanism by which Wnt signaling controls intestinal mucus formation is by regulating the transcriptional factor Hath1, a key regulator of mucin production in colon cells. Hath1 is negatively regulated by Wnt signaling and is reduced in CRC. Inhibition of Wnt signaling or expression of Hath1 results in increased Muc2 mRNA level in colon cells (41). Notably, we previously showed that untreated Apc<sup>fl/fl;NLS</sup> mice have reduced Hath1 expression in intestinal epithelium (19). Together with previous reports, these data suggest that the reduction in goblet cell number in Apc<sup>fl/fl;NLS</sup> mice could be attributed to a reduction in goblet cell formation. However, our data could not exclude the possibility of a higher rate of goblet cell death.

Inflammation and Wnt signal upregulation are not completely independent factors. Inflammation can activate Wnt signaling through many pathways including NF-kB (1). APC mutations can alter retinoic acid metabolism, leading to Wnt-independent upregulation of Cox-2. The induced Cox-2 increases prostaglandin E2 synthesis, which in turn, activates Wnt signaling (42,43). On the other hand, Wnt signaling can increase inflammation through upregulation of inflammatory mediators such as nitric oxide synthase-2 (44) and Cox-2 (37). Cox-2 is involved in colorectal tumor formation and often upregulated in colon cancer (43). Cox-2 inhibitors have shown some therapeutic benefits in CRC (2,45). Besides being the rate-limiting enzyme in the synthesis of inflammatory mediators, Cox-2 also induces the anti-apoptotic Bcl2 (46), the angiogenic vascular endothelial growth factor (47) and the pro-tumor cytokine, IL-23 and inhibits the tumor suppressor cytokine IL-12 (48).

In conclusion, we provide evidence that nuclear Apc contributes to suppression of colitis and colitis-associated CRC. Our data support a model whereby goblet cell alterations in Apc<sup>fl/fl;NLS</sup> mice increases inflammation susceptibility and tumorigenicity.

**Supplementary material**

Supplementary Methods can be found at http://carcin.oxfordjournals.org/

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