

ABSTRACT

Title of Document: THE EFFECT OF FOLIC ACID AND ITS METABOLITES ON HUMAN COLORECTAL CANCER CELLS

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In the United States, colorectal cancer (CRC) is the third leading cause of death from cancer. Promising research has shown the chemopreventive effects of folic acid (FA) on CRC. Folate is a water-soluble B vitamin that is essential in the transfer of one-carbon necessary for DNA synthesis and methylation reactions. Because folate is essential in DNA replication, a deficiency can predispose cells to neoplastic transformation. While folate depletion in normal tissues can predispose them to becoming cancerous, researchers discovered that folate depletion in colon cancer cells might suppress the progression of current neoplasms, decreasing cell proliferation. The current study investigated the role of FA and its metabolites on CRC cell proliferation, apoptosis, cell cycle regulation and metastasis. We demonstrated that FA did not regulate proliferation in several different colon cancer cell lines. FA did not influence cell cycle regulation, apoptosis or metastasis in HCT116 cells. In addition, we observed that FA decreased expression of proteins involved in epithelial-mesenchymal transition (EMT) in A549 lung cancer cells. Metabolites of FA, including dihydrofolic acid (DHF) and tetrahydrofolic acid (THF), did not significantly affect HCT116 cell proliferation or cell cycle regulation. This study suggests that FA and its metabolites do not regulate colon cancer. Further studies are warranted to investigate the potential role of FA in lung cancer EMT.

THE EFFECT OF FOLIC ACID AND ITS METABOLITES ON HUMAN
COLORECTAL CANCER CELLS

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2016

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Chapter 1: Background

1.1 Introduction

Cancer is among one of the leading causes of death in the world (1). In the United States, colorectal cancer is the third leading cause of death from cancer (2). In 2016, it is estimated that there will be about 134,000 new cases of colon and rectal cancer (3). Additionally, about 35 to 50% of Americans over 50 years old already have one or more adenomas in their colon. Many of these adenomatous growths lack any signs or symptoms, making the detection more difficult (4, 66). Therefore, early detection and chemoprevention is important to the colon cancer-related death rate. The incidence of colon cancer is highly associated with diet. The proposed strategies to reduce the risk of developing colorectal cancer (CRC) include limiting red and processed meats, avoiding alcohol and smoking, consuming more vegetables and fruits, avoiding obesity, and obtaining adequate calcium and vitamin D (2). Recently, a significant correlation between folate intake and occurrence of CRC has been reported, suggesting a role of adequate folate intake for colon cancer prevention (4, 23).

1.1.1 Diet and CRC

There is a strong relationship between dietary intake and the risk of developing CRC. A study by Doll and Peto found that dietary intake might be responsible for about 90% of colon cancer deaths in the US (5). A plant-based diet rich in vegetables and low in red meat is associated with a decreased risk of CRC (49). Some protective nutrients include vitamin D, folate, calcium, and certain antioxidants such as β -carotene, vitamin C and gamma-tocopherol (50). A study in women found that those who had diets rich in

fruits and vegetables had a significantly reduced risk of developing CRC than women who did not (51). In addition, a high consumption of meat and animal fat could increase the risk of developing CRC. A diet high in fruits, vegetables and whole grains was found to be protective against CRC (6).

1.1.2 Effect of folate on CRC

Folate has been shown to prevent the development of colon cancer (23). The potential chemopreventive role of folate may be due to its prevention of aberrations in DNA synthesis and methylation that can lead to colorectal carcinogenesis (7). However, studies have shown that after the mandatory folic acid (FA) fortification in flour and grains, the incidence of colon cancer increased (4, 23). These studies have only demonstrated an observational relationship and cannot prove causation. Due to the seemingly conflicting evidence to support this relationship, the effect of folate on CRC is still under exploration.

1.2 Folate Metabolism and health

1.2.1 Folate nutrition

Folate is a B vitamin that is fundamental in many biochemical reactions due to its ability to transfer one-carbon molecules. The body cannot synthesize this nutrient, so it must be obtained from dietary sources, such as leafy green vegetables and legumes (8). Folic acid is the synthetic form used for supplementation and fortification in foods while folate is the natural form. Folic acid is about 70% more bioavailable than folate for absorption in the small intestine (9). To assess short-term folate status in humans, serum folate concentration is measured, with a level of 3 ng/mL being sufficient. To assess

long-term folate status, erythrocyte folate concentration is measured. Recommended Dietary Allowance (RDA) of folate for adults is 400 µg DFE/d, with the Tolerable Upper Intake Level (UL) set at 1,000 µg DFE/d. According to the 2003–2006 National Health and Nutrition Examination Survey, most individuals obtain the recommended amount of folate, but some groups are still at risk of deficiency, such as women of childbearing age and non-Hispanic black women (10, 52).

1.2.2 Absorption

The absorption mechanism for FA is a complex process and has been under investigation during the last decade. Folates are primarily absorbed in the duodenum and jejunum, but also in the colon. The process involves a pH-dependent and carrier-mediated mechanism through the human colonic luminal membranes. There are two main carriers, reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT). RFC has a high affinity for 5-methyltetrahydrofolate and 5-formyltetrahydrofolate, while PCFT has a high affinity for folic acid. PCFT is expressed at a much lower level in the colon compared to the small intestine. However, high levels of folate transport occur in the proximal and distal colonic brush-boarder membranes (11).

1.2.3 Metabolism

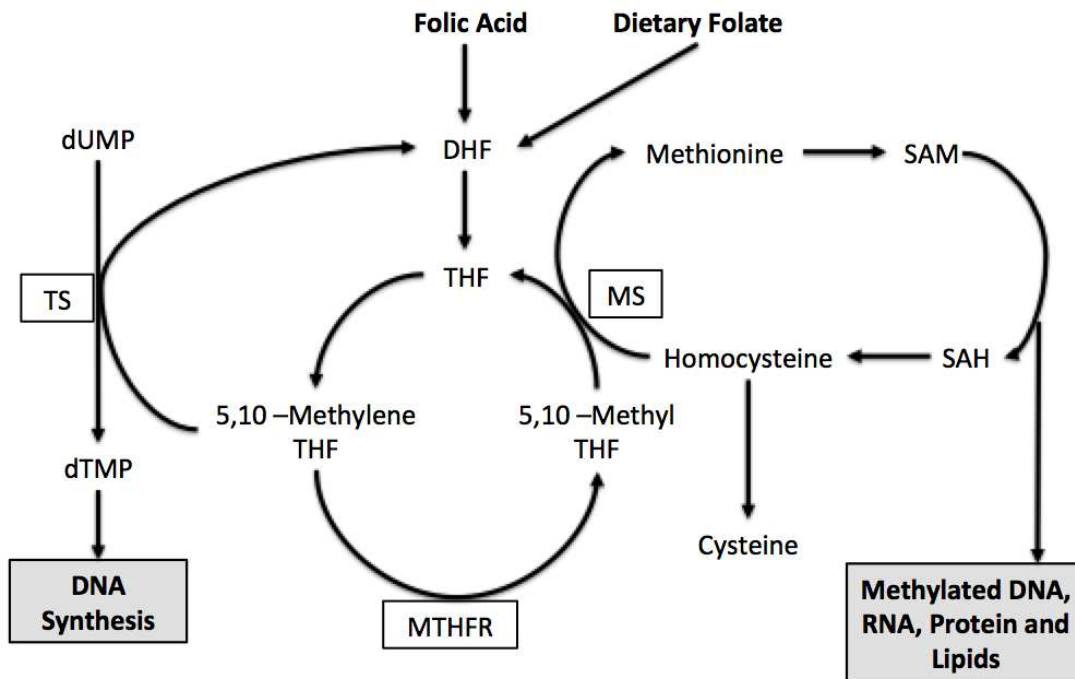


Figure 1.2.1 Folate One-Carbon Metabolism. Folate is fundamental in the process of DNA synthesis and methylation reactions. DHF, dihydrofolate; THF, tetrahydrofolate; MTHFR, methyltetrahydrofolate reductase; MS, methionine synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; TS, thymidylate synthase; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate

