THE IMPACT OF GENETIC AND NUTRITIONAL DISTURBANCES OF FOLATE METABOLISM ON TUMOURIGENESIS IN A MOUSE MODEL OF COLORECTAL CANCER

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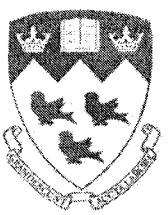

Your file Votre référence
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This thesis is dedicated to my family.

To my mom – for instilling in me the importance of an education, for working tirelessly to help me attain one, and for her unwavering belief in me.

To my dad – for his uncompromising pride, support, and love.

And to my sister – a true inspiration – the ultimate example of what hard work, determination, and fortitude can achieve.

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ABSTRACT

The relationship between colorectal cancer (CRC) and folate metabolism is complex. Dietary folate, depending on the timing and dose, may either prevent or enhance tumour initiation and/or growth, and polymorphisms in the genes encoding folate-metabolising enzymes may also modulate risk. In this thesis, the *Apc*$^{min/+}$ mouse model of CRC was used to investigate the effect of nutritional and genetic disturbances in folate metabolism on tumourigenesis and to examine various mechanisms.

The reduced folate carrier 1 (RFC1) is responsible for the cellular uptake and intestinal absorption of folate, primarily the 5-methyltetrahydrofolate (5-methylTHF) derivative. Methionine synthase (MTR) uses 5-methylTHF to remethylate homocysteine to methionine, which may be activated and used to methylate substrates such as DNA. 5-MethylTHF is also the product of the methylenetetrahydrofolate reductase (MTHFR)-catalysed reduction of 5,10-methyleneTHF, which is also used to convert dUMP to dTMP.

Adenoma number and load were reduced in *Rfc1*$^{+/-}$*Apc*$^{min/+}$ mice, compared with *Rfc1*$^{+/+}$*Apc*$^{min/+}$ mice, but were similar in *Mtr*$^{+/-}$*Apc*$^{min/+}$ and *Mtr*$^{+/+}$ *Apc*$^{min/+}$ mice. Neither *Rfc1* nor *Mtr* genotype affected global DNA methylation, apoptosis or plasma homocysteine (tHcy) levels. In the experiments involving *Mtr* mice, dietary folate deficiency increased adenoma number, plasma tHcy, and apoptosis, and decreased global DNA methylation. Neither *Mtr* nor *Rfc1* genotype affected the dUTP/dTTP ratio in the intestine of mice not predisposed to adenoma formation.

Adenoma number was decreased in *Mthfr*$^{+/-}$*Apc*$^{min/+}$ mice (compared with *Mthfr*$^{+/+}$*Apc*$^{min/+}$ mice) and in *Mthfr*$^{+/+}$*Apc*$^{min/+}$ offspring of *Mthfr*$^{+/-}$ mothers (compared with *Mthfr*$^{+/+}$*Apc*$^{min/+}$ offspring of *Mthfr*$^{+/+}$ mothers). A folate-deficient diet, when initiated prior to conception, significantly decreased adenoma number and decreased global DNA methylation. Overall, adenoma number was inversely correlated with plasma tHcy, dUTP/dTTP ratio and apoptosis. When initiated at three weeks of age, a folate-enriched diet significantly increased adenoma number

in *Apc*$^{min/+}$ mice. In the intestines of mice not predisposed to adenoma formation, *Mthfr* deficiency decreased, and folic acid deficiency increased, the dUTP/dTTP ratio.

These results support the evidence that *MTHFR* polymorphisms are protective in CRC tumourigenesis and that depending on stage or predisposition, folate may inhibit or enhance tumour growth.

RESUMÉ

La relation entre le cancer colorectal (CRC) et le métabolisme des folates est d'une grande complexité. Le folate ingéré, dépendant du moment d'ingestion et de la quantité assimilée, peut prévenir ou faciliter l'initiation des tumeurs ou leur croissance. Ces effets peuvent être modulés par des polymorphismes génétiques de gènes qui encodent des enzymes métabolisant les folates. Dans cette thèse, le modèle $Apc^{min/+}$, une souris souvent utilisée lors d'études du CRC, a été employée pour tester l'effet de désordres nutritionnels et génétiques du métabolisme du folate sur la tumorigénèse et pour examiner les mécanismes impliqués.

L'enzyme *reduced folate carrier 1* (RFC1) est responsable de l'importation cellulaire et de l'absorption intestinale du folate, principalement sous la forme du dérivé 5-méthyltétrahydrofolate (5-méthylTHF). La méthionine synthase (MTR) utilise le 5-méthylTHF pour reméthyler l'homocystéine en méthionine, laquelle peut être activée et utilisée pour méthyler divers substrats, tel l'ADN. Le 5-méthylTHF est aussi le produit de la méthylenetétrahydrofolate réductase (MTHFR), qui catalyse la réduction du 5,10-méthylèneTHF, qui est aussi utilisé pour convertir le dUMP en dTMP.

La quantité d'adénomes et le volume qu'ils occupent sont réduits dans le cas des souris $Rfc1^{+/-}Apc^{min/+}$, mais similaires à ceux obtenus avec les souris $Mtr^{+/-}$ $Apc^{min/+}$ et $Mtr^{+/+}Apc^{min/+}$. Le génotype de *Rfc1* ou de *Mtr* n'affecte pas la méthylation globale, l'apoptose ou les niveaux d'homocystéine plasmatique. Pour les expériences avec les souris *Mtr*, une déficience en folate augmente la quantité d'adénomes, l'homocystéine plasmatique, l'apoptose et diminue la méthylation de l'ADN. Le génotype de *Mtr* ou de *Rfc1* n'affecte pas le rapport dUTP/dTTP de l'intestin des souris qui ne sont pas prédisposées à la formation d'adénomes.

La quantité d'adénomes est diminuée pour les souris $Mthfr^{+/-}Apc^{min/+}$ (comparativement aux souris $Mthfr^{+/+}Apc^{min/+}$) et pour les $Mthfr^{+/+}Apc^{min/+}$ rejetons des mères $Mthfr^{+/-}$ (comparés aux rejetons des mères $Mthfr^{+/+}$). Une diète déficitaire en folate, lorsqu'elle est initiée avant la conception, diminue significativement la quantité d'adénomes et diminue aussi la méthylation de l'ADN.

Globalement, la quantité d'adénomes est inversément corrélée avec l'homocystéine plasmatique, le rapport dUTP/dTTP et l'apoptose. Lorsqu'elle est introduite à l'âge de trois mois, une diète enrichie en folates augmente significativement la quantité d'adénomes des souris *Apc*$^{min/+}$. Dans les intestins des souris qui ne sont pas prédisposées à la formation d'adénomes, une déficience en *Mthfr* diminue le rapport dUTP/dTTP alors qu'une déficience en acide folique augmente ce rapport.

Ces résultats appuient le concept que les polymorphismes de MTHFR protègent du cancer colorectal et que, selon le stade tumoral ou la prédisposition des souris, les folates peuvent inhiber ou favoriser la croissance tumorale.

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THESIS FORMAT

This thesis comprises 5 chapters. Chapter I is a review of the literature pertaining to this thesis. Chapters II through IV are data chapters in the form they have or will be submitted for publication and are linked by connecting text.

CONTRIBUTIONS OF THE AUTHORS

For chapters II, III and IV, the candidate designed the experiments, analysed and interpreted the data, and wrote the manuscripts in collaboration with her supervisor. Jeffery Cohn performed all homocysteine measurements described in these chapters as a service.

In chapter II, the candidate modified and adapted previously published methods for HPLC determination of nucleotide pools and DNA methylation. She performed all HPLC measurements. Qing Wu provided the animals.

In chapter III, the candidate carried out the animal experimentation, genotyping and tissue collection. She maintained a colony of *Rfc* mice derived from a breeding pair provided by Dr. Richard Finnell and a colony of *Mtr* mice derived from breeding pair provided by Dr. Barry Shane and Dr. Lawrence Brody. She conducted all the experiments described in this chapter with the exception of the DNA methylation determination, which she performed with Liyuan Deng.

In chapter IV, the candidate carried out the animal experimentation, genotyping and tissue collection. She conducted all the experiments with the exception of the DNA methylation determination, which was performed entirely by Liyuan Deng.

ABBREVIATIONS

BHMT	betaine homocysteine methyltransferase
CD	control diet
CI	confidence interval
CRC	colorectal cancer
DNMT	DNA methyltransferase
dUTP	deoxyuridine triphosphate
dTTP	deoxythymidine triphosphate
FAD	flavin adenine dinucleotide
FADD	folic acid-deficient diet
FAED	folic acid-enriched diet
HPLC	high performance liquid chromatography
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
OR	odds ratio
PCFT	proton-coupled folate transporter
PCR	polymerase chain reaction
RFC	reduced folate carrier
RR	relative risk
SAM	*S*-adenosylmethionine
SAH	*S*-adenosylhomocysteine
tHcy	total homocysteine
THF	tetrahydrofolate
TLC	thin layer chromatography

CONVENTIONS

In this thesis, the names of genes and transcripts are italicised; uppercase for human (*i.e., MTHFR*), titlecase for mouse (*i.e., Mthfr*). The names of proteins are not italicised; both human and mouse proteins are uppercase (*i.e.,* MTHFR).

CHAPTER I

Literature Review

1.1 FOLATE INTAKE, ABSORPTION AND TRANSPORT

1.1.1 Folates

The term folate encompasses a group of water-soluble compounds composed of pteroic acid and one or more L-glutamate residues. The various derivatives differ from one another in three main ways: by one-carbon substitutions in the N^5 and N^{10} positions of the pteroic acid (*i.e.*, methyl, methylene, methenyl, or formyl), by the level of reduction of the pteridine ring (at positions N^5,N^6,N^7,N^8) and by the number of glutamates (usually 1-6) (see **Figure 1.1**).

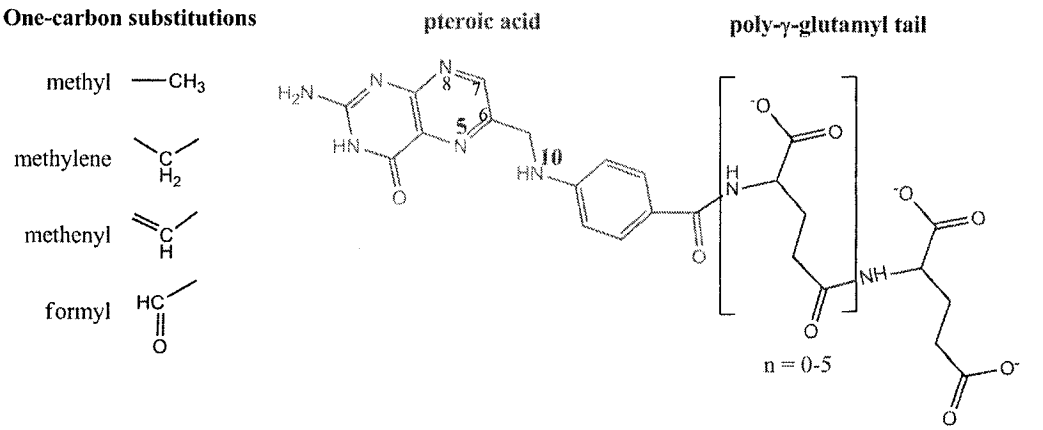


Figure 1.1 The structure of folates.

Folic acid is fully oxidised at positions 5,6,7 and 8. Dihydrofolate is reduced at positions 7 and 8. Tetrahydrofolate (THF) is reduced at positions 5,6,7 and 8. In tetrahydrofolate, positions N^5 and N^{10} may be substituted with one-carbon groups including methyl, methylene, methenyl and formyl. *Based on Figure 1 of Christensen et al. (1) and Stokstad et al.* (2).

Mammals cannot synthesise folates and thus require an exogenous supply. Naturally occurring folates are found in high amounts in leafy green vegetables, citrus fruits, yeast extracts, liver and kidney. They occur as a mixture of predominantly reduced polyglutamates and are considered relatively unstable to storage, processing and preparation as they readily undergo oxidative degradation

(3-5). There are also two common supplemental forms of folate: folic acid (pteroylmonoglutamic acid) and folinic acid (5-formylTHF). These compounds are highly stable and used to fortify cereal-grain foods and multivitamins. The bacterial flora of the large intestine can also produce folate, providing an additional source of this B-vitamin (6, 7).

When polyglutamyl folates are ingested, they undergo enzymatic deconjugation in the jejunal brush border membrane catalysed by a folate hydrolase (8). The monoglutamyl folates are then absorbed by a carrier-mediated transport process involving the reduced folate carrier and/or the proton-coupled folate transporter. At supraphysiological levels, folate may also permeate by unsaturable passive diffusion (9).

1.1.2 Reduced Folate Carrier – Folate Absorption

Intestinal folate absorption occurs through a saturable carrier; the pH optimum of transport is ~6.0, the same pH of the luminal surface of the jejunem where the majority of absorption occurs (9, 10).

The identity of the carrier was assigned to the reduced folate carrier 1 (RFC1) (11-13), a bidirectional transporter that displays high affinity for reduced folates (K_m ~2-4 μM) and a low affinity for folic acid (K_m ~200-400 μM).

RFC1 is ubiquitously expressed and subject to complex tissue-specific transcriptional and post-transcriptional regulation (14-16). Consistent with a role in absorption, immunohistochemical analysis showed that RFC1 is expressed in the brush-border membrane along the entire length of the small and large intestinal epithelium (strongest in the jejunum) in mice (17). Intestinal expression of *Rfc1* is transcriptionally upregulated by dietary folate deficiency (16, 18) and downregulated with age (19).

1.1.2.1 Proton-coupled Folate Transporter

The role of RFC1 in intestinal absorption has recently been questioned. The primary point of contention is the fact that the optimum pH for RFC1 is neutral and the pH of the jejunal lumen is acidic. Notwithstanding this fact, Balamurugan *et al.* found that silencing of *Rfc1* using short hairpin RNA in a rat-derived epithelial cell line abrogated folic acid uptake at pH 5.5 (20). This was in

contrast with the findings of Wang *et al.*, who found that in a chemically mutagenised version of the same cell line, the rate of methotrexate (a folate analogue) influx in *Rfc1*-null lines was reduced at neutral pH, but comparable to *Rfc* wild-type lines at pH 5.5 (21). This led the authors to speculate that an RFC-independent system facilitated transport at low pH. They have identified a previously designated heme carrier as a low pH folate carrier and named it the proton-coupled folate transporter (PCFT) (22). This transporter displays similar affinities for 5-methylTHF, 5-formylTHF, and folic acid (K_m ~0.5-1.0 μM) and has an acidic pH optimum. The relative contributions of the PCFT and RFC1 to intestinal folate absorption are not presently known.

1.1.3 Reduced Folate Carrier – Folate Transport

Following absorption across the brush border membrane, folates are metabolised within the intestinal epithelial cell (enterocyte) and exit across the basolateral membrane. The predominant circulatory form of folate is 5-methylTHF monoglutamate. The folates then travel through the portal circulation to the liver where they are either stored (as polyglutamates) or delivered via the systemic circulation to peripheral tissues where they are taken up by either reduced folate carriers or folate receptors.

In addition to the intestine, immunohistochemical analysis has shown that RFC1 is highly expressed in renal tubular epithelial cells, hepatocytes, dendrites and in the red pulp of the spleen of mice (17). In humans, *RFC1* transcripts are expressed in all tissues with the highest expression localised to placenta, liver, kidney, brain and duodenum (14).

1.1.4 Folate Receptors

Another class of folate transporters are folate receptors. Folate receptors, also called membrane folate binding proteins, are membrane-associated glycoproteins which function by receptor-mediated endocytosis for unidirectional transport into cells (23). In contrast with RFC1, they display a very high affinity for oxidised folates (such as folic acid) relative to reduced folates (such as 5-methylTHF), although their affinity for reduced folates is within the physiological concentration range (24).

Folate receptors are not highly expressed in the gastrointestinal tract and do not appear to serve an important role in the intestinal absorption of folate (17, 25, 26). They are, however, highly expressed in the renal tubule epithelium (27), the choroid plexes (28) and the placenta (24).

1.1.5 Folate Transport - Gestation

During gestation, the fetus obtains folate through transplacental absorption. Folate receptors are highly expressed on the brush border membrane of the placenta, facing the maternal circulation (29). These receptors bind 5-methylTHF to produce a concentration three times that of maternal plasma (30). The folates travel from the placenta to the fetal circulation, probably through RFC1, which is expressed on the basolateral membrane (29).

1.1.6 Folate Transport - Nursing

Nursing offspring obtain folate from their mother's milk. Milk contains large amounts of folate binding proteins (FBP) which bind folates with very high affinity (31, 32). The term folate binding protein encompasses soluble forms (found in milk or serum), their structurally related membrane forms (folate receptors) as well as certain folate-metabolising enzymes that bind folate intracellularly.

The purpose of soluble FBPs in milk may be to sequester folate; milk folate contains 5-10 times more folate than maternal plasma (33). They may also modify the intestinal absorption of folates in nursing offspring. Comparison of FBP-bound folic acid and free folic acid showed that the FBP-bound form was absorbed preferentially in the ileum by a mechanism unique from that of free folic acid (34). However, the majority of folate found in milk is polyglutamated 5-methylTHF, not folic acid (35). In a study of FBP-bound 5-methylTHF and free 5-methylTHF, the authors concluded that the same jejunal transporter (later identified as RFC1) was used by both the bound and free forms (36). They also found that at pH 6, the bound folate was transported at a slower rate than the free form, which they speculated could help regulate the nutritional bioavailability of folate. Finally, it has been shown that the stabilisation of tetrahydrofolates, which are normally quite labile, was greatly enhanced when bound to milk FBPs (37).

1.2 FOLATE METABOLISM

1.2.1 Overview

The only known role of folates is to transfer one-carbon groups for the production of nucleotides and methionine. The biologically active forms of folate are tetrahydrofolate (THF) derivatives, the forms generally found in nature. Folic acid, however, must be successively reduced (normally in the intestinal enterocytes or liver) by dihydrofolate reductase to dihydrofolate and THF. Intracelluar folates are polyglutamated by folylpoly-γ-glutamate synthetase in order to prevent cellular efflux and to increase their affinity for the folate-metabolising enzymes that interconvert the various coenzymatic forms.

The majority of folate metabolism takes place in the cytoplasm and mitochondria (1), although there is evidence for involvement of the nucleus as well (38). This thesis will focus on cytoplasmic metabolism and a simplified pathway detailing these reactions is found in **Figure 1.2**.

1.2.2 Nucleotide Synthesis

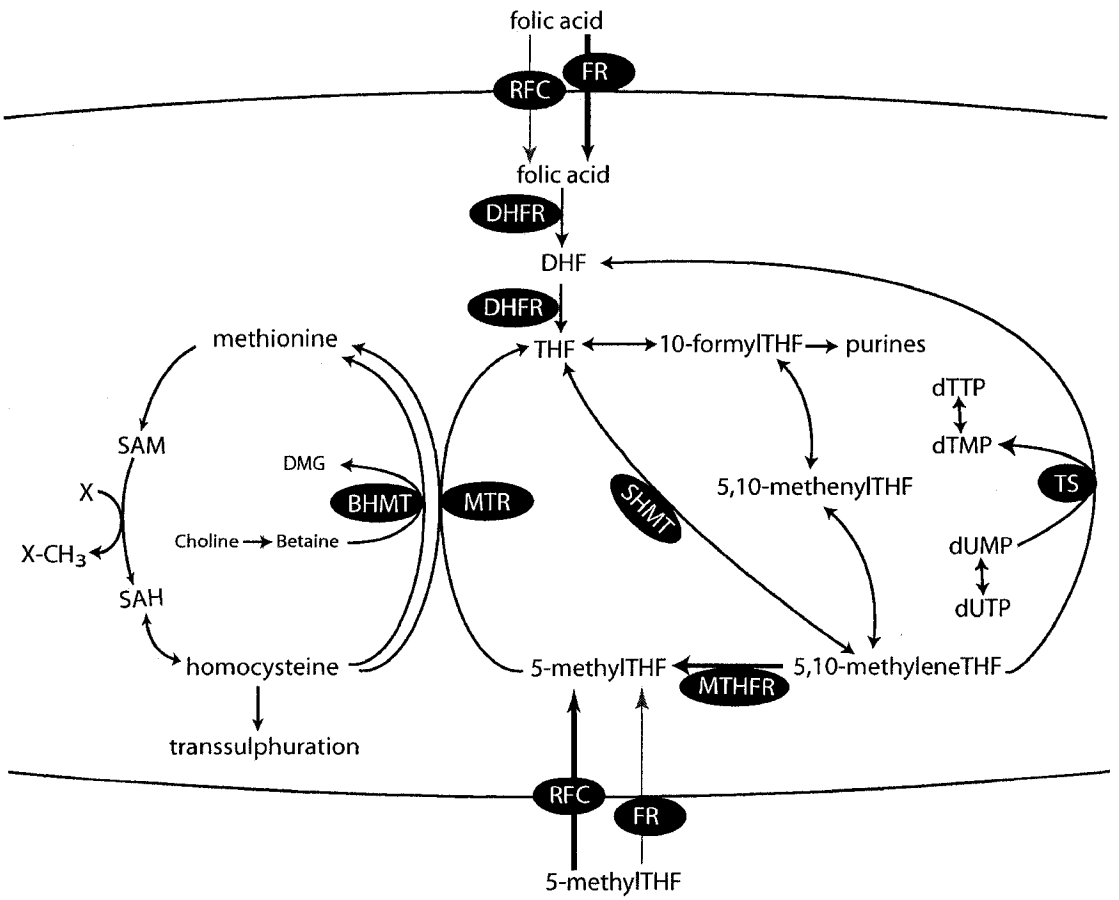
Nucleotides are essential to cellular metabolism. They are the monomeric units of nucleic acids (DNA and RNA), they are a source of chemical energy (primarily but not exclusively ATP), they are components of cofactors (including NAD, FAD, SAM) and they can act as biological regulators (cAMP and cGMP).

Nucleotides are composed of a nitrogenous base (either a pyrimidine or purine), a pentose sugar (either ribose or deoxyribose) and one to three phosphate groups. The term nucleoside refers to a molecule composed of the base and sugar alone. Uracil, cytosine and thymine comprise the major pyrimidine bases and adenine and guanine comprise the major purine bases (**Figure 1.3**).

Folate metabolism and nucleotide synthesis are linked by virtue of the fact that one-carbon substituted folates are required for the *de novo* synthesis of purines and the production of thymidylate. A simplified pathway is shown in **Figure 1.4**.

Figure 1.2 Folate metabolic pathway

Abbreviations: **BHMT** - betaine homocysteine methyltransferase; **DHFR** - dihydrofolate reductase; **DMG** - dimethylglycine; **dTMP** - deoxythymidine monophosphate; **dTTP** - deoxythymidine triphosphate; **dUMP** - deoxyuridine monophosphate; **dUTP** - deoxyuridine triphosphate; **FR** - folate receptor; **MTHFR** - methylenetetrahydrofolate reductase; **MTR** - methionine synthase; **RFC** - reduced folate carrier; **SAM** - *S*-adenosylmethionine; **SAH** - *S*-adenosylhomocysteine; **SHMT** - serine hydroxymethyltransferase; **TS** - thymidylate synthase.



1.2.2.1 Purines

Two steps of *de novo* purine synthesis require the incorporation of formyl groups from 10-formylTHF into the developing purine rings. The C8 carbon is derived from the incorporation of 10-formylTHF into glycinamide ribonucleotide (GAR) by GAR transformylase that produces the intermediate formylglycinamide ribonucleotide (FGAR). The C2 carbon is derived from the integration of 10-formylTHF into 5'-aminoimidazole-4-carboxamide ribonucleotide (AICAR) by AICAR transformylase to produce formylaminoimidazole-4-carboxamide ribonucleotide (FAICAR). FAICAR is then converted to inositol monophosphate (IMP), the parent purine compound. Both adenosine monophosphate (AMP) and guanosine monophosphate (GMP) are then produced from IMP by independent two-step pathways involving specific enzymes.

1.2.2.2 Pyrimidines

Just as IMP is the parent compound of purines, uridine monophospate (UMP) is the parent compound of pyrimidines. UMP is phosphorylated by UMP/CMP kinase to UDP and by nucleoside diphosphate kinase to UTP. CTP is produced from UTP by the action of CTP synthetase.

In general, ribonucleotide monophosphates (AMP, GMP, CMP, UMP) and deoxyribonucleotide monophosphates (dAMP, dGMP, dCMP, dUMP, dTMP) are converted to their diphosphate forms by specific kinases. All four ribonucleotide diphosphates (ADP, GDP, CDP and UDP) are converted to deoxyribonucleotide diphosphates by one enzyme, ribonucleotide reductase. Deoxyribonucleotide diphosphates (dADP, dGDP, dCDP, dUDP and dTDP) and ribonucleotide diphosphates (ADP, GDP, CDP, UDP) are phosphorylated to their triphosphate equivalents by the enzyme nucleoside diphosphate kinase.

Unlike the synthesis of dAMP, dGMP and dCMP, the *de novo* synthesis of dTMP involves only deoxyribonucleotides. Thymidylate synthase transfers a one-carbon unit from 5,10-methyleneTHF to dUMP to generate dTMP and DHF (**Figure 1.5**). 5,10-MethyleneTHF is also the substrate for methylenetetrahydrofolate reductase (MTHFR).

Figure 1.3 Bases found in DNA and/or RNA

[1]The "deoxy" prefix may be applied to nucleosides and nucleotides containing a deoxyribose sugar.

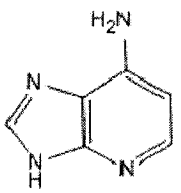
Example: dATP contains an adenine base, a deoxyribose sugar and three phosphate groups and may be called either deoxyadenylate or deoxyadenosine triphosphate.

Based on Figure 12-2 and Table 12-1 of Lehninger (39).

