

Low Dietary Folate Initiates Intestinal Tumors in Mice, with Altered Expression of G₂-M Checkpoint Regulators *Polo-Like Kinase 1* and *Cell Division Cycle 25c*

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Abstract

Clinical reports have suggested that low dietary folate increases risk for colorectal cancer. Animal studies for investigation of folate and tumorigenesis have used carcinogen induction or mice with germ-line mutations. We have developed a new spontaneous tumor model in which mice, with or without a null allele in a key folate-metabolizing enzyme, *methylenetetrahydrofolate reductase (Mthfr)*, develop intestinal tumors due to low dietary folate alone. On folate-deficient diets, 12.5% of *Mthfr*^{+/+} mice and 28.1% of *Mthfr*^{+/-} mice developed tumors; mice on control diets were negative. Dietary and genotype effects on tumor development were significant. To investigate mechanisms of folate-dependent tumorigenesis, we examined levels of DNA damage and gene expression of two genes involved in DNA damage response and G₂-M checkpoint regulation, *polo-like kinase 1 (Plk1)* and *cell division cycle 25c (Cdc25c)*. Folate deficiency increased DNA damage and decreased expression of both genes (assessed by quantitative reverse transcription-PCR and immunofluorescence) in normal intestine compared with levels in mice on control diets. An immunofluorescence assay for CDC25c activity (phosphorylated CDC2) also found CDC25c activity to be decreased in folate-deficient normal intestine. In tumors, however, *Plk1* and *Cdc25c* mRNA were found to be higher (11- and 3-fold, respectively) compared with normal intestine from folate-deficient mice; immunofluorescence studies of PLK1, CDC25c, and phosphorylated CDC2 supported these findings. Our data suggest that folate deficiency can initiate tumor development, that *Mthfr* mutation can enhance this phenomenon, and that altered expression of *Plk1* and *Cdc25c* may contribute to folate-dependent intestinal tumorigenesis. (Cancer Res 2006; 66(21): 10349-56)

Introduction

Colorectal cancer is estimated to develop in ~5% of the population and that figure increases to 50% if nonmalignant tumors are included. The majority of cases are sporadic and due largely to environmental factors. Hereditary forms may account for up to 15% of all colorectal cancers (1).

An insufficient dietary intake of folate has been associated with increased risk for the development of colorectal cancer. Studies suggest a 30% to 40% reduced risk in individuals consuming high dietary folate compared with those with low dietary folate (2).

Because one-carbon units within the folate metabolic pathway are required for conversion of dUMP to dTMP and for the conversion of homocysteine to methionine, dietary folate deficiency can reduce availability of methyl groups for DNA repair/synthesis and for methylation reactions. Abnormal DNA methylation patterns are commonly observed in a wide variety of tumors and are associated with altered expression of tumor suppressor genes or oncogenes. Insufficient conversion of dUMP to dTMP can also increase uracil misincorporation into DNA, which leads to double-strand breaks. These breaks can result in the formation of chromosomal abnormalities, such as translocations and gene amplification, which can disrupt gene expression and contribute to tumorigenesis (3).

Genetic disturbances in folate metabolism can also modulate risk for colorectal cancer. Methylenetetrahydrofolate reductase (MTHFR) synthesizes 5-methyltetrahydrofolate, the folate derivative used in homocysteine remethylation to methionine. A common variant in *Mthfr* (a C to T transition at nucleotide 677) results in a mild MTHFR deficiency that can confer protection against colorectal cancer when folate levels are adequate (4). However, this protection is not observed when dietary folate is low, and under these conditions, the variant may be associated with increased risk for colorectal cancer and other neoplasias (5).

To examine the mechanisms behind folate deficiency and the link to colorectal cancer, we and others have used the *Apc*^{min/+} mouse model of intestinal neoplasia. These mice have a germ-line mutation in the *adenomatous polyposis coli (Apc)* gene, the same gene that causes a hereditary form of colorectal cancer in man (*familial adenomatous polyposis*; reviewed in ref. 1). Somatic mutations in the *Apc* gene have also been found in up to 80% of sporadic colorectal cancers (1). Other genetic models for colorectal cancer include transgenic mice with a disruption in the mismatch repair genes *Mlh1* and *Msh2* (6). The nongenetic models that have been used to study colorectal cancer include chemically induced models (administration of dimethylhydrazine and azoxymethane), which can be administered alone or in conjunction with bile acid cocarcinogens, nonspecific injury, and surgical procedures known to enhance risk for colorectal cancer (6).

Several studies have reported an influence of folate levels on tumor numbers in *Apc*^{min/+} mice. These studies suggest that the timing of folate deficiency (or supplementation) may be critical. At early time points, folate supplementation may decrease polyp formation (7) and folate deficiency may increase tumor numbers in these mice (8). At later stages, folate supplementation may increase polyp number (7) and folate deficiency may decrease tumor numbers (8). We recently examined gene expression patterns by microarray analysis in *Apc*^{min/+} mice and identified 90 known genes with altered expression in tumors compared with that in normal intestine (9).

To determine whether low dietary folate alone or in combination with a genetic disturbance in folate metabolism can initiate

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doi:10.1158/0008-5472.CAN-06-2477

intestinal tumor development in mice, we fed control and folate-deficient diets to mice with and without a null allele in *Mthfr* (*Mthfr*^{+/-} mice). We had generated these mice in earlier work and showed that they are a good animal model for mild MTHFR deficiency in humans (the 677TT genotype) because of the similarity in residual enzyme activity and the degree of hyperhomocysteinemia. We examined the intestine in mice after ~1 year on diets and observed intestinal tumors in folate-deficient mice but not in mice fed control diets. From previous work in the *Apc*^{min/+} mouse model, we had discovered that folate deficiency may induce expression changes in two genes involved in G₂-M checkpoint control: *polo-like kinase 1* (*Plk1*) and *cell division cycle 25c* (*Cdc25c*). We therefore examined expression of these two candidate genes for tumorigenesis in our spontaneous model to begin to address the mechanisms by which folate deficiency can lead to intestinal neoplasia.

