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UMI
The effects of dietary folate on intestinal tumorigenesis in genetically predisposed murine models.

by

Jacquelin Song

A thesis submitted in conformity with the requirements for the degree of M.Sc.

Graduate Department of Nutritional Sciences

University of Toronto

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THE EFFECTS OF DIETARY FOLATE ON INTESTINAL TUMORIGENESIS IN GENETICALLY PREDISPOSED MURINE MODELS

Master of Science, 1999
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ABSTRACT

Epidemiologic and animal studies have shown dietary folate intake to have a dose-responsive inverse relationship with the risk of colorectal neoplasms.

Apc+/- (Min) and Apc+/-Msh2/- mouse models, which develop spontaneous intestinal tumors that are genotypically and phenotypically similar to human CRC, were used to study the effects of dietary folate on intestinal and colonic tumorigenesis. This study indicates that dietary folate supplementation at 10x and 4x the basal requirement, respectively, provided prior to neoplastic foci development, suppresses intestinal tumorigenesis. In contrast, in the Apc+/-Msh2/- model only, dietary folate deficiency, provided after the development of neoplastic foci, significantly reduces intestinal tumor number. DNA methylation and impaired mismatch repair, as mechanisms for the observed effects of folate, appear to not play major roles in this model.

Therefore, timing and dose of folate intervention is critical in providing safe and effective chemoprevention in these models.
ACKNOWLEDGEMENTS

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Finally, much love goes out to my family who has seen and endured the best and the worst of me, for which I am indebted. Thank you.
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<tr>
<td>5-aza-C</td>
<td>5-aza-cytidine</td>
</tr>
<tr>
<td>5mC</td>
<td>5-methyl-cytosine</td>
</tr>
<tr>
<td>ACF</td>
<td>aberrant crypt foci</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOM</td>
<td>azoxymethane</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>CHRPE</td>
<td>congenital hypertrophic retinal pigment-epithelial lesions</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestine phosphatase</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>DCC</td>
<td>deleted in colon cancer</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexanoic acid</td>
</tr>
<tr>
<td>DMH</td>
<td>dimethylhydrazine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ENU</td>
<td>ethylnitrosurea</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentanoic acid</td>
</tr>
<tr>
<td>F</td>
<td>female</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>FPGS</td>
<td>folylpolyglutamate synthetase</td>
</tr>
<tr>
<td>H and E</td>
<td>hemotoxylin and eosin</td>
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<tr>
<td>HNPCC</td>
<td>Hereditary Non-polyposis Colorectal Cancer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>HTP</td>
<td>tricalcium phosphate</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>M</td>
<td>male</td>
</tr>
<tr>
<td>MCR</td>
<td>mutation cluster region</td>
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<tr>
<td>MET</td>
<td>methylation</td>
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<tr>
<td>MI or MSI</td>
<td>microsatellite instability</td>
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<tr>
<td>Min</td>
<td>multiple intestinal neoplasia</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
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<tr>
<td>Mom-1</td>
<td>modifier of Min-1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MTase or Dnmt</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>MTHF</td>
<td>methylenetetrahydrofolate</td>
</tr>
<tr>
<td>NA</td>
<td>not available</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<tr>
<td>NTD</td>
<td>neural tube defect</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RER</td>
<td>replication error</td>
</tr>
<tr>
<td>RR</td>
<td>relative risk</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
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<td>THF</td>
<td>tetrahydrofolate</td>
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CHAPTER 1. INTRODUCTION

1.1 COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer and second most common cause of death, in Canada (1). In 1998 alone, there were over 16,000 new cases of CRC diagnosed and approximately 6,000 deaths from CRC (2). Areas at high risk for CRC tend to include developed, industrialized, urbanized and westernized countries within North America, Europe and Australasia (3), which suggests that environment may play a role in its development. The intercontinental difference in CRC risk would imply an inherited cultural bias, but this is disputed by migrational studies of native Japanese moving to the United States. Haenszel et al., found that the migrating generation had an increased risk of developing CRC as compared to their counterparts living in Japan; as well, they found that the offspring of the migrated generation were at comparable risk to the native white Americans (4). As well, Snowdon et al., studied white male, Seventh Day Adventists who had a 62% lower mortality of CRC compared to other Americans and attributed this, at least partly, to their increased consumption of cereal fiber and vegetables (5). The inter- and intra-regional differences in CRC incidence suggest a strong environmental influence on the development of the disease.

Environmental and genetic factors are both thought to play a role in the carcinogenic process. Known risk or predisposing factors include genetic predisposition, extensive ulcerative colitis of long duration, age, prior adenoma or adenocarcinoma of the colon, family history, smoking and dietary factors (3).
Dietary factors whose frequent consumption has been categorized as presenting a probable increased risk include red meat and alcohol (3). Other possible risk factors include obesity, high body mass index (BMI), frequent eating, increased consumption of simple sugars, total and saturated animal fats, eggs and processed meats (3). Foods whose consumption is associated with decreased risk of CRC include fiber, fruits and vegetables (6). Dietary factors such as selenium, omega-3 fatty acids, calcium and antioxidants are currently being investigated for their chemopreventive potential. One factor that appears promising as a potential chemopreventive agent is folate, a B vitamin.

Those who develop CRC may present with fatigue, anemia, a change in bowel movements, bowel obstruction, rectal bleeding, perforation and abdominal pain (7). Once patients experience these symptoms, extensive disease is already present, thus early detection by screening is extremely important. Early detection using screening techniques, such as a fecal occult blood test, sigmoidoscopy or colonoscopy, is a favored practice as a tremendous difference in prognosis has been reported between diagnosis at early and late stages of the disease (8). Sigmoidoscopy as a screening technique has been estimated to potentially prevent 5,500 cases of CRC per year in the United Kingdom (9). The prognosis of CRC is dependant upon the stage at which diagnosis occurs. The five year survival rate for those diagnosed at Dukes A and B is greater than 80%, for Dukes C it is approximately 50% and for Dukes D, the rate is less than 5-10%, where the Dukes' classification is correlated with the degree of penetration in the bowel wall (7).

The incidence of CRC in Canada has been decreasing over the last 15 years, with the rate of decrease being greater in women (2). Mortality has also been decreasing in Canada, possibly due to improved early detection and positive lifestyle changes, such as dietary habits (2,10). The
importance of prevention and early detection by screening is therefore becoming increasingly apparent as strategies to fight this disease.

1.1.1 Genetics and colorectal cancer

Carcinogenesis in the colon involves many steps and requires many years of development. The accumulation of genetic changes results in the progression of preneoplastic changes, benign neoplasm and malignant neoplastic mucosa (11). The majority of colorectal cancers arise from preexisting adenomas over several years to decades (11).

The genetic model, as presented by Vogelstein et al, (Figure 1.1) suggests that an accumulation of genetic alterations involving oncogenes (e.g. RAS and MYC), tumor suppressor genes (e.g. P53, APC, DCC) and mismatch repair genes are necessary for tumorigenesis. The initial step from normal epithelium to hyperproliferation is thought to be a result of the inactivation of the tumor suppressor APC (Adenomatous Polyposis Coli) gene on chromosome 5q (12). The similarity of APC mutation frequency found in adenomas when compared to carcinomas suggests that the inactivation of the gene likely occurs at an early stage in tumorigenesis (13). Another genetic event that is thought to play a role in early adenoma development is DNA hypomethylation, which has been found to occur very early in colorectal tumorigenesis (14). As well, at the early adenoma stage in 50% of primary colon cancers, mutations are found at codons 12,13 and 61 in the RAS oncogene (15). Mutations in the RAS gene, for example K-RAS, usually occur in a single cell within the small adenoma, which, through clonal expansion, develops into a larger adenoma (15). Following these changes are deletions at alleles located on loci 17p and 18q. Chromosome 17p is the location of the P53 tumor suppressor gene which has been implicated in the late development of colonic tumors (16). The genes thought to be of interest on chromosome 18q are DCC (deleted in colon
Figure 1.1 Genetic model for CRC. The accumulation of genetic changes results in a progression from normal epithelium to hyperproliferation, to adenoma and finally carcinoma. (Adapted from Fearon and Vogelstein, Cell, 61:758-767,1990)
cancer), *DPC4* and *MADR2*, which are also often altered in CRC. During the process of carcinogenesis, deletions at 17p and 18q are thought to frequently occur after mutations at 5q or *RAS*, suggesting their involvement in the late stages of colorectal carcinogenesis. However it is believed that importance of accumulating mutations exceeds that of the order of occurrence (16). Mismatch repair genes (MMR) such as *MSH2*, *MLH1*, *PMS1*, *PMS2*, *MSH6*, *MSH3* and *MSH5* also play a role in tumorigenesis and their effects are evident throughout the carcinogenic process. Mutations in MMR genes can result in a loss of repair function, resulting in hypermutability. This would have accelerating effects at any stage of carcinogenesis as the lack of crucial DNA repair mechanisms would hasten the effects of other somatic or germline mutations.

### 1.1.1.2 APC gene

*APC* is a tumor suppressor gene located on chromosome 5q21 that consists of 15 exons and encodes a protein 310 kD in size. The protein is located in the basolateral membrane in colorectal epithelial cells with increased expression as cells migrate up through the colonic crypt (17). The function of APC has yet to be clearly elucidated however, a number of potential mechanisms have been proposed through which APC controls cell growth. It has been suggested that wild type APC is associated with the microtubule cytoskeleton which plays an important role in maintaining cell structure and fidelity of mitosis by playing a crucial role in the segregation of chromosomes. If the *APC* gene is mutated, then it will not bind to the microtubule cytoskeleton, disrupt cell-cell interactions, and result in inappropriate cell proliferation (18,19). It has also been shown that APC may be associated with expression of an oncogene, *c-MYC* (20). Increased *c-MYC* expression is common in CRC (21) and is now postulated to be a result of mutations affecting the *APC* tumor suppressor gene pathway (22). Wild type APC binds with GSK-3β, a serine threonine glycogen synthase kinase, to regulate
intracellular levels of b-catenin, a putative modulator of cell-cell adhesion or contact associated growth regulation (23). The binding of wild type APC to GSK-3β results in a down regulation of the β-catenin/Tcf 4 (transcription factor 4) pathway, with a tumor suppressing effect. It has been suggested by He et al., that one of the genes regulated by this pathway is the c-MYC oncogene (20). c-MYC expression has been shown to be suppressed by APC induction (20) and increased with the loss of chromosome 5q where the APC gene is located (24). The increased expression of c-MYC seen in most CRC, may disrupt cell cycle control directly and promote entry into the S phase of the cell cycle (25).

Mutations in the APC gene have been observed in 60% of sporadic and 100% of familial tumors (13,26). These mutations have been detected at very early stages of neoplasia, which suggests that inactivation of APC is an early event in tumorigenesis. In support of this, inactivated APC has been found in aberrant crypt foci (ACF), precursors to adenoma and the earliest examinable neoplastic lesion (12,27,28). Thus, APC has been referred to as the 'gatekeeper' gene of colonic epithelial cell proliferation where inactivation or mutation results in a permanent imbalance in cell division over cell death (11). APC is therefore a very important gene, as cells with a normal gatekeeper gene, but mutations in other genes, would not undergo sustained cell proliferation (11). Inactivation of APC occurs when both alleles are mutated, as predicted by Knudson's two hit hypothesis, which suggests that the tumor suppressor gene functions in a “recessive” manner, such that both copies of the gene must be inactivated for the anti-proliferative function to be eliminated (29). Once one allele has been mutated, either by somatic or germline mutation, the remaining allele must be deleted or mutated for cancer to develop. In familial cancers, such as Familial Adenomatous Polyposis (FAP), a germline mutation may act as the primary step towards tumorigenesis with an additional mutation or deletion of the wild type allele occurring to promote the progression. Studies in human FAP
patients and mice have indicated that a somatic mutation in the wild type allele may act as a rate limiting step in initiating tumors from normal cells (27,28,30). In sporadic cancers, inactivation of both alleles is required by somatic mutation. Inactivation of the second wild type allele is often achieved by loss of heterozygosity (LOH), a complete elimination of the second allele (27,31). LOH occurs when the first copy of the gene is inactivated by mutation and the second copy is deleted or sometimes replaced by a copy of the mutant allele (32). In humans, mutations in the APC gene can result in FAP, which is an autosomal dominantly inherited disease predisposing patients to colon cancer. FAP accounts for less than 1% of CRC cases and although tumors are thought to develop at 15-20 years of age, colon cancer diagnosis in these patients tend to occur in the fourth decade of life (33). Patients with FAP develop millions of ACF and hundreds to thousands of polyps throughout their intestine that can progress to malignant carcinoma if not removed by polypectomy or colectomy (7). Other extraintestinal features seen in these patients include desmoid tumors, upper-gastrointestinal polyps, dental abnormalities, osteomas, congenital hypertrophic retinal pigment-epithelial lesions (CHRPE) and thyroid, brain and hepatobiliary cancers (34). However, not all tumors of FAP patients have loss of the second APC allele. LOH in the APC gene may thus be a common but unnecessary occurrence in tumorigenesis (11). It has been suggested that, in this case, the gene mutations act in a dominant negative manner where the truncated APC polypeptide binds to the wild type protein preventing its function without the required second mutation of the gene (35).

Mutations that occur most frequently on the APC gene are nonsense point mutations or microdeletions that result in the formation of a truncated protein product (33). Many of the point mutations that occur are alterations of a cytosine residue to another nucleotide; half of these cytosine changes are C to T changes at a CpG site (36), likely due to a spontaneous or enzymatic deamination of 5-methylcytosine (13). Interestingly, there are geographical
differences in somatic mutations. The frequency of CpG point mutations in American patients is much higher than that found in Japanese FAP patients (p<0.05) (13), suggesting a dietary or other environmental influence.

With regards to the *APC* gene, the relationship between phenotype and genotype is complex; patients with similar or identical mutations may have different clinical symptoms. Some studies have shown that patients with mutations proximal to the 5’ end of the gene have more polyps (37) than those with mutations closer to the 3’ end (13), whereas other studies have shown no difference in colorectal polyp number regardless of mutation location (38,39). These conflicting results are of concern as the majority of *APC* mutations occur within the 5’ half of the gene (40). There is also an area termed the mutation cluster region (MCR) at codons 1286 to 1513 which contains two thirds of the somatic mutations found in sporadic colorectal tumors, but not in FAP tumors (13,41). This MCR in colorectal tumors is very close to the region where germline mutations are found in FAP patients with a more severe phenotype (41). There is also a correlation between certain non-colonic features of FAP and *APC* mutation location. The occurrence of CHRPE is correlated with *APC* mutations in exons 9-15 (42) and desmoid tumors are correlated with the area spanning codons 1444-1578 (43).

A number of mouse models have been developed that are genetically predisposed to developing intestinal adenomas which have specific mutations in the Apc gene. Apc+/− heterozygous mice, with a mutation at codon 850, develop multiple intestinal adenomas and are thus known as Min mice. See Section 1.3 Animal models of CRC.

In summary, the *APC* gene functions as a gatekeeper of abnormal cell proliferation in the colon and in this capacity plays a crucial role in colorectal tumorigenesis.
1.1.1.2 RAS gene

The development from a small adenoma to a large adenoma is associated with a RAS gene mutation that occurs in one cell of an adenoma that becomes enlarged through clonal expansion (16). Oncogenes, such as RAS have been extensively studied in colon cancer. About 50% of large tumors (greater than 1 cm in diameter) have mutations in one of the three mammalian RAS genes (15): H-RAS, N-RAS and K-RAS. More than 90% of K-RAS mutations in CRC are found in codons 12 and 13. These mutations result in constitutively active protein and stimulate cell division. RAS genes encode proteins that are members of the GTP binding protein family that bind guanine nucleotides with GTPase activity. They are thought to be involved in signal transduction, cell proliferation and differentiation in membrane bound cellular compartments (44). Currently, there are two hypotheses on the role of RAS mutations in carcinogenesis. First, adenomas that are initiated by a RAS mutation may be more inclined to progress quickly to larger more dysplastic lesions than those adenomas without RAS mutations (45). Alternatively, RAS mutations may occur during the progression from small adenoma to large adenoma with more potential for malignancy (45).

Recently, mouse models with null ras genes have been developed. It was found that H-ras and N-ras genes are not essential for normal growth and are therefore dispensable (46). However, mouse embryos which have two null k-ras genes die during the embryonal stage. This is likely due to a defect in myocardial cell proliferation and neuronal apoptosis (44). The intestinal development in heterozygotes for k-ras is also unaffected and these mice only have abnormal hematopoietic development (46). To further investigate the potential role of k-ras in CRC development, mouse models with Apc (Min mice) and k-ras mutations were studied and no synergistic effects were seen (47). But in in vitro studies, it was found that one defective Apc
allele in addition to activated ras, was sufficient to induce transformation of normal colonic epithelial cells (48).

1.1.1.3 DCC gene

The DCC (deleted in colorectal cancer) gene is a putative tumor suppressor gene located at q21 position of chromosome 18. Loss of this allele has been found to occur in almost 50% of advanced adenomas and 70% of carcinomas (15). Loss of heterozygosity of DCC occurs in 30% of colorectal tumors during the progression of small adenomas to larger, more dysplastic adenomas (49). The DCC gene encodes a protein that has significant homology to neural cell adhesion molecules which mediate cellular recognition and adhesion (16). It has also been suggested that DCC is part of a netrin-1 axon receptor (50). Inactivation of the DCC gene may influence tumorigenesis by altering signal recognition, cellular adhesion properties and increasing the potential for metastases (45). DCC is expressed in normal colonic mucosa but is absent or diminished in most colorectal adenomas and carcinomas. This may be due to a somatic mutation in the DCC gene (16). Contrary to evidence suggesting DCC as a tumor suppressor gene involved in colorectal cancer in humans, inactivation of DCC in mice did not affect cell growth or differentiation to tumorigenesis in the intestines (50). As well, when DCC was nullified in Min (Apc+/-) mice, there was no acceleration of tumor development (50). It was concluded then that DCC has a small role, if any, in the physiology of the mouse intestine (50). Thus, DCC may not be the candidate gene involved in CRC; apparent loss of DCC in colorectal cancer may be due to its proximity to other genes located on chromosome 18 involved in CRC.

1.1.1.4 DPC4 and MADR2 genes

Other possible target genes on 18q21 are MADR2 and DPC4, both genes in the transforming growth factor β (TGFβ) signaling pathway. This pathway controls cell growth, by
supplying a growth inhibiting differentiation signal in colonic and breast epithelial cells (51). 

DPC4 and MADR2 are both members of the SMAD gene family, and are also known as SMAD4 and SMAD2, respectively. The corresponding proteins are involved in signal transduction pathways activated by the TGFβ family receptors (52). Mutations in both DPC4 and MADR2 have been observed in colon carcinomas (53) and both are candidate tumor suppressor genes. Recently, a Dpc4 mutation was introduced into Apc716 mice. These mice developed intestinal polyps which were larger and progressed more quickly to invasive carcinoma compared to controls (54). These results suggest that Dpc4 plays an important role in the progression of colorectal tumors to malignancy. Mice with inactivating mutations at MadR2 were generated which, in the heterozygous state, did not develop colorectal cancer (55).

1.1.1.5 P53 gene

The P53 tumor suppressor gene mediates the cell’s response to stressful stimuli. The P53 protein is activated when the cell is exposed to DNA damage, heat shock, or metabolic changes and results in programmed cell death or cell growth arrest to induce DNA repair (56). Mutations in P53 are found in up to 50% of a number of human tumor types, including up to 70% of colorectal cancer (57). Many of these P53 mutations are C to T transitions in mutational hotspots located in exons 5 through 8 (45). It has been suggested that loss of normal P53 would allow the perpetuation of abnormal, damaged cell reproduction by the failure of P53 to stop the accumulation of mutated tumor suppressor genes and oncogenes (56). As well, P53 mutations have been associated with genomic instability, development of aneuploidy and tumor progression (58-60). In colorectal carcinogenesis, P53 mutations are usually detected at later stages of development and are thus thought to act as a progression factor from adenoma to carcinoma (58). It has been reported that approximately 70% of human colon carcinomas have P53 mutations, whereas adenomas have only 30% (61). As well, P53 mutations were detected
in only one of 13 multifocal colonic ACF, in comparison to 10 of 13 carcinomas (62), therefore supporting the hypothesis that \( P53 \) mutations are important genetic events occurring late in the carcinogenic process.

Heterozygous mouse models with one null \( p53 \) allele are susceptible to spontaneous tumor development; however, the onset of these tumors is delayed and these tumors are most frequently osteosarcomas and soft tissue sarcomas (63). Mice which have two null \( p53 \) alleles have been generated and develop normally but are prone to develop spontaneous tumors, particularly malignant lymphoma. (64). Surprisingly, when these null mouse models of \( p53 \) were developed and crossed with \( Apc^{+/-} \) (Min) mice, which are, by themselves genetically predisposed to developing multiple intestinal neoplasms, there was no effect on number of tumors developed or number of tumors that progressed from adenoma to adenocarcinoma (65). Thus, despite the strong involvement of these two genes independently in human CRC, there does not appear to be any cooperative effects of \( Apc \) and \( p53 \) in the intestines of these mice (65). Perhaps necessary intermediate steps in colorectal carcinogenic development, such as the mutation of oncogenes or mismatch repair genes, are lacking in these animals, thus no cooperative effects were seen (65).

1.1.1.6 DNA methylation

DNA methylation, in mammals, occurs at the cytosine residue in the \( CpG \) dinucleotide sequence (66). However, \( CpG \) dinucleotides only occur at 5-10% of their predicted frequency, based on nucleotide composition (66). In the vertebrate genome, between 60-90% of \( CpGs \) are methylated, which means that approximately 4% of cytosine residues are methylated post-synthetically to 5-methylcytosine (5mC) (67). The majority of 5mC can be found within \( CpG \) dinucleotides. \( CpG \) sites which are dispersed in nontranscribed regions of DNA in germline and somatic cells are often methylated. This is in contrast to those sites located in \( CpG \) rich islands,
which are 0.5 to 5 kb long, occur approximately every 100 kb and contain a C+G content of greater than 60% (66). These islands are typically associated with promoters and coding regions of genes and are usually not methylated (67).

DNA methylation plays an important role in both normal cell development and carcinogenesis. It can influence DNA integrity and function by being involved with numerous processes in mammalian cells that include mutation, gene expression, chromatin structure, timing of DNA replication and genomic imprinting (68).