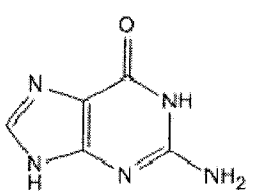
Nomenclature

Base	Nucleoside[1]	Nucleotide[1]
Adenine	Adenosine	Adenylate
Guanine	Guanosine	Guanylate
Cytosine	Cytidine	Cytidylate
Thymine	Thymidine	Thymidylate
Uracil	Uridine	Uridylate

Purines

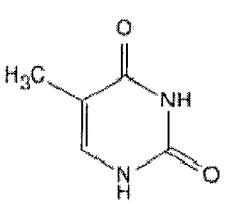


Adenine

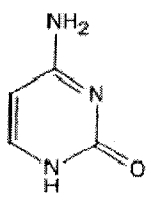


Guanine

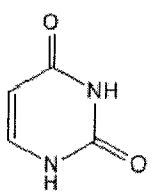
Pyrimidines



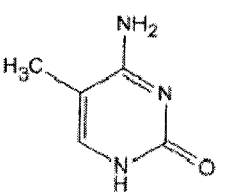
Thymine



Cytosine



Uracil



5-Methylcytosine

Figure 1.4 Pathways of *de novo* deoxynucleotide synthesis

Each pathway begins with the substrate of the first committed step of synthesis. Folate-dependent steps are shown in red.

Enzymes: **1** – GAR transformylase; **2** – AICAR transformylase; **3** – ribonucleotide reductase; **4** – nucleoside diphosphate kinase; **5** – CTP synthase; **6** – dCMP deaminase; **7** – thymidylate synthase.

Abbreviations: **AICAR** – 5'-aminoimidazole-4-carboxamide ribonucleotide; **ADP** – adenosine diphosphate; **AMP** – adenosine monophosphate; **dADP** – deoxyadenosine monophosphate; **dATP** – deoxyadenosine triphosphate; **dCMP** – deoxycytosine monophosphate; **dCDP** – deoxycytosine diphosphate; **dCTP** – deoxyuridine triphosphate; **dGDP** – deoxyguanosine triphosphate; **dGTP** – deoxyguanosine triphosphate; **dTMP** – deoxythymidine monophosphate; **dTDP** – deoxythymidine diphosphate; **dTTP** – deoxythymidine triphosphate; **dUMP** – deoxyuridine monophosphate; **dUDP** – deoxyuridine diphosphate; **dUTP** – deoxyuridine triphosphate; **FAICAR** – formylaminoimidazole-4-carboxamide ribonucleotide **FGAR** – formylglycinamide ribonucleotide; **GAR** – glycinamide ribonucleotide; **GMP** – guanosine monophosphate; **GDP** – guanosine diphosphate; **IMP** – inosine monophosphate; **PRPP** – phosphoribosylpyrophosphate; **UMP** – uridine monophosphate; **UDP** – uridine diphosphate; **UTP** – uridine triphosphate

Based on Figure 1 of Mathews (40), Figures 21-27 and 21-30 of Lehninger(39) and Figures 1 and 2 of Hatse et al. (41).

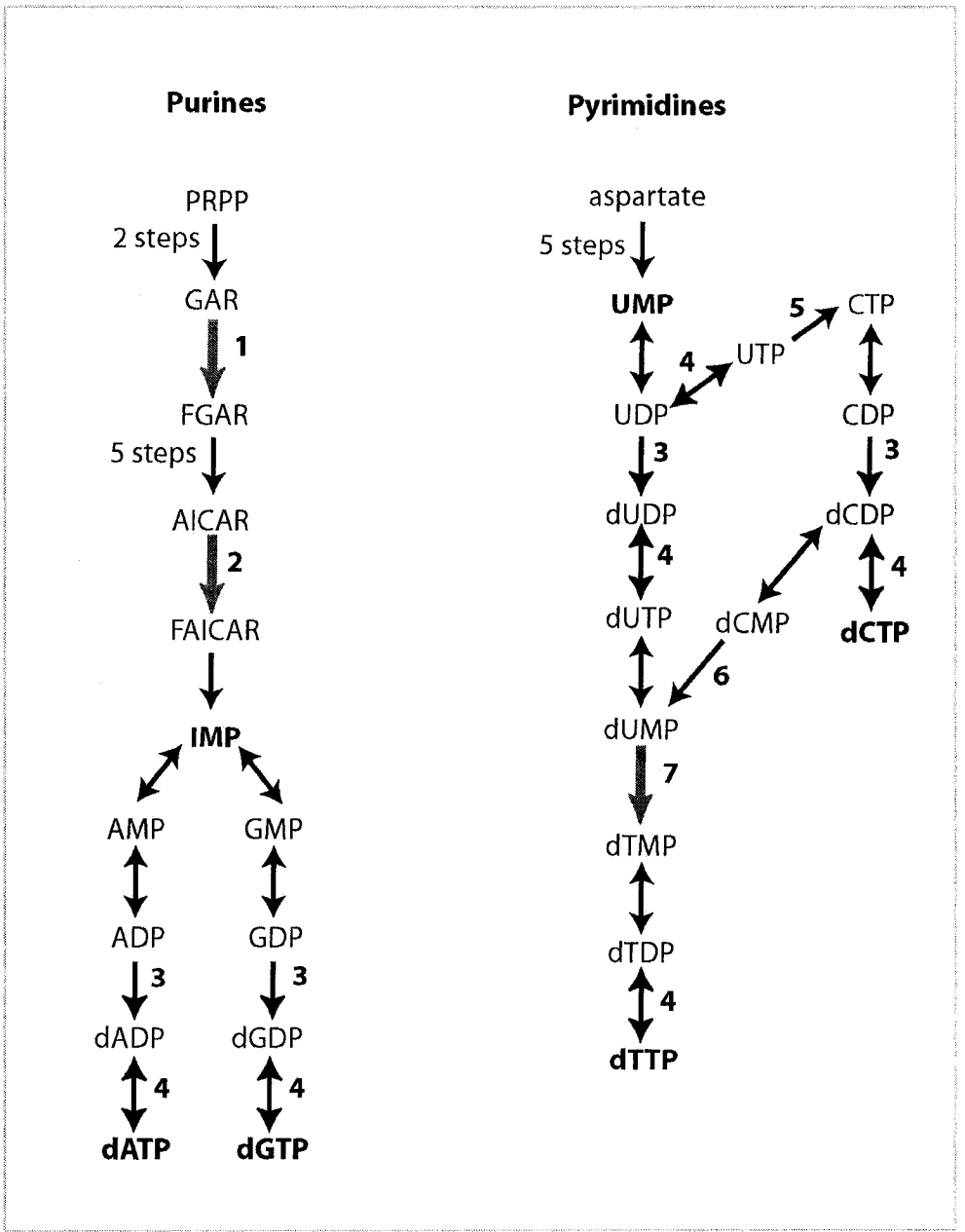
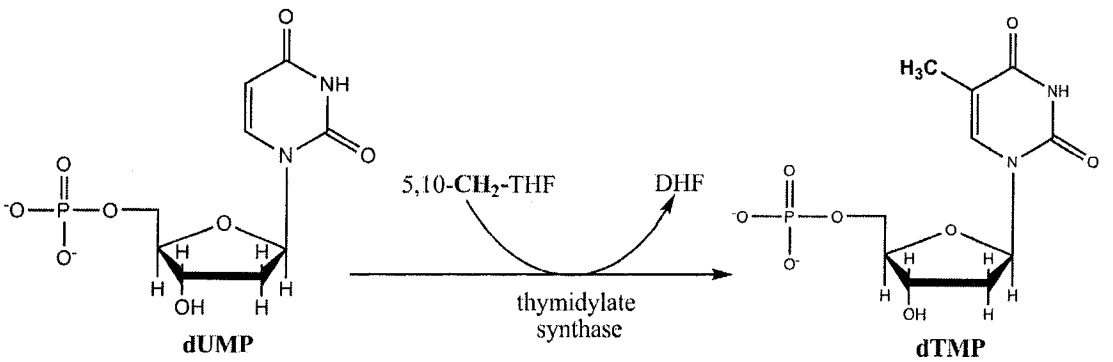

Purines
PRPP
2 steps
GAR
1
FGAR
5 steps
AICAR
2
FAICAR
IMP
AMP
GMP
ADP
GDP
3
3
dADP
dGDP
4
4
dATP
dGTP
Pyrimidines
aspartate
5 steps
UMP
5
CTP
4
UTP
UDP
CDP
3
3
dUDP
dCDP
4
4
dUTP
dCMP
dCTP
6
dUMP
7
dTMP
dTDP
4
dTTP

Figure 1.5 Conversion of dUMP to dTMP catalysed by thymidylate synthase



1.2.3 Methylenetetrahydrofolate Reductase

MTHFR catalyses the irreversible reduction of 5,10-methyleneTHF to 5-methylTHF. The majority of 5,10-methyleneTHF is derived as the product of the serine hydroxymethyltransferase-mediated addition of the β-carbon of serine to THF (42). MTHFR's product, 5-methylTHF, is used to remethylate homocysteine to methionine. Methionine may be activated to *S*-adenosylmethionine (SAM, see **section 1.2.6**), a methyl donor for numerous intracellular reactions, including DNA methylation.

Mammalian MTHFR is composed of an N-terminal catalytic domain and a C-terminal regulatory domain. It requires noncovalently bound flavin adenine dinucleotide (FAD) for catalysis and uses NADPH as a reducing agent.

MTHFR is regulated on a number of levels. *MTHFR* has at least two promoters and two isoforms (70 kDa and 77 kDa) which appear to exhibit tissue-specific expression (43, 44). Its activity may be inhibited by binding of dihydrofolate (45) and SAM (46); binding of *S*-adenosylhomocysteine (SAH) reverses the inhibitory effect of SAM (47). MTHFR can also be phosphorylated, a modification which decreases its activity by 20% and allows it to be more easily inhibited by SAM (48).

1.2.3.1 Severe MTHFR Deficiency

Severe MTHFR deficiency is the most common inborn error of folate metabolism. It is rare and caused by mutations resulting in 0-20% residual enzyme activity (49-51). Patients exhibit developmental delay, motor and gait dysfunction, seizures, and neurological impairment and have extremely high levels of homocysteine in their plasma and urine as well as low to normal plasma methionine levels (52).

1.2.3.2 Mild MTHFR Deficiency – 677C→T Polymorphism

Mild MTHFR deficiency is caused by a common genetic variant of *MTHFR*: 677C→T ($Ala^{222}Val$) (44). Compared with the lymphocytes of 677CC individuals, those of 677CT and 677TT have ~60% and ~30% specific MTHFR activity, respectively (44, 53). The degree of enzyme thermolability (assessed as residual activity after heat inactivation) is much greater in 677TT individuals (18-22%) compared with 677CT (56%) and 677CC (66-67%) (44, 53).

Low folate intake affects individuals with the 677TT genotype to a greater extent than those with the 677CC/CT genotypes. 677TT (but not 677CC/CT) individuals with lower plasma folate levels are at risk for elevated plasma homocysteine levels (54) and decreased methylation of peripheral blood mononuclear cell DNA (55).

The polymorphism does not directly affect MTHFR's catalytic activity or regulation by SAM. It is situated in a position that causes perturbations of the FAD binding site (56). In studies of human recombinant MTHFR, the protein encoded by 677T loses its FAD cofactor three times faster than the wild-type protein (47). 5-MethylTHF slows the rate of FAD release in both the wild-type and mutant enzymes, although it is to a much greater extent in the mutant enzyme. Low folate status with the consequent loss of FAD enhances the thermolability of the enzyme, thus providing an explanation for the normalised homocysteine and DNA methylation levels in folate-replete 677TT individuals.

The allele frequency of the polymorphism varies with geographical location and ethnic background (57-59). In North America, for instance, ~20-40% of Hispanics, 10-20% of whites and 0-3% of blacks are 677TT.

1.2.3.3 Mild MTHFR Deficiency – 1298A→C Polymorphism

Another common polymorphism in *MTHFR*, 1298A→C ($Glu^{429}Ala$), has been identified (53, 60). The MTHFR activity in lymphocytes of 1298CC individuals is ~65% of wild-type although the plasma homocysteine values of individuals carrying the polymorphism do not differ from wild-type values (53, 60). In studies of human recombinant MTHFR, the protein encoded by 1298C cannot be distinguished from 1298A in terms of activity, thermolability, FAD release, or the protective effect of 5-methylTHF (47). Although the mutation is situated in the regulatory domain, it does not affect regulation of MTHFR by SAM.

1.2.4 Methionine Synthase

Methionine synthase (MTR) catalyses the cobalamin (vitamin B_{12})-dependent remethylation of homocysteine to methionine (61). The methyl group from 5-methylTHF is transferred to MTR's bound cob(I)alamin cofactor to form MTR-methylcob(III)alamin and THF. It is then transferred from MTR-methylcob(III)alamin to homocysteine to form methionine. The sustained activity of MTR requires another enzyme, methionine synthase reductase. The oxidation of cob(I)alamin to cob(II)alamin may occur over time and inactivate MTR. Methionine synthase reductase transfers a one-carbon group from *S*-adenosylmethionine to MTR-cob(II)alamin to form MTR-methylcob(III)alamin (62).

MTR is the only enzyme that metabolises 5-methylTHF. Thus, when MTR activity is compromised (by genetic mutation or vitamin B_{12} insufficiency, for example), intracellular folates can be trapped as 5-methylTHF. The inability to regenerate other folate coenzymes impairs the synthesis of purines and thymidylate, causing megaloblastic anemia. Megaloblastic anemia is a feature of severe *MTR* deficiency, as are hyperhomocysteinemia, homocystinuria, hypomethioninemia, neural dysfunction and mental retardation (52).

A polymorphism, 2756A→G ($Asp^{919}Gly$), has been identified in *MTR* (61, 63). The 2756G variant has an allele frequency of ~15-20% (61, 64, 65). Some studies have shown that 2756GG individuals have lower plasma homocysteine

levels compared to 2756AA (66, 67) although others have not observed this effect (64, 68).

1.2.5 Betaine Homocysteine Methyltransferase

Homocysteine can also be remethylated to methionine by the folate-independent enzyme betaine homocysteine methyltransferase (BHMT). BHMT appears to be expressed exclusively in liver and kidney and uses betaine as a methyl group donor (69).

Betaine (*N,N,N*-trimethylglycine) may be obtained from the diet (wheat germ, wheatbran, shrimp and spinach are all excellent sources) (70). Alternatively, it is formed as a product of choline oxidation in the mitochondria through the activities of choline dehydrogenase and betaine aldehyde dehydrogenase (71, 72). Choline may be obtained from dietary sources (70) or from the hydrolysis of phosphatidylcholine by phospholipase D (73).

In humans, plasma betaine and homocysteine are strongly inversely correlated, especially in individuals with low serum folate levels (74). This suggests that the BHMT-catalysed remethylation of homocysteine can at least partially compensate for the folate-dependent MTR-catalysed reaction.

1.2.6 Methionine and *S*-Adenosylmethionine

Methionine is an essential amino acid. It is obtained from the diet through the digestion of proteins/peptides or it may be regenerated through the remethylation of homocysteine. It may be used in the synthesis of proteins, or it may be activated by ATP to form *S*-adenosylmethionine (SAM) in a reaction catalysed by methionine adenosyltransferase.

SAM participates as a methyl donor in over 120 cellular methylation reactions including the synthesis/modification of DNA, proteins, creatine, phospholipids, hormones and neurotransmitters (75, 76). *S*-adenosylhomocysteine (SAH) is the product of all SAM-dependent methylation reactions as well as a potent competitive inhibitor of most of them and therefore the SAM/SAH ratio is an important factor in the regulation of cellular methylation reactions (77).

Although the majority of SAM is used in methylation reactions (78), it can also contribute amino, ribosyl and aminoalkyl groups for the synthesis of a variety

of compounds (79). For instance, the aminopropyl group from decarboxylated SAM is critical for the production of polyamines, compounds that are essential for cellular proliferation and differentiation (80).

1.2.7 *S*-Adenosylhomocysteine and Homocysteine

Homocysteine is a sulphur-containing amino acid that is rarely found in proteins or food. Elevated plasma homocysteine levels have been implicated as an independent risk factor for cardiovascular disease (81) and high levels of homocysteine have been shown to induce oxidative stress, endoplasmic reticulum stress and the expression of pro-inflammatory cytokines (82-84).

Homocysteine is ultimately formed as a result of SAM-dependent transmethylation reactions, since it is the product of only one reaction, the hydrolysis of SAH. SAH hydrolase catalyses the bidirectional interconversion of SAH and homocysteine, a reaction that thermodynamically favours SAH synthesis but physiologically proceeds towards SAH hydrolysis (85). Since SAH is a potent inhibitor of transmethylation reactions, it has been suggested that its conversion to homocysteine provides a mechanism to remove SAH (86).

There are four fates for intracellular homocysteine. As discussed above, it may be remethylated to methionine by either MTR or BHMT or converted to SAH by the reversal of SAH hydrolysis. Homocysteine may also enter a transsulfuration pathway that ultimately produces cysteine, glutathione, taurine and inorganic sulphate. The first step of this pathway, which commits homocysteine for cysteine synthesis, is catalysed by cystathionine-β-synthetase, a vitamin B_6-dependent enzyme that is allosterically activated by SAM (87). A functional transsulphuration pathway is only found in 5 tissues: liver, kidney, pancreas, intestine, and brain (88, 89) with the highest levels of cystathionine-β-synthetase activity in liver, kidney and pancreas. Finally, homocysteine may be exported into the circulation. The presence of homocysteine in plasma probably reflects the export of homocysteine from peripheral tissues that are overwhelmed in their capacity to catabolise or remethylate it to tissues capable of transsulphuration or folate-independent remethylation, especially in the case of dietary folate deficiency.

1.3 MOUSE MODELS OF FOLATE METABOLISM AND TRANSPORT

1.3.1 Reduced Folate Carrier 1 Mouse Model

Two independent laboratories have generated knockout mice of the *Rfc1* gene (see **section 1.1.2** and **section 1.1.3**).

The Finnell group generated mice on an SWV/Fnn background. $Rfc1^{-/-}$ mice are embryonic lethal and there is no difference in the levels of plasma SAM, SAH or SAM/SAH ratio between $Rfc1^{+/-}$ and $Rfc1^{+/+}$ mice on an amino acid defined control diet (containing 2.7 mg folic acid/kg diet) (90). On a reduced folate diet (0.3 mg folic acid/kg diet), *Rfc1* heterozygotes have higher plasma SAH levels compared with *Rfc1* wild-types. In the colonic mucosa, there are no differences in the levels of folate or any homocysteine remethylation cycle intermediate (91).

The Goldman group generated mice on a C57Bl/6 background (92). They also found that the $Rfc1^{-/-}$ genotype is embryonic lethal, although treatment of pregnant dams with 1 mg folic acid per day allows ~10% of $Rfc1^{-/-}$ pups to survive until 12 days of age. The surviving knockouts exhibit a severe impairment of erythropoiesis and lymphopoiesis.

1.3.2 Methylenetetrahydrofolate Reductase Mouse Model

$Mthfr^{-/-}$ mice provide a model of severe MTHFR deficiency (see **section 1.2.3.1.**). They have a low rate of survival on the BALB/c background (~25%) and have hyperhomocysteinemia (10-fold greater than wild-type) (93). They have significantly lower levels of 5-methylTHF in plasma, liver and brain compared with $Mthfr^{+/-}$ and $Mthfr^{+/+}$ mice (94). As well, the DNA of certain tissues (brain, ovaries) is hypomethylated with respect to wild-type mice (93).

$Mthfr^{+/-}$ mice recapitulate the mild MTHFR deficiency in humans (see **section 1.2.3.2**). They have mild hyperhomocysteinemia (1.6-1.7 times greater than wild-type) and non-significantly lower levels of 5-methylTHF in plasma, liver and brain compared with $Mthfr^{+/+}$ mice (93, 94). The DNA of their brain and ovaries is also hypomethylated with respect to wild-type mice (93). They have lower levels of betaine in their plasma, liver and brain compared with $Mthfr^{+/+}$

mice, indicating an enhancement of BHMT-mediated remethylation of homocysteine (95).

Mthfr heterozygotes have been used to study the effect of mild MTHFR deficiency on the etiology of complex traits. Compared with $Mthfr^{+/+}$ mice, $Mthfr^{+/-}$ mice have impaired vascular structure and function (96-98) and lower levels of Apo-A1, a component of high density lipoprotein cholesterol (99). $Mthfr^{+/-}$ females are also more likely to have embryonic resorptions and embryos with congenital heart defects (100).

1.3.3 Methionine Synthase Mouse Model

Homozygous disruption of *Mtr* (see **section 1.2.4**) is embryonic lethal by embryonic day 8.5 (101). The hepatic and renal enzymatic activity of $Mtr^{+/-}$ mice is ~60% that of $Mtr^{+/+}$ mice. On the Black Swiss background, heterozygotes have slightly elevated homocysteine and methionine levels. In mice on a C57Bl/6 background, there are no genotype-dependent changes in SAM, SAH or SAM/SAH ratio in either liver or brain (102).

1.4 FOLATE SUPPLEMENTATION AND FORTIFICATION

The mandatory fortification of cereal-grain products with folic acid was implemented in the United States and Canada in 1998. The impetus for this policy was the demonstration that supplemental folate reduced the incidence of neural tube defects (103). In the decade since its inception, plasma folate levels have increased, plasma homocysteine levels have decreased and the incidence of NTDs has been lowered (104-106).

The level of enrichment (140 μg folic acid per 100g) was estimated to provide an additional 100 μg of folic acid per day. This was intended to supplement dietary intake to achieve the recommended intake of 400 μg per day (600 μg and 500 μg for pregnant and lactating females, respectively). However, the intake from fortified foods may be twice that which was originially estimated (107) and although folic acid is considered to be safe and non-toxic, no specific tests of its safety have been carried out (108).

In one study, unmetabolised folic acid was found in the serum of subjects supplemented with 400 μg folic acid/day, the level found in most multivitamins, but not 100 or 200 μg folic acid/day (109). This suggests that at doses above 400 μg folic acid/day, the cells of the intestinal epithelium may not be capable of metabolising the folic acid to 5-methylTHF. The effects of unmetabolised folic acid (as a marker of excess folic acid) are largely unknown, although a recent study found that natural killer cytotoxicity, an index of immune function, was inversely correlated with unmetabolised folic acid in plasma (110).

The primary concern with fortification has been the potential to mask vitamin B_{12} deficiency, a condition which may affect up to 15% of the elderly population (111). Both folate- and B_{12}- deficiency cause megaloblastic anemia. Folic acid supplementation will correct the anemia (an overt, early symptom), but not the severe neurological impairment that is also associated with chronic B_{12}-deficiency.

The impact of mandatory fortification on other aspects of public health has been the subject of several important reviews and commentaries in recent years (108, 112-116). There is concern that although folate can protect against the development of several cancers, it may promote the growth of pre-existing tumours. Such an "acceleration phenomenon" was reported in the folic acid supplementation of leukemia patients almost 60 years ago (117). Thus, individuals with premalignant, undiagnosed tumours may be unknowingly and adversely affected by fortification. There is particular concern for colorectal cancer, since folate metabolism appears to have a significant impact on cancer at this site. The epidemiological and experimental evidence of this will be discussed in **section 1.6** and the mechanisms will be discussed in **section 1.7**.

1.5 COLORECTAL CANCER

1.5.1 The Gastrointestinal Tract and its Development

The intestinal tract is a highly regenerating organ that digests and absorbs nutrients and water from ingested matter. The duodenum, jejunum and ileum comprise the small intestine and the colon and rectum comprise the large intestine.

Intestinal tissue is composed of four layers of tissue: connective tissue at the outside, smooth muscle, connective tissue (blood and lymphatic) and an epithelial layer which lines the lumen. To generate a very large absorptive area in the small intestine, the epithelial layer is organised with finger-like villi and tubular crypts of Lieberkühn. The colon is comprised only of deep crypts.

In mice, the undifferentiated endoderm is converted to a columnar epithelium from embryonic day 14.5 to 18.5 (118). During the first two postnatal weeks, the epithelium between the villi invaginates to form crypts. The highest rate of cell production and proliferation occurs between the second and third postnatal weeks (119).

Stem cells, which reside near the bottom of the crypts, give rise to transit-amplifying cells which differentiate into one of the four cell types which comprise the gut epithelium (120). The majority of cells, which are polarised and responsible for nutrient absorption, are called enterocytes (in the small intestine) or colonocytes (in the colon). Found interspersed among these cells are mucus-secreting goblet cells and hormone-releasing enteroendocrine cells. Following their differentiation, these three cell types migrate along the crypt-villus axis to the tip where they apoptose and are shed. The lifetime of epithelial cells is approximately 3-5 days (121). Paneth cells, which do not migrate but remain close to the stem cells, secrete protective antimicrobial compounds.

1.5.2 Colorectal Cancer

Intestinal tumourigenesis has been described by a well-known paradigm (122). Sequential progression from normal epithelium to early adenoma to late adenoma to adenocarcinoma requires sequential genetic and epigenetic changes in tumour suppressors and oncogenes that confer a growth advantage. It is presumed that stem cells acquire these mutations since they represent the longest living intestinal cell. However, it has also been proposed that transit-amplifying cells may have the capacity to acquire mutations, arrest and transform (commentary in (123)).

In terms of cancer mortality, colorectal cancer (CRC) ranks second overall in North America (124). Genetic factors account for approximately one fifth of

cases and environmental factors are implicated in the balance (125). The study of rare highly penetrant hereditary CRC syndromes, such as familial adenomatous polyposis, has provided invaluable clues into the etiology and pathology of this disease.

1.5.3 Familial Adenomatous Polyposis (FAP)

Familial Adenomatous Polyposis (FAP) is clinically characterised by the highly penetrant early onset of hundreds of colonic and rectal adenomatous polyps, and the majority of individuals will develop CRC by 40 years of age if left untreated (126). Germline mutations of the *Adenomatous Polyposis Coli* (*APC*) gene were identified as the primary cause of FAP in 1991 (127, 128). Since that time, over 1000 different mutations have been identified, the majority of which comprise frameshift and nonsense mutations which encode a truncated protein (129).