Materials and Methods

Mice. Animal experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital (Montreal, Quebec, Canada). *Mthfr*^{+/+} and *Mthfr*^{+/-} mice, generated in earlier work and backcrossed for at least 10 generations onto a BALB/c background (10), were housed at the Montreal Children's Hospital Research Institute animal facility. After weaning, mice were placed on amino acid-defined diets (Harlan Teklad, Madison, WI) with all the necessary components recommended by the American Institute of Nutrition (11). A control diet contained the recommended amount of folic acid for rodents (2 mg/kg diet), and the folate-deficient diet contained 0.3 mg/kg diet. Both diets contained 1% succinylsulfathiazole, an antibiotic, to prevent generation of folate by intestinal bacteria. These diets have been used in our previous reports, and the folate-deficient diet was shown to be effective in lowering folate or increasing homocysteine as expected (12, 13). Mice were fed these diets for 12 to 14 months until sacrifice. Body weight was recorded on sacrifice. Folate-deficient diet mice had a borderline significant increase in body weight compared with control diet mice by independent sample *t* test (*P* = 0.056).

The entire intestines were removed and examined for tumors under a dissecting microscope. Tumors were dissected and either snap frozen or fixed in 4% paraformaldehyde with surrounding normal intestine. The remaining normal intestine was either frozen or fixed. Fifty-seven mice were fed the control diet (31 *Mthfr*^{+/+} and 26 *Mthfr*^{+/-}), and 80 mice were fed the folate-deficient diet (16 *Mthfr*^{+/+} and 64 *Mthfr*^{+/-}).

Microarray analysis. Microarray analysis of RNA from tissues of *Apc*^{min/+} mice was reported in our earlier publication (9), which examined changes in gene expression between the normal intestine and tumors under several conditions. In this study, we compared the data by grouping the normal intestine data into control diet and folate-deficient diet groups and the folate-deficient diet data into normal intestine and tumor groups. The same analytic method was used as that in our previous report (9). This method was based on averaging probe set intensities for probes with a "present" call for all samples within the test group and dividing it by the average intensity for "present" probe sets for the control group to give a numerical fold change. Any fold change greater than two with an absolute difference >200 between the average probe intensities of the two groups was considered significant. Analysis of numerical fold changes between the four pairs of mice did not reveal any interesting results; a different approach was therefore adopted. The Affymetrix Microarray Suite 5.0 (MAS5, Santa Clara, CA) analysis that had been done on these arrays provides an overall indicator of the binding ability of a probe by giving a probe set a call value of "present" if the probe set binds above a certain threshold and if the binding is significantly higher for the specific probe compared with the mismatch control probe. An "absent" call indicates one of two situations: either the specific probe binding signal is equal to that of the mismatch control or the mismatch control has no signal and the specific probe intensity is below threshold (i.e., too low to reliably detect). The latter

situation can arise if the probe was indeed specific, but the transcript levels were very low/undetectable. We therefore hypothesized that if a probe set had a consistently "absent" call in all replicates of one group and a consistently "present" call in all replicates of the comparison group, then it could be assumed that the expression of the gene product was altered.

RNA extraction from normal intestine. RNA was isolated from 50 mg snap-frozen normal intestine of mice on control and folate-deficient diets using the Trizol reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's protocol. RNA was treated with 10 μL DNase I for 30 minutes before reextraction with equal volumes of a 25:24:1 mixture of phenol-chloroform-isoamyl alcohol. RNA pellets were washed in 75% ethanol and redissolved in diethylpyrocarbonate (DEPC)-treated water.

Laser capture microdissection and RNA extraction. Frozen tumors and normal intestines were sectioned from OCT (Sakura Finetek, Torrance, CA) blocks, cut into 7-μm sections, and kept at -80°C until use. A tumor section and a normal intestinal section from the same mouse were thawed in 75% ethanol for 30 seconds and in DEPC-treated water for 30 seconds and then stained with hematoxylin for 2 minutes. Slides were immersed in bluing solution for 30 seconds, in 70% ethanol for 30 seconds, and in 95% ethanol for 30 seconds and stained for 5 seconds in eosin. Slides were then dehydrated by immersion in 95% ethanol twice for 30 seconds each, in 100% ethanol twice for 30 seconds each, and finally in xylene twice for 5 minutes each. All solutions and plasticware were treated with RNase Away (ICN Biomedicals, Irvine, CA) or DEPC-treated water to maintain RNA integrity. Laser capture microdissection was done using a PixCell II Laser Capture System (Arcturus Biosciences, Sunnyvale, CA) microscope with CapSure LCM caps (Arcturus Biosciences). RNA was extracted from dissected samples using the PicoPure RNA isolation kit (Arcturus Biosciences) according to the manufacturer's instructions and then treated with DNase I (Invitrogen). RNA quantitation was done using the RiboGreen RNA quantitation reagent and kit (Molecular Probes, Burlington, Ontario, Canada) according to the manufacturer's instructions.

Reverse transcription-PCR. RNA (2 μg) from normal intestine, extracted with Trizol, was reverse transcribed. From laser capture microdissected tissue, equal amounts of RNA (ranging from 6 to 29 ng), representing the maximum possible amount per pair, were used. Reverse transcription was done using SuperScript II reverse transcriptase (Invitrogen) and a random hexamer for 50 minutes at 45°C. The enzyme was deactivated by incubation at 72°C for 15 minutes. Real-time PCR was done using the SyberGreen kit (Invitrogen) according to the manufacturer's protocol in an Mx3000P QPCR system (Stratagene, La Jolla, CA). For every gene, the analysis of real-time data was done by using *C_t* values obtained across eight serial dilutions of the same sample and normalizing the values against the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Semiquantitative PCR was done on a Biometra T-gradient system (Montreal Biotech, Montreal, Quebec, Canada). PCR products were run on 9% acrylamide gels and stained with ethidium bromide. Gels were visualized on a Bio-Rad GelDoc 2000 imaging system (Bio-Rad, Mississauga, Ontario, Canada), and bands were quantified using Quantity One 4.0.1 software. Normalization between samples was done by comparison to *GAPDH*. Statistical significance was assessed by independent sample *t* test for comparison of normal intestine on the two diets or by paired sample *t* test for comparison of normal intestine with tumor.

