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Use of a novel genetic mouse model to investigate the role of folate in colitis-associated colon cancer☆

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Abstract

Inflammatory bowel disease (IBD) patients are at high risk for developing folate deficiency and colon cancer. Since it is difficult to study the subtle global and gene-specific epigenetic mechanisms involved in folate-mediated tumor initiation and promotion, we have generated genetically modified mouse models by targeting the reduced folate carrier (RFC1) and folate-binding protein (Folbp1) genes. The transgenic mice were fed semi-purified diets for 8 weeks containing either normal (2 mg) or deficient (0.1 mg folate/kg diet) levels of folate. Compound heterozygous mice (Folbp1$^{+/-}$; RFC1$^{+/-}$) fed an adequate folate diet exhibited a reduction in plasma folate concentrations compared to heterozygous (Folbp1$^{+/-}$) and littermate wild-type mice ($P<.05$). In contrast, no differences were observed in colonic mucosa. Consumption of a low folate diet significantly reduced (three- to fourfold) plasma and tissue folate levels in all animal models, although plasma homocysteine levels were not altered. In order to elucidate the relationship between folate status and inflammation-associated colon cancer, animals were injected with azoxymethane followed by dextran sodium sulphate treatment in the drinking water. Mice were fed a normal folate diet and were terminated 5 weeks after carcinogen injection. The number of high multiplicity aberrant crypt foci per centimeter of colon was significantly elevated ($P<.05$) in compound Folbp1$^{+/-}$; RFC1$^{+/-}$ (3.5±0.4) mice as compared to Folbp1$^{+/-}$ (1.9±0.3) and wild-type control mice (1.1±0.1). These data demonstrate that the ablation of two receptor/carrier-mediated pathways for folate transport increases the risk for developing inflammation-associated colon cancer.

Keywords: Inflammatory bowel disease; Mouse models; Azoxymethane; Dextran sodium sulphate; Folate-binding protein; Reduced folate carrier

1. Introduction

Inflammatory bowel disease (IBD) patients are at high risk for developing folate deficiency and colon cancer [1,2]. Retrospective studies have shown that folate supplementation may have a protective role in IBD-associated colon cancer [3–6]. From a mechanistic standpoint, an insufficient supply of methyl group donors, e.g., folate, has been linked to altered methylation of colonic DNA and the promotion of colorectal tumorigenesis in mice/rats [7–10] and humans [4,11,12]. There is also mounting evidence that folic acid possesses dual modulatory effects on the initiation and promotion of colon cancer depending on the timing and dose of folate supplementation [12,13].

Since it is difficult to study the subtle global and gene-specific epigenetic mechanisms involved in folate-mediated

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tumor initiation and promotion, we have generated two genetically modified mouse models by gene targeting directed at the reduced folate carrier (RFC1) and folate-binding protein (Folbp1) genes [14–16]. These models are attractive for mechanistic studies because (a) alterations in the expression and function of Folbp1 and RFC1 have been reported in humans [17–20]; (b) these mice exhibit distinct perturbations in folate and *S*-adenosylmethionine status, the primary methyl donor for DNA methylation reactions [15,16]; (c) these mice exhibit an elevation in steady-state colonic cell proliferation [16]; (d) avoid the complication of anemia and premature death associated with a severe dietary folate restriction; (e) eliminate the compensatory up-regulation of DNA methyltransferase associated with a moderate short-term dietary folate deficiency [9]; (f) eliminate the need to use broad-acting antibiotic drugs, e.g., succinylsulfathiazole, which alter fecal pH and short-chain fatty acid concentrations [21]; and (g) mimic the subclinical but biochemically evident degree of folate deficiency in 40% of IBD patients [22,23]. Therefore, in this study genetically engineered compound heterozygous ($Folbp1^{+/-}$; $RFC^{+/-}$) mice were utilized to elucidate the intersection of folate metabolism and inflammatory pathways that contribute to colon cancer. Specifically, colitis-associated colon cancer was induced by exposure to dextran sodium sulphate (DSS) subsequent to a single azoxymethane (AOM) injection.