Typically, immediately following DNA replication, the newly synthesized strand is not methylated, however soon after the replication fork passes, maintenance methyltransferase methylates the CpG site on the nascent strand opposite the methylated parent strand utilizing the universal methyl donor S-adenosylmethionine (SAM) (69). This process, referred to as maintenance or hemi-methylation, maintains the symmetrical methylation pattern of DNA. The maintenance methylase is specific for hemimethylated sites such that only sites methylated prior to replication will be methylated (69). The one methylase enzyme that has been identified in mammalian cells thus far is the DNA (cytosine-5) methyltransferase (MTase or Dnmt) (70). The ability of the MTase enzyme to maintain the methylation pattern of the replicating DNA is dependent upon the capacity and fidelity of the MTase itself, the amount of SAM and the level of cell proliferation (69). Mechanisms of regulation for MTase are unknown (71).

Demethylation of DNA can occur either by two rounds of replication without maintenance methylation or by replication independent enzymatic demethylation (72), by a recently discovered demethylase (73). This demethylase is thought to catalyse the removal of the methyl group at 5mC and release it in the form of methanol in DNA demethylation (73). In order to recover the methylated sites, de novo methylation must occur. However, de novo methylation activity, methylation of previously non-methylated CpG sites, has been seen with the MTase in
vitro only; thus far, maintenance methylation appears to be the main activity of human MTase in vivo (74,75). The possibility of a de novo MTase in mammalian cells has not been ruled out as DNA methylation was seen in ES cells derived from MTase knock out mice (74). As well, a de novo MTase is known to exist in Ascomycota, which is similar to eukaryotic MTase but lacks the regulatory domain (76). Mice which were generated null for the MTase gene did not live past mid-gestation, which would imply that specific or global methylation patterns are necessary for embryonic development (74).

Changes in methylation patterns have been seen in a number of different tumor and cancer types. Genomic hypomethylation has been observed early in cancerous tissues when compared to normal (14,77-80). One study observed a correlation between hypomethylation and tumor progression such that metastatic tumors had less genomic 5MeC than benign or normal tissue (78). In contrast, another study showed that there was no difference between methylation levels in adenoma and cancerous tissues (11,77), suggesting that DNA hypomethylation is an early event in colorectal carcinogenesis. Global hypomethylation is thought to result in genetic instability due to chromosomal alterations which can ultimately lead to cell transformation and tumor development (81). Dietary deficiency of methyl donors (ie. methionine, choline, folate and vitamin B₁₂) in isolation or in varying combinations, has been shown to potentiate chemical carcinogenesis in the liver and other tissues in rats (82). Methyl donor deficiency has been associated with the spontaneous development of liver cancer in the setting of fatty liver in rats (82). Labile methyl donor deficiency is the only dietary deficiency known to be carcinogenic on its own (83). Although the exact mechanism has not yet been elucidated, one of the postulated mechanisms includes the role DNA hypomethylation in certain proto-oncogenes associated with hepatocarcinogenesis (82).
Laird et al. conducted a study where the results oppose this postulated role of DNA hypomethylation in carcinogenesis. The DNA methyltransferase (also known as Dnmt) enzyme activity was reduced in Min mice using genetic manipulation, resulting in a Apc+/-Dnmt+/- genotype, and combined with treatment of 5-aza-cytidine (5-aza-C), a methyltransferase inhibitor. In mice treated with 5-aza-C with documented global DNA hypomethylation, there was a reduction in tumor number compared to controls (84). Thus, fewer tumors occurred with decreased activity of methyltransferase, the enzyme responsible for methylating cytosine residues. This study supports the hypothesis that methyltransferase is involved in the high mutation rate of C to T transitions seen in human CRC (84). As well, in vitro studies have shown increased MTase gene mRNA levels in cultured cancer cells compared to non-tumorigenic cells (85) and increased MTase activity in transformed murine and human cells compared to normal cells (86). In vivo, MTase gene expression and activity have been shown to increase with tumor development from normal mucosa to polyps to carcinoma (86,87). In combination, these studies indicate that MTase gene expression plays an important role in neoplastic development.

Site specific hypomethylation of oncogene promoter regions has been seen in several types of cancer including colorectal cancer (14,88-90). Genomic hypomethylation with concurrent areas of site specific hypermethylation has also been identified in various human cancers (80). Site specific hypermethylation of the promoter region is thought to exert its effects by inhibiting tumor suppressor gene expression giving transformed cells a growth advantage (66,68,91,92). Thus, both hypo and hypermethylation play crucial roles in carcinogenesis.

There are two premises behind the hypothesis explaining the methylation cancer association. The first hypothesis suggests that DNA methylation and its effects on gene
expression play a role in tumorigenesis, whereas the second hypothesis proposes that the high mutation rate associated with methylated cytosine residues is of importance.

**Gene expression**

It has been proposed that changes in DNA methylation promote tumorigenesis by altering the levels of expression in proto-oncogenes and tumor suppressor genes. Cytosine methylation can alter gene transcription by directly affecting the binding ability of positive and negative transcription factors or indirectly by forming inactive chromatin (93). Methylation determines higher order chromatin structure and acts as a binding signal to transcription factors to prevent or induce transcription (94,95). It has been shown that a hypomethylated gene is more likely to be expressed when compared to one that has been hypermethylated (96). That is, there is thought to be an inverse relationship between methylation and gene expression (Figure 1.2), where hypomethylation increases gene expression of oncogenes, such as Ha-RAS, MYC and FOS, and hypermethylation results in gene silencing of tumor suppressor genes (66,88,89,97). However, changes in DNA methylation at CpG sites within the body of the gene correlate poorly with levels of gene expression (81). Methylation and gene expression are best correlated when referring to methylation patterns at CpG sites within or near the promoter regions (81).

In the context of oncogenesis, DNA hypomethylation has been proposed to act as a promoter by increasing the expression of proto-oncogenes (ie. c-MYC, K-RAS) to promote cell proliferation (96,98-100). In support, genomic and site specific proto-oncogene hypomethylation have been found to occur with high prevalence in hepatic tumors and leukemias (96,98-100). As well, the c-MYC oncogene is hypomethylated in human colorectal cancer (90). However, not all studies have shown an inverse relationship between DNA methylation and gene expression. Hypomethylation in the coding region of the p53 tumor
Figure 1.2 DNA methylation and gene expression. Open white boxes indicate promoter regions, blue boxes represent CpG islands, open circles are unmethylated cytosines, closed circles represent methylated cytosine.
suppressor gene has been shown in rats with chemically induced colorectal cancer (101). It is important to note that methylation status at the promoter region may or may not be indicative of gene expression as there are two possible scenarios. Typically, CpG sites within the promoter region are not methylated, therefore they would not be targeted for hypomethylation and transcriptional modification in transformed cells. Those genes without CpG islands in the promoter region are more likely to be transcriptionally modified by hypomethylation at individual CpG sites located near the transcription binding motifs as they are usually methylated (81).

DNA hypermethylation has also been observed in cancer cells and is thought to exert its effects by occurring at CpG islands located in the promoter region, inactivating tumor suppressor genes (ie. APC) to confer a growth advantage to transformed cells (68). Several tumor suppressor and imprinted genes in human tumors have been associated with promoter region CpG island hypermethylation, including Rb, p16 and H19/IGF2 (as reviewed by (81). Other genes, such as E-cadherin and estrogen receptor have also shown to be inactivated by hypermethylation of the promoter region (as reviewed by (81)). Specifically in CRC, promoter region hypermethylation has been found in the APC tumor suppressor gene (102) and Mlh1, a mismatch repair gene (92). However, no hypermethylation was found in MSH2 in sporadic primary colorectal cancers (103). The APC gene from tumor samples of patients with sporadic colorectal carcinoma were examined to study the role of methylation in colorectal carcinogenesis. It was revealed that the promoter region of the APC tumor suppressor gene was heavily methylated in patients with CRC compared to normal colonic mucosa and premalignant adenomas (102), suggesting a role for methylated cytosines in APC mediated CRC development. Hypermethylation of the hMLH1 promoter region has been correlated with gene silencing in both sporadic colon tumors and colon cancer cell lines, inactivating mismatch repair, resulting in
a mutator phenotype (92). It has been suggested that a consequence of the inactivation of DNA mismatch repair in these cell lines by hypermethylation is microsatellite instability (MI+ or MSI), often seen in sporadic CRC (103,104). In support, hypermethylation of MYF-3, a gene involved in muscle cell differentiation, was shown to occur prior to malignancy in colorectal cancers, increase during tumor progression and be most pronounced in tumors that demonstrate microsatellite instability (MI+) compared to those that do not (MI-) (105). The association of increased hypermethylation with tumor severity and MI+ suggest that the methylation imbalance may be responsible for the MI+ phenotype, however, further study is required to determine this relationship.

Although these studies indicate an association between methylation status and gene expression in carcinogenesis, the conflicting reports indicate more study is required to determine the exact relationship. Another possibility to consider is that methylation may be an indicator of transcription activity and not a regulatory mechanism for transcriptional control (106).

**Mutagenesis**

The mutation mediated model suggests that the hypermutability of 5-mC is associated with oncogenesis (84). The mutation rate of 5-mC is significantly higher than that of unmethylated cytosine residues (84). However, most CpG sites are mutational hotspots for cancer, including CRC (16,45,57,107); the majority of these mutations are C to T transitions. In colorectal cancer, 47% of point mutations found in P53 have been C to T transitions in the CpG dinucleotide which indicates an overrepresentation of mutations at CpG dinucleotides of approximately 7-fold (107).

There are four mechanisms by which 5mC or unmethylated cytosine can be mutated. First, there is spontaneous deamination of both unmethylated cytosine and 5-mC in CpG dinucleotides which occurs *in vitro* and *in vivo*, resulting in a uracil and a thymine respectively
The thymine can cause inefficient repair as the DNA repair system has difficulty discerning the mutated base in the (T:G) base-pair (109). The resulting uracil from a deamination of cytosine is an abnormal component in DNA and would therefore be easily recognized and repaired; the thymine from 5-mC however, is more likely to remain unnoticed (109). Second, there is enzymatic deamination of 5mC to thymine by MTase (110). Third, unmethylated cytosine is also vulnerable to deamination by MTase, which would result in a uracil residue (36,111). Subsequent MTase methylation and binding of the uracil residue would then yield a thymine residue and blockage of DNA repair, perpetuating the existence of the C to T transition (36,111). This mechanism is most likely to occur if there are low levels of S-adenosylmethionine (SAM), as it acts as the primary methyl donor for DNA methylation. With deficient SAM, DNA MTase catalyzed deamination would generate uracil residues from the unmethylated cytosine, and increase the affinity of MTase for uracil, blocking DNA repair (111). This situation, if coupled with inhibited DNA MMR, would secure the promutagenic lesion (111). Lastly, it has been suggested that 5-methylcytosine-DNA glycosylase may be involved in mutation at CpG sites. 5mC-DNA glycosylase removes 5mC residues, leaving a promutagenic apyriniminic site which may result in transversion (72).

Also, one study mentioned previously, developed an animal model which was heterozygous for the DNA MTase gene, with reduced MTase activity, and crossed it with a Min (Apc+/-) mouse. These animals were also treated with 5-azacytidine (5-aza-C), a DNA MTase inhibitor to severely decrease methylation activity (84). This group found a reduction in the number of intestinal adenomas in the treated mice compared to the control, which contradicts the role of DNA hypomethylation in carcinogenesis but supports the role of DNA MTase in the generation of C to T transitions. With decreased MTase activity in these mice, two of the
postulated mechanisms of C to T transitions would be inhibited, thus reduced cytosine
deamination by MTase may decrease the mutagenic potential of developing cells (84).

Genomic instability

Alterations in DNA methylation can also result in genomic instability by
changing the chromatin structure, resulting in deletions, inversions and chromosome loss (112). Two recent in vitro studies have demonstrated this relationship. One study conducted in Dnmt1-deficient cells, cells nullizygous for the MTase gene, demonstrated a mutator phenotype where elevated gene deletion mutation rates were a result of mitotic recombination or chromosomal loss (113). This study suggests that DNA methylation contributes to genomic stability by suppressing recombination and supporting precise chromosomal segregation during mitosis (113). More recently, DNA hypermethylation of the MLH1 promoter has also been observed to cause genomic instability in the form of microsatellite instability by inactivating the MMR gene (104).

Thus, genetic instability due to methylation abnormalities may lead to tumor
development through deviant mitosis or gain/loss of whole chromosomes.

Genomic Imprinting

Methylation is also thought to play a role in genomic imprinting which causes the
expression of a gene to vary according to its paternal or maternal origin. Imprinting is the
marking of specific genes for differential utilization by passage through maternal or paternal
germ line (106). Animal studies have shown that alleles can also have different DNA
modification patterns by being differentially methylated, therefore potentially having different
mutation rates (114). Whether this genomic imprinting process occurs in humans is still
unknown, however, it has striking similarities to the effects of DNA methylation. Both DNA
methylation and genomic imprinting are reversible, they alter transcription, they modify affected
sites (promoter sites or imprinting boxes) and they are both heritable in a chromosome specific manner (115). One possible example of genomic imprinting is Wilms' tumors which are characteristically homozygous on chromosome 11. In seven cases investigated, all of the lost alleles were maternal and in five of seven, there was a unilateral tumor where the new somatic mutation occurred on the paternal chromosome. Thus, it is possible that genomic imprinting is involved in the development of Wilms' tumors. Genomic imprinting then is another proposed mechanism by which methylation can alter gene expression. Thus it has been suggested that aberrant DNA hypomethylation is the mechanism by which genomic imprinting is lost in human cancer (114, 115).

There is still much controversy over the relationship between DNA methylation and carcinogenesis, more studies need to be conducted before we can determine whether these changes in methylation are a part of the malignancy process or merely an indication of altered metabolism or physiology.

1.1.1.7 MMR genes

Mismatch repair genes play a crucial role in correcting replicative mismatches which have eluded DNA polymerase proofreading, mismatches due to the spontaneous deamination of 5-methylcytosine and mispaired nucleotides that have occurred during genetic recombination (116). They have been associated with a number of human cancer syndromes as mutations in these genes can result in an increased spontaneous mutation rate or a mutator phenotype (117).

Mismatch repair studies began in E. Coli, identifying three genes, mutS, mutL and mutH, where MutS recognizes the mismatch, MutH is an endonuclease that makes a single strand break to direct the repair and MutL complexes with these two proteins to activate mismatch recognition (116). This MutHLS system repairs single base mismatches and small insertion/deletions in DNA (116) and a similar mismatch repair system has been found in
eukaryotes. Six genes (*MSH*) homologous to *mutS* and four genes (*MLH*) homologous to *mutL* have been found in yeast with similar genes in mammalian systems (118).

Mismatch repair genes discovered in humans thus far include *MSH2, MLH1, PMS1, PMS2, MSH6 (GTBP), MSH3* and *MSH5* (118). The prevalence of MMR deficiencies in sporadic colon adenomas, cancers and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) are 13%, 13-17% and greater than 90%, respectively (119). HNPCC is the most common familial cancer predisposition syndrome, which is characterized by the development of proximal colon tumors at an early age (120). Germline mutations in *MSH2* and *MLH1* constitute the majority of mutations in HNPCC kindreds, while mutations in *PMS1* and *PMS2* account for only 10% (121). In humans, mutations in *MSH2* specifically have been shown to occur in 45% of patients with HNPCC and 60% in those with sporadic colon cancer (119). Cells with a mutated *Msh2* gene result in Msh2 deficient cells that are tolerant to methylating agents, have lost mismatch binding and acquired microsatellite instability (mutator phenotype) (122).

Normally, proteins encoded by these mismatch repair genes complex to repair post mitotic mutations or replication errors (RER) which are small mutations that occur during mitosis (usually deletions of 1-5 base pairs). If these errors are allowed to persist, as expected if the cells are MMR deficient, the mutations will accumulate. The resulting MMR deficient tumors will show characteristic mutations throughout the genome called microsatellite instability (MI), also known as the RER phenotype (RER+), which indicates a failure to repair slipped replication intermediates (119). Microsatellite loci are 1 to 5 base pair tandem repeats found to number in the hundreds of thousands in the genome, often in intronic regions of genes. These loci are very polymorphic in that the pattern identifies the nature of those genes in the parental origin.
Microsatellite instability is characterized by the addition or deletion of bases within simple repeat sequences (123). In heterozygotes, these germline MMR mutations are relatively harmless as the remaining wild type allele is able to maintain adequate DNA repair (33). However, when the wild type allele is inactivated by a somatic mutation or by DNA hypermethylation at the promoter site there is severe genomic instability (33). This is of particular importance if these abnormalities occur within a stem cell where they may be accumulated through cell division, potentially resulting in carcinogenesis (33). Some of the MMR gene mutations found include in-frame deletions, frame shifts and nonsense permutations which can result in truncated gene products (58). The subsequent repair deficiencies could lead to more mutations through defects in growth control by disruption of control mechanisms, tumor suppressor genes and oncogenes, resulting in malignant cell development.

To illustrate the importance of these MMR genes, mouse models were developed that are homozygous for null alleles. Mice deficient in PMS2 have microsatellite instability and a predisposition to sarcoma and lymphoma development (117). MLH1 deficient mice also possess microsatellite instability and abnormal recombination during meiosis (124). Mice with a null MSH6 gene possess cells with defective single nucleotide mismatch repair, developed gastrointestinal tumors and lymphomas but did not develop microsatellite instability. Thus, MSH6 may be associated with a human cancer syndrome not characterized by microsatellite instability (118). In yeast and mammalian cells, it has been suggested that MSH3 may share partial function with MSH6 as they can both complex with MSH2 in mismatch recognition (125). In mice that are Msh2 deficient, a large proportion develop lymphoma and die at an early age. Microsatellite instability was found in lymphomas from all Msh2-/- mice analysed. The development of lymphoid tumors in this mouse model is how cancer predisposition is postulated to manifest itself (31).
The importance of functional mismatch repair genes in the prevention of cancer is becoming more apparent and with these mouse models, further studies can be conducted to investigate the progression of tumors and screen potential carcinogenic and chemopreventive agents.

1.2. FOLATE AND COLORECTAL CANCER

Folate is a water soluble B vitamin whose function involves the transfer of one-carbon groups important in biological methylation and the de novo synthesis of purines and thymidylate (Figure 1.3)(126). Folate is therefore important in DNA synthesis, cell proliferation, and repair mechanisms, as well as DNA methylation. Since folate is a required vitamin in many critical biological processes, it would seem as though a deficiency in folate would impair the replication and growth of cells within the body. The depletion of folate from rapidly dividing cancer cells using folate antagonists, such as Methotrexate and 5-fluorouracil (127,128), has been a method of chemotherapy for certain cancer patients, with much success. As well, depending upon the time of exposure or time at which deficiency is achieved, folate may have varying effects on the tumor development. When a cancerous tumor is transplanted into folate deficient mice, there is an inhibition of growth (129). Virally induced cancers have been shown to be reduced when deprived of folate (130). Transgenic mice predisposed to developing nerve sheath tumors experience delayed tumor development when dietary folate is restricted (131). The retarded tumor growth effect from folate deficiency has been seen in established tumors, however, folate deficiency in non-neoplastic tissues may have an opposite effect.

Many epidemiological studies have been conducted showing an average of 40% reduction in the risk of developing colorectal adenoma or cancer in groups taking the highest amount of folate compared to those taking the lowest (132-140). Interest in folate and CRC
Figure 1.3 Simplified folate metabolism including folate’s role in DNA synthesis and DNA methylation. THF - tetrahydrofolate; MTHFR - methylenetetrahydrofolate; B₁₂ - vitamin B₁₂; SAM - S-adenosylmethionine; SAH - S-adenosylhomocysteine (from Kim, Y.I., J Nutr Biochem, 1999).
began when Lashner et al. first observed an association between diminished folate status and increased colorectal dysplasia or cancer risk in patients with chronic ulcerative colitis (141). Those afflicted with chronic ulcerative colitis have a 10-40X increased risk of CRC and an increased risk of being folate deficient (142,143). These studies demonstrated the importance of investigating the role of folate in the chemoprevention of CRC. More recently, Lashner et al., found that subjects with ulcerative colitis and neoplasia had lower levels of RBC folate compared with controls (144). As well, his group also found an inverse dose response relationship between folate intake and risk of colorectal neoplasia in patients with ulcerative colitis (145).

*Dietary folate and adenoma risk.* There have been six studies that have investigated the relationship between dietary folate and adenoma risk (Table 1.1). Four case control studies indicated on average, a 35% decrease in risk of colorectal adenoma development when comparing individuals with the highest dietary folate (500 ng/d) compared to those with the lowest (100 ng/d) (132-135). In three of these studies, there was an inverse dose dependent relationship between dietary folate and colorectal adenoma risk (132-134). Two large prospective studies conducted by Giovannucci et al. provide the best epidemiological evidence to support the relationship between folate deficiency and colorectal adenoma development. These studies are the Nurses Health Study and the Health Professionals’ Study, which are composed of 121,700 US female nurses between 30-55 years of age and 41,529 US male health professionals aged 40-75 years, respectively. To determine nutrient intake, semiquantitative food frequency questionnaires were used and any polyps found were histopathologically confirmed. These studies found that increased dietary folate is associated with a lower incidence of colorectal adenomatous polyps in a dose dependant manner (146,147). After confounders for adenoma development were adjusted for, the relative risk for developing colorectal adenomas
Table 1.1 Dietary folate intake and colorectal adenoma

<table>
<thead>
<tr>
<th>Study</th>
<th>Location (year)</th>
<th>Study design</th>
<th>No. cases/controls</th>
<th>Odds ratio*</th>
<th>95% CI</th>
<th>P-trend for inverse association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benito et al (132)</td>
<td>Majorca, Spain (1993)</td>
<td>Case-control</td>
<td>101/242</td>
<td>0.27</td>
<td>NA</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bird et al (135)</td>
<td>USA (1995)</td>
<td>Case-control</td>
<td>M: 180/189 F: 152/161</td>
<td>0.70 1.47</td>
<td>0.36-1.34 0.73-2.95</td>
<td>NS NS</td>
</tr>
<tr>
<td>Tseng et al (133)</td>
<td>USA (1996)</td>
<td>Case control</td>
<td>M: 105/165 F: 131/245</td>
<td>0.84 0.39</td>
<td>0.29-2.43 0.15-1.03</td>
<td>NS 0.08</td>
</tr>
<tr>
<td>Boutron-Ruault et al (134)</td>
<td>France (1996)</td>
<td>Case-control</td>
<td>Small adenoma (&lt;1 cm) 154/426</td>
<td>0.50 0.80 F:0.40</td>
<td>0.30-1.00 M: 0.30-2.30 0.20-1.00</td>
<td>0.03 NS 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Large adenoma (≥1 cm) 208/426</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giovannucci et al (146)</td>
<td>USA (1993)</td>
<td>Prospective</td>
<td>M: 331/9159 F: 564/15420</td>
<td>0.63 0.66</td>
<td>0.41-0.98 0.46-0.95</td>
<td>0.03 0.04</td>
</tr>
<tr>
<td>Baron et al (256)</td>
<td>USA (1998)</td>
<td>Prospective</td>
<td>449/260</td>
<td>0.65</td>
<td>0.41-1.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*odds ratio for the highest dietary folate intake compared to the lowest intake. Adjusted for confounding factors.
CI-confidence interval. NA-not available. NS-not significant. M-male. F-female.
was 0.63 (95% C.I. 0.41-0.98) for males and 0.66 (95% C.I. 0.46-0.95) for females when comparing the highest quintile of folate intake (847 ng/d for males, 711 ng/d for females) to the lowest (241 ng/d for males, 166 ng/d for females) (146). The data was adjusted for age, family history, indications for endoscopy, history of previous endoscopy, total energy intake, saturated fat intake, dietary fiber and body mass index. When the moderate to high consumption of alcohol was compounded with low folate, the risk of colorectal adenoma development increased significantly (147). Ethanol consumption depletes the availability of 5-methylTHF, the form of folate required for methionine synthesis (146). It is important to note that the subjects who were most at risk were still at or above the recommended daily intake of folate.