Immunofluorescence. Intestines (with and without tumors), fixed in 4% paraformaldehyde, were embedded in paraffin and cut into 6- to 7-μm sections. Sections were treated according to the protocol provided by DakoCytomation, Inc. (Mississauga, Ontario, Canada). Protein block serum-free was used for the blocking step (DakoCytomation). One section per slide was incubated only with secondary antibody (negative control), and the other sections were covered in either a 1:50 (PLK1, phosphorylated histone H2AX), 1:800 (CDC25c), or 1:200 (phosphorylated CDC2) dilution of primary antibody in antibody diluent (DakoCytomation). All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), except for phosphorylated histone H2AX, which was purchased from Upstate Biotechnology (Lake Placid, NY). For visualization, slides were incubated with a 1:5,000 dilution of Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Molecular Probes). Slides were then washed in PBS and

counterstained with a 1:3,000 dilution of a 1 µg/µL stock of propidium iodide (Molecular Probes) according to the manufacturer's protocol, rinsed again in PBS, dried, and mounted in Prolong Gold Antifade reagent (Molecular Probes). Imaging was done using the Zeiss AxioImager.Z1 with the AxioVision 40 version 4.5.0.0 imaging program (Carl Zeiss Imaging Solutions, Toronto, Ontario, Canada). For ease of viewing, Adobe Photoshop software was used to enhance the brightness of all pictures equally.

To quantify the number of double-strand breaks, the number of foci containing the phosphorylated form of histone H2AX was determined. This protein has been shown to form nuclear foci around sites of double-strand breaks; the foci are quantifiable when immunostained (14). A focus was defined as a concentrated spot of staining inside the nucleus. Three individuals blinded to sample identity counted the number of foci and total number of nuclei in three pictures taken at random per sample. All cell types were included in the counts (e.g., cells in the villi, crypts, and underlying connective tissue). The number of foci was averaged over the three pictures per sample and divided by total number of nuclei. The results of the three independent counts were averaged per sample to achieve the average number of foci per 100 cells. Independent sample *t* test was used to assess significance.

Results

Tumor incidence and histology. A total of 57 mice (31 *Mthfr*^{+/+} and 26 *Mthfr*^{+/-}) was placed on the control diet (Table 1). None of these mice developed intestinal tumors. However, of the 80 mice (16 *Mthfr*^{+/+} and 64 *Mthfr*^{+/-}) that were fed folate-deficient diets, 25% (20 of 80) of these mice developed at least one adenoma or adenocarcinoma in the duodenum. By visual inspection, 13 mice had a single tumor, whereas four mice had two tumors each and three mice had three tumors each. This dietary effect was highly significant ($P < 0.001$, Fisher's exact test). Within the folate-deficient diet group, 12.5% (2 of 16) of *Mthfr*^{+/+} mice developed tumors compared with 28.1% (18 of 64) in the *Mthfr*^{+/-} group; this genotype difference was also significant ($P < 0.01$, Fisher's exact test).

Paraffin-embedded sections of normal intestine and tumors from mice fed control and folate-deficient diets were examined by

routine histology by an on-site pathologist. Seven intestines from control diet mice and 12 intestines from folate-deficient diet mice were examined (Table 1; Fig. 1). In the control diet group, one of the seven intestines was found to have hyperplastic polypoid tissue but no adenomas were observed; the other six intestines appeared normal (Fig. 1A). Of the 12 mice from the folate-deficient diet group, there were five tumors; two had a single adenoma each (Fig. 1C) and three had a single adenocarcinoma with or without invasion into the submucosa (Fig. 1D). Hyperplastic polypoid tissue (Fig. 1B) was found in another 3 of the 12 folate-deficient diet intestines examined. Hyperplastic polypoid tissue is noteworthy because the progression of neoplastic disease in the intestine often proceeds from polyps to adenomas, adenocarcinomas, and finally to metastatic disease (1).

Assessment of DNA damage. Studies have reported a link between folate deficiency and uracil misincorporation into replicating DNA (3). Uracil misincorporation has been linked to increased incidence of double-strand breaks and chromosomal instability, which could lead to cellular transformation (3). To assess DNA damage in our model, we stained control diet and folate-deficient diet normal intestines with an antibody against the phosphorylated form of histone H2AX, which is known to form nuclear foci around the sites of double-strand breaks (14). Using this method, we found that folate-deficient diet normal intestines had an average of 4.25 (± 0.44 , SE) foci per 100 cells, whereas control diet normal intestines had an average of 2.53 (± 0.69 , SE) foci per 100 cells. This increase in double-strand breaks in the folate-deficient diet normal intestine was found to be borderline significant ($P = 0.069$) by independent sample *t* test.