Although adenomas of the large bowel are the principal manifestation of FAP, extracolonic sites may also be affected. Desmoid tumours (130), congenital hypertrophy of the retinal pigment (131), thyroid cancer (132) and duodenal polyps (133) have all been reported. The primary treatment for FAP is prophylactic colectomy to prevent CRC development (134). Metastatic CRC is the main cause of death in FAP followed by small bowel cancer and desmoid tumours (135).

FAP is a very rare disease with a reported incidence of 1/8000 (126). However, loss of *APC* has also been recognised as a key early event in sporadic tumour formation (136). Functional disturbance of *APC* has been demonstrated in a large proportion of both colorectal adenomas and carcinomas (137, 138). In FAP tumours, in addition to the germline mutation in *APC*, the wild-type allele undergoes either somatic mutation (usually leading to a C-terminal truncation) or loss of heterozygosity (139). In sporadic tumours, *APC* expression may also be silenced by promoter hypermethylation (140). Although it is widely accepted that tumour formation necessitates disruption of both *APC* alleles (136), there is some evidence that certain mutations may exert a dominant-negative effect (141).

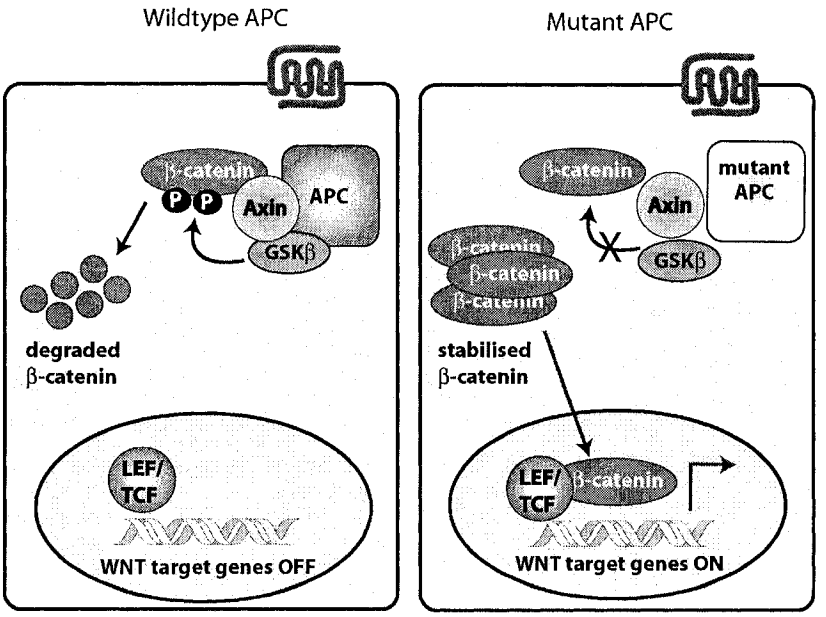
1.5.4 Functions of APC

APC is a ubiquitously expressed, predominantly cytoplasmic protein (142) which has been shown to interact with a variety of proteins in order to carry out a variety of functions.

In its most well-known role, it is a member of a protein complex involved in the canonical Wnt signaling pathway as depicted in **Figure 1.6** and reviewed in (143-145). Briefly, in unstimulated cells, cytoplasmic β-catenin is destabilised by a protein complex containing APC, Axin and glycogen synthase kinase 3 beta (GSK-3β). Axin serves as a scaffolding protein to facilitate the phoshorylation of β-catenin by GSK-3β, a modification which targets it for ubiquitination and degradation via the proteosome pathway. Although the precise role of APC in this process is unclear, it is known that APC must bind Axin for the destabilisation of β-catenin to occur (146) and that APC binds β-catenin. Stimulation by Wnt ligands and/or the absence of functional APC causes disruption of the protein complex and the stabilisation of β-catenin. Stabilised β-catenin can translocate to the nucleus where it is able to bind to T-cell factor proteins and constitutively co-activate the transcription of Wnt target genes. Expression of these genes is necessary for proper development; however, aberrant and constitutive expression may have pathogenic effects. For instance, the proto-oncogene *c-MYC* was identified as a Wnt target gene (147) and was found to be overexpressed in colon cancer (148).

Functional APC is also critical for proper cell migration. It has been shown that full-length APC associates in clusters with the distal ends of microtubles in cellular protrusions and moves along them towards the growing ends (149, 150). This ability may be dependent on interactions between APC and kinesin-2 (151) and/or EB1 (152). Furthermore, it was demonstrated that immediately following loss of both *Apc* alleles in mice, epithelial cells were unable to differentiate and migrate (153), a phenomenon that was recently attributed to compromised microtubule stability (154).

Figure 1.6 Canonical Wnt Signaling with and without functional APC.
Abbreviations: **APC** – adenomatous polyposis coli; **GSKβ** - glycogen synthase kinase 3 beta; **LEF**- lymphoid enhancer factor; **TCF**- T-cell factor. *Based on Figure 3 of Narayan et al. (155).*



APC is also implicated in cell adhesion. E-cadherin, a molecule which forms adherens junctions between epithelial cells, competes with APC for binding with β-catenin (156). The association between β-catenin and E-cadherin is required for cell adhesion and APC, through its role as a regulator of Wnt signaling, partially determines the amount of β-catenin available for this function. APC has also been localised at the plasma membrane in an association which is dependent on the actin cytoskeleton (157) and to bind to Asef, a guanine nucleotide exchange factor, an interaction which affects both E-cadherin-mediated cell adhesion and regulation of the actin cytoskeletal network (158, 159).

1.5.5 The *Apc*$^{min/+}$ Mouse Model of Colon Cancer

The generation of a mouse strain carrying an autosomal dominant germline mutation predisposing it to intestinal neoplasia was first described in 1990 (160). This strain, which was generated as part of an ethylnitrosourea mutagenesis screen, develops numerous intestinal and colonic tumours, anemia by 60 days of age, and has a reduced life-span of ~120 days. In 1992, the gene underlying this Min (multiplicity in neoplasia) phenotype was identified as the murine homolog of *APC* (161). These so-called *Apc*$^{min/+}$ mice were found to carry a nonsense mutation at codon 850 resulting in a truncated polypeptide.

Apc$^{min/+}$ mice phenotypically reflect FAP. They develop numerous small intestinal adenomas with 100% penetrance and small numbers of colonic adenomas with variable penetrance. Extraintestinal manifestations, such as desmoid tumours, epidermoid cysts and mammary tumours are rare but have been described (162). Female *Apc*$^{min/+}$ mice are occasionally healthy enough to sustain a pregnancy (160) although offspring carrying two *Apc* mutations are not born (163).

Apc$^{min/+}$ mice have been used in more than 270 studies to investigate the effect of carcinogens, pharmacological agents, vitamins and genetic modifiers (see http://jaxmice.jax.org/strain/002020rf.html). Rodent models, particularly inbred ones, allow the complete control of environment, nutrient intake and genetic background. They also facilitate an in-depth investigation of mechanisms in the mammalian system. This particular model, by virtue of carrying a mutation in a gene frequently mutated in hereditary and sporadic colorectal cancers, may be more biologically relevant than carcinogen-induced models. A much cited drawback of this model is that their adenomas are principally located in the small intestine rather than the colon. In addition, *Apc*$^{min/+}$ mice die at 4-6 months of age, as a consequence of anemia and cachexia, and therefore their adenomas rarely progress to metastatic adenocarcinomas.

In the adenomas of *Apc*$^{min/+}$ mice, the wild-type copy always undergoes allelic loss, not somatic mutation or promoter hypermethylation (164, 165). This allelic loss occurs principally by homologous somatic recombination (166). Adenomas in *Apc*$^{min/+}$ mice almost certainly arise from the stem cells in the crypts

(167). Once cells acquire two *Apc* mutations, they immediately acquire defects in differentiation, proliferation, migration and apoptosis (153). These phenotypes can be rescued by concurrent deletion of *c-Myc,* implying that deranged Wnt signaling contributes to tumourigenesis in mice (168).

1.5.6 Modifier of Min 1

In 1992, a major strain-dependent modifier of the Min phenotype was proposed following the discovery that intercrosses of $Apc^{min/+}$ mice (which are maintained on the C57Bl6/J genetic background) with AKR/J and MA/MyJ inbred strains produced F1 hybrids with considerably fewer adenomas compared with pure C57Bl/J $Apc^{min/+}$ mice (169). The locus, designated *Mom1* (for Modifier of the Min phenotype), was mapped (170) and identified as secretory type II phospholipase A2 (*Pla2g2a*) (171). *Pla2g2a* was shown to be polymorphic among different strains of inbred mice. Strains expressing full-length wild-type *Pla2g2a* were all shown to be resistant to the Min phenotype; the allele they carry was termed $Mom1^R$. The presence of one $Mom1^R$ allele decreased adenoma number by 50%. Strains expressing almost no functional *Pla2g2a* (due to a frameshift mutation and premature stop codon) were susceptible to adenoma formation; the allele they carry was termed $Mom1^S$. In humans, *PLA2G2A* does not appear to modify the FAP phenotype (172, 173) nor do variants appear to influence the formation of sporadic colon tumours (173). To date, seven *Mom* alleles have been identified (174).

1.6 FOLATE AND COLORECTAL CANCER

1.6.1 Dietary Folate and Colorectal Cancer – Humans

Numerous case-control, prospective and intervention studies have investigated the effect of folate status on colorectal adenoma or cancer risk. Folate status may be measured as food and/or supplement intake (using questionnaires or journals), red blood cell folate levels, serum folate levels or plasma homocysteine levels (a biomarker of folate deficiency).

The majority of prospective studies and retrospective case-control studies have demonstrated an inverse relationship between folate status and colorectal

adenoma or cancer risk (reviewed in (114, 175, 176)). In a *meta*-analysis of prospective studies, high versus low dietary folate status resulted in a 25% reduction in risk for CRC (RR=0.75; 95%CI = 0.64-0.89) (177). A similar result was obtained in the *meta*-analysis of case-control studies (OR=0.76; 95%CI = 0.62-1.05) (177).

Some studies do not support the assertion that folate is protective. De Vogel *et al.* found that in men, high folate intake was associated with an increased risk of CRC with a truncating *APC* mutation (RR=2.77; CI=1.29-5.95) and a decreased risk of CRC with no *APC* mutation (RR=0.58; CI=0.32-1.05) (178). Also, van Guelpen *et al.* suggested that low folate levels may protect against CRC risk (179). CRC risk was increased in the middle versus the lowest quintile of plasma folate levels (OR=2.00; 95% CI = 1.13-3.56).

There have been two randomised placebo-controlled folic acid intervention trials with colorectal adenoma recurrence as the primary endpoint. Folic acid (1mg/day) was supplied to individuals with previously diagnosed and resected colorectal adenomas. One study reported a nonsignificant decrease in adenoma recurrence following 2 years of supplemention (180). Another study found that after 5-8 years of supplementation, the overall recurrence incidence was unchanged but that supplemented individuals were at higher risk for developing greater than three adenomas (RR=2.32; 95% CI = 1.23-4.35) and advanced lesions (RR=1.67; 95% CI = 1.00-2.80) (181).

1.6.2 *MTHFR* and Colorectal Cancer

Numerous epidemiological studies have found a significant protective effect of the 677TT genotype compared to 677CC for CRC risk. These include Van Guelpen *et al.* (OR=0.41; 95% CI=0.19-0.85)(179), Ma *et al.* (OR= 0.45; 95% CI=0.24-0.86)(182), Chen *et al.* (OR=0.54; CI= 0.29-0.99)(183) and Ulvik *et al.* (OR=0.73; 95% CI=0.58-0.92)(184). A *meta*-analysis of 23 studies worldwide indicated that, overall, 677TT individuals are 14% less likely to develop CRC (OR=0.86; 95% CI=0.76-0.96, p<0.0001) but that *MTHFR* has no effect on colorectal adenoma risk (185). The lack of association between *MTHFR* genotype and adenoma risk has been observed in several studies (186-190).

1.6.2.1 *MTHFR* and Microsatelite Instability

Sporadic CRCs can be classified according to their microsatellite stability. Microsatellite instability (MSI) occurs in 10-15% of sporadic CRC and is associated with better prognosis compared with stage-matched microsatellite stable (MSS) tumours (191). Microsatellites are short, tandem DNA repeat sequences that can be expanded or contracted as a consequence of DNA repair defects which can introduce frameshift mutations that can alter gene expression. MSI is the hallmark of hereditary non-polyposis colon cancer (HNPCC), which is caused by germline mutations in DNA repair enzymes, including hMLH1. In sporadic forms, hypermethylation of the hMLH1 promoter region appears to underly the MSI phenotype (192, 193).

The effect of *MTHFR* genotype on microsatellite stability has been inconsistent. Three studies have found that 677TT individuals are more likely to develop MSI tumours than MSS tumours (194-196). Plaschke *et al.* found no effect of *MTHFR* genotype on risk for tumours with or without MSI (197). Eaton *et al.* found that folate-adequate individuals carrying at least one 677T allele were significantly less likely to have MSI tumours than MSS tumours (198). They also showed that folate intake (total, dietary and supplemental) did not influence the risk for MSI.

1.6.2.2 Interaction between Folate, *MTHFR* and Colorectal Cancer

Several studies have decribed an interactive effect of folate and *MTHFR* genotype on CRC risk. Ma *et al.* showed that the protective effect of the 677TT genotype is abolished when folate intake/status is low (182). Others have shown that the protective effect of the 677TT genotype is the strongest in subjects with high folate status (199, 200). In contrast, some studies have reported no interaction between *MTHFR*, folate status and risk (183, 184) or that the significant effect of 677TT genotype is equally protective in individuals with both high and low folate status (179). The combination of low folate status and 677TT genotype may also increase the risk of adenoma incidence (190, 201) and adenoma recurrence (202).

1.6.2.3 Interaction between Riboflavin, *MTHFR* and Colorectal Cancer

Riboflavin (vitamin B_2) is the precursor of flavin adenine dinucleotide (FAD), a cofactor for numerous enzymes including MTHFR. Biochemically, thermolabile MTHFR has reduced activity because of an increased rate of dissociation of the essential FAD cofactor which causes structural changes in the enzyme (47, 203). It has been shown that plasma homocysteine levels are inversely correlated with plasma riboflavin levels. This association may be independent of *MTHFR* genotype (204), although a number of studies have found it to be mainly confined to 677TT subjects (205, 206) or 677TT individuals with low folate status (207).

Few studies have investigated the interaction between riboflavin status, *MTHFR* genotype and CRC or adenoma risk. Le Marchand *et al.* found no interaction (199). Van den Donk *et al.* found that there was an inverse association between riboflavin intake and adenoma risk, especially in 677TT individuals (189).

1.6.2.4 Interaction between Alcohol, *MTHFR* and Colorectal Cancer

High alcohol intake interferes with folate metabolism and may cause folate deficiency. Chronic alcohol intake can cause folate malabsorption, increased excretion, increased catabolism and/or abherrent methionine metabolism (208, 209).

High alcohol intake may be an independent risk factor for colon cancer (*meta*-analysis; RR= 1.50; 95% CI = 1.25-1.79). This effect is exacerbated by low folate and methionine intake (210). Furthermore, in studies investigating the effect of *MTHFR* genotype on CRC risk, the protective effect of the 677TT genotype is ablated by high alcohol intake (182, 183).

1.6.3 *Methionine Synthase* and Colorectal Cancer

A number of studies have investigated the association between the *MTR* 2756A→G ($Asp^{919}Gly$) polymorphism and CRC risk. Five studies did not observe a significant effect of the polymorphism on CRC risk (188, 199, 211-213). However, Ma *et al.* found that the 2756GG genotype conferred a non-significant decreased risk for CRC compared to 2756AA (OR=0.59; 95% CI=0.27-1.27) (64). This finding was reinforced by a larger study which found a reduced risk in

2756GG compared with 2756AA individuals (OR=0.65; 95% CI=0.47-0.90) (184). The one study of colorectal adenoma (the precursor of CRC) risk found that 2756GG genotype nonsignificantly increased the risk in women (214).

The activity of methionine synthase reductase is essential for sustained MTR activity. Two polymorphisms, the functional significance of which have not yet been assessed, were found to be associated with an increased risk for CRC (213) as well as advanced colorectal adenoma (211).

1.6.4 *Reduced Folate Carrier* and Colorectal Cancer

A polymorphism, 80G→A ($Arg^{27}His$) has been described in *RFC1* (215). The 80A variant has an allele frequency of ~47% and does not significantly affect plasma homocysteine, plasma folate or red blood cell folate levels. Ulrich *et al.* did not observe an effect of the polymorphism on CRC risk (212).

1.6.5 Folate and Colorectal Cancer – Rodents

Animal studies have been used to investigate the effect of timing and dose of folic acid on intestinal neoplasia.

Two studies by Song *et al.* illustrated the disparate effects of folate supplementation and deficiency depending on the duration or time of initiation. In one study, $Apc^{min/+}$ mice were placed on diets varying in folic acid content from 3 weeks of age for either 3 or 6 months (216). There were no differences in the total number of adenomas, although in supplemented mice, the number of ileal adenomas was lower after 3 months of dietary treatment but higher after 6 months. In another study, $Apc^{+/-}Msh2^{-/-}$ mice were placed on either deficient (0 mg folic acid/kg diet) or supplemented (8 mg folic acid/kg diet) initiated at either 3 or 6 weeks of age (217). If the diets were started at 3 weeks of age (speculated to be prior to the establishment of neoplastic foci) the supplemented diet significantly decreased the number of adenomas relative to the deficient diet. However, if the diets were initiated at 6 weeks of age (speculated to be after the establishment of neoplastic foci), the deficient diet significantly decreased the number of adenomas relative to the supplemented diet. They did not identify any differences in DNA methylation or microsatellite instability.

Other reports have examined the effect of dietary folate content on SAM, SAH, DNA hypomethylation and adenoma number in *Apc*$^{min/+}$ mice. Sibani *et al.* examined these parameters in mice fed control (2 mg folic acid/kg diet, 3 mg choline/kg diet) and deficient (0 mg folic acid/kg diet, 1.5 mg choline/kg diet) diets (218). The dietary effect was inconsistent in three repeat experiments. The statistically significant finding, that deficient mice had higher adenoma numbers, occurred only in the group with low SAM levels and DNA hypomethylation. In control diet mice, adenoma multiplicity was positively correlated with SAM, SAH and DNA hypomethylation. Trasler *et al.* reported no effect of dietary folate on DNA methylation in *Apc*$^{min/+}$ mice, although deficient mice developed fewer and larger adenomas (219).

The injection of rodents with either azoxymethane (AOM) or dimethylhydrazine (DMH) can induce the development of colonic adenomas/adenocarcinomas as well as their precursor lesion, aberrent crypt foci. The effect of folate intake on AOM- or DMH-treated rats is inconsistent. Le Leu *et al.* found that in AOM-treated rats, those on a folate-deficient diet (0 mg folic acid/kg diet) had a lower incidence and number of tumours compared with rats on a control diet (8 mg folic acid/kg diet) (220). In contrast, Cravo *et al.* found that in DMH-treated rats, the folate-deficient diet increased the incidence of dysplasia and carcinoma compared with the supplemented group (8 mg folic acid/kg diet) (221). The main differences between the two studies were the diet (Le Leu *et al.* used a semi-purified rodent diet and Cravo *et al.* used high glutamate amino acid-defined diet) and the dosing protocol (Le Leu *et al.* injected 3 times, Cravo *et al.* injected 20 times).

The effect of AOM in mice with folate transport defects has also been investigated (91). Six weeks after azoxymethane injections, *Rfc1*$^{+/-}$ mice developed the same number of aberrent crypt foci as *Rfc1*$^{+/+}$ mice, although there were more aberrant crypts per focus. Thirty-eight weeks after injection, there were no differences in the number or incidence of colonic adenocarcinoma, although the size of the tumours was larger in the *Rfc1*$^{+/-}$ mice.

In mice without germline mutations and without carcinogen induction, folate deficiency has been shown to initiate tumours. Knock *et al.* showed that in the inbred BALB/c strain, 25% of mice fed a folate-deficient diet (0.3 mg folic acid/kg diet) developed intestinal adenomas compared with 0% of mice fed a control diet (2 mg folic acid/kg diet) (222).

1.6.6 Antifolate Drugs

The disruption of folate metabolism is the basis of antifolate chemotherapy. The relationship between folates and carcinogenesis was borne from the very early observation that treatment of leukemia patients with folic acid induced rapid and severe relapses (223). This observation was followed by the landmark study by Farber *et al.* who treated leukemia patients with a folate analogue (aminopterin) which resulted in temporary remissions (224). Since then, less toxic analogues, such as the widely used methotrexate (a tight-binding inhibitor of DHFR) have been developed. By inhibiting DHFR, methotrexate decreases the production of the biologically active folate compound, THF. This compromises the synthesis of purines and thymidylate, which inhibits the DNA replication of proliferative neoplastic cells (225). The pyrimidine analogue 5-fluorouracil (5-FU), which inhibits thymidylate synthase, is the mainstay of advanced and adjuvant treatment of CRC (226).

1.7 FOLATE AND COLORECTAL CANCER – MECHANISMS

Several hypotheses have been proposed to explain the effects of folate metabolism (dietary and genetic) on CRC risk: nucleotide imbalance, DNA methylation defects or apoptosis.

1.7.1 Nucleotide Imbalance and Uracil Misincorporation

The balance of dNTPs is crucial for the accuracy of DNA synthesis and repair. This is especially true of highly proliferative tissues, which place a high demand on nucleotide synthesis. Since they are energetically costly to produce, DNA precursor pools are very small, tightly coupled to DNA synthesis and highly regulated (227).

There are two points of allosteric control of dNTP production: ribonucleotide reductase and dCMP deaminase (see **Figure 1.4**). The balance of dNTPs is largely attributed to ribonucleotide reductase, the enzyme which reduces all NDPs to dNDPs (228). It contains an activity site which is activated by ATP (signaling a requirement for dNTPs) and repressed by dATP (signaling an oversupply of dNTPs). Binding of dTTP, dGTP and dATP/ATP to the specificity site regulates its substrate specificity. Additional regulation is achieved by the negative feedback inhibition of dTTP on dCMP deaminase, the enzyme responsible for the production of dUMP from dCMP.

The balance of dNTPs is also governed by substrate availability, which is affected by the rate-limiting enzymes of DNA synthesis. One such enzyme, thymidylate synthase, catalyses the production of dTMP by transferring a carbon group from 5,10-methyleneTHF to dUMP (see **Figure 1.5**). The availability of 5,10-methyleneTHF is largely determined by the activities of SHMT and MTHFR. As reviewed earlier, SHMT transfers a methylene group from serine to THF to generate 5,10-methyleneTHF. MTHFR, on the other hand, catalyses the irreversible reduction of 5,10-methyleneTHF, thus committing one-carbon groups to the methylation cycle. Therefore, if MTHFR activity is reduced (either by *Mthfr* knockout in mice or the 677TT polymorphism in humans), 5,10-methyleneTHF is expected to accumulate at the expense of 5-methylTHF. This increased availability of 5,10-methyleneTHF is hypothesised to favour dTMP (and dTTP) production relative to dUMP (and dUTP). In contrast, dietary folate deficiency, by limiting the availability of all folate derivatives including 5,10-methyleneTHF, is expected to increase the relative amounts of dUMP (and dUTP) with respect to dTMP (and dTTP). The significance of the dUTP/dTTP ratio will be discussed in the following section.

Uracil in DNA

Adenine basepairs with uracil and thymine in RNA and DNA, respectively. Uracil may, however, be found in DNA as a result of the spontaneous deamination of cytosine to uracil. This causes a premutagenic U:G mismatch which, if left unrepaired prior to replication, will cause a C→T transition mutation (229).

Uracils may also occur in DNA as a result of the inappropriate insertion of dUTP instead of dTTP during DNA polymerisation (230). This misincorporation is attributed to the close chemical structures of dUTP and dTTP; they differ solely by the single methyl group provided by 5,10-methyleneTHF. Although the resultant U:A mismatch is not harmful in and of itself (since it will be corrected in the next round of replication), the base excision repair processes which govern its removal may lead to detrimental consequences.

Uracils are removed from DNA by uracil DNA glycosylases (UDG) of which at least four have been described in mammalian cells: UNG, TDG, SMUG1 and MBD4 (reviewed in (231)). The functional distinction among these glycosylases is not entirely clear, but evidence suggests that the UNG family is responsible for the excision of misincorporated uracils (232) and SMUG1 is responsible for the removal of deaminated cytosines (233). After recognition of the inappropriate uracil, the UDG cleaves the N-glycosidic bond between the base and sugar, releasing uracil and creating an abasic site. The phosphodiester bond is then cleaved by an apurinic/apyrimidinic endonuclease which facilitates the ensuing insertion of the correct nucleotide. This step, which generates transient and benign single-stranded breaks, may cause harmful double-stranded DNA breaks if two uracils are found and repaired in close proximity. Double-stranded DNA breaks have been shown to cause genomic deletions, duplications, instability and ultimately transformation (234-237).

Dietary folate deficiency is hypothesised to increase the relative amount of dUTP to dTTP and therefore increase the likelihood of two closely spaced uracils. It has been demonstrated *in vitro* and in rodents that folate deficiency can increase the dUTP/dTTP ratio (238, 239) and induce uracil misincorporation (240). In contrast, MTHFR deficiency is hypothesised to decrease the relative amount of dUTP to dTTP and therefore decrease the likelihood of two closely spaced uracils. In human lymphocytes, the impact of *MTHFR* genotype on uracil DNA misincorporation has mainly yielded negative results (241-243) and it has not yet been studied in mice.

In terms of CRC, the increased risk for folate-deficient individuals may be a consequence of an increase in dUTP/dTTP ratio and uracil DNA misincorporation, which in turn may cause double-stranded DNA breaks which may lead to the initiation of tumours. On the other hand, *MTHFR* deficiency may be protective by decreasing the dUTP/dTTP ratio and uracil misincorporation therefore causing fewer double-stranded DNA breaks and decreasing the likelihood of tumour initiation.

1.7.2 DNA Methylation

Epigenetic modifications are heritable changes in gene expression that do not involve a change in DNA sequence and include histone modifications, microRNAs and DNA methylation.