*Dietary folate and CRC.* There have been ten studies that have examined the effects of folate and CRC risk (Table 1.2). Six of these ten studies showed a 35% reduction in CRC risk, on average, when comparing the highest folate intake group (>600 ug/d) to the lowest (<180 ug/d) (133,136-140), five of which showed a significant inverse dose dependent reduction in CRC risk (133,136,138-140). Of two negative studies, one found no significant decrease in risk with increased dietary folate (134) and one showed that only when the low folate was combined with high alcohol intake was the risk of developing CRC increased fourfold (148). These findings are supported by the prospective Health Professionals Study done by Giovanucci et al, which also showed an non-significant reduction in CRC risk (RR 0.86, 95% C.I. 0.54-1.36) when comparing high intake (>646 ug/d) to low intake (<269 ug/d) folate, but when high folate-low-alcohol-high methionine is compared to low folate-high-alcohol-low-methionine, the reduction in risk of developing CRC was 70% (147). The best evidence to date to support the inverse relationship of folate intake to CRC risk comes from the Nurses’ Health Study by Giovannucci et al. They showed that dietary and supplementary folate together have an inverse dose-dependent relationship to colon cancer (149). This study measured the baseline CRC risk of the
Table 1.2 Dietary folate intake and colorectal cancer

<table>
<thead>
<tr>
<th>Study</th>
<th>Location (year)</th>
<th>Study design</th>
<th>No. cases/controls</th>
<th>Odds ratio*</th>
<th>95% CI</th>
<th>P-trend for inverse association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benito et al (136)</td>
<td>Majorca, Spain</td>
<td>Case-control</td>
<td>286/295 (population)</td>
<td>0.53</td>
<td>NA</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>(1991)</td>
<td></td>
<td>286/203 (hospital)</td>
<td>0.56</td>
<td>NA</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Freudenheim et al (254)</td>
<td>USA (1991)</td>
<td>Case-control</td>
<td>Colon cancer</td>
<td>1.03</td>
<td>0.56-1.89</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M: 205/205</td>
<td>0.69</td>
<td>0.36-1.30</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F: 223/223</td>
<td>0.31</td>
<td>0.16-0.59</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rectal cancer</td>
<td>0.50</td>
<td>0.24-1.03</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M: 293/273</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F: 151/146</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meyer and White (139)</td>
<td>USA (1993)</td>
<td>Case-control</td>
<td>M: 238/224</td>
<td>1.24</td>
<td>NA</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F: 186/190</td>
<td>0.54</td>
<td>NA</td>
<td>OR=0.81 (0.66-1.00)</td>
</tr>
<tr>
<td>Ferraroni et al (137)</td>
<td>Italy (1994)</td>
<td>Case-control</td>
<td>1,326/2,024</td>
<td>0.52</td>
<td>0.40-0.68</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glynn et al (148)</td>
<td>Finland (1996)</td>
<td>Nested Case control</td>
<td>136/249</td>
<td>0.51</td>
<td>0.20-1.31</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M: 215/233</td>
<td>0.21†</td>
<td>0.06-0.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F: 193/194</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boutron-Ruault et al (134)</td>
<td>France (1996)</td>
<td>Case-control</td>
<td>171/309</td>
<td>1.00</td>
<td>0.50-2.0</td>
<td>NS</td>
</tr>
<tr>
<td>White et al (140)</td>
<td>USA (1997)</td>
<td>Case-control</td>
<td>441/427</td>
<td>0.51</td>
<td>0.34-0.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M: 251/233</td>
<td>0.59</td>
<td>0.34-1.01</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F: 193/194</td>
<td>0.44</td>
<td>0.24-0.80</td>
<td>0.007</td>
</tr>
<tr>
<td>LaVecchia et al (255)</td>
<td>Italy (1997)</td>
<td>Case-control</td>
<td>1,953/4,154</td>
<td>0.83</td>
<td>0.60-1.10</td>
<td>0.06</td>
</tr>
<tr>
<td>Giovannucci et al (147)</td>
<td>USA (1995)</td>
<td>Prospective</td>
<td>M: 251/47,680</td>
<td>0.86</td>
<td>0.54-1.36</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F: 442/88,756</td>
<td>0.30‡</td>
<td>0.14-0.63</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Giovannucci et al (149)</td>
<td>USA (1998)</td>
<td>Prospective</td>
<td>F: 442/88,756</td>
<td>0.53§</td>
<td>0.35-0.80</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*odds ratio for the highest dietary folate intake compared with the lowest intake. Adjusted for confounding factors.
†High folate-low alcohol-high protein intake compared with low folate-high alcohol-low protein intake.
‡High folate-low alcohol-high methionine intake compared with low folate-high alcohol-low methionine intake.
*RR (relative risk) for those taking multivitamins for ≥15 years
CI-confidence interval. NA-not available. NS-not significant. M-male. F-female.
participants and compared it to the present risk in relation to folate intake, while controlling for confounders such as age, family history of CRC, aspirin use and alcohol intake. Dietary folate alone was related to a modest reduction in risk of colon cancer, however, colon cancer risk was reduced significantly by about 70% when multivitamins containing ≥400 μg folic acid were used for 15 years (149). Multivitamin use for 4 years showed no decrease in risk, whereas multivitamin use for 5-9 or 10-14 years showed non-significant modest reductions in CRC risk (149). This sex specific result is supported by a case control study which found that women taking supplements with folate had a decreased risk of developing colon cancer (140).

**Blood and mucosal folate and cancer.** Studies have also been conducted investigating the relationship between blood and colonic mucosal folate to CRC risk, however these relationships are still not well defined (Table 1.3). The studies conducted thus far have produced conflicting results; four prospective studies indicated no difference in serum folate concentrations between subjects with and without cancer or adenomas (148,150-152). In contrast, a recent study done by Kato et al, using the New York University Women's Health Study cohort, found that the risk of colorectal cancer in subjects in the highest quartile of serum folate (≥31.04 nmol/l) was half that of those in the lowest quartile (≤12.23 nmol/l) (153). When RBC folate is used as a measure of folate concentration, only one of the three studies found it to be significantly decreased in patients with colorectal adenoma compared to those without (150). In support of this, one population based case control study also showed an inverse dose response relationship with RBC folate and colorectal adenoma risk in men only (135). RBC folate is a better indicator of folate status than serum folate as serum folate levels tend to fluctuate more readily than RBC folate. Therefore, if tissues are depleted in folate, there will be a decrease in serum folate, however, low serum folate may not necessarily reflect the folate status of tissues (154). One explanation for the conflicting results obtained with regards to the relationship between blood
Table 1.3  Blood and colonic mucosal concentrations of folate and colorectal neoplasia.

<table>
<thead>
<tr>
<th>Study</th>
<th>Location (year)</th>
<th>No. cases/control</th>
<th>Case diagnosis</th>
<th>Case diagnosis</th>
<th>Folate level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Case</td>
</tr>
<tr>
<td>Paspatis et al (150)</td>
<td>Greece (1995)</td>
<td>62/50</td>
<td>Adenoma</td>
<td>Serum (ng/mL)</td>
<td>4.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RBC (ng/mL)</td>
<td>536.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasma (ng/mL)</td>
<td>M: 9.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F: 8.8</td>
</tr>
<tr>
<td>Bird et al (135)</td>
<td>USA (1995)</td>
<td>332/350</td>
<td>Adenoma</td>
<td>Plasma (ng/mL)</td>
<td>M: 9.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F: 8.8</td>
</tr>
<tr>
<td>Glynn et al (148)</td>
<td>Finland (1996)</td>
<td>144/276 smokers</td>
<td>Cancer</td>
<td>Serum (ng/mL)</td>
<td>4.0</td>
</tr>
<tr>
<td>Kim et al (151)</td>
<td>USA (1998)</td>
<td>20/10</td>
<td>Adenoma*</td>
<td>Serum (ng/mL)</td>
<td>22.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RBC (ng/mL)</td>
<td>307.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasma homocysteine (nmol/mL)</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Colon (ng/mg tissue)</td>
<td>343.9</td>
</tr>
<tr>
<td>Meenan et al (152)</td>
<td>Ireland (1997)</td>
<td>12 cancer 7 adenoma 8 control</td>
<td>Cancer or adenoma</td>
<td>Serum (ng/mL)</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RBC (ng/mL)</td>
<td>361</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Colonocytes (pg/μg DNA)</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenoma Cancer</td>
<td>15.1</td>
</tr>
<tr>
<td>Kato et al (153)</td>
<td>USA (1999)</td>
<td>105/523</td>
<td>Cancer</td>
<td>Serum (nmol/L)</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serum homocysteine (μmol/L)</td>
<td>10.35</td>
</tr>
</tbody>
</table>

*Hyperplastic polyps were used as controls
NS-not significant. RBC-red blood cell.
Folate and CRC risk is that it has been indicated previously that a small reduction in folate status, even maintaining levels at or above 'normal' is often sufficient to increase the risk of developing CRC. Thus, these reductions in folate may be so small that they may not be easily detected using blood tests (151). Alternative measures of folate status include serum or plasma homocysteine and mucosal folate concentrations. Homocysteine is an amino acid synthesized during the metabolism of methionine and has often thought to be a more sensitive indicator of cellular folate status than blood levels (155). Serum homocysteine has an inverse relationship to folate status and, accordingly, has been found to be greater in patients with adenomas than in controls (151). As well, one study found that subjects in the highest quartile of serum homocysteine had a 70% increase in colorectal cancer risk compared to those in the lowest quartile (153). Mucosal folate measurements have also been used as an indicator of CRC risk. One study has shown a lower concentration of folate in the normal rectal mucosa of patients with colorectal adenomas when compared to those with non-neoplastic polyps (151), in spite of similar serum and RBC folate concentrations between these two groups. Also, a study done by Meenan et al., found that both serum and RBC folate were poorly correlated with folate concentrations measured in colonic epithelial cells (156). However, this latter study did not show lower mucosal folate concentrations in cancerous compared to normal tissue (156).

These studies suggest serum folate is not an accurate indicator of systemic folate status and assessment of folate status is best determined by measuring either serum homocysteine or mucosal folate concentrations.

Folate intervention studies. In order to investigate the chemopreventive potential of folate, human intervention studies must be conducted. Of the studies conducted so far, many of them have been small randomized placebo-controlled double blind protocols with only 20-60 subjects (Table 1.4). Three studies have shown no significant overall effect of folate on DNA
Table 1.4 Randomized, double-blind, placebo-controlled intervention trial of folate and colorectal neoplasia

<table>
<thead>
<tr>
<th>Study</th>
<th>Location (year)</th>
<th>No. subject</th>
<th>Case diagnosis</th>
<th>Folate dose (mg/d)</th>
<th>Duration</th>
<th>Primary end point</th>
<th>End point modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cravo et al (160)</td>
<td>Portugal (1994)</td>
<td>22</td>
<td>Cancer Adenoma</td>
<td>10</td>
<td>6 months</td>
<td>DNA methylation</td>
<td>Significant increase in DNA methylation</td>
</tr>
<tr>
<td>Paspatis et al (150)</td>
<td>Greece (1995)</td>
<td>60</td>
<td>Adenoma</td>
<td>1</td>
<td>2 years</td>
<td>Adenoma recurrence</td>
<td>40% reduction at 1 year (P=NS) 46% reduction at 2 years (P=NS)</td>
</tr>
<tr>
<td>Cravo et al (160)</td>
<td>Portugal (1994)</td>
<td>20</td>
<td>Adenoma</td>
<td>5</td>
<td>3 months</td>
<td>DNA methylation</td>
<td>Overall: no change Subgroup analysis: significant increase in DNA methylation only in those with single polyp</td>
</tr>
<tr>
<td>Kim et al (161)</td>
<td>USA (1998)</td>
<td>20</td>
<td>Adenoma</td>
<td>5</td>
<td>1 year</td>
<td>-DNA methylation -p53 exons 5-8 strand breaks</td>
<td>-Significant increase in both groups compared with baseline -Significant decrease in both groups compared with baseline</td>
</tr>
<tr>
<td>Fenech et al (158)</td>
<td>Australia (1998)</td>
<td>63</td>
<td>Healthy volunteers</td>
<td>700 μg folate + 7μg B_{12} in cereal then 2 mg folate + 20 μg B_{12} in tablet</td>
<td>3 months</td>
<td>-DNA methylation -Micronucleated cell frequency</td>
<td>No significant change</td>
</tr>
<tr>
<td>Cravo et al (159)</td>
<td>Portugal (1995)</td>
<td>25</td>
<td>Chronic ulcerative colitis</td>
<td>5</td>
<td>6 months</td>
<td>DNA methylation</td>
<td>No significant change</td>
</tr>
<tr>
<td>Biasco et al (162)</td>
<td>Italy (1997)</td>
<td>24</td>
<td>Chronic ulcerative colitis</td>
<td>15+*</td>
<td>3 months</td>
<td>Rectal cell proliferation</td>
<td>Significant reduction in cell proliferation in the upper 40% of crypts</td>
</tr>
</tbody>
</table>

*Folinic acid was used.
NS-not significant. B12-vitamin B12
methylation, a CRC biomarker (157-159). The patient population in these studies included those with previous adenoma (157), healthy volunteers (158) and chronic ulcerative colitis patients (159). However, two studies using the same biomarker, found a significant increase in methylation with folate treatment in patients previously diagnosed with adenoma (160,161). Adenoma recurrence (150), occurrence of p53 strand breaks (161) and rectal cell proliferation (162) are other CRC biomarkers studied in chronic ulcerative colitis patients which were affected by folate. There was a non-significant 40% decrease in adenoma recurrence and a significant decrease in strand breaks in patients treated with folate, respectively (150,161). As well, in patients given folate there was a significant decrease in crypt cell proliferation (162).

Several large randomized, double-blind, placebo controlled, multi-center folate chemoprevention trials are currently being performed in the U.S., which should help clarify the relationship between dietary folate and colorectal cancer and adenoma risk.

Animal studies. In support of the epidemiological studies done thus far, several animal studies were conducted in 1,2 dimethylhydralazine dihydrochloride (DMH) treated rats (Table 1.5). DMH is a procarcinogen whose metabolites alkylate DNA to promote tumor development. Two studies found that there was a significant decrease in colorectal tumors when comparing rats on folate repleted and folate depleted diets. The Cravo et al. study found that all of the mice on the folate deplete diet (0 mg folate/kg diet) developed microscopic tumors 20 weeks after the DMH injections were initiated, whereas only 29% in the replete diet (8 mg folate/kg diet) did, which suggests an early effect of folate on carcinogenesis (133). There was also a non-significant decrease in macroscopic tumor incidence in rats fed the folate supplemented diet compared to those on the folate deficient diet, from 86% to 43% (p=0.09)(133). In addition, no cancers were observed in either of the saline injected controls, which supports the suggestion that folate deficiency only potentiates the risk factor and alone cannot initiate carcinogenesis (163). In a
<table>
<thead>
<tr>
<th>Study</th>
<th>Model</th>
<th>Folate dose (mg/kg diet)</th>
<th>Duration</th>
<th>End point</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cravo et al (163)</td>
<td>Sprague-Dawley rats DMH</td>
<td>0 mg</td>
<td>20 weeks</td>
<td>Tumor incidence</td>
<td>0 mg/kg vs. 8 mg/kg 100% 29% (P=0.005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 mg</td>
<td></td>
<td>Microscopic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Macroscopic</td>
<td></td>
</tr>
<tr>
<td>Kim et al (164)</td>
<td>Sprague-Dawley rats DMH</td>
<td>0 mg</td>
<td>15 weeks</td>
<td>Tumor incidence</td>
<td>no difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mg</td>
<td></td>
<td>Microscopic</td>
<td>0 mg: 70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 mg</td>
<td></td>
<td>Macroscopic</td>
<td>2 mg: 40%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 mg</td>
<td></td>
<td>Microscopic</td>
<td>8 mg: 10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40 mg: 50% (P&lt;0.03)</td>
</tr>
<tr>
<td>Shivapurkar et al (167)</td>
<td>Fischer 344 rats AOM</td>
<td>3 mg + high fat + low fiber vs. high fat + low fiber</td>
<td>10, 14, 18 weeks</td>
<td>Aberrant crypt foci</td>
<td>No effect</td>
</tr>
<tr>
<td>Reddy et al (168)</td>
<td>Fischer 344 rats AOM</td>
<td>2,000 mg vs. control</td>
<td>50 weeks</td>
<td>Tumor incidence</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tumor size</td>
<td>Increased with folate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tumor multiplicity</td>
<td>Increased with folate</td>
</tr>
<tr>
<td>Wargovich et al (169)</td>
<td>Fischer 344 rats AOM</td>
<td>2,500 mg vs. 2 mg</td>
<td>2 weeks</td>
<td>Aberrant crypt foci</td>
<td>Increased effect compared to 2 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5,000 mg vs. 2 mg</td>
<td></td>
<td></td>
<td>No effect compared with 2 mg</td>
</tr>
</tbody>
</table>

DMH-dimethyldrazine, AOM-azoxymethane.
study done by Kim et al, results showed an inverse dose dependant response between dietary folate (from 0 up to 8 mg folate/kg diet) and macroscopic tumor incidence, suggesting a late phase folate effect of tumorigenesis. There was no difference in tumor incidence of microscopic tumors in this study (164). This is because the DMH dose used to induce microscopic tumor foci in the colorectum was 20x normal as this study was designed to see the effect of folate on tumor progression from micro to macroscopic tumors. At a supraphysiologial dose of folate, (20x the basal requirement, 40 mg/kg) there was a non-significant trend towards an increase in the number of tumors when comparing the controls (2 mg folate/kg diet) to the 20x repleted group. Therefore, it appears that folate has dual effects: one protective effect, if supplemented in modest amounts and one promoting effect if given at high levels of supplementation in a strongly procarcinogenic environment (164). This "acceleration phenomenon" has been seen previously where extremely high doses of folate in the context of a procarcinogenic environment have resulted in increased growth of microscopic neoplasms (129-131,165,166).

There have also been several studies in rats using azoxymethane (AOM), a metabolite of DMH, which report no protective effect of folate supplementation on carcinogenesis when compared to controls (167-169). One study did find that folate supplementation increased tumor size and multiplicity, however, the dose given was 2,000 mg/kg diet, 1000X higher than the usual supplemented dose (168). Of the three studies, one was not properly adjusted for dietary confounders of high fat, low fiber (167), while two others used exceptionally high doses of folate (168,169).

Although there is not enough data to make conclusive associations, dietary folate and its subsequent functions and metabolites have been linked with other forms of cancer, including lung, cervix, breast, pancreas and prostate. In lung cancer, folate has been shown to reverse
bronchial metaplasia, a precursor to bronchial squamous cell carcinoma, if used in conjunction with vitamin B12 (170). Studies investigating the relationship between dietary folate and cervical neoplasia have found that the risk of developing cervical dysplasia is decreased with increased dietary folate intake (171), however, there is little or no effect of folate on the more advanced cervical cancers (172). As well, once confounding factors for risk are corrected for, the effect on cervical dysplasia is no longer seen (172). In the breast, a recent large, prospective study found that total folate intake was not associated with overall risk for breast cancer, however, adequate folate intake may reduce increased risk due to alcohol consumption (173). In the pancreas, serum folate was shown to have a significant inverse relationship to pancreatic cancer risk (OR=0.45; 95% CI = 0.24-0.82; P trend=0.04) (174). Folate metabolism has also been discovered to play a role in prostate cancer, whereby the prostate-specific membrane antigen (PSM) strongly expressed in prostate cancer has been identified as a folate hydrolase (175). The presence of this folate hydrolase makes a current folate antagonist chemotherapy agent, Methotrexate, ineffective in the fight against this disease (175); therefore, future therapies for prostate cancer using folate antagonists may have to be reconsidered. All of these studies reiterate the potential modulatory role of folate in the carcinogenic process.

Investigations of folate deficiency and its clinical implications are important as previous studies have shown that up to 30% of healthy, ambulatory populations have a subclinical, but biochemically evident degree of folate deficiency (176). Folate deficiency has been associated with increased risk of cardiovascular and cerebrovascular disease (177,178) and neural tube defects (179,180). In 1996, the U.S. Food and Drug Administration required that all enriched grain products be fortified with folic acid at 140 μg/100g in order to prevent neural tube defects (181). Since then, a large cohort study has shown that mean folate concentrations have increased from 4.6 to 10 ng/ml and the prevalence of low folate concentrations (<3 ng/ml), has
decreased from 22 to 1.7% (182). Mean total homocysteine was observed to decrease significantly, as did the prevalence of high homocysteine concentrations (182). In general, the fortification of enriched grain products with folic acid significantly improved the folate status of middle-aged and older adults (182).

Folic acid fortification was originally implemented in order to reduce the risk of neural tube defects, but may also have a beneficial effect on cardiovascular disease, as increased risk has been associated with elevated concentrations of circulating homocysteine (182). It is possible that fortification may also have an effect on the population risk of CRC, as decreased folate intake is associated with increased risk of CRC development. Therefore, an overall increase in plasma folate concentrations may reduce the risk of CRC development in those that are predisposed. However, because of the possibility that folate can increase growth and proliferation of already established colonic neoplastic foci (ie. adenomas and ACF), the safety for folate fortification with regard to CRC risk needs to be evaluated.