Folate is an essential factor in one-carbon metabolism in the body. The pathway involves DNA synthesis and methylation. In the first step, folate is converted to dihydrofolate (DHF) by dihydrofolate reductase (DHFR) in a slow reaction. DHF is then converted to tetrahydrofolate (THF) by DHFR in a fast reaction, and is subsequently converted into 5,10-methyltetrahydrofolate (5,10-methylTHF) through several steps of chemical modifications (12). In the methionine cycle, 5,10-methylTHF acts as a methyl donor of methionine synthase (MS), converting homocysteine to methionine and producing S-adenosylmethionine (SAM). SAM is an essential methyl donor in many reactions, such as the methylation of DNA, RNA, proteins and lipids (8). 5-methylTHF is

converted to tetrahydrofolate (THF) and then to 5,10-methyleneTHF by serine hydroxymethyltransferase (SHMT). SHMT also catalyzes the interconversion of serine to glycine. 5,10-methyleneTHF is used for the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), catalyzed by thymidylate synthase (TS) (12). dTMP is the only source of thymidine in the cell and is the rate limiting step for DNA synthesis (13). In addition, both THF and 5,10-methyleneTHF can be used for purine synthesis via addition of a formyl group (12).

1.2.4 Unmetabolized folate

An excess or deficiency of folic acid (FA) can have negative impacts on the health of individuals by interrupting folate homeostasis. A high intake of FA can increase the release of unmetabolized FA into the plasma (14). For FA to be incorporated into one-carbon metabolism, it must first be reduced to tetrahydrofolate (THF) (12). When there is an excess amount of FA, the body cannot metabolize it fast enough and unmetabolized FA can accumulate. Although some studies suggest that unmetabolized FA could increase the risk of certain conditions, other studies suggest that there are no harmful effects (14, 60, 61). One study demonstrated that unmetabolized FA was linked to a decline in immune function in postmenopausal women (60). Additionally, circulating unmetabolized FA was linked to lower cognitive test scores in older adults (61). In a case-control study conducted by Cho et al. (2015), unmetabolized FA was not associated with an increased colon cancer risk. Future studies are essential to understand the impact that unmetabolized FA has on the risk of chronic disease.

1.2.5 Health complications

Several negative health consequences are associated with folate deficiencies, including neural tube defects (NTDs) in newborns. In 1996, the Food and Drug Administration (FDA) published regulations requiring the addition of FA to flour to help reduce NTDs (13). After the mandatory fortification of FA, the incidence of NTDs significantly declined. It is estimated that fortification averts about 1,000 cases of NTDs each year (15). A study by Mulinare et al. found vitamin supplements containing 0.8 mg of FA reduced the risk of NTDs by about 60% (16).

Inadequate folate intake is also associated with megaloblastic anemia. This form of anemia occurs when the bone marrow creates immature and unusually large red blood cells, ultimately leading to a low red blood cell count. Folate is essential for the maturation of precursor megaloblastic cells to mature red blood cells. As a result of folate deficiency, an increase of immature megaloblasts and decrease of red blood cells leads to anemia (17).

Additionally, folate deficiency has been linked to an increased risk of cognitive impairment (18). This relationship may be due to elevated serum homocysteine levels, also known as hyperhomocysteinemia (53). Normally, homocysteine is converted to methionine through a folate-dependent remethylation reaction (8). Another nutrient essential in this process is vitamin B12. When folate or vitamin B12 levels are low, the reaction is compromised and homocysteine builds up in the plasma (53). Studies have shown that plasma homocysteine levels are positively correlated with cognitive decline (56-58). In addition, a systematic review of case-control studies demonstrated that folate

and vitamin B12 levels were lower and homocysteine was higher in Alzheimer's disease subjects than in the healthy controls (54). Additionally, a large cohort of older adults was analyzed to examine the effects of dietary B vitamins and the long-term incidence of dementia. The researchers found that higher intakes of folate reduced the risk of dementia, while vitamin B6 and B12 had no effect (59). Although there is promising research on the effect of folic acid on cognition, a causal relationship has not been established.

Poor folate status is also associated with heart disease and stroke due to hyperhomocysteinemia, defined as levels above 15 $\mu\text{mol/L}$ in the blood (8, 19). Inadequate folate and vitamin B12 intake can increase homocysteine levels (53). In the 1990's, homocysteine was first recognized as a risk factor of heart disease. As research has developed, the mechanism behind this relationship has been unveiled. Researchers believe that hyperhomocysteinemia causes endothelial cell damage and increased permeability and decreased flexibility of blood vessels, which increases the risk of heart disease and stroke (19). A study in older adults demonstrated that poor folate and vitamin B12 status was related to cardiovascular disease due to elevated homocysteine levels (55). Overall, intake of optimal folate remains a beneficial public health intervention in the US to prevent against numerous conditions.

1.3 Folate and CRC

A deficiency of folate can increase the risk of developing several malignancies, specifically CRC. Due to its involvement in DNA synthesis, a FA deficiency can lead to DNA strand breaks, uracil misincorporation, chromosomal breakage, and impaired DNA

repair (20). The misincorporation of uracil is likely due to the reduced methylation of dUMP to dTMP, increasing the levels of deoxyuridine triphosphate (dUTP) in the cell. This subsequently causes the incorporation of uracil into the DNA in place of thymidine, leading to compromised DNA integrity and increased cancer risk (21). In addition, a folate deficiency can increase homocysteine levels due to the lack of 5'-methyl-THF, a compound essential for the conversion of homocysteine to SAM (9). The increase in homocysteine causes an increase in *S*-adenosylhomocysteine (SAH) levels, which is an inhibitor of methylation reactions (13). Low folate status can also cause hypomethylation in DNA, proteins and lipids due to a reduction in SAM and an increase in SAH. Hypomethylation may upregulate the expression of oncogenes and lead to cancer progression (8). It is evident that consumption of appropriate folate is essential in the prevention of cancers, including CRC.

FA is known to play an essential role in cancer prevention. Some epidemiological studies have shown an inverse relationship between adequate folate intake and colon cancer risk (4, 23). An *in vitro* study using HCT116 colon cancer cells showed that FA acted as a chemopreventive agent by downregulating insulin-like growth factor-I receptor (IGF-IR) (22). Another proposed chemopreventive mechanism is the prevention of uracil misincorporation and DNA breakage by allowing normal synthesis of thymidylate and purine. These nucleotides are crucial in the synthesis, replication and repair of DNA. Proper DNA replication and repair is necessary to prevent mutations that ultimately can cause cancer. FA also allows for the production of SAM, which is the methyl donor for DNA methylation (23). Adequate FA prevents alterations in DNA methylation, which

can promote tumor formation and cancer. While adequate folate intake is known to be chemopreventive, a folate deficient diet can lead to the development of CRC.

