Effects of short-term supplementation of folic acid and L-5-methyltetrahydofolate on cell proliferation and the expression of folate transporters in human colorectal adenocarcinoma (Caco2) cells

by

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Abstract

Folate plays a role in the synthesis and repair of DNA and the generation of methyl groups. Folic acid (FA) is a synthetic oxidized form of folate used in food fortification and supplements in Canada. Increased colon cancer incidence has been correlated with FA fortification in several countries. The effect of FA on the development of colon cancer is controversial as other research shows a lack of association between FA fortification and colon cancer incidence. I hypothesize that FA affects proliferation and folate transporter expression in colon cancer cells differently than L-5-methyltetrahydrofolate (5MTHF). In addition, the forms of folate, reduced versus oxidized, would differentially affect the activity of the Wnt signaling pathway. The overall objective of my research is to investigate the effect of FA and 5MTHF on cell proliferation, the expression of selected folate transporters, and the activity of the Wnt signalling pathway in human colorectal adenocarcinoma (Caco2) cells. Caco2 cells were cultured for 3 or 5 days in folate-free RPMI 1640 medium supplemented with 10% dialyzed FBS and treated with 0, 0.9, 2.3, or 3.4 μM FA or MTHF. Cell viability was assessed using WST-1 colourimetric assay. Cell proliferation was assessed by BrdU colourimetric assay and cell cycle analysis with BrdU incorporation was measured by flow cytometry. The abundance of reduced folate transporter (RFC), folate receptor-α (FRα), proton-coupled folate transporter (PCFT), breast cancer resistance protein (BCRP) was assessed by Western blotting. β-Catenin nuclear localization was assessed by measuring the fluorescence of Alexa Fluor 488® using confocal microscopy. FA treatment increased cell proliferation compared to treatment with MTHF at all concentrations after 3 days. After 5 days, there was no difference in cell viability or cell

proliferation. Cell cycle analysis after 5 days of 3.4 μM FA and 5MTHF treatment showed spikes in the pre-G1 phase compared to the control. Neither folate transporter expression nor β-Catenin nuclear localization was affected by FA and 5MTHF treatment under the conditions tested. This lack of effect of FA and 5MTHF on cell proliferation and the expression of selected folate transporters was possibly due to relatively short treatment duration.

Preface

This graduate thesis was prepared in accordance to the University of British Columbia Faculty or Graduate Studies requirements. I was responsible for performing all experiments. The research design, interpretation of the results, and preparation of this thesis were accompanied with the assistance and guidance of Dr. Zhaoming Xu.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

5MTHF	5-methyltetrahydrofolate
ABC	ATP binding cassette protein
AICAR	Phosphoribosylaminoimidazolecarboxamide transformylase
ANOVA	Analysis of variance
AOM	Azoxymethane
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CamKII	Calcium/calmodulin-dependent kinase II
CI	Confidence interval
CK1	Casein kinase 1
CNS	Central nervous system
CRD	Cysteine rich domain
Daam1	Dishevelled associated activator of morphogenesis 1
DAPI	4',6-diamidino-2-phenylindole
dFBS	Dialyzed fetal bovine serum
DFE	Dietary folate equivalents
DRI	Dietary reference intake
DSH	Dishevelled protein
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
DHF	Dihydrofolate
DKK	Dickkopf protein
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid
EAR	Estimated average requirement
ER	Endoplasmic reticulum
EDTA	Ethylenediaminetetraacetic acid
FA	Folic acid
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FR	Folate receptor
FZD	Frizzled protein
GAR	Phosphoribosylglycinamide transformylase
GPI	Glycosyl phosphatidylinositol
GSK3	Glycogen synthase kinase 3
HCl	Hydrochloric acid
HCP1	Heme carrier protein 1
HFM	Hereditary folate malabsorption
HR	Hazard ratio
ISAM	Intestinal surface acidic microclimates
LRP5/6	Lipoprotein receptor-related protein 5/6

mRNA	Messenger RNA
MRP	Multidrug resistance protein
MTHFR	Methylenetetrahydrofolate reductase
NTD	Neural Tube Defect
PBS	Phosphate buffered saline
PBT	PBS containing Tween-20
PBTB	PBS containing Tween-20 and BSA
PBTG	PBTB containing normal goat serum
PCFT	Proton-coupled folate transporter
PCP	Planar cell polarity
PDE	Phosphodiesterase
PI	Propidium iodide
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl-fluoride
Porc	Porcupine protein
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
RDA	Recommended daily allowance
RFC	Reduced folate carrier
ROCK	Rho associated kinase
RNA	Ribonucleic acid
RNase	Ribonucleases
RR	Risk Ratio
SAH	*S*-Adenosylhomocysteine
SAM	*S*-Adenosylmethionine
SDS	Sodium dodecyl sulphate
SFRP	Soluble frizzled-related proteins
SHMT	Serine hydroxymethyltransferase
siRNA	Small interfering RNA
TBS	Tris-buffered Saline
TCF/LEF	T cell factor/Lymphoid enhancer-binding factor
TTBS	Tris-tween buffered saline
THF	Tetrahydrofolate
VEGF	Vascular endothelial growth factor
Wg	Wingless protein
WIF	Wnt inhibitory protein
Wls	Wntless/Wnt sorting receptors
Wnt	Wingless type protein
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

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Introduction

Folate is a generic term for the water-soluble B-vitamin that plays an important role in DNA synthesis and repair, methylation, and amino acid metabolism. Folate deficiency in humans is characterized by impaired DNA synthesis, which disrupts cell division, and presents clinically as megaloblastic anemia (Vilter *et al.*, 1963; Herbert, 1964). Folate supplementation has been associated with a decreased incidence of neural tube defects (NTDs; MRC Vitamin Study Research Group, 1991). Higher folate intakes have been suggested as protective against certain types of cancer, especially colorectal (Blount *et al.*, 1997; Giovannucci *et al.*, 1998; Sanjoaquin *et al.*, 2005; Kim *et al.*, 2010; Lee *et al.* 2011). The relationship between folate intake and colorectal cancer is controversial as there are studies correlating the start of fortification of folic acid (FA), the oxidized form of folate, with an increase in colorectal cancer rates and studies pointing to over-supplementation of FA contributing to the development of cancer (Mason *et al.*, 2007; Ebbing *et al.*, 2009; Figueiredo *et al.*, 2009; Hirsch *et al.*, 2009; Nyström and Mutanen, 2009). The reasons for the apparent contradictory effects of FA intake and cancer are as yet unknown, although it is thought that the timing of folate exposure or supplementation is important to determine whether folate will be associated with an increased or decreased risk of cancer (Ulrich and Potter, 2007).

Since 1998, flour and grain products such as cereal, pasta and cornmeal have been fortified with FA to prevent folate-related NTDs in Canada and the United States. At the time it was also theorized that FA fortification could have beneficial effects on cardiac events (by lowering homocysteine) and cancer incidence. In addition to FA fortification, Health Canada also recommended that all women of childbearing age to take a daily

supplement of 400 μg FA. FA is used in food fortification and vitamin supplements due to its chemical stability against oxidation and therefore is used in food products requiring cooking or long term storage. However, FA is a synthetic form of the vitamin and is not biologically active. Presently, it is not known whether this synthetic form of folate has different effects on cancer cell growth compared to the naturally occurring form of folate.

The Wnt signalling pathway is constitutively active in most colon cancers. It is so prevalent that the next generation of colon cancer treatments will be aimed at preventing or controlling Wnt signalling to stop cancer progression. Loss of regulation of Wnt signalling is likely the starting point for many colon cancers. Since this pathway is also aberrant in NTDs (some of which are folate-sensitive), a connection between Wnt signalling and folate needs to be investigated.

CHAPTER 1
Literature Review, Hypothesis, and Objectives

1.1 Folate

1.1.1 Dietary Sources

Folate is a water-soluble B vitamin. Folate is a term that refers to reduced forms of the vitamin that occur naturally in foods and tissues in humans (Figure 1.1). FA is a synthetic, oxidized derivate of the vitamin that is used in supplements and in fortification of flour, pasta and cereals (Figure 1.1).

Dietary sources of folate (Table 1.1) are leafy green vegetables (e.g. spinach, broccoli, okra and asparagus), legumes (e.g. black, kidney, navy and pinto beans, chick peas and lentils), fruit (e.g. oranges) and organ meat (Cuskelly *et al.,* 1996; West Suitor and Bailey, 2000). Folate is present mostly as dihydrofolate (DHF) in foods of plant origin and as 5-methyltetrahydrofolate (5MTHF) in foods of animal origin. Naturally occurring folates have a polyglutamate tail.

In Canada, flour, pasta, and cereals are fortified with FA due to its chemical stability. FA has also been used in supplements. Because of its monoglutamate tail, FA is more readily absorbed than naturally occurring folates and therefore is considered to have a higher bioavailability.

1.1.2 Recommended Daily Allowance (RDA) and Folate Intake in Canada

Dietary folate equivalents (DFE) are used to account for the differences of bioavailability between food folates and FA (West Suitor and Bailey, 2000). A DFE is defined as 1 μg of naturally occurring food folate, which is equal to 0.6 μg of FA from a

supplement or fortified food consumed with a meal, or 0.5 μg of FA from a supplement consumed on an empty stomach (DRI, 1998).

The bioavailability of natural food folates appears to be 50% of that of FA, based on a study in which 10 non-pregnant, healthy women were monitored in a metabolic unit for 92 days and the ability of naturally occurring food folates or FA to raise or maintain plasma folate levels after a 28 day folate depletion period was examined (Sauberlich *et al.*, 1987). Another study involving a 4-week dietary intervention, determined that the bioavailability of food folates (from fruit, vegetables, and liver) is 80% of that of FA after comparing 4 diets supplemented with varying levels of [$^{13}C_{11}$]-labelled FA (Winkels *et al.*, 2007). Actually, true bioavailability of folates cannot be determined because of modifications such as formylation or methylation that occur in the intestinal mucosa after absorption, so the bioavailability of the folate relative to the bioavailability of the fully oxidized monoglutamate form of the vitamin (FA) is determined (Brouwer *et al.*, 2001). Evidently, FA is more biologically available than folate.

The RDA of folate for Canadians aged 14 and above is 400 μg DFE/day, for pregnant women it is 600 μg DFE/day, and for lactating women it is 500 μg DFE/day (Health Canada DRI reference tables, 2010). In addition, Health Canada also recommends that women of childbearing age take a supplement containing 400 μg FA daily (Health Canada, 2010).

The Canadian Community Health Survey cycle 2.2 (CCHS 2.2) reported that in some groups of Canadians, over 10% of people had an inadequate intake of folate. Over 10% of men (≥ 51 y) and women (≥ 14 y) had inadequate folate intake (CCHS 2.2, 2004). The worst inadequacies are found in women over the age of 14 and men aged 71 and

over, whose inadequate folate intakes reached an incidence of more than 20% (CCHS 2.2, 2004).

It is estimated that the number of British Columbians with folate intakes less than the estimated average requirement (EAR) is lower than the national average (15.5% versus 15.7%; CCHS 2.2, 2004). Even with mandatory FA fortification of flours, cereals, pasta and cornmeal, there are still groups of Canadians that are not meeting the EAR for folate intake. This is important in the case of women of childbearing age (approximately 20% of women aged 14 – 50 years do not meet the requirements of the EAR for folate; CCHS 2.2, 2004), the original target of FA fortification. A survey of women of child-bearing age (aged 18 – 45 years) from Vancouver, Canada showed that 86% meet the EAR for folate, but only 26% of women met the recommended levels of folate for women who are capable of getting pregnant (French *et al.*, 2003). Another study focusing on Canadian women (aged 18 – 25 years), showed that only 17% of women met the recommended levels of folate for women capable of getting pregnant (Shuaibi *et al.*, 2008). Analysis of the Canadian Health Measures Survey shows that less than 1% of Canadians have folate deficiency according to their red blood cell folate and more than 40% showed high red blood cell folate (>1360 nmol/L; Colapinto *et al.*, 2011). Looking at women of childbearing age, 22% do not meet the red blood cell folate recommendations for maximal reduction of risk of NTDs (<906 nmol/L; Colapinto *et al.*, 2011). Women of childbearing age were the original targets of the folate fortification program.

1.1.3 Digestion and Absorption

Folate is absorbed in monoglutamate form (Reisenauer *et al.*, 1977). Naturally occurring folates have a polyglutamate tail that is cleaved by glutamate carboxypeptidase II before absorption (Reisenauer *et al.*, 1977). FA does not require glutamate carboxypeptidase II-mediated cleavage before absorption because it is in the monoglutamate form. The lack of a polyglutamate tail makes FA more bioavailable than naturally occurring folates.

Proton coupled folate transporter (PCFT). Folates are currently thought to be absorbed mainly in the duodenum and proximal jejunum *via* PCFT, a membrane-bound transport protein. PCFT was initially identified as heme carrier protein 1 (HCP-1; *SLC46A1*; Qiu *et al.*, 2006). PCFT is expressed at the highest levels in the duodenum and jejunum, and lower levels in the ileum and colon. It exhibits greatest folate transport activity at pH 5.5 (Qiu *et al.*, 2006).

PCFT exerts its function in the intestine in humans and rats because of the presence of intestinal surface acidic microclimates (ISAM; Lucas and Blair, 1978; Said *et al.*, 1987). The pH of ISAM has been found to increase from the duodenum to the ileum with further increases in the colon (Lucas and Blair, 1978; Said *et al.*, 1987). In rat small intestine the ISAM pH ranges from 5.5 (in the duodenum) to approximately 6.5 (in the colon; Lucas and Blair, 1978). This acidic microclimate is maintained by Na^+/H^+ exchangers on the brush border that pump H^+ ions into the lumen creating the acidic microclimate (Thwaites and Anderson, 2007). PCFT works as a folate/H^+ symporter, using the gradient created by the Na^+/H^+ exchanger to bring folate into the cells (Zhao *et al.*, 2009).

There are some individuals born with a non-functional variant of PCFT and this genetic disorder is known as hereditary folate malabsorption (HFM; Qiu *et al.*, 2006). Individuals with HFM are unable to absorb a sufficient quantity of folate to meet their daily needs for folate. These individuals depend on large oral or parenteral doses of FA to meet their folate requirement (Zhao *et al.*, 2009).

When *Pcft* is knocked out in mice (*Pcft* $^{-/-}$), the mutation is not embryonic lethal and the mice develop normally until about 4 weeks of age when severe macrocytic normochromic anemia and pancytopenia develops (Običan *et al.*, 2010). *Pcft*$^{-/-}$ mice cannot be rescued by oral administration of FA, but an intraperitoneal injection of folate rescues them from the development of megaloblastic macrocytic anemia (Običan *et al.*, 2010). *Pcft*$^{-/-}$ mice as well as individuals suffering from HFM provide strong evidence for PCFT as the main route of intestinal folate uptake because both the mice and the humans suffer symptoms of folate deficiency if they are left untreated.

Reduced folate carrier (RFC). Before the identification of PCFT, RFC (*SLC19A1*) was thought to be the main folate transporter in the small intestine and this is still a point of scientific disagreement (Balamurugan and Said, 2006). RFC is a member of the SLC family and an anion exchanger. It transports folate into the cell driven by the energy gradient derived from the downstream transport of organic anions out of the cell (Goldman, 1971). RFC is ubiquitously expressed in human tissues, including the intestine, with higher levels of expression in tissues such as the placenta, liver, leukocytes and central nervous system (CNS) and lower levels of expression in the skeletal muscle and heart (Whetstine *et al.*, 2002). The main function of RFC is cellular uptake of folate, as demonstrated by its ubiquitous expression in human tissue (Whetstine *et al.*, 2002).

RFC knockout mice (*Rfc*$^{-/-}$) are not viable because of embryonic lethality; the embryos die shortly after implantation (Gelineau-van Waes *et al.*, 2008). However, if the mother receives a daily low-dose of subcutaneous FA injection (25 mg/kg/day), the *Rfc*$^{-/-}$ embryos may survive past embryonic day 6.5, but they are developmentally delayed along with a number of disorders such as NTDs and failure of hematopoiesis (Gelineau-van Waes *et al.*, 2008*)*. In mothers injected with a daily high-dose subcutaneous FA injection (50 mg/kg/day), 22% of *Rfc*$^{-/-}$ fetuses survive until embryonic day 18.5. These fetuses are morphologically normal, but have cardiac and lung abnormalities (such as ventricular septal defects, and small, pale lungs with less branching morphogenesis), pale colour and various other defects (Gelineau-van Waes *et al.*, 2008*)*. Because *Rfc*$^{-/-}$ mice are embryonic lethal it can be inferred that RFC is very important for fetal folate uptake and transport.

Folate receptors (FR). FR are glycosyl phosphatidylinositol (GPI)-linked proteins that have 3 isoforms, FRα, FRβ and FRγ (*FOLR1, FOLR2,* and *FOLR3,* respectively; Antony, 1996). FRα is a membrane-linked form of folate receptor and is differentially expressed in different tissues (Ross *et al.*, 1994). FRα is highly expressed in kidney, lung, ovary and placental tissue, and its expression is greatly elevated in carcinomas of many different tissue types (Ross *et al.*, 1994).

Conversely, FRβ, the most common form of FR, is expressed at lower levels in many normal tissues and carcinomas, but its expression is increased in nonepithelial tumours such as meningiomas and sarcomas (Ross *et al.*, 1994).

FRγ has an alternate mRNA splice site that produces a protein product called FRγ' (Shen *et al.*, 1994). FRγ has an amino terminal signal peptide for insertion into the

membrane but this process may be rendered less efficient due to a carboxyl terminal sequence of hydrophobic amino acids interrupted by two charged amino acids (Shen *et al.*, 1994). FRγ is expressed in tissues containing hematopoietic cells such as the spleen, bone marrow and thymus, and is also found in ovarian, cervical and uterine carcinomas (Shen *et al.*, 1994). A deletion of 2 base pairs from the intact FRγ mRNA results in a truncated version of FRγ, denoted as FRγ', that lacks an amino terminal signal peptide (Shen *et al.*, 1994) and therefore is not a membrane-anchored protein.

Functionally, FRα facilitates the uptake of folates by receptor-mediated endocytosis (Kamen *et al.*, 1988). Folates bind to the FR forming a FR-folate complex, which is then endocytosed into an acid-resistant endosome (Kamen *et al.*, 1988). Within the endosome, the acidic pH causes the release of folate from the receptor. Subsequently, folate is thought to be exported into the cytoplasm by PCFT (Anderson *et al.*, 1992; Zhao *et al.*, 2009). The FR is then recycled back to the surface membrane (Kamen *et al.*, 2004; Paulos *et al.*, 2004).

The importance of the FRα versus FRβ is illustrated by their corresponding knockout mice. Folbp1 is the murine equivalent of FRα and Folbp2 is the murine equivalent of FRβ. Fetuses of *Folbp1* knockout mice (*Folbp1*$^{-/-}$) develop NTDs whereas fetuses of *Folbp2* knockout mice (*Folbp2*$^{-/-}$) develop normally with their neural tubes properly closed (Piedrahita *et al.*, 1999).

PCFT, RFC and FRα have all been shown to be differentially expressed in human cell lines in the presence of different folate conditions (Ashokkumar *et al.*, 2007; Crott *et al.*, 2008). The relative expression of *PCFT* mRNA is increased to 125% of the control in Caco2 cells treated with 0.25 μM FA, but is decreased to 50% of the control in cells

treated with 100 µM FA (Ashokkumar *et al.*, 2007). Long-term exposure (maintained for 5 passages) of Caco2 cells to 0.25 µM FA increases the relative expression of *RFC* mRNA to 110% and relative RFC protein levels to 135% compared to the control (Ashokkumar *et al.*, 2007). Treatment of Caco2 cells with 100 µM FA decreases *RFC* mRNA expression to 80% and RFC protein levels by 50% compared to the control (Ashokkumar *et al.*, 2007). In HK-2 cells, FA treatment at 0.25 µM increases *FOLR* mRNA levels to125% and FR protein levels to 135% compared to the control (Ashokkumar *et al.*, 2007). Similarly, *FOLR* mRNA and protein levels are reduced to ~60% in HK-2 cells treated with 100 µM FA compared to the control (Ashokkumar *et al.*, 2007). Clearly, the expression of folate transporters is downregulated at high extracellular concentrations of FA and upregulated at lower concentrations of FA.

PCFT expression appears to be regulated by promoter methylation in human leukemia cell lines (Gonen *et al.*, 2008) as well as by extracellular folate concentrations. A CpG island located in the promoter region of *SLC46A1* (PCFT) is 85-100% methylated and correspondingly low levels of *PCFT* mRNA transcripts and proteins are the result (Gonen *et al.*, 2008). When treated with a demethylating agent (5-Aza-2'-deoxycytidine), expression of PCFT is restored to 50 times of that observed in untreated cells (Gonen *et al.*, 2008).

Once folate is absorbed, it is transported across the basolateral membrane entering the portal vein through a transporter-mediated process. The transporters that are thought to play this role include multidrug-resistance-associated protein (MRP) 1-5 (Kruh and Belinsky, 2003) and breast cancer resistance protein (BCRP; Lemos *et al.*, 2008; Maubon *et al.*, 2007). In particular, BCRP is able to export mono-, di- and tri-glutamate forms of

folates (Chen *et al.*, 2003). BCRP is expressed in Caco2 cells and also in pooled samples of human small intestine (Maubon *et al.*, 2007). BCRP expression is affected by cellular folate status in Caco2 cells. For example, in the presence of low extracellular folate conditions (1 nM folate, after 69 days of treatment), BCRP expression is induced 3.9 - 5.7 fold compared to the control (Lemos *et al.*, 2008). BCRP and MRP1-5 are members of the ATP-Binding Cassette (ABC) transporter family. This family of transporters transports folates and other molecules using the chemical energy derived from the hydrolysis of adenosine triphosphate (Stewart *et al.*, 1996; Zhao *et al.*, 2009).

1.1.4 Physiological Functions

Folate exerts its physiological role by participating in the transfer of a single carbon unit in cellular reactions (one-carbon metabolism). Thus it plays an important role in DNA synthesis and repair, methylation, amino acid metabolism, and protein metabolism.

Folate participates in DNA synthesis by donating a methyl group from 5,10-methylenetetrahydrofolate to deoxyuridine monophosphate (dUMP) to produce deoxythymidine monophosphate (dTMP; Figure 1.2). Eventually dTMP is incorporated into DNA. During folate deficiency, the ratio of dTMP to dUMP could decrease, resulting in a higher proportion of uracil to thymine in the cell (Blount *et al.*, 1997). This in turn can lead to uracil misincorporation into DNA and possibly DNA damage, such as double strand breaks (Blount *et al.*, 1997).

A second important role for folate is in DNA methylation: 5MTHF donates a methyl group to cobalamin (vitamin B_{12}, a cofactor for methionine synthase) to form methyl-cobalamin, which subsequently donates the methyl group to homocysteine producing

methionine (Figure 1.2). Methionine is then converted to *S*-adenosylmethionine (SAM), which can donate the methyl group directly to DNA. DNA methylation occurs predominantly on a cytosine residue where it is followed by a guanine residue (CpG; Cedar, 1988). DNA methylation in the promoter regions of genes plays a role in regulating gene expression (Rountree *et al.*, 2001; Siegfried and Simon, 2010).

As well, folate participates in purine synthesis *via* transferring a formyl group from 10-formylfolate to phosphoribosyl glycinamide to produce inosine monophosphate, which is then used in purine synthesis (Figure 1.2). Purines (nucleotides such as deoxyguanosine monophosphate and deoxyadenosine monophosphate) are important for the synthesis of DNA and RNA.

In addition, folate is also involved in the metabolism of some amino acids, such as the conversion of serine to glycine (Figure 1.2) and the synthesis of methionine from homocysteine as described above.

1.1.5 Interactions with Vitamin B_{12}

A potentially negative nutrient-nutrient interaction between folate and vitamin B_{12} is that a deficiency of vitamin B_{12} can induce a functional folate deficiency (Vilter *et al.*, 1963). The biochemical basis of this interaction is that 5,10-methylene THF (Tetrahydrofolate) is converted to 5MTHF, a one-way reaction catalyzed by 5,10-methyleneTHF reductase (MTHFR; Figure 1.2). 5MTHF is then converted to THF, a reaction catalyzed by methionine synthase with vitamin B_{12} acting as a co-enzyme (Figure 1.2). In the absence of vitamin B_{12}, folate is trapped as 5MTHF with a diminished cellular pool of THF, a phenomenon known as the 'methyl trapping hypothesis' (Sauer

and Wilmanns, 1977). Consequently, less THF is available to support normal DNA synthesis and impaired methylation can occur due to a diminished synthesis of SAM, a major cellular methyl donor. Clearly, if megaloblastic macrocytic anemia is developed due to a deficiency in folate, vitamin B_{12}, or a combination of both, treatment with just folate can alleviate the anemia by normalizing DNA synthesis; however, if the anemia is caused by vitamin B_{12} deficiency, methylation remains impaired leading to the development of irreversible neurological consequences. Data from the NHANES (1999-2002) survey for seniors (≥60 years) revealed that seniors with low serum vitamin B_{12} (Serum vitamin B_{12} <148 pmol/L or serum methylmalonic acid >210 nmol/L) and high serum folate (>59 nmol/L; the 80th percentile) were more likely to have anemia and cognitive impairment (Morris *et al.*, 2007; Selhub *et al.*, 2009). Thus care must be taken not to mask a potential vitamin B_{12} deficiency with high doses of folate when treating megaloblastic macrocytic anemia that could lead to cognitive decline.

1.1.6 Deficiency

Folate deficiency increases the risk of hyperhomocysteinemia and megaloblastic macrocytic anemia (in severe cases). Folate deficiency has also been linked to the development and progression of certain types of cancer.

The incidence of NTDs such as spina bifida and anencephaly can be lowered by ensuring women of a child bearing age take FA supplements pre- and peri-conceptionally (MRC Vitamin Study, 1991; Berry *et al.*, 1999). Studies have also found that the risk of other birth defects (such as cleft palate or cleft lip) appear to decrease when mothers increase their folate intake (Czeizel *et al.*, 1999; van Rooij *et al.*, 2004). Prevention of

folate-related NTDs was the main reason for the decision to fortify grains and flours with FA in Canada. Health Canada's stance is that it is important that women of childbearing age have an adequate folate status to prevent NTDs, especially in the case of unplanned pregnancies where prenatal vitamins may not be used. Therefore women of childbearing age are directed by Health Canada to take a supplement containing 400 μg FA daily, over and above the added FA in food.

Hyperhomocysteinemia is a condition that has been linked to an increased risk for ischemic heart disease and stroke (The Homocysteine Studies Collaboration, 2002). At the metabolic level, homocysteine plays a role in the development of endothelial dysfunction and atherothrombosis by inducing proinflammatory factors, oxidative stress and endoplasmic reticulum stress (Austin *et al.*, 2004). Heatlhy people aged 18-59 years with a normal fasting plasma homocysteine level (9.5 ± 0.9 μmol/L) were given oral L-methionine (10, 25, 100 mg/kg) or lean chicken (551 ± 30 g, containing 3.2 ± 0.2 g methionine) and their plasma homocysteine response and brachial artery flow dilatation was measured (Chambers *et al.*, 1999). After 4 hours, dose-dependent increases of plasma homocysteine and decreases in brachial artery flow-mediated dilatation were observed, indicating endothelial dysfunction (Chambers *et al.*, 1999).