Identification of candidates by gene expression in *Apc*^{min/+} mice on control and folate-deficient diets. In an earlier report (9), we had done microarray analysis of RNA from normal intestine and tumors of *Apc*^{min/+} mice; the goal of that study was to identify candidates that could contribute to tumor growth in the *Apc*^{min/+} model (9). These mice had been fed the same control and

Table 1. Tumor incidence in intestine of mice on control diet or folate-deficient diet with and without a null allele in *Mthfr*

No. mice examined for tumors	Diet		Total mice	% Mice with tumor
	Control	Folate deficient		
<i>Mthfr</i> ^{+/+}	31 (0)	16 (2)	47	12.5
<i>Mthfr</i> ^{+/-}	26 (0)	64 (18)	90	28.1*
Total mice	57 (0)	80 (20)	137	
% Mice with tumor	0	25 [†]		
Histologic examination of intestine	Total examined	Adenoma	Adenocarcinoma	Polyp
Control diet	7	0	0	1
Folate-deficient diet	12	2	3	3

NOTE: The numbers in parentheses indicate the number of mice that developed tumors in each group. None of the mice on control diet developed tumors compared with 25% of mice on folate-deficient diet. Within the folate-deficient diet group, 12.5% (2 of 16) of mice with no *Mthfr* mutation and 28.1% (18 of 64) of *Mthfr*^{+/-} mice developed between one and three duodenal tumors. Both diet and genotype effects were significant. Histologic examination of intestines revealed one intestinal polyp and no adenomas or adenocarcinomas in the control diet group. Of the 12 folate-deficient diet intestines, five tumors were observed and further examined. Of these, two were adenomas and three were adenocarcinomas, some of which showed invasion into the submucosa. Three other folate-deficient diet intestines showed a single micropolyp.

* $P < 0.01$ for comparison of *Mthfr*^{+/+} and *Mthfr*^{+/-}.

[†] $P < 0.001$ for comparison between control diet and folate-deficient diet.

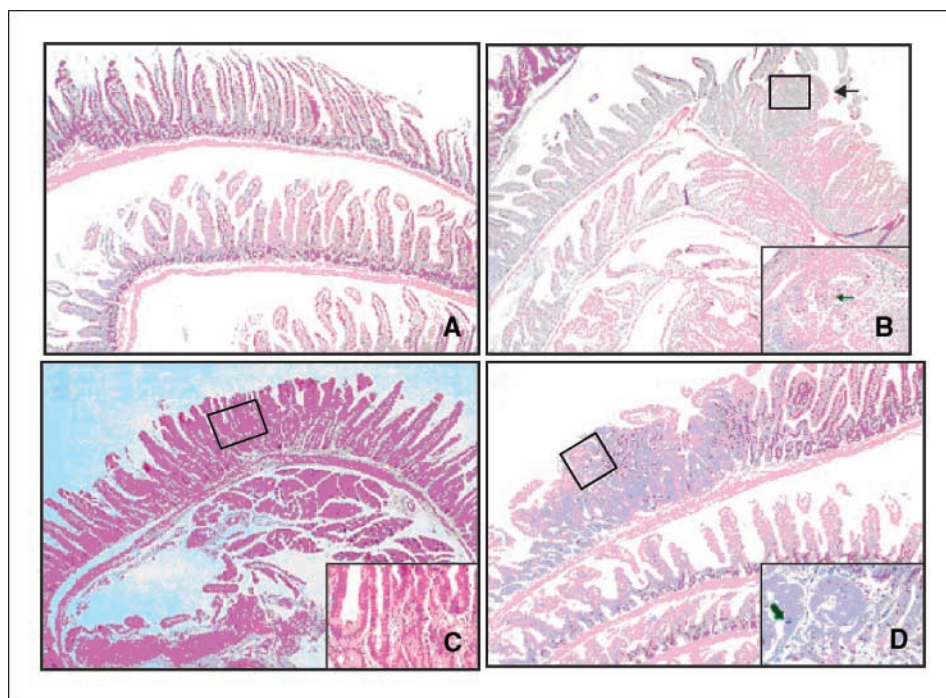


Figure 1. Histologic characterization of intestines. *A*, H&E-stained normal intestine from a mouse fed control diet for 12 months. Magnification, $\times 40$. *B*, H&E-stained polypoid hyperplasia of the epithelium (micropolyp; arrow) covered by normal mucosa from a folate-deficient diet mouse. Magnification, $\times 40$. *Inset*, higher magnification of the boxed area ($\times 400$) with some dark staining proliferating cells (green arrow). *C*, H&E-stained villous adenoma from a folate-deficient diet mouse showing the long, villous, glandular fronds. Magnification, $\times 40$. *Inset*, higher magnification of the boxed area ($\times 400$). *D*, H&E-stained adenocarcinoma from a folate-deficient diet mouse showing the lesions *in situ* without invasion into the submucosa. Magnification, $\times 40$. *Inset*, higher magnification of the boxed area ($\times 400$), with intensely stained nuclei indicative of proliferating cells (green arrow).

folate-deficient diets that were used in this study. We therefore compared the data from the normal intestine of *Apc*^{min/+} mice fed control diet with data from the normal intestine of *Apc*^{min/+} mice fed folate-deficient diet. The goal of this comparison was to identify selected candidates altered in expression by the folate-deficient diet that could then be examined in the tissues of our new spontaneous model of tumorigenesis described in this report. Application of the numerical fold change analysis did not yield readily interpretable results. We then included as a significant result all probe sets that displayed a consistent call value of “present” or “absent” for the control diet group and a consistent opposite call for the folate-deficient diet group. Using this approach, we found several interesting candidate genes that met these variables (Table 2). Although 12 genes were found to be consistently “present” in control diet normal intestines and “absent” in folate-deficient diet normal intestines, no genes were found for the reverse situation. Both *Plk1* and *Cdc25c* were found to be “present” in the normal intestine of *Apc*^{min/+} mice fed control diet and consistently “absent” in the normal intestine of *Apc*^{min/+} mice fed folate-deficient diet (Table 2). Semiquantitative reverse transcription-PCR (RT-PCR) was used to confirm the expression changes of *Plk1* in *Apc*^{min/+} normal intestine of the two dietary groups. In four pairs of RNA, done in duplicate, *Plk1* showed decreased expression with an average fold change of -1.77 ± 0.45 , but the change was not statistically significant. *Plk1* and *Cdc25c* are of interest because *Plk1* is a regulator of *Cdc25c* in the DNA damage response pathway, and the aforementioned results with phosphorylated histone H2AX suggested that the folate-deficient diet was increasing DNA damage in the normal intestine of our mice. *Plk1* is inactivated during the DNA damage response, whereas *Plk1* activation is required for reentry into mitosis once DNA damage has been repaired (15). In undamaged cells, PLK1 activates CDC25c, which can proceed into the nucleus to dephosphorylate CDC2; CDC2 can then complex with cyclin B to initiate mitosis. Because folate deficiency seemed to have increased

DNA damage, one might expect *Plk1* inactivation to allow cells to repair DNA; the decrease in *Plk1* mRNA by microarray and RT-PCR analyses was consistent with this hypothesis.