1.2.1. Mechanisms of folate

Although the exact mechanism by which folate can exert its mediating effects on the development of cancer has not yet been established, several hypothesis have been brought forth (Table 1.6) (183).

At the forefront of the postulated mechanisms are hypotheses on the role of folate in DNA methylation, mutation and DNA synthesis and repair.

1.2.1.1. DNA methylation

Folate has one known biochemical function, that is to mediate the transfer of one-carbon moieties (126). Folate, in the form of methyltetrahydrofolate (MTHF), is involved in the synthesis of methionine, a precursor of S-adenosylmethionine (SAM) - the primary methyl
Table 1.6  Folate mechanisms in carcinogenesis

1. DNA methylation
2. DNA damage and DNA repair
3. DNA mutations
4. MTHFR* polymorphism
5. Impaired immune function
6. Secondary choline deficiency
7. Interacting with tumorigenic viruses

*MTHFR - methylenetetrahydrofolate reductase
donor to most of the methylation reactions within the body, including that of DNA (Figure 1.3). Therefore, it has been hypothesized that a deficiency in the methyl donor, folate, would result in hypomethylation. Hypomethylation, both globally and of specific oncogenes, is thought to be an early event in carcinogenesis including that of the colorectum (106,184). Mechanisms to explain the relationship between methylation and carcinogenesis have not yet been determined, however, as previously described in Section 1.1.1.6, DNA methylation is important in many aspects of cell development, including gene expression, conformation and stability of DNA, transcription factor binding, mutations and genomic imprinting (as reviewed in (185)).

Numerous animal studies have demonstrated that diets deficient in methyl donors have enhanced the development of hepatocellular carcinoma (83) and decreased genomic and specific oncogene methylation status (98,99). Fewer studies have been conducted using folate deficiency alone to affect DNA methylation, with conflicting results. In vivo studies have shown that folate deficiency causes low hepatic SAM concentrations (186-188) but studies investigating whether these low concentrations result in genomic and protooncogene specific DNA hypomethylation are conflicting (189-191). One study found a trend for increased methylation in severely depleted rats (189). This may be explained by other observations which have suggested that the decreased SAM induces an increase in MTase methylation activity. Taken together, these studies indicate that dietary folate deficiency alone may not be sufficient to induce a significant degree of DNA hypomethylation, globally or at specific oncogenic loci in the liver or colon.

However, folate deficiency does appear to be sufficient to cause hypomethylation of specific regions of an important tumor suppressor gene, p53 (189). Kim et al. found that in the rat p53 gene exons 6 and 7, areas which are highly conserved and contain abundant CpG sites, had reduced levels of DNA methylation following a folate deficient diet. The amount of
methylation decreased over time from a 15% reduction to a 40% reduction compared to controls after 4 and 6 weeks of folate deficient diet, respectively (189). In addition, administration of folate was shown to reverse genomic hypomethylation and site specific colonic p53 hypomethylation induced by procarcinogen DMH (101,160). The p53 gene may be more susceptible to the methylating effects of folate as it has an abundant number of CpG sites in exons 5 through 8 (56). Thus, folate does appear to have modulating effect on DNA methylation in the specific areas of the p53 tumor suppressor gene.

Hypermethylation has also been implicated in tumorigenesis (91,92), where areas within or upstream from the promoter region of tumor suppressor genes are hypermethylated, resulting in gene silencing. However, no studies have been performed to date, that have investigated or observed a relationship between dietary folate intake and DNA hypermethylation.

Several hypotheses have been brought forth to explain the methylation-cancer association, as previously described in Section 1.1.1.6 DNA methylation. One hypothesis suggests DNA methylation and its effects on gene expression are responsible for tumorigenesis. A second hypothesis proposes that a high mutation rate, associated with unmethylated cytosine residues when SAM is limiting or methylated cytosine residues, is of greater importance (84).

1.2.1.2 DNA damage and repair

Folate, in the form 5,10-methylenetetrahydrofolate (THF), is necessary for the de novo synthesis of purines and thymidylate (126). 5,10-methylene THF contributes to the synthesis of DNA in two roles: it is responsible for converting deoxyuridylate to thymidylate; and it can be oxidized to form 10-formyl THF, which is required for the synthesis of purines (Figure 1.3) (192). Therefore, any deficiencies in folate may cause abnormalities to occur during DNA synthesis. It has been shown previously that when folate deficiency reduces 5,10-methylene THF levels, there is a subsequent buildup of uracil and shortage of thymidine, resulting in the
incorporation of uracil in place of thymidine. Most of these effects can be reversed by folate (193,194). Also, folate deficiency has been implicated in genomic (195,196) and site specific strand breaks (189,197). Patients with megaloblastic anemia, commonly found in those with folate deficiency, have been found to possess chromosomal gaps and breakage in their DNA (198). Strand breaks are also a common occurrence in folate deficient cells. Breaks have been shown to occur in mammalian cells treated with Methotrexate, a folate antagonist (196) and in vivo in lymphocytes of rats fed folate deficient diets (199). As well, more strand breaks are shown to occur in Chinese hamster ovary cells treated with alkylating agents when the media is free of folate and nucleotide precursors (194). Site specifically, in the p53 gene, a tumor suppressor gene frequently associated with cancer, folate deficiency has been shown to increase strand breaks in exons 5-8 in rat colon (189) and liver (200) DNA. It has been postulated that folate deficiency induced strand breaks disables the tumor suppressor function of the p53 gene allowing tumorigenesis to proceed. From a nutritional perspective, dietary folate has been shown to cause strand breaks but only if it is severe deficiency and maintained long term (189). These strand breaks may be a result of folate deficiency induced DNA hypomethylation, which makes DNA susceptible to nucleases and strand breaks (201). In general, strand breaks can result in many chromosomal abnormalities: chromosomal recombination and aberration (202), loss of heterozygosity (203), increased mutation rate (195) and neoplastic transformation (202).

The occurrence of strand breaks places an increased reliance on the DNA repair system which is in turn dependent upon a balanced nucleotide pool, however folate deficiency causes an increase in the uridine to thymidylate ratio, which, as previously shown, can result in misincorporation of uracil for thymidine (193,200). It has also been recently shown that DNA excision repair is impaired in the colonic mucosa of rats fed folate deficient diets (204). Excision repair is responsible for removing damaged short sequences or bases and replacing...
them with normal bases using the complementary strand as a template (205). A recent study by Cravo et al., investigated the relationship of folate deficiency and DNA mismatch repair. Mismatch repair differs from excision repair, in that it corrects errors made during DNA replication by searching the DNA for mispaired bases, removing those found and replacing them with the correct nucleotides. This study reported a trend (p=0.10) towards an inverse relationship between colonic mucosal folate and microsatellite instability, an indicator of failed mismatch repair, in patients with ulcerative colitis (157). As well, following treatment with folate supplementation, one of the patients demonstrated a partial disappearance of microsatellite instability. However, given the small number of subjects involved with microsatellite instability (n=3), this study can only suggest a relationship between folate depletion and DNA mismatch repair function, more studies are required before it can be definitively determined.

1.2.1.3. DNA mutations

Folate deficiency has also been associated with increased mutations, independent of CpG methylation. CpG sequences within certain genes have been identified as mutational hot spots for several cancers, including CRC (16,45,57,107). Typically in cancer, the mutations involved with these hot spots are cytosine to thymine transitions. There are several postulated mechanisms for these transitions. These mutational hot spots may be induced by enzymatic (methyltransferase) or spontaneous deamination of 5-methylcytosine (108,110). Alternatively, the transitions may be initiated by enzymatic deamination of unmethylated cytosine to uracil by methyltransferase, methylation of uracil to thymine by methyltransferase, and then blockage of repair of DNA mismatches by the binding of methyltransferase when SAM concentrations are limited (36,111). The latter mechanism is particularly relevant as folate deficiency has been shown to induce decreases in SAM concentrations (186-188,190). One study has shown that folic acid supplementation prevents p53 mutations in ulcerative colitis patients at risk for
coloected cancer development (206). However, a relationship between folate deficiency and DNA mutations has yet to be clarified.

As well, an in vitro study done by Duthie et al., showed that in lymphocyte DNA, DNA strand breakage, uracil misincorporation and inefficient repair of oxidative DNA damage increased with folate deficiency (207). These observations help determine the mechanisms of folate effects on genetic instability (207).

1.2.1.4. MTHFR polymorphism

Methylenetetrahydrofolate reductase is the enzyme responsible for converting 5,10-methylene THF to 5-methylTHF, the predominant form of folate in the plasma. 5-methylTHF provides the methyl group for de novo synthesis and DNA methylation (126). A common mutation in MTHFR is a C to T transition, resulting in an amino acid change from alanine to valine. The mutation reduces enzyme activity, leading to lower levels of circulating 5-methylTHF, an accumulation of 5,10-methyleneTHF, and increased plasma homocysteine (208,209).

Two studies have shown that individuals that were homozygous for the MTHFR polymorphism (val/val) had a 40-50% reduction in CRC risk compared to those with heterozygote (val/ala) and normal, wild type genotypes (ala/ala) (210,211). Those who have a reduced enzyme activity as a result of this mutation may be at reduced risk due to the critical role of 5,10-methyleneTHF in DNA synthesis. 5,10-methyleneTHF is required for DNA biosynthesis and maintaining the deoxynucleotide pool balance (126). However, the protective effect of homozygosity was nullified by other high risk factors, such as high alcohol and low methionine/folate intake (210,211). It was suggested that with these risk factors, there is an additional depletion of 5-methylTHF. Abnormal DNA synthesis, deoxynucleotide pool imbalance and abnormal DNA methylation may result, offsetting the benefit of the mutation
phenotype (210,211). The folate-MTHFR interactions is an excellent paradigm of gene-nutrient interactions in carcinogenesis.

1.3. ANIMAL MODELS

In order to examine the validity of these folate mechanisms on CRC, it is important to have controlled, randomized, prospective studies. In the studies listed previously, the relationship between folate and CRC carcinogenesis in humans and chemically induced animal models was successfully established. However, in spite of the contributions of the chemical carcinogen model, there are drawbacks that lie within how human tumor development differs from these models. There is a lack of evidence to suggest that the substances used to induce cancer in these animals, ie. DMH, AOM, are colorectal carcinogens in humans (212). In addition, no human CRC develops as a result of genotoxic exposure to these types of carcinogens as the dose required is too high. As well, although the tumors developed in this model are similar in their histological and proliferative characteristics to those found in humans, many of the tumors appear to develop de novo – making them ineffectual in the study of the adenoma-carcinoma development typically found in human tumors (213). There are also differences in the molecular genetics of tumors found in humans compared to chemical animal models. In both humans and mice, APC has been identified as a gene with up to 60% involvement in causal effects on CRC in a tumor suppressing role. However, in AOM or heterocyclic amine induced rat models, only 14-29% of colon tumors had Apc mutations (214,215). This is in contrast to humans, where germline and somatic APC mutations are seen in approximately 70% of colorectal adenomas and carcinomas (11). Similarly, the frequency of p53 mutations in primary tumors of DMH or AOM rats and mice are either absent or uncommon (107,216). In human tumors, inactivation of P53 usually involves the mutation of one allele and
loss of the other (217), however allelic loss is almost impossible to determine in these inbred animal models as they have a homozygous genome. K-ras is, however, involved in the DMH and AOM models of CRC, similar to that found in humans (219). There are also unique genes that predispose DMH treated rats and mice to CRC (220,221).

In order for data from animal studies to be extrapolated to clinical human use differences between the models must be minimized. Recently genetically modified mouse models have been generated that spontaneously develop intestinal and colorectal tumors that are similar genotypically and phenotypically to certain human forms of CRC. These genetically predisposed murine models do not require chemical carcinogens, as colonic ACF, adenomas and adenocarcinomas spontaneously develop; thus the possibility of high dose carcinogens interfering with the study of the adenoma-carcinoma sequence is eliminated. Two genetic mouse models used, Min (Apc+/-) and Apc+/-Msh2/- involve genes whose human homologs have been implicated in the development of CRC. The method of generating the Apc+/-Msh2/- mice is described in Section 2.3 Both models have one mutated Apc allele, which has been frequently observed in FAP (13,30,41,222), sporadic cancers and HNPCC (13,41,120,222). The tumors that develop in these models are similar to human colorectal tumors in that they show a loss of wild type Apc due to LOH or somatic mutation in intestinal tumorigenesis. The Apc+/-Msh2/- mouse model carries a null Msh2 mismatch repair gene, a gene in humans that is thought to be responsible for many of the HNPCC cases (121). The Apc+/- model was used in addition to the Apc+/-Msh2/- model due to time and financial constraints. Unlike the Min mouse, the Apc+/-Msh2/- mouse model is difficult to generate. Thus, the number of Apc+/-Msh2/- mice required for adequate statistical power in a dose-response experiment would have been difficult to achieve in the time available.
These models allow us to investigate the effects of environmental and genetic factor on the development and progression of CRC in a necessarily strictly controlled environment.

1.3.1 Apc mouse models

Min (Apc+/−850) mice are heterozygotes of the Apc gene, characterized by an autosomal dominant mutation that predisposes them to multiple intestinal neoplasms. In these mice, a specific mutation at codon 850 has been introduced by ethynitrosurea (ENU) mutagenesis to the mouse homolog of the human APC gene, resulting in a truncated protein product (223). These heterozygotes begin to develop benign adenomas in the intestinal tract at less than one month of age (224). When these mice reach 160-180 days of age, they become moribund and die of anemia or bowel obstruction (223). Min mice bred in our facility typically develop approximately 30 small intestinal tumors, no ACF and 3-5 colonic tumors, before dying at 6 months of age. No adenocarcinomas have been observed. Homozygotes of this Apc mutation are not viable as the ectoderm does not develop, thus functional Apc is required for normal growth (225).

Apc acts in typical tumor suppressor gene fashion in that it requires both copies of the gene to be inactivated in order for tumors to develop. That is, each cell in the body of a Min mouse has only one wild type Apc allele, but only some of the cells will develop into a tumor. This suggests that heterozygosity of Apc alone is not sufficient to induce neoplasia and that there is some initiating second event (either genetic or epigenetic) that must occur (27). In every Min mouse adenoma, there is loss of heterozygosity (LOH) (28), loss of the homologue carrying the wild type Apc allele, unlike human adenomas where some but not all have allelic loss (226,227).

There are complex associations of genotype to phenotype with respect to the APC gene, this is clearly illustrated in Apc mouse models. First, all Min mice have a truncating mutation in
the Apc gene at codon 850, however, depending upon the strain and breed of the mouse, the amount of intestinal polyps varies significantly (11). The number of polyps also varies significantly between mice with different Apc mutations. There are two other mouse models of CRC with mutations in the Apc gene (Table 1.7), including the Apc716 and 1638. Apc716 mice contain a truncation mutation at codon 716 (228). Heterozygotes for Apc716 on a C57B16/J strain, develop 200-500 intestinal adenomas and produce a stable 80 kDa truncated protein (228). Similar to Min mice, only adenomas were observed in these mice which were characterized by the loss of the wild type Apc allele (228). Mice with a frameshift mutation at codon 1638 have a less severe phenotype where Apc+/Apc1638 mice develop 5-6 small intestinal and colonic tumors and live to 8-16 months of age (229). The tumors developed in the Apc1638 mice included both benign adenomas and malignant adenocarcinomas, located predominantly in the duodenum and the jejunum (229). This extended life span enables us to study the progression to carcinoma in the intestines of these mice.

Previous studies using the Min mice have investigated the relationship of dietary factors and NSAIDS to colorectal cancer risk. Dietary factors often associated with colorectal cancer risk include fat, fruit and vegetable intake. A recent study in Min mice examined the effects of these factors on intestinal tumor formation (230). The results indicated that neither a low fat diet nor a high fat diet supplemented by a vegetable-fruit mixture decreased the incidence of polyps in Min mice. Studies in Min which have found tumor suppressing effects include some involving short chain fructo-oligosaccharides (231), soybean derived Bowman-Birk protease inhibitor (232) and a fish oil derivative rich in omega-3 fatty acids (233). Other dietary regimens have been tested in Apc1638 and Apc716 mice for their effects on colorectal tumorigenesis. In Apc1638 mice, a Western style diet was found to increase the incidence of carcinomas and invasive tumors in the colon and intestine (234). Similarly, in Apc716 mice, a low-fat, high-
<table>
<thead>
<tr>
<th>Mutation site (codon)</th>
<th>Number of intestinal tumors</th>
<th>Homozygote viability</th>
<th>Adenoma/carcinoma</th>
<th>Age at tumor development</th>
<th>Lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>850 (223)</td>
<td>50-100</td>
<td>No</td>
<td>Adenomas</td>
<td>&lt;1 month</td>
<td>6 months</td>
</tr>
<tr>
<td>716 (228)</td>
<td>250-500</td>
<td>No</td>
<td>Adenomas</td>
<td>5-7 weeks</td>
<td>&gt;6 months</td>
</tr>
<tr>
<td>1638N (229)</td>
<td>5-6</td>
<td>No</td>
<td>Adenoma/carcinoma</td>
<td>2 months</td>
<td>8-12 months</td>
</tr>
</tbody>
</table>
fiber diet was shown to decrease intestinal polyposis when compared to a high-fat, low fiber diet (235). Also in the Apc716 model, females fed docosahexanoic acid (DHA), an omega-3 fatty acid, only developed one-third the number of polyps found in the controls, with no effect of DHA seen in males (236). Thus, dietary modulation of CRC is possible in Apc mouse models.

Treatment of these mice with NSAIDS, such as aspirin and peroxicam, have decreased the incidence of small intestinal tumors in these Apc mouse models (237,238). NSAIDs are thought to exert their effects through an inhibition of cyclooxygenase (COX) and induction of apoptosis (237). One of the two isoforms of COX, COX-2, has been shown to be elevated in intestinal polyps and cancers, but when treated with an inhibitor, tumor formation is suppressed. Specific COX-2 inhibitors in Min mice, such as sulindac and nimesulide, have been shown to act as effective chemopreventive agents (239,240) and sulindac alone has been shown to cause regression of tumors by returning apoptosis levels to normal (240,241).

The severity of the intestinal neoplastic phenotype is strain specific and this is thought to be due to different gene modifiers in each of the strains that reduce tumor multiplicity (242). In the AKR strain, a modifier has been identified as Modifier of Min-1 (Mom-1). Mom-1 has been identified as a semi-dominant modifier of both tumor multiplicity and tumor size such that homozygosity for Mom-1 resulted in a fourfold reduction in tumor number (242,243). The Mom-1 locus maps to an area with a secretory phospholipase gene Pla2g2a which has also been found to reduce tumor multiplicity and tumor size in Min mice (244), the mechanism for the relationship between the phospholipase and decrease in tumor number is still unknown. However, in humans, Pla2g2a is not altered in colorectal tumorigenesis (245). Another gene that has been shown to modify the phenotype is DNA methyltransferase (MTase) (84). Min mice were crossed with a DNA methyltransferase (Dnmt)-deficient strain of mouse. The mutation in the Dnmt+-/- mice results in a 50% decrease in DNA methylation levels. When Min mice were
crossed with these heterozygous mice, tumor multiplicity decreased by 2.5X and when the genotype was compounded with treatment with 5-azacytidine, a potent MTase inhibitor, the tumor number was reduced by 57X. These findings are contrary to other observations which suggest global DNA hypomethylation is associated with increased risk of colorectal cancer (14). From this study, it is thought that the methylation status affects tumor initiation, as treatment after 2 months of age, had no effect on tumor number. Mismatch repair genes also have a modifying effect on the Min phenotype.

Min mice are similar genotypically to the human FAP situation, in that FAP patients also have a germline mutation in the APC gene. Mutation in APC is the most frequently observed somatic event in FAP (13,30,41,222) and it also occurs in sporadic cancer and HNPCC (13,41,120,222). As well, the human and mouse APC genes are 86% identical at the nucleotide level and 90% identical at the amino acid level (246). Phenotypically, both Min and FAP patients develop multiple neoplasms in their intestines, however, in mice they are usually found in the small intestine, whereas in humans, they are found predominantly in the colon (223). The phenotypic similarities allow further study of the human disease using the mouse model. Being able to experimentally manipulate the mouse, genetically and environmentally, will enable us to study further the role of Apc in carcinogenesis. Thus, Min mice are a good model for human CRC.

1.3.2. Apc+/-Msh2-/- mouse

Mismatch repair genes, such as MSH2, MLH1, PMS1, PMS2, MSH3, MSH6 and MLH2, are frequently associated with hereditary cancer syndromes. Specifically, mutations in MSH2 have been shown to occur in 45% of patients with Hereditary Non-polyposis Colorectal Cancer (HNPCC) and 60% in those with sporadic colon cancer (119). MSH2 encodes a component
required for DNA mismatch recognition; without it, cells become tolerant to methylating agents, lose mismatch binding and acquire microsatellite instability (122).

As described previously, inactivation of APC acts as an early and crucial step towards FAP and sporadic CRC development (222). Mutations in MSH2, however, appear to accelerate the progression from adenoma to carcinoma in HNPCC patients (247). Clearly, both genes play important roles in the carcinogenic process, and yet the relationship of the APC and MSH2 genes have not been well established. Recently, mice were generated with Apc+/-Msh2+/-, Apc+/Msh2+/- and Apc+/-Msh2-/- genotypes to investigate this relationship (247). All three genotypes developed tumors in the first 10 days of age. Colon tumors developed in all three genotypes, however, Apc+/-Msh2-/- mice developed the most (8) within the shortest time frame (27 days), compared to Apc+/-Msh2+/+ and Apc+/Msh2+-/- which were older than 70 days of age before >5 colon tumors developed. ACF, which are proposed precursors to colonic adenomas, were also counted in the colon and found to be abundant (approximately 55 per mouse colon) in the Apc+/-Msh2-/- mice but rare occurrences in the other two genotypes. The accelerated intestinal phenotype of the Apc+/-Msh2-/- mice is even more evident in the small intestine, where these mice developed on average, 333 adenomas compared to the 48 and 42 adenomas seen in the Apc+/-Msh2+/+ and Apc+/Msh2+/- mice respectively. The average time required for a nascent tumor to develop into a macroscopically visible adenoma in these mice was estimated to be 42 and 27 days in the small intestine and colon respectively (247). The lifespan of these mice are a reflection of the severity of disease, such that Apc+/-Msh2+/+ and Apc+/-Msh2+/- mice live to about 160 days of age, compared to the Apc+/-Msh2-/- mice which only live to 80 days before dying of anemia or bowel obstruction (247). In the tumors that have been analyzed thus far, no carcinomas have been detected. In comparison, mice which are Msh2+/- only did not develop tumors, however, the Msh2-/- mice did develop lymphoid tumors
(31). Resulting defects in mismatch repair were assessed by microsatellite instability and found to be present in the lymphoid tumors developed in Msh2-/- mice (31).