In one-carbon metabolism, homocysteine accepts a methyl group from 5MTHF *via* vitamin B_{12} to produce methionine, which is subsequently converted to SAM (Figure 1.2). Thus folate deficiency can result in hyperhomocysteinemia. Folate deficiency-induced hyperhomocysteinemia can be reversed by administration of FA (Ubbink *et al.*, 1994). It has also been shown that folate supplementation can lower plasma homocysteine. The Heart Outcomes Prevention Evaluation (HOPE) 2 is a randomized,

double-blind, placebo-controlled trial investigating the effect of a B vitamin mix (2.5 mg FA, 50 µg vitamin B_6, and 1 mg vitamin B_{12}) on the reduction of cardiovascular events in patients (≥ 55 years) with prior vascular disease or diabetes (HOPE-2 investigators, 2006). After an average of 5 years of the study, mean plasma homocysteine decreased in the treatment group and increased in the placebo group, while treatment did not reduce the primary outcomes (death from cardiovascular causes, myocardial infarction, and stroke) in the treatment group compared to the placebo group (HOPE-2 investigators, 2006). The Homocysteine Lowering Trialists' Collaboration performed meta-analyses of randomized controlled trials assessing the effects of FA supplements on blood homocysteine concentrations (1998) and to determine the lowest amount of FA associated with the highest reduction in plasma homocysteine concentrations (2005). The first meta-analysis determined that folic acid supplementation at any doseage (0.5 – 5 mg/d) resulted in an approximate 25% reduction in plasma homocysteine (Homocysteine Lowering Trialists' Collaboration, 1998). The second meta-analysis determined that supplementation with ≥ 0.8 mg FA daily produced the maximum reduction in plasma homocysteine (23%, 95% CI: 21 – 26%; Homocysteine Lowering Trialists' Collaboration, 2005). As well as lowering plasma homocysteine, folate supplementation has been shown to improve endothelial dysfunction. 29 healthy subjects (aged 40 – 70 years) with hyperhomocysteinemia (mean: 9.0 ± 1.7 µmol/L, at baseline) received FA (10 mg/d) for 1 year (Woo *et al.*, 2002). FA supplementation resulted in higher plasma folate levels (40 ±5 vs. 24 ± 5 nmol/L, $p<0.001$), lower total plasma homocysteine levels (9.0 ± 1.7 vs 7.9 ± 2.0 µmol/L, $p<0.001$) and improved flow-mediated endothelium-dependent

dilatation of the brachial artery (8.9 ± 1.5% vs 7.4 ± 2.0%, mean difference=1.5%; 95% CI: 1-2%; p<0.0001), compared to the baseline (Woo *et al.*, 2002).

Folate deficiency can also lead to the development of megaloblastic macrocytic anemia (Herbert, 1964) due to altered DNA synthesis and impaired repair mechanisms (Das and Hoffbrand, 1970). Decreased synthesis of thymidine during folate deficiency can lead to the production of red blood cells that are not fully matured (Koury *et al.*, 1997). As a result of impaired thymidylate synthesis and DNA synthesis, the blood cells continue to grow without dividing, resulting in large, immature red blood cells (Koury *et al.*, 1997; Vilter *et al.*, 1963). Megaloblastic macrocytic anemia can be caused by a deficiency in folate alone or a combined deficiency of vitamin B_{12} and folate (Vilter *et al.*, 1963), as discussed in the previous section.

1.1.7 Neural Tube Defects and Folic Acid Fortification

NTDs affect 0.5-2 births/1,000 live births (Greene *et al.*, 2009). They are caused by a failure of the neural tube to close (primary neurulation) during embryogenesis (Blom, 2009). In humans, primary neurulation occurs 3-4 weeks post conception (Blom, 2009). The process of neurulation occurs before most women even become aware of their pregnancy. A study conducted by the MRC Vitamin Study Research Group found that administration of 4 mg of FA daily to women before and up to 12 weeks of pregnancy results in a 72% reduction in the incidence of NTDs compared to the women given a placebo (MRC Vitamin Study Research Group, 1991). Another study by Berry *et al.* (1999) reported a 79% decrease in the rate of NTD-affected pregnancies in women from

a northern region in China who took a 400 μg FA supplement daily starting after last menstrual period compared to women who did not take a daily FA supplement.

Mandatory FA fortification was introduced in Canada in 1998 at the level of 150 μg FA / 100 g flour or 200 μg FA / 100 g pasta (Public Health Agency of Canada, 2004). The fortification levels are designed so that the average person receives approximately 100 μg FA daily from the consumption of fortified foods (Public Health Agency of Canada, 2004). This mandatory FA fortification is implemented to ensure that the majority of women of childbearing age will have adequate folate status due to strong evidence that pre- and peri-conceptional FA supplementation reduces the incidence of NTDs (MRC, 1991; Berry *et al.*, 1999). Unfortunately, the mechanism by which FA reduces the incidence of NTDs remains to be elucidated.

In addition, FA fortification has also been implicated in lowering colon cancer incidence (Giovannucci *et al.*, 1998; Su and Arab, 2001; La Vecchia *et al.*, 2002) and the potentially lowering the risk of cardiovascular disease by decreasing plasma homocytsteine levels (Homocysteine Lowering Trialists' Collaboration, 1998 and 2005; Brouwer *et al.*, 1999).

There are some concerns associated with FA fortification. Because of the structure of FA and its metabolism, FA enters the folate cycle as THF (Figure 1.1 & 1.2). As discussed earlier, THF is metabolized to 5,10-methyleneTHF, which is required for the synthesis of dTMP and subsequently DNA synthesis. Thus, FA fortification provides a means to bypass an integral step in the vitamin B_{12}-dependent folate recycling. Consequently, FA prevents vitamin B_{12} deficiency-induced megaloblastic anemia while disguising vitamin B_{12} deficiency-induced neurological lesions (Vilter *et al.*, 1963).

The rate of NTD-affected pregnancies in Canada had dropped from 1.58 / 1,000 births before FA fortification to 0.86/1,000 births after the implementation of FA fortification in 1998 (De Wals *et al.*, 2007). Since FA fortification is designed for women of child-bearing age, the health impacts of long-term high FA intakes on other subpopulations in our society is largely unknown.

1.2 Wnt Signalling Pathway

1.2.1 Overview

Wingless-type proteins (Wnts) are a family of secreted glycoproteins that are involved in the Wnt signalling pathway (Moon *et al.*, 2004). A number of recombinant and native Wnt proteins are glycosylated before secretion in several murine cell lines (Smolich *et al.*, 1993). This pathway is responsible for many developmental processes (e.g. closure of the neural tube during neurulation and convergent extension during gastrulation in zebrafish (Ungar *et al.*, 1995) and *Xenopus* (Moon *et al.*, 1993), homeostasis of bone mass (Baron *et al.*, 2006), and neurogenesis in adults (Malaterre *et al.*, 2007). In addition, dysregulation of Wnt signalling has been implicated in the development of a wide range of diseases, for example, hereditary and sporadic colon cancer (Bienz and Clevers, 2000), birth defects (Zeng *et al.*, 1997; Hamblet *et al.*, 2002; Carter *et al.*, 2005) and nervous system disease (Freese *et al.*, 2010).

Wnt was first identified as *int1*, an activated proto-oncogene involved in the aberrant formation of tumors in mice infected with the mouse mammary tumour virus (Nusse and Varmus, 1982). This was also the beginning of research into the involvement of Wnt in cancer development (Polakis, 2011).

From its humble beginnings as wingless and *int1*, Wnt is now known to play the part of a secreted signalling molecule in the Wnt signalling pathway. 'Wnt signalling pathway' is a general term covering two main distinct pathways: the canonical Wnt signalling pathway or β-Catenin-dependent Wnt signalling pathway and the non-canonical Wnt signalling pathway or β-Catenin-independent Wnt signalling pathway. The β-Catenin-independent Wnt signalling pathways includes the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (Chien *et al.*, 2009).

1.2.2 β-Catenin-dependent Wnt Signalling Pathway

The canonical Wnt/β-Catenin-dependent signalling cascade is initiated when a secreted Wnt protein binds to the frizzled (FZD) receptor and low density lipoprotein receptor-related protein 5/6 (LRP5/6), the co-receptor (Figure 1.3). The binding of both FZD and LRP5/6 by Wnt is necessary for signal transduction (Tamai *et al.*, 2000).

FZD acts as a G-protein coupled receptor when it is activated upon Wnt binding (Katanaev, 2010). Katanaev (2010) has shown that the trimeric G protein G_o binds to FZD in the presence of Wnt. The βγ subunit of G_o activates dishevelled (DSH). DSH and the α_o subunit then converge to inhibit axin. The inhibition of axin prevents the phosphorylation and eventual destruction of β-Catenin (Katanaev, 2010), allowing β-Catenin to move into the nucleus. In the nucleus, β-Catenin complexes with transcription factors of the T cell factor (TCF) and lymphoid enhancer-binding factor (LEF) families, exerting its effect on the transcription of target genes. When this pathway is not stimulated by Wnt, antagonized, or blocked, axin is not inhibited by DSH. Axin, along with glycogen synthase kinase 3 (GSK3) and adenomatous polyposis coli (APC), then

bind to and phosphorylate β-Catenin. This phosphorylation marks β-Catenin for ubiquitination and subsequent degradation by the proteosome.

Wnt proteins are post-translationally modified in the endoplasmic reticulum (ER) before extracellular release (Komiya and Habas, 2008). Wnt proteins, specifically murine *Wnt3a*, have also been shown to be palmitoylated (Willert *et al.*, 2003) and palmitoleated (Takada *et al.*, 2006). Mutation of the palmitoylation site in Wnt proteins leads to loss of signalling activity (Willert *et al.*, 2003; Galli *et al.*, 2007; Komekado *et al.*, 2007; Kurayoshi *et al.*, 2007). In contrast, mutation of the palmitoleation site inhibits secretion and results in a buildup of the mutated Wnt protein in the ER (Takada *et al.*, 2006). Thus, these two lipid modifications in Wnt proteins are responsible for the hydrophobicity of Wnts and are required for both signalling and secretion (Harterink and Korswagen, 2012).

Post-translational modifications of Wnt proteins appear to be mediated by Porcupine (Porc), a member of the membrane-bound *O*-acyltransferase family (Harterink and Korswagen, 2012). In *Drosophila*, Porc is localized to the ER at the same location that Wnt modification is thought to take place (Zhai *et al.*, 2004). In *Drosphila*, Porc interacts with the N-terminal domain of wg (in a region that is also conserved in Wnt proteins) and is essential to the process of *N*-glycosylation because it anchors wg/Wnt to the ER membrane for subsequent glycosylation (Tanaka *et al.*, 2002). If Porc is depleted, Wnt secretion is inhibited and a buildup of Wnt proteins in the ER occurs (van den Heuvel *et al.*, 1993).

Wnt sorting receptors (Wntless; Wls) are also necessary for Wnt secretion (Harterink and Korswagen, 2012). Wls proteins are highly conserved 7-pass

transmembrane proteins (Banzinger *et al.*, 2006). When Wls binds to Wnt as illustrated by co-immunopreciptation experiments and in cells with non-functioning Wls, Wnt proteins build up in the cell (Banzinger *et al.*, 2006; Bartscherer *et al.*, 2006). Further analysis of non-functioning Wls proteins in *Drosophila* reveals that Wnt builds up in the Golgi (Harterink and Korswagen, 2012). Thus Wls probably functions in Wnt secretion from cells by acting as a shuttle, transporting Wnt proteins from the Golgi apparatus to the plasma membrane of cells (Harterink and Korswagen, 2012). In a model proposed by Harterink and Korswagen (2012), Wnt proteins are post-translationally modified, which requires Porc, and form a complex with Wls in the ER. The Wnt-Wls complex is then translocated to the Golgi apparatus and secreted from the Golgi in secretory vesicles. Wnt is translocated to the plasma membrane and then secreted into the extracellular space via exocytosis. Wnt proteins appear to be released from Wls by endosomal acidification en route to the plasma membrane, as inhibition of V-ATPase prevents β-Catenin-dependent and independent signalling from occurring (Coombs *et al.*, 2010). Inhibitors of acidification increase intracellular Wnt, Wls and Wnt-Wls complex levels and inhibit the release of Wnt from the Wnt-Wls complex (Coombs *et al.*, 2010).

β-Catenin-dependent Wnt signalling is constitutively inhibited by the β-Catenin destruction complex, made up of GSK3, casein kinase I (CKI), axin, and APC (Ikeda *et al.*, 1998; Archbold *et al.*, 2012). This complex binds to β-Catenin resulting in GSK3-dependent phosphorylation of β-Catenin, marking it for ubiquitination and subsequent destruction by the proteasome (Ikeda *et al.*, 1998; Archbold *et al.*, 2012). This pathway can also be inhibited by secreted proteins that interfere with or compete for receptor binding (Komiya and Habas, 2008). These secreted proteins include Dickkopf (DKK;

Glinka *et al.*, 1998), Wnt-inhibitor protein-1 (WIF-1; Hsieh *et al.*, 1999), soluble frizzled-related proteins (SFRPs; Hoang *et al.*, 1998) and FrzB (Frizzled motif associated with bone development; Lin *et al.*, 1997; Wang *et al.*, 1997). DKK proteins bind to LRP5/6, the co-receptor for FZD, preventing the association of the two proteins and subsequently β-Catenin-dependent Wnt signalling (Bafico *et al.*, 2001). WIF-1, SFRP and FrzB are examples of Wnt signalling inhibitors that bind to Wnt proteins and inhibit Wnt signalling regardless of dependence on β-Catenin (Kawano and Kypta, 2003). SFRPs (including FrzB, otherwise known as SFRP3) contain an N-terminal cysteine-rich domain (CRD) that shares 30-50% similarity to the CRD found in FZD proteins (Melkonyan *et al.*, 1997). WIF-1 has a WIF domain and 5 epidermal growth factor repeats (Hsieh *et al.*, 1999). This WIF domain is similar to an extracellular domain of RYK, a tyrosine kinase that was later discovered to have a role in Wnt signalling in *Drosophila* (Patthy, 2000).

1.2.3 β-Catenin-independent Wnt Signalling Pathways

The planar cell polarity pathway (PCP pathway) is a β-Catenin-independent Wnt signalling pathway first observed in *Drosphila* that appears to be conserved in mammalian tissue (Mlodzik, 2002; Veeman *et al.*, 2003). As the classification implies, PCP signalling is mediated through the binding of Wnt to a receptor and transduction through DSH, but β-Catenin is not involved. Signalling in the PCP pathway occurs when a Wnt (Wnt4, Wnt5a or Wnt11; Komiya and Habas, 2008) protein binds to FZD. This binding does not involve LRP5/6 as in the canonical pathway. As a result of the binding, DSH is activated and complexes with Daam1 (Dishevelled associated activator of morphogenesis), leading to the activation of Rho (small G protein) and ROCK (Rho

associated kinase). The PCP pathway is especially important in tissues that have a defined polarity, for example, stereocilia in the inner ear, organization of hair follicles (Wang *et al.*, 2007) and convergent extension during gastrulation (Keller *et al.*, 2003).

The Wnt/Ca^{2+} pathway is another β-Catenin-independent signalling pathway that shares components with the PCP pathway, but is distinct enough that it is categorized as the third Wnt signalling branch (Komiya and Habas, 2008). The role of Ca^{2+} as a second messenger in the Wnt pathway is realized when the frequency of calcium transients in the enveloping layer of the blastodisc of zebrafish embryos is doubled in response to injections of *Wnt5a* (Slusarski *et al.*, 1997) and *Wnt11* (Westfall *et al.*, 2003) mRNA. In this pathway, Wnt binding to FZD and possibly a co-receptor (Ror2 or Kynpek) results in the activation of calcium/calmodulin-dependent kinase II (CamKII; Kühl *et al.*, 2000) and protein kinase C (PKC; Sheldahl *et al.*, 1999). Binding of Wnt to FZD receptors can also activate phospholipase C (PLC) and phosphodiesterase (PDE) *via* G protein binding (Kohn and Moon, 2005).

The Wnt/Ca^{2+} pathway is important for body plan specification (Kühl *et al.*, 2000) and it seems to have a role in slow twitch muscle fibre formation in adults (Naya *et al.*, 2000). An increase in intracellular calcium release and PLC signalling results in the downstream activation of cdc42, a regulator of cell adhesion, migration and tissue separation (Choi and Han, 2002). Intracellular calcium increase activates CamKII, which activates TAK1 and NLK. Activation of TAK1 and NLK in turn causes phosphorylation of TCF and LEF, interfering with binding of these transcription factors to DNA (Ishitani *et al.*, 1999). In this way Wnt/Ca^{2+} signalling can inhibit the canonical Wnt signalling

pathway and Wnt5a, a stimulator of the non-canonical pathway, can act as a tumour suppressor (Ishitani *et al.*, 1999).

1.3 Linking Folic Acid and Wnt Signalling Pathways to Colorectal Cancer

1.3.1 Folic Acid and Colorectal Cancer

FA fortification has been implicated in both the development of and the protection against colon cancer. There are animal studies as well as epidemiological studies that show a protective role of folate against colorectal cancer. However, there have also been animal and epidemiological studies showing the opposite, that higher folate intake promotes cancer development. The mechanism behind folate's potential role in cancer protection or promotion is not yet elucidated, however the timing of folate exposure appears to play an important role in whether folate will be protect against or promote cancer (Ulrich and Potter, 2007).

1.3.1.1 Protective Effects of Folate Intakes Against Cancer

There are both animal studies and epidemiological studies showing the protective effect of folate against cancer. A study with Sprague-Dawley rats fed diets containing either 0 or 8 mg/kg FA found that folate deficiency promotes the development of colonic neoplasia (2/7 rats vs. 7/7 rats) and carcinoma development (1/7 rats vs. 6/7 rats) after 20 weeks of treatment with dimethylhydrazine, a colon carcinogenic (Cravo *et al.*, 1992).

Another study had similar results using the same animal model of colorectal cancer: microscopic colorectal neoplastic foci were induced in Sprague-Dawley rats by treatment with dimethylhydrazine (Kim *et al.*, 1996). This study used the same diets as

the Cravo *et al.*, (1992) study, and found that increasing levels of dietary folate up to 8 mg/kg diet, decreases the percentage of rats with macroscopic tumours ($p<0.03$) and the average number of tumours per rat ($p<0.04$; Kim *et al.*, 1996). In rats fed a high folate diet (40 mg folate/kg diet), there was a nonsignificant trend towards increasing macroscopic tumour carcinogenesis compared to the other groups (Kim *et al.*, 1996).

In humans, pooled analyses of 13 prospective cohort studies from Canada, the US, Netherlands and Sweden investigating folate intake and the incidence of colon cancer found that folate intake in the highest quintile is mildly protective against colon cancer when compared to the lowest quintile of intake (0.92 risk ratio (RR); 95% CI 0.84-1.00 for dietary folate and 0.85 RR; 95% CI 0.77-0.95 for dietary and supplemental folate; Kim *et al.*, 2010). Similarly a meta-analysis of cohort studies and case-control studies from Canada, the US, Netherlands, Italy, France, Finland and Switzerland examining the relationship between folate intake and colorectal cancer risk also found that folate at the highest intake (The highest tertile, quartile or quintile from the studies in the meta-analysis: ranging from >249 µg/day to >2430 µg/day) is protective compared to the lowest intake (The lowest tertile, quartile, or quintile: ranging from <103 µg/day to <301 µg/day; 0.75 RR; 95% CI 0.64-0.89 for intake from foods alone and 0.95 RR; 95% CI .81-1.11 for intake from foods and supplements; Sanjoaquin *et al.*, 2005). The meta-analysis shows that there is more protection from naturally occurring folate in food as opposed to FA from fortified foods and supplements.

The Nurses' Health Study (1980-1994) reported that women who have taken FA-containing vitamins for less than 15 years have no reduction in the risk of colon cancer across the ranges of folate intake (≤200, 201-300, 301-400, and >400 µg/d), but women

have a 31% reduced risk (RR of 0.69, 95% CI 0.52-0.93) if they consume >400 µg folate/d compared to intakes of ≤200 µg folate/d (Giovannucci *et al.*, 1998). Another study examining the latency between folate intake and colorectal cancer risk found that high folate intakes (≥ 800 µg/d) for 12-16 years before cancer diagnosis offers protection compared to low folate intakes (<250 µg/d; RR 0.69; 95% CI 0.51-0.94); however, folate intake during the 4 years prior to the study is not associated with colorectal cancer risk (Lee *et al.*, 2011). The Nurses' Health Study and the Health Professionals Follow-up Study has also found that both long-term (8 – 16 year prior to diagnosis) and short-term (0 – 8 years prior to diagnosis) total folate intake before diagnosis offers protection against colorectal adenomas comparing high (≥800 µg/d) and low folate intakes (<250 µg/d; Lee *et al.*, 2011). The strongest inverse association was observed with high total folate intake (≥800 µg/d) compared to low intake (<250 µg/d) 4-8 years before adenoma diagnosis (Odds Ratio (OR) 0.68; 95% CI 0.60 – 0.78; Lee *et al.*, 2011). Lee's study (2011) shows that colorectal cancer and colorectal adenomas do not have the same risk factors, as it seems that shorter term folate exposure is important for adenoma risk and longer term folate exposure is important for colorectal cancer risk. These studies build a solid case for the protective effect of adequate folate status. However it cannot be concluded from these studies that folate deficiency contributes to colorectal carcinogenesis.

1.3.1.2 Promoting Effects of Folate Intake on Cancer Development

While it has been shown in Sprague-Dawley rats that folate deficiency promotes the development of colorectal neoplasia, another study shows that dietary folate

deficiency is protective. For example, folate deficiency protects Sprague-Dawley rats against azoxymethane (AOM)-induced intestinal tumors (Le Leu *et al.*, 2000). In this study weanling rats were fed diets containing either 0 or 8 mg FA/kg for 4 weeks, at which point AOM was administered (Le Leu *et al.*, 2000). After 26 weeks, rats fed the 0 FA diet have significantly lower incidence of total intestinal tumours compared to rats fed diets with the control diet containing FA ($p < 0.01$; Le Leu *et al.*, 2000). Rats fed the 0 FA diet also have fewer adenocarcinomas than the control ($p < 0.01$) and show a 71% decrease in tumour malignancy (Le Leu *et al.*, 2000). According to Kim (2004), this study did not actually show that 0 FA diet was protective against colorectal cancer. The animals were fed a casein-based diet, containing 20% fat and measurable levels of folate (Kim, 2004). The colonic folate concentrations were much higher than usual levels achieved by the folate levels used in amino acid defined diets, suggesting the rats in the study were actually exposed to supraphysiologic doses of FA, and this is further supported by the low plasma homocysteine values of the rats in the study (Kim, 2004).

Another study in Sprague-Dawley rats examined the link between maternal dietary folate supplementation and post-weaning dietary folate supplementing in rat pups with AOM-induced colorectal cancer (Sie *et al.*, 2011). This study found that pups born from mothers fed with the control diet (2 mg FA/kg diet) whose diets were supplemented with FA (5 mg/kg diet), had higher tumour multiplicity and burden than rat pups in other diet groups (Sie *et al.*, 2011). The authors theorized that pups born from mothers fed the control diet possibly developed more microscopic precursor lesions in the colorectum (Sie *et al.*, 2011).

A study focusing on the link between development of aberrant crypt foci (ACF; an early precursor to colorectal cancer) and FA supplementation revealed that male Sprague-Dawley rats with AOM-induced ACF, develop 54% more ACF (p=0.011) when their diet is supplemented with 8 mg FA/kg diet compared to the 0 folate control-diet fed animals (Lindzon *et al.*, 2009). This study also revealed a correlation between folate supplementation and tumour multiplicity (r=0.32, p=0.002), tumour burden (r=0.35, p=0.001), and rectal epithelial proliferation (r=0.39, p<0.001) in Sprague-Dawley rats with AOM-induced colorectal cancer (Lindzon *et al.*, 2009). From their study, Lindzon *et al.* (2009) concluded that FA supplementation after treatment with AOM, may promote the progression of ACF to tumours.

A recent study looking at unmetabolized FA in the serum of participants in NHANES studies from 1999-2002 (2 cycles) and serum folate concentrationss in participants from 5 cycles of NHANES studies (1999-2008) found that in adults older than 60, those whose serum did not contain unmetabolized FA had a 24% reduced risk of developing all cancer (Bauldauff, 2013). The study also found that individuals in the highest quartile of serum folate (quartiles not defined) were 1.4 times more likely to have cancer than those with lower serum folate concentrations (Bauldauff, 2013). Women over age 60 in the highest quartile of serum folate were 1.9 times more likely to have breast cancer than women in lower quartiles (Bauldauff, 2013). Another study found that among healthy, post-menopausal women, those with detectable plasma FA had natural killer cell cytoxicity 23% lower than those without detectable plasma FA (Troen *et al.*, 2006). Furthermore, among women who consumed a folate-rich (> 233 µg folate/d) diet and consumed supplements containing > 400 µg FA/d, natural killer cell cytoxicity was lower

compared with women who consumed a low-folate (<233 µg folate/d) diet and used no FA supplements (Troen *et al.*, 2006). These two studies in older adults show that more research needs to be done on the effect of FA on the body and on cancer.

A study looking at American (The US Surveillance, Epidemiology and End Result registry, 1986 - 2002) and Canadian (Canadian Cancer Statistics, 2006) colon cancer incidence show a decline in the incidence of colorectal cancer in both the US and Canada; however this downward trend was interrupted by a sudden upturn starting in 1996 for the US and 1997 for Canada (Figure 1.4; Mason *et al.*, 2007). Hirsch *et al.* (2009) observed similar trends to the US and Canada when examining pre- and post-FA fortification colorectal cancer incidence in Chile. Data, including length of hospital stay, diagnosis and discharge, showed that in patients over age 45, colon cancer-caused hospital discharges are increased (45-64 years: rate ratio 2.61; 99% CI 2.58-2.93; 65-79 years: rate ratio 2.9; 99% CI 2.86-3.25) during the post-fortification period (2002-2004) compared to pre-fortification (1992, 1993 and 1996; Hirsch *et al.*, 2009). In a Norwegian intervention study, the hazard ratio (HR) for developing any cancer is 1.21 (95% CI 1.03-1.41) for participants who received a multivitamin supplement, containing FA (800 µg/day), vitamin B_{12} (400 µg/day) and vitamin B_6 (40 mg/day), or FA (800 µg/day) and vitamin B_{12} (400 µg/day) compared to patients who received vitamin B_6 (40 mg/day) alone or a placebo, suggesting that FA increases the overall risk of getting any cancer (Ebbing *et al.*, 2009). Furthermore, participants receiving FA and vitamin B_{12} also have increased risk of cancer death (HR, 1.38; 95% CI 1.07-1.79) and all-cause mortality (HR, 1.18; 95% CI 1.04-1.33; Ebbing *et al.*, 2009). It appears that FA may play a role in both preventing and promoting cancer development depending on the timing of exposure.

1.3.1.3 Possible Mechanisms of Folate's Role in Colorectal Cancer

The proposed mechanisms of colorectal carcinogenesis due to insufficient folate intake or status include decreased production of SAM for DNA methylation causing global DNA hypomethylation (Kim, 1999) and the potential for misincorporation of uracil during DNA synthesis (Blount *et al.*, 1997). Cell culture studies with human colonic epithelial cells have shown that folate deficiency causes increased uracil incorporation into DNA, increased DNA double strand breaks, impaired cellular response to oxidative and alkylation damage and decreased global DNA methylation (Duthie *et al.*, 2000). Folate deficiency altered the expression of genes involved in cell cycle control (e.g. *p53*), DNA repair, apoptosis and angiogenesis (e.g. *VEGF*) in a cell-specific manner in HCT116, Caco2, HT29 and LS513 colon cancer cells (Novakovic *et al.*, 2006).

In Caco2 cells, *p53*, which is involved in the regulation of apoptosis and cell cycle, and *VEGF*, which is involved in angiogenesis, are both upregulated after 20 days culture in folate deficient conditions (0 folate RPMI 1640 plus 10% dialyzed fetal bovine serum containing approximately 0.6 nM folate) relative to higher folate conditions (2.3 µM FA RPMI 1640 plus 10% fetal bovine serum); however, expression of these genes is down regulated in HCT116 cells cultured in folate deficient conditions (Novakovic *et al.*, 2006). In people at high risk of developing colon cancer due to their history of developing recurrent adenomatous colorectal polyps, FA supplementation at 2 mg/day reduces proliferation of cells obtained from rectal biopsies compared to that in the placebo control group (Khosraviani *et al.*, 2002). Cell culture studies are generally consistent with prospective cohort studies showing that folate deficiency is associated

with higher risk for colorectal cancer development whereas supplementation seems to protect against it.