2. Materials and methods

2.1. Diets and animals

The generation of Folbp1 and RFC1 knockout mice on an SWV background has been described previously [16]. To ensure genetic homogeneity, mice were interbred onto an SWV background for more than 10 generations and littermates used in all experiments. A separate colony of SWV mice was also examined for comparative purposes. $Folbp1^{+/-}$, $Folbp1^{+/-}$; $RFC^{+/-}$ compound heterozygous mice; and their wild-type controls ($Folbp1^{+/+}$, $RFC^{+/+}$, $Folbp1^{+/+}$ and SWV) were fed a semi-purified diet and exposed to either (i) saline or (ii) AOM+one cycle of DSS. For each treatment, 4- to 6-week-old specific pathogen-free animals were provided experimental AIN-93 M diets (Supplemental Table 1) containing 1.25 g/kg choline and either folic acid at (deficient) 0.1 mg/kg or (adequate) 2 mg/kg diet for 4 weeks prior to AOM injection. The basal diet contained a similar level of folic acid relative to earlier experiments [15] and met the folic acid requirement (0.5 mg/kg diet) for mice [16]. In contrast, folic acid at 0.1 mg/kg diet was selected because it has been shown to enhance the development of colonic tumors in rodents [9] and does not retard growth [24]. Blood for plasma folate and RBC analysis was collected at 8 weeks post-DSS treatment at the time of termination. Colonic mucosa and tissues were collected at 8 weeks. Food and water were freely available. Body weights were recorded weekly throughout the study. In a separate study, animals were treated with a single injection of AOM (12.5 mg/kg body weight), a colon-specific carcinogen [25] followed 1 week later by DSS at 2.5% for 5 days. DSS is directly toxic to colonic epithelial cells of the basal crypts and is considered to be an indispensable model of intestinal inflammation [26]. Mice were fed a normal folate (2 mg/kg) diet and were terminated 5 weeks after carcinogen injection.

2.2. Folate and homocysteine analyses

Red blood cell (rbc) and plasma folate and homocysteine status as well as tissue folate levels in brain, liver and colonic mucosal were determined using the method of Bottiglieri [27].

2.3. Aberrant crypt analyses

To detect aberrant crypt foci (ACF), colons were fixed in 70% ethanol and briefly stained with methylene blue (0.3%) as previously described [16]. The following criteria were used to designate a crypt as "aberrant" (crypts are usually enlarged; considerably darker in comparison to surrounding crypts; enlarged lumens generally have elongated, serrated or small round lumens; may appear to be raised above the surrounding mucosa; may have an enlarged pericryptal zone). Location within the colon, size and number of crypts/foci were scored and grouped by number of aberrant crypts per foci: 1–2 aberrant crypts were classified as low multiplicity, 3+ were high multiplicity.

3. Results

3.1. Perturbation of systemic (plasma and rbc) and colonic mucosa folate levels in compound mice

We have previously demonstrated that allelic ablation of Folbp1 and RFC1 results in the expected reduction in level of mRNA for each respective gene in colonic mucosa [16]. In this study, genetically engineered compound heterozygous ($Folbp1^{+/-}$; $RFC^{+/-}$) mice were utilized to elucidate the intersection of folate metabolism and inflammatory pathways that contribute to colon cancer. The combined effect of reduced expression of RFC1 and Folbp1 on plasma folate status is shown in Fig. 1. Consistent with a reduction in Folbp1 and RFC1, compound mice exhibited a significant ($P<05$) reduction in plasma folate levels compared to all other groups. As expected, diet significantly ($P<05$) affected plasma folate levels. Specifically, all mice fed a diet low in folate (0.1 mg/kg) had reduced folate levels as compared to animals fed a normal folate (2 mg/kg) diet. In general, similar trends were observed with respect to rbc folate levels with compound heterozygous mice exhibiting the lowest levels of folate, regardless of dietary treatment (Supplemental Table 2). We also evaluated a key folate metabolic intermediate in plasma (i.e., homocysteine), given that folate can directly affect homocysteine status [28]. Interestingly, the concentration of