In contrast to the chemopreventive effect of FA, high folate intakes may lead to an increased cancer risk (62, 65). After the mandate of FA fortification in flour, levels of folate in the plasma increased by about 2-fold (63-64). The increase in plasma folate levels in the US corresponded to an increase in the rate of CRC (4). An animal study demonstrated that increased FA intake increased the formation of tumors in animals that already had neoplastic foci (65). Additionally, in a double blind placebo-controlled trial, researchers reported that individuals consuming 1 mg/day of FA had an increased risk for advanced lesions and adenomas compared to a control group (62). Mason et al. (2007) speculated that the increase of FA in the food supply might lead to the conversion of adenomas into cancer.

Additionally, folate depletion in cancerous tissues may suppress the progression of colon cancer (20). A study led by Novakovic et al. (2006) examined the expression of specific cancer-related genes that regulate cancer development during folate depletion. The folate deficient cancer cells demonstrated a significant decrease in growth compared to the folate sufficient cells. They proposed that the inhibitory role of folate depletion in cancer invasion and metastasis was due to the down regulation of VEGF, a growth factor known to promote migration of endothelial cells (20). Further studies should be conducted to explain this correlation.

Folate supplementation can have dual effects depending on the stage of colon cancer development. While folate deficiency in normal tissues can predispose them to neoplastic

transformation, folate depletion in cancerous cells may suppress the progression of current neoplasms (20). In colon cancer cells, DNA replication and repair occur at an accelerated rate compared to normal healthy cells. When folate metabolism is disrupted during folate depletion, DNA synthesis in the cancer cells is disrupted leading to an inhibition of cancer cell growth (12). Therefore, the function of folate in DNA synthesis makes it a likely growth factor for cancer cells (4). Several studies have shown that the timing and dose of folate during carcinogenesis can be very important in chemotherapy (23). One chemotherapeutic approach is to use anti-folate agents to suppress the progression of existing neoplasms (20). For example, methotrexate is an analog of FA used to inhibit dihydrofolate reductase for chemotherapy (24). Folate depletion may also act synergistically with other medications, such as alkylating agents, to increase DNA strand breaks. Therefore, folate deficiency can have different effects on colon cancer development depending on the state of the individual (20).

In contrast, various studies have demonstrated that FA has no effect on CRC risk (14, 42, 43, 47). In a cohort study by the American Cancer Society, researchers measured intakes of both natural folate and FA and examined their relationship to CRC risk. The researchers determined that dietary fortification with FA did not increase the risk of CRC (42). Additionally, a case-control study was conducted to determine the relationship between plasma folate levels, a sensitive indicator of dietary folate, and the risk of CRC. Over 38,000 individuals were followed for 11.5 years. The researchers reported that plasma folate was not related to the risk of CRC (47). Overall, there is conflicting evidence on the role of FA in CRC. Therefore, more research is warranted to understand this complex relationship.

1.4 Hallmarks and mechanisms of CRC progression

1.4.1. General features of CRC

Cancer arises from cells when they undergo aberrant gene expression and transformation. These genetic defects cause abnormal gene expression that provide tumor cells with special properties that normal cells do not have, such as rapid proliferation, resistance to apoptosis, formation of new blood vessels, and the ability to invade and metastasize from the primary tumors (26). The development of CRC starts as a benign, non-cancerous polyp inside the lining of the colon or rectum. Then the polyp invades the muscle layer and develops into an adenoma. It subsequently progress to an adenocarcinoma and carcinoma, which can metastasize or move to distant regions of the body (2). Currently, researchers are focusing on cancer spread because it has poor prognosis and is associated with 90% of cancer mortality (25).

1.4.2 Apoptosis

Apoptosis is a process of programmed cell death occurring when normal cells undergo DNA-damage and have defective DNA repair. One of the main hallmarks of cancer is the ability of cells to evade apoptosis and undergo malignant transformation. One mechanism by which cells evade apoptosis is through the genetic disruption and up- or down-regulation of apoptosis-regulating genes. For example, when p53 is mutated or downregulated, it makes the cancer cells resistant to apoptosis in the later stages of tumorigenesis (27).

There are two main apoptotic pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The intrinsic pathway involves non-receptor-

mediated stimuli that send intracellular signals to targets. These targets are mitochondrial-initiated events. The extrinsic pathway involves receptor-mediated interactions, including death receptors that are ligands of the tumor necrosis factor (TNF) superfamily. Proteins in the TNF receptor family contain “death domains”, which transmit death signals from the cell surface to intracellular pathways. There are several ways that apoptosis can be inhibited, including stimulation of inhibitors of apoptosis (IAP) family of proteins, caspase inhibition, poly [ADP-ribose] polymerase (PARP) inhibition, and Bcl-2 protein inhibition (28). PARP is a protein responsible for DNA repair when the cell is damaged. It is a target of caspase so PARP cleavage has been used as a molecular marker of apoptosis (29).

1.4.3 Cell cycle

The cell cycle is a process that is tightly regulated by specific cellular proteins. The cell cycle is divided into G1, S, G2, and M stages. G1 is the gap phase where the cell prepares to enter S phase for DNA replication. The cell can either proceed to S phase, pause, or exit the cell cycle. The transition from one cell cycle phase to another is tightly regulated by expression of cyclins that activate certain cyclin-dependent kinases (CDKs) (30). The protein cyclin D1 activates CDK4/6 to allow transition from G1 to S phase. There are also several CDK inhibitors, such as p21, that inhibit CDKs and prevent the transition to S phase. In cancer cells, cell cycle regulation is disrupted and cells proliferate uncontrollably (31). For example, aberrant expression of cyclin D1 can directly increase cell division and contribute to tumor formation, while up-regulation of p21 is related to cell cycle arrest at G1/S phase (30-31).

1.4.4 Angiogenesis and metastasis

Metastasis is another hallmark of cancer characterized by the process of cancerous cells spreading from the original tumor location to new regions of the body and developing secondary tumors. The process can be divided into several main steps: invasion, intravasation, circulation, extravasation and formation of micro- and macrometastasis. In the process of invasion, malignant tumor cells lose cell-cell adhesion, detach from the primary tumor mass and invade the surrounding stroma. The process involves the release of proteases such as MMP-9 and MMP-2 that break down the basement membrane and extracellular matrix. (32).

Angiogenesis is the process to form new blood vessels. As the volume of primary tumors increase, the cancer cells undergo the deficiency of oxygen and hypoxia-associated necrosis. To overcome this limitation and survive, the cancer cells form new blood vessels through which invasive cells obtain oxygen and essential nutrients, permitting them to travel to distant sites. Angiogenesis is controlled by interactions between malignant cancer cells and normal endothelial cells. Hypoxia stimulates the expression of vascular endothelial growth factor (VEGF), which is a growth factor for endothelial cells. VEGF directly binds to its receptor (VEGFR) in the membrane of endothelial cells and stimulates their proliferation and subsequent formation of new blood vessels (33).

1.4.5 EMT

Recently epithelial-mesenchymal transition (EMT) is generally accepted as a promising mechanism of metastasis. The process permits polarized epithelial cells to

undergo changes to allow them to assume a mesenchymal phenotype, which promotes migratory and invasive characteristics. During EMT, cells lose adhesive properties, change the expression profile of surface proteins and release matrix-degrading enzymes. A decrease of E-cadherin and increase of N-cadherin is an important hallmark in the process of EMT and metastatic progression. E-cadherin is downregulated in almost all epithelial cancers and can be categorized as a tumor suppressor. In contrast, N-cadherin is a mesenchymal cadherin, which stimulates migration and invasion of cancer cells (32).