Many epidemiologic studies point to a protective role for folate; however studies have linked rises in colon cancer incidence to the initiation of a FA fortification program. It is theorized that the timing of folate exposure can predict whether folate is protective or it promotes colorectal cancer (Ulrich and Potter, 2007).

Timing refers to whether the patient has precancerous lesions, polyps, adenomas, or neoplastic foci during the exposure to folate (Ulrich and Potter, 2007). In Apc^{Min} mice, a model of mice genetically predisposed to developing small intestintal and colonic adenomas, folate deficiency (0 mg FA/kg diet) reduced the occurrence of ileal polyps by 62-76% at 6 months compared to the other folate supplemented diets (2, 8, and 20 mg FA/kg diet; Song *et al.*, 2000). In this model of mouse, 3 months is the time of maximum tumour development and 6 months is considered the point at which the tumours are established (Song *et al.*, 2000). The number of ileal polyps was also correlated to serum folate levels in the mice after 6 months (r=0.44, p=0.006), suggesting that after tumours are established in the mice, folate deficiency causes a regression of the ileal polyps in Apc^{Min} mice (Song *et al.*, 2000). Considering the role of folate in DNA synthesis, it makes sense that restricting folate in rapidly proliferating tissue would cause growth to slow down or stop.

A previously discussed study in Sprague-Dawley rats investigating maternal and post-weaning folate supplementation showed that pups from mothers fed the control diet (2 mg FA/kg diet) that were given the FA supplemented diet (5 mg/kg diet) develop higher tumour multiplicity and burden than any other group (Sie *et al.*, 2011). The

explanation given for this observation was that pups from mothers fed the control diet may have had more microscopic precursor lesions in the colorectum than pups from dams fed the supplemented diet (Sie *et al.*, 2011). The pups fed the supplemented diet then developed more tumours because the FA promoted tumourigenesis (Sie *et al.*, 2011). This is an example where timing of folate exposure can cause FA to promote tumourigenesis. In fact, this study also shows that maternal supplementation significantly reduces the risk of colorectal adenocarcinomas in rat pups with AOM-induced colorectal cancer (OR 0.36; 95% CI 0.18-0.71, p=0.003; Sie *et al.*, 2011). This study showed that timing of folate exposure is very important, as folate supplementation *in utero* ias protective against AOM-induced colorectal cancer, whereas post-weaning supplementation does not offer protection, and in certain cases, increases tumour multiplicity and burden (Sie *et al.*, 2011).

A randomized, double-blind, placebo-controlled study conducted on patients with a recent history of colorectal adenomas, but no previous invasive large instestine carcinoma, found that FA supplementation (1 mg/d) had higher risks for having 3 or more adenomas and development of noncolorectal cancers compared to the placebo group (Cole *et al.*, 2007). This study had two follow-up intervals, at the second follow-up interval, participants receiving folic acid were more likely to develop multiple adenomas (≥3; RR: 2.32, 95% CI: 1.23-4.35, p<0.02) and more likely to have advanced lesions (RR: 1.67, 95% CI: 1.00-2.80, p<0.05) compared to the placebo group (Cole *et al.*, 2007). The patients in this study had a history of adenomas, so FA supplements were intended to treat secondary occurrences. In this case, FA appeared to promote carcinogenesis, rather

than protect against it in patients at higher risk for colorectal cancer. This makes sense as rapidly proliferating tissue requires folate for nucleotide synthesis.

The two aforementioned studies in Apc^{Min} mice (Song *et al.*, 2000) and in people with a recent history of colorectal adenomas (Cole *et al.*, 2007), show how timing affects the outcome of folate on carcinogenesis. Since a significant proportion of the population over age 50 have colorectal polyps, the issue of folate exposure and timing is very important. One study conducted at 3 American medical centres found that 50.4% of the asymptomatic adults (aged 50-79 years) in the study had polyps (Pickhardt *et al.*, 2004).

In subjects with otherwise normal healthy tissues, folate deficiency appears to predispose them to cancer (Kim, 2004). Folate is important for DNA synthesis, stability, repair and integrity, so it stands to reason that deficiency could compromise DNA allowing DNA errors to go uncorrected and cancer to occur (Kim, 2004). So timing of exposure to folate seems to be an important key in predicting how folate will affect carcinogenesis.

1.3.2 Wnt Signalling and Colorectal Cancer

Wnt was initially identified as a protooncogene (Nusse and Varmus, 1982). Later it was discovered that *APC*, a tumour suppressor located on chromosome 5, is mutated in familial adenomatous polyposis (FAP), an inherited colorectal cancer (Bodmer *et al.*, 1987; Leppert *et al.*, 1987; Solomon *et al.*, 1987). In many cases of sporadic colon cancer both *APC* alleles are inactivated (Bienz and Clevers, 2000). APC as part of the β-Catenin destruction complex binds with β-Catenin (Su *et al.*, 1993) and the formation of the complex leads to the destabilization of β-Catenin. Therefore, APC is posited as a

negative regulator of β-Catenin-dependent Wnt signalling (Munemitsu *et al.*, 1995). In colorectal cancer where *APC* is inactivated, β-Catenin is stabilized resulting in constitutive activation of Wnt signalling (Morin *et al.*, 1997; Korinek *et al.*, 1997). Axin is another member of the β-Catenin destruction complex (Ikeda *et al.*, 1998) that is also mutated in some colorectal cancer cases (Fearnhead *et al.*, 2004; Suraweera *et al.*, 2006). Patients with mutation to *AXIN2* are predisposed to developing colon cancer (Lammi *et al.*, 2004). Loss of function alterations to components of the β-Catenin destruction complex leads to accumulation of stabilized β-Catenin within the cytoplasm. Subsequently, β-Catenin enters the nucleus where it forms a complex with the transcription factors TCF/LEF and binds to DNA, activating transcription of target genes (Bienz and Clevers, 2000).

β-Catenin mutations can also occur in colorectal cancer (Fearnhead *et al.*, 2004), most commonly in patients with hereditary non-polyposis colorectal cancer (HNPCC; Johnson *et al.*, 2005). Mutations in β-Catenin interfere with the ability of the β-Catenin destruction complex to bind and phosphorylate β-Catenin and therefore deregulation of Wnt signalling occurs (Polakis, 2007).

APC, *AXIN*, and *CTNNB1* are the main genes that are mutated in genetic activation of many cancers. Mutations of some of the other components of the Wnt signalling pathway have also been shown to cause or contribute to the development of colorectal cancer. For example, mutation of *TCF4*, a transcription factor that is responsible for activation of Wnt targeted transcription, occurs in about half of colorectal cancer cases with microsatellite instability (Duval *et al.*, 1999; Shimizu *et al.*, 2002).

One of the consequences of constitutively active β-Catenin-dependent Wnt signalling in normal cells is the development of cancer. One of the downstream targets of the Wnt signalling pathway is *c-MYC*. c-MYC, a helix loop helix transcription factor (Soucek and Evan, 2010), is upregulated in a wide variety of cancers, including Burkitt's Lymphoma, breast cancer, prostate cancer, gastrointestinal cancer, melanoma, multiple myeloma and myeloid leukemia (Nesbit *et al.*, 1999). In experimentally induced murine epithelial hyperplasia, the loss of *Apc* activates *c-Myc* expression (Sansom *et al.*, 2004), when *Apc* and *c-Myc* are both lost, the overgrowth caused by original *Apc* removal is inhibited despite high cellular β-Catenin levels (Sansom *et al.*, 2007). *c-MYC* is also upregulated in colon cancer cell lines such as HT29, HCT116 and SW480 cells (He *et al.*, 1998).

The expression of many genes is upregulated in colon cancer directly by the Wnt signalling pathway, such as cyclin D1 (*CCND1*; Tetsu and McCormick, 1999; Shtutman *et al.*, 1999), peroxisome proliferator-activated receptor-δ (*PPARD*; He *et al.*, 1999), *c-JUN* (Mann *et al.*, 1999), matrix metalloproteinase-7 (*MMP7*; Brabletz *et al.*, 1999; Crawford *et al.*, 1999), *CD44* (Wielenga *et al.*, 1999), claudin-1 (*CLDN1*; Miwa *et al.*, 2001), Survivin (*BIRC5*; Zhang *et al.*, 2001), *VEGF* (Zhang *et al.*, 2001), *MET*, a Receptor Tyrosine Kinase (Boon *et al.*, 2002) and inhibitor of differentiation/DNA binding-2 (*ID2*; Rockman *et al.*, 2001; Willert *et al.*, 2002). Expression of some components of the Wnt signalling pathway is also upregulated in colorectal cancer, such as the *TCF1*, *LEF1*, and *AXIN2*. The overall effect of these changes is impaired apoptosis and differentiation, and the promotion of cell growth, proliferation, angiogenesis and metastasis.

Suppression of Wnt signalling in cancer cells can stop the growth of cancer cells. S100A4 is a calcium binding protein and a target of the Wnt signalling pathway (Sack *et al.*, 2011). In colorectal cancer cells, S100A4 serves as a metastatic regulator. When the transcription of *S100A4* is inhibited in various colon cancer cell lines (HCT116, SW620, LS174T, SW480 and DLD-1 cells) by calcimycin (an inhibitor of β-Catenin-dependent Wnt signalling), cell growth, viability, migration, invasion and proliferation are all decreased (Sack *et al.*, 2011). In NOD/SCID-IL2R^{-} mice xenografted with human colorectal cancer HCT116 cells, calcimycin reduces the metastatic potential compared to that of the controls (Sack *et al.*, 2011). LRP6 is a co-receptor required for β-Catenin-dependent Wnt signalling. Targeting LRP6 with niclosamide results in the inhibition of β-Catenin-dependent Wnt signalling, reduction in cell viability, and increased apoptosis in some types of breast and prostate cancer cells (Lu *et al.*, 2011).

1.3.3 Evidence for Wnt and Folate Interactions in Colorectal Cancer

There are some indications that folate and Wnt signalling pathway intersect. In several human colonic epithelial cell lines, folate depletion increases the expression of β-Catenin (*CTNNB1*) and *APC* (Crott *et al.*, 2008). In contrast, folate depletion also upregulates the expression of *p16, p21,* and *p53* tumour suppressor genes (Crott *et al.*, 2008). These results are consistent with earlier findings showing an upregulated expression of *CTNNB1* and downregulation of *VEGF* and *p53* in four colorectal cell lines, including Caco2 cells (Novakovic *et al.*, 2006). These observations suggest that folate deficiency affects the expression of genes involved in cell cycle regulation, D NA repair, apoptosis and angiogenesis in a cell-specific manner (Novakovic *et al.*, 2006). In

C57BL/6J mice, folate deficiency (0 dietary folate, but no antibiotic administration) induces down regulation of *Apc* expression, upregulation and increased nuclear localization of β-Catenin, and upregulation of the downstream target cyclin D1 (*Ccnd1*) in the colon (Liu *et al.*, 2007). Normal human fibroblasts cultured in 0 folate medium for 7 days show an altered distribution of cells in various phases of the cell cycle compared to the folate sufficient control, suggesting that proliferation and growth are inhibited by 0 folate conditions (Katula *et al.*, 2007). Further, culturing in 0 folate conditions for 7, 10 or 14 days also upregulates the expression of *WNT5a* and *WISP1* (Wnt1 inducible signalling pathway protein 1) and downregulates the expression of *DKK1* (an inhibitor of Wnt signalling), which results in increased Wnt signalling activity (Katula *et al.*, 2007).

A possible mechanism for the effect of folate on gene expression is through affecting DNA methylation. 5MTHF is a major methyl donor *via* SAM in the one-carbon metabolism pathway (Figure 1.2). One of the important functions of SAM is the methylation of CpG dinucleotides in DNA (Cedar, 1988). Of particular importance is the methylation of promoter regions of genes because an increase in methylation can lead to gene silencing by inhibiting the binding of transcription factors to the promoter region while decreased methylation could lead to gene activation (Cedar, 1988; Rountree *et al.*, 2001). Aberrant DNA methylation is observed in a variety of cancers, in particular the hypermethylation of CpG dinucleotides in genes resulting in gene silencing has been intensely studied (Das and Singal, 2004). For example, genes involved in apoptosis (e.g. *DAPK* and *TMS1*), cell cycle regulation (e.g. *p16* and *p15*), and DNA repair (e.g. *BRCA1* and *MGMT*) are frequently found to be hypermethylated in cancer (Das and Singal, 2004).

In colon cancer cells, hypermethylation induces silencing of *SFRP* (Suzuki *et al.*, 2002), *DKK* (Aguilera *et al.*, 2006) and *WIF* (He *et al.*, 2005). As mentioned previously, these genes are all involved in inhibition of the β-Catenin-dependent Wnt signalling pathway. For example, hypermethylation of the *SFRP1* promoter has been reported in various colorectal cancer cell lines, including RKO, Caco2, Colo205, DLD-1, HCT116, HT29, LoVo and SW480 (Suzuki *et al.*, 2002). Stronger evidence linking hypermethylation to colorectal cancer is from human studies. In healthy individuals with no detected colorectal cancer, there is an absence of *SFRP1* hypermethylation (Suzuki *et al.*, 2002). In contrast, *SFRP1* is hypermethylated in primary colorectal cancer tissues (Suzuki *et al.*, 2002). Similarly, other members of the *SFRP* family (*SFRP 1-5*) are also hypermethylated (Suzuki *et al.*, 2002). To further understand the effects of hypermethylated *SFRP* in colorectal cancer cells, Suzuki *et al.* (2004) examined whether the correction of *SFRP* hypermethylation could downregulate the Wnt signalling pathway in colon cancer HCT116 cells, which possess mutated *CTNNB1*, and SW480 cells, which possess mutated *APC*. Equal expression of hemagglutinin-tagged *SFRP1*, *SFRP2* and *SFRP5* suppresses β-Catenin-mediated Wnt signalling in HCT116 and SW480 colorectal cancer cells (Suzuki *et al.*, 2004). In these cell lines, stable overexpression of *SFRP1*, *SFRP2* and *SFRP5* results in a downregulation of *MYC*, a downstream target of Wnt signalling pathway (Suzuki *et al.*, 2004). Similarly, the promoter region of *SFRP1* is hypermethylated in 9 colorectal cancer cell lines examined (Aguilera *et al.*, 2006). These studies have established that Wnt signalling can be constitutively upregulated in colorectal cancer cells when the promoters of *SFRP* genes are hypermethylated and silenced; however, the possible role of folate in the hypermethylation was not studied.

DKK1 is epigenetically silenced in several colorectal cancer cell lines (SW480, LS174T, HT29, LoVo and SW620), but not in normal lymphocytes and normal primary colorectal mucosal tissue (Aguilera *et al.*, 2006). When the colorectal cancer cells with low-level expression of *DKK1* are treated with a demethylation agent, *DKK1* expression is restored (Aguilera *et al.*, 2006). Further, *DKK1* silencing appears to be correlated to colorectal cancer stage (Aguilera *et al.*, 2006). In cell lines derived from advanced or late stage colorectal cancer, the expression of *DKK1* is silenced; however in cell lines derived from earlier stage colorectal cancers, the promoter region for *DKK1* is not hypermethylated, and therefore not silenced (Aguilera *et al.*, 2006). This same relationship is also observed in primary tumours: *DKK1* is hypermethylated in the most clinically advanced tumours (Aguilera *et al.*, 2006). This is in contrast to the methylation of *SFRP1*, which is independent of cell lines and the cancer stage of the primary tissue from which the cells are derived (Aguilera *et al.*, 2006). Also in contrast to *SFRP1*, restoration of *DKK1* expression does not inhibit basal Wnt signalling in either late stage- or early stage-derived colorectal cancer cell lines (Aguilera *et al.*, 2006). However, restoration of *DKK1* expression inhibits the growth of DLD-1 colorectal cancer cell colonies (Aguilera *et al.*, 2006). Similarly, tumor size is reduced in mice xenografted with DLD-1 cells transfected with *DKK1* compared to the control (Aguilera *et al.*, 2006).

WIF1 is another gene where methylation status and expression have been altered in colorectal cancer cells (Aguilera *et al.*, 2006; He *et al.*, 2005). *WIF1* is expressed in normal colon cells, but its expression is much lower in SW480 colorectal cancer cells and non-existant in HCT116 colorectal cancer cells (He *et al.*, 2005). Methylation-specific PCR showed that *WIF1* is not methylated in normal colon cells, but is hypermethylated in

both SW480 and HCT116 colorectal cancer cells (He *et al.*, 2005). In SW480 colorectal cancer cells, restoration of *WIF1* expression or inhibition of *WNT1* with siRNA induces apoptosis (He *et al.*, 2005). In primary advanced-staged colorectal cancer tissues, expression of *WIF1* is either downregulated or absent in 5 out of 7 individuals (He *et al.*, 2005). This status of *WIF1* expression correlates with the methylation status of the *WIF1* promoter (He *et al.*, 2005). Epigenetic silencing of *SFRP, WIF1* and *DKK1* suggests that upstream Wnt signalling is important in carcinogenesis. In the case of *SFRP* and *WIF1*, restoring expression of these genes in colorectal cancer cells inhibits the Wnt/β-Catenin signalling pathway. Both SFRP and WIF1 exert their inhibitory functions on the Wnt signalling pathway by directly binding to Wnt proteins. Restoring expression of *DKK1* in colorectal cancer cells did not inhibit Wnt signalling. However in DLD-1 colorectal cancer cells, tumour growth is inhibited when *DKK1* expression is restored, suggesting that anti-growth effect of DKK-1 is independent of the Wnt signalling pathway.

Expression of several other genes encoding proteins in the Wnt signalling pathway is regulated by methylation. For example, *WNT5A* (a tumour suppressor) is hypermethylated in 5 out of 6 colorectal cancer cell lines, including Caco2 cells, and unmethylated in a normal colon cells (Ying *et al.*, 2008). Treatment of the colorectal cancer cells exhibiting hypermethylated *WNT5A* promoters with a demethylating agent (5-Aza-2'-deoxycytidine) restores *WNT5A* expression (Ying *et al.*, 2008). Hypermethylation in the *WNT5A* promoter is present in 48% of primary colorectal cancer samples, compared to only 13% of normal primary colon tissue samples (Ying *et al.*, 2008). *WNT5A* is not expressed in HCT116 colon cancer cells. Transfecting HCT116 cells with *WNT5A* significantly inhibits tumour colony formation compared to the

control, while both β-Catenin protein levels and luciferase reporter activity and mRNA levels of *CCND1*, a downstream target of Wnt/β-Catenin signalling, are significantly reduced (Ying *et al.*, 2008).

Increased global DNA methylation has been shown in several clinical trials in patients with colorectal adenomas when they are treated with daily folate supplements (400 µg, 5 mg and 10 mg FA/d; Kim, 2005). In one study conducted on patients with colonic adenomas, supplementation with 5 mg/d FA promotes global DNA methylation in rectal mucosa if the patient has 1 adenoma (Cravo *et al.*, 1998). Cravo *et al.* (1994) reported that cancerous tissues from patients with colorectal carcinomas are hypomethylated compared to normal colonic tissue and adenoma tissue, but both patients with adenomas and carcinomas exhibit increased global DNA methylation after FA treatment (10 mg/d). In a clinical intervention trial, colorectal cancer patients with adenomas were randomized to receive either FA supplements (5 mg/d) or a placebo for 1 year with the outcomes being global DNA methylation of rectal mucosa and *p*53 strand breaks assessed at 6 months and 1 year (Kim *et al.*, 2001). Patients receiving FA have increased global DNA methylation and decreased *p53* strand breaks, while adenoma recurrence is not affected after 1 year (Kim *et al.*, 2001). In the most recent study looking at global DNA methylation in patients with colorectal adenomas, FA supplementation (400 µg/d) was non-significantly associated with higher or greater global DNA methylation in the rectal mucosa by 25% (95% CI, 0.11 – 39%, p=0.09) as compared to the placebo group (Pufulete *et al.*, 2005). These studies all have similar results with differing supplementation levels of FA: increase in global DNA methylation. The authors state that global DNA hypomethylation may lead to the development of colorectal cancer;

however these studies only looked at people who already had cancer. The consequences of increasing global DNA methylation in patients who do not already have cancer are not established. As *in vitro* evidence has shown, hypermethylation of certain genes is observed in cancer cells lines and primary culture. It is not known whether daily folate supplementation could increase promoter hypermethylation of these genes.

The link between folate, colorectal cancer and perhaps Wnt signalling is widely hypothesized to be attributed to the role of folate in DNA methylation; however there are other studies showing that nuclear localization of β-Catenin is affected by folate as well. Translocalization of β-Catenin from the cytoplasm to the nucleus is essential for Wnt signalling pathway activity. In NIH3T3 cells, folate deficiency was shown to increase β-Catenin nuclear localization (Morillon II, 2008). However, β-Catenin protein levels do not differ significantly between the folate sufficient and deficient cells (Morillon II, 2008). This observation supports an earlier observation that folate supplementation in patients with colorectal adenomas decreases β-Catenin localization in the nucleus of rectal mucosa cells (Jaszewski *et al.*, 2004).

1.4 Summary

FA fortification may have adverse effects in relation to colorectal cancer in certain groups of the population. There is a need for research to establish whether FA has a differential effect between normal cells and cancerous cells compared to 5MTHF. Introduction of FA fortification in several nations has been linked to a rise in colon cancer incidence. However, this issue is controversial because studies also point to folate deficiency being a risk factor for cancer progression. Most cases of colorectal cancer

have genetic mutations that result in constitutive upregulation of the Wnt signalling pathway, leading to increased proliferation, vascularization, metastatic potential and decreased apoptosis. There is evidence that folate and Wnt signalling are linked: inhibitors of Wnt signalling are epigenetically silenced in many colorectal cancer cases and β-Catenin nuclear translocation is increased during folate deficiency.

1.5 Hypothesis

The ***hypothesis*** for my thesis research is that folic acid will affect the proliferation and folate transporter expression in colon cancer cells differently than L-5-methyltetrahydrofolate. In addition, the forms of folate, reduced versus oxidized, will also differentially affect the activity of the Wnt signaling pathway.

1.6 Overall Objective and Specific Aims

The objective of my research is to investigate the effect of folic acid and L-5-methyltetrahydrofolate on the proliferation, expression of selected folate transporters, and the activity of the Wnt signalling pathway in human colorectal adenocarcinoma Caco2 cells.

The specific **aims** of my thesis research are:

1) To establish the effect of the form and concentration of folate on the proliferation and viability of Caco2 cells.

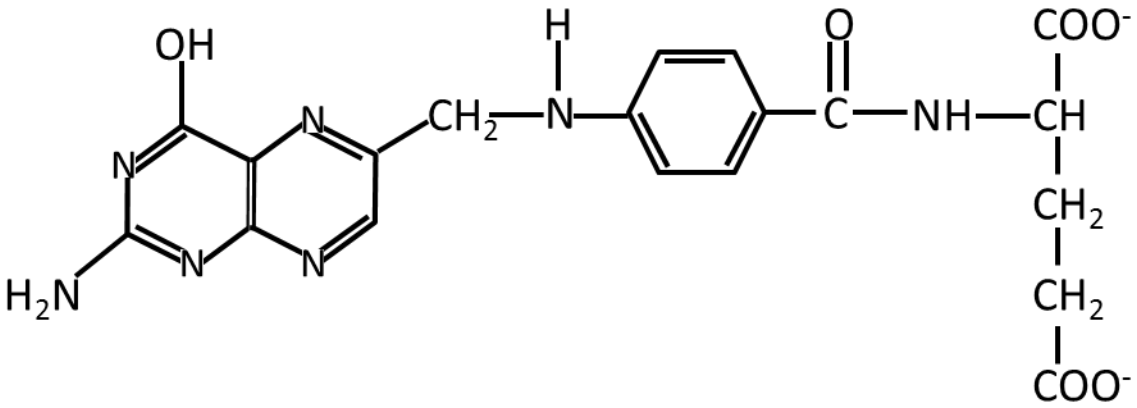
2) To assess the effect of the form and concentration of folate on the expression of folate transporters in Caco2 cells.

3) To investigate the effect of the form and concentration of folate on the intracellular translocation of β-Catenin, a key molecule in the Wnt signalling pathway, in Caco2 cells.

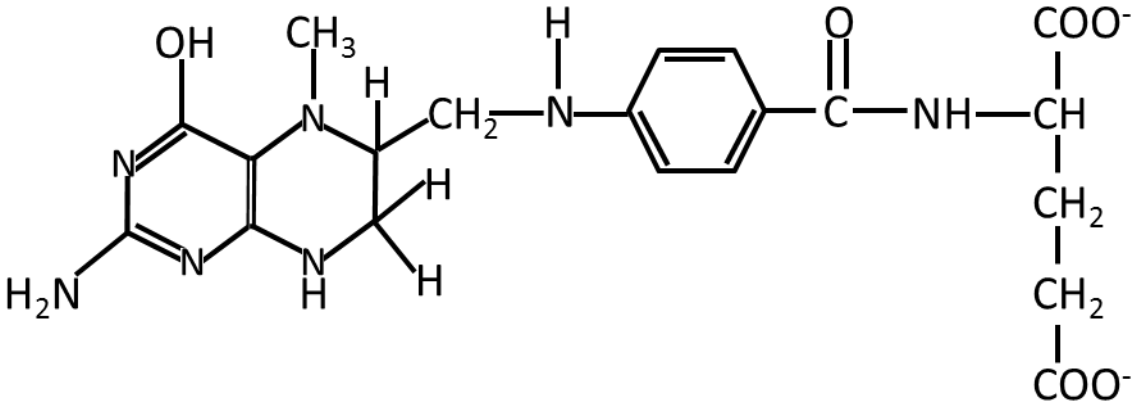
Table 1.1: Food Sources of Folate (Canadian Nutrient File, 2010)

Food Source	**Serving Size**	**Folate (µg)**
Asparagus, cooked	4 spears	80-88
Baby soybeans, cooked	125 mL	106-255
Bagel, plain	½ bagel (44.5 g)	101
Beans, cranberry/roman, cooked	175 mL	271
Beans (kidney, great northern), cooked	175 mL	157-170
Beans (mung, adzuki), cooked	175 mL	234-238
Beans (navy, black, small white), cooked	175 mL	181-190
Beans (pink, pinto), cooked	175 mL	210-218
Bread, white	1 slice (35 g)	60
Bread, whole wheat	1 slice (35 g)	18
Broccoli, cooked	125 mL	89
Lentils, cooked	175 mL	265
Liver, (beef, pork), cooked	75 g	122-195
Liver (lamb, veal), cooked	75 g	262-300
Liver (turkey, chicken) cooked	75 g	420-518
Okra, frozen, cooked	125 mL	142
Orange juice	125 mL	58
Pasta, egg noodles, enriched, cooked	125 mL	138
Pasta, white, enriched, cooked	125 mL	83-113
Peas (chickpeas, black-eyed/cowpeas/adzuki),cooked	175 mL	180-263
Peas, pigeon, cooked	175 mL	138
Spinach, cooked	125 mL	121-139
Turnip greens or collards, cooked	125 mL	68-93

*According to the Canadian Food Inspection Agency, a food must contain ≥5% of the RDI of a vitamin or mineral to be labelled a source (information updated 2014-03-25).



A)



B)

Figure 1.1: The structures of A) folic acid (FA) and B) 5-methyltetrahydrofolate (5MTHF).