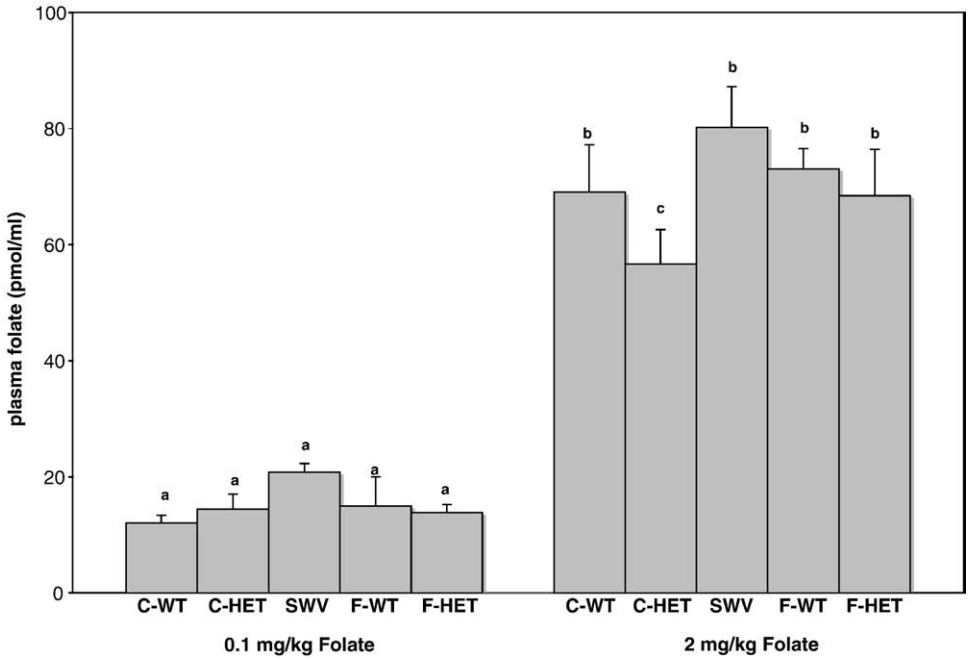

Fig. 1. Plasma folate levels. Data (pmol/ml) represent mean±S.E. for n=6 animals per group. Values with different symbols are significantly different (P<.05). C-WT, (Folbp1$^{+/+}$; RFC$^{+/+}$) littermate control mice; C-HET, genetically modified (Folbp$^{+/-}$; RFC$^{+/-}$); SWV, a separate colony of control mice on an SWV background; F-WT, (Folbp1$^{+/+}$) littermate control mice; F-HET, (Folbp1$^{+/-}$) genetically modified mice.

homocysteine was not altered by either diet or genotype (Supplemental Table 2).

Mucosal folate levels were not altered by gene ablation; however, similar to plasma findings, folate levels in colonic mucosa were significantly (P<.05) suppressed in all mouse models fed a low folate diet (Fig. 2). In contrast, brain (Fig. 3) and, to some degree, liver (Supplemental Table 3) folate levels were refractive to both genetic and dietary manipulation. With respect to all clinical outcomes, as expected, SWV and Folbp1$^{+/+}$ littermate control mice behaved similarly.

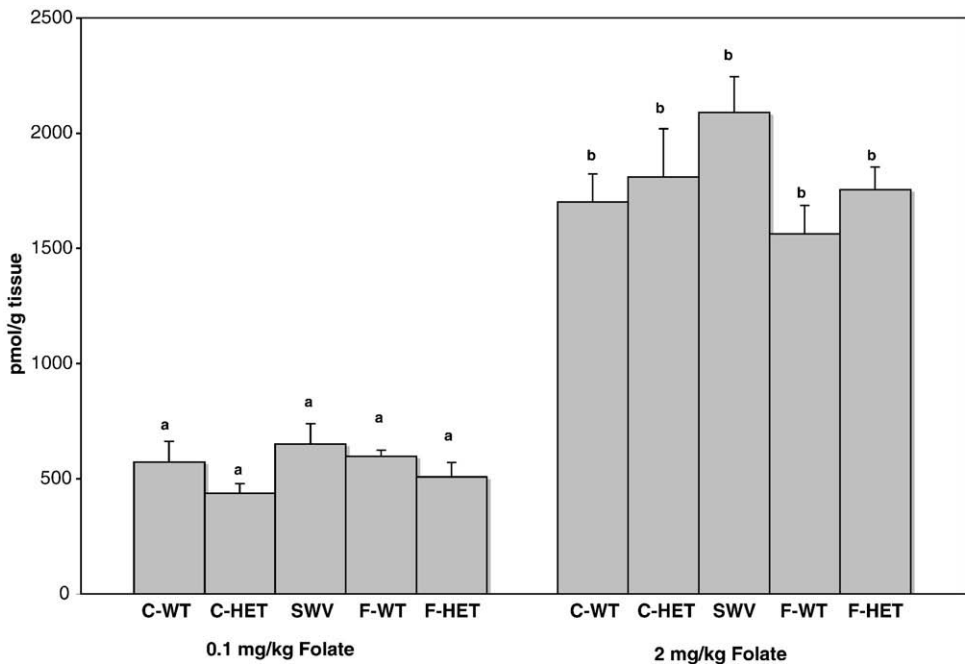

Fig. 2. Colonic mucosa folate levels. Values (pmol/g tissue) represent mean±S.E. for n=6 animals per group. Values with different symbols are significantly different (P<.05). Refer to Fig. 1 for legend details.