EMT can be induced by various growth and differentiation factors, including transforming growth factor beta (TGF- β). Upon treatment with TGF- β , epithelial cells decrease expression of epithelial markers, such as E-cadherin, and increase expression of mesenchymal markers, such as N-cadherin. TGF- β signaling occurs through a complex of type I and type II transmembrane serine-threonine kinase receptors. The activation of the receptor complex leads to the phosphorylation, and subsequent activation, of SMAD2 and SMAD3. The phosphorylated SMAD2 and SMAD3 then bind to SMAD4 and translocate to the nucleus to control the transcription of certain target genes, such as Snail and Slug. Therefore, increased phosphorylation of SMAD2 and SMAD3 has been used as a molecular indicator to induce EMT. In addition, increased Snail levels have been associated with more invasive tumor types (34). Overall, understanding the process of EMT and metastasis in CRC could provide insight into how metastasis could be delayed or prevented.

90% of colorectal cancers develop as a result of loss-of-function mutations in tumor suppressor genes, including the truncation of the APC gene. The truncated APC gene

fails to suppress cellular overgrowth and allows for the formation of polyps in the colon that can develop into malignant tumors (35). The encoded protein for APC interacts with a variety of proteins in the cytoplasm and is involved in several cellular processes, such as the WNT signaling pathway. APC forms a complex with glycogen synthase kinase 3 (GSK3), axin and casein kinase-1 (CK-1) that lead to the phosphorylation of β -catenin and its resulting proteasomal degradation through the 26S proteasome. Therefore, the dysfunction of the APC gene inhibits β -catenin phosphorylation and protects it from proteasomal degradation. As a result, free β -catenin translocates to the nucleus and binds to the TCF/LEF transcription factors to stimulate their oncogenic target genes (26).

Due to the conflicting evidence on the role of FA in CRC, we chose to study this complex relationship. In the present study, we examined if treatment with FA or its metabolites could alter CRC cell proliferation, apoptosis, cell cycle regulation, and metastasis. We utilized different FA amounts, including deficient, adequate and surplus doses, to represent different nutritional statuses.

Chapter 2: Methods and Materials

2.1 Materials

Folic acid (FA) and dihydrofolic acid (DHF) were purchased from Sigma Aldrich (St. Louis, MO) and dissolved in phosphate-buffered saline (PBS). Tetrahydrofolic acid (THF) was purchased from Cayman Chemical Company (Ann Arbor, MI) and dissolved in PBS. TGF- β (240B002) was purchased from R&D Systems (Minneapolis, MN). Antibodies for p-GSK3 β (9336s), P21 (2947s), SMAD3 (9513s), Phospho-SMAD3 (9520s), Snail (3879s), N-cadherin (4061s) were purchased from Cell Signaling (Beverly, MA). Antibodies for cyclin D1 (sc-718), β -catenin (sc-1496), E-cadherin (sc-21791), ATF3 (sc-188), GSK3 β (sc-9166), and actin (sc-1615) were purchased from Santa Cruz (Santa Cruz, CA). Dulbecco's modified Eagle medium (DMEM/F-12), folate-deficient and folate-sufficient RPMI 1640 medium were purchased from Fisher Scientific (Hampton, NH).

2.2 Cell Culture

Human colon adenocarcinoma cells (HCT116, SW480, HCT15, HT29, and CaCO2) and human lung cancer cells (A549 and H358) were purchased from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM/F-12). The medium was supplemented with 10% fetal bovine serum (FBS) under a humidified atmosphere of 5% CO₂ at 37°C. During cell treatment, cells were grown in folate-deficient RPMI 1640 medium and treated with different concentrations of FA, DHF or THF.

2.3 Cell Proliferation

Cell proliferation was assessed using the MTT [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) method. Cells were plated at 8000 cells/well onto a 96-well plate in four replicates and grown overnight. Human colon adenocarcinoma cells HCT116, SW480, HCT15, HT29, and CaCO2 were treated with different concentrations of FA (0-50 $\mu\text{g}/\text{mL}$), DHF (0-20 $\mu\text{g}/\text{mL}$) or THF (0-20 $\mu\text{g}/\text{mL}$) in complete folate-deficient RPMI 1640 medium for 24, 48 and 72 hours at 37°C under 5% CO₂. FA, DHF and THF were dissolved in PBS for treatment. Cells were incubated with 100 μL of MTT solution for 3 hours at 37°C. The optic density was recorded at 540 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc., Winooski, VT).

2.4 Apoptosis Assay

HCT116 colon cancer cells were treated with different levels of FA (0-20 $\mu\text{g}/\text{mL}$) for 48 hours at 37°C under 5% CO₂. Apoptosis was measured using a Cell Death Detection ELISA Kit (Roche Diagnostics, Indianapolis, IN). Attached cells were collected and suspended in RPMI 1640 medium. The following steps were conducted following manufacturer instructions. The optic density was recorded at 490 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc., Winooski, VT).

2.5 SDS-PAGE and Western Blot

Cells were washed with cold 1 \times phosphate-buffered saline (PBS), placed on ice for 15 minutes in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA), supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis,

MO) and phosphatase inhibitor cocktail (Sigma Aldrich), and then harvested. The cell lysate was centrifuged at $12,000 \times g$ for 15 min at 4°C . Protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN). The membranes were blocked for non-specific binding in 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1h at room temperature. Membranes were probed with specific primary antibodies in 3% Bovine Serum Albumin (Santa Cruz) at 4°C overnight and then with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature. Chemiluminescence was detected with Pierce ECL Western blotting substrate (Thermo Scientific) and visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA).

2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS, and the data was analyzed using an independent sample t-test. Data was expressed as means \pm SD and differences were considered significant at $p \leq 0.05$.

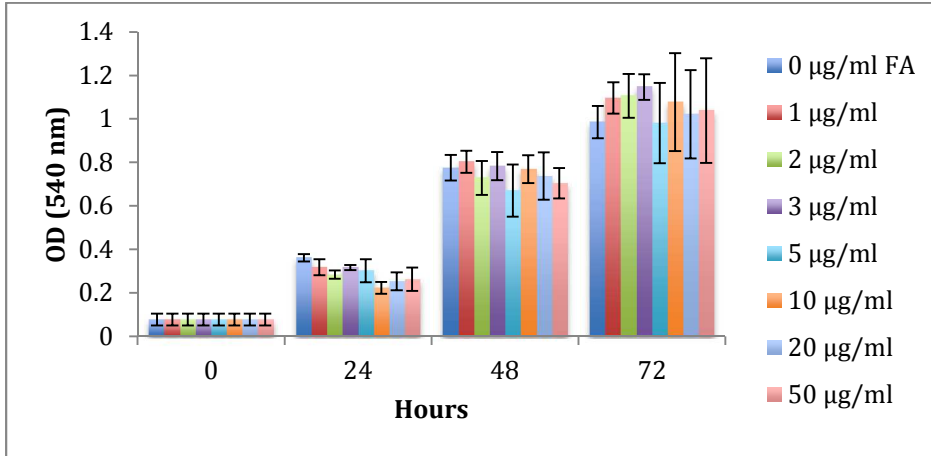
Chapter 3: Folic acid (FA) and its metabolites do not affect phenotypic expression profiles of human colon cancer cells