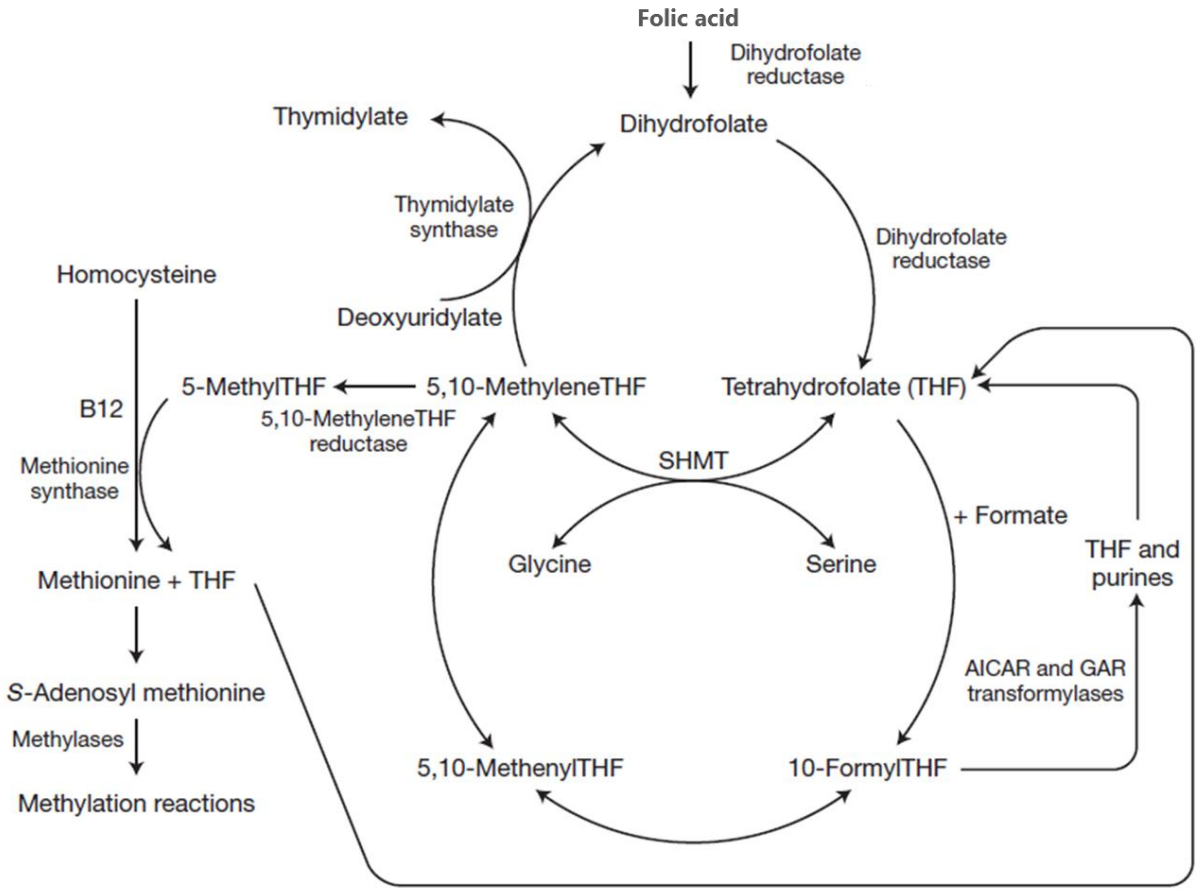
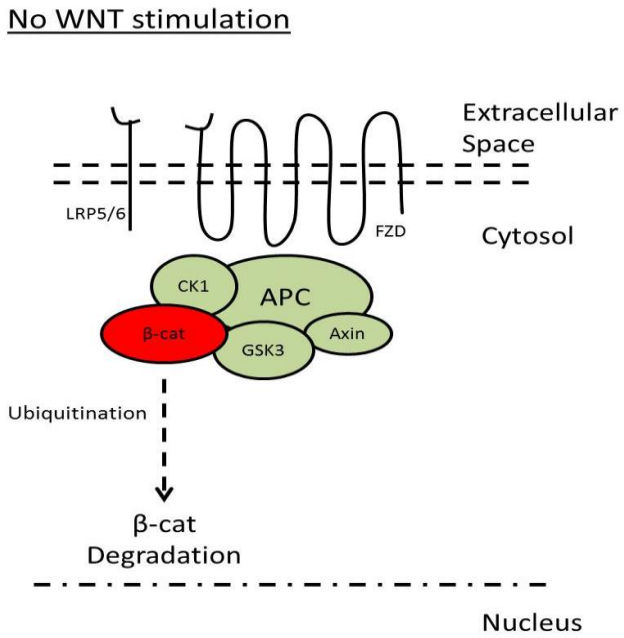
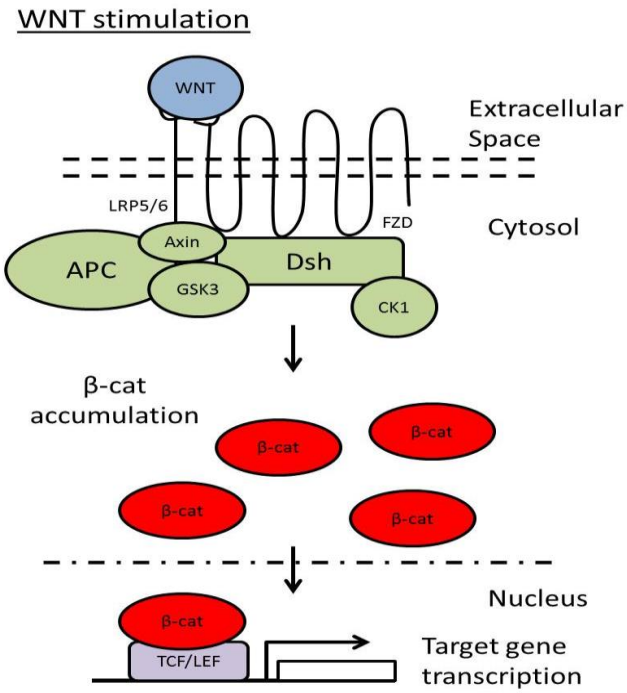


Figure 1.2: The folate metabolism pathway (Zhao *et al.*, 2009. Adopted with modifications by permission from Expert Reviews in Molecular Medicine). *Abbreviations: AICAR (phosphoribosylaminoimidazolecarboxamide transformylase), B12 (Vitamin B_{12}), GAR (phosphoribosylglycinamide transformylase), SHMT (serine hydroxymethyltransferase), THF (tetrahydrofolate).*



A)



B)

Figure 1.3: Schematic of canonical Wnt/β-Catenin-dependent Wnt signalling. A) No Wnt stimulation and B) with Wnt stimulation. *Abbreviations: APC (Adenomatous polyposis coli), β-cat (β-Catenin), CK1 (Casein kinase 1), DSH (Dishevelled), FZD (Frizzled), GSK3 (Glycogen synthase kinase 3), LRP5/6 (Lipoprotein receptor-related protein 5/6), TCF/LEF (T cell factor/Lymphoid enhancer-binding factor), Wnt (Wingless type proteins).*

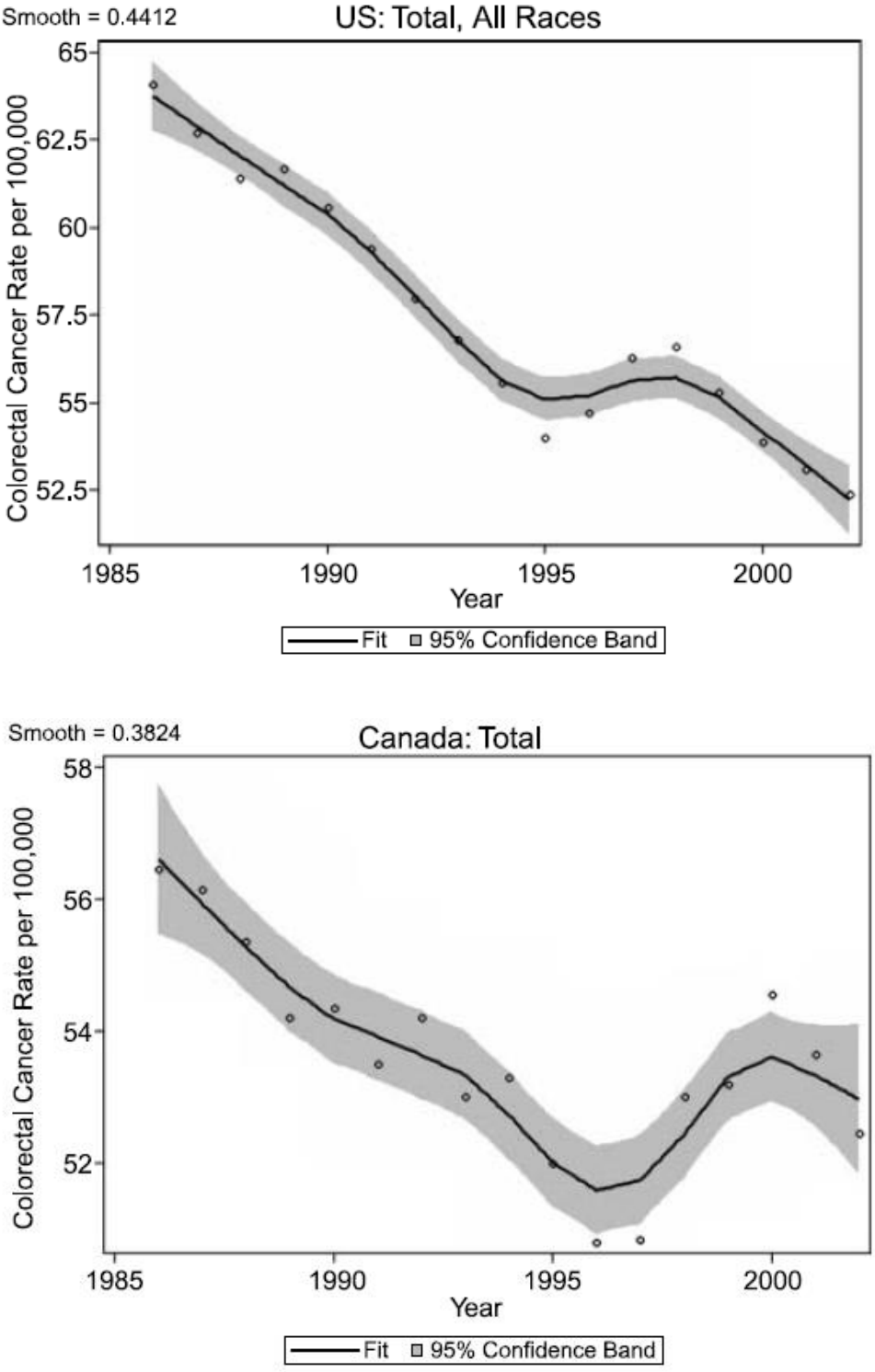


Figure 1.4: Colorectal Cancer rates pre- and post-fortification in the U.S. and Canada (Mason *et al.*, 2007. Reproduced with permission from Cancer Epidemiology, Biomarkers & Prevention)

CHAPTER 2
Effects of Short-term Supplementation of Folic Acid and L-5-methyltetrahydofolate on Proliferation, Viability and the Expression of Folate Transporters in Human Colorectal Adenocarcinoma Caco2 Cells

2.1 Introduction

An unexplained increase in colorectal cancer incidence has been observed in Canada, the United Sates and Chile post-folic acid fortification (Mason *et al.*, 2007; Hirsch *et al.*, 2009). These observations, as well as reported rises in all cancer incidence and mortality observed in patients who received FA and vitamin B_{12} supplements (Ebbing *et al.*, 2009), give cause for the concern that FA fortification may be harmful for certain sub-populations.

Dietary folate has been shown to affect the expression of PCFT, FR, and RFC, a group of major folate importers, and BCRP (Zhao *et al.*, 2009), a folate exporter in the small intestine, colon, liver, ovary and placenta (Maliepaard *et al.*, 2001). These receptors transport folate through various mechanisms. For example, PCFT is a folate-proton symporter (Qiu *et al.*, 2006) while RFC is an anion exchanger (Goldman, 1971). In contrast, FRs transport folate through receptor-mediated endocytosis (Kamen *et al.*, 1988).

FA is an oxidized form of folate whereas 5MTHF is a reduced form of the vitamin. This difference in their chemical structure influences their binding affinity towards these folate transporters. For example, PCFT, which transports folate best at an acidic pH, has a K_m of 0.53, 0.83, and 2.01 µM for 5MTHF, FA, and methotrexate, respectively, at pH 5.5 (Qiu *et al.*, 2006), indicating that PCFT has a higher affinity for 5MTHF than FA and methotrexate. The difference in affinities becomes more apparent if

the pH is increased to 6.5, where K_m for 5MTHF, FA, and methotrexate is 0.78, 2.99, and 8.07 µM, respectively (Qiu *et al.*, 2006). 5MTHF and other reduced folates are transported by RFC with a higher affinity (K_m = 1 – 3 µM) than FA (K_m = 200 – 400 µM; Jansen *et al.*, 1997). In contrast, FRα has a higher affinity for FA (K_D = 1 pM) than 5MTHF ($K_D \approx$ 1 nM; Kamen and Smith, 2004). Clearly, folates, such as 5MTHF, and FA are likely transported by these transporters with different efficiencies.

The Wnt signalling pathway is important for many developmental processes in the body (Logan and Nusse, 2004). Aberrant Wnt signaling has been linked to the development and progression of many diseases, especially cancer (Logan and Nusse, 2004). In sporadic or hereditary colorectal cancer, Wnt signalling is often aberrant. Therefore folate could affect the Wnt signaling pathway, possibly through gene promoter methylation (Suzuki *et al.*, 2004). In fact, it has been shown that a number of Wnt signaling pathway inhibiters are hypermethylated in primary colorectal cancer samples and colon cancer cell lines (Suzuki *et al.*, 2002; Suzuki *et al.*, 2004; He *et al.*, 2005; Aguilera *et al.*, 2006; Ying *et al.*, 2008). It has previously been shown that folate deficiency affects β-Catenin (an effector of Wnt signalling) localization into the nucleus in NIH3T3 cells (Morillon II, 2008). β-Catenin movement to the nucleus is essential for the activity of the Wnt signaling pathway. Therefore folate could be involved in the Wnt signaling pathway over and above its role in DNA methylation.

I hypothesize that folic acid will affect the proliferation and folate transporter expression in colon cancer cells differently than L-5-methyltetrahydrofolate. In addition, the forms of folate, reduced versus oxidized, will also affect the activity of the Wnt signaling pathway differently. The specific aims of my thesis research are: 1) to establish

the effect of the form and concentration of folate on the proliferation and viability of Caco2 cells; 2) to assess the effect of the form and concentration of folate on the expression of folate transporters in Caco2 cells; and 3) to explore the effect of the form and concentration of folate on the translocation of β-Catenin, a key molecule in the Wnt signaling pathway, in Caco2 cells.

2.2 Materials and Methods

2.2.1 Cell Culture System and Folate Treatments

Human colorectal adenocarcinoma Caco2 cells (HTB-37; ATCC, Manassas, VA), passage number 20 – 30, were selected as the cell culture system for this research.

The Caco2 cells were routinely maintained in Dulbecco's modified essential medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS), sodium pyruvate (1 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) in a humidified atmosphere at 37°C, 10% CO_2.

In preparation for the 5 day experiments, cells were initially cultured in a folate-free RPMI 1640 medium (ff-RPMI 1640; Gibco, Grand Island, NY) containing 10% dialyzed FBS (dFBS; Gibco, Grand Island, NY), sodium pyruvate (1 mM), pencillin (100U/mL), and streptomycin (100 µg/mL) for 5 days in a humidified atmosphere at 37°C, 5% CO_2. To examine the effect of folate on cell proliferation and viability, FA (Sigma-Aldrich, St. Louis, MO) or 5MTHF (Calcium salt; Merck, Whitehouse Station, NJ) was added to the treatment medium at 0, 0.9, 2.3, or 3.4 µM.

These supplementation levels were designed to deliver 400, 1,000 and 1,500 µg folate/L (0.9, 2.3, and 3.4 µM folate, respectively) to mimic adequate folate intake, and medium and high folate supplementations, respectively.

In order to prevent oxidation of 5MTHF, sodium ascorbate (5%; Sigma-Aldrich, St. Louis, MO) was added to the 5MTHF stock solutions (1 mM). The stock solution was sterilized with a 0.22 µm syringe filter and stored at -20^{o}C until use for up to 1 month.

The treatment duration of 3 days was initially chosen to investigate the acute response to folate supplementation. Akoglu *et al.* (2001, 2004) have shown effects of different folates (1.4 – 22.6 µM) on the growth and viability of Caco2 cells after 24 hours and 48 hours.

2.2.2 Assessment of Cell Viability and Proliferation

Caco2 cells were sub-cultured in ff-RPMI 1640 supplemented with 0 (5% sodium ascorbate), 0.9, 2.3 or 3.4 µM FA or 5MTHF for 3 or 5 days in a 96-well plate. For the 5 day experiment, Caco2 cells were initially cultured in ff-RPMI 1640 for 5 days in a 10 cm culture dish. The initial seeding density was 5,000 and 2,000 cells/well for the 3 and 5 day experiments, respectively. At the end of the treatment duration, cells were 60 – 80 % confluent. Cell viability following folate treatment was assessed using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Roche, Indianapolis, IN) according to the manufacturer's instruction. The WST-1 assay measures the reduction of WST-1, a water-soluble tetrazolium salt, to formazan, a water-soluble dye, by mitochondrial succinate-tetrazolium reductase present in viable cells

(Ngamwongsatit *et al.*, 2008). Briefly, WST-1 (20 µL) was added to each well and mixed by placing the plate on a Thermomixer (400 rpm, 55°C, 1 min; Thermomixer R, Eppendorf, Mississauga, ON) followed by incubation at 37°C, 5% CO_2 for 4 h. Subsequently, the reaction mixture was mixed again with the Thermomixer under the same conditions described above. The formazan produced was measured using a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA) at 450 nm with a reference wavelength at 690 nm.

To assess cell proliferation, cells were cultured and treated with FA or 5MTHF as described above. At the end of the 3 or 5 day treatment period (cells were 70 – 80% confluent), the cells were subjected to the 5-bromo-2'-deoxyuridine (BrdU) colourimetric assay (Roche, Indianapolis, IN) according to the manufacturer's instruction. The intensity of the color developed was determined at 405 nm using the same microplate reader described above with a reference wavelength of 490 nm. The BrdU assay measures the incorporation of BrdU (a nucleotide analogue) into newly synthesized DNA and therefore, only cells undergoing active DNA synthesis will be labelled with BrdU (Terry and White, 2006).

2.2.3 Cell Cycle Analysis

Caco2 cells were cultured in ff-RPMI-1640 medium at an initial seeding density of 500,000 and 250,000 cells/10 cm plate (3 and 5 day experiments, respectively) and treated with either FA or 5MTHF at the concentrations described above for 3 or 5 days. For the 5 day experiement, cells were first cultured in ff-RPMI 1640 for 5 days. At the end of the treatment period, the cells were 70 – 80% confluent. Subsequently, the cells

were incubated with BrdU (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 µM (10 µL of BrdU stock solution (1 mM) / mL of culture medium) for 1 h at 37°C, 5% CO_2. The cells were protected from light after addition of BrdU until the completion of the assay. At the end of the incubation period, cells were subjected to cell cycle analysis using a flow cytometric assay (Terry & White, 2006) with modifications. Briefly, cells were harvested with 2 mL 0.25% Trypsin-EDTA (Gibco, Grand Island, NY), neutralized with an equal volume of ff-RPMI 1640 medium. Cells were then counted by diluting 0.1 mL of cell suspension in 9.9 mL of IsoFlow™ diluent (Beckman-Coulter, Indianapolis, IN) using a particle counter (Z1 Coulter® Particle Counter, Beckman-Coulter, Indianapolis, IN), and pelleted by centrifugation (400 x *g*, 4 min, 20°C). The supernatant was then aspirated and cells were fixed by slowly adding cold PBS at 0.8 mL/2 million cells and subsequently trickling in 100% ethanol (-20°C) at 1.2 mL/2 million cells while vortexing to a final concentration of 1 million cells/mL. Cells were then left at 4°C overnight.

After fixing overnight, the cells were resuspended by vortexing and collected by centrifugation (400 x *g*, 4 min, 20°C). After removing the supernatant, HCl (2 N; 3 mL) was added to each tube while vortexing followed by incubation at 37°C for 30 min. During the incubation, the cells were mixed by vortexing every 10 min (twice). After the incubation, sodium borate (0.1 M; 6 mL) was added to each tube while vortexing and pelleted by centrifugation (400 x *g*, 4 min, 20°C). The cell pellet was slowly suspended in 6 mL PBTB (PBS containing 0.5% Tween-20 and 0.5% bovine serum albumin (BSA)) while vortexing followed by centrifugation (400 x *g*, 4 min, 20°C). The cells were subsequently incubated with anti-BrdU mAb (sc-20045, Santa Cruz Biotechnology, Santa

Cruz, CA) at 1:100 dilution (0.3 mL for up to 20 million cells in PBT (PBS containing 0.5% Tween-20) for 60 min at room temperature in the dark.

After incubation with the primary antibody, the cells were rinsed with 3 mL PBTB while vortexing, pelleted by centrifugation (400 x *g*, 4 min, 20°C), and incubated with goat-anti mouse IgG_1-FITC antibody (sc-2078, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution (0.2 mL for up to 20 million cells in PBTG (PBTB containing 1% normal goat serum)) for 45 min at room temperature in the dark. At the end of incubation period, 3 mL PBTB was added to the incubation solution and counted using the same particle counter described above. The volume of the cell suspension was adjusted to yield 1 million cells/tube. The labelled cells were either analyzed on the same day or stored at 4°C overnight in the dark and analyzed the next day.

Before analysis, the cells were collected by centrifugation (400 x *g*, 4 min, 20°C) and stained with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) in PBTB containing RNase A. The final concentration of PI and RNase A was 10 and 20 µg/1 million cells/mL, respectively. After incubating the cells for 30 min in the dark at room temperature, the cells were analyzed using a flow cytometer (FACScalibur; Beckton-Dickinson and Company, Franklin Lakes, NJ) with excitation at 488 nm (5-W argon-ion laser operating at 200 mW). BrdU (FITC, green fluorescence) was measured after blocking incident laser light using a logarithmic amplifier with a 530 nm short-pass filter. Linear DNA content was measured using PI (red fluorescence) with a 610 nm long-pass filter. Data was collected using Cell Quest (Beckton-Dickinson and Company, Franklin Lakes, NJ) and analyzed using FlowJo software (Version 7.6.5, Tree Star Inc., Ashland, OR).

2.2.4 Whole Cell Lysate Preparation and Western Blot Analysis

Caco2 cells were cultured in ff-RPMI-1640 for 5 days with an initial seeding density of 250,000 cells/10 cm dish and subsequently treated with FA or 5MTHF for 5 days as described above. At harvest, cells were 70 – 80% confluent.

To prepare the whole cell lysate, cells were rinsed with warm PBS (pH 7.4; 37°C) and harvested with 0.25% Trypsin-EDTA followed by neutralization with equal volume of the culture medium containing FBS. The harvested cells were pelleted with centrifugation (700 rpm, 5 min, 4°C). The cell pellet was resuspended in PBS and counted as described above. The cells were then repelleted with centrifugation (700 rpm, 5 min, 4°C), resuspended in 1 mL PBS and subsequently transferred to a microcentrifuge tube. After pelleting (14,000 x *g*, 5 min, 4°C), the cells were suspended in whole cell lysis buffer (pH 7.4; 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulphonyl-fluoride (PMSF)) in the presence of 10% protease inhibitor cocktail (AEBSF, 104 mM; Aprotinin, 80 µM; Bestatin, 4 mM; E-64, 1.4 mM; Leupeptin, 2 mM; Pepstatin A, 1.5mM; P8340; Sigma-Aldrich, St. Louis, MO) at 30 µL whole cell lysis buffer/10^6 cells. The cell suspension was incubated on ice for 30 min. After the incubation, the lysis buffer-cell mixtures were centrifuged at 14,000 x *g* for 10 min at 4°C and the supernatant was transferred to a new microcentrifuge tube. Total cellular protein was quantified using BioRad DC System (BioRad, Hercules, CA, USA).

Total cellular protein (50 µg/well) was prepared by diluting the volume to 10 µL with whole cell lysis buffer (without protease inhibitors or PMSF) and adding 10 µL 2X loading buffer (0.125 M Tris (pH 6.8), 5% SDS, 3.73 mM bromophenol blue, 10%

glycerol, ddH_2O; For RFC and PCFT, 2 µL dithiothreitol was added to 8 µL 2X loading buffer, for FRα and BCRP, no dithiothreitol was used), for a total of 20 µL/well). Protein and loading buffer were then boiled for 5 minutes and then electrophoretically separated on a 10% (RFC1, FR-α, and PCFT) or 12% (BCRP) SDS-PAGE at 175 V until the dye front reached the bottom of the gel (approximately 55 min). The proteins were electrotransferred at constant current (200 mA) to a PVDF membrane (0.2 µm; Immobilon®-PSQ; Millipore, Billerica, 3 h) using a Tris-glycine transfer buffer (25 mM tris, 0.192 M glycine, 20% methanol and ddH_2O; stored for maximum 1 week at 4°C). After transfer, the membrane was washed with TBS (50 mM Tris and 150 mM NaCl) twice (10 min/wash) and blocked with 2.5% skim milk powder in TTBS buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 75 min. To detect the targets, the membrane was incubated with the appropriate primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 2.5% skim milk powder in TTBS buffer overnight at appropriate dilution (Table 2.1) followed by washing with TTBS (3 X; 10 min/wash). Subsequently, the membrane was incubated with the appropriate secondary antibody in 1% skim milk powder in TTBS buffer for 1 h and washed with TTBS (3 X, 10 min/wash). The primary antibodies and their corresponding secondary antibodies and the dilution factors are listed in Table 2.1. Presence of the target proteins was detected using the enhanced chemiluminescence kit (ECL; Thermo Scientific, Rockford, IL) and visualized with chemiluminescence scientific imaging film (Kodak BioMax Light film, Sigma-Aldrich, St. Louis, MO) or using BioRad Chemidoc (BioRad, Hercules, CA). Band intensity was quantified with ImageJ software (version 1.45s, US NIH, Bethesda, MD).

2.2.5 Cellular localization of β-catenin

Caco2 cells were initially grown in ff-RPMI 1640 for 5 days and then subcultured onto a glass coverslip (12 x 12 x 1.5 mm), which was placed in a well in a 24-well plate at an initial seeding density of 20,000 cells/well, and treated with FA or 5MTHF as described above. At seeding, the cells were well dispersed, and not confluent. After the treatment period, the cells were 70 – 80% confluent.

After 5 days of treatment with FA or 5MTHF, the cells were rinsed with PBS and fixed with 100% methanol for 20 min at -20°C. After fixation, the cells were rinsed with ice cold PBS twice. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min followed by washing with PBS three times (5 min/wash).

Subsequently, cells were incubated with 0.5% BSA in PBT for 30 min to block unspecific binding of antibodies and labelled with rabbit anti-β-Catenin polyclonal antibody (AHO0462; Invitrogen, Grand Island, NY) at 15 µg antibody/mL hybridization solution (0.5% BSA in PBT) at room temperature for 1 h. The cells were then washed with PBS three times (5 min/wash). After washing, the cells were incubated with Alexa Fluor® 488 goat anti-rabbit IgG (A11008; Invitrogen, Grand Island, NY) at 5 µg antibody/mL hybridization solution at room temperature, dark, for 1 h followed by 3 PBS washes (5 min/wash).

Finally, the cells were counterstained with DAPI and mounted on a glass microscope slide in the same step using ProLong® Gold Antifade Reagent with DAPI (P-36931; Invitrogen, Grand Island, NY). Three drops of the reagent were placed on a glass microscope slide and a coverslip was placed on top of each drop with the side that cells grown on faced down. After curing overnight at room temperature in the dark, each

coverslip was sealed with clear nail polish and left to dry at room temperature before examining samples.