In Apc+/Msh2-/- mice, the relationship between ACF and colon tumors is unclear. It is reported that ACF, despite being a postulated colonic adenoma precursor, which develop in these Apc+/Msh2-/- mice are not directly related to the number of adenomas found in the colon (31). This is because ACF are thought to develop in adult Min and Apc+/Msh2-/- mice, whereas colon adenomas arise perinatally (247,248). These observations are different from human data, which still supports the possibility of ACF as an adenoma precursor.

Unlike Min mice with proficient Msh2, the mechanism responsible for the loss of wild type Apc function in the Msh2 deficient mice is not LOH, as only 15% of inactivated Apc in these mice was due to LOH. It was suggested that a somatic mutation due to inactivated mismatch repair may be responsible for the loss of wild type Apc, accelerated ACF and additional tumors found in the Apc+/Msh2-/- mice (247). This is supported by a recent study in mice homozygous for mismatch repair gene Mlh1, which develop lymphomas and tumors in their intestinal tract (249). When these Mlh1 mutant mice were crossed with Apc1638N/- mice, there was accelerated tumor formation accompanied by loss of APC function, the basis of which was the loss of MMR activity (249). Apc1638N mouse generated previously, developed tumors when Apc function was lost through LOH, whereas in the Mlh1/Apc1638N mice the loss of Apc function occurred as a result of mutations in the wild type allele (249).

There are several differences between the tumorigenesis observed in the Apc+/Msh2-/- mouse model and humans. These mice develop most of their adenomas in the small intestine, unlike humans with FAP that develop colonic tumors. In addition, this model does not develop adenocarcinomas in either the small intestine or the colon as there is insufficient time for cancer development in the short life span of these mice. Unlike the human situation, the contribution of
ACF to colonic adenoma development in the Apc+/-Msh2-/- mouse model has not yet been established. Widespread microsatellite instability, commonly found in HNPCC, is absent in the tumors of this model. The Apc+/-Msh2-/- model, similar to the Min model, involve genes commonly mutated in familial cancers, such as FAP and HNPCC. However, in sporadic cancers, the rate of mutations in Apc and Msh2 are less frequent. Thus, these models, particularly Apc+/-Msh2/-, may better reflect the familial human intestinal tumorigenic process than sporadic colorectal carcinogenesis in humans.

However, the Apc+/-Msh2-/- mouse model is still a clinically relevant model for human colorectal carcinogenesis as it is similar both phenotypically and genotypically to the human situation. These mice develop intestinal adenomas, ACF and colonic adenomas spontaneously without the need for carcinogens. In contrast to other genetic models of colorectal cancer, Apc+/-Msh2-/- mice develop ACF, the probable earliest precursor of colorectal cancer observed in humans (250), particularly HNPCC. In addition, the accelerated nature of tumor development in the small intestine allows for the study of treatments and the nature of tumor progression in the bowel within a short time period.
1.4 HYPOTHESIS

Folate has a modulating effect on intestinal and colorectal tumorigenesis in genetically predisposed murine models.

1.5 OBJECTIVES

1. Determine whether folate deficiency accelerates the development of intestinal neoplasms in genetic murine models of intestinal tumorigenesis.

2. Determine whether dietary folate supplementation has a protective effect on intestinal tumorigenesis in the same genetic model.
CHAPTER 2: THE EFFECTS OF FOLATE ON TUMORIGENESIS IN

\textit{Apc+/-Msh2/-} MICE.

2.1 INTRODUCTION

Several genetic murine models for human colorectal carcinogenesis have been developed which are similar genotypically and phenotypically to the human situation. The \textit{Apc+/-Msh2/-} mouse model was developed to investigate the synergistic effect of the \textit{APC} tumor suppressor gene and a mismatch repair gene, \textit{MSH2}. These genes are associated with both familial and sporadic human colorectal cancer (27,30,41,120,251). The \textit{APC} tumor suppressor gene is associated with Familial Adenomatous Polyposis (FAP), a disease characterized by the development of hundreds to thousands of polyps in the intestine (27,30). \textit{MSH2} is associated with Hereditary Non-polyposis Colorectal Cancer (HNPCC), where affected individuals develop colon cancer in the proximal colon at significantly younger ages than the general population (120). The \textit{Apc+/-Msh2/-} mouse model is good representation of the human colorectal tumorigenesis situation because the mice develop colon tumors and ACF, which are both found in FAP and HNPCC. It is also an excellent model to study as the tumors develop spontaneously without the use of potentially confounding carcinogens and the accelerated intestinal tumorigenesis allows for study of treatment given at varying stages of tumor growth. This feature is particularly relevant for the study of folate which has been shown to have dual effects in colorectal carcinogenesis. Folate deficiency in normal tissues enhances the development of tumors whereas folate depletion in established tumors retards or regresses tumors (163,164), resulting in a decrease in tumor number (127-131). Many epidemiological studies have also implicated folate supplementation as having potential chemopreventive effects on colorectal
carcinogenesis (136-140, 164). However, epidemiological studies often rely upon retrospective recollection of food intake where the potential for error is quite substantial. Although prospective studies are considered to be the gold standard, the variability can be quite large as the environment and genetics of human subjects cannot be strictly controlled. Therefore, with human subjects there are many sources of variability and potential confounders. The variability of genetically altered mouse models, such as Min and Apc+/−-Msh2−/−, is limited to their environment, which can be strictly controlled. Thus, many aspects of intestinal tumorigenesis can be investigated with the Apc+/−-Msh2−/− models, including the relationship of small intestinal and colon tumor development to the genetic alterations in the Apc+/−-Msh2−/− mouse and the dietary influences of folate supplementation and deficiency.

This study investigates effects of dietary folate deficiency and supplementation on small intestinal and colorectal tumorigenesis in Apc+/−-Msh2−/− mice, a mouse model genetically predisposed to developing intestinal adenomas.
2.2 SPECIFIC OBJECTIVES

1. Investigate the effects of dietary folate deficiency and supplementation on small intestinal and colonic tumorigenesis when implemented *prior to* the establishment of neoplastic foci.

2. Investigate the effects of dietary folate deficiency and supplementation on small intestinal and colonic tumorigenesis when implemented *post* neoplastic foci development.

3. Determine possible molecular effects of dietary folate responsible for the development of small intestinal and colonic tumors.
2.3 MATERIALS AND METHODS

Materials: Min \( (Apc^{-/-}\text{-}Msh2^{+/+}) \) mice used for the original breeding pair were purchased from Jackson Laboratory (Bar Harbor, ME). Taq polymerase, buffers and the PE2400 PCR machine used for PCR based genotyping were purchased from Perkin Elmer (Applied Biosystems, Mississauga, Ontario). Diets were amino acid defined diets containing different levels of folic acid and are purchased from Dyets (Bethlehem, PA, USA). Heparin, red top Vacutainers, 18 gauge needles, formalin, Whatman paper and methylene blue were purchased from Fisher Scientific (Springfield, NJ). Sodium ascorbate, folic acid, Tris-HCl, Tween 20 and Bis-Tris were from Sigma (St. Louis, MO.). Lactobacillus casei used for the folate assay was from ATCC (#7649) (Rockville, Maryland). Folic acid media and chicken pancreas conjugase were from Difco (Detroit, MI). NaOH, glycerol and the potassium phosphates for the potassium phosphate buffer, both dibasic and monobasic, were from ACP Chemical (Montreal, Quebec). Hydroxylapatite (Gel HTP) and Dowex-1 were from BioRad (Hercules, CA). Toluene and \( \beta \)-mercaptoethanol were from BDH (Toronto, Ontario) and Caledon (Georgetown, Ontario), respectively. A SpectraMAX 96-well plate reader spectrophotometer was used in conjunction with SOFTmax software for the folate assay, both of which were from Molecular Devices (Sunnyvale, CA). The Polytron tissue homogenizer was from Brinkmann Instruments (Westbury, NY), while the preparatory centrifuge and the spectrophotometer (DU 640B) were from Beckman (Allendale, NJ).

Proteinase K, HpaII, MspI, T4 Kinase, Taq DNA polymerase, calf intestine phosphatase (CIP), DNA ligase, EcoRI, XbaI, ClaI and XhoI enzymes, 123 bp ladder and agarose were all purchased from Canadian Life Technologies (Mississauga, Ontario). KCl, NaCl, sodium acetate, urea, boric acid and NaOH were obtained from BDH (Toronto, Ontario). EDTA and
\(\text{MgCl}_2\) were from ACP Chemical (Montreal, Quebec). Phenol:chloroform and isoamyl alcohol are from Bioshop (Burlington, Ontario). Acrylamide gel reagents, including 40% acrylamide (19:1), TEMED, SDS, xylene cyanol and bromophenol blue were obtained from BioRad (Hercules, CA).

**Mice:** All animals have been bred at the Samuel Lunenfeld Research Institute on the C5BL/6J strain in contained, sterile microunits housing 5-12 mice per cage. The development of the Min \((Apc^+/-)\) mice has been previously described (223). A second genotype, \(Apc^+/^/-Msh2^+/-\), was generated by crossing male Min \((Apc^+/-)\) mice with female \(Msh2^+/-\). The male \(Apc^+/-Msh2^+/-\) offspring were then crossed with female \(Msh2^+/-\) mice to generate \(Apc^+/-Msh2^/-\) mice (247).

**Genotyping:** Ear punch tissue was processed for genotyping using PCR based assays to determine \(Apc\) and \(Msh2\) status as previously described (247,252).

**Diet:** There were two amino acid defined diets with 2 different levels of folic acid used in this \(Apc^+/-Msh2^/-\) study. The 0 mg folate/kg diet produces progressive folate deficiency of a moderate degree without anemia, growth retardation or premature death through week 5 after which systemic folate indicators stabilize (253). The degree of folate deficiency produced by this diet is comparable to that associated with the increased risk of colorectal cancer in humans (132-137,139-141,144-149,254-256). The 8 mg folate/kg diet represents 4x the basal dietary requirement for rodents (i.e. 2 mg folate/kg diet) and has been consistently shown to have significant protective effects on colorectal carcinogenesis in rodent chemical models of colon cancer (163,164,253). The amino acid defined diet contained 50 g cellulose/kg diet, 60%
calories as carbohydrates, 23% fat, and 17% L-amino acid (253). The amount of methyl donors, methionine, choline and vitamin B12, were 8.2 g, 2.0 g and 10 mg per kg diet, respectively.

Study design. *Apc*+/--*Msh2*-/- mice were randomized to receive the amino acid defined diet with either 0 or 8 mg folate/kg diet at either 3 or 6 weeks of age. They were sacrificed at the end of their lifespan at 11 weeks of age (Figure 2.1). The three week time point was chosen to represent a time prior to the establishment of neoplastic foci, given the average time for a nascent tumor to develop into a macroscopically visible adenoma is estimated to be 42 and 27 days in the small intestine and colon, respectively (247). The six week time point represents the time following the establishment of neoplastic foci. There were seven mice in each diet group at the 3 week diet start; and ten and seven mice in the 0 and 8 mg folate/kg diets, respectively in the 6 week diet start. Diet and water were provided *ad libitum*. Mice were weighed weekly.

Study termination: The study mice were sacrificed by cervical dislocation at scheduled time points, blood was collected from the heart using a pre-heparinized 18 gauge needle and centrifuged at 2500xg for 10 minutes at room temperature. Serum was aliquotted into Eppendorf tubes in 0.5% ascorbic acid and stored at −20°C for the serum folate assay. Any macroscopically abnormal extraintestinal neoplastic lesions or organs were excised and fixed in 10% buffered formalin for histological examination. The liver was quick frozen on dry ice and stored at −70°C for DNA analysis and liver folate determination. The intestines were removed and flushed with formalin to remove fecal debris and to fix the intestinal villi in a spread position. A longitudinal incision was made so that the intestine could be opened, laid flat on Whatman paper and fixed in 10% neutral buffered formalin in a petri dish.
Figure 2.1 Apc+/- Msh2/- study design

Weanling period

Weanling

Birth 3 weeks 6 weeks 11 weeks

0 mg folate/kg diet (n=7) Sacrifice

8 mg folate/kg diet (n=7) Sacrifice

0 mg folate/kg diet (n=7) Sacrifice

8 mg folate/kg diet (n=10) Sacrifice

Standard lab chow
Enumeration of intestinal adenomas and colonic ACF: Intestines fixed in formalin were stained in methylene blue and examined for tumors and aberrant crypt foci (ACF) by gross inspection and light microscopy as previously described (247). ACF were not graded, however, were easily identifiable as clusters of crypts that were darker and larger, with thicker epithelial lining than the normal surrounding crypts (257,258). All small intestinal and colonic tumors were counted and recorded in a blinded fashion. Representative small intestinal tumors and all colonic tumors were chosen and embedded in paraffin for histological verification and later DNA extraction. Slides of 5μm thickness were cut and stained with hematoxilyn and eosin (H&E) for verification of colonic adenomas by a gastrointestinal pathologist blinded to the treatment groups.

Folate concentration determination: Serum and liver folate concentrations were determined using a standard microbiological Lactobacillus casei microtiter plate technique as described by Tamura et al (259). The L. casei bacteria grows in proportion to the amount of folic acid in the medium, therefore, the turbidity of the media as measured by the spectrophotometer is an indication of the concentration of folic acid in the sample.

Folate extraction: Serum was prepared as stated above. Liver tissue folate was extracted with 10X volume of extraction buffer (1 g sodium ascorbate, 1 g Bis-Tris, 50 mL dH₂O, 35 uL β-mercaptoethanol), respectively (260). Mixture was boiled for 15 minutes then cooled in an ice water bath. Tissue was then homogenized and spun down at 17,500 rpm at 4°C for 20 minutes. The supernatant was then transferred to a Vacutainer with no additive using an 18 gauge lumbar puncture (LP) needle, to prevent oxidation of folate, and stored at -70°C. Colonic mucosal folate concentrations were not measured as collection of the mucosa would have interfered with tumor enumeration. Therefore, only the systemic measures of folate were determined.
However, previous rodent experiments and human studies have demonstrated significant direct
correlations between colonic mucosal and blood folate concentrations (151, 164).

**Folic acid standard preparation:** Folic acid was dissolved in H₂O with 0.1M NaOH to a
concentration of 10 mg folate/10 mL. The concentration was verified using a spectrophotometer
at 282 nm. The solution was diluted to 2 ng/mL in sterile filtered 0.1M potassium phosphate
buffer (1.05 g KH₂PO₄, 0.4 g K₂HPO₄ and 1 g NaAscorbate in 100 mL H₂O). Standard was
stored in aliquots at -70°C.

**Lactobacillus Casei Preparation:** L. casei was grown in 1 mL of folic acid media (9.4 g folic
acid media in 200 mL of dH₂O and 50 mg NaAscorbate; boiled for 2 minutes and autoclaved)
with 0.3 ug/L folic acid. Then 0.25 mL of the suspension was added back to the remaining 199
mL of folic acid media. Bacteria was allowed to grow for 18 hrs at 35°C. An equal volume
(200mL) of 80% autoclaved cold glycerol was added, the solution was mixed and stored in
aliquots at -70°C.

**Chicken pancreatic conjugase preparation:** Chicken pancreas acetone powder was dissolved
2.5g/75 mL 0.1 M KPO₄, pH 7.0 and stirred for 1 hour at room temperature. It was then
incubated at 37°C under a blanket of toluene for 6 hours. Toluene was then removed and
solution was centrifuged at 10,000 x g for 15 minutes. Supernatant was collected and an equal
volume of tricalcium phosphate was added (BioRad Gel HTP was rehydrated: 1 part HTP to 6
parts buffer, mixed and allowed to settle. Supernatant was decanted and HTP was resuspended
in 100 mL 0.1 M KPO₄, pH 7.0 per 10g HTP.). Mixture was stirred at 4°C for 30 minutes then
spun down at 25,000 x g for 15 minutes. Supernatant was collected and cooled to 4°C. An
equal volume of 95% ethanol was added, mixed and left overnight at -20°C. Solution was then
spun at 25,000 x g for 15 minutes. Supernatant was removed and precipitate resuspended in 50
mL of cold 0.1M KPO₄, pH 7.0. Solution was then mixed with 10 g Dowex-1 (BioRad AG1-
X8) and stirred for 1 hour at 4°C. The solution was filtered at 4°C through a Whatman #1 filter and then aliquotted into tubes for storage at -70°C.

**Folic acid concentration determination assay:** Fresh folic acid media (9.4 g folic acid media in 100 mL of dH₂O, boiled and autoclaved) and fresh potassium phosphate buffer (as above) were made prior to each assay. To all of the wells on a 96 well microtiter plate, 150 uL of potassium phosphate buffer was added. An equal volume of standard was added (150 uL) and then serially diluted along the plate to create a standard curve. Samples were run in duplicate and serially diluted three times per plate. Serum samples were thawed and 5 uL per well was used. Liver extract was mixed: 200 µL of extract with 50 µL conjugase and 750 µL phosphate buffer. The liver mixture was again diluted, 100 µL diluted extract with 900 µL phosphate buffer. For liver samples, 5 uL of the diluted extracts were added to the plate for assay. The *L. casei* was diluted 1 mL to 24 mL of folic acid media and following the addition of samples, 150 uL of diluted *L. casei* was added to each well. Plate was covered with a mylar sealer and mixed. Bacteria was allowed to grow at 37°C for 28 hours. Plate was then read on a spectrophotometer at 650 nm.

**Nucleic acid extraction:**

**Slide DNA extraction:** Both normal and tumor DNA were extracted from paraffin blocks of intestinal tissue using proteinase K digestion (31,261). Areas of interest were marked on a hemotoxylin and eosin (H and E) stained slide, slides were aligned with respective unstained slides and desired areas were cut out with a sterile scalpel blade. Paraffin samples were put into lysis buffer (10 mM Tris-HCl pH 8.0, 100 mM KCl, 2.5 mM MgCl₂ and 0.45% Tween 20), incubated at 95°C for 10 minutes and then cooled to room temperature. Then 15 uL of 20 mg/mL Proteinase K was added, samples were incubated at 65°C for 1 hour, then 95°C for 10 minutes. DNA was then organically extracted once with phenol:chloroform:isoamyl alcohol
(25:24:1), once with chloroform then ethanol precipitated on dry ice for 30 minutes. Samples were then spun down at 4°C for 30 minutes, pellets were washed with 70% ethanol and resuspended in 30 μL dH2O. DNA concentration was determined on a Beckman DU 640B spectrophotometer at wavelengths 260 and 280 nm.

Frozen tissue DNA extraction: DNA was also extracted from quick frozen tissue samples using standard techniques (245). Frozen liver tissue was weighed, added to 0.5 mL of tissue lysis buffer (100 mM Tris HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 ug proteinase K/mL). Tissue was digested overnight at 55°C, centrifuged and the supernatant was added to an equal volume of isopropanol. After mixing until no viscosity, the aggregate was removed and dissolved in 30 to 50 uL of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA pH 7.5) at 55°C for several hours. DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation with sodium acetate before use in assays and concentration was determined by spectrophotometer at 260 and 280 nm.

DNA methylation determination:

Restriction digest method: Genomic methylation status was assessed in liver DNA tissue using a methyl-sensitive restriction digest method. Three samples from folate depleted and folate supplemented groups at 3 and 6 week diet starts were selected as representatives of their respective groups because their serum folate concentrations were closest to the respective means of their groups. Genomic liver DNA (5 μg) was digested with HpaII for 8 hours at a concentration of 20 U/μg DNA at 37°C. One additional sample from each group was selected and digested with MspI. HpaII and MspI are isoschizomers that cleave the sequence 5′-CCGG-3′ between the two cytosine residues. HpaII is unable to cleave CCGG if the internal cytosine is methylated whereas MspI cleaves CCGG regardless of the methylation status of the internal
cytosine (14). A HpaII specific oligo (5'-TAT AGC CGG CTA TA-3') was used in 10-fold molar excess of genomic DNA to enhance the cutting efficiency of the enzyme (232). Digested DNA fragments were separated on an 0.8% agarose gel which was then denatured and neutralized before DNA was transferred in 20X SSC to a positively charged nylon membrane (Boehringer Mannheim) using Southern blotting under standard conditions (262). Membranes were then UV cross-linked. The membranes were then hybridized with a mouse centromeric minor satellite repeat sequence derived from plasmid pMR150 (262). pMR150 is a cloned mouse probe of repetitive DNA sequences 130 nucleotides long which identify centromeric repeating sequences that are characterized by a banding pattern in Southern blots following restriction digestion of MspI (262). pMR150 was generously provided by Dr. Janet Roussant (Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, Ontario). pMR150 was labelled with digoxigenin-11-dUTP using the PCR DIG Probe Synthesis kit (Boehringer Mannheim, Montreal, Quebec, Canada) and hybridized with the membrane overnight at 65°C, before being washed and developed according to the manufacturers protocol.

**Probe preparation:** The pMR150 was cloned into pBluescriptIIkst. The insert was amplified using primers: sense: 5' ACA CTC CTC GAG ACT GAA AAA CAC ATT CGT 3' and antisense: 5' CTC AAA GAA TTC AGT GTG GTT TTC ATC ATT 3'. The conditions for PCR were 95°C for 5 minutes, then 35 cycles of 54°C, 72°C and 95°C for 45 seconds each, then elongation for 10 minutes at 72°C. The insert and pBSTIIkst (2.9 kb) were each digested with XhoI and EcoRI, dephosphorylated with CIP and then gel purified using the QIAEX kit (Qiagen, Mississauga, Ontario). The two were then ligated together to make pBSTmR150, 3.0 kb in length.