3.1 Effect of FA on cell proliferation in human colorectal cancer (CRC) cells

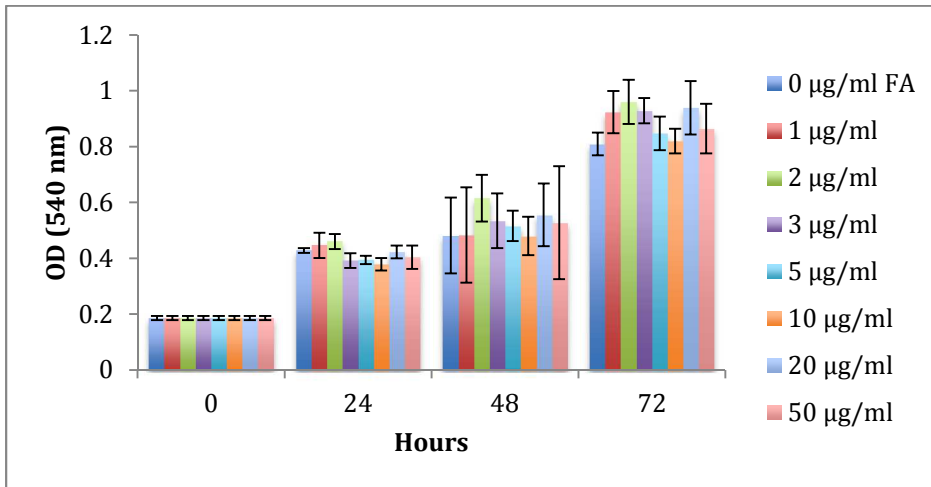
One of the fundamental features of cancer is the ability of cells to proliferate abnormally. In the earliest stages of CRC, colon epithelial cells develop an increased proliferative potential (36). Previous studies have demonstrated that a FA deficiency may suppress the progression of existing neoplasms (20). In contrast, other studies have demonstrated that FA supplementation can decrease proliferation of human CRC cells (22). It is still unclear if FA influences the ability of colon cells to proliferate uncontrollably.

To examine the effect of FA on proliferation of human CRC cells, HCT116, HT29, HCT15, SW480 and CaCO2 cells were treated with a range of FA concentrations (0-50 µg/ml). Several different cell lines with different genetic alterations were selected to encompass a variety of cancer cell mutations. For example, HCT116 cell line contains wild type APC, while SW480 and HT29 have truncated APC gene. SW480, HCT15 and HT29 express intact β-catenin, and HCT116 and CaCO-2 are β-catenin mutant cell lines. In addition, HCT116 and HCT15 are p53 wild type while others express mutated p53. A level of 0 µg/ml FA represented a deficiency, a level of 1 µg/ml FA represented an adequate amount, and levels above 1 µg/ml FA represented an excess amount. As shown in Fig 3.1 A-E, the treatment of FA did not change cell proliferation rates in all treatment amounts of FA. This result was seen in all five colon cancer cell lines.