Samples were visualized with a Fluoview FV10i confocal microscope (Olympus, Center Valley, PA). Images were taken with the 60 x objective, zoomed in 2 x for a total of magnification of 120 x (Olympus Fluoview FV1000, version 2.1c; Olympus, Center Valley, PA). Image resolution is 512 x 512 pixels and aperture was x 1.0. β-Catenin localization was represented by the fluorescence of Alexa fluor 488® excited at 473 nm and counterstaining with DAPI was excited at 405 nm. Images were taken as z stacks with each slice 0.819 µm thick. Images were analyzed using Volocity 3D Image Analysis Software (Version 6.1.1; PerkinElmer, Waltham, MA).

2.2.6 Statistical Analysis:

All data was analyzed with two-way ANOVA. The ANOVA showed that there was a significant effect of the form of folate on 3 day cell viability and both form and concentration of folate on 3 day cell proliferation; but there was no significant effect of the concentration and interaction between the form and concentration of folate tested on all other end points. One-way ANOVA was performed followed by Tukey's Honestly Significant Difference Test for determining the effects among the folate concentrations within the same form of folate and T-test for determining the effects between FA and 5MTHF folate at the same concentration ($p < 0.05$; SPSS Statistics 22, IBM, Armonk, NY).

2.3 Results

2.3.1 Cell Viability

3-day folate treatment. Cell viability was not affected by FA and 5MTHF at the concentrations tested compared to the control (Figure 2.1). Cell viability was 15.8% higher in cells treated with 0.9 μM of FA than that in cells treated with 5MTHF at the same concentration (p = 0.044; Figure 2.1). Cell viability did not differ between FA and 5MTHF treatment at 2.3 and 3.4 μM (Figure 2.1).

5-day folate treatment. Cell viability was not affected by treating the cells with FA and 5MTHF at 0.9, 2.3, and 3.4 μM compared to the control (Figure 2.2). There was no difference in cell viability between the cells treated with FA or 5MTHF (Figure 2.2).

2.3.2 Cell Proliferation

3-day folate treatment. Cell proliferation was increased by 11 (p = 0.018), 12 (p = 0.001), and 21% (p = 0.021) in Caco2 cells treated with 0.9, 2.3, and 3.4 μM of FA, respectively, for 3 days compared to that in cells treated with same concentrations of 5MTHF (Figure 2.3). Cell proliferation was 23% higher in cells treated with 3.4 μM FA compared to the control (p = 0.008), but was not affected by treating the cells with 0.9 and 2.3 μM of FA (Figure 2.3). Cell proliferation in Caco2 cells was also not affected by 5MTHF treatment at all three concentrations tested (Figure 2.3).

5-day folate treatment. Cell proliferation was not affected by treating Caco2 cells with 0.9 and 3.4 μM of FA or 5MTHF at all three concentrations tested for 5 days compared to the control (Figure 2.4). There was also no effect of cell proliferation in cells treated with FA or 5MTHF for 5 days (Figure 2.4).

2.3.3 Cell Cycle Analysis

3-day folate treatment. Results of cell cycle analysis are shown in Figure A.1 and summarised in Table 2.2. After 3-day folate treatment, there was no significant difference in the proportion of cells in pre-G1 phase (Table 2.2). Over half (52.5%) of the control cells were in the G1 phase while 34.1 and 8.6% of the cells had progressed into the S and G2/M phases, respectively (Table 2.2). Treating the cells with 0.9 and 2.3 μM of FA or 5MTHF had no effect on the cell cycle progression compared to the control. However, treating the cells with 3.4 μM of FA resulted in an 8.4 and 7.9% reduction in the proportion of cells in the G1 phase compared to the control and cells treated with 5MTHF at the same concentration, respectively (Table 2.2). Furthermore, treating the cells with 3.4 μM of FA also resulted in a 15.9% increase in the proportion of cells in the S phase compared to the cells treated with 5MTHF at the same concentration, while the proportion of cells in the G2/M phases remained the same between these two groups of cells. Treating the cells with 3.4 μM of 5MTHF had no effect on the proportion of cells in the G2/M phase compared to the cells treated with 3.4 μM of FA.

5-day folate treatment. Results of the cell cycle analysis are shown in Figure A.2 and summarised in Table 2.3. Treating the cells with FA or 5MTHF at 0.9, 2.3, and 3.4 μM for 5 days had no effect on cell cycle progression compared to the control, except in the pre-G1 phase (Table 2.3). At 3.4 μM of 5MTHF, there was a 49.3% increase in the proportion of cells in the pre-G1 phase compared to the cells treated with FA at the same concentration. Treating the cells with 3.4 μM of FA and 5MTHF increased the proportion of cells in the pre-G1 phase by 3.6 and 5.3 times, respectively, compared to the control. Treating the cells with FA and 5MTHF at 0.9 μM resulted in an 8% increase in the

proportion of cells in the G1 phase compared to the control. In contrast, treating the cells with FA and 5MTHF at 0.9 µM resulted in 19.6 and 17.4% reduction in the proportion of cells in the S phase compared to the control, respectively. At 3.4 µM, 5MTHF treatment resulted in a 26.6% decrease in the proportion of cells in G2/M compared to the control.

Comparing the data from 3 day and 5 day folate treatment showed a large difference in cell cycle analysis. The proportion of cells in the G1 phase is much higher in Caco2 cells treated with either FA or 5MTHF for 5 days (61.9 – 67.3 %; Table 2.3) compared to the cells treated with the same treatments for 3 days (48.1 – 52.5 %; Table 2.2). On day 5, the folate treated groups had percentages of cells in the S phase ranging from 18 – 22.4% (Table 2.3). The percentage of cells in the S phase of the 3 day folate-treated Caco2 cells was 30.8 – 35.7% (Table 2.2).

2.3.4 Folate Transporters

Treating Caco2 cells with FA and 5MTHF at 0.9, 2.3, and 3.4 µM had no effect on the abundance of RFC (Figure 2.5), FRα (Figure 2.6), and BCRP (Figure 2.7) compared to the control. Due to very low abundance of the PCFT in non-confluent cells, there was no detectable signal in all treatment groups (Results are not shown).

2.3.5 Nuclear Localization of β-Catenin

The nuclear localization of β-Catenin was not affected by folate treatment, regardless of the form and concentrations of folate that Caco2 cells were exposed to (Figures 2.8 and 2.9).

2.4 Discussion

2.4.1 Folate Treatment Did Not Affect Caco2 Cell Viability and Proliferation

It has been well documented that folate deficiency decreases cell viability and proliferation. Folate deficiency affects DNA stability through its role as a carbon donor in the synthesis of thymine from uracil (Duthie *et al.*, 2002). In times of cellular folate deficiency, the metabolism of one-carbon units is reduced. In theory, treating Caco2 cells treated with FA or 5MTHF should have increased cell viability and proliferation compared to the cells cultured in the control medium, which contained very small levels of folate due to the dFBS. In this study, there was no difference in cell viability between the control and the FA or 5MTHF treatment groups after 3 days (Figure 2.1). In terms of cell proliferation, treating the cells with FA at 3.4 μM significantly increased DNA synthesis compared to the control (Figure 2.3); however treating the cells with FA at lower concentrations had no effect on DNA synthesis compared to the control. Further, treating cells with 5MTHF at all three concentrations tested had no effect on DNA synthesis. This lack of consistency in folate-induced promotion of DNA synthesis suggests that three days may not be sufficiently long to deplete cellular folate level in the control cells to suppress DNA synthesis in these cells.

Cell proliferation was also investigated with cell cycle analysis. After 3 days folate treatment, compared to the control, there were no differences in the proportion of cells in each phase of the cell cycle (Table 2.2). This finding was consistent with the cell proliferation and cell viability results: after 3 days, there is no effect of folate treatment on the growth of Caco2 cells compared to the control.

Since 3 day folate treatment did not elicit an effect, a longer treatment period of 5 days was investigated. Caco2 cells were first grown in folate free medium for 5 days, followed by a 5 day treatment with either FA or 5MTHF. This 5 day depletion was done to "reset" the cells so their response to FA and 5MTHF could be better observed. In studies with Caco2 and other colon cancer cells lines, 20 days culture in folate free medium depleted the intracellular folate to 98-99% of control (Novakovic *et al.*, 2006). Following 5 days culture in 0 folate and 5 days treatment with FA or 5MTHF, there was no difference in cell viability or cell proliferation compared to the control cells (Figure 2.2, Figure 2.4). Cell cycle analysis, on the other hand, revealed 3.6 and 5.3-fold differences in the proportion of cells in the pre-G1 between the treated cells (3.4 µM FA and 5MTHF, respectively) and the control (Table 2.3).

After 5 days treatment, Caco2 cells treated with 3.4 µM FA or 5MTHF the proportion of cells in pre-G1 phase were much higher than the control (Table 2.3; Figure A.2). Caco2 cells treated with 5MTHF had 20.3% of cells in pre-G1 whereas FA treated cells had 13.6% of cells in pre-G1 phase, compared to the control (3.8%; Table 2.3). A possible explanation for the spikes in the proportion of cells in the pre-G1 phase was that the Caco2 cells were becoming confluent. As Caco2 cells approach confluency the proportion of cells in G1 increases while the proportion of cells in the S phase decreases (Ding *et al.*, 1998). As Caco2 cells grow towards confluence they begin to differentiate into a cell with a small intestinal enterocyte phenotype and they start to exhibit contact inhibition and subsequently go into cell cycle arrest (Stierum *et al.*, 2003).

Though the proportion of cells in the pre-G1 phase was higher after treatment with 3.4 µM FA or 5MTHF, the proportion of cells in G1 phase did not change after

folate treatment (Table 2.3). However, treatment with 3.4 µM 5MTHF decreased the proportion of cells in the S phase by 6.7% and the G2/M phase by 26.6% compared to the control. Treatment with 3.4 µM FA also decreased the proportion of cells in the S phase and G2/M phase compared to the control by 4.9 and 9.4%, respectively. This is consistent with Caco2 cells approaching confluency, according to Ding *et al.* (1998).

Comparing the data from 3 day and 5 day folate treatment showed a large difference in cell cycle analysis. The proportion of cells in the G1 phase is much higher in Caco2 cells treated with either FA or 5MTHF for 5 days (61.9 – 67.3 %; Table 2.3) compared to the cells treated with the same treatments for 3 days (48.1 – 52.5 %; Table 2.2). On day 5, the folate treated groups had percentages of cells in the S phase ranging from 18 – 22.4% (Table 2.3). The percentage of cells in the S phase of the 3 day folate-treated Caco2 cells was 30.8 – 35.7% (Table 2.2). As explained earlier, as cells approach confluency, the proportion of cells in the G1 phase increases while the proportion of cells in the S phase decreases (Ding *et al.*, 1998). Compared with Caco2 cells treated with FA and 5MTHF at all concentrations for 3 days, 5 day treated Caco2 cells appeared to corroborate this observation. However, the 3 and 5 day treatments were separate experiments. They were done at different times and the 5 day treatment was preceded by a 5 day folate depletion period. These differences in folate treatment regimen made any direct comparison between the two experiments unreliable.

A study in normal human fibroblast cells (GMS03349) explored the effect of folate deficiency on the cell cycle and found that by day 7 of culturing cells in folate-deficient media, there is a change in the proportion of cells in the S phase (Katula *et al.*, 2007). This difference may be attributed to the degree of confluency: as mentioned

above, the proportion of Caco2 cells in the G1 phase is inversely related to the proportion of cells in the S phase as cells grow more confluent (Ding *et al.*, 1998).

After 3 and 5 days, both FA and 5MTHF at the concentrations tested did not have an effect on pre-confluent Caco2 cell proliferation determined using the BrdU incorporation assay compared to the control. However, cell cycle analysis shows a spike in the proportion of the cells in the pre-G1 phase after 5 days of 3.4 µM folate treatment compared to the control. Both assays assess cell proliferation using BrdU incorporation; however the BrdU incorporation assay looks at the whole population of cells while cell cycle analysis uses BrdU as a marker to indicate individual cells engaged in DNA synthesis, a hallmark of the S phase. So the BrdU incorporation assay can be swayed by the number of cells: the more cells there are, the more intense the green signal. A more intense signal may indicate a growth promoting effect or if seeding density was different among treatments or if cells were lost during the assay, it could cause a misleading result.

The results of the BrdU incorporation assay and cell cycle analysis together are an indication of cell proliferation. Based on the results, compared to the control folate treatment, regardless the form and concentrations tested, did not have an effect on cell proliferation. Likely, the lack of an effect is due to the treatment duration. In other experiments, treatments are much longer, starting at 20 days and increasing. Perhaps 3 and 5 day periods were too short to have a significant effect on cell proliferation.

2.4.2. Apparently Differential Effect of FA and 5MTHF on Cell Viability and Proliferation

Caco2 cells treated with FA at all concentrations tested resulted in a higher level of cell proliferation compared to the cells treated with 5MTHF at the same concentrations

after 3 days (Figure 2.3). The differential effects of FA on cell proliferation compared to 5MTHF have not been well explored in cell culture or animal studies, let alone in humans. Perhaps this difference can be attributed to high levels (0.101 mM) of methionine in the culture medium. Total plasma methionine in healthy human adults should be less than 10.5 – 11.7 µmol/L to ensure no increased risk for vascular disease (Stampfer *et al.*, 1992), which is much less concentrated than the cell culture medium. Methionine loading in healthy human subjects leads to increased plasma concentrations of SAM and homocysteine, and a decrease in plasma 5MTHF (Loehrer *et al.*, 1996). The authors theorized that the decreased serum 5MTHF concentration is caused by increased turnover of the homocysteine remethylation reaction that requires 5MTHF as a methyl donor (Loehrer *et al.*, 1996). However, in rat liver, when methionine concentrations are high, it leads to increased SAM levels, which inhibits MTHFR (Krebs *et al.*, 1976). The inhibition of MTHFR increases levels of 5,10-methyleneTHF and other C_1-THF derivatives, including 10-formylTHF (Krebs *et al.*, 1976). The increased level of 10-formylTHF increases the activity of formylTHF dehydrogenase (catalyzes the converson of 10-formylTHF to THF) and causes a drop in 5MTHF levels (Krebs *et al.*, 1976). In this way, in the presence of high dietary or extracellular methionine, the cell disposes of excess C_1 units and decreases 5MTHF concentrations (Krebs *et al.*, 1976).

Akoglu *et al.* (2004) observed that hyperproliferation caused by high concentrations of homocysteine (2 µmol/L) was reversed by folate supplementation (5MTHF had a more anti-proliferative effect than folic acid) in Caco2 cells. This study also showed that folate treatment after exposure to high levels of homocysteine led to an increased proportion of Caco2 cells in the G1 phase (Akoglu *et al.*, 2004). In addition,

SAM is an allosteric inhibitor of MTHFR, which would lower the amount of 5,10-methyleneTHF converted to 5MTHF (Brosnan and Brosnan, 2006). Perhaps 5MTHF treatment did not have a proliferative effect on Caco2 cells because of the presence of methionine in the medium. According to the manufacturer, RPMI 1640 medium is formulated with 0.101 mM L-methionine (Gibco, Grand Island, NY). Perhaps methionine doesn't have the same effect on FA treated cells because FA is reduced first to DHF and then to THF, and it can be used in DNA synthesis reactions.

FA must first be reduced to DHF and further to THF to be able to carry out biological functions in cells. THF is then able to participate in the conversion of deoxyuridylate to thymidylate (as 5,10-methyleneTHF) and the synthesis of purines (as 10-formylTHF) before it acts as a methyl donor to homocysteine (Figure 1.2). 5MTHF, on the other hand, must donate a methyl group to homocysteine to produce methionine before it is able to participate in any of the nucleotide synthesis functions. Perhaps this accounts for the differences in BrdU incorporation observed in Caco2 cells after 3 days treatment with FA or 5MTHF (Figure 2.3). FA enters the cycle in a form that is the precursor for any of the functions of folate. If this were the case, it would take a longer period of time for 5MTHF supplementation to have the initial effects on proliferation decrease. This is exactly what was observed in Caco2 cells after 5 days treatment with either FA or 5MTHF (Figures 2.3, 2.4). After 3 days, cell proliferation was significantly higher at all concentrations of FA compared to the same concentrations of 5MTHF. However, after 5 days of FA treatment there was no difference in cell proliferation compared to cells treated with 5MTHF (Figure 2.4).

In lymphocytes isolated from healthy females, a higher frequency of apoptosis and lower cell viability was observed in the cells treated with 5MTHF at 12 or 120 nM for 9 days; but not in cells treated with FA at the same concentrations (Wang and Fenech, 2003). The lowest amount of apoptosis was observed in lymphocytes treated with 120 nM FA and the highest apoptosis levels were observed after treatment with 5MTHF (Wang and Fenech, 2003). In this thesis study, treating Caco2 cells with FA at 0.9 µM for 3 days increased cell viability by 15.8 % compared to the cells treated with 5MTHF at the same concentration (Figure 2.1). In HT29 colon cancer cells, exposure to FA at 0.23 µM for 3 weeks exhibited a faster growth and higher metabolic activity (as measured by the concentration of folate metabolites: SAM, SAH and 5MTHF) than cells exposed to lower levels of FA (Pellis *et al.*, 2008). Thus, this observation fits in with folate's role in DNA synthesis and the observed increases in cell proliferation and viability in Caco2 cells treated with FA in this research.

Pellis *et al.* (2008) reported much higher levels of apoptosis in HT29 cells treated with 0.23 µM FA for 3 weeks compared to lower concentration treatments (10 ng/mL; 0.023 µM). This same phenomenon was not observed at 3 days with Caco2 cells (Table 2.2). There was no difference in the proportion of cells in the pre-G1 phase (apoptosis) regardless of the treatment (Table 2.2). The previously mentioned studies in lymphocytes (Wang and Fenech, 2003) and HT29 cells (Pellis *et al.*, 2008) had much longer treatment duration (9 days and 3 weeks, respectively); It seems that a 3 day folate treatment is not long enough to bring about a difference in the proportion of cells in pre-G1 phase.

Caco2 cells treated with a higher concentration of FA (3.4 µM) exhibited a 15.9% increase of cells in the S phase compared to cells treated with 5MTHF. FA treatment

resulted in increased cell proliferation at all concentrations tested and increased cell viability at 0.9 µM compared to 5MTHF treatment over 3 days.

A study looking at the effects of different folates in Caco2 cells concluded that 5MTHF, along with DHF, are growth-inhibitory compounds in colon cancer cells (Akoglu *et al.*, 2001). They observed a larger decrease in cell proliferation relative to the control after 48 hour treatment compared to 24 hours (Akoglu *et al.*, 2001). The authors reasoned that after 48 hours, FA had been reduced to the active compounds of growth inhibition, 5MTHF and DHF (Akoglu *et al.*, 2001). If their reasoning holds correct then a longer folate treatment should result in less difference in cell proliferation between cells treated with FA and 5MTHF. After 3 days treatment with 3.4 µM FA or 5MTHF, 5MTHF treated cells had decreased proliferation. After 5 days there was no statistical difference among any of the groups.

One of the reasons for using FA as a fortificant is because of its stability: FA can stand long periods of storage, light, and cooking temperatures. 5MTHF is much more susceptible to oxidation than FA. The observed difference in cell proliferation and cell viability could be attributed to the stability of 5MTHF in the treatment medium. 5MTHF is oxidized first to 5-methyldihydrofolate (5MDHF) and then to *p*-aminobenzoylglutamate (the result of C_9-N_{10} scission), after which reducing agents cannot rescue it back to its original form (Ng *et al.*, 2008). 5MTHF is more stable at acidic pH than at neutral or basic pH (Ng *et al.*, 2008) and its thermal stability is higher as well (Liu *et al.*, 2011). Addition of ascorbic acid to 5MTHF-containing solutions increases the salvage of 5MTHF and reduces oxidative break down, however after 426 minutes in pH 7 buffer, 50% of 5MTHF is lost, a significant amount (Ng *et al.*, 2008).

Sodium ascorbate can regenerate 5MTHF after is has been exposed to heat (Liu *et al.*, 2011. Addition of sodium ascorbate can regenerate 5MTHF to 93 ± 3% and 87 ± 4% after heating at 50°C for 60 and 150 minutes, respectively (Liu *et al.*, 2011). A study measuring serum folate response to oral doses of 5MTHF in healthy adult men found that administering 5MTHF with L-ascorbic acid results in significantly higher area under the curve folate response compared to administering 5MTHF alone (Verlinde *et al.*, 2008). When treating Caco2 cells, the utmost care was taken to ensure as little damage to 5MTHF as possible. Stock solutions were made containing 5% sodium ascorbate, stock solutions were aliquotted and frozen immediately (aliquotted stock was used within 1 month) and stock solutions were protected from the light. Despite precautions taken, some 5MTHF was likely oxidized in the treatment medium, meaning less 5MTHF was available for Caco2 cells in the experiments than FA, which is stable in the medium. This could have resulted in reduced viability and proliferation in the 5MTHF treated Caco2 cells.

Difference in the bioavailability of 5MTHF and FA likely cannot explain differences in observed cell proliferation, as 5MTHF is equal to or more bioavailable than FA. As discussed in Chapter 1, natural folate has a polyglutamate tail which must be hydrolyzed before the folate can be absorbed. As a result naturally occurring dietary folates (with polyglutamate tails) have a lower bioavailability than FA. The 5MTHF used in this thesis research is a synthetic, monoglutamylated form. Studies comparing the bioavailability of 5MTHF and FA in humans have shown that 5MTHF has equivalent or higher bioavailability compared to FA. In women of childbearing age there was no difference in plasma folate or red blood cell (RBC) folate after supplementation with FA

and 5MTHF (Venn *et al.*, 2002). In a double-blind, crossover study, 13 healthy men who were presaturated with FA received placebo capsules, 500 µg FA capsules, or 500 µg 5MTHF capsules for a 1 week interval in random order (Pentieva *et al.*, 2004). There was no difference in maximum plasma folate response or area under the curve for plasma folate between the two folate treatments (Pentieva *et al.*, 2004). Lamers *et al.* (2006) conducted a double-blind, randomized, placebo-controlled intervention study in which healthy women aged 19-33 years received 400 µg FA, 416 µg 5MTHF, 208 µg 5MTHF, or placebo daily for 24 weeks. RBC folate increased significantly more with 416 µg 5MTHF supplementation compared to 400 µg FA and 208 µg 5MTHF (Lamers *et al.*, 2006). It was also found that the short term bioavailability of 5MTHF was higher compared to FA in healthy men and women (in a repeated measures crossover design experiment) as measured by plasma folate (Harvey, 2011). In a randomized, crossover study, healthy women received a single oral dose of 400 µg FA and 416 µg 5MTHF (Prinz-Langenohl *et al.*, 2009). Prinz-Langenohl *et al.* (2009) found that area under the curve of plasma folate and maximum plasma folate concentration were both significantly higher for 5MTHF compared to FA. Studies involving 5MTHF and FA in humans suggest equal or better bioavailability of 5MTHF compared to FA. However, in Caco2 cells, no folate uptake studies were performed, so it cannot be ruled out that folate uptake differences played a role in the differences observed in cell proliferation after 3 days treatment with FA and 5MTHF.

2.4.3 The Effect of Folate Treatment on the Abundance of Folate Transporters

In this study, folate form and concentration had no significant effect on the abundance of PCFT, RFC (Figure 2.5), FRα (Figure 2.6) and BCRP (Figure 2.7). These observations differ from previous studies that have shown that these transporters are affected by extracellular folate concentration.

The presence of PCFT protein in pre-confluent Caco2 cells was not detectable in this study. A possible cause was a low abundance of PCFT in pre-confluent Caco2 cells. Subramanian *et al.* (2008) reported low levels of PCFT in pre-confluent Caco2 cells, which possess a colonocyte phenotype. Upon confluence PCFT expression increases as the cells differentiate into a phenotype resembling that of a small intestinal enterocyte (Subramanian *et al.*, 2008). Since this study used undifferentiated Caco2 cells that don't possess a small intestinal enterocyte phenotype, PCFT may not have been expressed in high enough levels to be detected. The lack of signal for PCFT could also be due to the assay not working. This remained as a possibility as there was no positive control used during the assay.

It was observed that *Pcft* mRNA in laying hens was downregulated in jejunal tissue when the chickens were fed a diet supplemented with 5MTHF compared to chickens fed the control diet, whereas chickens fed a FA-supplemented diet showed no change compared to chicken fed the control diet (Jing *et al.*, 2010). Other groups have shown the folate status affects the expression of PCFT, for example in Caco2 cells supplemented with 0.25, 9 and 100 µM FA, *PCFT* mRNA expression decreases with rising folate concentration (Ashokkumar *et al.*, 2007). However, both studies only

reported *PCFT* mRNA expression, which does not necessarily correlate with protein abundance.

RFC abundance was not affected by folate concentration or form (Figure 2.5). These observations are inconsistent with what has been previously observed. For example, Caco2 cells treated with 0.25, 9 and 100 µM FA for 5 passages showed an inverse relationship between FA concentration and RFC protein and mRNA abundance (Ashokkumar *et al.*, 2007). The relative abundance of protein between cells treated with 0.25 µM FA (140%) and 100 µM FA (50%) is approximately 3:1 (Ashokkumar *et al.*, 2007). This ratio is similar to what was observed in Caco2 cells after 5 days treatment with 3.4 µM 5MTHF compared to the control (Figure 2.5). The Caco2 cells in the Ashokkumar *et al.* (2007) study were harvested after 5 generations of treatment and 3-4 days post-confluence. At this point the Caco2 cells have begun to differentiate. Other groups have shown that *RFC* mRNA is upregulated in Caco2 cells exposed to 0 folate conditions (Cockman, 2009; Subramanian *et al.*, 2003) and also in human colonic epithelial cell lines (Crott *et al.*, 2008).

As RFC is upregulated in folate deficiency, one would expect that RFC would be most abundantly expressed after 10 days culture in very low folate conditions (control group). The expression of RFC in Caco2 cells changes depending on the confluency of the cells (Subramanian *et al.*, 2008). In Caco2 cells, confluent and post-confluent cells have much higher relative mRNA expression of RFC compared to preconfluent cells, and this was confirmed by Western blot (Subramanian *et al.*, 2008). No difference was observed in RFC relative protein abundance between the treated groups and the control. Based on the evidence in the literature, a difference in RFC protein levels between folate

concentrations was expected. Given that the cells were harvested before confluency and thus before differentiation took place, this could explain the lack of difference in RFC protein levels among different treatments. The degree of differentiation is important as the cells take on a different phenotype, transforming from a cancerous colonocyte to a cell with a small intestinal cell phenotype. Folate is absorbed largely in the small intestine, where the folate transporters are found in higher abundance than in the large intestine.

FRα abundance was not significantly affected by folate treatment in Caco2 cells in this study (Figure 2.6). FRα has been previously shown to be upregulated in folate deficient Caco2 cells (Cockman, 2009), in folate deficient renal HK-2 cells (Ashokkumar *et al.*, 2007) and human colonic epithelial cell lines treated with 25 nM FA compared to 50, 75 and 150 nM FA (Crott *et al.*, 2008). With this in mind, it was expected that the control group would have the highest relative abundance of FRα, however no differences were observed among any of the groups.

BCRP expression is not affected by Caco2 confluency and it is found on the apical membrane (Xia *et al.*, 2005). After 5 days culture in free folate medium followed by folate treatment for 5 days, expression of BCRP was not affected by folate form or concentration (Figure 2.7). BCRP has been found to be induced in folate deficient Caco2 cells (Lemos *et al.*, 2008). It has also found to be either up-regulated or down-regulated by methylation of the promoter region of the *BCRP* gene in lung cancer cells (Nakano *et al.*, 2008), renal carcinoma (To *et al.*, 2006), pancreatic cancer cell lines (Chen *et al.*, 2012) and multiple myeloma cell lines and patient plasma cells (Turner *et al.*, 2006). The evidence from other cancer cell lines points to a possibility that BCRP abundance

depends on the methylation status of CpG islands in the promoter region. If this is the case in colon cancer, it could explain the upregulation in folate deficiency. However, in this study, the control Caco2 cells did not show increased BCRP expression relative to folate supplemented cells (Figure 2.7). If BCRP is indeed regulated by promoter methylation, the control cells would be expected to have the highest abundance of BCRP, especially considering that BCRP expression is apparently not affected by Caco2 cell confluency. However, BCRP is regulated by promoter methylation in different tissues, for example, lung cancer, renal carcinoma, and pancreatic cancer. Promoter methylation is tissue specific, so what is true for lung cancer cells, for example, is not necessarily true for colorectal adenocarcinoma cells.