DNA methylation is linked to folate metabolism through SAM (see **section 1.2.6**). DNA methyltransferase (DNMT) enzymes covalently attach the methyl group from SAM to cytosines *in situ* (see **Figure 1.3**). The DNMT1 isoform is responsible for maintaining methylation patterns after replication (thereby ensuring the propagation of tissue specific patterns) whereas the DNMT3a and DNMT3b isoforms are responsible for *de novo* methylation (usually during development) (reviewed in (244)).

In most cases, the consequence of DNA methylation is reduced transcriptional activity. This appears to be achieved through the recognition of methylated cytosines by specific proteins (i.e., MeCP2) and the subsequent recruitment of a histone deacetylase-containing complex, the activity of which promotes a transcriptionally repressive chromatin state (245). Alternatively, methylation may directly block the binding of transcriptional activators to their recognition sites (246).

DNA methylation generally occurs in the context of a CpG dinucleotide; 4-6% of cytosines are methylated in normal tissue (247). Short segments of DNA with a high CpG content are called CpG islands. They comprise a small percentage (~6-7%) of all CpGs, normally occur at the 5'-region of genes and are usually unmethylated (248). CpG methylation is important for X chromosome inactivation in females and for the expression of imprinted genes (genes that

contain differentially methylated regions and are expressed depending on the parental origin of the allele) (249). Finally, almost half of CpGs occur in repetitive parasitic sequences, such as transposons and endogenous retroviruses, which comprise about a third of the genome (250-252). In this context, the majority of CpGs are methylated. This may prevent the activation of parasitic sequences (which have the ability to "jump" around the genome and cause mutations) and the unmasking of repeats (which may cause chromosomal recombination) (250-252).

DNA Methylation and Colorectal Cancer

Relative to normal tissue, global DNA hypomethylation and regional hypermethylation appear to paradoxically coexist in tumours (253, 254). Although it is clearly an important process, in the field of cancer epigenetics it remains unresolved whether DNA methylation changes are the cause or consequence of tumourigenesis (commentary in (255)).

There are a number of possible consequences of global DNA hypomethylation (reviewed in (249)). Although it is likely quite rare, it may cause reactivation of transposable elements. For example, the disruption of *APC* by the retroviral insertion of a repetitive element has been demonstrated in a human colon cancer (256).

DNA hypomethylation may also promote the activation of proto-oncogenes. In rats fed methyl-deficient diets, global DNA hypomethylation was associated with the overexpression of *c-myc* and *c-fos* (257).

DNA hypomethylation may also lead to the loss of imprinting, which may play a role in the etiology of CRC (258). One study showed that hypomethylation of a differentially methylated region of insulin-like growth factor 2 (*IGF2)* in colonic and lymphocyte tissue was associated with a higher risk for CRC (259). This finding was complemented by a study using the $Apc^{min/+}$ model; mice overexpressing *Igf2*, a model for its loss of imprinting, developed ~2.2 times the number of intestinal adenomas (260).

Finally, DNA hypomethylation may induce chromosomal instability. This may be mediated by the hypomethylation of pericentromeric regions (normally heavily methylated) that may result in chromosomal abnormalities including

rearrangements, translocations or deletions (261). One study showed that DNA hypomethylation in *Dnmt1* hypomorphic mice (~10% expression) was associated with an increased incidence of T-cell lymphomas containing chromosome 15 trisomy (262).

In the majority of rodent studies to date, folate deficiency did not affect global DNA hypomethylation in the colon or intestine (217, 219, 263-267) and in studies of $Apc^{min/+}$ mice, global DNA hypomethylation has been shown to be associated with both an increase (218) or a decrease (268) in adenoma number.

In addition to global DNA hypomethylation, regional hypermethylation is often observed in tumours. In CRC, the hypermethylation of CpG islands in the promoter region of a number of tumour suppressors, including *APC* and *hMLH1* has been described (reviewed in (269)). In $Apc^{min/+}$ mice, *Dnmt1* deficiency was found to significantly decrease adenoma number (219, 268, 270, 271). One of these studies found that mice harbouring *Dnmt1* mutations had reduced levels of CpG island methylation in three tumour suppressor genes in both their normal intestinal mucosa and adenomas (270). Another study found that neither folate deficiency nor *Dnmt1* deficiency altered global methylation levels, *E-cadherin* promoter methylation or methylation of exons 5-8 in *p53* (219).

1.7.3 Apoptosis

Apoptosis, or programmed cell death, is an important process in tissue homeostasis and development. Apoptosis is triggered by signals that activate either an intrinsic (mitochondrial) or an extrinsic (death receptor) pathway depending on their origin or nature. The central mediators of apoptosis are caspases, members of a family of cysteine proteases that cleave selected substrates which ultimately cause cell death (272). All caspases are initially synthesised as zymogens (procaspases) that are activated upon cleavage. A "caspase cascade" (cleavage of downstream procaspases by activated upstream caspases) leads to the activation of the effector caspases (caspases-3, -6 and –7). Both the intrinsic and extrinsic pathways converge at caspase-3 activation (272).

Apoptosis is essential for the elimination of damaged cells and the prevention of tumour formation. For example, germline mutations in the tumour

suppressor *TP53* (a pro-apoptotic protein) causes Li Fraumeni syndrome, a disease characterised by the early onset of cancer (273). Once a tumour has initiated, genetic and epigenetic strategies are employed in order for it to avoid cell death. Various chemotherapeutics are designed to enhance or promote apoptosis in order to counter this.

In the normal intestinal epithelium, a highly proliferative tissue, the balance between cell renewal and cell death is achieved through the apoptosis of the short-lived epithelial cells (274-276). The rate of apoptosis may also play an important role in the prevention of tumours in this tissue. One study found that the intestinal mucosa of patients with a history of adenomas exhibited a marked decrease in apoptosis compared with controls (277). In studies of $Apc^{min/+}$ mice, a decrease in adenoma number was attributed in part to enhanced apoptosis in response to aspirin (278), regulatory lymphocytes (279), polyethylene glycol (280) and the overexpression of the ZNF148 transcription factor (281). Importantly, it has been demonstrated that caspases –3 and –7 are downregulated in $Apc^{min/+}$ mice compared with $Apc^{+/+}$ mice, a finding hypothesised to contribute to tumour growth and the resistence to apoptotic stimuli (282).

There is evidence supporting a relationship between folate metabolism and apoptosis. Apoptosis levels were increased by folate deficiency in a liver hepatoma cell line (283) and by homocysteine in an endothelial cell line (284). The antisense inhibition of *Mthfr* both *in vitro* (CRC cell lines) and *in vivo* (tumour xenografts) was shown to reduce cell survival and growth by enhancing apoptosis (285). Finally, in response to 5-fluorouracil treatment (see **section 1.6.6**), CRC cells die by apoptosis in a "thymineless death" caused by dTTP depletion and DNA damage (286).

1.8 THESIS RATIONALE

Although the evidence supporting a role for folate metabolism in CRC is clear, the definitive mechanisms through which it influences tumour initiation and growth is not. Such knowledge would be beneficial for chemotherapeutic design and application and for nutritional guidelines, especially in the era of folic acid supplementation and fortification. Epidemiological studies have provided invaluable insight, but they are limited in their ability to provide mechanistic information. In this thesis, animal models will be used to complement such studies through the examination of genetic and nutritional variables in a controlled environment.

In Chapter II, the effect of genetic deficiency of *Mthfr*, *Rfc* and *Mtr* and dietary folate deficiency on dUTP/dTTP ratios will be investigated in mice not predisposed to intestinal neoplasia.

In Chapter III, the effect of genetic deficiency of *Rfc* and *Mtr* and dietary folate deficiency on tumourigenesis in the $Apc^{min/+}$ model will be addressed. Two mechanisms, DNA methylation and apoptosis, will be also be examined.

In Chapter IV, the effect of genetic deficiency of *Mthfr* and dietary folate deficiency on tumourigenesis in the $Apc^{min/+}$ model will be investigated. The effects of maternal genotype and the timing of diet initiation will also be examined, as will DNA methylation, apoptosis and dUTP/dTTP ratios.

CHAPTER II

Low dietary folate and methylenetetrahydrofolate reductase deficiency alter dUTP/dTTP ratios and DNA methylation in mouse intestine

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2.1 ABSTRACT

A polymorphism in methylenetetrahydrofolate reductase (*MTHFR*) is associated with decreased risk of developing CRC when folate levels are adequate. One hypothesis involves the role of the MTHFR substrate (5,10-methyleneTHF) in the methylation of dUMP to dTMP, the precursor for thymidine. Imbalances in the relative amounts of their triphosphates (dUTP/dTTP) can lead to uracil misincorporation into DNA, DNA damage, genomic instability and tumourigenesis. Decreased MTHFR activity may be protective by lowering the dUTP/dTTP ratio and thus limiting the amount of DNA damage, especially in highly proliferative tissues such as the intestine. Using $Mthfr^{+/+}$, $Mthfr^{+/-}$ and $Mthfr^{-/-}$ mice, we investigated this mechanism by measuring the dUTP/dTTP ratios in intestine and liver. Mice were fed rodent chow (8 mg folic acid/kg diet) or a folic acid- deficient diet (FADD, 0.3 mg folic acid/kg diet). Compared to rodent chow, the FADD elicited a two-fold increase in the dUTP/dTTP ratio in intestine and liver. Mutation in *Mthfr* was associated with a lower dUTP/dTTP ratio in the intestine, compared to wild-type mice, with borderline significance (P=0.06). Since the MTHFR product (5-methylTHF) is used for the production of methionine and SAM, we measured DNA methylation. We found that folate deficiency significantly decreased the amount of methylation in intestine and liver; $Mthfr^{-/-}$ mice showed decreased methylation in the intestine but not in liver. Our findings support the hypothesis that MTHFR maintains the balance between folate derivatives for nucleotide synthesis and for methylation reactions *in vivo*, and are consistent with the protective effect of MTHFR deficiency in some neoplasias.

2.2 INTRODUCTION

Folates facilitate the transfer of one-carbon units in several intracellular pathways including nucleotide and amino acid synthesis, and methylation reactions. Low dietary intake of the vitamin folic acid, which decreases the levels of all folate forms, has been associated with an increased risk for various cancers, including CRC (175, 210, 287). A polymorphism (677C→T) in an important enzyme of folate metabolism, methylenetetrahydrofolate reductase (MTHFR),

which causes a redistribution of folate derivatives, confers protection against colorectal cancer and certain leukemias (182, 184, 288). The protective effect of the polymorphism in colorectal cancer is not observed in the presence of folate deficiency, whereas the influence of folate status on leukemia risk has not yet been examined. The mechanisms by which disturbances in folate metabolism modify cancer risk have not been conclusively established. To this end, efforts have been primarily focused on the role of folate derivatives as providers of one-carbon units for DNA and RNA precursor production and for methylation reactions.

5,10-Methylenetetrahydrofolate (5,10-methyleneTHF) is required for the methylation of dUMP to dTMP, a rate-limiting step in DNA synthesis catalysed by thymidylate synthase. 5,10-MethyleneTHF is also the substrate for methylenetetrahydrofolate reductase (MTHFR). Low dietary intake of folates results in a decrease of 5,10-methyleneTHF as well as other folate derivatives, whereas decreased MTHFR activity, as has been reported for individuals who are homozygous for the 677 C→T polymorphism (44), may result in an increase of 5,10-methyleneTHF. 5,10-MethyleneTHF cannot be measured directly at physiological pH, but it has been previously demonstrated that 677TT individuals have altered distributions of methylated and formylated folate forms (289). It is thought that lower levels of 5,10-methyleneTHF may lead to a decrease in deoxythymidine monophosphate (dTMP) and the DNA precursor deoxythymidine triphosphate (dTTP), as well as an accumulation of deoxyuridine monophosphate (dUMP) and deoxyuridine triphosphate (dUTP). Because dUTP and dTTP are similar in structure and DNA polymerases do not distinguish between the two with high fidelity, dUTP may be misincorporated into DNA as uracil. It has been demonstrated *in vitro* and in rodents that dietary folate deficiency can increase the dUTP/dTTP ratio (238, 239) and can result in uracil misincorporation, double-stranded DNA breaks and genomic instability (237, 240, 290). The hypothesised endpoint of these deleterious changes, cancer, is supported by epidemiologic studies in humans (reviewed in (175)) as well as in more direct studies in mice (222), which have shown that a dietary folate deficiency is associated with increased risk for CRC.

It has also been hypothesised that decreased activity of MTHFR may be protective in neoplasias, especially in highly proliferative tissues such as the intestine and lymphocytes, because the dUTP/dTTP ratios and levels of uracil misincorportion might be lowered. This possibility has been suggested because MTHFR deficiency might increase the levels of 5,10-methyleneTHF for thymidylate synthesis, as discussed above. Although it has been shown that *MTHFR* 677TT individuals are at a decreased risk for developing colorectal cancer and certain leukemias (182, 288), studies on the effect of the polymorphism on uracil misincorporation into human lymphocyte DNA have been mainly negative (241-243) although dUTP/dTTP ratios have not been measured *in vivo*.

The product of the MTHFR-catalysed reaction is 5-methyltetrahydrofolate (5-methylTHF), which is used to remethylate homocysteine (Hcy) to methionine by methionine synthase (MTR). Methionine is converted to *S*-adenosylmethionine (SAM), a methyl donor which participates in numerous cellular reactions including DNA methylation, a modification that can alter gene transcription. Changes in DNA methylation, either global or site-specific, may permit aberrant oncogene expression and/or silencing of tumour suppressors. Both dietary folate deficiency and MTHFR deficiency may compromise DNA methylation, since they both decrease the availability of 5-methylTHF (291).

In earlier work, we generated genetically altered mice in which the *Mthfr* gene has been inactivated by homologous recombination (93) and demonstrated that $Mthfr^{-/-}$ mice have altered distributions of folates in plasma, liver and brain (94). $Mthfr^{+/-}$ and $Mthfr^{-/-}$ mice also have 1.7 and 10-fold higher levels of plasma Hcy compared to $Mthfr^{+/+}$ mice (93). These mice ($Mthfr^{-/-}$ and $Mthfr^{+/-}$) are good animal models for the severe MTHFR deficiency observed in patients with homocystinuria and for the mild MTHFR deficiency observed in individuals with the 677TT genotype, respectively. Since MTHFR deficiency has been suggested to protect against colorectal carcinogenesis through an alteration in uracil: thymidine levels, we investigated this possibility by examining dUTP/dTTP ratios in the intestine of *Mthfr*-deficient mice on a regular diet and on a low folate diet. We compared these findings with those from two other mouse models that have

disturbances in folate metabolism: *Rfc1*$^{+/-}$ mice with a deficiency of the reduced folate carrier 1 (90, 91), which is involved in folate transport, and *Mtr*$^{+/-}$ mice with a deficiency of methionine synthase (101), the enzyme that utilises 5-methylTHF to remethylate homocysteine for generation of methionine.

2.3 MATERIALS AND METHODS

Mice and dietary intervention

Animal experimentation was approved by the Montreal Children's Hospital Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care.

The generation and genotyping of mice with mutations in *Mthfr*, reduced folate carrier 1 (*Rfc1)* and methionine synthase (*Mtr)* genes were performed as previously described (91, 93, 101) and shown in Appendix I. The genetic backgrounds of the *Mthfr*, *Rfc* and *Mtr* mice used in these experiments were BALB/c, SWV/Fnn and Black Swiss, respectively. *Mthfr* mice were weaned at 3.5 weeks and fed either standard mouse chow containing 8 mg folic acid/kg diet (Mouse Chow, Laboratory rodent diet 5001, Agribrands Purina) or an amino-acid defined folate-deficient diet containing 0.3 mg folic acid/kg diet that was supplemented with 1% succinylsulfanthiozole to inhibit folate synthesis by intestinal flora (FADD, TD 01546, Harlan Teklad, Madison WI). The *Rfc* and *Mtr* mice were fed mouse chow from weaning until sacrifice. The animals were sacrificed by carbon dioxide asphyxiation at 5-7 months of age.

dUTP/dTTP analysis

Free deoxyribonucleotides were separated using a previously-described HPLC method (292) that was slightly modified. Briefly, duodenal tissue was flash frozen in liquid nitrogen, ground to powder using a mortar and pestle, treated with 0.6 M trichloracetic acid, neutralised with trioctylamine and injected onto an Econosphere C18 column (particle size 5 μm, length 250 x 4.6mm, Waters instrument Part No. 70071, Alltech). Separation of dUTP and dTTP was achieved by isocratic elution with 100% Buffer A (0.1 M $NH_4H_2PO_4$, 0.33 M KCl, 0.25% methanol, pH 5.35) for 12 minutes followed by a linear gradient to 25% Buffer B

(0.1 M $NH_4H_2PO_4$, 0.4 M KCl, 20% methanol, pH 5.0) for 18 minutes followed by a linear gradient to 80% Buffer B for 10 minutes, then 10 minutes of 80% Buffer B, followed by re-equilibration with 100% Buffer A for 20 minutes. The flow rate for the entire run was 0.8 mL/min. A UV detector monitored peaks at wavelengths of 254 nm and 280 nm. Peaks were assigned by co-elution with known standards (Sigma-Aldrich).

DNA methylation analysis

DNA bases were separated using a previously-described HPLC method (293) with some modifications. Briefly, 10 μg of RNA-free genomic DNA obtained from duodenal tissue was treated with 1.2 U nuclease P1 (US Biological) and 2 U calf intestinal alkaline phosphatase (Invitrogen), and injected onto an Econosphere C18 column (Alltech). Separation of bases was achieved with 40 minutes of isocratic elution with 0.01 M $NH_4H_2PO_4$ containing 2.5% methanol (pH 5.3) followed by 30 minutes of isocratic elution with 0.01 M $NH_4H_2PO_4$ containing 10% methanol (pH 5.1). The flow rate was maintained at 1mL/min. A UV detector, monitoring at wavelengths of 254 nm and 280 nm, was used. Peaks were identified by co-elution with known standards (Sigma Aldrich). DNA methylation was calculated from the integration of the peaks as methylated cytosines/(methylated cytosines + unmethylated cytosines).

Statistical analyses

Two-factor analysis of variance (ANOVA) and independent sample *t*-tests were performed using SPSS for WINDOWS software version 11.0. *P*-values<0.05 were considered significant. All data are reported as mean ± S.E.M.

2.4 RESULTS

dUTP/dTTP ratios

Intestinal extracts were prepared from normal and mutant mice, and analysed by HPLC for dUTP/dTTP ratios (**Figure 2.1A**). In the mice fed standard mouse chow (**Figure 2.1A,** left panel), the ratios were lower in mutant mice (*Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ combined), compared to *Mthfr*$^{+/+}$ mice, with borderline significance (*P*=0.068). We also examined these ratios in the intestine of mice fed the folic

acid-deficient diet (FADD) (**Figure 2.1A,** right panel); only two genotype groups were evaluated on FADD, since there is a low rate of survival of *Mthfr*$^{-/-}$ mice, as previously reported, and there were limited numbers of mice with this genotype. The folate-deficient diet increased dUTP/dTTP ratios by approximately two-fold in the two genotype groups, as previously shown in rats (239). Two-factor ANOVA confirmed the significant effect of diet ($P<0.001$) and maintained the borderline significant effect of genotype (comparison of *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ groups only; $P = 0.06$).

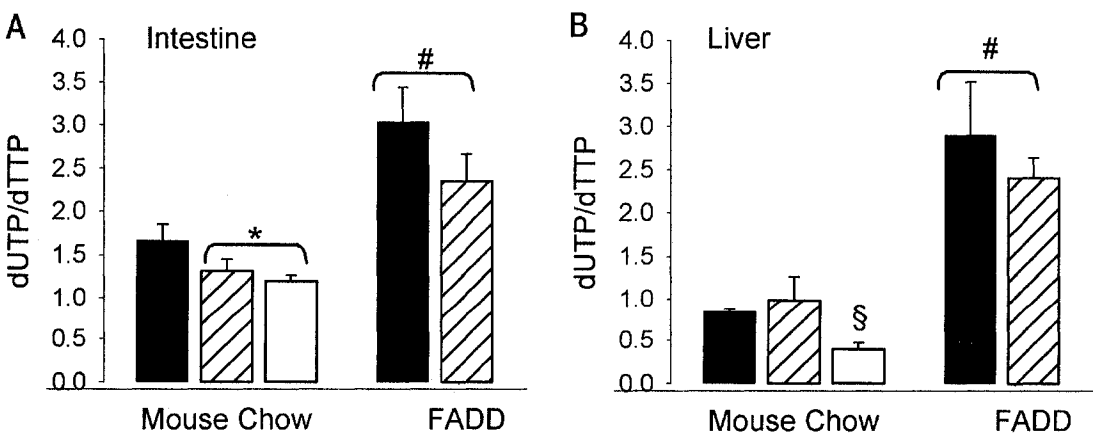
Figure 2.1 Effect of *Mthfr* genotype and diet on dUTP/dTTP ratios in intestine and liver

dUTP/dTTP ratios in the intestine (A) and liver (B) of *Mthfr*$^{+/+}$ (■), *Mthfr*$^{+/-}$(▨) and *Mthfr*$^{-/-}$ (□) mice. Bars represent the mean ± S.E.M. of at least 5 animals per group.

#$P<0.001$ for dietary effect (two-factor ANOVA).

*$P=0.068$ for genotype effect in mouse chow group (*t*-test, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ versus *Mthfr*$^{+/+}$).

§$P<0.05$ for genotype effect in mouse chow group (*t*-test, *Mthfr*$^{-/-}$ versus *Mthfr*$^{+/+}$).

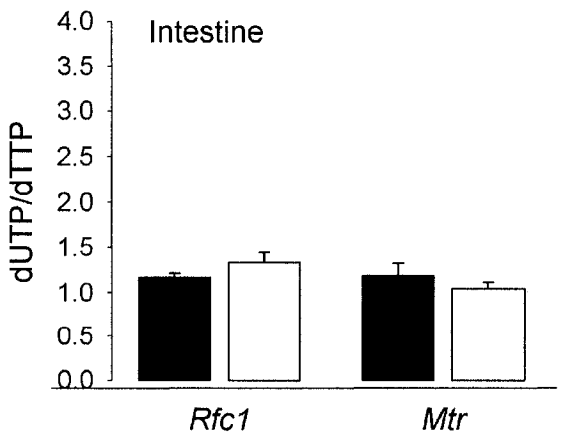


We also examined liver (**Figure 2.1B)** to determine whether the altered ratios were tissue specific. Folate deficiency significantly increased the dUTP/dTTP ratios in liver **(Figure 2.1B,** right panel). *Mthfr*$^{-/-}$ mice on mouse chow showed a significant decrease in this ratio in liver when compared to their *Mthfr*$^{+/+}$ littermates **(Figure 2.1B,** left panel).

Since MTHFR directly competes for 5,10-methyleneTHF with thymidylate synthase, mouse models with other disturbances in folate metabolism might not exhibit the same changes in nucleotide ratios. To test this hypothesis, we measured dUTP/dTTP ratios in intestine of mice with *Rfc1* and *Mtr* deficiency (*Rfc1*$^{+/-}$ and *Mtr*$^{+/-}$ mice) compared to their respective wild-type littermates; the homozygous knockout mice for both of the above models were not studied since they are lethal *in utero*. Neither *Mtr* nor *Rfc1* deficiency altered the dUTP/dTTP ratios in the intestine **(Figure 2.2)**.

Figure 2.2 Effect of *Mtr* and *Rfc* genotypes on dUTP/dTTP ratios in the intestine

dUTP/dTTP ratios in the intestine of wild-type (■) and heterozygous knockout (□) mice. Bars represent the mean ± S.E.M. of 5 animals per group.



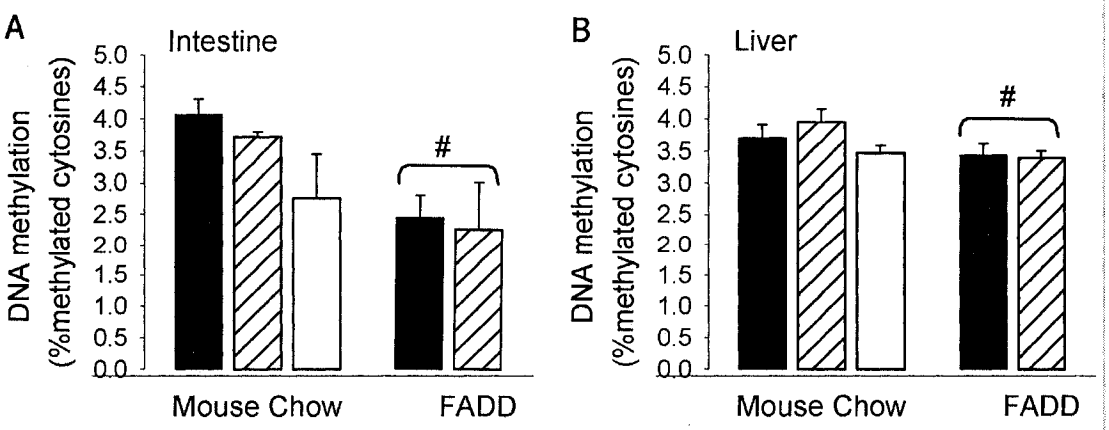
DNA methylation

Both dietary folate deficiency and *Mthfr* disruption might be expected to decrease DNA methylation. Some studies have already shown that there is decreased global DNA methylation in the presence of folate deficiency (294, 295) whereas others have demonstrated that there is no effect (219, 265). For *Mthfr*-deficient mice, we had previously shown that there was a significant decrease in DNA methylation in brain, testes and ovaries, with no significant differences in liver (93). However, in our earlier publication, we had not examined DNA methylation in intestine. Here we observed that $Mthfr^{-/-}$ mice have a 30 % decrease in DNA methylation (from 4.0% to 2.8%) when fed the standard diet **(Figure 2.3A,** left panel) but this difference was statistically significant ($P<0.05$) only when compared with the other two genotype groups combined. The folate-deficient diet had a significant decrease in methylation in both genotype groups, as expected **(Figure 2.3A,** right panel).