**In vitro methyl acceptance method:** Genomic methylation status was also determined by the in vitro methyl acceptance capacity of DNA using [3H]-methyl] SAM as a methyl donor and a
prokaryotic CpG DNA methyltransferase, Sss1 (190). This assay produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous $^3$H-methyl incorporation. Liver DNA (500 ng) is incubated with 2.0 $\mu$Ci of $[^3$H-methyl] S-adenosylmethionine (New England Nuclear, Boston, MA), 3 units of Sss1 methylase, 1x Sss1 methylation buffer (120 mM NaCl, 10mM Tris-HCl, pH 7.9, 10 mM EDTA, 1mM dithiothreitol) in a total volume of 30 $\mu$L for 1 hour at 30°C. The Sss1 is then inactivated at 65°C for 10 minutes. The in vitro methylated DNA was isolated from 15 $\mu$L of the reaction mixture by filtration onto Whatman DE-81 ion exchange filters (Fisher Scientific. Springfield, NJ) which were air dried for 12 minutes. Once dried, the filter was washed three times for ten minutes each, in a scintillation vial with fresh 20 mL of sodium phosphate wash buffer, pH 7.0. The filters were then rinsed once with 70% ethanol and then once with 100% ethanol before drying under a lamp. Radioactivity of the filters was then measured by using 10 mL non-aqueous scintillation fluor (Cytoscint, ICN, Costa Mesa, CA) and a 1217 RakBeta scintillation counter (Fisher Scientific, Springfield, NJ). The amount of radiolabel bound to the filter in a control (without DNA) was taken as background and subtracted from values obtained from filters with DNA. Background was <1% of uptake observed with DNA samples. All samples were performed in duplicate.

Microsatellite instability assay: Microsatellite instability is a marker for DNA replication error in tumor tissues, an indicator of MMR deficiency. Microsatellite instability was measured to determine the effects of folate on mismatch repair in small intestinal and colonic neoplasms. Microsatellite instability was determined by comparing the electrophoretic mobility of amplified normal and neoplastic DNA using primers from 5 loci on mouse chromosomes 1 (D1Mit4), 2 (D2Mit16), 5 (D5Mit10), 6 (D6Mit8) and 10 (D10Mit2) (Research Genetics, Huntsville, AL) as
described by Reitmair et al. (31,252). Matched normal and neoplastic DNA from the small intestine and colon were selected from each dietary group at two time points. Primers were labeled with [(-3)p] ATP (NEN Life Science) using T4 polynucleotide kinase, reactions were incubated for 1 hour at 37°C, cooled to room temperature for 30 minutes and then heat inactivated for 5 minutes at 94°C. The PCR reaction conditions were as follows: denaturing at 94°C for 5 minutes; then amplification with denaturing at 94°C for 15 seconds, annealing at 58°C for 20 seconds and then extension at 72°C for 20 seconds, for a total of 35 cycles; then further extension at 72°C for 10 minutes. The reactions then had 10 uL of formamide dye (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) added before further denaturing at 94°C for 3 minutes. After the 4% polyacrylamide gel had been pre-run for about 30 minutes, 4 uL of each sample was loaded with a labeled 123 bp ladder separating the samples. The gel was run at 80W for approximately 1.5 hours, then dried at 80°C for 1 hour using a Savant Gel Dryer. Once dry, the film was exposed to Kodak BioMAX MR autoradiographic film overnight at -70°C. A positive case was defined as one showing instability at one or more loci, confirmed in two independently performed PCR reactions.

Statistics: The distribution of each variable was assessed graphically to determine whether it was normally distributed. Those variables that were not normally distributed were subjected to logarithmic transformation before performing significance test. Comparisons of means between the folate-depleted and supplemented groups were assessed by Student’s t-test. Statistical analyses were performed using SYSTAT 5 for Macintosh (Systat, Evanston, IL). All significance tests were two sided, and were considered statistically significant if the observed significance level was less than 0.05. Results are expressed as mean ± standard error of the mean (SEM) of the untransformed data.
2.4 RESULTS

2.4.1 *Apc*+/−*Msh2*−/− mice on folate deficient and supplemented diets *prior* to neoplastic foci development – *intervention* beginning at 3 weeks of age.

2.4.1.1 Body weight

Growth curves were similar between the two diet groups during the study period (data not shown); there was never a significant difference between the average weights of the mice at any time point. This indicates that the folate deficiency in this experiment was mild to moderate. Otherwise, growth retardation or premature death would have been observed.

2.4.1.2. Serum and liver folate concentrations

Mice placed on the folate deficient diet had significantly lower mean concentration of folate in their serum than those mice on the supplemented diet (*p*<0.001, Table 2.1). Although the liver folate concentration in the folate deficient mice was lower than that in the supplemented mice, it fell short of statistical significance (*p*=0.07, Table 2.1), this is likely because of the small number of samples (type II error). Similar to studies completed previously, the serum and liver folate concentrations of the mice in our study reflected the level of dietary folate ingested.

2.4.1.3. Development of small intestinal adenomas and colonic ACF and adenomas.

In the small intestine, mice fed the folate supplemented diet had a 2.7X lower number of intestinal adenomas compared to those fed the folate depleted diet (mean # of polyps ± SEM; 8mg/kg, 110.3 ± 45.2; 0 mg/kg, 299.2 ± 27.4 (*p*=0.004), Figure 2.2). This pattern of intestinal adenoma development between the two diet groups was consistent throughout the length of the small intestine (*p*<0.02). The consistency of small intestinal tumor number throughout the length of the small intestine in our study indicates that folate effects in this model are not site specific.
Table 2.1. Apc+/-Msh2-/- serum and liver folate concentrations, global DNA methylation

<table>
<thead>
<tr>
<th>Age at diet start</th>
<th>Dietary folate</th>
<th>Serum folate</th>
<th>Liver folate</th>
<th>DNA methylation$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg folate/kg diet</td>
<td>ng/ml</td>
<td>μg/g tissue</td>
<td>dpm x 10$^3$</td>
</tr>
<tr>
<td>3 weeks</td>
<td>0</td>
<td>15.0 ± 2.65$^a$</td>
<td>0.82 ± 0.11</td>
<td>163 ± 1.46</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>63.5 ± 7.71$^b$</td>
<td>1.12 ± 0.10</td>
<td>136 ± 16.8</td>
</tr>
<tr>
<td>6 weeks</td>
<td>0</td>
<td>19.8 ± 3.20$^a$</td>
<td>0.70 ± 0.08$^*$</td>
<td>98.4 ± 8.96$^*$</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>95.6 ± 27.0$^b$</td>
<td>1.14 ± 0.18$^b$</td>
<td>224 ± 6.22$^b$</td>
</tr>
</tbody>
</table>

$^1$ mean ± SEM. Means with different superscripts within each age group are significantly different between diets, $p<0.005$ for serum, $p=0.03$ for liver, $p<0.05$ for DNA methylation (independent t-test)

$^2$ there should be an inverse relationship between dpm and level of global methylation
Figure 2.2 Small bowel adenoma enumeration in *Apc*+/-*Msh2*+/- mice started on folate diet prior to the establishment of neoplastic foci. a indicates significant difference p=0.004, b: p<0.02
There were significantly fewer colonic ACF found in the folate supplemented group than in the deficient group (mean # of ACF ± SEM; 8 mg/kg, 19.4 ±7.34; 0 mg/kg, 55.1 ±12.3 (p<0.03), Figure 2.3).

In contrast, there was no significant difference in the number of colon adenomas between the two diet groups (Figure 2.3). However, there was a non-significant trend towards fewer colon adenomas in the folate supplemented group compared to the folate deficient group (p=0.1).

2.4.1.4. Genomic DNA methylation status

The extent of genomic methylation, as assessed by the in vitro methyl acceptance assay was not significantly different between the two dietary groups (Table 2.1). Also, the extent of genomic DNA methylation status, as assessed by the restriction digest method, was not significantly different between the two groups (Figure 2.4). This represents that a moderate degree of folate deficiency given for 8 weeks did not induce a significant degree of genomic DNA hypomethylation.

2.4.1.5. Microsatellite instability

From both diet groups combined, there were 21 small intestinal and colonic tumors from 10 mice analysed for microsatellite instability. There was no difference in electrophoretic mobility between normal and tumor tissue from either of the diet groups at the five loci tested (Figure 2.5). This suggests that microsatellite instability did not play a major role in the development of small intestinal and colonic adenomas. Also, this suggests that superimposed dietary folate deficiency and sufficiency did not affect microsatellite instability in these tumors.
Figure 2.3 Colon ACF and adenoma enumeration of $Apc^{+/-}Msh2^{-/-}$ mice started on folate diet prior to neoplastic foci establishment. a indicates significant difference, $p<0.02$. b: $p=0.1$
Figure 2.4 Genomic DNA methylation in hepatic Apc+/-Msh2-/- mouse tissue on folate diet as assessed by the methyl sensitive restriction digest method. A. Folate intervention prior to neoplastic foci development: no difference in digestion pattern is observed between the two dietary folate groups when intervention provided for 8 weeks. B. Folate intervention post neoplastic foci development: lower molecular weight HpaII digested fragments in the folate supplemented group compared to the folate deficient group, suggesting a higher degree of genomic DNA methylation in the folate deficient group. M and H indicate digestion by MspI and HpaII, respectively.
Figure 2.5 Microsatellite instability in Apc+/-Msh2-/ mice with dietary folate intervention. A. D10Mit2 locus assessed in mice fed diet prior to the establishment of neoplastic foci development. B. D5Mit10 locus assessed in mice fed diet post neoplastic foci development. There was no difference in electrophoretic mobility between corresponding normal and small intestinal or colonic adenoma samples at either loci, indicating a lack of microsatellite instability. S_n indicates normal small bowel tissue; S_{tn} - small bowel adenoma tissue, number n; C_n - normal colon tissue and C_{tn} - colon adenoma tissue, number n.
2.4.2. *Apc*+/−-*Msh2*−/− mice on folate deficient and supplemented diets post neoplastic foci development, diet intervention beginning at 6 weeks of age.

2.4.2.1. **Body weight.**

The mean weight of the supplemented mice was 20% greater than that of the deficient mice at the start of the experiment. The average weight of the mice in the deficient group increased in parallel to that of the folate supplemented mice during the study period, although it remained 20% lower than that of the folate supplemented group at each time point (p<0.03, data not shown). No growth retardation occurred in the folate deficient group and the difference in the mean weight between the two groups was not due to dietary folate deficiency.

2.4.2.2. **Serum and liver folate concentration.**

Serum folate in the mice given the folate deficient diet was significantly lower than that of the folate supplemented mice (p=0.004) (Table 2.1). As well, liver folate was significantly lower in the deficient mice compared to the supplemented mice (p<0.03) (Table 2.1). Similar to studies completed previously, the serum and liver folate concentrations of the mice in our study accurately reflected the level of dietary folate.

2.4.2.3. **Development of small intestinal adenomas and colonic ACF and adenomas.**

In the small intestine, mice placed on the folate deficient diet had a 4.2X lower number of intestinal adenomas compared to those fed the supplemented diet (0mg/kg, 70.0± 17.1; 8mg/kg, 295.0± 59.6 (p=0.001), Figure 2.6). When each of the segments in the small intestine were examined individually, this pattern was maintained throughout the length of the intestine (p<0.02). The consistency of small intestinal tumor number throughout the length of the small intestine in our study indicates that folate effects in this model are not site specific. (Figure 2.6).

In contrast, there was no significant difference in colon adenoma or ACF number when comparing the two dietary folate groups (Figure 2.7).
Figure 2.6 Small bowel adenoma enumeration of Apc+/− Msh2−/− mice started on folate diet following neoplastic foci establishment. a indicates significant difference, \( p=0.001 \), b: \( p<0.02 \).
Figure 2.7 Colon ACF and adenoma enumeration of *Apc*+/−*Msh2*−/− mice started on folate diet following the establishment of neoplastic foci.
2.4.2.4. Genomic DNA methylation status.

The extent of genomic DNA methylation, as determined using the in vitro methyl acceptance assay, was significantly higher in the folate deficient mice compared to the folate supplemented mice (p<0.05)(Table 2.1). This observation was confirmed by the restriction digest method of genomic DNA methylation. There were more lower molecular weight fragments with folate supplemented samples digested with methyl-sensitive HpaII compared to the folate deficient samples (Figure 2.4).

2.4.2.5. Microsatellite instability.

From both diet groups, there were 21 small bowel and colonic tumors from 10 mice analysed for microsatellite instability. There was no difference in electrophoretic mobility between the tumor and normal samples taken from any of the mice, regardless of dietary treatment. This indicates there is no microsatellite instability in the tumors of these Apc+/- Msh2-/- dietary folate treated mice, suggesting that superimposed folate deficiency and sufficiency did not affect microsatellite instability (Figure 2.5).
2.5 DISCUSSION

The results of the present study demonstrated the dual effects of dietary folate deficiency and supplementation on intestinal tumorigenesis in Apc+/Msh2/- mice depending upon timing of intervention.

2.5.1 Intestinal tumor and ACF development in Apc+/Msh2/- mice fed folate diets prior to establishment of neoplastic foci

Folate supplemented diets, 4x the basal requirement, fed to Apc+/Msh2/- mice at three weeks of age (ie. prior to the establishment of neoplastic foci) appears to have a protective effect on small intestinal tumorigenesis and colonic ACF development. There also appears to be a protective effect of folate on the colorectal adenomas, however, this fell short of statistical significance.

There was an almost 300% (2.7X) reduction in the number of small intestinal adenomas in mice fed the folate supplemented diet (4X the basal requirement) from the total observed in mice fed the folate deficient diet. The number of small intestinal adenomas in the folate deficient mice was similar to the number typically found in Apc+/Msh2/- mice on standard lab chow containing the usual basal dietary requirement of folate at 11 weeks of age observed in previous experiments (247). Folate deficiency did not cause an increase in tumor number beyond the maximum number of adenomas observed in this model on control diet (ie. 300-350) in the present study, as the intestines were likely already maximally saturated with tumors and it was not possible to grow more. The incidence curve of small intestinal tumors in Apc+/Msh2/- mice on standard lab chow plateaus at 300-350 at approximately 11 weeks of age (247), thus despite being fed folate deficient diets, these mice were unable to grow more tumors. Both epidemiologic (132-137,139,140,146-149,254-256) and rat DMH studies (163,164) have observed an enhancing effect of a moderate degree of folate deficiency on the development of
colorectal cancer. However, the long term use of the folate antagonist, Methotrexate, as treatment for other non-neoplastic diseases, such as inflammatory arthritis and autoimmune disorders, has not resulted in any reports of increased risk of tumor development (263,264). Nonetheless, the possibility that folate deficiency can enhance the small intestinal adenoma development cannot be ruled out for certain. An important follow-up study would be to sacrifice the mice at 7 weeks of age, before the age the tumor incidence begins to plateau. Then, any accelerating effects of folate might be observed.

These results, illustrating the protective effect of folate provided prior to the establishment of neoplastic foci, support findings from previous epidemiological studies, both case-control and prospective, which showed an average reduction of 35-40% in colorectal adenoma and adenocarcinoma risk when comparing the highest dietary folate group to the lowest (132-140). A recent large human prospective study conducted in 88,756 female nurses, observed a 75% reduction in colorectal cancer risk in women with longterm (≥ 15 years) multivitamin use (with ≥ 400 μg folic acid) compared to those not taking supplements, following adjustments for confounders (149). Confounders corrected for in this study included vitamins A, C, D, E and calcium (149). The present study also corroborates the findings from earlier chemical carcinogen rat studies, where a moderate degree of dietary folate deficiency (0 mg folate/kg diet) has been observed to enhance, whereas folate supplementation at 4X the basal requirement protects against, the development of colonic neoplasms in the DMH rat model of colon carcinogenesis (133,164).

There were almost three times fewer ACF in the folate supplemented mice when compared to the folate deficient mice. This indicates that folate may have a preventative effect on the early stages of colonic tumorigenesis as ACF are proposed earliest precursors to colonic adenomas (250). This finding is supported by a previous study, conducted in the DMH rat
model, which found that 100% of the folate depleted rats developed microscopic tumors, compared to only 29% in the control diet group, suggesting an early effect of folate in colorectal carcinogenesis (160). However, despite fewer ACF in the folate supplemented group, there was no difference seen in the number of colonic tumors found between the two dietary folate groups. The lack of observable effect may be due to the small number of colonic adenomas observed in the present study or the number of mice included in each dietary group was too small for a statistically significant difference to be observed (ie. a type II error). These results may also be related to the fact that the progression of ACF to adenoma is not well established in this model as most mice die at an early age, and likely there is not enough time for tumors to develop in the colon (247). The accelerated intestinal adenoma development seen in the small intestine does not appear to occur in the colon of these Apc+/−Msh2−/− mice.

2.5.2 Intestinal tumor and ACF development in Apc+/−Msh2−/− mice fed folate diets following establishment of neoplastic foci

In the mice started on folate diets at six weeks of age, after the establishment of neoplastic foci, there was a different effect of folate on small intestinal tumorigenesis to that observed in mice started on diet prior to the establishment of neoplastic foci. In this six week diet start study, the folate deficient diet decreased the number of tumors compared to the folate replete diet by 4.2X (p<0.001). Thus, in this case, folate depletion has a role in inhibiting the progression of small intestinal neoplastic foci to adenomas. Since folate is a required vitamin in many biological processes, including DNA synthesis, cell proliferation, and repair mechanisms, in a state of folate depletion, the body would be lacking cofactors and precursors necessary for cell replication during tumorigenesis. The folate deficiency would hinder DNA synthesis by limiting the amount of thymidylate available, thus inhibiting tumor growth. This is particularly
important in a neoplastic cell where cell replication is occurring at an accelerated rate. The depletion of folate from rapidly dividing cancer cells using folate antagonists, such as Methotrexate and 5-fluorouracil (127,128), has been a method of chemotherapy for certain cancer patients with much success. Several other studies have been conducted which support the effect of folate deficiency on established cancers. When a cancerous tumor is transplanted into folate deficient mice, there is an inhibition of growth (129); virally induced cancers are reduced when deprived of folate (130) and transgenic mice predisposed to developing nerve sheath tumors experience delayed tumor development when dietary folate is restricted (131). Thus, there is a retardation of growth of established tumors by folate depletion. Previous work in the rat DMH model also showed a non-significant increase in the progression of microscopic to macroscopic tumors in mice fed diets with 20× the basal requirement of folate (164). However, the results from the present Apc+/-Msh2-/- study cannot confirm the suggested promotory effect of folate supplementation on tumor progression. The Apc+/-Msh2-/- mice fed the 8 mg folate/kg diet in our six week diet start study, did not increase the number of tumors in the small bowel beyond the maximum number observed in this model fed standard lab chow. This may be the small bowel had already been saturated with adenomas (247). This is similar to the effects of the 0 mg folate/kg diet in the 3 week diet start study. Thus, there may have been an accelerating effect of these diets at their respective time points, but the effect of the genotype was already too overpowering for any effects of folate to be seen. In order to observe the potential accelerating effect of folate, we suggested in the 3 week diet start study to sacrifice earlier, prior to the tumor incidence plateau. However, this would likely be ineffective in the 6 week diet start study as intervention would then be only one week in duration. One week is not long enough for folate to have its effects on tumorigenesis in these mice.
In the colon, there was no effect on the number of ACF or the number of adenomas. The lack of effect of folate on established ACF, may be due to the short 11 week life span of these mice (247), or perhaps dietary folate has no significant modulatory effect on ACF after they have been established. It may be that in these mice, small intestinal adenomas with accelerated tumorigenesis are susceptible to the effect of folate depletion, while colonic ACF with a normal or slower growth rate are not. Again, dietary folate had no observable effect on the development of colonic adenomas. This may be related to the fact that progression of ACF to adenoma is not well established in this model as most mice die at an early age, and likely there is not enough time for adenomas to develop in the colon (247). The accelerated intestinal adenoma development seen in the small intestine does not appear to occur in the colon of these Apc+/-Msh2/- mice. The lack of observable effect may also be due to a type II error, where there were not enough adenomas and too few mice in each group for significant differences to be observed.

2.5.3. Genome-wide DNA methylation status

The extent of genomic DNA methylation, in our study, varied depending upon the duration of dietary folate intervention in these Apc+/-Msh2/- mice. The mice in the three week diet start study (ie. 8 weeks of folate intervention) showed no difference in the extent of genomic DNA methylation, regardless of the level dietary folate they were provided. However, in the mice started on folate deficient or folate supplemented diets at six weeks of age (ie. 5 weeks of folate intervention), there was a significant increase in the extent of genomic hepatic methylation compared to the folate supplemented mice (p<0.05).

In normal eukaryotic cells, the majority of cytosines in the CpG dinucleotide are methylated (106). Genomic and site-specific DNA hypomethylation has been shown to be a consistent occurrence in colorectal carcinogenesis (14,16). Studies have shown genomic DNA
methylation is lower in tumor cells compared to normal cells (96,98-100) and site-specific hypomethylation often occurs at proto-oncogenes in neoplastic transformation (106,115). Hypermethylation has also been associated with the carcinogenic process and is thought to exert its effects by occurring at CpG islands within or upstream from the promoter region, inactivating tumor suppressor genes (ie. APC) to confer a growth advantage to transformed cells (68). Also, hypomethylation resulting from a reduction in DNA MTase activity in Min mice due to a combination of heterozygosity for DNA MTase and use of a DNA MTase inhibitor resulted in a decrease in tumor formation (84).

The inverse relationship of dietary folate deficiency to an increased extent of genomic DNA methylation observed in the six week diet start study is in contrast to previous studies conducted in rodents thus far, where only studies involving diets deficient in all methyl donors (ie. methionine, choline, folate, B12) (98,99), choline and methionine only (265) or severe folate deficiency (191) have shown genomic DNA hypomethylation. In contrast, in a previous rodent study, a moderate folate deficiency (where the degree is the same as the present study) given for 25 weeks did not induce a significant degree of genomic or protooncogenic specific DNA hypomethylation (190). However, in a subsequent rat study, severely folate depleted rats had a 59% decrease in the extent of genomic DNA methylation compared to control rats, after six weeks of dietary intervention (189). In the present study, a moderate folate deficiency for five weeks resulted in a greater extent of genomic DNA methylation than folate supplementation. This increase in genomic DNA methylation may be due to an increase in DNA MTase activity, induced by the cell in an effort to increase methylation to compensate for the lack of folate available as a methyl donor (189). There are several studies which have demonstrated an increase in DNA MTase activity resulting from dietary methyl donor deficiency (112,266).
Also, although the diet used in this study had 0 mg folate/kg, the amino acid defined diet still provided other methyl sources, including choline and methionine (253). Methionine is the immediate precursor to SAM, the primary methyl donor for the majority of methylation reactions within the body and can either be ingested through dietary sources or generated through the methylation of homocysteine (Figure 1.3 in Chapter 1)(267). Greater than 50% of homocysteine in the body is remethylated, depending upon the dietary content of methionine and choline (267). Choline is a precursor to betaine, another one carbon methyl donor which catalyzes the homocysteine remethylation reaction (Figure 1.3 in Chapter 1) (267,268). The methyl donors that are available, such as methionine and choline, are favored to enter the methylation pathway, which, coupled with increased MTase activity, may account for the hypermethylation observed in the folate deficient mice.