The abundance of folate transporters was unaffected by folate form or concentration in Caco2 cells after 5 days of folate treatment. Perhaps 5 days of folate treatment was not enough time to elicit a change in the abundance of folate transporters. In other experiments, folate treatment has been considerably longer than 5 days. For example, Ashokkumar *et al.* (2007) found a difference in the expression of RFC, PCFT and FRα after maintaining the cells in folic acid for 5 generations in Caco2 and HK-2 cells. Crott *et al.* (2008) found differences in the expression of RFC (*SLC19A1*) and FRα (*FOLR1*) after treating cells for 32-34 days.

The response of Caco2 cells to folate treatment was surprising as it did not correlate with what was previously been found in the literature for each transporter. Perhaps there is something else at work. Due to the instability of 5MTHF, it is likely that some was oxidized in the medium. Perhaps the oxidative breakdown products of 5MTHF have an effect on the abundance of folate transporters. Unfortunately, there seems to be

no published studies about the effect of 5-methylDHF and *p*-aminobenzoylglutamate on folate transporter abundance at this time.

2.4.4 Nuclear Localization of β-Catenin was Unaffected by Folate Treatment

β-Catenin nuclear localization can be used as a measure of Wnt signalling pathway activity because translocation of this protein into the nucleus is an essential step in the pathway (Archbold *et al.*, 2011). Once inside the nucleus, β-Catenin complexes with transcription factors TCF/LEF1 to regulate gene transcription (Archbold *et al.*, 2011).

Studies have shown that the localization of β-Catenin can be affected by folate treatment. Morillon II (2008) showed that β-Catenin nuclear localization increased in NIH3T3 cells treated with 0 folate for 10 days compared to cells treated with a "sufficient" [sic] amount of folate. Another study found that FA supplementation (5 mg daily for 1 year) in patients with adenomatous polyps decreased β-Catenin nuclear localization in primary colorectal tissue when compared to baseline measurements (Jaszewski *et al.*, 2004). However, in this research, folate treatment had no significant effect on β-Catenin nuclear localization (Figures 2.8, 2.9). This absence of decreased β-Catenin nuclear localization in response to folate treatment could be due to a combination of a relatively short duration of folate treatment and low folate concentration. Furthermore, the responsiveness of cells to folate status-related β-Catenin cellular translocation may also be cell type specific. To date, there have been no studies investigating the effect of different forms of folate on β-Catenin nuclear localization. This

should be investigated more thoroughly involving longer duration of folate treatment and higher folate concentration.

2.4.5 Summary

In summary, this thesis project showed that, after 3 days of folate treatment, Caco2 cells treated with FA had increased cell proliferation at all three concentrations tested and increased cell viability at 0.9 μM compared to the cells treated with 5MTHF at the same concentration. After 5 days of folate depletion followed by 5 days folate treatment, there was no difference of cell viability or proliferation among Caco2 cells treated with FA or 5MTHF at any concentration. The mechanism behind the increases in cell proliferation and cell viability after treatment with FA compared to 5MTHF is unclear. The relative abundance of RFC, FRα and BCRP was not affected by the form of folate at the concentrations tested, which was contrary to what has been previously observed in the literature. β-Catenin nuclear localization was also not affected by the form of folate at the concentrations tested. However, many studies used longer folate treatment duration at higher folate concentrations; perhaps these differences in the folate treatment regimen played a role in the incongruous results. Reflecting back, treatment duration was too short and a chronic folate treatment model should have been used instead.

Table 2.1: Antibodies used in the Western blots.

Target Protein	**Antibody**[1]	**Dilution**	**Catalogue Number**
BCRP	1°: Rabbit anti-ABCG2 polyclonal IgG	1:1,000	sc-25821
	2°: Bovine anti-rabbit IgG-HRP	1:5,000	sc-2370
RFC	1°: Rabbit anti-RFC1 polyclonal IgG	1:400	sc-98971
	2°: Bovine anti-rabbit IgG-HRP	1:2,500	sc-2370
FRα	1°: Rabbit anti-FR polyclonal IgG	1:300	sc-28997
	2°: Bovine anti-rabbit IgG-HRP	1:5,000	sc-2370
PCFT	1°: Goat anti-HCP1 polyclonal IgG	1:300	sc-54204
	2°: Donkey anti-goat IgG-HRP	1:2,500	sc-2020
α-Tubulin (loading control)	1°: Mouse anti-α tubulin monoclonal IgG_{2A}	1:300	sc-5286
	2°: Goat anti-mouse IgG_{2A}-HRP	1:1,000	sc-2061

[1] All antibodies were obtained from Santa Cruz Biotechnology.

Table 2.2: Cell cycle analysis of Caco2 cells following 3 day treatment with FA or 5MTHF.

Sample	Pre-G1 phase	G1 Phase	S phase	G2/M Phase
Control	5.7	52.5	34.1	8.6
0.9 µM FA	6.0 (+5.3 %)	49.9 (-5 %)	35.4 (+3.8 %)	9.3 (+8.1 %)
2.3 µM FA	6.5 (+14 %)	51.1 (-2.7 %)	33.6 (-1.5 %)	9.1 (+5.8 %)
3.4 µM FA	6.2 (+8.8 %)	48.1 (-8.4 %)	35.7 (+4.7 %)	12 (+39.5 %)
0.9 µM 5M	7.7 (+35.1 %)	52.2 (0 %)	33.5 (-1.8 %)	10.6 (+23.2 %)
2.3 µM 5M	5.1 (-10.5 %)	49.9 (-5 %)	32.9 (-3.5 %)	11.9 (+38.4 %)
3.4 µM 5M	6.2 (+8.8 %)	52.2 (0 %)	30.8 (-9.7 %)	12.4 (+44.2 %)

Data from cell cycle analysis of Caco2 cells after 3 days treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or MTHF for 3 days. Cell cycle was determined using BrdU incorporation for S phase detection and PI staining for total DNA and analyzed by flow cytometry. The table shows proportion of cells in each phase. Change relative to the control is in brackets. Scatter plots and histograms for cell cycle analysis are shown in Figure A.1.
Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).

Table 2.3: Cell cycle analysis of Caco2 cells following 5 day treatment with FA or 5MTHF.

Sample	Pre-G1 phase	G1 phase	S phase	G2/M phase
Control	3.8	62.3	22.4	12.8
0.9 µM FA	3.7 (-2.6 %)	67.3 (+8 %)	18 (-19.6 %)	11.8 (-7.8 %)
2.3 µM FA	2.8 (-26.3 %)	61.9 (-0.6 %)	21.9 (-2.2 %)	12.5 (-2.3 %)
3.4 µM FA	13.6 (+ 3.6 fold)	63.4 (+1.8 %)	21.3 (-4.9 %)	11.6 (-9.4 %)
0.9 µM 5M	3.8 (0 %)	67.3 (+8 %)	18.5 (-17.4 %)	11.9 (-7 %)
2.3 µM 5M	3.5 (-7.9 %)	63.6 (+2.1 %)	20.6 (-8 %)	13.5 (+5.5 %)
3.4 µM 5M	20.3 (+ 5.3 fold)	64 (+2.7 %)	20.9 (-6.7 %)	9.4 (-26.6 %)

Data from cell cycle analysis of Caco2 cells after 5 days treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. Cell cycle was determined using BrdU incorporation for S phase detection and PI staining for total DNA and analyzed by flow cytometry. The table shows proportion of cells in each phase. Change relative to the control is in brackets. Scatter plots and histograms for cell cycle analysis are shown in Figure A.2. *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

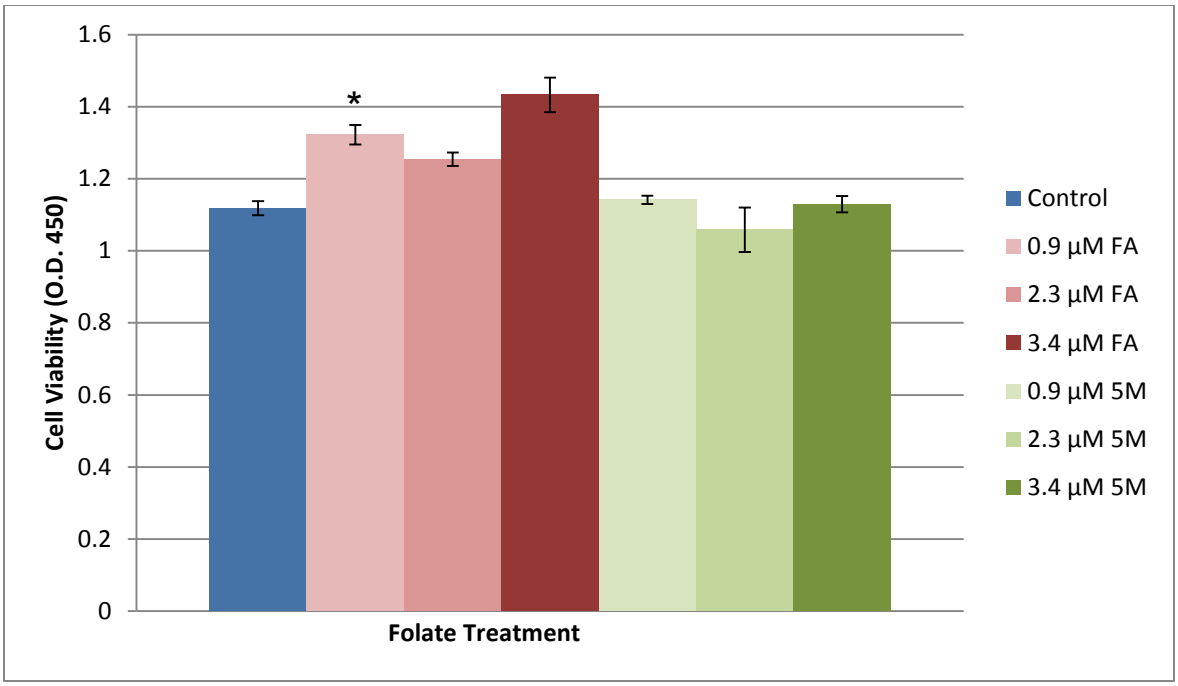


Figure 2.1: Cell viability of Caco2 cells after 3 day treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 3 days. Cell viability was quantified using the WST-1 colorimetric assay where absorbance is equivalent to cell viability. The values represent mean ± SEM (n=10). Means marked with * are significantly different from the corresponding concentration of 5MTHF ($p<0.05$). *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

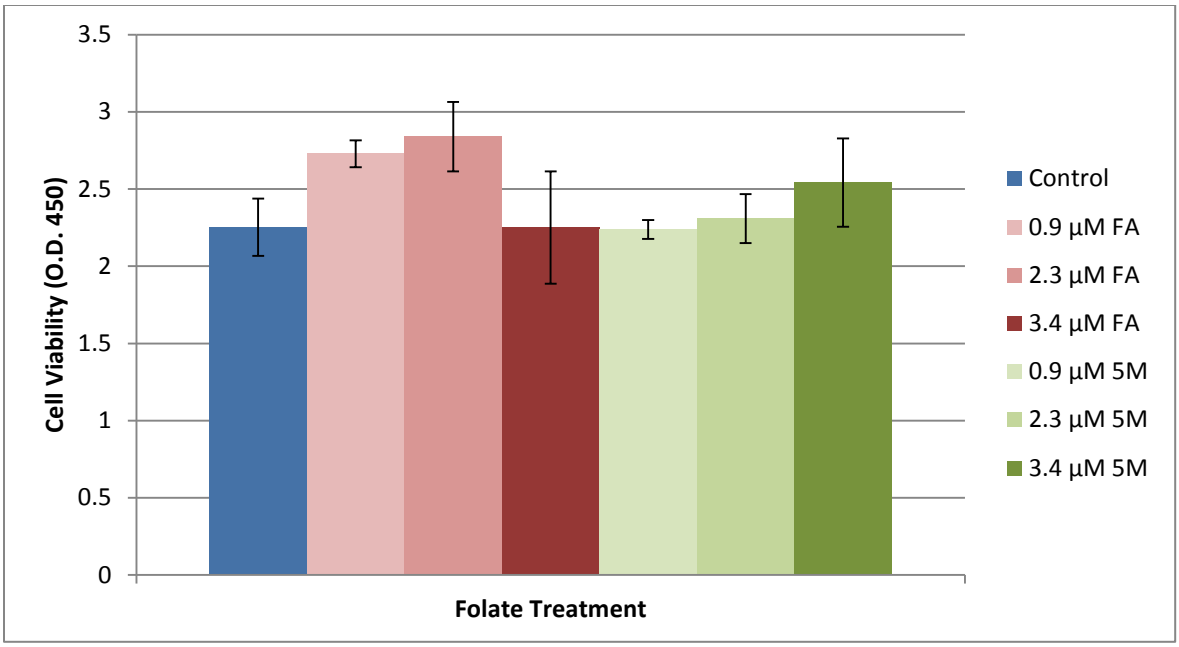


Figure 2.2: Cell viability of Caco2 cells after 5 day treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. Cell viability was quantified using the WST-1 colorimetric assay where absorbance is equivalent to cell viability. The values represent mean ± SEM (n=10). No significant differences were observed among the treatments ($p<0.05$). *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

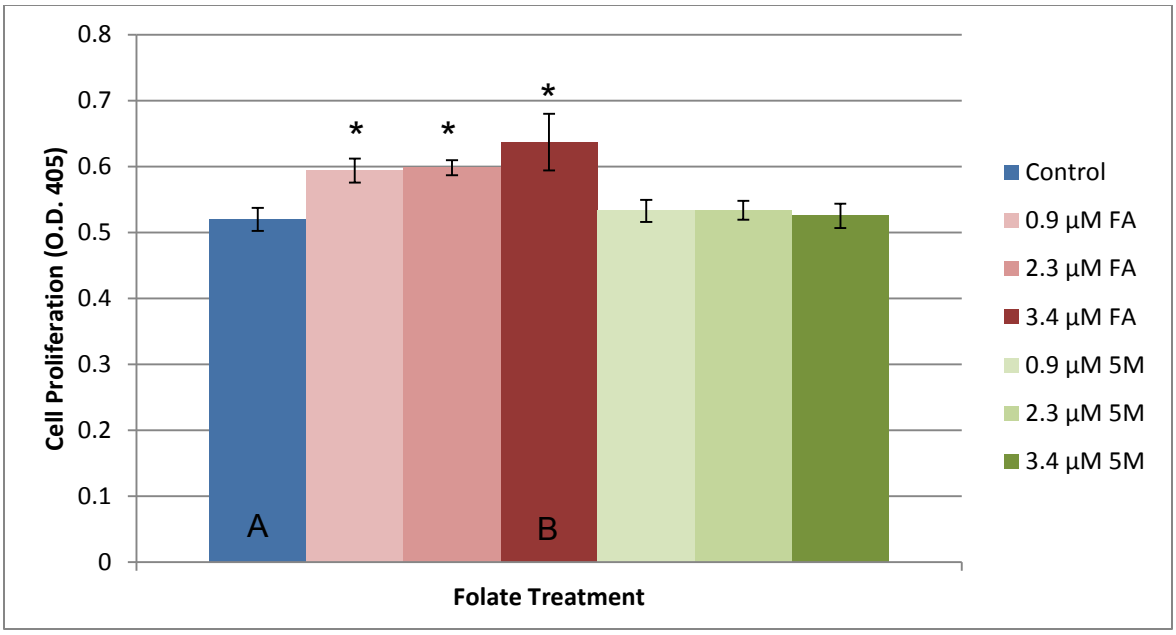


Figure 2.3: Cell proliferation of Caco2 cells after 3 day treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 3 days. Cell proliferation was quantified using the BrdU incorporation assay where absorbance is a measure of BrdU incorporation. The values represent mean ± SEM (n=10). Means marked with capital letters are significantly different from the control, while means marked with * are significantly different from the corresponding concentration of 5MTHF ($p<0.05$). *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

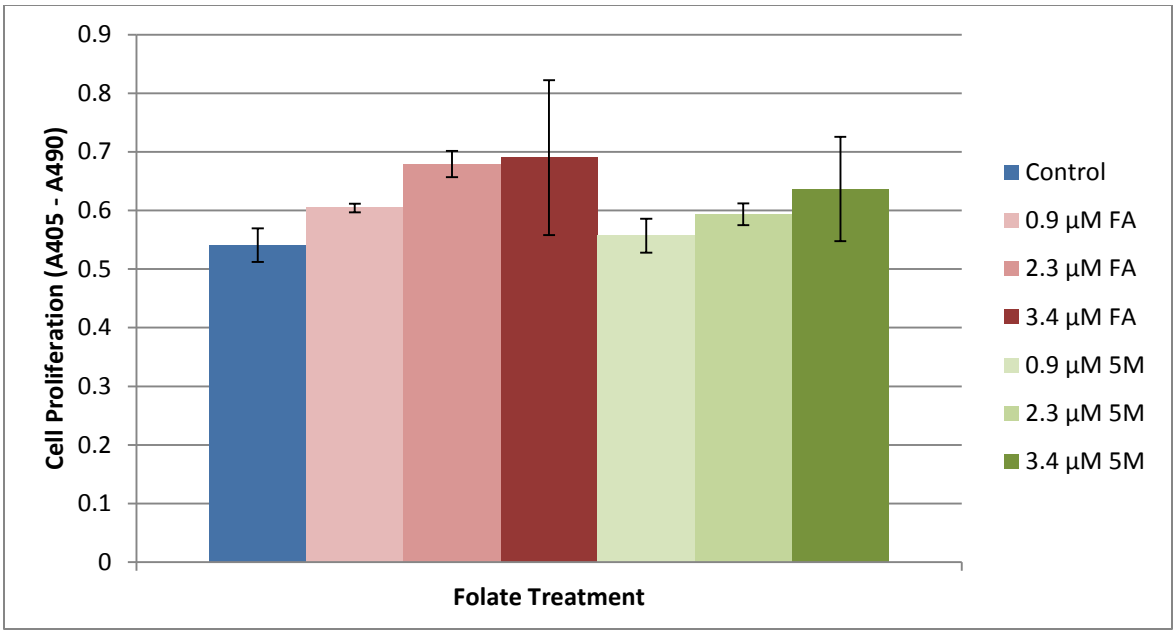
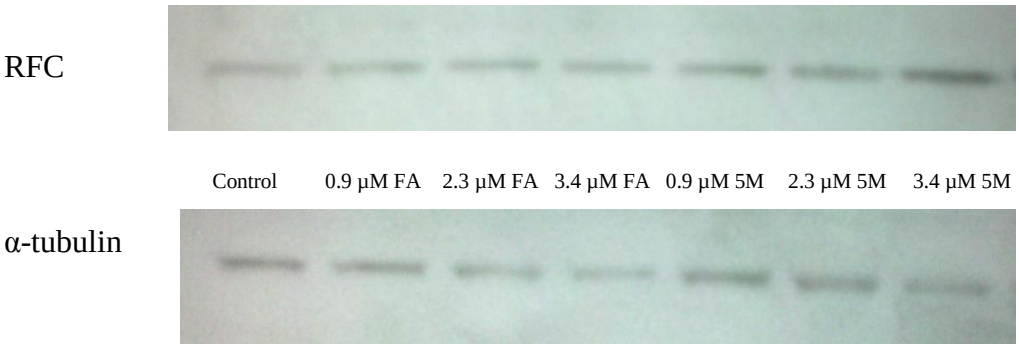


Figure 2.4: Cell proliferation of Caco2 cells after 5 day treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. Cell proliferation was quantified using the BrdU incorporation assay where absorbance is a measure of BrdU incorporation. The values represent mean ± SEM (n=10). No significant differences were observed among the treatments ($p<0.05$). *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

A)



B)

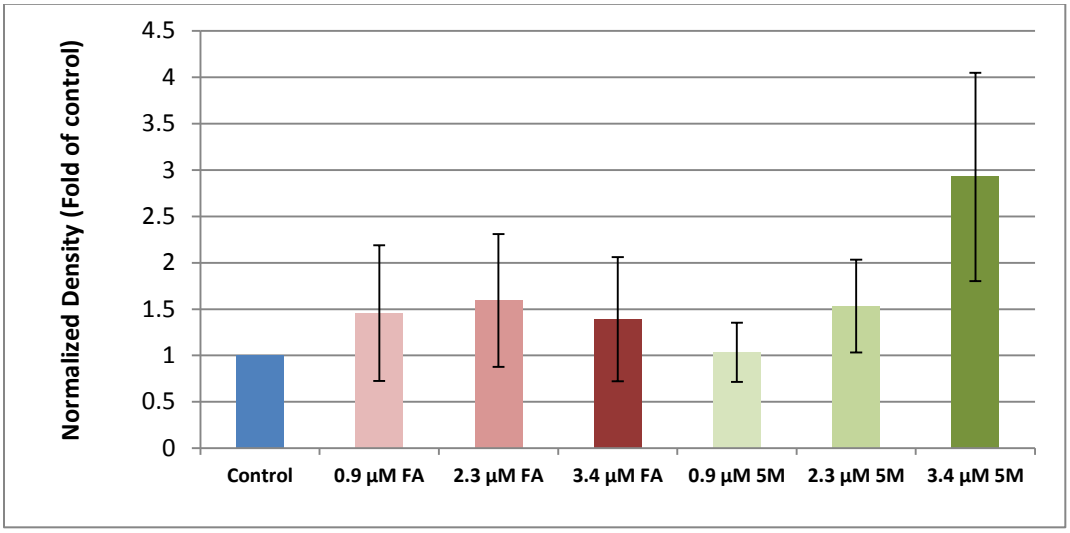
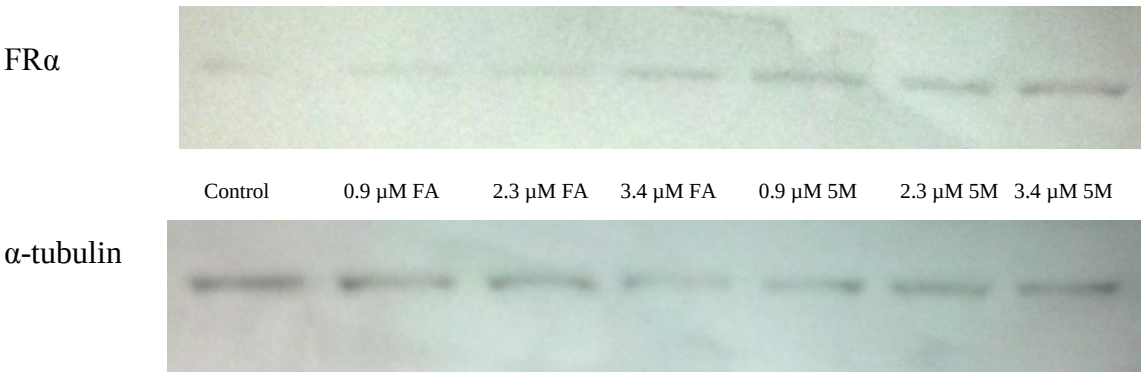


Figure 2.5: Relative protein abundance of RFC in Caco2 cells after 5 day treatment with FA or MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. RFC in whole cell lysate was determined using Western blot. Values represent the mean ± SEM (n=3). Western blot results (A) were analyzed for the ratio of RFC/α-tubulin (B). No significant difference among the treatment groups was observed ($p<0.05$) *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

A)



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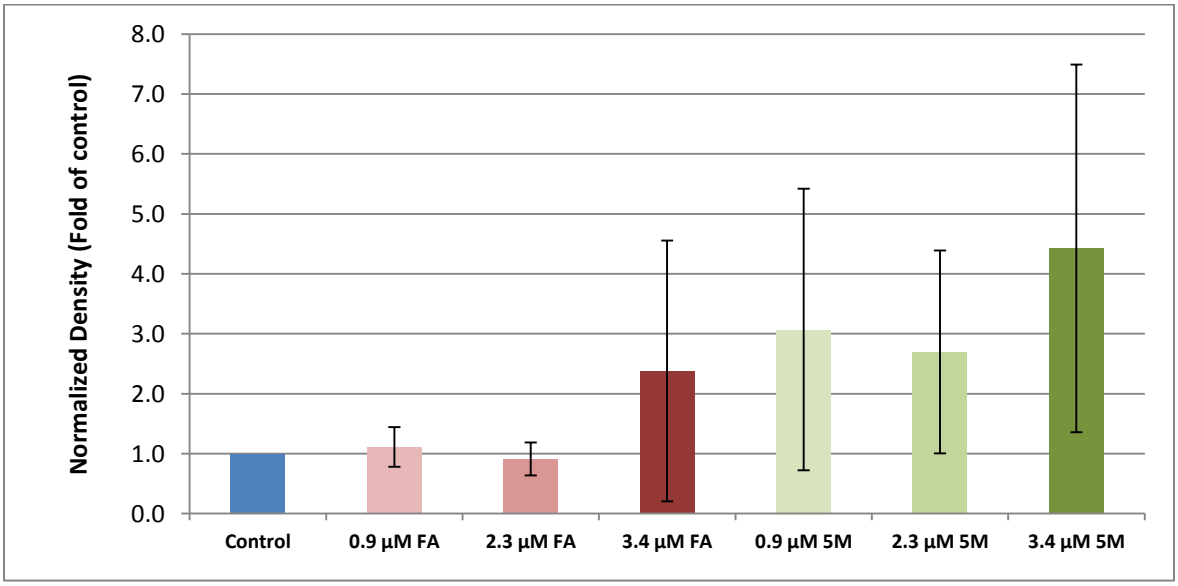
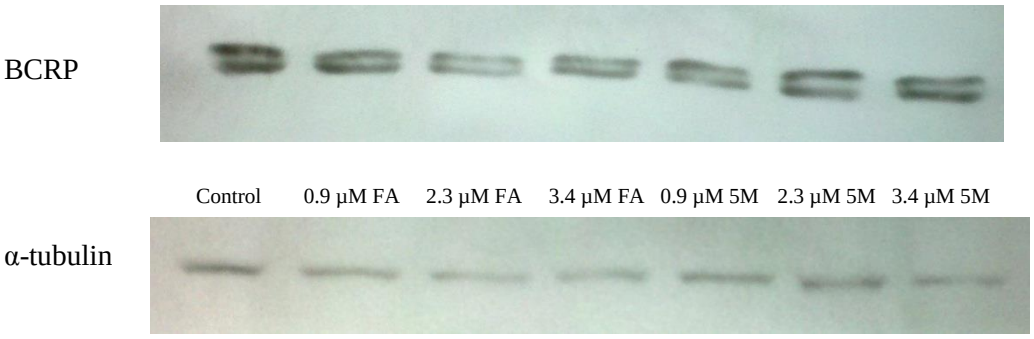


Figure 2.6: Relative protein abundance of FRα in Caco2 cells after 5 day treatment with FA or MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. FRα in whole cell lysate was determined using Western blot. Values represent the mean ± SEM (n=3). Western blot results (A) were analyzed for the ratio of FRα/α-tubulin (B). No significant difference among the treatment groups was observed ($p<0.05$) *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

A)



B)