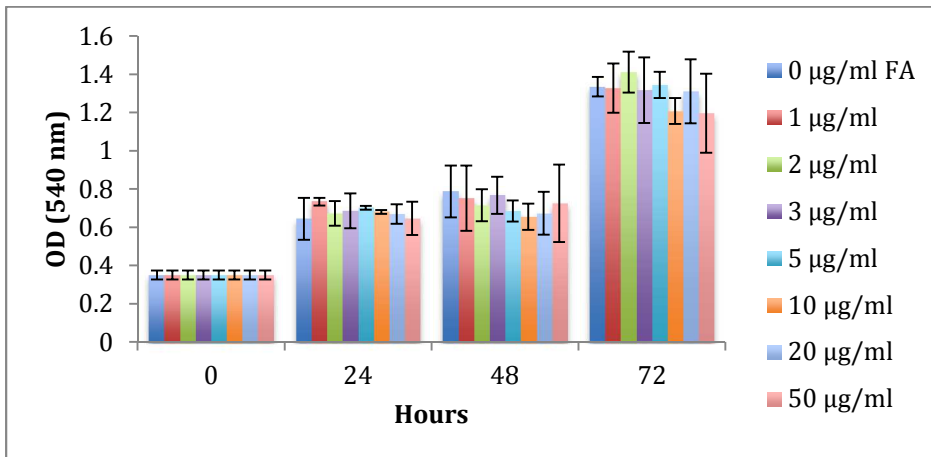
(A) HCT116



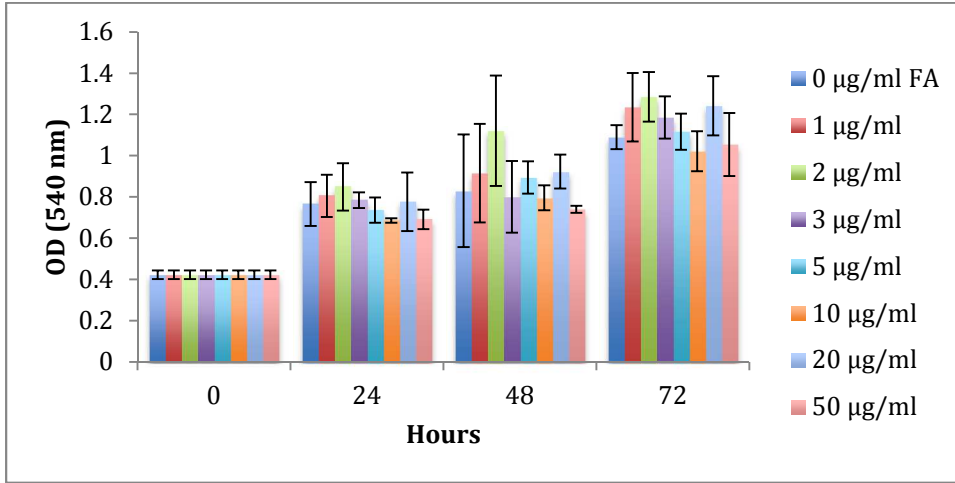
(B) HT29



(C) HCT15



(D) SW480



(E) CaCO2

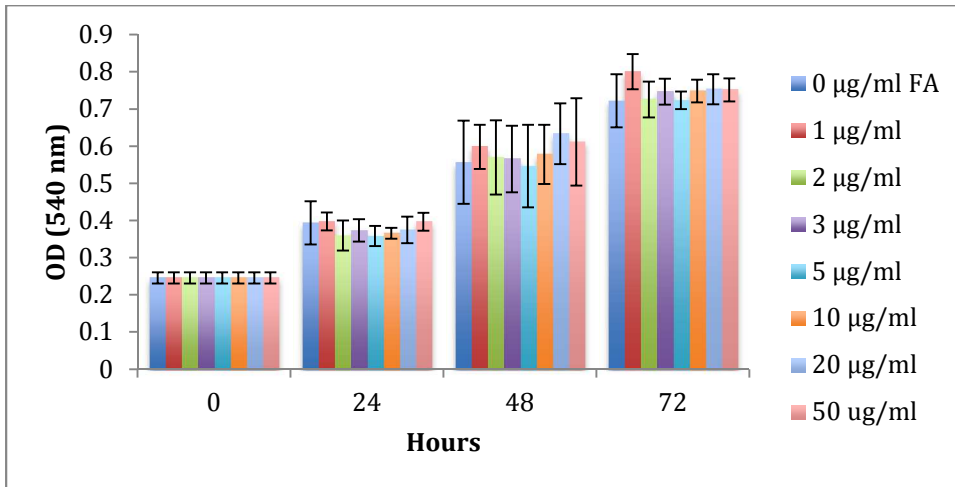


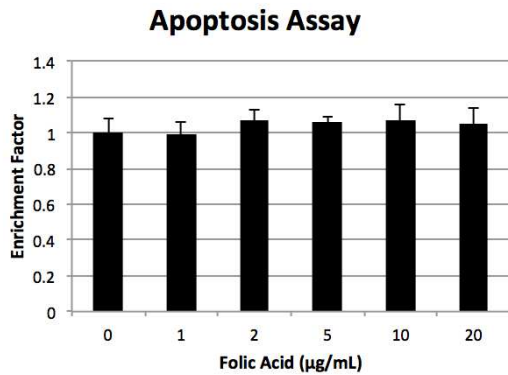
Figure 3.1 FA had no effect on cell proliferation in 5 human colon cancer cell lines. Human colon cancer cells HCT116 (A), HT29 (B), HCT15 (C), SW480 (D), and CaCO2 (E) were plated onto a 96-well plate and grown overnight. The cells were treated with different concentrations of FA (0-50 µg/mL) in complete RPMI Medium containing no FA for 24, 48 and 72 hours at 37°C under 5% CO₂. Cell proliferation was assessed by the MTT [(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] method. The optic density was recorded at 540 nm using an enzyme-linked immunosorbent assay plate reader. Values are means ± SD, n = 4.

3.2 Effect of FA on apoptosis in HCT116 cells

Apoptosis is an essential process for the body to eliminate cells that are damaged or unneeded. When transformed cells are able to evade apoptosis, they can survive and undergo malignant transformation and become cancerous (37). One of the main hallmarks of apoptosis is the cleavage of PARP by caspases. At its full length, PARP is 116 kD. When it is cleaved by caspase, it forms 89 kD and 24 kD fragments. The 24 kD fragment binds to nicked DNA and attenuates DNA repair (38). An increase in the 89 kD fragment serves as a marker for cells undergoing apoptosis.

To analyze the effect of FA on programmed cell death in colon cancer cells, an apoptosis assay was conducted using HCT116 colon cancer cells. HCT116 cells were treated with 0-20 $\mu\text{g/ml}$ FA for 48 hours in RPMI 1640 serum free medium. Apoptosis was measured using a Cell Death Detection ELISA Kit. As shown in Fig 3.2A, there was no significant difference in the amount of apoptosis in the different cell treatments of FA. Cleavage of PARP was subsequently measured to confirm the Apoptosis Assay. A consistent amount of the full length PARP (116 kD) was observed, but the cleaved form (89 kD) was not detected with any of the treatment levels of FA (Fig 3.2B).

(A)



(B)

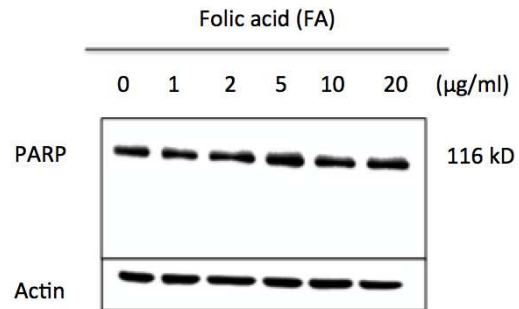


Figure 3.2 FA had no effect on apoptosis in HCT116 colon cancer cells. HCT116 cells were treated with different concentrations of FA (0-20 µg/mL) in serum free and folate-deficient medium for 48 hours at 37°C under 5% CO₂. Apoptosis was assessed using a Cell Death Detection ELISA Kit (Roche Diagnostics, Indianapolis, IN). The optic density was recorded at 490 nm. Values are means ± SD, n = 6 (A). HCT116 cells were treated with different concentrations of FA (0-20 µg/mL) in serum- free and folate-deficient medium and incubated for 48 hours. The cells were harvested and cell lysates were analyzed using Western Blot. Protein levels of PARP were measured (B).

3.3 Effect of FA on cell cycle regulation in HCT116 cells

The cell cycle is a process that is tightly regulated by different cellular proteins. Cyclin-dependent kinases (CDKs) and cyclins are major regulators of the cell cycle and influence the cell's fate by directing transition to S phase or G1 arrest. The protein cyclin D1 activates certain CDKs to allow for transition into G1 phase. There are certain CDK inhibitors, such as p21, that prevent the transition to G1 phase when DNA in the cell is damaged. Mutations in proto-oncogenes or tumor suppressor genes can promote tumor growth. Specifically, aberrant expression of cyclin D1 can promote G1 to S transition and activate cell cycle, while up-regulation of p21 inhibits the activity of CDK4/6 and leads to arrest of G1/S phase (30-31).

To determine if FA influences the expression of cell cycle regulating proteins, expression of p21 and cyclin D1 were measured using Western Blot. The results indicate that there was a significant decrease in p21 protein levels at 5 $\mu\text{g/mL}$ FA ($p=0.03$), but no other FA level led to a significant change (Fig 3.3A-B). Additionally, there was no significant change in cyclin D1 protein levels (Fig 3.3C) with increasing levels of FA.

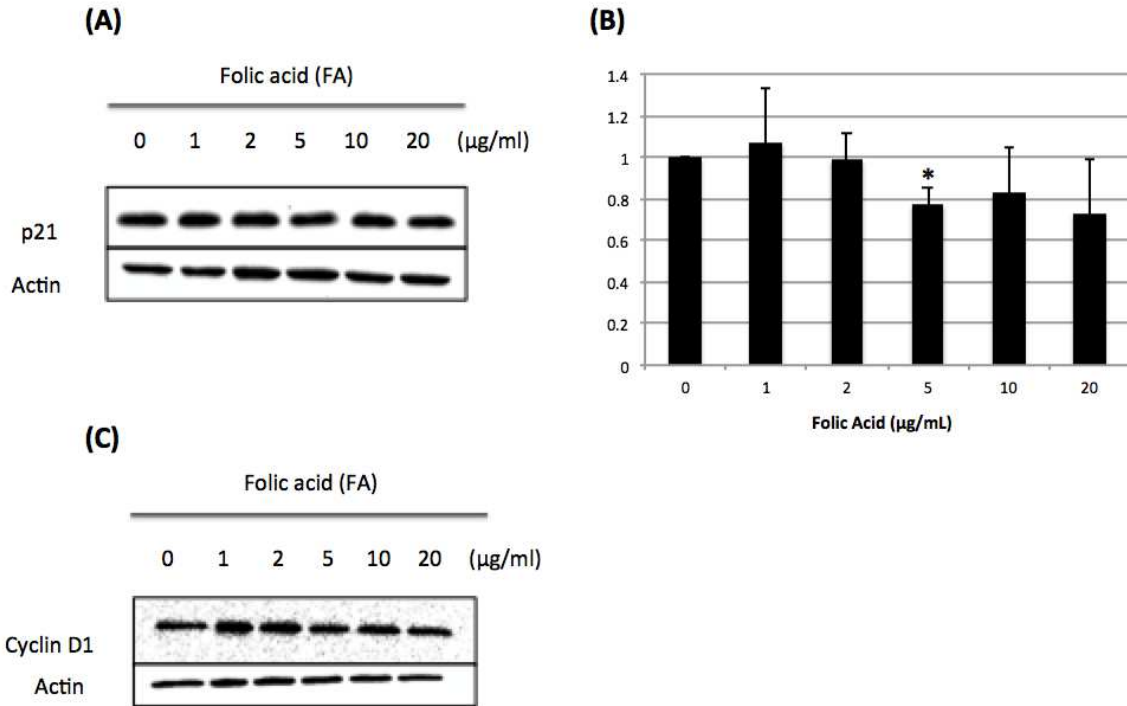


Figure 3.3 FA did not affect protein levels of p21 or cyclin D1. HCT116 cells were treated with different concentrations of FA (0-20 $\mu\text{g/mL}$) in serum-free and folate-deficient medium for 48 hours. The cells were harvested and cell lysates were analyzed using Western Blot. Protein levels of p21 were measured (A) and analyzed. Values are means \pm SD, n = 3 (B). Protein levels of cyclin D1 were measured (C).