Based on these results, we were interested in determining whether folate deficiency altered the expression of *Plk1* and *Cdc25c* in tumors of the *Apc*^{min/+} model. Our original report (9) did not show any differences in expression between normal intestine and tumors for these genes; however, this comparison grouped tissues of both diets together. We therefore did a new comparison separating the control diet normal intestine and tumors from the folate-deficient diet normal intestine and tumors. On the control diet, there was no change in gene expression between normal intestine and tumor. However, it was interesting to note that *Plk1* was found to be consistently “present” in folate-deficient diet tumors and consistently “absent” in folate-deficient diet normal intestine. In addition, *Cdc25c* was 2.13-fold increased in folate-deficient diet tumors compared with folate-deficient diet normal intestine (Table 2).

Expression of *Plk1* and *Cdc25c* RNA in spontaneous model of folate-dependent tumorigenesis. Our results in the *Apc*^{min/+} model led us to believe that folate deficiency altered expression of *Plk1* and *Cdc25c*, two important mitotic regulators. Furthermore, the *Plk* family of kinases is up-regulated in several cancers (16–18), and human studies have shown an increase in *Plk1* mRNA and protein levels in colorectal carcinoma (19–22). We therefore examined expression of these genes in the normal intestines and tumors of our new mouse model.

Using quantitative RT-PCR, *Plk1* and *Cdc25c* expression were assessed in six pairs of normal intestines from mice on folate-deficient diet and mice on control diet; three pairs were *Mthfr*^{+/+} and three pairs were *Mthfr*^{+/-} mice. *Plk1* and *Cdc25c* both showed decreases in expression in folate-deficient diet normal intestine; the changes were significant for *Plk1* (-1.41 ± 0.12 ; $P = 0.045$) and there was borderline significance for *Cdc25c* (-1.61 ± 0.20 ; $P = 0.06$). These findings are consistent with those seen in *Apc*^{min/+} mice.

Table 2. Microarray results in normal intestine and tumors from *Apc^{min/+}* mice on control and folate-deficient diets

Gene	Accession no.	Probe set*	Gene product	CD NI call	FD NI call	FD T call or fold change (FD T/FD NI)
<i>Capg</i>	X54511	160106	Gelsolin, actin capping	P	A	P
<i>Igg3</i>	D14625	102721	Immunoglobulin γ 3	P	A	No change
<i>Ent</i>	L17324	100120	Entactin	P	A	No change
<i>Tacc3</i>	AW209238	97238	Transforming acid coiled coil 3	P	A	P
<i>Elp3</i>	AI851229	95717	Elongation protein 3	P	A	No change
<i>Oprs1</i>	AF004927	94828	Sigma 1 receptor	P	A	P
<i>Plk1</i>	U01063	93099	Polo-like kinase 1	P	A	P
<i>Tomm40</i>	AF043249	160653	Mitochondrial outer membrane protein	P	A	No change
<i>Cdc23</i>	AA657164	104090	Cell division cycle 23	P	A	No change
<i>Th9</i>	AI845815	103343	α -Catenin binding protein	P	A	No change
<i>Cdc25c</i>	U15562	102935	Cell division cycle 25c	P	A	2.13
<i>Tpd52l1</i>	AF004428	101446	Tumor protein D53	P	A	P

NOTE: Four pairs of RNA were used to compare expression in normal intestine of *Apc^{min/+}* mice fed control diet or folate-deficient diet and two pairs for the folate-deficient diet mice to compare expression in normal intestine with that in tumor. Numerical fold changes were calculated from the average probe set intensities of one condition (diet or tissue type) compared with the other. Nonnumerical fold changes are defined as a consistent call of "absent" (A) or "present" (P) for the probe set for all repeats of one condition and the opposite call consistently noted for the probe set for all repeats of the opposite condition.

Abbreviations: CD NI, control diet, normal intestine; FD NI, folate-deficient diet, normal intestine; FD T, folate-deficient diet, tumor.

*Description of probe sets can be obtained at <http://www.affymetrix.com> by adding "_at" to the end of the numbers presented in this table.

Laser capture microdissection was used to isolate tumor tissue and normal intestine for RNA isolation and quantitative RT-PCR from three *Mthfr^{+/-}* mice. *Plk1* was found to be increased 11.6-fold (± 5.1 , SE) in tumors compared with normal intestine, whereas *Cdc25c* was found to be increased 3.1-fold (± 1.8 ; Fig. 2B). The changes in *Plk1* RNA were significant ($P < 0.02$), whereas the changes in *Cdc25c* were borderline significant ($P = 0.066$) by paired *t* test.

Expression of PLK1, CDC25c, and phosphorylated CDC2 immunoreactive proteins in normal intestine and tumors. To confirm the expression changes observed by RT-PCR, we examined the protein levels of PLK1 and CDC25c in normal intestine from control diet and folate-deficient diet mice as well as in tumors from folate-deficient diet mice. We also assessed CDC25c activity by

examining phosphorylated CDC2 levels. Immunofluorescence was used to measure the three immunoreactive proteins on serial sections of tissues from three mice per group.