Figure 2.3 Effect of *Mthfr* genotype and diet on DNA methylation in the intestine and liver

The effect of *Mthfr* genotype and diet on intestinal (A) and hepatic (B) DNA methylation in $Mthfr^{+/+}$ (■), $Mthfr^{+/-}$ (▨) and $Mthfr^{-/-}$ (□) mice. Bars represent the mean ± S.E.M. of 3-5 animals per group.

#$P<0.05$ for dietary effect (two-factor ANOVA).



DNA methylation in liver was also decreased due to the folate-deficient diet (**Figure 2.3B,** right panel) but there were no significant differences due to MTHFR genotype (**Figure 2.3B,** left panel); the absence of DNA methylation changes in liver of *Mthfr*-deficient mice was also observed in our earlier study (93).

2.5 DISCUSSION

Folate deficiency has been shown to increase dUTP/dTTP levels *in vitro* and in rodent models (238, 239). However, the effect of *MTHFR* genotype on this ratio is unclear since only human lymphocytes have been examined and the results were largely negative (241-243). In this study, we showed that folate deficiency increases dUTP/dTTP ratios in intestine and liver in our mouse model, as previously reported in other model systems. In addition, we observed that $Mthfr^{-/-}$ mice have a lower dUTP/dTTP ratio in intestine and liver. The mice analysed in the lab chow group were the same mice that we had examined earlier for folate distribution (94). In that study, we reported significant decreases in 5-methylTHF in livers of $Mthfr^{-/-}$ mice compared with $Mthfr^{+/-}$ and $Mthfr^{+/+}$; intestine was not examined. Although 5,10-methyleneTHF could not be measured directly, there was an increase in unsubstituted tetrahydrofolate or formylated tetrahydrofolate, suggesting that MTHFR deficiency alters the distribution between methylated and non-methylated folate. dUTP/dTTP ratios reflect the levels of 5,10-methyleneTHF, since this folate derivative is utilised in the conversion of dUTP to dTTP. Our findings support the fact that MTHFR deficiency alters folate distribution and enhances conversion of dUTP to dTTP. This phenomenon may contribute to the protective effect of mild MTHFR deficiency on intestinal tumourigenesis, by decreasing the amount of uracil misincorporation into DNA.

We also measured dUTP/dTTP in the intestine of other mouse models of folate metabolism and found that neither methionine synthase (*Mtr)* nor reduced folate carrier 1 (*Rfc1)* heterozygosity had an effect on the ratio. MTR catalyses the methylTHF-dependent conversion of homocysteine to methionine. Inhibition of MTR can trap folates in the form of 5-methylTHF and thus reduce the participation of nonmethylfolates in the production of purines and thymidylate. A common

polymorphism in *MTR* has also been reported to decrease the risk of colorectal cancer (184), but mechanisms have not been examined. Although we did not find a disturbance of dUTP/dTTP ratios in this study for *Mtr*, heterozygosity for *Mtr* disruption may not have sufficiently reduced enzyme activity to alter the ratio. Since $Mtr^{-/-}$ mice die *in utero*, we were not able to test these mice.

RFC1 transports predominantly 5-methylTHF into cells and is expressed in the intestine (11, 91). Although $Rfc1^{+/-}$ mice might be expected to experience a mild folate deficiency and thus a slightly higher dUTP/dTTP ratio, this was not observed in our study. Again, a single null allele may not have been adequate to alter nucleotide pools and we were unable to measure the effect of severe *Rfc1* deficiency since $Rfc1^{-/-}$ mice also die *in utero*.

DNA methylation, an important epigenetic modification, occurs at 4-6% of cytosines. We observed that folate deficiency decreased the extent of global DNA methylation in intestine and liver; the effect of MTHFR genotype was more pronounced in the intestine compared to liver. The lack of a significant effect of genotype on methylation in liver is consistent with our earlier findings (93) as well as those by another group (96). The liver may be less vulnerable to methylation changes since homocysteine can be remethylated to methionine and SAM using a folate-independent enzyme, betaine-homocysteine methyltransferase (BHMT) which utilises betaine as a methyl donor. BHMT activity has been demonstrated in the kidney and liver, but not the small intestine (69). Rapid proliferation in tissues such as the intestine may also serve to make them more sensitive to availability of methyl donors.

$Mthfr^{+/-}$ mice are a good model for 677TT individuals based on their levels of enzyme activity, methylated folate and homocysteine elevation, compared to their respective controls (93). However, none of the differences between $Mthfr^{+/+}$ and $Mthfr^{+/-}$ mice were significant, although there was a non-significant decrease in the intestine for the dUTP/dTTP ratio and for DNA methylation. Our system may not be sensitive enough to detect small fluctuations which could be important over the lifetime of an individual. Furthermore, we conducted these experiments at a single timepoint during the life of adult mice. *Mthfr* genotype may exert a greater

effect during periods of rapid growth and development or in response to environmental stress.

We did not observe any genotype differences for nucleotide pools or for DNA methylation when the mice were fed low folate diets. The severe folate deficiency may have overwhelmed any potential genotype effect, as previously suggested in other studies of these mice (100). It is important to note that in some studies, the protective effect of the MTHFR 677TT genotype on colorectal cancer was not observed in individuals with low folate status (182); in fact, low folate in combination with the 677TT genotype may increase risk for certain cancers (296-298). Low dietary folate appears to be a stronger determinant for colorectal cancer risk and may overwhelm the effect of the polymorphism in human studies. Our data on dUTP/dTTP ratios are consistent with the clinical studies *i.e.*, low dietary folate dramatically increases this ratio and MTHFR deficiency may only affect the ratio when folate levels are replete, as in mouse chow.

In conclusion, we have demonstrated *in vivo* the proof of principle that MTHFR deficiency can affect uracil/thymidine interconversion, an observation that is consistent with the epidemiological evidence suggesting that the *MTHFR* 677 polymorphism is protective in terms of colorectal cancer risk.

2.6 ACKNOWLEDGEMENTS

This work was supported by the Canadian Institutes of Health Research (CIHR) to RR. AKL was the recipient of a Studentship from the Montreal Children's Hospital Research Institute.

CONNECTING TEXT – Chapter II-III

The results of Chapter II demonstrate that dietary folate deficiency adversely affects nucleotide balance and DNA methylation in the intestine of BALB/c mice. It has been previously reported by our laboratory that one quarter of the mice of this strain will develop intestinal adenomas after being fed a folate-deficient diet for one year (222). This tumour initiation occurs in the absence of a germline tumour suppressor mutation or exposure to a chemical carcinogen. The following chapter describes the effect of disturbances in folate metabolism in a model predisposed to adenoma formation, the *Apc*$^{min/+}$ mouse. In this study, the effect of dietary folate and genetic deficiency of either the reduced folate carrier 1 or methionine synthase on tumourigenesis will be examined.

CHAPTER III

Genetic and nutritional deficiencies in folate metabolism influence tumourigenicity in *Apc*$^{min/+}$ mice

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3.1 ABSTRACT

Epidemiological studies indicate that adequate dietary folate is protective against colon cancer, although mechanisms remain largely elusive. We investigated the effects of genetic disruptions of folate transport and metabolism and of dietary folate deficiency in a mouse model of colon cancer, the *Apc*$^{min/+}$ mouse. *Apc*$^{min/+}$ mice with a heterozygous knockout of the gene for the reduced folate carrier 1 (*Rfc1*$^{+/-}$) developed significantly fewer adenomas compared to *Rfc1*$^{+/+}$*Apc*$^{min/+}$ mice [30.3 ± 4.6 versus 60.4 ± 9.4 on a control diet (CD), and 42.6 ± 4.4 versus 55.8 ± 7.6 on a folic acid-deficient diet (FADD), respectively]. *Rfc1*$^{+/-}$*Apc*$^{min/+}$ mice also carried a lower tumour load, an indicator of tumour size as well as tumour number. In contrast, there were no differences in adenoma formation in *Apc*$^{min/+}$ mice carrying a knockout allele for methionine synthase (*Mtr*$^{+/-}$), an enzyme that catalyses folate-dependent homocysteine remethylation, and *Mtr*$^{+/+}$*Apc*$^{min/+}$ mice. However, in both *Mtr* groups of mice, dietary folate deficiency significantly increased adenoma number (from 32.3 ± 3.8 on the CD to 48.1 ± 4.2 on the FADD), increased plasma homocysteine, decreased global DNA methylation in the preneoplastic intestine and increased apoptosis in this tissue. There were no genotype-associated differences in these parameters in the *Rfc1* group, suggesting that the protection conferred by *Rfc1* deficiency is through a different mechanism. In conclusion, genetic and nutritional disturbances in folate metabolism can have distinct influences on tumourigenesis in *Apc*$^{min/+}$ mice; altered levels of homocysteine, global DNA methylation and apoptosis may contribute mechanistically to the dietary influence.

3.2 INTRODUCTION

Folate derivatives participate in several important cellular processes including amino acid interconversions and DNA methylation, synthesis and repair. Epidemiological studies have demonstrated that dietary folate status and the activity of folate-dependent enzymes can alter the risk of certain cancers. Specifically, several studies have shown that high folate intake is inversely correlated with the risk of developing colorectal adenomas and carcinomas, and

that an activity-reducing polymorphism in an enzyme central to folate metabolism (methylenetetrahydrofolate reductase, MTHFR) has a protective effect against colon cancer incidence when adequate folate status is maintained (182, 210, 299, 300). The mechanisms by which folates modulate cancer risk are not completely understood, although several hypotheses have been proposed.

Folate is required for the generation of *S*-adenosylmethionine (SAM), an important methyl donor involved in the DNA methyltransferase (DNMT)-catalysed methylation of DNA, a means of transcriptional regulation. Folate deficiency may cause global DNA hypomethylation (potentially permitting oncogene expression) or aberrant methylation patterns such as promoter hypermethylation (preventing expression of tumour suppressor genes). For example, a severely methyl-deficient diet was shown to induce DNA hypomethylation and increase mRNA levels of the *c-myc* and *c-fos* proto-oncogenes in rat liver (257). In contrast, another study using a rat colon cancer model did not observe any changes in DNA methylation during folate deficiency, although the number and size of colonic aberrant crypt foci was reduced (264).

The *de novo* synthesis of nucleotides is also dependent on the availability of folates. 10-FormylTHF contributes one-carbon units for the generation of purines and 5,10-methyleneTHF provides the methyl group for the methylation of dUMP to dTMP. *In vitro* studies suggest that the latter reaction is critical since a high dUTP to dTTP ratio may lead to the misincorporation of uracil into DNA and ultimately to DNA double-strand breaks, genomic instability and DNA fragmentation (239, 240, 290, 301-303) . Folate deficiency therefore leads to uracil misincorporation and DNA damage, as well as a reduced pool of nucleotides for DNA synthesis and repair.

Disturbances of folate metabolism can also adversely affect the rate of apoptosis in animal tissues (239, 304). This may be a result of methylation or nucleotide pool changes (as discussed above), an accumulation of cytotoxic homocysteine, or a deficiency of choline, a precursor of betaine (the alternate carbon donor for homocysteine remethylation to methionine). Enhanced apoptosis can be beneficial in eliminating tumourigenic cells, thus inhibiting the formation –

or restricting the size – of tumours. However, it may also induce a chronic increase in cell turnover rate, decreased repair time, and increased DNA damage with a selection pressure that favours cells with transformation potential (238).

Mouse models provide an opportunity to explore the etiology of complex diseases. An established model for intestinal neoplasia, the *Apc*$^{min/+}$ mouse, develops multiple small intestinal adenomas within a few months of age (161). These mice harbour a germline mutation in the tumour suppressor, *Apc*, a gene shown to be frequently mutated in sporadic and hereditary forms of colon cancer in humans (137). The effect of dietary folate has been investigated in these mice with variable outcomes that might be dependent on timing and duration of intervention. In one study, there was no difference in total adenoma number between folate-supplemented and folate-deficient mice although there was a decrease in the number of ileal adenomas in the folate-supplemented group at 3 months and a decrease in ileal adenomas in the folate-deficient group at 6 months (216). In our previous work with *Apc*$^{min/+}$ mice, we concluded that folate deficiency exerts different effects depending upon the transformation state of the cell. When folate intervention occurs at an early stage, it may promote tumourigenesis; if it occurs during later stages of transformation, it may inhibit tumour growth (218).

Apc$^{min/+}$ mice have also been used to investigate genetic modifiers of cancer. In multiple reports, *Dnmt1* deficiency significantly reduced adenoma multiplicity (219, 270, 271), whereas *Myh*- or *Msh2*- null mutations increased adenoma numbers dramatically (217, 305). In one study, a moderately folate-deficient diet in *Apc*$^{min/+}$ mice carrying a *Dnmt1* mutation did not alter global DNA methylation or promoter-specific methylation of the *E-cadherin* gene (219). In another report, *Dnmt1* deficiency was associated with a decrease in the methylation of CpG islands of *Itga4*, *Mgmt* and *Timp3* with a decrease in tumour number (270).

Despite the critical role of folate metabolism in tumourigenesis, crosses between mouse models of folate transport or metabolism and *Apc*$^{min/+}$ mice have not been reported. We therefore examined the influence of genetic and nutritional disturbances in folate metabolism on tumourigenicity in *Apc*$^{min/+}$ mice by crossing them with mice harbouring a knockout allele in one of two key genes in

folate transport and metabolism, and by administering control and folate-deficient diets to these animals. The mouse models were heterozygous for a disruption of the gene for the reduced folate carrier 1 (RFC1), or for the gene encoding methionine synthase (MTR). RFC1 transports the predominant plasma folate, 5-methylTHF, into cells. MTR transfers the methyl group from 5-methylTHF to homocysteine to generate methionine, which is subsequently converted to SAM. We investigated the impact of these metabolic disruptions on tumour multiplicity and size in $Apc^{min/+}$ mice, in addition to cellular processes that might contribute to tumourigenesis, specifically global DNA methylation and intestinal apoptosis.

3.3 MATERIALS AND METHODS

Mice and dietary intervention

Animal experimentation was approved by the Montreal Children's Hospital Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care.

The generation and genotyping of $Rfc1^{+/-}$ and $Mtr^{+/-}$ was performed as described previously (91, 101) and shown in Appendix I. *Rfc1* mice were established on the SWV/Fnn background (91). *Mtr* mice were established on a mixed Black Swiss and 129/Sv background (101). Male $Apc^{min/+}$ mice (C57Bl/6J background) were obtained from The Jackson Laboratory (Bar Harbor, Maine) and bred with female $Rfc1^{+/-}$ or $Mtr^{+/-}$ mice. Offspring were weaned at 3-3.5 weeks and fed amino-acid-defined diets (Harlan Teklad, Madison WI, USA) until they were 10 weeks old. These diets complied with the recommendations of the American Institute of Nutrition guidelines for rodents (306). The mice were randomly assigned to receive either a control diet (CD), containing 2 mg folic acid/kg diet (recommended amount), or a folic acid-deficient diet (FADD) containing 0.2 – 0.3 mg folic acid/kg diet. All diets were supplemented with 1% succinylsulfanthiozole to inhibit folate synthesis by intestinal flora.

All mice were genotyped for *Pla2g2a*, the candidate gene for *Mom1*, an established modifier of the Min phenotype that can affect adenoma formation, using described procedures (170). This was performed to ensure that the four

experimental groups for each mouse strain were of the same *Pla2g2a* genotype. There are two possible alleles: $Mom1^R$ (wild-type *Pla2g2a*), which confers resistance to adenoma formation, and $Mom1^S$ (mutant *Pla2g2a*), which sensitises to adenoma formation. Every mouse in the *Rfc1* group was $Mom1^{S/R}$ (the $Apc^{min/+}$ strain is $Mom1^{S/S}$ and the *Rfc1* strain is $Mom1^{R/R}$, as genotyped in our laboratory). *Mtr* mice were either $Mom1^{S/S}$ or $Mom1^{S/R}$ due to their mixed background (genotyping of the parental strains in our laboratory revealed that the Black Swiss strain is $Mom1^R$ and the 129/Sv is $Mom1^S$). Since $Apc^{min/+}$ mice are $Mom1^{S/S}$, we included *Mtr* mice that were $Mom1^{S/S}$ for our experiments. For this reason, one group ($Mtr^{+/-}$ FADD) was left with only 3 mice and further attempts at breeding mice of the same *Mom1* genotype were not successful.

Adenoma scoring

The number and size of small intestinal adenomas were determined as in our previous reports (218, 219). Mice were sacrificed by asphyxiation. Blood was obtained by cardiac puncture for plasma homocysteine evaluation. Intestines were removed, opened longitudinally and flushed with phosphate-buffered saline. Selected adenomas and preneoplastic (normal) intestinal tissues were snap-frozen in liquid nitrogen and stored at –70°C. The remaining intestine was placed flat between two pieces of filter paper and fixed in 10% formalin solution for at least 24 hours. The intestine was then stained with a 0.1% methylene blue solution and assessed for adenoma number and size by two different individuals blinded to genotype and diet using a dissecting microscope and a micrometer. Adenoma load refers to the sum of the areas of the tumours for each mouse.

Total plasma homocysteine evaluation

Cardiac blood was collected in potassium-EDTA tubes and centrifuged at 6000×*g* for 5 minutes at 4°C to separate plasma. Measurements were performed by HPLC as described (307).

Methylation analysis

To assess methylation of CCGG sites in preneoplastic (normal) intestine, a thin layer chromatography assay was performed as previously described (308). Briefly, 5 μg of RNA-free genomic DNA obtained from duodenal tissue was

treated with *Msp1* (which digests both methylated and unmethylated CCGG sequences). The DNA was then dephosphorylated with calf intestinal alkaline phosphatase, end-labelled with γ–[^{32}P]dATP, hydrolysed with nuclease P1, spotted on a cellulose TLC plate and developed in isobutyric acid-water-ammonium hydroxide (66:33:1). The images were quantified by a phosphorimager. The amount of methylation was calculated as the percent of methylated cytosines/(methylated cytosines + unmethylated cytosines).

Caspase-3/7 activity assay

Preneoplastic duodenal tissue was ground into powder in liquid nitrogen and lysed in buffer (50 mmol/L potassium phosphate, 0.3 mmol/L EDTA, pH 8.0). Total protein concentration was determined using Bio-Rad Protein Assay solution (Bio-Rad, Montreal, Canada). Caspase-Glo® 3/7 Assay kit (Promega, Nepean, Canada) was used to measure caspase-3/7 activities according to the manufacturer's instructions. Two μg of total protein was used per assay. Two different intestinal extracts from the same mouse, each assayed in duplicate, were used to generate a mean of four assays per mouse.

Statistical analysis

Two-factor analysis of variance (ANOVA) and independent sample *t*-tests were performed using SPSS for WINDOWS software Version 11.0. $P< 0.05$ was considered significant. All data are reported as mean ± S.E.M.

3.4 RESULTS

Rfc1 deficiency reduces adenoma number and load in $Apc^{min/+}$ mice

$Rfc1^{+/-}Apc^{min/+}$ mice developed fewer adenomas than $Rfc1^{+/+}Apc^{min/+}$ mice. (**Figure 3.1A**; two-factor ANOVA, $*P<0.05$). On the control, folate-replete diet, $Rfc1^{+/-}Apc^{min/+}$ mice had 50% fewer tumours than their wild-type $Rfc1^{+/+}Apc^{min/+}$ counterparts (30.3 ± 4.6 versus 60.4 ± 9.4). The values for the folate-deficient diet were 42.6 ± 4.4 versus 55.8 ± 7.6. The total area of the adenomas (expressed as adenoma load) was also lower in $Rfc1^{+/-}Apc^{min/+}$ mice compared to $Rfc1^{+/+}Apc^{min/+}$ mice (**Figure 3.1B**; two-factor ANOVA, $*P<0.05$). The load on the CD for

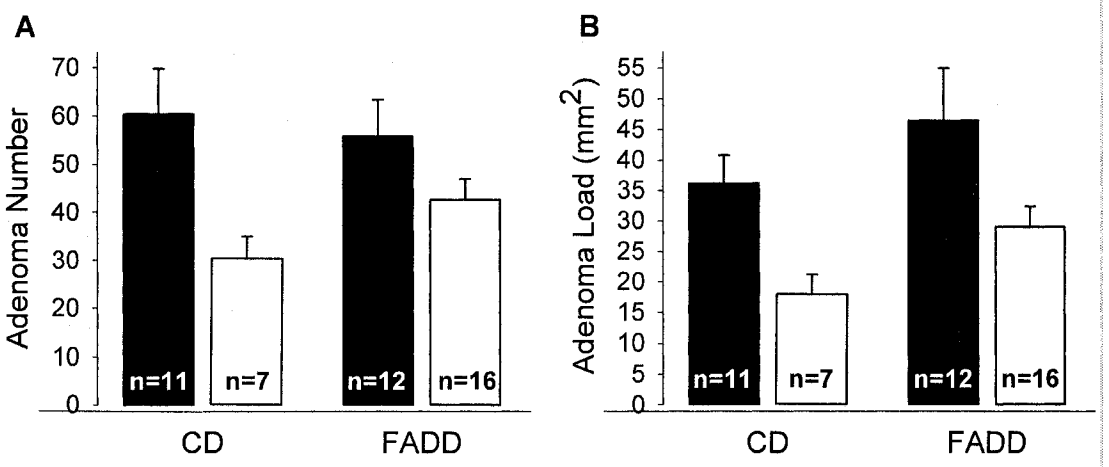
Rfc1$^{+/-}$*Apc*$^{min/+}$ and *Rfc1*$^{+/+}$*Apc*$^{min/+}$ was 17.9 ± 3.3 mm^2 and 36.1 ± 4.7 mm^2; the load on the FADD was 29.0 ± 3.3 mm^2 and 46.5 ± 8.5 mm^2, respectively.

The adenoma load was slightly but nonsignificantly increased by the FADD (**Figure 3.1B**; two-factor ANOVA, P=0.07). Since the average adenoma size was also slightly but not significantly increased (data not shown), it is likely that the increase in load is attributable to an increase in adenoma size.

To examine the potential effects of gender, we repeated each ANOVA with gender as a covariate; both number and load remained significant for genotype (*Rfc1*$^{+/+}$, 10 females, 13 males; *Rfc1*$^{+/-}$, 12 females, 11 males).

Figure 3.1 Effect of *Rfc1* genotype and dietary folate on adenoma number and adenoma load in *Apc*$^{min/+}$ mice

Effect of *Rfc1* genotype and dietary folate on A) adenoma number and B) adenoma load in *Rfc*$^{+/+}$*Apc*$^{min/+}$ (■) and *Rfc1*$^{+/-}$*Apc*$^{min/+}$ (□) mice on a control diet (CD) or a folic acid-deficient diet (FADD). Values represent mean ± S.E.M.. n= number of mice.



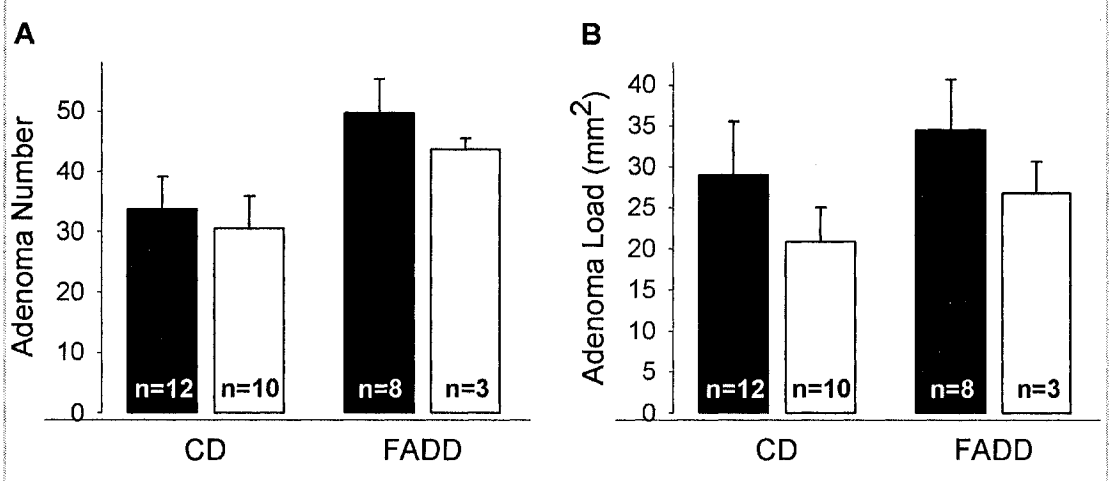
***Dietary folate deficiency increases adenoma number in Mtr*$^{+/+}$*Apc*$^{min/+}$ and *Mtr*$^{+/-}$*Apc*$^{min/+}$ mice**

Mtr genotype did not significantly affect either adenoma number or load (**Figures 3.2A** and **3.2B**). However, adenoma number was increased in both

genotype groups ($Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice) receiving the FADD (**Figure 3.2A**; two-factor ANOVA, $*P<0.05$), from an average of 32.3 ± 3.8 on the CD to 48.1 ± 4.2 on the FADD. There was no significant dietary influence on adenoma load (**Figure 3.2B**). When analyses were repeated with gender as a covariate, the significant effect of diet on adenoma number was retained (CD, 6 females, 16 males; FADD, 6 females, 5 males).

Figure 3.2 Effect of *Mtr* genotype and dietary folate on adenoma number and adenoma load in $Apc^{min/+}$ mice

Effect of *Mtr* genotype and dietary folate on A) adenoma number and B) adenoma load in $Mtr^{+/+}Apc^{min/+}$ (■) and $Mtr^{+/-}Apc^{min/+}$ (□) mice on a control diet (CD) or a folic acid-deficient diet (FADD). Values represent mean ± S.E.M.. *n*=number of mice.