3.4 Effect of FA on beta-catenin expression

WNT signaling is an important intracellular pathway regulating progression of colon cancer. The tumor suppressor gene APC is one of the key components of WNT signaling and is commonly (>90%) inactivated in colorectal cancer patients. In the WNT signaling pathway, APC binds to GSK3- β and ultimately leads to the phosphorylation of β -catenin and its resulting degradation. When the activity of APC is suppressed by a mutation, β -catenin becomes active, translocates to the nucleus, and binds to certain transcription factors (TCFs/LEFs). This leads to the activation of certain oncogenes that promote tumorigenesis (26).

HCT116 cell line contains wild type APC, making it an ideal cell line to study the WNT signaling pathway. Protein levels of phosphorylated GSK3- β (inactive form) were measured to observe the changes in WNT signaling activity (Fig 3.4A). Protein levels of non-phosphorylated β -catenin (active form) were also measured (Fig 3.4B). There was no significant difference in phospho-GSK3- β (inactive form) or non-phosphorylated β -catenin (active form) protein levels with increasing treatments of FA.

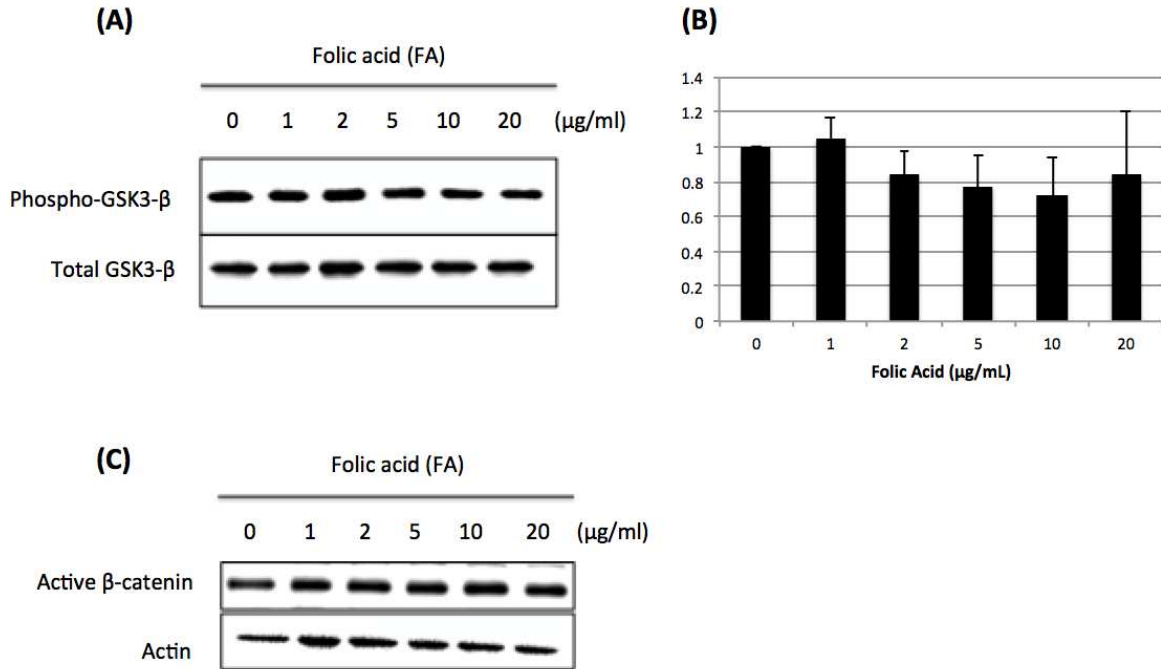


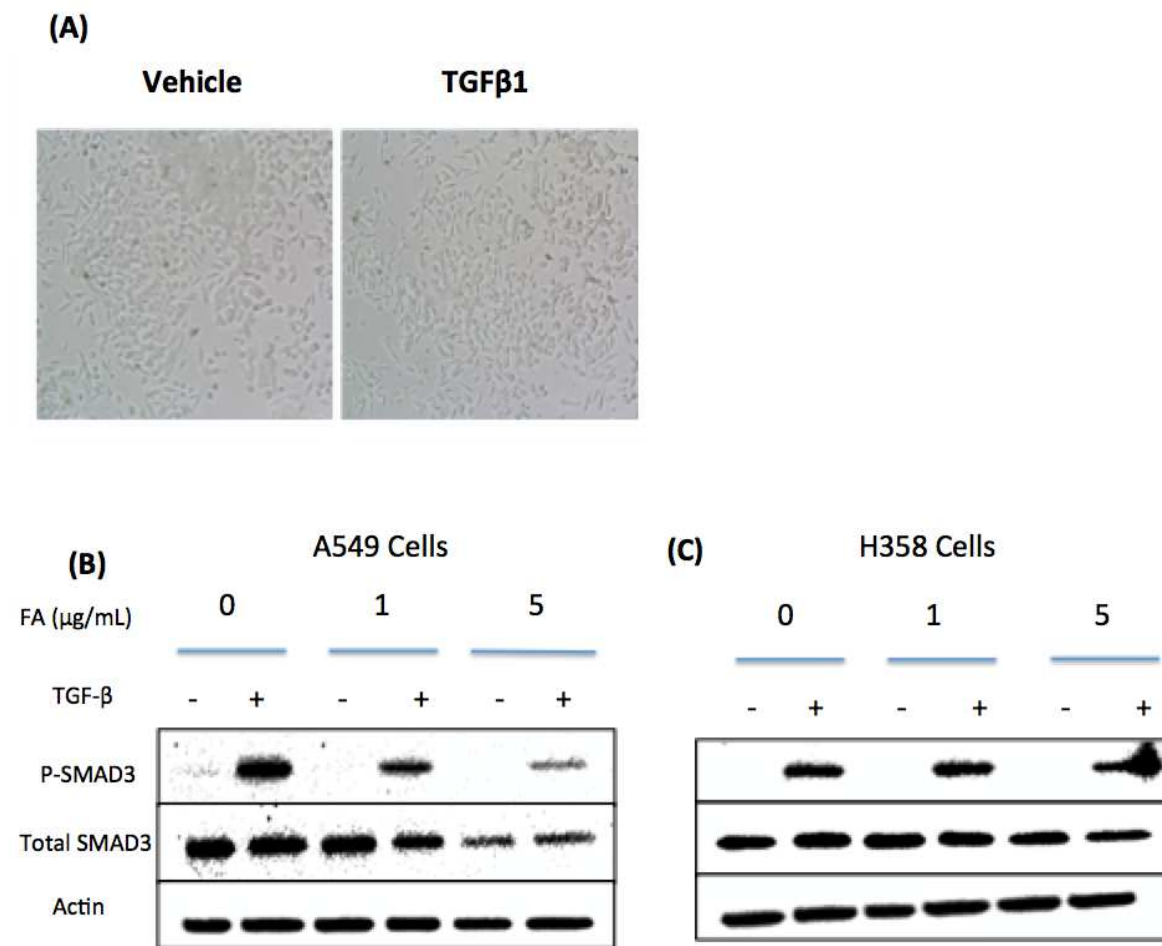
Figure 3.4 FA did not affect protein levels of phospho-GSK3- β or β -catenin. HCT116 cells were treated with different concentrations of FA (0-20 $\mu\text{g/ml}$) in serum free and folate deficient medium for 48 hours. The cells were harvested and cell lysates were analyzed using Western Blot. Protein levels of phospho-GSK3- β were measured (A) and analyzed. Values are means \pm SD, n = 3 (B). Protein levels of non-phosphorylated (active) β -catenin were measured (C).