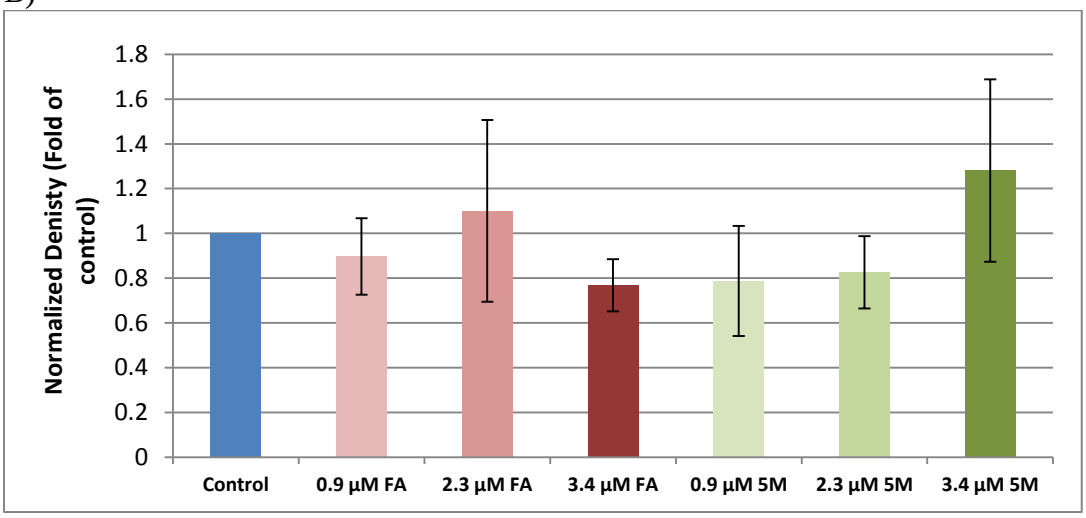


Figure 2.7: Relative protein abundance of BCRP in Caco2 cells after 5 day treatment with FA or MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. BCRP in whole cell lysate was determined using Western blot. Values represent the mean ± SEM (n=4). Western blot results (A) were analyzed for the ratio of BCRP/α-tubulin (B). No significant difference among the treatment groups was observed ($p<0.05$) *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

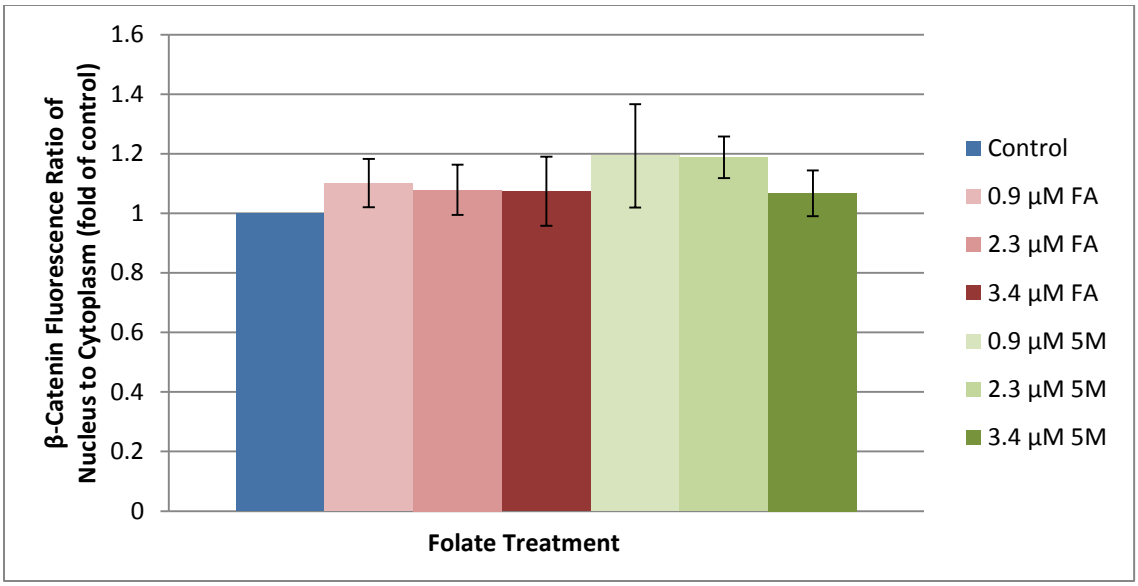
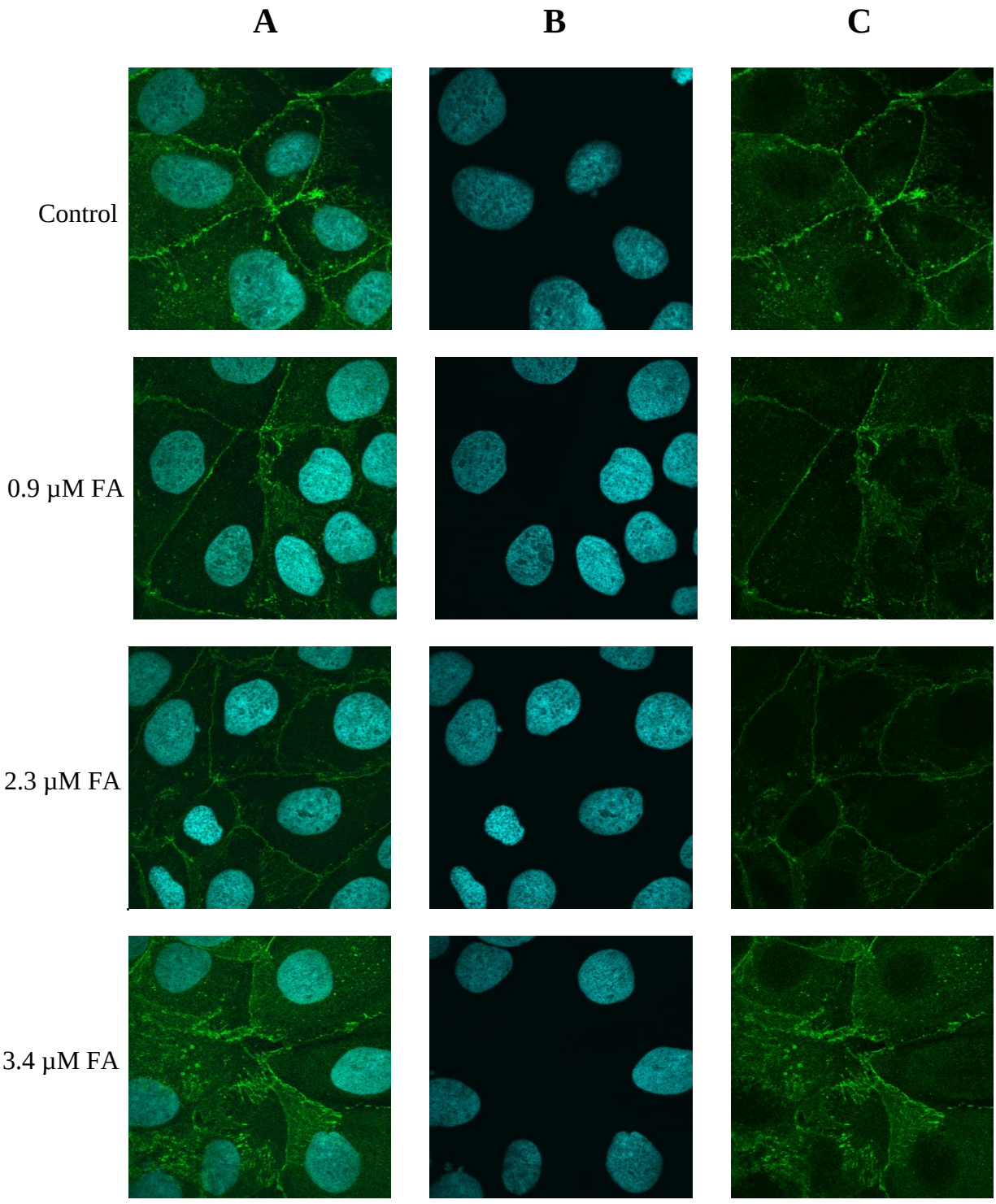


Figure 2.8: β-Catenin fluorescence (ratio of nucleus to cytoplasm) in Caco2 cells after 5 days treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. β-Catenin was visualized with Alexa Fluor 488® and nuclei were counterstained with DAPI. β-Catenin fluorescence is represented by the ratio of nuclear to cytoplasmic fluorescent and normalized to the control. All values are mean ± SEM (n=5 except: n=3 for 0.9 µM 5M, n=4 for 3.4 µM FA and 5M). No significant difference among the treatment groups was observed ($p<0.05$).
Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).


A
B
C
Control
0.9 µM FA
2.3 µM FA
3.4 µM FA

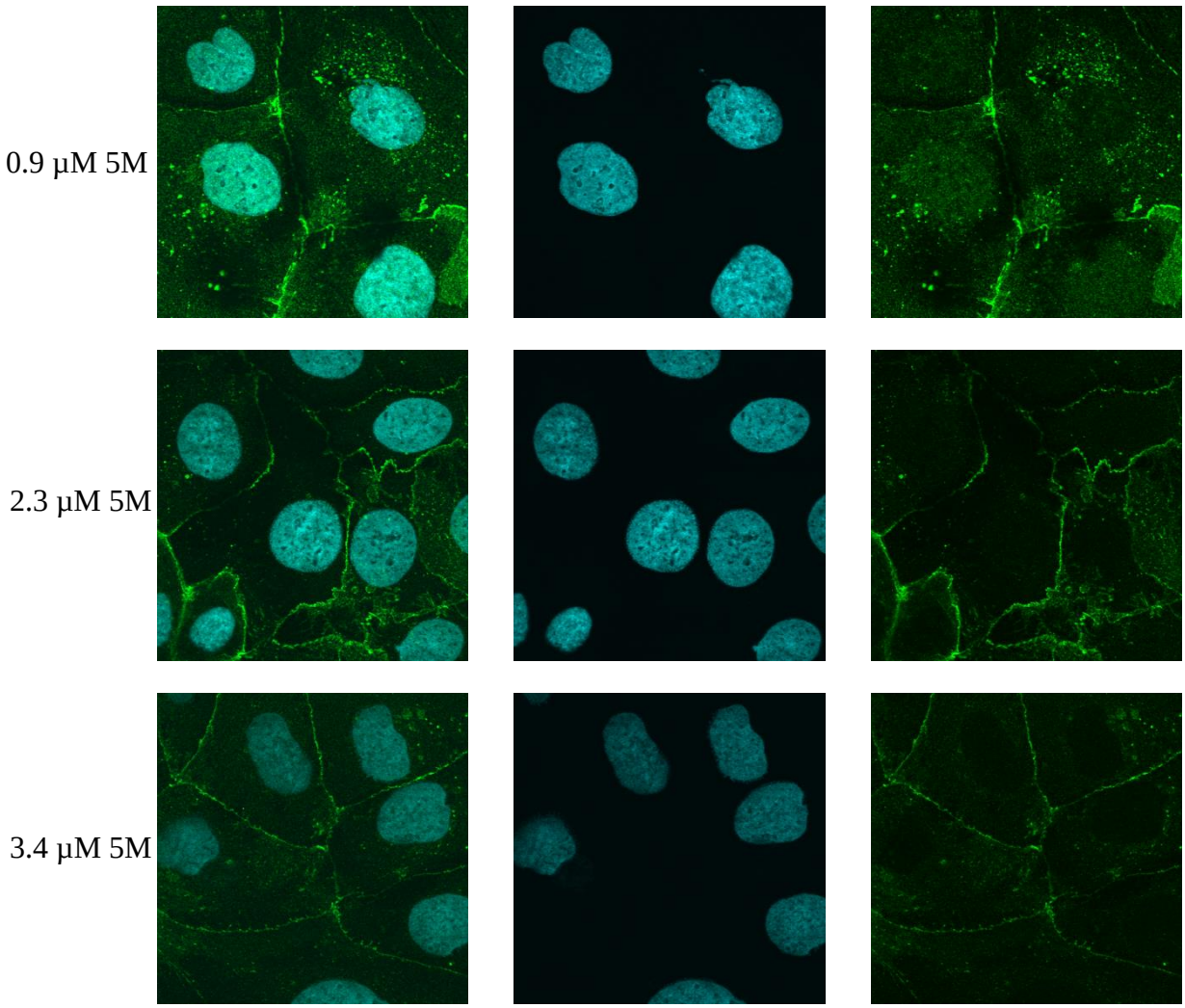


Figure 2.9: β-Catenin fluorescence in Caco2 cells after 5 days treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. β-Catenin was visualized with Alexa Fluor 488® (green) and nuclei were counterstained with DAPI (blue). Images captured using confocal microscopy. (A) Alexa Fluor 488® and DAPI; (B) DAPI only; (C) Alexa Fluor 488® only. *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

CHAPTER 3
Limitations and Future Directions

3.1 Limitations

There are several limitations of this research project. One of the limitations was the use of an *in vitro* system. Although a single-cell system is useful in identifying cellular and molecular targets in the early phase of research, it eliminates the cell-cell communication, interactions, and coordination, and regulation as seen in a multicellular organism system. It is advantageous that the test conditions are relatively easier to control in an *in vitro* system and thus specific phenomena can be examined in more detail, but it is a major limitation because results obtained from an *in vitro* system cannot be extrapolated to whole organisms or even other cell types or cell lines of the same tissue. So results obtained from studies in *in vitro* systems need to be confirmed in *in vivo* afterwards. This research needs to be repeated in other colon cancer cell lines as well as in primary culture and hopefully in the future, an *in vivo* model such as rats or mice.

Another limitation of using an *in vitro* system is that the cell lines have often been transformed to make them immortal. Transformed cells may not have the same characteristics as the original cells *in vivo*. They may have altered biochemical and cellular characteristics than their *in vivo* counterparts. Therefore, results obtained from studies with *in vitro* system are to be treated with caution.

A third limitation is the stability of 5MTHF. 5MTHF is very labile to light, heat and oxidation. Though precautions were taken to prevent oxidation of the compound, it is impossible to fully prevent it from occurring. The oxidation of 5MTHF may have resulted in less 5MTHF in the culture medium and it could have a direct influence on the

results obtained. 5MTHF is first oxidized to 5MDHF, a reversible reaction, and then further oxidized to *p*-aminobenzoylglutamate, which cannot be rescued by the presence of reducing agents (Ng *et al.*, 2008). These oxidation products are not functional as folates.

The conditions within the lumen of the small intestine (where folate is absorbed) and the large intestine are much different than what the Caco2 cells used in this research are exposed to. These conditions would affect the folate treatments differently. For example, intestinal cells can produce a reducing environment which would affect the stability of 5MTHF. These conditions were not recreated in this study. Caco2 cells were used in my thesis research not for their differentiation potential, but because they are colorectal adenocarcinoma cells. *In vivo*, colorectal adenocarcinoma cells would be subjected to the conditions within the lumen of the large intestine, as opposed to the conditions in the experiments: 37°C humidified incubator, 5% CO_2 atmosphere, and cell medium. Therefore, the conditions of the experiments do no replicate the *in vivo* conditions that an actual colorectal adenocarcinoma would be exposed to.

Another limitation related to folate treatment is that high folate concentrations (0.9 – 3.4 µM) were used. These concentrations were chosen to represent concentrations that the cells may be exposed to on the apical membrane, in other words, supplemental folate. However, systemic folate levels are much lower than the concentrations of folate used in this research, for example, the mean serum folate in healthy young women (18 – 25 years) was 33.1 nmol/L (Shuaibi *et al.*, 2008). So serum folate levels are much lower than the treatment concentrations used in this research.

Many studies performed with colon cancer cell lines have longer treatment and culture times. For this project, treatment time was capped at 5 days. Caco2 cells have a relatively slow doubling time of 62 hours, according to the ATCC® product specifications for Caco2 (ATCC® HTB-37™). Perhaps treating the cells for a longer time could reduce variability and bring about more significant results.

Other studies examining the link between folate and cancer cell growth have found an effect of folate after longer treatment periods. Therefore, longer treatment durations should be investigated. For example, a study in HT29 cells showed that treatment with 0.23 µM FA for 3 weeks had growth and metabolism promoting effect compared to treatment with 0.023 µM FA (Pellis *et al.*, 2008). Another study in Caco2 cells found an inverse relationship with FA concentration in the medium and RFC mRNA and protein abundance after 5 passages of Caco2 cells (Ashokkumar *et al.*, 2007). The growth rate of Caco2 cells was decreased when grown in folate deficient [sic] conditions compared to folate sufficient [sic] conditions after 15 and 30 days (Cockman, 2009). This same study also found differences in *RFC* mRNA abundance, β-Catenin transcripts, and β-Catenin nuclear localization in Caco2 cells in folate deficient or sufficient conditions after 30 days (Cockman, 2009).

3.2 Future Directions

The main hypothesis for the interactions between folate, Wnt signalling and colon cancer is thought to be related to the regulation of genes. Future directions for this research should include increased treatment length, different types of cells, studying the

expression of key genes in folate transport and metabolism, the Wnt signalling pathway as well as colorectal cancer progression.

3.2.1 Treatment Length

As rationalized above, the treatment duration for the current study was probably too short. Results of other studies have shown an effect of folate with longer treatment durations. Therefore, the present study should be repeated using longer treatment times. Based on the results of other studies, a range of time points would be ideal, for example, 0, 7, 14, 21, 28, and 35 days. However, if using Caco2 cells it must be ensured that the cells are not allowed to differentiate or else they will lose their adenocarcinoma phenotype. After looking at that range, depending on the results, a narrower span of treatment times can be chosen.

3.2.2 Cell Type

In this thesis research, only one cell type was used. In future studies, other cell lines should be used. ATCC® offers a spectrum of colon cancer cell lines, grouped together specifically because of genomic mutations affecting specific genes. For example, Colon Cancer Panel 2, BRAF (ATCC® TCP-1007™) is a panel of 8 colon cancer cell lines with mutations in one or more of *BRAF, APC, CTNNB1, EGFR, FBXW7, NF1, PIK3CA, PIK3R1, SMAD4,* and *TP53*. *APC* and *CTNNB1* are genes that are essential in the Wnt signalling pathway. This panel contains SNU-C1 (ATCC® CRL-5972™), SW48 (ATCC® CCL-231™), RKO (ATCC® CRL-2577™), COLO 205 (ATCC® CCL-222™), SW1417 (ATCC® CCL-238™), LS411N (ATCC® CRL-2159™), NCI-H508

(ATCC® CCL-253™), and HT-29 (ATCC® HTB-38™). Testing other cell lines is important because what occurs in one cell type may not in another. Each cell line has its own unique features, for example SW48 is classified as a Duke`s type C, grade IV colorectal adenocarcinoma with an epithelial cell morphology, isolated from an 82-year old Caucasian female (Product Specifications; ATCC® CCL-231™). The present study only examined the effects of folate type in Caco2 cells. Researching one cell type gives limited data that can't be readily applied to other cell lines or cell types.

It would be interesting to contrast findings from the cancerous cell lines with healthy cells. There are 5 "normal" cell lines available from ATCC®: CCD 841 CoN (ATCC® CRL-1790™), FHC (ATCC® CRL-1831™), CCD-19Co (ATCC® CRL-1459™), CCD-33Co (ATCC® CRL-1539™), and CCD-112CoN (ATCC® CRL-1541™). These cell lines are all isolated from very young patients, the oldest being 7 years (CCD-112CoN) and the youngest a 13 week gestation fetus (FHC), according to the product specifications (ATCC®).

3.2.3 Gene Expression

One of the intended endpoints of this thesis research was gene expression of the folate transporters and of Wnt signalling pathway components in response to different folate treatments. Because other studies have showed that extracellular folate concentrations affect the expression of folate transport genes, future research projects should examine whether there is a difference in expression of folate transport genes after treatment with different forms of folate. In addition, to further study the involvement of Wnt and folate in colon cancer, the original plan in this thesis research was to examine

the effect of folate type and concentration on expression of genes within the Wnt signalling pathway using a human Wnt signalling pathway PCR array (PAHS-043A; SA Biosciences, Frederick, MD).

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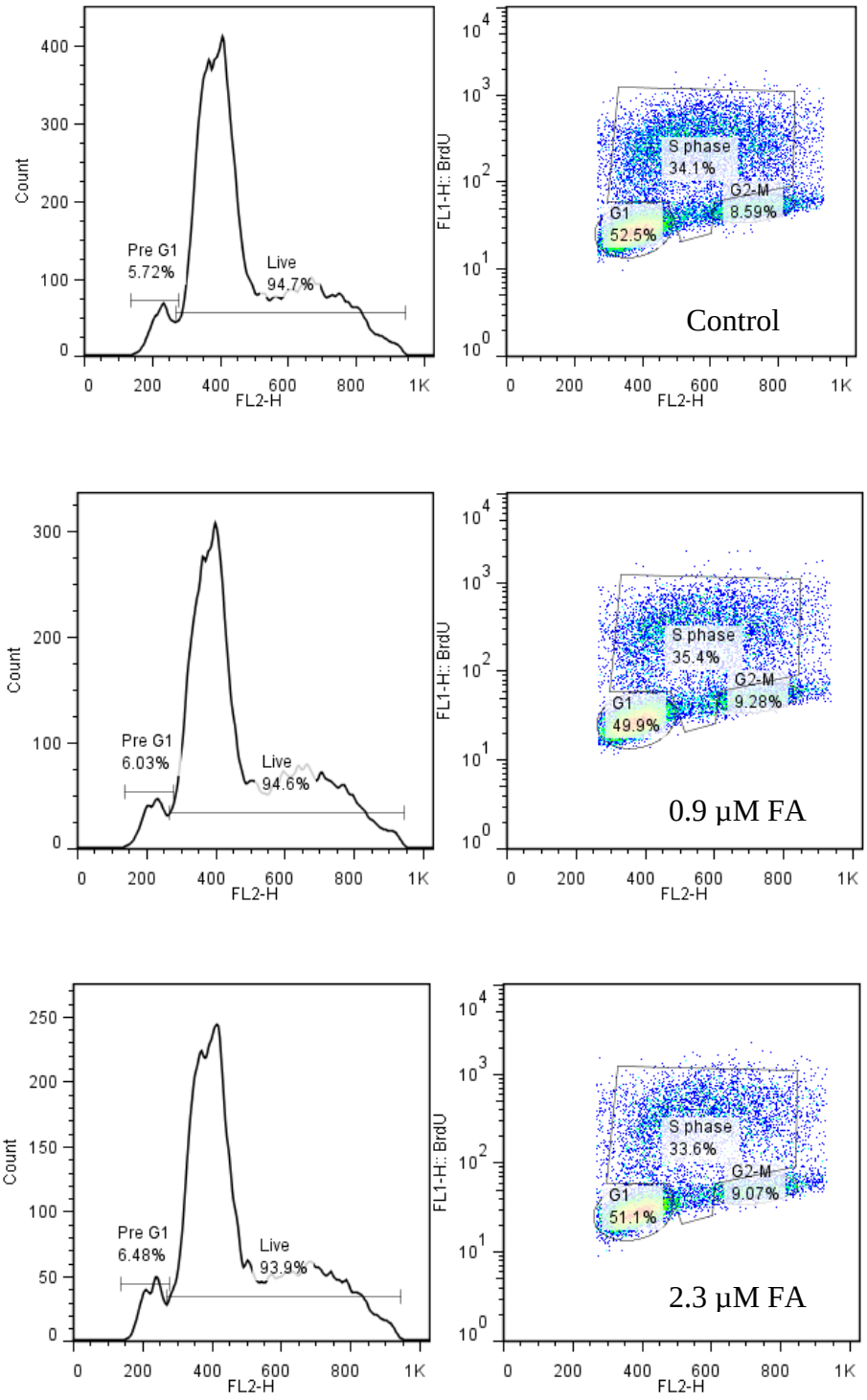
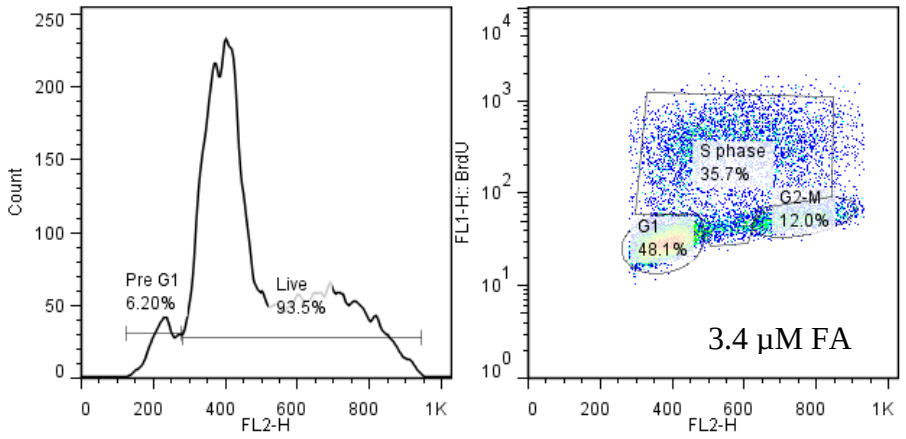
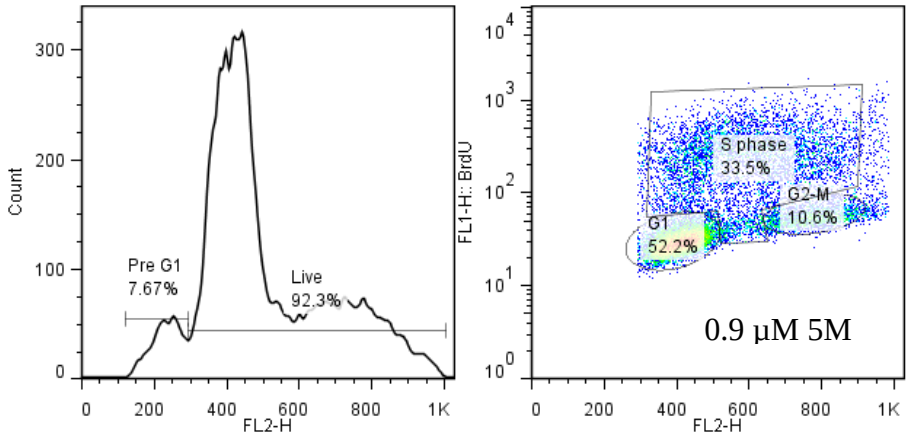
Appendices

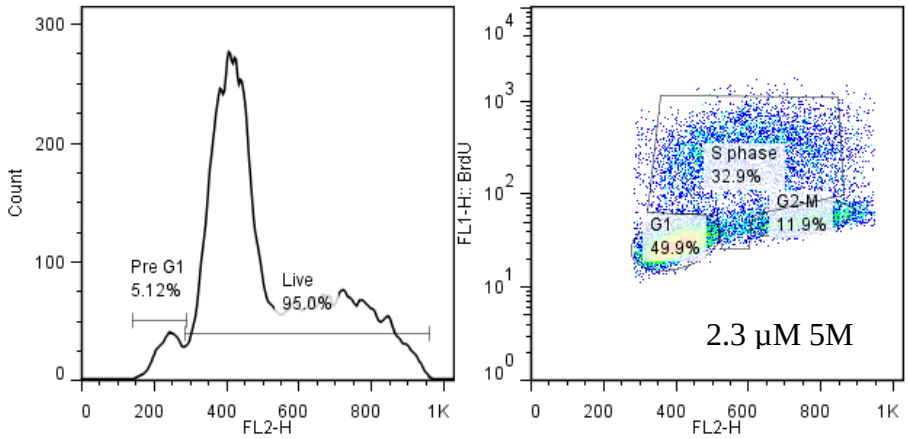
Appendix A: Flow Cytometry Diagrams

Histogram Scatter Plot




Count
FL2-H
Pre G1
6.20%
Live
93.5%
FL1-H:: BrdU
S phase
35.7%
G2-M
12.0%
G1
48.1%
3.4 µM FA


Count
FL2-H
Pre G1
7.67%
Live
92.3%
FL1-H:: BrdU
S phase
33.5%
G2-M
10.6%
G1
52.2%
0.9 µM 5M


Count
FL2-H
Pre G1
5.12%
Live
95.0%
FL1-H:: BrdU
S phase
32.9%
G2-M
11.9%
G1
49.9%
2.3 µM 5M