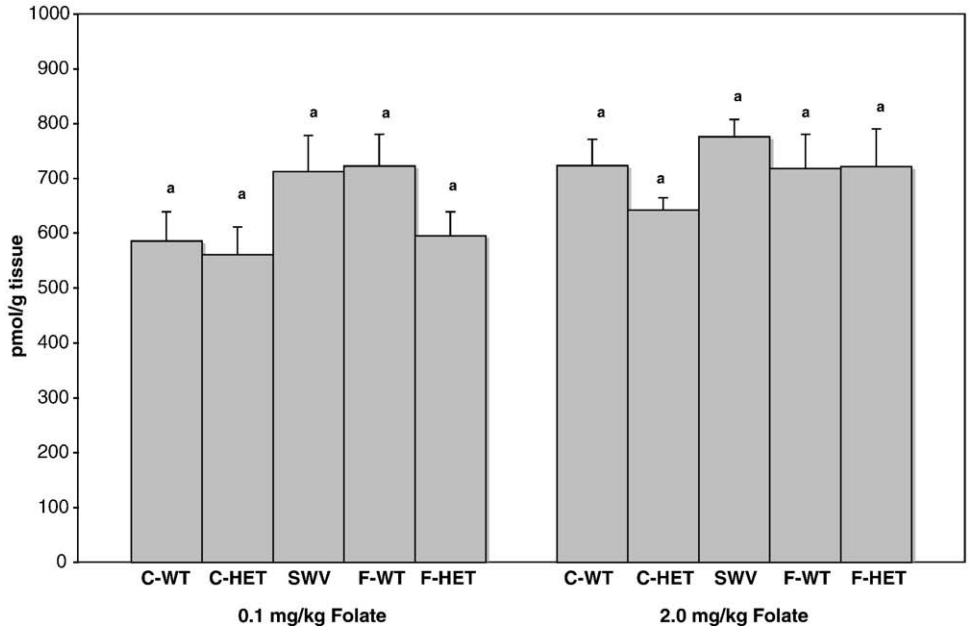

Fig. 3. Brain folate levels. Folate levels (pmol/g tissue) were unchanged, regardless of diet or genotype ($P>.05$). Values represent mean±S.E. for $n=6$ animals per group. Refer to Fig. 1 for legend details.

3.2. Reduced expression of RFC1 and Folbp1 promotes the progression of low to high multiplicity ACF

The combined effect of Folbp1/RFC1 targeted deletion on colonic ACF levels was examined. All animals were placed on a normal folate (2 mg/kg) diet and treated with AOM and DSS in order to recapitulate the phenotypic features associated with inflammation-associated malignant transformation [25]. ACF were readily recognized by their enlarged size, pericryptal area and thickened epithelial lining (Fig. 4). At 5 weeks following AOM treatment, there was no difference in the number of ACF per centimeter of colon (Table 1). However, when ACF multiplicity was assessed at 8 weeks, there were significantly ($P<.05$) more high