When comparing the immunostaining of the control diet (Fig. 3A and B, top row) and folate-deficient diet (Fig. 3A and B, middle row) normal intestines, there seems to be a decrease in protein levels for both PLK1 and CDC25c corresponding to the RT-PCR results. The examination of immunoreactive proteins in tumor sections (Fig. 3A and B, bottom row) suggests that both proteins seem to have increased expression in tumor tissue compared with corresponding normal intestine (Fig. 3A and B, middle row).

To obtain independent confirmation of CDC25c activity, we used an immunofluorescent stain for phosphorylated CDC2. The function of CDC25c in checkpoint control is to dephosphorylate and activate CDC2 to allow cell cycle progression (23). Therefore, cells progressing into mitosis would be expected to have lower levels of phosphorylated CDC2 and cells that are arrested in G_2 would have higher levels of phosphorylated CDC2. Figure 4 shows phosphorylated CDC2 immunofluorescence in sections of normal intestine from control diet mice (top row), normal intestine from folate-deficient diet mice (middle row), and corresponding tumors from folate-deficient diet mice (bottom row). It is clear that the levels of phosphorylated CDC2 are low in the undamaged normal intestine from control diet mice, where normal proliferation is occurring, and that they increase in the folate-deficient diet normal intestine where we have observed increased DNA damage and the cells are likely arrested in G_2 . This finding is consistent with the decreases in *Cdc25c* mRNA (reported above) and protein levels shown in Fig. 3. In tumor tissue, there is a decrease in phosphorylated CDC2 staining (bottom row) compared with the normal intestine of folate-deficient diet mice (middle row). This observation suggests that the tumors have managed to bypass the G_2 checkpoint and continue into mitosis. Again, this finding is

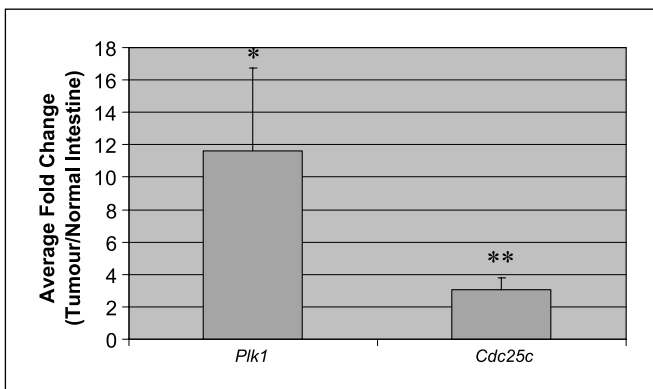


Figure 2. Quantitative RT-PCR for *Plk1* and *Cdc25c*. Comparison of laser capture microdissected tumors with normal intestine from folate-deficient diet *Mthfr^{+/-}* mice. Columns, mean of three pairs of RNA for each gene; bars, SE. *Plk1* shows an 11-fold (± 5.1) increase in expression in tumors. *Cdc25c* shows a 3-fold (± 1.8) increase in expression. *, $P = 0.019$; **, $P = 0.066$ by paired sample *t* test.