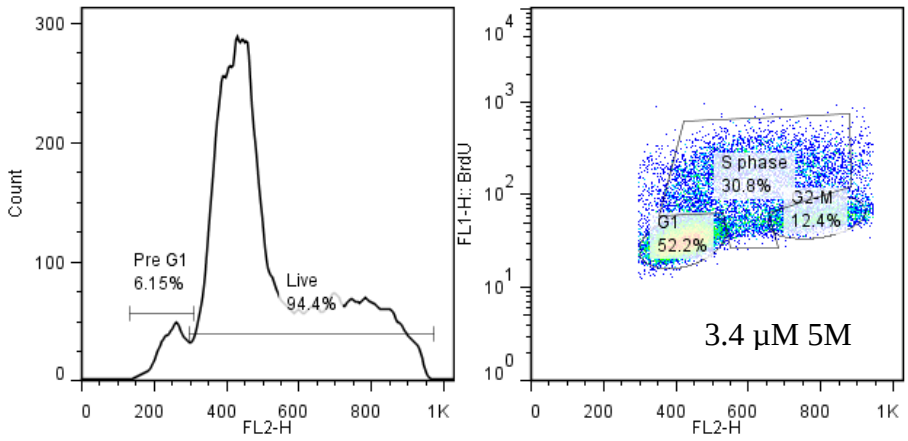
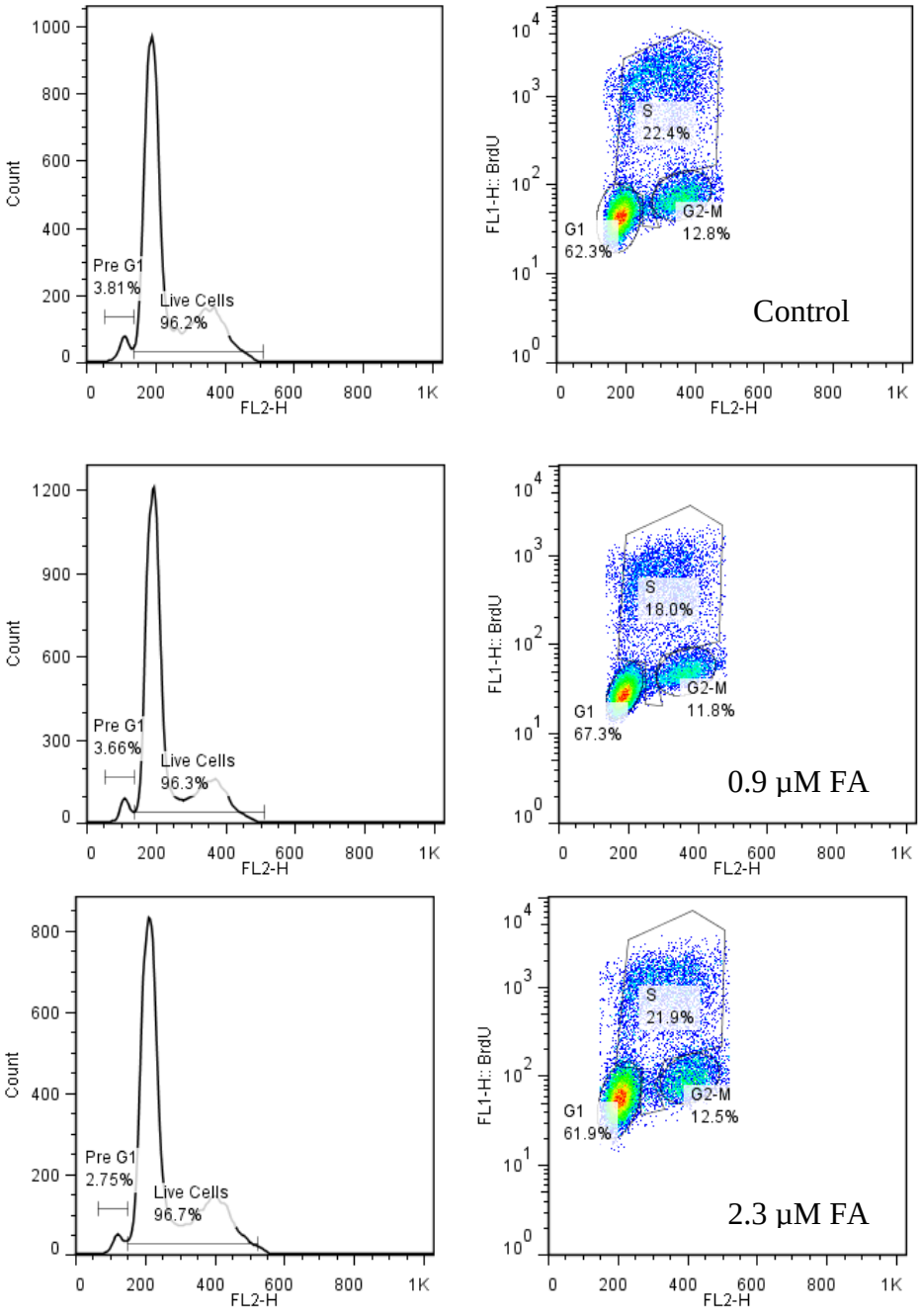
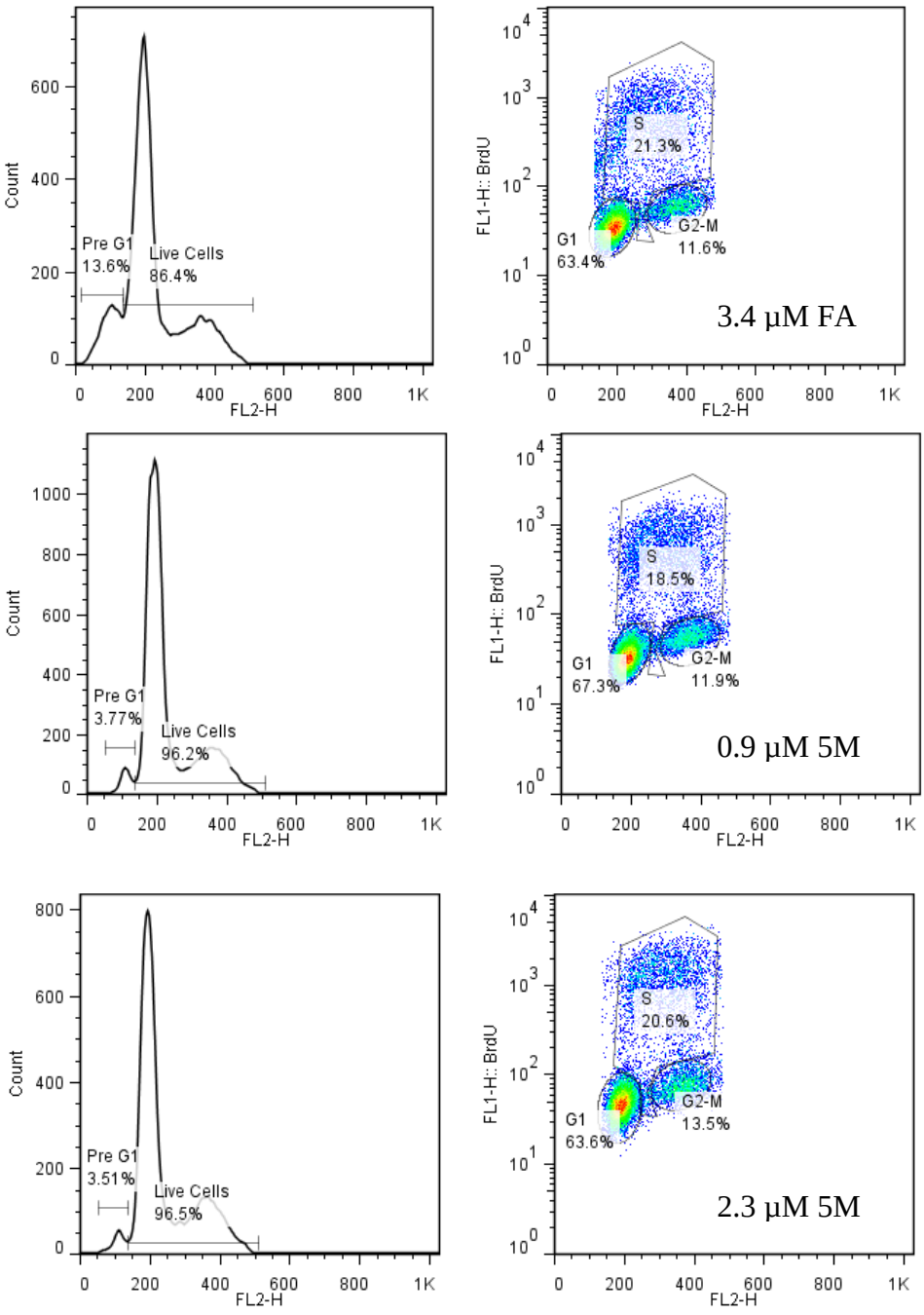


Figure A.1: Cell cycle analysis of Caco2 cells after 3 days treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or MTHF for 3 days. Cell cycle was determined using BrdU incorporation for S phase detection and PI staining for total DNA and analyzed by flow cytometry. The first column shows the histogram with percentage pre-G1 cells. The second column shows the corresponding scatter plot with the G1, S and G2/M phase gated and quantified. *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

Histogram Scatter Plot




Count
Pre G1
13.6%
Live Cells
86.4%
FL2-H
FL1-H:: BrdU
S
21.3%
G1
63.4%
G2-M
11.6%
3.4 µM FA
Count
Pre G1
3.77%
Live Cells
96.2%
FL2-H
FL1-H:: BrdU
S
18.5%
G1
67.3%
G2-M
11.9%
0.9 µM 5M
Count
Pre G1
3.51%
Live Cells
96.5%
FL2-H
FL1-H:: BrdU
S
20.6%
G1
63.6%
G2-M
13.5%
2.3 µM 5M

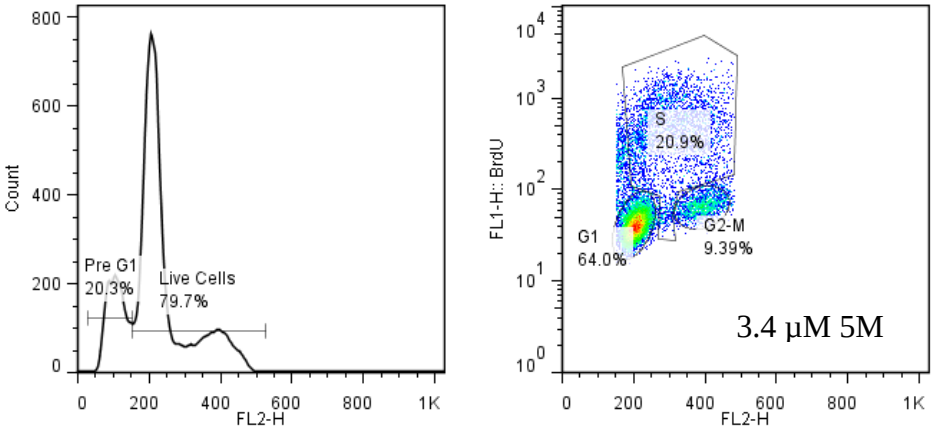


Figure A.2: Cell cycle analysis of Caco2 cells after 5 days treatment with FA or 5MTHF. Caco2 cells were cultured in folate-free RPMI-1640 medium for 5 days and then transferred to folate-free RPMI-1640 medium containing 0, 0.9, 2.3, or 3.4 µM FA or 5MTHF for 5 days. Cell cycle was determined using BrdU incorporation for S phase detection and PI staining for total DNA and analyzed by flow cytometry. The first column shows the histogram with percentage pre-G1 cells. The second column shows the corresponding scatter plot with the G1, S and G2/M phase gated and quantified. *Abbreviations: FA (folic acid), 5M (5-methyltetrahydrofolate).*

Appendix B: Raw Data for β-Catenin Nuclear Localization

Table B.1 Raw Data for β-Catenin Nuclear Localization

Sample	Nucleus Fluorescence	Nucleus Volume	Nucleus (Fluor/vol)	Whole Cell Fluorescence	Whole Cell Volume	Cytoplasmic Fluorescence	Cytoplasmic Volume	Cytoplasm (Fluor/vol)	Adj. Nucleus: Cytoplasm
1-1-1a	97133246	10574	9186	529000000	67501	431866754	56926	7586	1.2
1-1-1b	87538356	9479	9235	485000000	67501	397461644	58022	6850	1.3
1-1-1c	109001374	11980	9099	443000000	56251	333998626	44271	7544	1.2
1-1-1d	71393268	8477	8422	404000000	56251	332606732	47774	6962	1.2
1-1-1e	147353877	16197	9098	512000000	67501	364646123	51304	7108	1.3
1-1-1f	119223099	14061	8479	523000000	67501	403776901	53440	7556	1.1
								Average	**1.2**
1-1-2a	42966069	7943	5409	291000000	45001	248033931	37057	6693	0.8
1-1-2b	56282387	7756	7257	332000000	45001	275717613	37245	7403	1.0
1-1-2c	28156159	4082	6897	279000000	45001	250843841	40918	6130	1.1
1-1-2d	48549351	4725	10274	461000000	67501	412450649	62776	6570	1.6
1-1-2e	88135577	5767	15282	471000000	56251	382864423	50483	7584	2.0
1-1-2f	34767978	4840	7184	344000000	56251	309232022	51411	6015	1.2
								Average	**1. 3**
1-1-3a	40615740	6703	6059	266000000	45001	225384260	38297	5885	1.0
1-1-3b	52570978	9392	5598	393000000	67501	340429022	58109	5858	1.0
1-1-3c	58812574	8696	6763	312000000	45001	253187426	36305	6974	1.0
1-1-3d	79078763	11979	6601	341000000	56251	261921237	44271	5916	1.1
1-1-3e	47873448	6468	7402	292000000	45001	244126552	38533	6336	1.2
								Average	**1.1**
1-2-2a	17510091	3288	5326	226000000	45001	208489909	41713	4998	1.1

1-2-2b	134387549	14029	9579	528000000	67501	393612451	53472	7361	1.3
1-2-2c	97227575	15561	6248	464000000	67501	366772425	51940	7061	0.9
1-2-2d	30920913	6070	5094	230000000	45001	199079087	38931	5114	1.0
1-2-2e	46297279	6153	7525	399000000	67501	352702721	61348	5749	1.3
								Average	**1.1**
1-2-3a	43058888	6958	6188	352000000	56251	308941112	49293	6267	1.0
1-2-3b	37966496	6032	6294	399000000	67501	361033504	61468	5873	1.1
1-2-3c	33937314	5382	6306	320000000	44998	286062686	39616	7221	0.9
1-2-3d	23956619	4141	5785	344000000	67501	320043381	63360	5051	1.1
1-2-3e	36768230	7221	5092	250000000	45001	213231770	37779	5644	0.9
								Average	**1.0**
2-1-1a	31964606	5006	6385	401000000	67501	369035394	62494	5905	1.1
2-1-1b	60429838	12108	4991	253000000	56251	192570162	44142	4362	1.1
2-1-1c	49498520	9804	5049	305000000	67501	255501480	57696	4428	1.1
2-1-1d	68953110	13285	5190	381000000	78751	312046890	65466	4767	1.1
2-1-1e	33510717	6267	5347	304000000	67501	270489283	61234	4417	1.2
								Average	**1.1**
2-1-2a	65797498	11793	5579	402000000	78751	336202502	66957	5021	1.1
2-1-2b	49381492	8426	5861	384000000	67501	334618508	59075	5664	1.0
2-1-2c	63679531	8627	7381	423000000	61369	359320469	52742	6813	1.1
2-1-2d	68051807	7582	8975	434000000	55868	365948193	48286	7579	1.2
2-1-2e	135341391	11004	12299	713000000	90001	577658609	78997	7312	1.7
								Average	**1.2**
2-1-3a	58374483	10297	5669	400000000	67501	341625517	57204	5972	0.9
2-1-3b	60017663	8868	6768	362000000	67501	301982337	58633	5150	1.3
2-1-3c	24447966	3828	6386	303000000	56251	278552034	52422	5314	1.2
2-1-3d	35964677	5516	6521	361000000	67501	325035323	61985	5244	1.2

2-1-3e	56617398	6415	8825	368000000	67501	311382602	61085	5097	1.7
								Average	**1.3**
2-2-1a	43412970	7365	5894	411000000	67501	367587030	60136	6113	1.0
2-2-1b	52863507	10341	5112	332000000	67501	279136493	57160	4883	1.0
2-2-1c	22266482	3697	6023	347000000	78751	324733518	75054	4327	1.4
2-2-1d	65895154	7718	8538	460000000	78751	394104846	71033	5548	1.5
2-2-1e	65867448	9076	7258	337000000	56251	271132552	47175	5747	1.3
								Average	**1.2**
2-2-3a	66474840	7698	8636	482000000	78751	415525160	71053	5848	1.5
2-2-3b	50780335	7513	6759	374000000	67501	323219665	59988	5388	1.2
2-2-3c	47613029	6861	6939	425000000	67501	377386971	60640	6223	1.1
2-2-3d	51942751	9724	5342	323000000	67501	271057249	57777	4691	1.1
2-2-3e	26055651	3230	8066	325000000	56251	298944349	53020	5638	1.4
								Average	**1.3**
3-1-2a	33898649	6332	5353	335000000	67501	301101351	61168	4922	1.1
3-1-2b	37248467	6669	5586	347000000	67501	309751533	60832	5092	1.1
3-1-2c	74346990	9983	7448	421000000	56251	346653010	46268	7492	1.0
3-1-2d	128519264	12627	10178	545000000	67501	416480736	54874	7590	1.3
3-1-2e	63750179	8527	7476	434000000	67501	370249821	58973	6278	1.2
3-1-2f	79799183	9336	8547	506000000	67501	426200817	58165	7327	1.2
								Average	**1.2**
3-1-3a	23132478	4614	5014	270000000	56251	246867522	51637	4781	1.0
3-1-3b	64824532	13029	4975	420000000	87259	355175468	74229	4785	1.0
3-1-3c	49921658	9127	5469	388000000	78751	338078342	69623	4856	1.1
3-1-3d	80063738	11191	7154	499000000	90001	418936262	78810	5316	1.3
3-1-3e	38778888	6614	5863	378000000	67501	339221112	60887	5571	1.0
								Average	**1.1**

3-2-1a	19362885	3068	6311	317000000	67501	297637115	64433	4619	1.4
3-2-1b	25776249	5446	4733	268000000	56251	242223751	50804	4768	1.0
3-2-1c	36113874	3632	9944	381000000	56251	344886126	52619	6554	1.5
3-2-1d	36913422	6930	5327	358000000	56251	321086578	49321	6510	0.8
3-2-1e	56728371	8594	6601	475000000	78751	418271629	70157	5962	1.1
								Average	**1.2**
3-2-2a	21459899	3887	5520	313000000	67501	291540101	63613	4583	1.2
3-2-2b	20337504	3114	6530	343000000	67501	322662496	64387	5011	1.3
3-2-2c	38823178	5095	7620	332000000	67501	293176822	62406	4698	1.6
3-2-2d	52090974	6220	8375	391000000	78751	338909026	72531	4673	1.8
3-2-2e	128966570	21887	5892	429000000	67501	300033430	45614	6578	0.9
								Average	**1.4**
3-2-3a	41291279	7328	5635	362000000	67501	320708721	60173	5330	1.1
3-2-3b	109701022	16361	6705	502000000	78751	392298978	62390	6288	1.1
3-2-3c	91488341	14537	6294	464000000	67501	372511659	52964	7033	0.9
3-2-3d	67593382	9457	7147	483000000	78751	415406618	69294	5995	1.2
3-2-3e	81426632	4802	16955	513000000	56251	431573368	51448	8388	2.0
								Average	**1.3**
4-1-2a	45902675	9878	4647	310000000	67501	264097325	57623	4583	1.0
4-1-2c	62208312	11721	5307	395923863	89885	333715551	78163	4269	1.2
4-1-2d	113216079	22733	4980	339000000	67501	225783921	44768	5043	1.0
4-1-2e	61188503	10535	5808	392000000	67501	330811497	56966	5807	1.0
4-1-2f	29218432	5390	5421	326000000	67501	296781568	62111	4778	1.1
								Average	**1.1**
4-1-3a	79220405	13771	5752	419000000	78751	339779595	64980	5229	1.1
4-1-3b	67608575	4518	14965	572000000	67501	504391425	62983	8008	1.9

4-1-3c	86804939	10868	7987	530000000	67501	443195061	56633	7826	1.0
4-1-3d	46344823	9101	5092	364000000	78751	317655177	69650	4561	1.1
4-1-3e	49148420	8489	5789	418000000	78751	368851580	70262	5250	1.1
4-1-3f	36515749	6773	5391	329000000	56251	292484251	49477	5911	0.9
								Average	**1.2**
4-2-1a	65680083	10963	5991	399000000	78751	333319917	67788	4917	1.2
4-2-1b	113331551	19156	5916	490000000	78748	376668449	59592	6321	0.9
4-2-1c	50865453	6224	8173	362000000	67501	311134547	61277	5077	1.6
4-2-1d	32768577	5145	6369	292000000	56251	259231423	51106	5072	1.2
4-2-1e	56554405	5632	10041	455000000	67501	398445595	61869	6440	1.6
								Average	**1.3**
4-2-2a	55111779	6497	8483	443000000	67501	387888221	61004	6358	1.3
4-2-2b	97309509	15588	6243	442000000	78751	344690491	63163	5457	1.1
4-2-2c	21156893	2094	10104	327000000	56251	305843107	54157	5647	1.8
4-2-2d	40173567	4688	8569	386000000	67501	345826433	62813	5506	1.6
4-2-2e	58082212	9837	5904	379000000	67501	320917788	57664	5565	1.1
								Average	**1.4**
5-1-2a	92246842	8755	10537	626000000	90001	533753158	81246	6570	1.6
5-1-2b	86230894	7100	12145	698000000	90001	611769106	82901	7380	1.6
5-1-2d	198893991	22512	8835	882000000	101251	683106009	78739	8676	1.0
5-1-2e	103314251	13648	7570	635000000	78751	531685749	65103	8167	0.9
5-1-2f	207932335	29081	7150	790000000	90001	582067665	60920	9555	0.7
								Average	**1.2**
5-1-3b	66105310	3263	20260	519000000	56251	452894690	52988	8547	2.4
5-1-3c	72770074	5294	13745	605000000	56251	532229926	50956	10445	1.3
5-1-3d	325740169	21021	15496	1100000000	78751	774259831	57730	13412	1.2
5-1-3e	121972571	7744	15750	776000000	67501	654027429	59756	10945	1.4

5-1-3f	89055720	7846	11351	570000000	56251	480944280	48405	99356	1.1
								Average	**1.5**
5-2-2a	90362686	5098	17724	693000000	45001	602637314	39902	151023	1.2
5-2-2b	83116484	11223	7406	439000000	67501	355883516	56278	6324	1.2
5-2-2c	47756214	2982	16017	427000000	56251	379243786	53269	7119	2.2
5-2-2d	78714269	8058	9769	426000000	56251	347285731	48193	7206	1.4
5-2-2e	50761821	3048	16652	421000000	56251	370238179	53202	6959	2.4
5-2-2f	50556755	4626	10930	392000000	56251	341443245	51625	6614	1.6
5-2-2g	80083076	10146	7893	412000000	56250	331916924	46104	7199	1.1
5-2-2h	46460343	6794	6838	355000000	56251	308539657	49456	6239	1.1
								Average	**1.5**
6-1-1a	50078320	6422	7798	290000000	56251	239921680	49829	4815	1.6
6-1-1b	94156045	12222	7704	570000000	78751	475843955	66529	7152	1.1
6-1-1c	60275432	5677	10616	414000000	67501	353724568	61823	5722	1.9
6-1-1d	43623927	4449	9805	417000000	67501	373376073	63052	5922	1.7
6-1-1e	81736180	7452	10969	526000000	67501	444263820	60049	7398	1.5
								Average	**1.6**
6-1-2a	50670187	6872	7373	447000000	78751	396329813	71879	5514	1.3
6-1-2b	102664729	9033	11365	441000000	56251	338335271	47217	7165	1.6
6-1-2c	39833820	6079	6552	358000000	67501	318166180	61421	5180	1.3
6-1-2d	81106521	9559	8484	537000000	78751	455893479	69192	6589	1.3
6-1-2e	59775928	9963	6000	329000000	56251	269224072	46287	5816	1.0
								Average	**1.3**
6-1-3a	92270956	9755	9458	568000000	787501	475729044	68996	6895	1.4
6-1-3b	98876988	18179	5439	418000000	78751	319123012	60571	5268	1.0
6-1-3c	53566927	9714	5514	283000000	56251	229433073	46537	4930	1.1
6-1-3d	26955735	5071	5315	368000000	78751	341044265	73680	4629	1.1

6-1-3e	48122576	7557	6368	427000000	67501	378877424	59944	6320	1.0
								Average	**1.1**
6-2-1a	89899710	7318	12285	692000000	90001	602100290	82683	7282	1.7
6-2-1b	55573194	7413	7497	484000000	78751	428426806	71338	6006	1.2
6-2-1c	88334931	10636	8305	759000000	101251	670665069	90615	7401	1.1
6-2-1d	80077390	4959	16147	613000000	78751	532922610	73792	7222	2.2
6-2-1e	41686957	5756	7243	381000000	67501	339313043	61745	5495	1.3
								Average	**1.5**
6-2-3a	33150850	5854	5663	312000000	67501	278849150	61647	4523	1.2
6-2-3b	46717347	7800	5990	356000000	67501	309282653	59701	5180	1.2
6-2-3c	54882526	7656	7168	389000000	67501	334117474	59844	5583	1.3
6-2-3d	56096138	7384	7597	467000000	67501	410903862	60116	6835	1.1
6-2-3e	48639256	7260	6700	308000000	56251	259360744	48991	5294	1.3
								Average	**1.2**
7-1-1a	42386135	7452	5688	362000000	67501	319613865	60048	5323	1.1
7-1-1b	173152651	27341	6333	593000000	101251	419847349	73910	5680	1.1
7-1-1c	34081518	4729	7207	338000000	56251	303918482	51521	5899	1.2
7-1-1d	43408291	6943	6252	431000000	67501	387591709	60557	6400	1.0
7-1-1e	65195846	7051	9246	411000000	67501	345804154	60450	5720	1.6
								Average	**1.2**
7-1-2a	20800308	3260	6381	298000000	56251	277199692	52991	5231	1.2
7-1-2b	71746390	12215	5873	371000000	67501	299253610	55285	5413	1.1
7-1-2c	28770018	4210	6834	340000000	67501	311229982	63291	4917	1.4
7-1-2d	57566914	9622	5982	368000000	67501	310433086	57878	5364	1.1
7-1-2e	49654506	6422	7732	406000000	67501	356345494	61079	5834	1.3
								Average	**1.2**

7-1-3a	38166885	4459	8560	387000000	67501	348833115	63042	5533	1.5
7-1-3b	24717780	4269	5791	291000000	56251	266282220	51982	5123	1.1
7-1-3c	31498136	5390	5844	320000000	67501	288501864	62111	4645	1.3
7-1-3d	77286769	8784	8799	466000000	67501	388713231	58717	6620	1.3
7-1-3e	23662420	4022	5883	317000000	67501	293337580	63479	4621	1.3
								Average	**1.3**
7-2-1a	88045587	10300	8548	521000000	78751	432954413	68451	6325	1.4
7-2-1b	61850907	8116	7621	431000000	67501	369149093	59385	6216	1.2
7-2-1c	56192294	9971	5636	359000000	67501	302807706	57530	5263	1.1
7-2-1d	43578441	6949	6271	377000000	78751	333421559	71802	4644	1.4
7-2-1e	112884890	18472	6111	598000000	101251	485115110	82779	5860	1.0
								Average	**1.2**

*Data is represented in Figure 2.8 and 2.9

Sample identity is organized by folate treatment (1=control, 2=0.9 µM FA, 3=2.3 µM FA, 4=3.4 µM FA, 5=0.9 µM 5MTHF, 6=2.3 µM 5MTHF, 7=3.4 µM 5MTHF), slide number (1-2), position on slide (1- 3), and locations sampled within a sample (a-h).