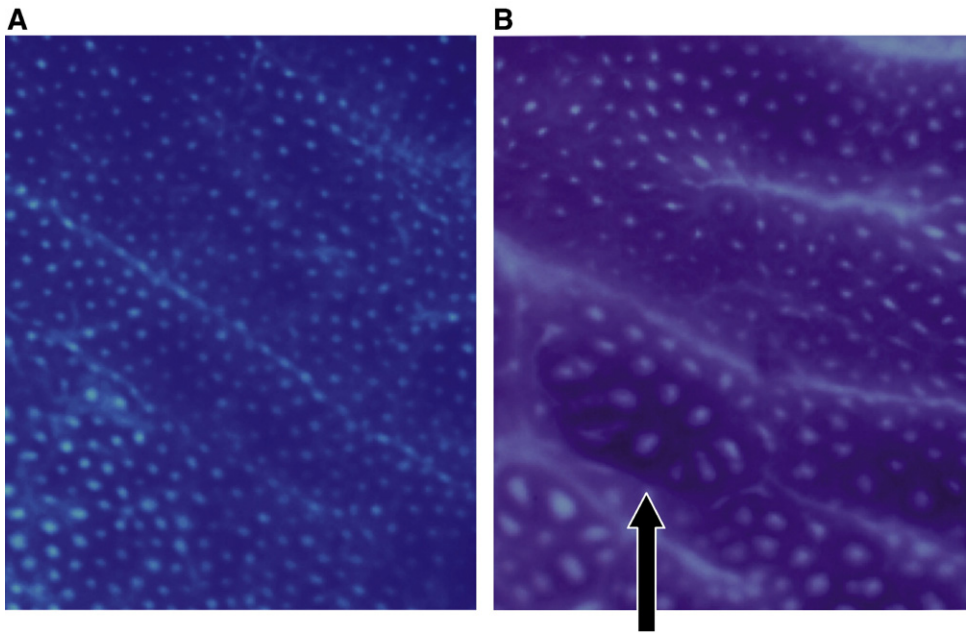

Fig. 4. Effect of combined AOM and DSS treatment on ACF development. (A) Normal colon from untreated control animals. (B) Representative high multiplicity ACF characterized by enhanced staining, an enlarged lumen and perinuclear region. *Arrow* indicates ACF. Images were captured under low magnification, 40×.

Table 1
Aberrant crypt foci levels 8 weeks following carcinogen (AOM) and DSS treatment

Genotype	Total ACF/cm	Low multiplicity ACF/cm	Hi multiplicity ACF/cm
SWV	9.3±1.1	7.5±1.0[a]	1.1±0.1[a]
F-HET, (Folbp1$^{+/-}$)	8.7±0.8	6.5±0.5[a]	1.9±0.3[a]
C-HET, (Folbp$^{+/-}$; RFC$^{+/-}$);	7.4±1.2	4.3±0.9[b]	3.5±0.4[b]

Data represent mean±S.E. from six animals per group. Low multiplicity, 1–2 ACF per foci; high multiplicity, 3+ ACF per foci. Values with different letters indicate a significant difference at $P < .05$.

multiplicity ACF in heterozygous compound mice relative to Folbp1$^{+/-}$ and SWV control mice. Interestingly, this corresponded with a decrease in low multiplicity ACF levels, suggesting that the progression of dysplasia is enhanced in compound heterozygous (Folbp1$^{+/-}$; RFC$^{+/-}$) mice. These data are noteworthy because both the number and multiplicity of ACF are considered useful biomarkers in the prediction of colon tumor development [29].

4. Discussion

Human inflammatory bowel diseases are chronic, relapsing inflammatory conditions of unknown etiology. Both genetic and environmental factors have been implicated [2,30]. These diseases are clinically characterized by two overlapping phenotypes, i.e., ulcerative colitis (UC) and Crohn's disease (CD). CD affects more than 500,000 individuals in the US and represents the second most common chronic inflammatory disorder after rheumatoid arthritis. In addition, approximately 20–50% of patients with chronic intestinal inflammation (>8 years) will develop colon cancer [1]. IBD patients have a tendency to become folate deficient which may exacerbate their susceptibility for colorectal cancer development [5,6,31]. Therefore, it is important to identify overlapping regulatory relationships among folate-responsive genes considered to drive inflammation-associated colonic tumor development. Over the past decade, a variety of animal models have been generated for the purpose of developing novel approaches to control IBD-type inflammation [25,32]. The DSS method of induced inflammation is an excellent preclinical model of colitis that exhibits many phenotypic characteristics relevant to the human disease [33]. Colitis-induced colon cancer can be induced by repeated feeding cycles of DSS subsequent to a single AOM pretreatment [32,34]. Macroscopically, a dysplasia-invasive adenocarcinoma sequence is observed, resulting in both flat and polypoid tumors. This is analogous to the dysplasia-associated lesion or mass seen in patients with UC [35]. Thus, the combined use of AOM and DSS-induced colitis represents exposure to an external carcinogen with an inflammatory process that, in principle, is relevant to IBD in humans.

In this study, we used compound heterozygous knockout (Folbp1$^{+/-}$; RFC$^{+/-}$) mice in order to elucidate the relationship between folate status and inflammation-associated colon cancer. The reduced folate carrier (RFC1) is a facilitative anion exchanger that mediates folate delivery into a variety of cells including colonocytes [16]. RFC1 has a high affinity for reduced folates, including the primary physiological substrate (5-methyltetrahydrofolate) [36]. In the absence of RFC1, neonatal mice die due to failure of hematopoietic organs [37,38]. In comparison, folate receptors (Folbp1 and Folbp2 in mice; a and b in humans) are coupled to the membrane via a glycosylphosphatidylinositol linkage and transport oxidized folates, e.g., folic acid, with very high affinity [39]. Folbp1 is highly expressed in the kidney proximal tubule, choroids plexus and placenta [39,40]. Comparatively, very low levels are detected in the colon [16]. Both receptors may play a role in the creation or relief of localized, tissue-specific folate deficiencies [40,41].