Therefore, moderate folate deficiency after 5 weeks of dietary treatment, results in hypermethylation due to a compensatory increase in DNA MTase activity, but by 8 weeks of treatment this effect is no longer observed.

The implication of global hypermethylation resulting from folate deficiency is important as there was a significant finding of small intestinal tumor inhibition when folate intervention occurs following the establishment of neoplastic foci. In mice with tumors already developed, there is an increased requirement of folate by these tumor cells, in order to sustain DNA synthesis necessary for accelerated tumor cell growth. However, since these animals are folate deficient and have an increased compensatory increase in MTase activity, we hypothesize that all of the methyl donors that are available are shunted into the methylation pathway. This would account for the increase in methylation in folate deficient mice. Folate, as 5,10-methylene tetrahydrofolate, is an immediate substrate for thymidylate synthetase, the enzyme used for the formation of thymidylate from deoxyuridylate in DNA synthesis (Figure 1.3). Choline is a
cofactor in the transmethylation of homocysteine to methionine, which is an immediate precursor to S-adenosylmethionine, the primary methyl donor in DNA methylation reactions (Figure 1.3). Therefore, we hypothesize that folate deficiency induces a compensatory increase in MTase activity which uses the other methyl donors, such as methionine and choline, to maintain methylation. However, as a result, tumor cells have insufficient folate and cannot support the increased requirement for DNA synthesis, thus cells are unable to replicate and there is a retardation in tumor growth in the small intestine.

Laird et al., generated a mouse model which is heterozygous for the DNA methyltransferase gene and the Apc gene and further treated it with 5-aza-2’-deoxycytidine, DNA MTase inhibitor, to study the effects of reduced DNA MTase activity and resulting methylation levels on intestinal tumorigenesis in the Min mouse model (84). They observed a significant reduction in the number of tumors developed in mice with reduced DNA MTase activity, resulting from a combination of being heterozygous for the DNA MTase and treatment with 5-aza-2’-deoxycytidine (84). This study is in contrast to our study in that in our Apc+/−Msh2−/− mice, hypermethylation induced by folate deficiency resulted in a decrease in tumor number. However, this is when intervention occurred following the establishment of neoplastic foci. In the three week diet start study where folate intervention occurred prior to the establishment of neoplastic foci, there was no effect of diet on methylation. Hypo or hypermethylation as a result of folate deficiency or supplementation did not occur, despite the observation of tumor suppression in mice on the folate supplemented diet. Thus, this study does not support the Laird paper, however, the relationship among folate, methylation and tumorigenesis is still unclear.
2.5.4. Microsatellite instability

The microsatellite instability (RER+) phenotype is characterized by DNA replication errors occurring in simple repeat sequences due to a deficiency in mismatch repair (MMR). Errors in the DNA, not corrected by MMR, would cause alterations in the size of these repeat sequences, compared to normal DNA; thus electrophoretic mobility shifts in tumor DNA compared to normal DNA would be an indication of mismatch repair failure. In humans, microsatellite instability is associated with the majority of HNPCC tumors (269-271) and approximately 15-20% of sporadic colorectal carcinomas (269,270). Patients with HNPCC carry germline mutations of mismatch repair gene, including $MSH2$, $MLH1$, $hPMS1$, $PMS2$ and $MSH6/GTBP$ (118,120,272). Tumors from the MMR deficient $Apc^{+/-}Msh2^{-/-}$ mouse model do not share the microsatellite instability characteristic typically found in hereditary human colorectal cancer (247). It was thought that an additional damaging factor, such as folate deficiency, which has the potential to compound the mutations resulting from genetic manipulation by interfering with DNA repair, might increase the occurrence of microsatellite instability resulting in a positive finding of RER.

Folate deficiency has been recently shown to interfere with DNA excision repair (204), the mechanism of which is related to the crucial role of folate in the de novo synthesis of purines and thymidylate. However, excision and mismatch repair are repair systems that serve different functions. Excision repair is responsible for removing damaged short sequences or bases and replacing them with normal bases using the complementary strand as a template (205), whereas mismatch repair recognizes and excises mismatches and then replaces them with the appropriate nucleotide (116).

The results in both the three and six week diet start studies indicate that widespread microsatellite instability does not occur in these accelerated intestinal adenoma models. The
appearance of microsatellite instability is only expected with the folate deficient mice, as it is unlikely folate supplementation would cause a disruption in mismatch repair. In the six week diet start study, where the results show a decrease in tumor number with folate deficiency, we would expect no indication of RER+ as microsatellite instability is unlikely to result in less tumor development. In the three week diet start study, there were also no RER+ tumors despite the superimposition of folate deficiency, which has been associated with defects in MMR (157). A recently published human study (157) found that serum and colonic concentrations of folate were 30-50% lower in ulcerative colitis patients with RER+ non-neoplastic rectal mucosa, however, this result was not statistically significant (157). Although the values of serum folate were lower, they were still within the range of folate considered normal. Cravo et al. hypothesized that folate depletion would contribute to the accumulation of genomic mutations by impairing DNA repair in patients with long standing ulcerative colitis. One patient showed a change in three of six previously positive MI markers when supplemented with 5 mg folate/day for 6 months (157). This study suggests that folate may have an important role in DNA mismatch repair. Thus, the lack of folate effect on the microsatellite stable phenotype of these mice does not eliminate the possibility of an association between folate and mismatch repair.

It is expected that without Msh2, these mice would have ineffective mismatch repair, indicated by microsatellite instability, but this is not the case. This suggests that mismatch repair may not be dependant upon Msh2 to function. It has been shown previously that there are redundancies within the mismatch repair mechanism. In yeast, it has been suggested that MSH3 and MSH6 may partially substitute for each other (125), as both proteins bind with MSH2 to recognize mismatches. Since the yeast mismatch repair system is quite similar to the eukaryotic system (118), this explanation could be extrapolated to our mismatch repair deficient mouse model. A parallel redundancy in the mouse system would help to explain the lack of the RER+
phenotype in our \textit{Apc+/-Msh2-/-} mice. This RER- phenotype is also seen in \textit{Msh6} null mice which are also predisposed to tumor development but do not develop the microsatellite instability characteristic (118), unlike other mismatch repair animal models such as mice which are deficient in \textit{Pms2} or \textit{Mlh1} that do express the RER+ phenotype (117,124). The activation of this redundant system may be mediated by other genes, such as \textit{Apc}, as mice which are \textit{Msh2-/-} only, do express the RER+ phenotype and develop RER+ lymphoid tumors (31), but the \textit{Apc+/-Msh2-/-} do not (247). This hypothesis is not supported by the development of a \textit{Pms2-/-;Min} mouse which does have an accelerated intestinal tumor phenotype accompanied by an RER+ phenotype (273). Nonetheless, despite the lack of microsatellite instability in our \textit{Apc+/-Msh2-/-} mice, the lack of Msh2 may still participate in increasing somatic mutations in the \textit{Apc} gene. In the control mice of \textit{Apc+/-Msh2-/-}, although all tumors had inactive Apc, only 15\% of them demonstrated LOH (247). This is in contrast to Min mice whose tumors are characterized by LOH of \textit{Apc} (28). In tumors from both the \textit{Mlh1/Apc1638} and the \textit{Pms2-/-;Min} mouse models, there was a lack of Apc protein due to somatic mutations in the wild type copy of the \textit{Apc} gene (249,273). Thus, a similar mechanism may occur in the \textit{Apc+/-Msh2-/-} model, where a lack of mismatch repair has an effect on the mechanism of \textit{Apc} inactivation in the tumorigenic process.

In these \textit{Apc+/-Msh2-/-} mice, other mechanisms of repair may compensate for the loss of Msh2, explaining the absence of the replication error phenotype. Folate deficiency and supplementation had no significant effect on microsatellite instability in these mice, suggesting that microsatellite instability is not a mechanism by which folate modulates small intestinal and colonic tumorigenesis.

Before attempts can be made to extrapolate our data to the human situation, there are several limitations to the \textit{Apc+/-Msh2-/-} mouse model that need to be identified. This model differs from humans in that the majority of intestinal adenomas that develop do so in the small
intestine, compared to the colon tumors that develop in FAP patients. These mice do not develop adenocarcinoma at any location in their intestine, as they die from bowel obstruction and anemia before it can develop. In addition, the relationship of colonic ACF to adenoma has not been established, but, ACF does not appear to be a precursor to adenoma development. Unlike human HNPCC, microsatellite instability does not appear to play a role in the tumorigenesis of these \( Apc^+/-Msh2^-/- \) mice. Thus, these mice may be best suited as a model for familial types of accelerated tumorigenesis, such as FAP, as opposed to sporadic colorectal carcinogenesis. In spite of these limitations, the \( Apc^+/-Msh2^-/- \) mouse model still provides an excellent medium for the study of dietary or pharmaceutical chemopreventive agents on CRC. This model spontaneously develops small and large intestinal adenomas and colonic ACF, it is similar genetically to human CRC and its accelerated intestinal tumorigenesis phenotype allows us to study the effects of agents prior to and following the establishment of neoplastic foci in a short time period.
2.6 CONCLUSION

Timing of intervention is important in determining the effects of folate as it has dual effects, depending upon whether folate intervention begins prior to or following establishment of neoplastic foci. The results of this study indicate that when a folate supplemented diet is provided prior to neoplastic foci development in the Apc+/-Msh2-/- mice, there is a preventative effect of folate on intestinal tumorigenesis. In contrast, when folate deficient diets are provided after the development of neoplastic foci, there is a decrease in tumor number. The mechanism responsible for these effects has yet to be elucidated. Global DNA methylation was only transiently affected by dietary folate. In folate deficient mice, after 5 weeks of treatment, there was an increase in genomic hepatic DNA methylation due to an increase in MTase activity, brought on to compensate for the lack of folate available for DNA methylation. Despite being both Msh2 and folate deficient, there was no evidence of MI in tumor or normal tissue at three or six week diet starts. Thus, the results of this study suggest that folate deficiency appears to have no effect on the ability of Msh2 mediated DNA MMR to function. The negative RER phenotype may also suggest that the mismatch repair gene Msh2 does not play a large role in the ability of mismatch repair to function normally in the Apc+/-Msh2-/- mouse model.

Thus, more study is required to accurately determine a therapeutic dose and appropriate time of intervention of folate which provides an effective chemopreventive effect on colorectal carcinogenesis. As well, the mechanisms by which folate mediates these effects should be further investigated so that they may be clearly defined.
CHAPTER 3. THE EFFECTS OF DIETARY FOLATE IN MIN MICE

TUMOR DEVELOPMENT

3.1. INTRODUCTION

Min mice, with an Apc+/- genotype, develop spontaneous small intestinal tumors and carry a genetic mutation which reflects that of human FAP. Min mice begin to develop benign adenomas in the intestine, predominantly in the small intestine, at less than one month of age. At our institution, they develop about 30 tumors in their small intestines, no ACF. 5 colorectal tumors and have a lifespan of about 6 months before they die of anemia or bowel obstruction. The Min mouse model is similar genotypically and phenotypically to the human situation in several ways. The Apc gene in mice is almost identical to the human APC gene (246), the Apc gene is frequently mutated in both human and mouse intestinal tumor development (13,27,41,226,246) and Min mice develop multiple spontaneous intestinal adenomas similar to human FAP patients (227).

In the previous Apc+/-Msh2/- mouse model, we identified the dual effects of folate on intestinal tumorigenesis depending on the timing of folate intervention. However, we were only able to determine the effects of two folate diets, one folate deficient and the other folate supplemented with 4x the basal requirement. This was due to the difficult nature of breeding these mice and time constraints that did not allow the generation of a sufficiently large number of these mice to achieve adequate statistical power. Therefore, we decided to use the Min mouse model of intestinal tumorigenesis to determine the effects of four different levels of dietary folate given at three weeks of age. In addition to the diets used in the first study, diets with the basal requirement of folate (2 mg folate/kg diet) and 10X the basal requirement (20 mg folate/kg
diet) were used to determine the chemopreventive effect of folate over a wide range of dietary folate levels. The three week diet start was chosen as the Min mice do not share the accelerated intestinal tumorigenesis phenotype demonstrated by the Apc+/−Msh2−/− mice and an equivalent time point for post neoplastic foci development in the Min mice had not been determined. In order to compare with previous animal studies, the diets and the sacrifice time chosen were similar to those previously reported (101,164,230,237,240). These studies involving chemically induced CRC rat models have previously shown an inverse relationship between dietary folate intake and tumor incidence (163,164).
3.2 SPECIFIC OBJECTIVES

1. To determine whether sustained folate deficiency of a moderate degree accelerates the development of intestinal neoplasms in Min (Apc+/-) mice.

2. To determine whether dietary folate supplementation prevents or retards the development of intestinal neoplasms in this model and to determine what level of dietary folate is needed for such an effect.
3.3 MATERIALS AND METHODS

Study design: Min (Apc+/-) mice purchased from Jackson Laboratory (Bar Harbor, ME) were generated as previously described (223). These mice were randomized to receive one of the four folate amino acid defined diets. There were 20 animals used per diet group (10 per time point), for a total of 80 animals. Two time points of sacrifice were used to determine the effects of folate on tumor incidence at an early and late stage of intestinal tumorigenesis in this model (Table 3.1). Min mice have been found to develop both intestinal and colorectal adenomas at less than one month of age before eventually dying from anemia and intestinal obstruction at 6 months (247). Thus, the animals were put on diet at 3 weeks of age and early and late time points were chosen as 3 and 6 months, respectively. These time points have been used previously in this Min mouse model; several previous studies have utilized 90-110 days of age as time of sacrifice (230,237,240), as well, one study used 200 days of age as a late time point of sacrifice (237). Diet and water were provided ad libitum. Mice were weighed weekly. Upon completion of the study, mice were killed by cervical dislocation, serum and liver were harvested for folate concentrations and the intestines were removed for tumor and ACF enumeration. For detailed description of procedures, please see Chapter 2, Section 3, Materials and Methods.

Statistics: The distribution of each variable was assessed graphically to determine whether it was normally distributed. Those variables that were not normally distributed were subjected to logarithmic transformation before performing significance test. Comparisons of means between the folate-depleted and supplemented groups were assessed by ANOVA, then further analysed using least significant difference. Statistical analyses were performed using SYSTAT 5 for Macintosh (Systat, Evanston, IL). All significance tests were two sided, and were considered
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<th>Age on diet</th>
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Table 3.1: Min study design
statistically significant if the observed significance level was less than 0.05. Results are expressed as mean ± standard error of the mean (SEM) of the untransformed data. Correlation was tested using the Pearson’s Correlation test.
3.4 RESULTS

3.4.1 Body weight

There were no significant differences in the average body weights of the mice among the groups at each time point during the study period (data not shown). There did not appear to be any growth retardation or early morbidity in the mice fed the folate deficient diet, indicating that the deficiency of folate was mild to moderate.

3.4.2 Serum and liver folate concentrations

3.4.2.1 At the three month sacrifice

Serum folate concentrations showed that those mice fed the deficient diet had concentrations significantly lower than mice on the basal and 10x basal requirement of folate diets (p<0.001) (Figure 3.1). Mice fed the basal requirement of folate had significantly less serum folate than mice fed the 4x basal diet (p<0.03). There was no difference between the serum folate measured in the mice fed the 10x basal diet compared to the other folate supplemented diets. Thus, there was a stepwise increase in the serum folate concentrations with increasing dietary folate, to a maximum of 4x the basal requirement of folate.

Folate deficient mice had lower liver folate concentrations than mice on the basal and 10x basal requirement of folate (p<0.04) (Figure 3.1). Also, mice on the 10X basal diet had more folate than the other less supplemented diet mice (p<0.006). There was a moderate but significant correlation between the serum and liver folate concentration, with a Pearson correlation coefficient of 0.42 (p<0.01).

3.4.2.2 At the six month sacrifice

Serum folate concentrations showed that those mice fed the deficient diet had concentrations significantly lower than mice on the other three diets (p<0.001) (Figure 3.2). The mice fed 2 mg folate/kg diet had significantly lower serum folate concentrations than those mice
Figure 3.1  A. Serum and B. Liver folate concentrations in Min mice sacrificed at three months. Different letters indicate significant difference, p<0.04.
Figure 3.2 A. Serum and B. Liver folate concentrations in Min mice sacrificed at six months. Different letters indicate significant differences (p<0.02).
fed the 8 mg folate/kg diet ($p<0.02$). There was no difference between the serum folate concentrations measured in the mice fed the 10x basal requirement of folate and the two other folate supplemented diets. Thus, there was a stepwise increase in the serum folate concentrations with increasing dietary folate, to a maximum of 4x the basal requirement of folate. There was no significant effect of the diet being administered for an additional three months, that is, the folate concentrations in the serum stayed the same at three months and at six months.

Liver folate concentrations indicated that the deficient mice had significantly less folate in their livers than the other diets ($p<0.02$) (Figure 3.2). As well, mice on the basal diet had significantly less folate in their livers than mice in the 4X basal group ($p<0.05$). There is adequate correlation between the serum and liver folate concentrations in these mice, as evidenced by a Pearson correlation coefficient of 0.67 ($p<0.001$).

### 3.4.3. Small intestinal tumor and colonic ACF and tumor development

#### 3.4.3.1. At the three month sacrifice

There was no significant difference in the number of duodenal, jejunal or overall small bowel tumors among the diet groups. However, in the ileal portion of the small bowel, there were significantly fewer tumors in the 20 mg folate/kg diet group when compared to the other three diets ($p<0.008$) (Figure 3.3). In the colon, there was significantly more ACF with 0 mg/kg dietary folate when compared to the other three diets ($p<0.04$) (Figure 3.4). There was no significant difference in the number of colonic tumors between the four diet groups.

#### 3.4.3.2. At the six month sacrifice

There was no significant difference in the number of duodenal, jejunal or overall small bowel tumors among the diet groups. However, in the ileum, there were significantly fewer
Figure 3.3  Min small intestinal tumor enumeration at 3 months time point. a indicates significant difference, p<0.008
Figure 3.4 Colon ACF and tumor enumeration in Min mice sacrificed after three months of dietary folate treatment. a indicates significant difference, p<0.04
tumors observed in the folate deficient group when compared to the other three diet groups (p<0.004) (Figure 3.5). In the colon, there were no differences observed in the number of ACF or tumors among the four diet groups (Figure 3.6).
Figure 3.5. Min small bowel tumor enumeration at 6 month time point. a indicates significant difference p<0.004
Figure 3.6 Colon ACF and tumor enumeration in Min mice sacrificed after six months of dietary treatment.
3.5 DISCUSSION

3.5.1 Serum and liver folate concentrations

In Min mice sacrificed at three months and at six months, there was a stepwise increase in serum folate concentrations with increasing dietary folate, up to 4x the basal requirement of folate. This result would imply that in these mice there is plateau, where folate supplementation cannot increase serum folate concentrations beyond 8 mg folate/kg diet. The plateau result observed in the Min mice is consistent with previous dietary folate studies in rats which have shown stepwise increases in plasma and colonic mucosal folate concentrations correlating to the increases in dietary folate intake (101,164). In those rats, plasma folate increases up to a dietary intake of up to 40 mg folate/kg diet, however, colonic mucosal folate reaches a plateau in rats at the 4X basal requirement, 8 mg folate/kg diet (164). Mucosal folate was not measured in the Min study as the intestines of mice are quite delicate and obtaining colonic mucosa would interfere with adenoma enumeration.

In the liver of these Min mice, the stepwise increase in folate concentration is observed with a plateau effect at 4X the basal requirement at the six month time point, but not at three months. The plateau effects and small discrepancies between and within serum and liver folate may be attributed to folate metabolism in mice. Folic acid is composed of pteroic acid with one or more glutamates attached (185). Typically, dietary folates are polyglutamate derivatives which are hydrolyzed to monoglutamate forms in the gut before being absorbed in the intestinal mucosa (185). Folate then circulates in the plasma as monoglutamates before being taken up by the liver and metabolized back to its polyglutamate derivatives, folate is then retained in the liver or released to the blood or bile (274). Polyglutamates are preferentially retained by tissues and are more effective substrates than monoglutamates (185). Biliary excretion of folate in humans has been estimated to be almost 100 μg per day (275), however much of it is reabsorbed into the
small intestine. One possible explanation for the plateau effect seen in the Min mice in this present experiment, is that there is a similarly high rate of folate excretion into the bile, but due to the presence of tumors in the intestine, there is malabsorption, resulting in a loss of folate (185). Also, it has been reported that there is limited ability of tissues to accumulate and store levels of folate greater than what is required for normal metabolism (276). This is because folylpolyglutamate synthetase (FPGS), the enzyme required to synthesize the polyglutamate storage form of folate, will be limited by the competition brought on by the excess substrate (276). Thus, much of the transported folate will not be in the retainable polyglutamate form of folate. Therefore, Min mice on folate supplemented diets, particularly the diet with 10x basal requirement of folate, may be unable to retain the excess folate due to overwhelming demand for FPGS.

Another study found that large doses of folate increase tissue and folate levels only slightly, due to the varying levels of FPGS activity in different tissues (277). That same study found that, unlike the folic acid used in our Min study, 5-formyl-tetrahydrofolate (5-formylTHF) is more effective at elevating tissue folate levels (277).

There has been previous discussion that the blood folate measurement used is not sensitive enough to detect small differences in folate concentration, which are often enough to cause differences in tumor development (144). Several studies have shown that although there is increased risk with lower dietary folate, the folate concentration in the ‘folate deficient’ group is still within normal range (144,150). Possibly, a more sensitive indicator would be the use of homocysteine as a measure of folate status (151). Homocysteine is an amino acid synthesized during the metabolism of methionine and has often thought to be a more sensitive indicator of cellular folate status than blood levels (155). Serum homocysteine has an inverse relationship to
folate status and, accordingly, has been found to be greater in patients with intestinal adenomas than in controls (151).