Dietary folate, but not Rfc1 or Mtr genotype, affects total plasma homocysteine (tHcy), global DNA methylation and levels of apoptosis in $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice

Homocysteine is a cytotoxic amino acid that is either eliminated via transsulfuration or remethylated to form methionine, using either 5-methylTHF or betaine as a methyl donor. Since mice have been shown to develop mild

hyperhomocysteinemia in response to folate-deficient diets (100), we measured plasma total homocysteine (tHcy) to ensure the effectiveness of the FADD and to determine if hyperhomocysteinemia or its consequences could contribute to the observed decreases in adenoma numbers or load. There was no influence of genotype on plasma tHcy in the two strains on either diet. In contrast, the folate-deficient diet significantly increased plasma tHcy in the $Rfc1^{+/+}Apc^{min/+}$ and $Rfc1^{+/-}Apc^{min/+}$ mice as well as in the $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice, but the increase was much more dramatic in the *Mtr* group of $Apc^{min/+}$ mice, which demonstrated a three-fold increase in plasma tHcy on the FADD compared to the value on the CD; the increase due to diet in the *Rfc1* group of $Apc^{min/+}$ mice was approximately 50% (**Figures 3.3A** and **3.4A**).

SAM is the methyl donor for DNMT-catalysed DNA methylation, an important process in epigenetic gene regulation. Increased levels of homocysteine will generate SAH, an inhibitor of SAM-dependent methyltransferases. In the *Mtr* group, which demonstrated a dramatic diet-dependent increase in plasma tHcy, there was a concomitant decrease in DNA methylation in the preneoplastic intestinal tissue (**Figure 3.4B**). The *Rfc1* mice, which did not respond as dramatically to the FADD in terms of plasma tHcy, did not exhibit any significant changes in global DNA methylation in the intestine (**Figure 3.3B**). There were no significant effects of *Mtr* or *Rfc1* genotype on methylation in the preneoplasic intestinal DNA of these $Apc^{min/+}$ mice; this observation is consistent with the absence of an effect of genotype on plasma tHcy levels.

Since homocysteine has been reported to increase apoptosis in some experimental systems, we also assessed caspase-3/7 activities as a marker of apoptosis in preneoplastic intestinal tissue. In the $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice, caspase-3/7 activities were increased on the FADD diet (**Figure 3.4C**) indicating an increased rate of apoptosis in this tissue. $Rfc1^{+/+}Apc^{min/+}$ and $Rfc1^{+/-}Apc^{min/+}$ mice did not exhibit any significant effects of diet on caspase activities (**Figure 3.3C**). As mentioned for methylation, there was no effect of genotype on apoptosis; this is also consistent with the absence of a genotype effect on plasma tHcy.

Figure 3.3 Effect of *Rfc1* genotype and dietary folate on homocysteine, DNA methylation and apoptosis in $Apc^{min/+}$ mice

Effect of *Rfc1* genotype and dietary folate on A) plasma tHcy, B) intestinal global DNA methylation and C) intestinal caspase-3/7 activity in $Rfc^{+/+}Apc^{min/+}$ (■) and $Rfc^{+/-}Apc^{min/+}$ (□) mice.

Values represent mean ± S.E.M.. *n*=number of mice.

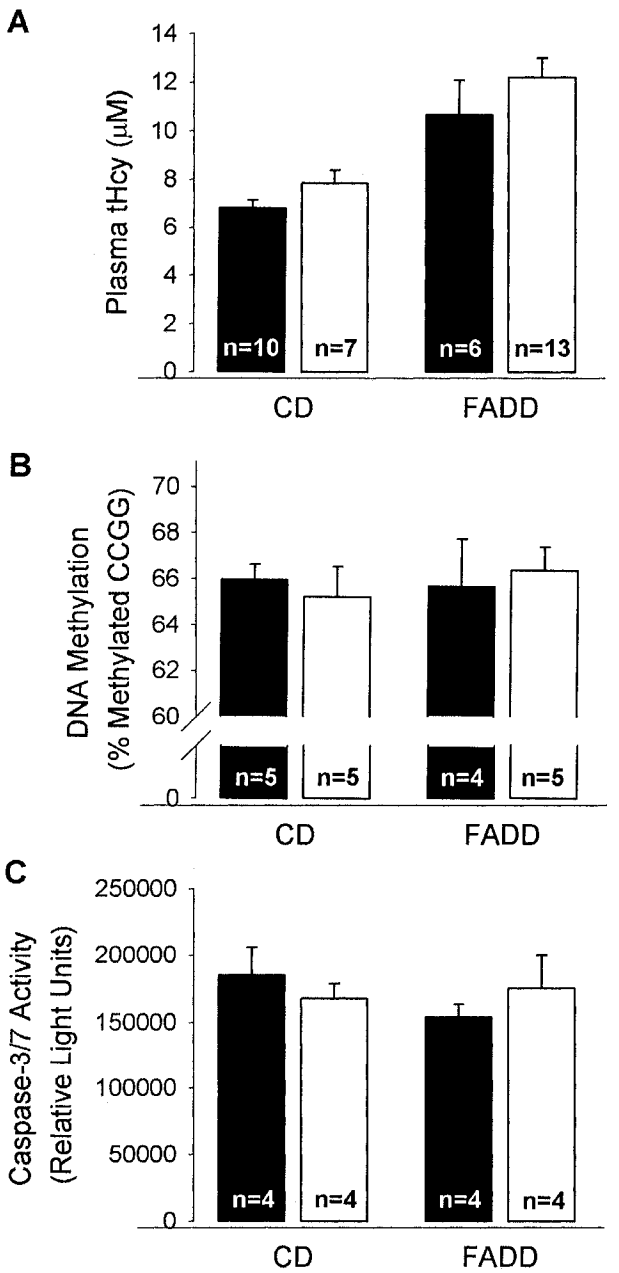
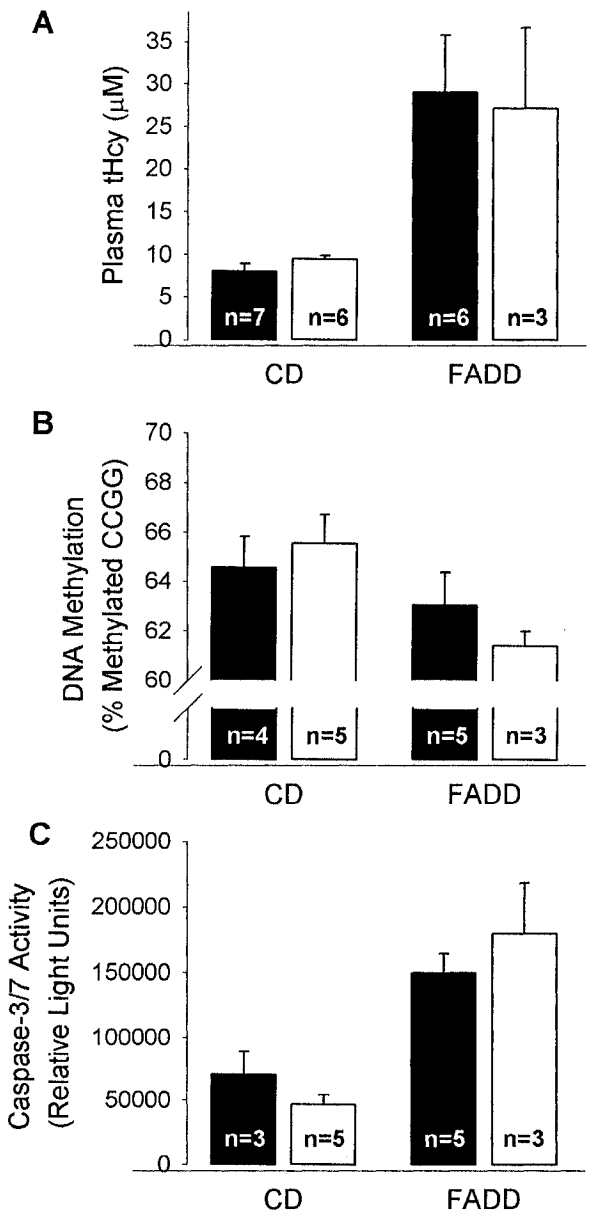


Figure 3.4 Effect of *Mtr* genotype and dietary folate on homocysteine, DNA methylation and apoptosis in $Apc^{min/+}$ mice

Effect of *Mtr* genotype and dietary folate on A) plasma tHcy, B) intestinal global DNA methylation and C) intestinal caspase-3/7 activity in $Mtr^{+/+}Apc^{min/+}$ (■) and $Mtr^{+/-}Apc^{min/+}$ (□) mice.

Values represent mean ± S.E.M.. *n*=number of mice.



Basal levels of intestinal apoptosis on the CD were different between the *Rfc1* $Apc^{min/+}$ mice and *Mtr* $Apc^{min/+}$ mice but this could be attributable to strain differences; we tested additional strains and obtained variable strain-dependent levels of apoptosis (data not shown).

3.5 DISCUSSION

This study demonstrates that both genetic and nutritional disturbances in folate metabolism can influence adenoma formation and growth in $Apc^{min/+}$ mice. Genetic disruption of the *Rfc1* gene reduced adenoma number and load in this strain, whereas the low dietary folate did not have a significant impact. In contrast, the dietary folate deficiency increased tumour number in the $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice whereas genetic mutation had little or no influence. Direct comparisons between the two mutant strains cannot be made since they are on different genetic backgrounds. Nonetheless, our work suggests that folate metabolism can influence tumourigenesis in a multifactorial manner, depending on the genetic variation and nutritional status of the host. The effect of the genetic variation was underscored further by the fact that the $Rfc^{+/+}Apc^{min/+}$ mice, all of which carry one $Mom1^{R}$ allele, developed more adenomas overall than the $Mtr^{+/+}Apc^{min/+}$ group, which did not. The genetic background of the *Rfc* mice may contain additional modifiers of the Min phenotype that can increase adenoma number.

The *Rfc1* mice are on a uniform genetic background (SWV/Fnn) (91), whereas the *Mtr* mice are on a mixed background (Black Swiss and 129SV) (101). Since strong genetic determinants of tumourigenesis may be less evident on a mixed background, this factor may have contributed to the dramatic effect of diet in the $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice.

The genetic deficiency of *Rfc1*, a reduced folate transporter, resulted in fewer adenomas with no concurrent changes in plasma homocysteine, global DNA methylation, or intestinal cell apoptosis. Low dietary folate, administered to rodents postnatally, has been examined for its role in tumour formation in the $Apc^{min/+}$ mouse model as well as in other colon cancer models (218-220). Here we

show that a very early disruption, in the form of a genetic defect in folate transport, inhibits adenoma formation in these mice, although the mechanism remains speculative.

Plasma tHcy in the *Rfc1* strain was not influenced by the *Rfc1* mutation and only modestly affected by low dietary folate (~ 50% increase on the FADD). These findings, which may relate to the background of the strain, are consistent with those of a recent study which showed that these mice were refractory to folate deficiency with respect to changes in the levels of SAM and SAH – other biomarkers in the homocysteine/methylation cycle (90).

In adult mice, the intestinal expression of *Rfc1* in $Rfc1^{+/-}$ mice, based on RT-PCR, is lower than that of their wild-type littermates (data not shown), and there are modest differences in colonic mucosal SAM and SAH metabolites (91). Developmentally, however, *Rfc1* expression may be more critical; homozygous mutants are embryonic lethal (90) and nursing mice receive 5-methyTHF from their mother's milk (309). This is noteworthy for studies involving the $Apc^{min/+}$ mice, since their intestinal cells have an age-specific sensitivity to adenoma formation. Younger (5- to 15-day-old) mice are more sensitive to adenoma initiation by chemical carcinogen exposure than older mice (162). Throughout development and in the first few postnatal weeks, the mice are dependent on maternal sources of folate and their own uptake mechanisms. Consequently, a genetic defect in the *Rfc1* transporter might manifest itself earlier than a dietary deficiency that is initiated at weaning. The modulation of adenoma initiation rates could occur through several potential mechanisms. For example, polyamines are necessary for cell proliferation and can be formed from the decarboxylation of SAM. *Rfc1* heterozygosity has previously been shown to decrease the SAM/SAH ratio (91). By genetically depleting polyamines through increased catabolism, it was shown that $Apc^{min/+}$ mice develop 75% fewer adenomas than do wild-type mice (310). Although we did not observe changes in global DNA methylation in this strain, *Rfc1* heterozygosity could potentially result in methylation changes within critical oncogenes or tumour suppressor genes. In addition, the pool of DNA

precursors for proliferation may have been reduced, inhibiting the DNA synthesis of burgeoning adenomas.

A decrease in adenoma number was also found in folate-deficient *Rfc1*$^{+/-}$ *Apc*$^{min/+}$ mice, compared to *Rfc*$^{+/+}$*Apc*$^{min/+}$ mice, although this was not statistically significant if measured by *t*-test (55.8 ± 7.6 versus 42.6 ± 4.4; *t*-test +/+ vs. +/-, *P*=0.12). The decrease in adenoma numbers in *Rfc1*$^{+/-}$*Apc*$^{min/+}$ mice, compared to *Rfc1*$^{+/+}$*Apc*$^{min/+}$ mice, was more striking when they were on the control diet (60.4 ± 9.4 versus 30.3 ± 4.6; *t*-test +/+ vs. +/-, **P*=0.03). It is possible that the dietary intervention after weaning ablated the decrease in adenoma number resulting from *Rfc1* deficiency. Low dietary folate is an important risk factor for colorectal cancer in human populations (210, 300); despite the fact that a polymorphism in the folate-metabolising enzyme MTHFR may be protective, this decreased risk is observed only when folate status is adequate (1). Low dietary folate overcomes any potential benefit from the genetic variant in human populations and may also be a risk factor under certain conditions in these animal studies (as discussed for the *Mtr* mice below).

A recent study investigated the effect of *Rfc1* on azoxymethane-induced aberrant crypt folic (ACF) formation. In this model, certain subtypes of ACF are thought to represent colorectal cancer precursors (311). They showed that *Rfc1* status did not affect either the absolute number of ACF or the incidence of adenocarcinoma in the colon (91). However, when crypt multiplicity was considered, they found that *Rfc1*$^{+/-}$ mice had a higher number of ACF with more than one crypt per focus (larger ACF). The disparity between this finding and our own may be attributable to the model (genetic *Apc* disruption versus chemical carcinogen) or localisation (small intestine versus colon). The *Apc*$^{min/+}$ mouse model is associated primarily with small intestinal tumours and tumour formation is initiated early since the mutation is present in the germline. The effect of an *Rfc1* mutation that is also present in the germline may have different consequences than those of dietary deficiency or carcinogen treatment administered to older animals.

The genetic deficiency of *Mtr* did not elicit the same response as that of *Rfc1*. Although two epidemiological studies have suggested that individuals

carrying an *Mtr* polymorphism have decreased colorectal cancer risk (64, 184), *Mtr* deficiency in this animal model did not affect tumourigenesis. $Mtr^{-/-}$ mice are embryonic lethal and heterozygous mice have decreased enzyme activity and a moderate increase in homocysteine in female mice (101). There is an alternative homocysteine remethylation pathway, catalysed by betaine-homocysteine methyltransferase (BHMT), which may partially compensate for *Mtr* deficiency. BHMT is expressed in hepatic and renal tissue (69) but the BHMT products, methionine or SAM, may circulate and offset a disturbance in folate-dependent remethylation of homocysteine, as we suggested for *Mthfr* knockout mice, and for humans with hyperhomocysteinemia (312). The mixed genetic background of these mice is a limitation of this study and precludes the drawing of any definitive conclusions regarding the effect of genetic disruption of *Mtr* in this model.

In $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice, dietary folate deficiency significantly increased the number of adenomas. This increase is consistent with the epidemiological studies of low dietary folate in human populations, as mentioned above (210, 300). In these mice, folate deficiency caused a significant three-fold increase in plasma tHcy, a decrease in DNA methylation and an increase in intestinal cell apoptosis. Since hyperhomocysteinemia is known to increase SAH levels through a reversal of the SAH hydrolase reaction and SAH is a potent inhibitor of SAM-dependent methylation reactions, marked hyperhomocysteinemia may be directly responsible for hypomethylation. In previous work, we found that tumour number in $Apc^{min/+}$ mice was positively correlated with levels of DNA hypomethylation (218).

The increase in apoptosis due to the folate deficiency in $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice may also be related to hyperhomocysteinemia. Elevated homocysteine has been shown to increase the rates of apoptosis (313) and to induce hyperproliferation of colon cancer cells (314). High levels of apoptosis may lead to high cell turnover with decreased repair time and selection pressure for cells capable of transformation (238).

Folate deficiency results in lower levels of SAM (315), which is required for the *de novo* synthesis of choline, the precursor for betaine. Folate deficiency

may produce a secondary choline deficiency, both by inhibiting its synthesis and by enhancing its depletion by activating BHMT-catalysed homocysteine remethylation (316). This may contribute to tumourigenesis since choline deficiency has been shown to induce apoptosis and promote carcinogenesis (317).

In this study, we have shown that an early disruption in folate metabolism, in the form of a germline mutation in the folate transporter *Rfc1*, resulted in fewer intestinal adenomas in $Apc^{min/+}$ mice predisposed to form large numbers of tumours. A later disruption, in the form of dietary folate deficiency administered on weaning, resulted in an increase in tumour numbers in a different strain with or without a mutation in *Mtr*. These findings recapitulate the complex relationship between folate metabolism and tumourigenesis, and highlight the utility of genetic animal models for these types of studies.

3.6 ACKNOWLEDGEMENTS

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CONNECTING TEXT – Chapter III-IV

The results of Chapter II showed that *Mthfr* genotype affected both intestinal dUTP/dTTP ratio and DNA methylation, two candidate mechanisms for the protective effect of *MTHFR* 677TT genotype in CRC. In the following chapter, the effects of *Mthfr* genotype in the $Apc^{min/+}$ model will be described. Furthermore, the results of Chapter III demonstrated that a genetic defect in an intestinal folate transporter reduced adenoma number in $Apc^{min/+}$ mice. Since this may represent an early form of folate deficiency, we hypothesised that the early initiation of folate-deficient diets may have a similar effect. Song *et al.*, had previously suggested that neoplastic foci are established after weaning in $Apc^{+/-}Msh2^{-/-}$ mice (217). However, another study reported that younger (5- to 15-day-old) mice are more sensitive to adenoma initiation by chemical carcinogen exposure than older mice (318), suggesting that adenoma number is amenable to intervention prior to weaning. Since mice are dependent on maternal sources of nutrition during this time period, we proposed that by supplying their mothers with specialized diets before their conception, $Apc^{min/+}$ offspring will experience the dietary effects for their entire lives. Similarly, since the pre-weaning time period may be crucial, we hypothesised that maternal *Mthfr* genotype may also influence adenoma formation. The following chapter describes the effects of early and late initiation of diets varying in folate content as well as the effects of *Mthfr* genotype and maternal *Mthfr* genotype on adenoma formation.

CHAPTER IV

Methylenetetrahydrofolate reductase deficiency and low dietary folate inhibit tumourigenesis in *Apc*$^{min/+}$ mice

Andrea K Lawrance, Liyuan Deng, Rima Rozen

4.1 ABSTRACT

Epidemiological studies suggest a protective effect of high folate intake and *MTHFR* 677TT genotype on colorectal cancer risk. Using the $Apc^{min/+}$ mouse model, we examined the effect of *Mthfr* genotype, maternal *Mthfr* genotype and dietary folic acid content. When diets were initiated prior to conception, mice fed a folic acid-deficient diet (FADD) developed fewer adenomas than those fed a control diet (CD). In both dietary groups, $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/-}$ mothers developed fewer adenomas than $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/+}$ mothers. As well, in offspring of $Mthfr^{+/-}$ mothers, $Mthfr^{+/-}Apc^{min/+}$ mice developed fewer adenomas than $Mthfr^{+/+}Apc^{min/+}$ mice. MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate (which is used for the production of thymidylate from uridylate) to 5-methyltetrahydrofolate (which is used for the remethylation of homocysteine to methionine). Overall, adenoma number was inversely correlated with plasma homocysteine levels, the dUTP/dTTP ratio and apoptosis (borderline significant). When diets were initiated at weaning, mice fed a folic acid-enriched diet developed significantly more adenomas than those fed the FADD or CD. Together these results demonstrate that in mice predisposed to adenoma formation, *Mthfr* deficiency is protective, but dietary folate is not.

4.2 INTRODUCTION

The mandatory fortification of flour/cereal products with folic acid (a synthetic form of folate) in North America has raised some important questions about the possible detrimental effects of folate supplementation on colorectal cancer (CRC) risk (113, 114). By virtue of the essential role of folate in the production of purines, thymidylate and methionine, inhibitors of folate metabolism have used as chemotherapeutic agents (*eg.* methotrexate). There is concern that individuals with undiagnosed premalignant tumours or with a genetic predisposition to cancer development may be adversely affected by high folic acid intake. It is therefore imperative to clarify the relationship between folate metabolism and CRC.

Although the majority of epidemiological studies have demonstrated that dietary folate is protective in terms of CRC risk (210, 287, 319-321), exceptions have been reported. In a subgroup of a large prospective study from Sweden, plasma folate levels were strongly positively related to CRC risk (179) and another study found that high dietary folate intake increased the risk of a subtype of colon tumours in men (178). One recently published intervention study investigated the effect of folate supplementation on adenoma recurrence in individuals with previously diagnosed and resected colorectal adenomas (181). They found that following 5-8 years of supplementation, the overall recurrence incidence was unchanged but that individuals in the supplemented group were at higher risk for developing greater than three adenomas as well as advanced lesions.

In a mouse model predisposed to adenoma formation, the $Apc^{min/+}$ mouse, the timing, degree and duration of dietary folate supplementation or deficiency significantly affect adenoma multiplicity. Song *et al.* reported that the number of ileal adenomas was decreased by folate supplementation when the mice were sacrificed at 3 months of age and decreased by folate deficiency when the mice were sacrificed at 6 months of age (216). In another report they showed that in $Apc^{+/-}Msh2^{-/-}$ mice, folate deficiency enhanced tumourigenesis if initiated early and folate supplementation enhanced tumourigenesis if implemented later (217). We have shown that an early genetic form of folate deficiency, a mutation in the intestinal reduced folate carrier resulted in fewer adenomas in $Apc^{min/+}$ mice (295).

A critical enzyme in folate metabolism, methylenetetrahydrofolate reductase (MTHFR), has also been implicated as a modifier of CRC risk. A common polymorphism 677C→T, encodes a thermolabile enzyme with reduced activity. Numerous studies have found a significant protective effect of the 677TT genotype on CRC risk (179, 182, 184). In some studies, low folate intake/status abolished the genotype protection (182) whereas in others it was maintained (179).

The mechanism through which MTHFR exerts its protective effect has not been firmly established. MTHFR catalyses the irreversible reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF). 5-MethylTHF is used by methionine synthase to remethylate

homocysteine to methionine. Methionine can be subsequently activated to *S*-adenosylmethionine (SAM) a methyl donor that participates in numerous cellular methylation reactions, including the DNA methyltransferase-catalysed methylation of DNA.

Both folate deficiency and MTHFR deficiency may affect tumourigenesis through altered DNA methylation either by promoting global hypomethylation or aberrant methylation patterns such as promoter hypermethylation. In addition, by restricting the availability of 5-methylTHF, deficiency of both dietary folate and MTHFR activity result in high plasma homocysteine levels. Homocysteine has been reported to exert a variety of toxic effects (82-84).

Disturbances in folate metabolism may also affect tumourigenesis through alteration of DNA precursor synthesis. MTHFR's substrate, 5,10-methyleneTHF, contributes the methyl group for the production of dTMP from dUMP and can be converted to 10-formyltetrahydrofolate which is used in the synthesis of the purine ring. It has been hypothesised that this reaction may be enhanced by MTHFR deficiency and impaired by folate deficiency. The relative amount of the triphosphates (dTTP and dUTP) is important for proper DNA synthesis and repair. A high dUTP/dTTP ratio may cause uracil misincorporation into DNA, the consequences of which may include DNA double-strand breaks, genomic instability and transformation potential (234-237, 302).

We have previously generated a mouse model for 677TT individuals; *Mthfr*$^{+/-}$ mice have similar decreases in MTHFR activity and increases in homocysteine levels relative to their wild-type counterparts (93). The purpose of this study is to investigate the impact of *Mthfr* deficiency and dietary folic acid on adenoma multiplicity. The results of a previous study have indicated that adenoma initiation in *Apc*$^{min/+}$ mice occurs in the first two weeks of life and that during this time, multiplicity can be optimally modified (318). For this reason, we initiated diets at two time-points: at weaning (3 weeks of age) or pre-natally, by placing their mothers on diets prior to mating. In the pre-natal group, we also investigated the effect of maternal *Mthfr* genotype on adenoma formation.

4.3 MATERIALS AND METHODS

Mice

Animal experimentation was approved by the Montreal Children's Hospital Animal Care Committee (Montreal, Quebec, Canada), in accordance with the guidelines of the Canadian Council on Animal Care. Male *Apc*$^{min/+}$ mice (C57Bl/6J background) were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred with female *Mthfr*$^{+/+}$ or *Mthfr*$^{+/-}$ mice according to the experimental designs described below. Offspring were genotyped for *Apc*$^{min/+}$ by PCR (217) as shown in Appendix I. Our *Mthfr* colony was originally established on the BALB/c background as previously reported (93). The *Mthfr* mice used in this study were the result of 13-15 generations of backcrossing to the C57Bl/6 strain (Charles River). Since *Mthfr* and *Pla2g2a* (the candidate gene for *Mom1,* an established modifier of the *Apc*$^{min/+}$ phenotype) are both located on distal mouse chromosome 4 and because *Pla2g2a* is polymorphic between the BALB/c and C57Bl/6 inbred strains, mice were genotyped for *Pla2g2a* using described procedures (170) as summarised in Appendix I. All mice were confirmed to harbour the *Mom1*^{S} allele (mutant *Pla2g2a,* the allele found in C57Bl/6 mice), and not the *Mom1*^{R} allele (wild-type *Pla2g2a*, the allele found in the BALB/c strain).