The partial ablation of both Folbp1 and RFC1 was associated with a reduction in plasma and rbc folate levels consistent with its role in the reabsorption of folate by the kidney [36]. In contrast, colonic mucosa folate levels were only modestly decreased (P=.28), indicative of a moderate, not severe, degree of folate depletion. Perhaps the contribution of passive diffusion and other proton-coupled carriers within the small intestine explains why overt systemic folate deficiency was not observed [36,42]. In complementary experiments, heterozygous (Folbp1$^{+/-}$ and RFC1$^{+/-}$) compound mice were fed a diet partially deficient in folate in order to further manipulate folate stores. Consistent with previous studies [43], dietary folate deficiency significantly suppressed plasma, rbc and colonic folate levels in all mouse models. In contrast, liver and brain were not affected. Interestingly, neither the partial ablation of both Folbp1 and RFC1 nor dietary deficiency altered plasma homocysteine levels, an indicator of cellular folate depletion [43]. These data are consistent with previous observations [16] indicating that in the absence of any sulfonamides in the diet, which prevent the generation of folate by intestinal bacteria, Folbp1 and RFC1 gene inactivation establishes a mild, chronic state of folate deficiency mimicking the subclinical degree of folate deficiency in IBD patients [22,23].

Interestingly, despite only modest folate depletion, we show for the first time that the reduced expression of folate transporters (RFC1 and Folbp1) promoted the development of high multiplicity ACF in a colitis-associated colon cancer mouse model. This is a highly relevant biological end point given that high multiplicity ACF are considered to be early preneoplastic lesions in humans and rodents [29]. Only a single study to date has examined the effect of folate supplementation on colon cancer within the context of chronic colitis [44]. Our observations are consistent with this previous study using the interleukin-2 and beta$_2$-macroglobulin deficient (IL-2 null×β_2m^{null}) mouse, confirming

that moderate perturbations in folate status modulate colitis-associated colorectal carcinogenesis. Additional studies are in progress to determine whether the promotive effect of RFC1 and Folbp1 gene ablation is in part mediated by changes in colonic inflammation.

It is becoming increasingly clear that folate possesses multiple modulatory effects on the initiation and progression of colon cancer depending on the timing and dose of folate intervention [9]. There are several mechanisms to explain the ability of two receptor/carrier-mediated pathways for folate transport to increase the risk for developing inflammation-associated colon cancer. Since folate plays an important role in CpG island methylation, aberrant methylation has been considered as a link between folate metabolism and colon cancer. However, currently, there are no conclusive data linking folate deficiency and the perturbation of genomic DNA methylation and/or site-specific DNA methylation in the colon [9]. Undoubtedly, the cyto-protective properties of folate are in part related to its effects on DNA stability [45]. Folate deficiency, induced by either a deficient diet, IBD, methotrexate therapy, competitive inhibition of folate absorption by sulfasalazine used for the treatment of IBD [23] or possibly loss of function mutations associated with RFC1, Folbp1 and proton-coupled folate transporter, may generate a block in the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate leading to the accumulation of deoxyuridine triphosphate. This can result in uracil misincorporation into DNA, which in itself is mutagenic [46]. If folate is continually limiting, excision repair occurs repeatedly resulting in a "catastrophic repair cycle," which may lead to double-strand breaks, chromosomal damage and cancer [47]. Interestingly, endogenous levels of repair enzyme, i.e., uracil DNA glycosylase, are highest in actively proliferating cells [48], raising the possibility that differential damage may occur in distinct regions of the colonic crypt. For example, stem cells and actively proliferating cells which are localized to the base of the crypt may be adversely affected [49]. Further studies investigating the effects of folate deficiency on epithelial lineages and stem cells in the colon are warranted.

In conclusion, the partial ablation of Folbp1 and RFC1 folate receptor/carrier pathways involved in folate transport increased the risk of developing inflammation-associated colon cancer. We posit that the genetically engineered folate transporter knockout mouse model will provide valuable insight into the role of folate in modulating the initiation and progression of colitis-associated colon cancer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2008.07.001.

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