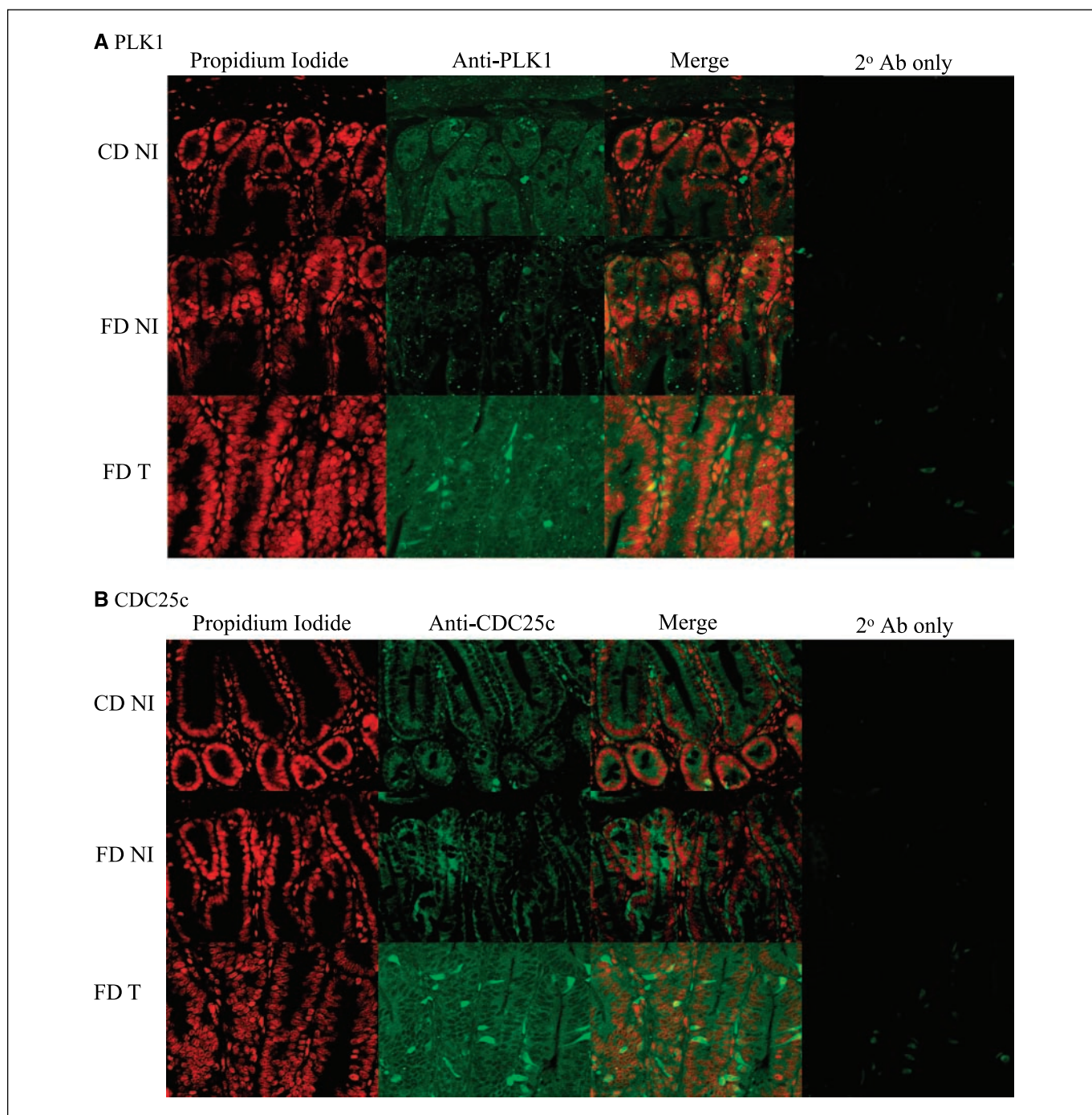


Figure 3. Immunofluorescence staining of PLK1 and CDC25c in normal intestine and tumors. A representative experiment (from three mice per group) is shown at $\times 40$ magnification. Anti-PLK1 (A; column 2) and anti-CDC25c (B; column 2) were used to stain paraffin-embedded sections of normal intestine from a control diet mouse (top row), normal intestine from a folate-deficient diet mouse (middle row), and a tumor from a folate-deficient diet mouse (bottom row). Column 1, propidium iodide was used as a nuclear counterstain. AxioVision 40 software was used to merge the propidium iodide signal with the immunofluorescent signal. Both PLK1 and CDC25c seem to decrease in folate-deficient diet normal intestine compared with control diet normal intestine and then increase in tumors from folate-deficient diet mice. Column 4, negative controls containing secondary antibody, but no primary antibody, for each sample. CD NI, control diet, normal intestine; FD NI, folate-deficient diet, normal intestine; FD T, folate-deficient diet, tumor.

supported by the increases in *Cdc25c* mRNA and protein levels in tumors as discussed for Fig. 3.

Discussion

Our model of environmentally induced colorectal cancer may be more physiologically relevant than previous animal models for

examining dietary effects on neoplasia. Chemically induced models of colorectal cancer use doses of carcinogens that are much higher than those that would be applicable to human populations. Furthermore, the series of molecular events set in motion by carcinogen exposure do not necessarily reflect the events that occur in the progression of human cancer (6). Although genetic

models (such as the *Apc^{min/+}* mouse) can mirror human hereditary cancer disorders, these cancers occur in only a minority of cancer cases, with the majority due largely to environmental factors (1).

We have developed a new model of intestinal tumorigenesis due to a deficiency of dietary folate. Mice, with or without a mutation in *Mthfr*, can develop duodenal tumors after ~1 year on the diet. We are not aware of other animal models that develop spontaneous intestinal tumors due to a dietary deficiency alone, without radiation/chemical induction or a germ-line mutation. Our findings provide a clear link between folate deficiency and tumor initiation. Although epidemiologic studies have shown an inverse relationship between folate intake and risk for colorectal cancer, there has been no definitive biological evidence to link folate deficiency and colon cancer (24).

In beginning to address the mechanisms of folate-induced tumorigenesis, we identified altered expression of two important G₂-M checkpoint control genes, *Plk1* and *Cdc25c*, in the normal intestine as well as in the tumors of folate-deficient diet mice. In normal intestine, both *Plk1* and *Cdc25c* have decreased expression in folate-deficient diet tissue. We hypothesize that decreased folate leads to an accumulation of uracil due to decreased availability of 5,10-methyleneTHF, which is required for dUMP conversion to dTMP. The accumulation leads to uracil misincorporation into DNA and double-strand breaks, which increases risk for chromosomal abnormalities (25). DNA damage, such as double-strand breaks, will arrest the cell at the G₂-M boundary by activating the ataxia-telangiectasia mutated (ATM)/ATR and Rad3-related (ATR) pathway. Indeed, our results show that the folate-deficient diet seems to increase the amount of double-strand breaks in the normal intestine as shown by increased formation of phosphorylated histone H2AX foci. Downstream targets of the ATM/ATR

pathway include checkpoint kinases 1 and 2, both of which function to phosphorylate CDC25c on inhibitory residues, keeping it in an inactive conformation (23). PLK1 has also been shown to be inactivated by checkpoint kinase 1 during the ATM/ATR DNA damage response pathway; once the DNA damage has been repaired, PLK1 is necessary for reentry into mitosis (15). Because both *Plk1* and *Cdc25c* are down-regulated in response to DNA damage (15, 23), it is reasonable to conclude that the observed decrease in their mRNA and protein levels in folate-deficient diet normal intestine is due to the normal response of the cells to the increase in DNA damage. Decreasing both *Plk1* and *Cdc25c* results in cell cycle arrest, presumably to allow cells to repair their DNA before continuing into mitosis (15).

More interesting, however, is the large increase in mRNA and protein expression for both *Plk1* and *Cdc25c* in tumor tissue compared with the surrounding normal intestine. The *Plk* family of kinases and *Cdc25c* are up-regulated in several cancers and are targets of several antisense and small-molecule inhibitors currently under investigation as antitumor agents (16–18). Two studies have examined *Plk1* mRNA (19, 20) or PLK1 protein (21, 22) levels in human colorectal carcinoma. *Cdc25c* levels (mRNA or protein) have not previously been examined in colorectal tumors. Our study links the overexpression of these two critical proteins in the initiation of intestinal carcinogenesis. In the folate-deficient diet normal intestine, *Plk1* and *Cdc25c* are under tight regulation and seem to be responding in the expected way to DNA damage. However, under the extended chronic stress of folate deficiency and DNA damage, the tight regulatory mechanisms controlling their expression seem to break down, allowing increased expression and abnormal progression into mitosis.