Post-weaning (late) dietary initiation

Male *Apc*$^{min/+}$ mice were bred with female *Mthfr*$^{+/-}$ mice fed standard mouse chow throughout pregnancy and nursing (laboratory rodent diet 5001, Agribrands Purina; containing 8 mg folic acid/kg chow). The *Apc*$^{min/+}$ offspring were weaned at 3 weeks and randomly assigned to receive one of three amino acid-defined diets: a control diet (CD) containing 2 mg folic acid/kg diet (the recommended amount for rodents), a folic acid-deficient diet (FADD) containing 0.3 mg folic acid/kg diet or a folic acid-enriched diet (FAED) containing 20 mg folic acid/kg diet. All diets were supplemented with 1% succinylsulfanthiozole to inhibit folate synthesis by intestinal flora. These diets complied with the recommendations of the American Institute of Nutrition guidelines for rodents. *Mthfr*$^{+/+}$*Apc*$^{min/+}$ and *Mthfr*$^{+/-}$*Apc*$^{min/+}$ mice were sacrificed at 10 weeks of age.

Pre-natal (early) dietary initiation and effect of maternal genotype

Four weeks prior to mating with male *Apc*$^{min/+}$ mice, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ female mice were randomly assigned to either the CD or FADD (described above) and were maintained on these diets throughout pregnancy and nursing. After weaning, the *Mthfr*$^{+/-}$*Apc*$^{min/+}$ and *Mthfr*$^{+/+}$*Apc*$^{min/+}$ offspring were continued on the same diets their mothers had received, until they reached 10 weeks of age when they were sacrificed.

Adenoma scoring

Adenoma scoring was performed as previously described (218, 219). Intestines were dissected, opened longitudinally and flushed with phosphate-buffered saline. Selected adenomas and preneoplastic (normal) intestinal tissues were snap frozen in liquid nitrogen and stored at –70°C. The remaining intestine was placed flat between two pieces of filter paper and fixed in 10% formalin solution for at least 24 hours. The intestine was then stained with a 0.1% methylene blue solution and assessed for adenoma number and size using a dissecting microscope and micrometer by two individuals blinded to genotype and diet.

Total plasma homocysteine evaluation

Cardiac blood was obtained at sacrifice, collected in potassium-EDTA tubes and centrifuged at 6000xg for 5 minutes at 4°C to separate plasma and frozen until analysis. Measurements were performed by HPLC as described (307).

Methylation analysis

To assess methylation of CCGG sites in preneoplastic (normal) intestine, a thin layer chromatography assay was performed as previously described (308). Briefly, 5 μg of RNA-free genomic DNA obtained from duodenal tissue was treated with *Msp1* (which digests both methylated and unmethylated CCGG sequences). The DNA was then treated with calf intestinal alkaline phosphatase, end-labelled with γ–[^{32}P]dATP, hydrolysed with nuclease P1, spotted on a cellulose TLC plate and developed in isobutyric acid-water-ammonium hydroxide (66:33:1). The images were quantified by a phosphorimager. The amount of

methylation was calculated as the percent of methylated cytosines/(methylated cytosines + unmethylated cytosines).

dUTP/dTTP analysis

Free deoxyribonucleotides were separated using an HPLC method previously described with slight modifications (292). Briefly, duodenal tissue was flash frozen in liquid nitrogen, ground to powder using a mortar and pestle, treated with 0.6 M trichloracetic acid, neutralised with trioctylamine and injected onto an Econosphere C18 column (particle size 5μm, length 250 x 4.6mm, Waters instrument Part No. 70071, Alltech). Separation of dUTP and dTTP was achieved by isocratic elution with 100% Buffer A (0.1 M $NH_4H_2PO_4$, 0.33 M KCl, 0.25% methanol, pH 5.35) for 12 minutes followed by a linear gradient to 25% Buffer B (0.1 M $NH_4H_2PO_4$, 0.4 M KCl, 20% methanol, pH 5) for 18 minutes followed by a linear gradient to 80% Buffer B for 10 minutes, then 10 minutes of 80% Buffer B, followed by re-equilibration with 100% Buffer A for 20 minutes. The flow rate for the entire run was 0.8 mL/min. A UV detector monitored peaks at wavelengths of 254 nm and 280 nm. Peaks were assigned by co-elution with known standards (Sigma-Aldrich).

Preparation of protein extracts

Frozen normal duodenal tissue was homogenised in ice-cold 0.1 M KPO_4 (pH 6.3) buffer containing a protease inhibitor mixture (Complete mini, Roche) using a Polytron (Brinkmann Instruments) and incubated on ice for 30 minutes. Supernatants were collected following centrifugation (4°C, 13000 rpm, 15 minutes) and protein concentrations were determined using the Bio-Rad protein assay.

Active Caspase-3 immunoblotting

60 μg of total protein was loaded onto a 12% SDS polyacrylamide gel and electrophoresed. Proteins were transferred to nitrocellulose membranes (Hybond ECL membrane, Amersham Pharmacia Biotech) at 4°C for 90 minutes at 70V. The membrane was blocked with 4% non-fat skim milk in Tris buffered saline/Tween (TBS-T) buffer for 1 hour at ambient temperature and then cut to allow incubation with the following primary antibodies overnight: rabbit anti-mouse active Caspase-3 (Chemicon) and rabbit anti-β-Actin (Sigma). Membranes were washed in TBS-T and incubated with an anti-rabbit HRP-conjugated secondary antibody

(Amersham Biosciences) for one hour at room temperature. The immunocomplexes were visualised by enhanced chemiluminescence with an ECL kit (Amersham Biosciences). Signals were quantified with Quantity One 4.1.0 software. The amount of active-Caspase 3 was normalised to β-Actin. To control for inter-blot variation, each of the six experimental groups was represented on each blot and values were calculated relative to the *Mthfr*$^{+/+}$*Apc*$^{min/+}$ offspring of *Mthfr*$^{+/+}$ mothers on the CD (considered to be the control group for these experiments).

Statistical analyses

Two-factor analysis of variance (ANOVA), independent sample *t*-tests (two-tailed) and regression analysis were performed using SPSS for WINDOWS software Version 11.0. *P*-values < 0.05 were considered significant. All data are reported as mean ± S.E.M..

4.4 RESULTS

Post-weaning (late) effects of diet

Body Weight

Three diets differing only in folic acid content were administered to *Mthfr*$^{+/+}$*Apc*$^{min/+}$ and *Mthfr*$^{+/-}$*Apc*$^{min/+}$ mice from 3-10 weeks of age. Neither diet nor *Mthfr* genotype influenced the body weight of the *Apc*$^{min/+}$ mice at sacrifice (data not shown). Body weight was not correlated with either adenoma number or adenoma size.

Adenoma number

When diets were introduced at weaning, very high amounts of folic acid (FAED) resulted in an increase in the total number of adenomas (**Figure 4.1A**). There was no difference in adenoma number between the CD and FADD groups. *Mthfr* genotype also did not have an effect on any one diet, nor overall. Mean adenoma size and size distributions were also not affected by either diet or *Mthfr* genotype (data not shown).

Plasma homocysteine levels

The *Mthfr*$^{+/-}$ genotype significantly increased plasma tHcy levels compared with the *Mthfr*$^{+/+}$ genotype in every dietary group (**Figure 4.1B**). The FADD caused a modest, but significant increase on this parameter. No correlation was found between plasma tHcy and adenoma number, mean adenoma size or size distributions (data not shown).

Pre-weaning (early) effects of diet and maternal genotype

Adenoma number

When diets were initiated before conception, folate deficiency dramatically decreased adenoma number (**Figure 4.2A**). This was borne out in every group; when diet was the only variable and *Mthfr* genotype and maternal *Mthfr* genotype remained constant, folate deficiency decreased the number of adenomas (comparison between bars of the same colour). The most dramatic diet-dependent decrease was in *Mthfr*$^{+/-}$*Apc*$^{min/+}$ offspring of *Mthfr*$^{+/-}$ mothers; mean adenoma number decreased from 27.0 ± 2.7 on the CD to 14.8 ± 1.1 on the FADD.

Maternal *Mthfr* genotype significantly affected adenoma formation in their *Apc*$^{min/+}$ offspring (**Figure 4.2A**). If the *Mthfr* genotype of the *Apc*$^{min/+}$ offspring is kept constant (by only considering only *Mthfr*$^{+/+}$*Apc*$^{min/+}$ mice), *Mthfr* deficiency of the mother resulted in a decrease in adenoma number in their offspring (comparisons between black and grey bars on either the CD or FADD). In *Mthfr*$^{+/+}$*Apc*$^{min/+}$ mice on the CD, adenoma number decreased from 46.0 ± 4.1 in offspring of *Mthfr*$^{+/+}$ mothers to 32.6 ± 2.1 in offspring of *Mthfr*$^{+/-}$ mothers. In *Mthfr*$^{+/+}$*Apc*$^{min/+}$ mice on the FADD, adenoma number decreased from 27.7 ± 2.4 in offspring of *Mthfr*$^{+/+}$ mothers to 18.8 ± 2.2 in offspring of *Mthfr*$^{+/-}$ mothers.

The *Mthfr* genotype of the *Apc*$^{min/+}$ mice had a modest but significant effect on adenoma number (**Figure 4.2A**). If maternal *Mthfr* genotype is kept constant (by considering only *Mthfr*$^{+/-}$ mothers), *Mthfr* deficiency of the *Apc*$^{min/+}$ mice decreased adenoma number (comparisons between grey and white bars on both the CD and FADD).

There were no differences in either mean adenoma size or size distributions in any comparison group (data not shown).

Figure 4.1 Post-weaning effects: the impact of *Mthfr* genotype and dietary folate in $Apc^{min/+}$ mice

The effect of *Mthfr* genotype and dietary folic acid on adenoma number (**A**) and plasma total homocysteine (tHcy) levels (**B**) in $Apc^{min/+}$ mice. Bars represent the mean ± S.E.M. for $Mthfr^{+/+}Apc^{min/+}$ (▒) and $Mthfr^{+/-}Apc^{min/+}$ (□). All mice were offspring of $Mthfr^{+/-}$ mothers fed mouse chow prior to conception, during pregnancy and nursing. $Apc^{min/+}$ mice were placed on diets at three weeks of age (FAED, folic acid-enriched diet; CD, control diet; FADD, folic acid-deficient diet). The total numbers of animals per group are indicated in (**A**). 5-6 animals per group were analysed for plasma tHcy levels.

*P<0.05; dietary effect (two-factor ANOVA).

#P<0.0001; genotype effect (t-test, $Mthfr^{+/+}$ versus $Mthfr^{+/-}$).

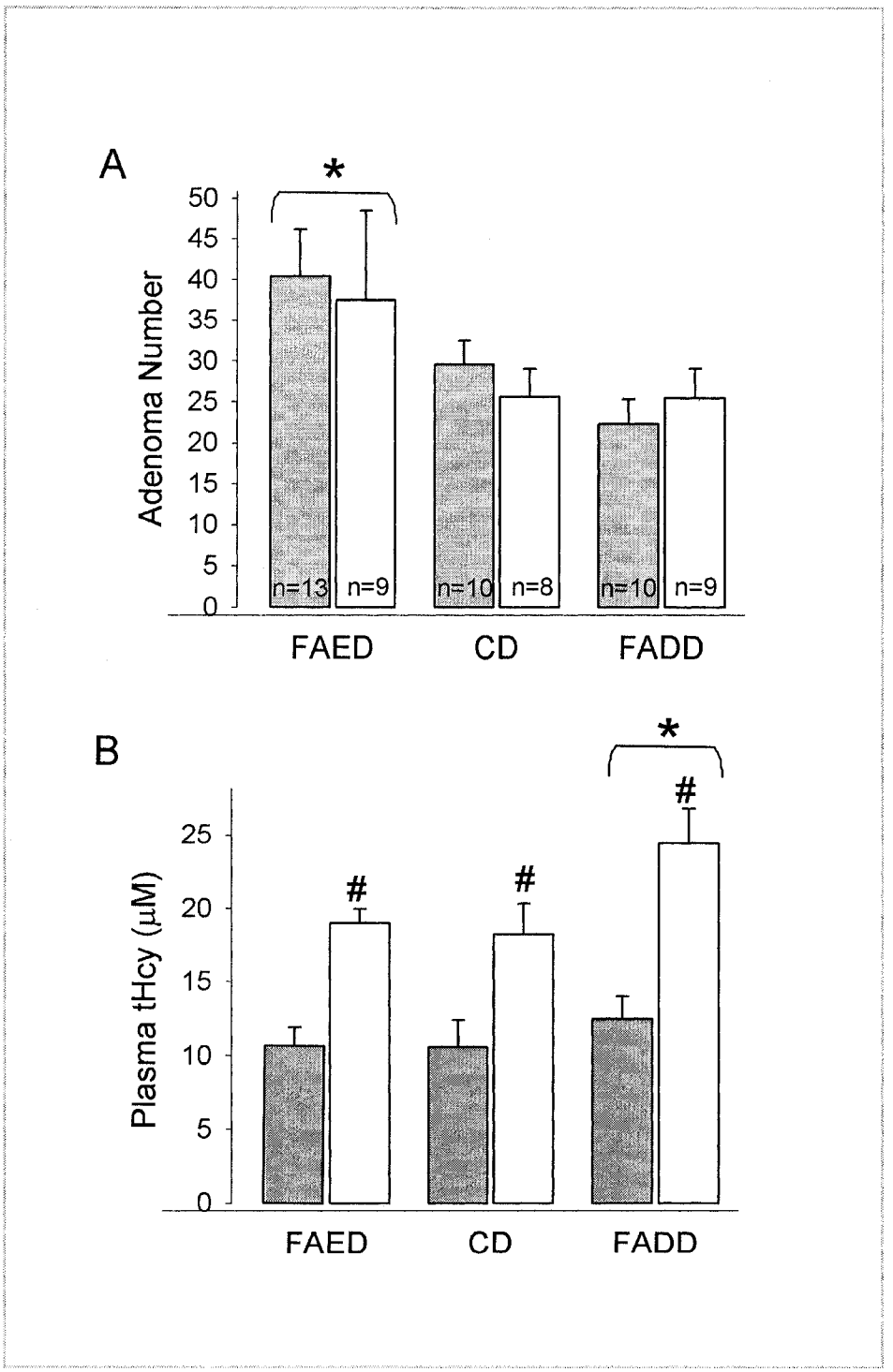

A
*
Adenoma Number
50
45
40
35
30
25
20
15
10
5
0
n=13
n=9
n=10
n=8
n=10
n=9
FAED
CD
FADD
B
*
#
#
#
Plasma tHcy (μM)
25
20
15
10
5
0
FAED
CD
FADD

Figure 4.2 Pre-weaning effects: the impact of *Mthfr* genotype, maternal *Mthfr* genotype and dietary folate in $Apc^{min/+}$ mice.

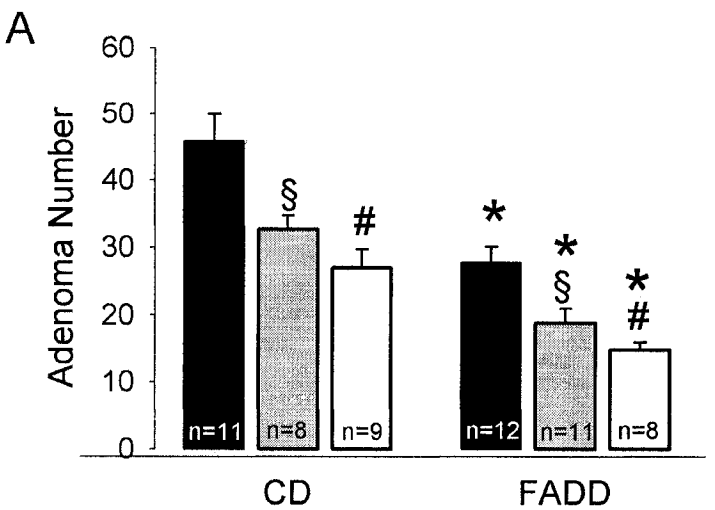
The effect of folic acid, *Mthfr* genotype, and maternal *Mthfr* genotype on adenoma number (**A**) and plasma total homocysteine (tHcy) levels (**B**) in $Apc^{min/+}$ mice and the correlation between adenoma number and plasma tHcy levels (**C**).

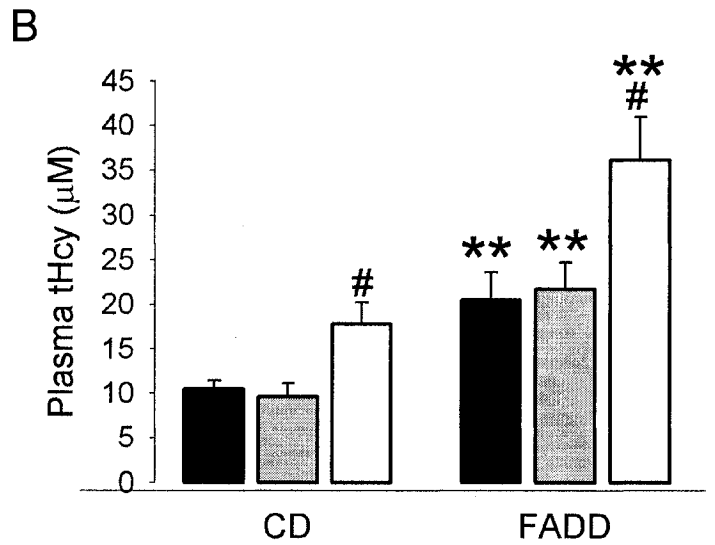
Bars represent the mean ± S.E.M. for $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/+}$ mothers (■), $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/-}$ mothers (▩), and $Mthfr^{+/-}Apc^{min/+}$ offspring of $Mthfr^{+/-}$ mothers (□). Mothers were placed on diets prior to mating with $Apc^{min/+}$ males (CD, control diet; FADD, folic acid-deficient diet) and diets were continued until the $Apc^{min/+}$ offspring were sacrificed at 10 weeks of age.

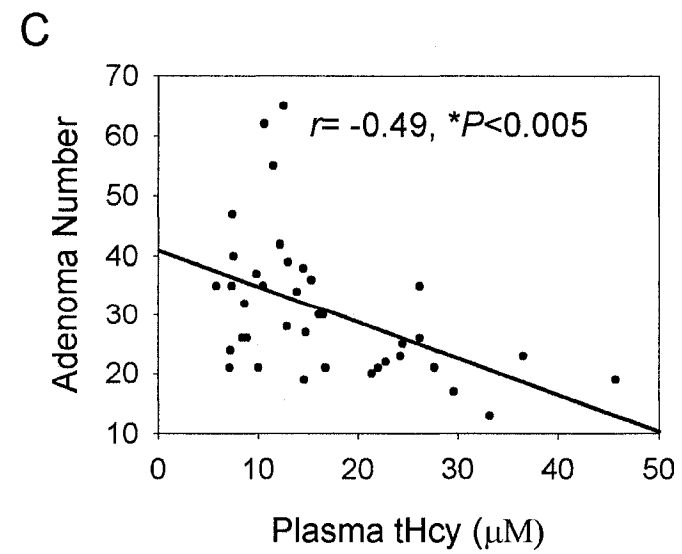
*$P<0.05$, **$P<0.005$; dietary effect (*t*-test, CD versus FADD for mice with the same *Mthfr* genotype and same maternal *Mthfr* genotype)

#$P<0.05$; genotype effect (adenoma number: two-factor ANOVA; plasma tHcy: *t*-test, $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/-}$ mothers versus $Mthfr^{+/-}Apc^{min/+}$ offspring of $Mthfr^{+/-}$ mothers).

§$P<0.05$; maternal *Mthfr* genotype effect (*t*-test, $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/+}$ mothers versus $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/-}$ mothers)


A
Adenoma Number
60
50
40
30
20
10
0
n=11
n=8
n=9
n=12
n=11
n=8
§
#
*
*
§
*
#
CD
FADD


B
Plasma tHcy (μM)
45
40
35
30
25
20
15
10
5
0
#
**
**
**
#
CD
FADD


C
Adenoma Number
70
60
50
40
30
20
10
0
10
20
30
40
50
r= -0.49, *P<0.005
Plasma tHcy (μM)

The effect of folate enrichment was also investigated using this experimental design in a small number of mice (data not shown). Adenoma numbers and sizes of the three groups of FAED mice were comparable to the three groups of CD mice (*t*-tests, $P>0.05$, comparisons between groups with same *Mthfr* genotype and same maternal *Mthfr* genotype). The maternal genotype effect was also maintained; $Mthfr^{+/+}Apc^{min/+}$ (offspring of $Mthfr^{+/+}$ mothers) developed 47.8 ± 6.5 adenomas (n=5) compared with $Mthfr^{+/+}Apc^{min/+}$ (offspring of $Mthfr^{+/-}$ mothers), which developed 30.8 ± 3.3 adenomas (n=4) (*t*-test, $*P<0.05$).

Plasma total homocysteine (tHcy) levels

Elevated plasma tHcy levels are indicative of disturbances in folate metabolism. Low levels of 5-methylTHF (due either to *Mthfr* deficiency or dietary folate deficiency) increase homocysteine by impairing its remethylation to methionine. Both *Mthfr* deficiency and the FADD increased tHcy levels (**Figure 4.2B**), as expected. Maternal *Mthfr* genotype alone did not exert an effect at the time of these measurements (10 weeks of age) since the mice were considerably past the nursing period. tHcy levels were strongly inversely correlated with adenoma number (**Figure 4.2C**). This negative correlation was maintained (albeit not always significantly) when various subgroups were analysed separately (*i.e.*, by diet, genotype or maternal genotype) but was especially strong in the $Mthfr^{+/-}Apc^{min/+}$ subset (r= -0.813, $*P<0.005$) (data not shown).

Litter size

Both *Mthfr* deficiency and folate deficiency have been shown to adversely affect reproductive outcomes in mice on the BALB/c strain background (100). Since our present study demonstrates that maternal influences (both dietary and genetic) dramatically affect tumourigenesis in this model, we compared litter sizes to ensure that this factor, which may affect *in utero* growth and milk availability, was not an issue. Indeed, there were no significant differences in litter size among any of the groups (data not shown), which is consistent with our unpublished data of reproductive outcomes in the C57Bl/6 strain.

Body weight

The body weights of the *Apc*$^{min/+}$ mice were recorded at 10 weeks of age (data not shown). Although the mice were exposed to the diets from conception until sacrifice, there were no differences in body weight attributable to diet. Body weight was not affected by either maternal *Mthfr* genotype or *Mthfr* genotype of the *Apc*$^{min/+}$ mouse in any group. Body weight was, however, positively correlated with mean adenoma size (r=0.428, *P<0.005), but not adenoma number. Timing of diet (pre-weaning versus post-weaning group) did not affect the body weights of female mice, but post-weaning group males had slightly elevated body weights compared with pre-weaning group male mice (~10% difference in body weight; *P<0.05, two-factor ANOVA).

The effect of timing: initiation of diets pre-natally (before conception) versus post-weaning (at 3 weeks of age).

Analysis of the temporal effects of dietary initiation can only include comparisons of groups represented in both the pre-weaning (early) and post-weaning (late) experiments. Thus, only *Apc*$^{min/+}$ offspring of *Mthfr*$^{+/-}$ mothers and mice fed the CD and FADD can be considered. Note that prior to weaning, mice in the post-weaning group were exposed to the dietary effects of mouse chow (containing 8 mg folic acid/kg chow), both *in utero* and in milk provided by their mother.

The effect on adenoma number can be appreciated by comparing the grey and white bars of **Figure 4.1A** (CD and FADD) with **Figure 4.2A**. The mice that were most significantly affected were the *Mthfr*$^{+/-}$*Apc*$^{min/+}$ mice on the FADD. Early initiation of folate deficiency compared with later initiation of diet decreased adenoma number from 25.4 ± 3.6 to 14.7 ± 1.1 (t-test, *P<0.05). Early initiation of folate deficiency in *Mthfr*$^{+/+}$ mice resulted in a non-significant decrease in adenoma number from 22.3 ± 3.1 to 18.8 ± 2.2 (t-test, P=0.36).

The duration of the diet also had a dramatic effect on plasma tHcy levels at 10 weeks of age in folic acid-deficient mice which can be seen by comparing the grey and white bars of **Figure 4.1B** (CD and FADD) with **Figure 4.2B**. *Mthfr*$^{+/+}$ mice were particularly affected; early onset of folate deficiency translated into an almost two-fold increase in tHcy in this group (21.8 ± 3.0 versus 12.5 ± 1.6; t-test,

*P<0.05). tHcy was also increased as a result of the early versus late inititation of the FADD in *Mthfr*$^{+/-}$ mice (36.2 ± 4.9 versus 24.4 ± 2.4; t-test, P=0.07). In mice fed the CD, the duration of the diet did not influence tHcy levels, indicating that the folic acid content of the CD was comparable to mouse chow in terms of the ability to lower tHcy relative to the FADD.

Investigation of Mechanisms in the Pre-weaning Group

Intestinal global DNA methylation was decreased in FADD mice compared with CD mice (**Table 4.1**). This factor was not affected by either *Mthfr* genotype nor maternal *Mthfr* genotype nor was it correlated with either adenoma number or plasma tHcy levels.

Table 4.1. Pre-weaning effects in *Apc*$^{min/+}$ mice: mechanisms

Global intestinal methylation, dUTP/dTTP ratio and relative levels of apoptosis were measured in 4-6 mice per group. Mean ± S.E.M. are shown.

NS = not significant, P>0.05.

*P<0.05 for dietary effect (two-factor ANOVA, diet and *Mthfr* genotype).

#P<0.001 for genotype effect (t-test, *Mthfr*$^{+/+}$ versus *Mthfr*$^{+/-}$)

Diet	Maternal *Mthfr* Genotype	*Mthfr* Genotype	DNA Methylation		dUTP/dTTP ratio		Apoptosis	
CD	+/+	+/+	64.6 ± 2.0		0.9 ± 0.3		1.0	
	+/-	+/+	66.5 ± 2.3		1.3 ± 0.3		0.9 ± 0.3	#
	+/-	+/-	65.9 ± 2.6		1.2 ± 0.3		2.4 ± 0.6	#
FADD	+/+	+/+	60.2 ±2.1		1.8 ± 0.3		1.4 ± 0.5	
	+/-	+/+	62.5 ± 2.9		1.6 ± 0.1	*	2.8 ± 0.9	
	+/-	+/-	62.1 ± 1.6		1.3 ± 0.3		3.1 ± 0.9	
Correlation with plasma tHcy			r= -0.15, NS		r= -0.18, NS		r = 0.48, *P<0.05	
Correlation with adenoma number			r= 0.10, NS		r= -0.42, *P<0.05		r= -0.36, P=0.08	

The dUTP/dTTP ratio, an indication of the rate of the thymidylate synthase-catalysed methylation of dUMP to dTMP, was not significantly altered in response to diet, genotype or maternal genotype (**Table 4.1**). However, it was significantly inversely correlated with adenoma number.