Serum folate, which has been established as an indicator of systemic folate status, may not be a good indicator of cancer risk as studies have shown that despite systemic folate status, deficiency in specific tissues is what dictates the potential of neoplastic development in these tissues (278). Thus, there is question as to whether serum folate or blood folate in general is indicative of the concentration of folate in colonic mucosa where the neoplastic changes are taking place. One human study found no relation between blood and colonic cell folate concentrations, thus it is possible blood is not the only source of folate for colonic mucosal cells, some may come from the bacteria located in the intestinal lumen (152). However, Kim et al. found a significant correlation between folate concentrations in human colonic biopsy samples and serum and RBC folate (151). Also, in rats, Kim et al. found that plasma and colonic mucosal folate concentrations both correlated directly with dietary folate (164).

3.5.2 Small intestinal tumor and colonic ACF and tumor enumeration

Dietary folate given for three months had no significant effect on the development of duodenal, jejunal and total small intestinal adenomas. However, the 10x basal requirement level of folate resulted in a decrease in the number of adenomas in the ileum. Because the serum level of folate on 10X dietary folate was not significantly different from that of the 8 mg folate/kg diet, other factors other than folate might have mediated this protective effect. Our finding is in contrast to a previous DMH rat study which found a non-significant trend towards increased tumor incidence associated with the supraphysiological dose of 20x the basal requirement (279). In that rat study, this trend was attributed to the strong procarcinogenic milieu induced by a DMH dose much higher than the conventional dose used. In this environment the high doses of
Folate would accelerate the growth of already established microscopic neoplastic foci induced by high doses of DMH. However, in the Min mouse situation, tumor development is not as severe or accelerated as the DMH model as Min do not develop carcinoma of the intestine. Therefore, treatment with high levels of dietary folate may not have the same effects.

The protective effect of dietary folate supplementation on intestinal tumorigenesis has been observed previously (136-140,148,255). However, what was unexpected was the site specificity of the protective effect of 10X basal folate diet on intestinal tumorigenesis in the ileum of the Min mouse. Site specificity of chemopreventive agents concerning small intestinal and colonic tumorigenesis in Min mice has been previously observed (7,230,237,238). In Min mice, the distal small bowel was observed to be susceptible to the actions of chemopreventive agents (237), which may explain the effects of folate on the ileum of the Min mouse only. In spite of the observation that, at three months, mice fed the 10X basal requirement of folate showed no difference in serum or liver folate concentrations compared to other folate supplemented diets, there may be a localized folate increase in the ileal mucosa that is exerting its protective effects.

In Min mice that were sacrificed at the late time point of 6 months, the modulatory effects of folate were again only seen in the ileum. At 6 months of age, Min mice fed the folate deficient diet had fewer ileal adenomas than mice on other folate supplemented diets. Since the effects of folate in the small intestine at the three month sacrifice were only seen in the ileum, it is not surprising that this was the only affected location at the six month sacrifice. The effect of fewer ileal adenomas in mice fed folate deficient diets may be a result of tumor regression in this tissue. The six month time point is long enough so that ileal tumors have developed and depletion of folate can cause a regression of adenomas, similar to the effect of anti-folate chemotherapeutic agents, such as Methotrexate and 5-fluorouracil. This would also be similar to
the *Apc+/−Msh2−/−* mice in Chapter II, where the presence of folate deficiency after tumors have begun to develop decreases the number of tumors. This would suggest that tumors have already started to develop in the Min mice at some point early in their lifespan.

In the present study, folate deficiency induced early in the tumorigenic process has been found to induce an increase in ACF, an intermediate biomarker for CRC in Min mice at three months. This is consistent with an earlier animal study utilizing the dimethylhydrazine (DMH) rat model of CRC which showed that 100% of rats fed a folate deficient diet developed microscopic neoplasms compared to 29% of rats fed an 8 mg folate/kg diet (163). This present study also corroborates other epidemiological studies which suggested that mild to moderate folate deficiency results in increased CRC risk (136-140,148,255). Although it has not yet been established when folate would exert its mediating effects on CRC development, the results of this study would suggest that it would have early effects on the carcinogenic process.

However, there was no significant difference in the number of ACF among the three folate supplemented groups. There are several possible explanations for this. First, this result may indicate that 2 mg folate/kg diet is adequate to protect against ACF development. Second, the number of mice involved in each group of this study may have been too small for the detection of differences in ACF number, that is, a type II error may have occurred. Finally, the number of ACF developed per Min mouse fed the folate supplemented diets may have been too small for adequate statistical analysis, again, resulting in a type II error.

Our data also suggest that there is no significant effect of dietary folate on tumorigenesis in the colon. The lack of modulatory effects of folate in the colon, despite effects on the number of ACF, is not surprising as ACF do not contribute significantly to the colon adenoma population in adult *Apc+/−* mice (248). Based upon a mathematical model created for the *Apc+/−Msh2−/−* mouse model, it is possible that the adenomas that develop in the colon arise
perinatally (247). If this is true, then folate intervention at three weeks of age would be too late to have an effect on tumor initiation in the colon. Another possible explanation for the lack of effect of folate on colon tumorigenesis may relate to the varying characteristics of the intestine itself. One study conducted in Min mice treated with the NSAID piroxicam found that the duodenum and colon were relatively resistant to chemoprevention (237). They suggested that this regional bias may be due to varying concentrations of drug in the intestine or possibly biological mechanisms within the intestine, as the tumors seen in these areas are morphologically different to the distal small intestine (237). The latter explanation may be more accurate as other studies have reported a similar colonic resistance to chemopreventive agents, such as NSAIDs (238,240,241) and dietary factors, such as vegetable-fruit mixtures (230) and soybean Bowman-Birk inhibitor (232).
3.6 CONCLUSION

In Min mice, the effects of dietary folate on intestinal and colonic tumorigenesis are complex. However, it appears that dietary folate deficiency enhances the development of the earliest precursor of CRC in the colon, ACF. In the small bowel, folate supplementation at 10X basal requirements had a protective effect on ileal tumorigenesis. Sustained folate deficiency, for 6 months, had a regressive effect on adenomas that had already developed in the ileum. The distal small bowel, in contrast to the proximal small bowel and large colon, has been previously shown to be sensitive to the actions of chemopreventive agents in CRC. In Min mice, small intestinal and colorectal tumorigenesis are different processes in terms of the extent, speed and susceptibility. The small bowel develops a greater number of adenomas in an accelerated fashion with greater susceptibility to chemopreventive agents, compared to the large bowel. Thus, it is not surprising that folate has different effects on the small intestine and colon. Our results from this study support our previous findings in the Apc+/-Msh2-/- mice. Future studies are required to determine the mechanism and timing of folate’s mediating effects on intestinal and colonic tumorigenesis in Min mice.
CHAPTER 4. EFFECTS OF FOLATE DEFICIENCY AND SUPPLEMENTATION IN UTERO IN MIN MICE

4.1 INTRODUCTION

As shown in Chapters 2 and 3, timing of folate intervention can play a crucial role in the effects of folate on intestinal tumorigenesis in genetically predisposed mice. Depending upon whether the intervention occurs prior to or following the establishment of neoplastic foci, dietary folate can have dual or opposing effects on tumor development. Since disease prevention is the ultimate goal of research, it is important to accurately determine the optimal dose and timing of folate supplementation in preventing tumor development. In Chapter 3, we conducted a study utilizing Min mice to determine what effects on tumorigenesis different levels of folate supplementation would have, if given at three weeks of age. The results from this study also support the dual effects of folate. An early promoting effect of folate deficiency on ACF and a decrease in ileal tumors with the 10X basal requirement of folate was observed, while a regressive effect of tumor development in the ileum was seen at a later time point in folate deficient mice. However, the lack of folate effect on tumorigenesis in other areas of the intestine may be explained by the suggestion that the initiation of colon adenomas in the Min and Apc+/-Msh2-/- models occur perinatally (247). Thus folate intervention at three weeks of age might be too late for modulating effects to be seen in this Min intestinal tumorigenesis model.

Therefore, the present study is designed to investigate the effects of folate deficiency and supplementation on intestinal tumorigenesis in Min mice if induced perinatally at the third trimester of gestation. The third trimester was chosen as the time for intervention as folate deficiency induced prior to the third trimester results in embryonic resorptions (280). It was
previously reported that giving folate deficient diets one week prior to parturition results in pups with decreased brain folate, severe decreases in liver folate, reduction in body, liver and brain weights, lower brain SAM and pups which were less viable (281). However, currently with a better diet formulation, inadequate dietary folate in utero, provided one week prior to parturition, results in smaller and fewer embryos which are normally developed (280) which have reduced liver folate (281). Another animal study indicated that neonatal folate status related significantly to dietary folate intake of the mother during pregnancy and lactation (5). This is in contrast to the human situation, where mothers who are deficient in folate will preferentially channel their folate stores to their babies. That is, folate levels of babies are maintained at the expense of the mothers' stores through pregnancy and during lactation. Therefore, during these times, folate supplementation is recommended to preserve the adequate reserves of the mother (282). Supplementation is important as folic acid is necessary for reproduction and deficiencies in maternal folate can result in embryonic resorptions or malformed embryos (280). Folic acid supplementation has also been shown to be important in the prevention of neural tube defects (NTDs) in both humans and mice. Folic acid supplementation prevents 70% of human neural tube defects (283). Women taking multivitamins containing folic acid periconceptually have a significantly lower occurrence risk for having a baby with neural tube defects (284). However, in mice, one study concluded that folate deficiency alone does not cause neural tube defects, but does result in fewer and smaller normally developing embryos (264). This result is supported by another mouse study which found that in mice genetically predisposed to developing NTDs, administration of folic acid did not reduce the incidence of the defect (285).

Since previous study has shown that colon adenomas in Min mice may be initiated perinatally, folate deficiency will be induced in Min mice, in utero, by feeding pregnant females
folate deficient diets during their last trimester of gestation. This study is designed to determine the effects of reduced maternal folate on the tumorigenesis of Min pups.
4.2. SPECIFIC OBJECTIVE

To examine the effects of dietary folate deficiency and supplementation on intestinal tumorigenesis in Min mice, if first administered in utero.

4.3 MATERIALS AND METHODS

Study design: Breeding pairs were composed of a male Min (Apc+/-) mouse and a female wild type (Apc+/-) mouse. Pairs were housed together for four days before separation. Two weeks following the separation, females were examined visually and by palpation for signs of pregnancy. Those that appeared pregnant upon examination were put on one of two diets for the last trimester: 0 mg folate/kg diet or 8 mg folate/kg diet (Figure 4.1). For the last trimester of gestation, through parturition and through the weaning period (in utero-weaning), the dams were fed their respective folate diets. At three weeks of age, the pups were genotyped and the Min mice were then divided into two subgroups in each folate group (0 vs. 8 mg folate/kg diet). One subgroup continued to feed on their present diet which began with their mothers, and mice in the other subgroup were changed to the other folate diet. Thus, there were four groups of Min pups (Table 4.1).

The Min mice pups then remained on diet for three months before being sacrificed. Diet was provided ad libitum. Mice were weighed weekly. Mice were killed by cervical dislocation. Blood was taken for serum folate concentration determination, livers were quick frozen on dry ice and the intestines were opened and fixed in formalin for later tumor and ACF enumeration. Description of mice, diet, tumor enumeration and folate concentration determination can be found in Chapter 2, Section 3, Materials and Methods.
Figure 4.1. Study design of dietary folate intervention implemented *in utero* in Min mice.
Table 4.1 *Min in utero* group designations

<table>
<thead>
<tr>
<th>Group</th>
<th>Folate diet <em>in utero</em> and <em>during</em> weaning (mg folate/kg diet)</th>
<th>Folate diet for 3 months <em>after</em> weaning (mg folate/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0/8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>8/0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>8/8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
4.4 RESULTS

4.4.1 **Body weight**

There were no significant differences throughout the study in the average body weights of the mice fed the folate deficient diets in utero-weaning (ie. groups 0/0 and 0/8) (Figure 4.2). Mice fed folate supplemented diets in utero-weaning (ie. groups 8/0 and 8/8) weighed significantly more than those fed folate deficient diets in utero-weaning (ie. groups 0/0 and 0/8) for the first five weeks of post weaning diet (p<0.04). After those first five weeks, only group 8/0 was significantly greater than groups 0/0 and 0/8 (p<0.03). When compared to the Min study in Chapter 3, the weights of these mice are quite similar at the respective ages (data not shown). Therefore, there was little or no growth retardation or early morbidity in the mice fed the folate deficient diet, indicating that these Min mice were in a mild to moderate state of folate deficiency.

4.4.2 **Serum folate concentration**

The serum folate concentrations in these Min mice indicated that the mice fed folate deficient diets for the three months following birth (ie. groups 0/0 and 8/0) had significantly lower serum folate than those mice on the folate supplemented diets following birth (ie. groups 0/8 and 8/8)(p<0.008) (Table 4.2), regardless of the diet provided in utero-weaning. That is, there was no difference between mice fed the same diets after weaning regardless of the diet provided in utero-weaning.
Figure 4.2 Body weights of mice with folate intervention in utero. a indicates significant difference between group 8/8 and groups 0/0, 0/8 (p<0.04); b indicates significance between group 8/0 and groups 0/0,0/8 (p<0.03)
Table 4.2. Serum folate of pups with folate intervention *in utero*

<table>
<thead>
<tr>
<th>Diet (mg folate/kg diet)</th>
<th>Serum folate (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In utero and weaning</td>
<td>Post three week wean</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
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</table>

Different letters indicate significant difference (p<0.05).
4.4.3 Small intestinal tumor and colonic ACF and tumor enumeration

In the small intestine, there were no significant differences observed in the overall number of tumors among the four groups (Figure 4.3). However, there was a non-significant trend in mice fed the 8 mg folate/kg diet in utero-weaning, which was dependent upon the diet given following the weaning period; whereby mice fed the folate deficient diet after weaning had fewer tumors than those fed a folate supplemented diet (ie. group 8/0<8/8) (p=0.1).

In the colon, there were no significant differences in the number of ACF or adenomas found in mice on any of the four diet treatments (Figure 4.4).
Figure 4.3 Small bowel tumor enumeration in Min pups with folate conditions induced in utero. Diets are indicated by order of feeding, i.e. in utero and weaning diet/three month post wean diet.
Figure 4.4 Colon ACF and adenoma enumeration for Min pups with folate conditions induced \textit{in utero}. Diets are indicated by order of feeding, i.e. \textit{in utero} and weaning diet/three month post wean diet.
4.5 DISCUSSION

4.5.1. **Serum folate concentration**

Serum folate is an indicator of recent folate intake and the folate concentrations measured in these mice demonstrate that. It appears that the diet fed *in utero* and during weaning have no effect on serum folate measurements three months later as there is no difference between groups of mice on the same post weaning diet but different *in utero*-weaning diets. Also, the mean serum folate concentrations at three months of age in the post weaning folate deficient fed mice are similar to those seen in the Chapter 3 Min mice. The Min mice were fed the deficient diet for three months and began diet at three weeks of age following weaning (group 0/0: 15.08 ng/ml ± 3.41; group 8/0: 20.37 ± 3.16 ng/ml vs. group I: 13.19 ± 9.93 (0 mg folate/kg diet for 3 months)). This would support the suggestion that the diet fed *in utero*-weaning had no effect on serum folate concentrations three months later. However, in the mice fed folate supplemented diets after weaning, the serum folate was less than that measured in the Chapter 3 Min mice fed the same diet at three weeks of age (group 0/8: 29.59 ± 3.28; group 8/8: 36.28 ± 3.74 vs. group III: 61.51 ± 9.42 (8 mg folate/kg diet for three months)).

4.5.2. **Small intestinal tumor and colonic ACF and tumor enumeration**

In the small bowel as a whole, there were no significant differences in tumor number between any of the groups. However, there was a non-significant trend towards fewer intestinal tumors in mice deficient for folate following weaning, when compared to mice fed a folate supplemented diet following weaning, regardless of the diet fed *in utero*-weaning (ie. group 0/0<0/8 and group 8/0<8/8). That is, mice fed either 0 or 8 mg folate/kg diet *in utero*-weaning and fed 0 mg folate/kg diet for the three month period after weaning, developed fewer tumors than mice fed the 8 mg folate/kg diet post weaning (Figure 4.3). The results suggest that the folate deficient diet was having a regressive effect on tumors that had developed in the intestine.
During the intervention period. Folate deficiency has been shown previously to regress tumors as folate antagonists, such as Methotrexate and 5-fluorouracil, have been used as chemotherapeutic agents to reduce tumors in cancer (127,128). The lack of statistical significance may be due to the low number of tumors developed and the small number of mice (ie. a type II error). The mechanism for the decrease in tumor number would be the deprivation of the tumor of folate, a vitamin necessary for DNA synthesis and cell reproduction.

Typically there are few, if any, aberrant crypt foci found in the large intestine of Min mice (223). The Min mice in this study as well as the mice in Chapter 3 had, on average, less than one ACF in the large intestine. Thus, there are too few ACF for statistical analysis to have any validity with the number of animals used in this study.

In the colon, there was no significant difference in adenoma number observed among mice in any of the four diet groups. The mean number of adenomas identified in each mouse was less than one, therefore it is possible that there were too few adenomas for statistical significance to be established. There was however, a trend towards a regressive effect of folate deficiency compared to folate supplementation when the in utero-weaning diet was folate deficient (p=0.1). It appears as though the folate deficiency in utero-weaning 'primes' the intestine for future effects of folate on tumorigenesis. Although not statistically significant, this would suggest a folate deficient in utero-weaning diet had an effect on colonic tumorigenesis in Min mice. The effects of folate on tumorigenesis seen here is similar to what would be expected if tumors had already begun to develop perinatally, prior to folate intervention. That is, the decrease in tumor number during folate deficiency and the increase with folate supplementation in utero-weaning is reflective of the post neoplastic foci development effects of folate. The regression of tumors with folate deficiency has been observed previously (127,128) and therefore these results tend to support the hypothesis that colonic tumors are developed
perinatally (247). Thus far, one study has looked at chemoprevention in utero in Min mice, investigating the effects of dietary fat and a vegetable-fruit mixture. This study found significant accelerating effects of the vegetable fruit mixture combined with low fat diet in females on intestinal polyp multiplicity, however, no significant results were seen in the colon (230).
4.6. CONCLUSION

When folate intervention is implemented in utero in Min mice, there are no significant effects on intestinal tumorigenesis. Although a non-significant trend was observed in the small and large intestine towards folate deficiency post weaning to have a protective or regressive effect on tumorigenesis compared to folate supplementation. It is possible that there were not enough mice, colonic adenomas and ACF for statistical significance to be observed. Further study is required to determine the time of the initiation to promotion transition in the Min mice, as timing appears to play an important role in determining the effects of folate intervention on colorectal tumorigenesis.
5.1 SUMMARY

Folate has been established as a mediating factor in colorectal tumorigenesis, in both human situations and animal models. However, the relationship is complex as its timing of intervention, levels of effectiveness and affected site specificity are all aspects to be considered before the exact role and mechanisms of folate in CRC can be determined.

The present study conducted in Apc+-Msh2-/- demonstrated the dual effects of folate depending upon the timing of intervention. Folate deficiency and supplementation induced prior to the establishment of neoplastic foci resulted in a null and protective effect on intestinal tumorigenesis, respectively. However, when these conditions are induced after neoplastic foci have developed, there is a decrease in tumor incidence with folate deficiency and a null effect with folate supplementation. Therefore, timing of folate intervention is critical in exerting the desired effect. The null effect seen was likely due to a saturation effect in the intestines as the number of tumors observed was not greater than the maximum number of adenomas observed in this model on control diet (ie. 350) (247).

In order to determine the most effective level of dietary folate as a chemopreventive agent, Min mice were provided with different levels of dietary folate. The results demonstrated the dual effects of folate only in the ileum of the small bowel at 10X the basal requirement. This site specificity in tumor development is not uncommon, particularly in this Min mouse model, where the colon and duodenum have been shown to be resistant to chemopreventive agents (237) when given at the three week weaning time point. Colon resistance to chemopreventive agents in Min mice may be due to the timing of intervention and adenoma development in the colon. It
has been suggested that colon adenomas develop perinatally in Min mice, therefore, studies where intervention occurs at three weeks of age may be too late for an effect to occur. When folate intervention was implemented in utero in Min mice, however, there were no significant effects of folate observed in either the colon or the small intestine.

Two mechanisms were investigated to explain the folate-CRC relationship using the Apc+/-Msh2-/- mouse model. First, our study suggests genomic DNA methylation may be associated with dietary folate through the activity of DNA MTase. Second, when the role of folate was studied in conjunction with mismatch repair (MMR) deficiency using microsatellite instability (MI), a measure of functional MMR, there was no evidence of MI in any of the intestinal adenomas evaluated in spite of a loss of Msh2 in these mice.

In summary, the results indicate that although folate does have mediating effects on colorectal tumorigenesis in genetic mouse models, the relationship is complex requiring the consideration of many factors, including timing of intervention, level of effective dose(s) and affected site-specificity. The mechanism for the folate-tumorigenesis relationship may involve methylation, however in the Apc+/-Msh2-/- mouse model, deficient Msh2 does not appear to be a factor.

5.2 FUTURE STUDIES

Although the dual role of folate has been observed, it would be important to determine whether the null effect seen with the folate deficient and supplemented mice post and prior to tumor development is actually an accelerating effect. Using the same mouse model with 0 and 8 mg folate/kg diet, the mice would be sacrificed at approximately 40 days of age, before the
intestine becomes saturated with tumors (247). Therefore, an accelerating effect could be observed, if any.

The determination of localized concentrations of folate within the mucosa of the intestine would be advantageous since systemic measures, such as serum folate, are not necessarily indicative of the folate status in the affected tissue.

Although an inverse relationship was observed between dietary folate and genomic methylation, it would be interesting to determine the site specific methylation status of crucial CRC genes, including different tumor suppressor genes (eg. Apc, p53) and oncogenes (eg. c-myc). In addition, to further investigate the relationship observed in our study, the levels of DNA MTase activity in these tissues should also be determined.

In the Apc+-/-Msh2-/- mice, there did not seem to be any effect on mismatch repair with folate deficiency or supplementation. Thus, to ensure these mice are truly Msh2 deficient, perhaps levels of Msh2 activity should be measured, along with the activity of other MMR gene products, in order to determine if there are any redundancies within the system. Also, using this model, it would be beneficial to determine whether somatic mutations of the wild type Apc allele are responsible for the loss of wild type Apc and whether these mutations are a result of the inactivated mismatch repair gene, Msh2.

These studies in Apc+-/-Msh2-/- and Min mice support previous studies demonstrating the mediating effects of folate on intestinal tumorigenesis. Due to the complex nature of this relationship, however, further studies are required to clarify safe and effective dose(s) and timing of folate chemoprevention and to elucidate potential mechanisms.
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