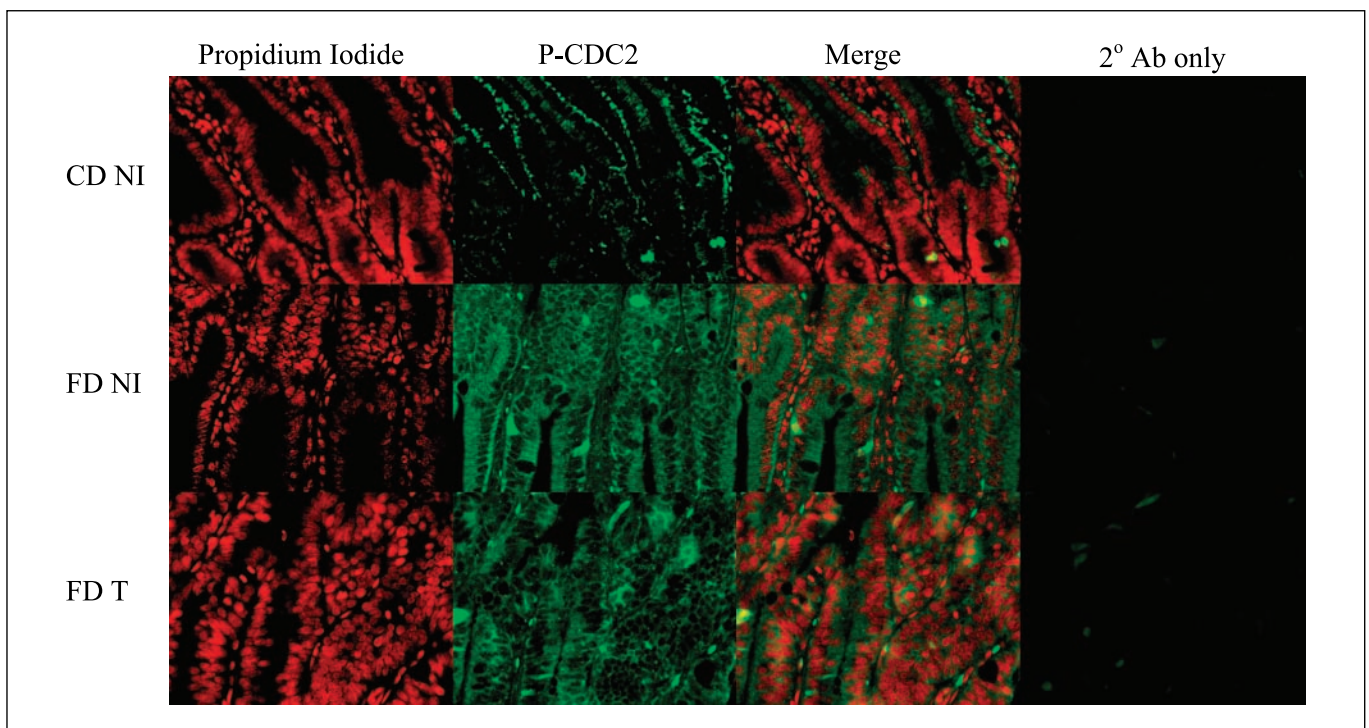


Figure 4. Phosphorylated CDC2 (*P-CDC2*) immunofluorescence as a measure of CDC25c activity in normal intestine and tumors. A representative experiment (from three mice per group) is shown at $\times 40$ magnification. As in Fig. 3, paraffin-embedded sections of normal intestines and tumors were stained with anti-phosphorylated CDC2 and counterstained with propidium iodide. The two images were then merged. Phosphorylated CDC2 seems to be increased in folate-deficient diet normal intestine compared with control diet normal intestine or folate-deficient diet tumors. *Column 4*, negative controls containing secondary antibody, but no primary antibody, for each sample.

The mechanisms that lead to increased expression of these genes require elucidation. One possibility is that the deregulation of *Cdc25c* expression is due to a decrease in p53 expression and/or function. The *Cdc25c* promoter has been shown to have consensus sequences that are responsive to p53, and increased p53 expression has been shown to down-regulate *Cdc25c* as would occur in response to DNA damage (26). It is possible that folate deficiency leads to decreased expression or increased mutation of p53, which would increase expression of *Cdc25c* and allow cell cycle progression. The corresponding increase in *Plk1* expression could be explained by feedback mechanisms in the G₂-M checkpoint (27). Another possible explanation for the aberrant expression of *Cdc25c* and *Plk1* (or of p53) is abnormal DNA methylation. Many studies have shown that folate deficiency can alter methylation patterns in DNA, resulting in global DNA hypomethylation and regional promoter hypermethylation (28). In tumors, altered methylation patterns have been shown to affect expression of key growth control genes (29). For example, studies of hepatocarcinogenesis have revealed methylation changes within the p53 promoter region in livers of methyl-deficient rats (30).

In summary, dietary folate deficiency can initiate tumors in the intestine. The deficiency leads to increased DNA damage and decreased expression of *Plk1* and *Cdc25c* in normal intestine presumably to repair the damage. However, the chronic stress leads to deregulation of these genes in the tumor and increases their expression, allowing cells to proceed to mitosis without cell cycle control. The establishment of a nutritionally deficient animal model for intestinal tumorigenesis provides the biological evidence for the important role of folate in prevention of colorectal cancer. The elucidation of the tumorigenic mechanisms in this more physiologically relevant model may lead to the design of novel therapies for this common cancer.

Acknowledgments

Received 7/6/2006; revised 9/5/2006; accepted 9/11/2006.

Grant support: Canadian Institutes of Health Research. E. Knock was the recipient of a Montreal Children's Hospital Research Institute Studentship.

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We thank Dr. Nada Jabado and Damien Faury for their assistance.

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