Levels of apoptosis were measured using an antibody directed against the active form of Caspase-3, an effector molecule in the programmed cell death cascade. *Mthfr* genotype had the greatest effect on this factor (**Table 4.1**). On the CD, $Mthfr^{+/-}$ mice had higher levels of intestinal apoptosis compared with $Mthfr^{+/+}$ mice. This was also true in the FADD, although it was not statistically significant. Overall, apoptosis was positively correlated with tHcy levels ($^{*}P<0.05$) and inversely correlated with adenoma number ($P=0.08$).

4.5 DISCUSSION

This study addresses some critical issues surrounding the effect of folate on intestinal tumourigenesis. Specifically, how do the dose and timing of dietary folate affect adenoma formation and growth and what are the consequences of genetic disturbances of folate metabolism? We approached this question by using the $Apc^{min/+}$ mouse model of colon cancer in conjunction with an *Mthfr* knockout mouse and diets differing in folic acid content which were initiated at two time-points (prior to conception and at weaning).

Our results strongly suggest that early initiation of folate-deficient diets inhibits tumourigenesis in the $Apc^{min/+}$ mouse model. By placing their mothers on amino acid-defined diets prior to mating, the $Apc^{min/+}$ mice experienced the dietary effects throughout their entire lives. Despite the severity of this dietary regimen, folate deficiency did not have deleterious effects on either *in utero* survival or on body weight, an indicator of general health status. Folate deficiency did, however, reproducibly decrease adenoma number in three independent experimental groups.

The protective effect of the FADD may be relevant when there is a propensity towards adenoma growth, as is the case for $Apc^{min/+}$ mice. In mice without germline mutations or chemical carcinogen induction, we have previously shown that the same FADD used in this report initiated intestinal adenomas. In the

BALB/c strain, a strain not predisposed towards adenoma formation, 25% of mice fed the FADD developed intestinal adenomas compared with 0% of mice fed the CD (222).

When the diets were initiated at weaning (a later timepoint), there were no differences between the FADD and CD groups. Since we were able to modulate adenoma number by introducing the diets prior to weaning, we conclude that this is a critical time-point in adenoma initiation. We further conclude that initiation of diets subsequent to this time may have limited our ability to detect significant differences. Notwithstanding this, we did observe that very high levels of folic acid increased the number of adenomas. It is possible that microadenomas, which were unobservable but nonetheless present in the CD and FADD groups, were encouraged to grow larger when supplied with excessive amounts of folic acid, making them visible. This is consistent with Song *et al.*, who reported that folate supplementation after the establishment of neoplastic foci increased the adenoma number in $Apc^{+/-}Msh2^{-/-}$mice (217).

In this study, we demonstrated that maternal genotype significantly influenced adenoma formation in their $Apc^{min/+}$ offspring. We showed that the $Apc^{min/+}$ offspring of $Mthfr^{+/-}$ mothers developed significantly fewer adenomas compared with the $Apc^{min/+}$ offspring of $Mthfr^{+/+}$ mothers on both the FADD and CD. Maternal genotype would presumably exert its most direct effects during pregnancy and nursing, the two time-points during which the offspring is critically dependent on its mother for nutrients. It is possible that there are important differences in the levels of metabolites passed on from the $Mthfr^{+/+}$ and $Mthfr^{+/-}$ mothers to their offspring, either across the placenta or through their milk. MTHFR catalyses the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF and thus directs one-carbon units to either the methylation cycle or thymidylate synthesis. We have previously shown that the MTHFR activity of $Mthfr^{+/-}$ mice is 60% of that of $Mthfr^{+/+}$ mice (93) and, although not statistically significant, that $Mthfr^{+/-}$ mice have lower tissue levels of 5-methylTHF, the major circulating folate derivative (94). $Mthfr^{+/-}$ mothers may transfer a higher proportion of 5,10-methyleneTHF with respect to 5-methylTHF to their offspring

during this critical window for adenoma initiation. Such a situation would be especially relevant since the number of intestinal crypts increases dramatically in the second and third postnatal weeks (119). This rapid proliferation places high demands on the production of DNA precursors such as dTTP. If the synthesis of dTTP is impaired, uracil misincorporation, DNA strand breaks and genomic instability may occur. We measured the ratio of dUTP to dTTP at 10 weeks of age and did not observe a maternal effect on this parameter. Investigation of this theory may be better served by analysing the dUTP/dTTP at earlier time-points.

Maternal genotype may also affect the methylation status of specific genes in their offspring, a change that may affect gene expression and the $Apc^{min/+}$ phenotype, a phenomenon that is not unprecedented. It has been shown that in a specific strain of mouse, coat color in offspring could be altered by supplementing the mother's diet with methyl donors including folic acid. The diet changed the methylation status and expression of a particular gene governing this trait (322). In our study, *Mthfr* heterozygosity may decrease the amount of 5-methylTHF and ultimately affect the amount of SAM produced for DNA methylation. Our analysis of global DNA methylation was insufficient to address this hypothesis which requires the identification of specific methylation targets.

Our study design also does not allow us to determine whether the maternal genotype effect we observed was manifest during gestation or nursing (or a combination of both). This issue could be addressed by cross-fostering experiments, *i.e.*, by placing pups of $Mthfr^{+/+}$ mothers with $Mthfr^{+/-}$ mothers at birth, and *vice versa*. This strategy could also be used to determine at which developmental stage the inhibitory effect of folate deficiency was most pronounced.

We have also demonstrated that the *Mthfr* genotype of the $Apc^{min/+}$ mouse modifies tumourigenesis, although this finding is overshadowed by the more marked maternal and dietary effects. It is plausible that the mechanism underlying the maternal *Mthfr* genotype effect in early development (when it has a stronger impact in this model) is also effective in adulthood (when it has a lesser impact in this model) through the *Mthfr* genotype of the $Apc^{min/+}$ mouse. It is also possible

that the mechanism governing the *Mthfr*-dependent effect may be different from that of the diet-dependent effects.

In an attempt to define these mechanisms in the pre-weaning group, we measured plasma tHcy levels, intestinal DNA methylation, dUTP/dTTP ratio, and apoptosis.

We found a very significant negative correlation between tHcy levels and adenoma number. To suggest that homocysteine, which has already been identified as an independent risk factor for cardiovascular disease (323), may play a protective role in tumourigenesis is controversial. It is entirely possible, if not probable, that the homocysteine changes are coincident with another process which is modifying adenoma number. On the other hand, homocysteine, by virtue of its cytotoxic nature, may have an indirect effect on adenoma growth by triggering apoptosis. In fact we did observe that apoptosis levels (as measured by active Caspase-3 levels) were positively correlated with plasma tHcy levels and negatively correlated with adenoma number (borderline significant).

Mthfr heterozygotes displayed higher levels of intestinal apoptosis than wild-type mice which may or may not be related to high homocysteine levels. We have previously shown that antisense inhibition of *MTHFR* both *in vitro* (colorectal cancer cell lines) and *in vivo* (tumour xenografts) decreased cell survival and growth by enhancing apoptosis (285). We have also demonstrated higher levels of apoptosis in the cerebella of $Mthfr^{-/-}$ mice, who have dramatically elevated plasma tHcy levels, compared to $Mthfr^{+/+}$ (324).

Global DNA methylation was most affected by dietary folic acid deficiency. In previous studies, genetic disruption of the enzyme that catalyses DNA methylation decreased adenoma number in $Apc^{min/+}$ mice (219, 268, 270, 271), as did the pharmacologic inhibition of DNA methylation by 5-azacytidine (268). One group demonstrated that promoter CpG island methylation was affected (270) whereas another group suggested that the protective effect of DNA hypomethylation was based on the lower probability of mutagenic C→T transitions originating from the deamination of 5-methylcytosine residues (268).

We also observed a negative correlation between dUTP/dTTP ratio and adenoma number. A high dUTP/dTTP ratio may also be construed as low dTTP levels which may be conducive to lower levels of proliferation and restricted adenoma growth. Alternatively, high levels of dUTP may lead to DNA damage and increased apoptosis which would lead to tumour cell death. In response to 5-fluorouracil treatment (a thymidylate synthase inhibitor), colorectal cells have been shown to die by apoptosis in a "thymineless death" caused by dTTP depletion and DNA damage (286).

In conclusion, we have found that deficiency of both *Mthfr* and folic acid is protective in the $Apc^{min/+}$ mouse model. Our data on *Mthfr* provides biological evidence for the hypothesis generated by numerous epidemiological studies, that suggest a protective effect of the 677TT genotype in terms of CRC risk. We have begun to address possible mechanisms through which this protection is conferred. Our dietary results complement the findings of one folic acid intervention trial (181). Folic acid supplementation increases adenoma number in predisposed mice ($Apc^{min/+}$) and individuals (with a history of adenoma) (181). In contrast, folic acid supplementation may decrease risk in non-predisposed mice (BALB/c) (222) and humans (175). Therefore, folic acid fortification may be advantageous for healthy individuals. However, if our mouse data can be extrapolated to humans, then it is possible that the subset of the population with undiagnosed pre-malignant tumours or a genetic predisposition for them may be adversely affected. Further studies are clearly warranted to address this important health issue.

4.6 ACKNOWLEDGEMENTS

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CHAPTER V

General Discussion

5.1 THE *Apc*$^{min/+}$ MODEL

In this thesis, the *Apc*$^{min/+}$ model of CRC was used to investigate the impact of nutritional and genetic disturbances in folate metabolism on tumourigenesis. In these mice, the majority of adenomas are initiated early in life, prior to two weeks of age (318). Since the term tumourigenesis by definition refers to the initiation of neoplasia, it most accurately describes the effects of germline mutations (*i.e.*, in *Rfc*, *Mtr* or *Mthfr*) as well as dietary initiation prior to conception in this model. Dietary manipulation at weaning may affect later stages, such as progression to larger adenomas. In either case, the context is important: these mice have a germline *Apc* mutation and a genetic predisposition towards adenoma formation.

The primary advantage of this model is that the mice develop numerous adenomas in a short period of time that are microscopically similar to those in humans (325). There are, however, several limitations in this model. Unfortunately, the benign adenomas of *Apc*$^{min/+}$ mice rarely progress to adenocarcinomas nor do they metastasise, probably due to the short lifespan (120 days) of these mice (169). In the experiments described in this thesis, the mice were sacrificed at 10 weeks of age (70 days). In the literature, the age at sacrifice varies significantly and is somewhat arbitrary. However, it has been previously shown that tumour multiplicity does not significantly change from 67-97 days of age in these mice (318), thus a later time-point would be unlikely to dramatically affect numbers, although it may have allowed differences in size to become evident. Another limitation of the *Apc*$^{min/+}$ models is that the majority of adenomas are formed in the small intestine and not the colon, a more relevant organ in terms of human cancer risk. Nonetheless, an improvement in the experimental design would have been to analyse the colon for the formation of adenomas and aberrent crypt foci.

5.2 GENETIC DISTURBANCES OF FOLATE METABOLISM

A primary goal of this thesis was to determine which aspects of folate metabolism are most important for tumour growth in *Apc*$^{min/+}$ mice. The availability of folate pathway knockout mice provided an excellent opportunity to

address this objective. Unfortunately, the genetic heterogeneity of the *Mtr* and *Rfc* strains was a limitation of these studies. Backcrossing of both the *Mtr* and *Rfc* strains to C57Bl/6 for at least 10 generations, as was done for the *Mthfr* strain, would have been ideal. Nevertheless, the information gained from each of these studies contributed to the overall understanding in this field.

5.2.1 Reduced Folate Carrier 1

Early (germline mutation) deficiency of a folate transporter is protective in $Apc^{min/+}$ mice. On both the CD and FADD, $Rfc1^{+/-}Apc^{min/+}$ mice developed fewer adenomas than $Rfc1^{+/+}Apc^{min/+}$ mice. This finding is consistent with the dietary results of the *Mthfr* experiment, *i.e.*, that the early initiation of folate deficiency decreased adenoma number

We were unable to identify the mechanism through which *Rfc1* deficiency decreased adenoma number as there were no genotype-related changes in plasma tHcy, global DNA methylation or apoptosis. The dUTP/dTTP ratio was not analysed in $Apc^{min/+}$ mice, but in adult mice without *Apc* mutations there were no changes relating to *Rfc* genotype. Since we speculate that the events modifying adenoma number occur very early in $Apc^{min/+}$ mice, it may be constructive to examine these mechanisms at an earlier time-point.

The recent identification of another intestinal transporter, the PCFT, requires consideration. In rats, the pH of the luminal membrane of the gastrointestinal tract produces a gradient from the duodenum (pH 6.2) to the jejunem (pH 6.3-6.5) to the ileum (pH 7.0) (326). If PCFT, which has an acidic pH optimum, is indeed responsible for the majority of folate absorption in the proximal intestine, it is possible that RFC1, which has a neutral pH optimum, may be responsible for folate absorption in the distal intestine, where it may support local tissue (and adenoma) growth. Some studies have shown that, compared with other regions, adenoma number is highest in the ileum in control mice (327-329) and that dietary folate content modifies adenoma number only in that region (216). Unfortunately, we did not test this directly as adenoma location was not assessed in this thesis.

5.2.2 Methionine Synthase

Although an *MTR* polymorphism is associated with a decreased risk for CRC in some human studies (64, 184), we did not observe an affect of *Mtr* deficiency on adenoma number or load in $Apc^{min/+}$ mice. The reason for this may be technical; the outbred genetic background of this strain may have obscured genotype-related effects. Alternatively, *Mtr* heterozygosity may not have a large impact in this model or may be compensated for by BHMT activity.

Hypothetically, *Mtr* heterozygotes have higher levels of 5-methylTHF and plasma tHcy, and lower levels of SAM and DNA methylation compared to wild-type mice. Since MTR is the only enzyme that uses 5-methylTHF, the only consequence of its accumulation is the sequesteration of one-carbon groups which could affect DNA synthesis. We measured the dUTP/dTTP ratio in *Mtr* mice and found no effect of genotype, nor did we see differences in either DNA methylation or plasma tHcy levels. Since we have found that early intervention is important in this model, an experimental design including different maternal *Mtr* genotypes may be useful for future work.

5.2.3 Methylenetetrahydrofolate Reductase

Early (germline mutation) and earlier (maternal germline mutation) deficiency in *Mthfr* is protective in $Apc^{min/+}$ mice. The maternal effect was more pronounced, probably because it was effective during the time period (before weaning) when adenoma number can be optimally modified.

We did not observe an effect of genotype on global DNA methylation which indicates that *Mthfr* deficiency does not significantly affect adenoma number through this mechanism. We did find, however, that adenoma number was negatively correlated with plasma tHcy, intestinal dUTP/dTTP ratio and intestinal apoptosis and that the $Mthfr^{+/-}$ genotype was associated with higher levels of apoptosis. The fact that tissues were harvested at 10 weeks of age, rather than at an earlier and perhaps more relevant time-point, is a limitation of this study.

The effect of the *Mthfr* genotype was only evident in mice fed amino acid-defined diets for their entire lives and not when the diets were initiated at weaning. Since the pre-weaning period has been identified as a crucial point for intervention, it could be argued that a genotype effect should be apparent regardless of the dietary exposure during this period. However, there is an important difference between amino acid-defined diets and rodent chow. Amino acid-defined diets are composed of pure ingredients with standardised composition, whereas standard rodent chow contains potentially confounding substances, such as phytoestrogens, in concentrations which can vary from batch to batch (330, 331). Moreover, inter-experimental variability has been reported in studies where diets were initiated at weaning and attributed to differences in the transformation state of the cells at the onset of diets (218, 219). Lifelong dietary exposure removes any such ambiguity.

The protective effect of *Mthfr* genotype is not usually seen in folate-deficient humans (182). Similarly, it was not observed in the BALB/c model where a low folate diet initiated tumours in 25% of mice (222). Folate-deficient $Mthfr^{+/-}$ mice were twice more likely to develop tumours than were $Mthfr^{+/+}$ mice. In this model, adenomas are only observed after one year of exposure to the FADD whereas they are observed much earlier in $Apc^{min/+}$ mice. In the BALB/c model, the FADD is the initiator and the $Mthfr^{+/-}$ genotype may exacerbate its effects. In the $Apc^{min/+}$ model, MTHFR reduces tumourigenesis, as evidenced by the protective and early effect of the maternal $Mthfr^{+/-}$ genotype.

5.3 DIETARY FOLATE

The effect of dietary folate content and timing was examined in the $Apc^{min/+}$ model in this thesis. Tissue folate levels were not assessed in this thesis. However, a study has shown a dose-dependent increase in folate in the gastrointestinal tract of rats fed diets ranging from 0-40 mg folic acid/kg diet (332). We used plasma tHcy levels as a surrogate marker of folate levels and they were increased in response to folate deficiency in every experiment. Tissue homocysteine levels were not measured and since the intestine is capable of transsulphuration (catabolism of homocysteine), it is possible that it does not accumulate in this

organ. However, the transsulphuration pathway enzymes are present in lower concentrations in the intestine relative to other sites (89) and tracer studies in a folate-deprived CRC line indicated that the capacity of the enterocyte transsulphuration pathway can be exceeded (333).

Initiation of a FADD at weaning did not affect adenoma numbers in the *Rfc* or *Mthfr* studies. In the *Mtr* experiment, the FADD increased adenoma number, a result which is incongruous with other findings of this thesis. However, the effect of genetic background cannot be underestimated as illustrated by the fact that folate deficiency initiates intestinal adenomas in BALB/c mice, but not C57Bl/6 mice (334).

Initiation of a FADD prior to conception dramatically reduced adenoma numbers in the *Mthfr* experiment and a FAED increased adenoma number when initiated at weaning, after the majority of adenomas had begun to grow. Together these results suggest that in predisposed mice, dietary folate increases adenoma number, a finding is consistent with a folic acid intervention trial in which supplementation increased adenoma number in individuals with a history of adenoma (181).

5.4 MECHANISMS

Throughout this thesis, several candidate mechanisms were explored: nucleotide imbalance, DNA methylation and apoptosis. It should be noted that a limitation of these mechanistic studies is the use of duodenal pieces, and not isolated mucosa.

The dUTP/dTTP ratio was increased in response to folate deficiency in non-predisposed mice and inversely correlated with adenoma number in $Apc^{min/+}$ mice. Uracil misincorporation may be protective, perhaps by inciting apoptosis, or dTTP depletion may be protective, either by inciting apoptosis or inhibiting proliferation. The effects of genotype were not pronounced, and may be related to the tight regulation of nucleotide synthesis. A significant improvement to this technique would be the inclusion of an internal standard, so that absolute values of nucleotides could be obtained.

We observed that DNA hypomethylation was associated with dietary folate deficiency and with both higher adenoma number (in the *Mtr* experiment) and lower adenoma number (in the *Mthfr* experiment), a disparity which may be related to timing. The *Mthfr* experiment involved the early initiation of diets; early germline *Dnmt1* mutations also decrease adenoma number in $Apc^{min/+}$ mice (219, 268, 270, 271). In the *Mtr* experiment, the diets were initiated at weaning which may account for the opposite effect. There was no appreciable effect of *Rfc1*, *Mtr* or *Mthfr* heterozygosity on global DNA methylation in any experiment, which may be due to the low sensitivity of the techniques used.

In the *Mthfr* experiment, apoptosis was inversely correlated with adenoma number and may have been triggered by either homocysteine or nucleotide imbalance. Corrobatory techniques, such as terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, could strengthen our findings and allow us to identify the affected cell populations.

5.5 FOLATE AND COLORECTAL CANCER – MODEL

The data presented in this thesis advance the current understanding of folate and CRC and allow us to formulate the following model.

In healthy humans (in epidemiological studies) and mice (BALB/c), dietary folate is protective through a mechanism which may involve the prevention of DNA damage accumulation (222). In predisposed humans (history of adenoma) and mice ($Apc^{min/+}$), increased dietary folate may be harmful. The evidence in this thesis suggests that dietary folate may enhance DNA synthesis by increasing the relative dTTP levels. There may also be a beneficial effect of DNA hypomethylation, as in *Dnmt1*-hypomorphic $Apc^{min/+}$ mice. In healthy humans (in epidemiological studies) and predisposed mice ($Apc^{min/+}$), deficiency in MTHFR is protective. In this thesis, lower levels of MTHFR reduced the dUTP/dTTP ratio in folate-replete non-predisposed mice, which may be protective over time. In $Apc^{min/+}$ mice, *Mthfr* deficiency did not significantly affect the dUTP/dTTP ratio, however the apoptosis in $Mthfr^{+/-}$ mice may be protective by killing tumour cells.

5.6 FOLATE AND COLORECTAL CANCER – IMPLICATIONS

In the decade since its inception, the beneficial effects of folic acid fortification are incontrovertible; neural tube defect (NTD) rates have declined and plasma homocysteine and folate levels have been favourably affected (104-106). However, as Kim astutely remarked in his recent commentary, 50% of 50 year olds have colorectal adenomas whereas NTDs affect less than 1% of births annually (114). Furthermore, although homocysteine is an established risk factor for cardiovascular disease, which are also common, a B-vitamin (folic acid, B_{12} and B_6) intervention trial investigating the effects on myocardial infarction and vascular disease risk failed to report positive outcomes on these primary endpoints. In fact, non-significant increases in the rate of total cancer (335) and colon cancer (336) were reported. In terms of public health concern, the cancer-promoting properties of folic acid may outweigh its positive effects. The genetic and nutritional studies described in this thesis have contributed to the understanding of this issue.

5.7 CLAIMS TO ORIGINALITY

1. *Mthfr* deficiency decreased the dUTP/dTTP ratio in the intestine of folate-replete BALB/c mice.
2. *Mthfr* deficiency decreased DNA methylation in the intestine of folate-replete BALB/c mice.
3. $Rfc1^{+/-}Apc^{min/+}$ mice developed fewer intestinal adenomas than $Rfc1^{+/+}Apc^{min/+}$ mice. *Rfc1* genotype did not affect plasma tHcy, intestinal DNA methylation or intestinal apoptosis in these mice.
4. *Mtr* genotype did not affect intestinal adenoma number in $Apc^{min/+}$ mice.
5. $Mtr^{+/+}Apc^{min/+}$ and $Mtr^{+/-}Apc^{min/+}$ mice fed folate-deficient diets from 3-to-10 weeks of age developed more intestinal adenomas than those fed control diets for the same period of time. They also had higher levels of plasma tHcy and intestinal apoptosis and lower levels of intestinal DNA methylation.
6. $Mthfr^{+/-}Apc^{min/+}$ mice developed fewer intestinal adenomas than $Mthfr^{+/+}Apc^{min/+}$ mice when amino acid-defined diets were initiated prior to conception, but not when diets were initiated at 3 weeks of age. In mice fed the control diet, $Mthfr^{+/-}Apc^{min/+}$ mice had higher levels of intestinal apoptosis than $Mthfr^{+/+}Apc^{min/+}$ mice.
7. $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/-}$ mothers developed fewer intestinal adenomas than $Mthfr^{+/+}Apc^{min/+}$ offspring of $Mthfr^{+/+}$ mothers. This was true for mice fed control, folate-deficient and folate-enriched diets.
8. $Apc^{min/+}$ mice exposed to a folate-deficient diet for their entire lives developed fewer adenomas than $Apc^{min/+}$ mice exposed to a control diet. Their intestinal DNA was hypomethylated relative to that of control mice.
9. In mice exposed to control and folate-deficient diets for their entire lives, adenoma number was inversely correlated with plasma homocysteine levels, intestinal dUTP/dTTP and apoptosis (borderline significant).
10. $Mthfr^{+/+}Apc^{min/+}$ and $Mthfr^{+/-}Apc^{min/+}$ mice fed folate-enriched diets from 3-to-10 weeks of age developed more intestinal adenomas than mice fed control or folate-deficient diets.

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APPENDIX I

Genotyping Protocols

	Primer Sequences (5'→ 3')	PCR and Digest Conditions
Mthfr	GAA GCA GAG GGA AGG AGG CTT CAG AGC CTG AAG AAC GAG ATC AGC AGC GAC TAG CTG GCT ATC CTC TCA TCC	94°, 3 min; 6x (94°, 1 min; 69°, 1.5 min); 30x (94°, 1 min; 68°, 2 min); 72°, 5 min; 4° hold
Rfc	GAT TCC AAG CAT GTC CAC TAC CC CAG AAG GCA AGT GTC TGT ATG TG AAG GCA ATG CAG GCA GAT ACG TGG GCT CTG TCC TTA TAG GGG TTG TGA	95° 5 min, 30x (95° 0.5 min, 55° 0.5 min, 72° 0.5 min); 72° 10 min, 4° hold
Mtr	TCC ATT GCT CAG CGG TGC TGT C CAC ACT GTT GTT ATA TGA CTC TTG C AAG ATG TGG TTG GCT GTT AGT GAC	95°, 7 min; 30x (64°, 1 min; 72°, 2 min; 95°, 1 min); 64°, 1 min; 72° 10 min; 4° hold
Apc$^{min/+}$	TTC CAC TTT CCG ATA AGG C TTC TGA GAA AGA CAG AAG CT	35x (94°, 1min; 53°, 1 min; 72°, 2 min); 4° hold digest: 18.5 uL PCR product + 1.5 uL MaeI; 45°, 2 hours
Pla2g2a	GGA AAC CAC TGG GAC ACT GAG GTA GTG CTG GCT TTC CTT CCT GTC AGC CTG GCC	94°, 2 min; 35x (94°, 0.5 min; 62°, 0.5 min; 72°, 0.5 min); 4° hold digest: 20 uL PCR product + 1 uL BamHI; 37°, 1 hour

APPENDIX II

Compliance Forms

Building Insights.
Breaking Boundaries

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