3.5 Effect of FA on EMT

The process of epithelial-mesenchymal transition (EMT) is an essential process allowing cancer cells to metastasize or move to distant sites in the body. The process transforms polarized epithelial cells to cells containing a mesenchymal phenotype. This process increases the cancer cell's migratory capacity, invasiveness and resistance to apoptosis. EMT is induced by various growth factors, including transforming growth factor beta (TGF- β). Upon treatment with TGF- β , epithelial cells have been shown to decrease expression of epithelial markers, such as E-cadherin, and increase expression of mesenchymal markers, such as N-cadherin. TGF- β signaling activates a receptor complex, which induces the phosphorylation, and subsequent activation, of SMAD2 and SMAD3. The phosphorylation SMAD2 and SMAD3 leads to the transcription of TGF- β target genes, such as Snail. In addition, increased Snail levels have been associated with more invasive cancers (34).

To examine if FA can influence the ability of cancer cells to undergo EMT, we treated several human cancer cell lines with TGF- β and compared the morphological changes and TGF- β signaling. We found that all of the human cancer cell lines we tested showed no EMT phenotype, specifically SW480 cells (Fig. 3.5A). We speculate that all colon cancer cell lines we tested are insufficient models for observing TGF- β signaling, probably due to mutations of the type II TGF- β receptor. Therefore, we used two lung cancer cells line (A549 and H 358), which contain an intact TGF- β pathway and are commonly used as a research model for EMT. In the A549 cells, pretreatment of FA decreased TGF- β -induced phosphorylation of SMAD3, although there was decrease of total Smad3 at high doses of TGF- β (5 μ g/mL) (Fig 3.5B). In the H358 cells, FA

treatment slightly suppressed TGF- β -induced phosphorylation of Smad 3 at high doses (5 $\mu\text{g/mL}$) (Fig. 3.5C). As shown in figure 3.5D, treatment with TGF- β induced Snail expression, downregulated E-cadherin expression, and upregulated N-cadherin expression. In the A549 cells, FA treatment did not influence TGF- β mediated upregulation of snail and downregulation of E-cadherin, but blunted TGF- β induced expression of N-cadherin. (Fig 3.5D).



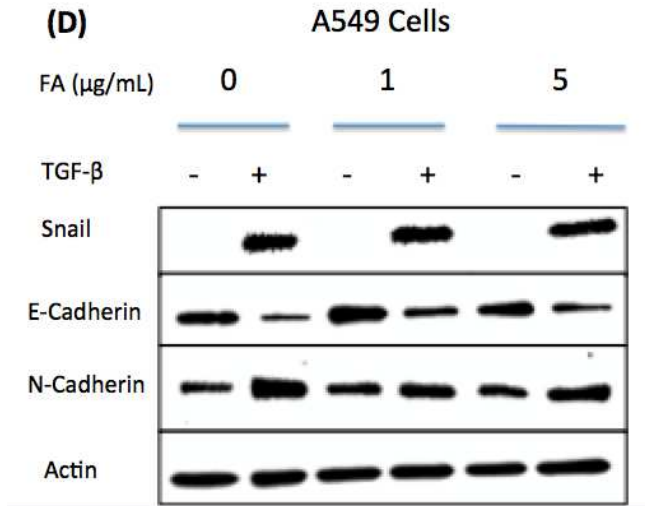


Figure 3.5 FA did not affect expression of EMT regulating genes. SW480 colon cancer cells were treated with $5\mu\text{L}$ of TGF- β 1. Morphological changes were observed with microscopic imaging (A). A549 and H258 lung cancer cells were treated with different concentrations of FA (0, 1 and $5\mu\text{g/mL}$) in serum free and folate-deficient medium for 6 hours. Cells were co-treated with $5\mu\text{L/well}$ of TGF- β 1. The cells were harvested and cell lysates were analyzed using Western Blot. Protein levels of phospho-SMAD3, total SMAD3 were measured (B-C). Protein levels of Snail, E-cadherin and N-cadherin were also measured (D).

3.6 Effect of FA on ATF3 protein levels in HCT116 cells

Throughout the course of tumor progression, cells can face various stressors. Activating transcription factor 3 (ATF3) is a member of the ATF/CREB family of transcription factors, and can be induced by numerous stress signals. Recently, dichotomous activity of ATF3 has been reported, including apoptotic and metastatic activity (41). A study using HT29 and CaCO2 colon cancer cells showed that ATF3 promoted *in vitro* motility and invasion (45). In an *in vivo* study, overexpression of ATF3 significantly reduced the size of tumor xenografts in mice (46). Recently we found that ATF3 overexpression suppressed anti-apoptotic protein, Bcl-2, while it increased collective cell migration (39). Therefore, ATF3 may play a pro- and anti-tumorigenic role in colon cancer cells in a context-dependent manner.

Protein levels of ATF3 were measured in HCT116 cells with different levels of FA treatment. There was no significant difference in protein levels of ATF3 with different treatment amounts of FA (Fig3.6A-B).

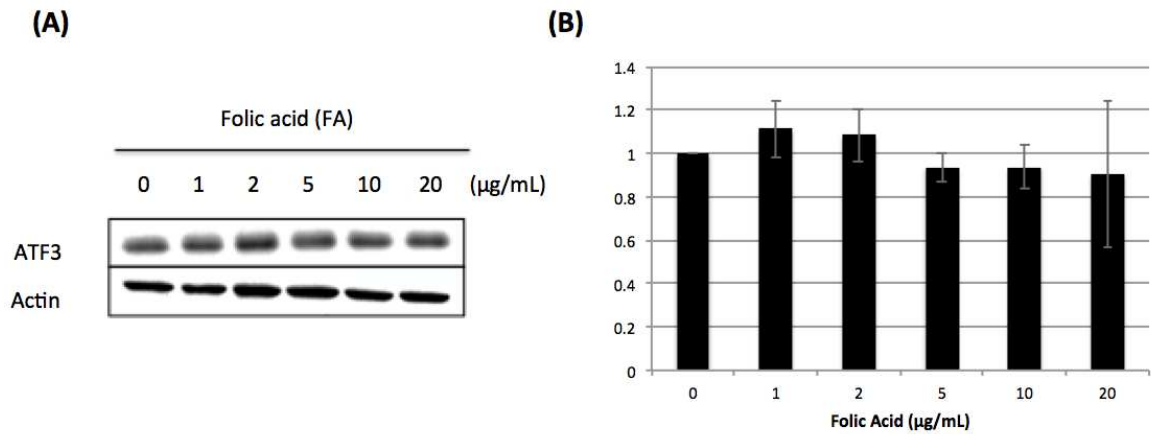
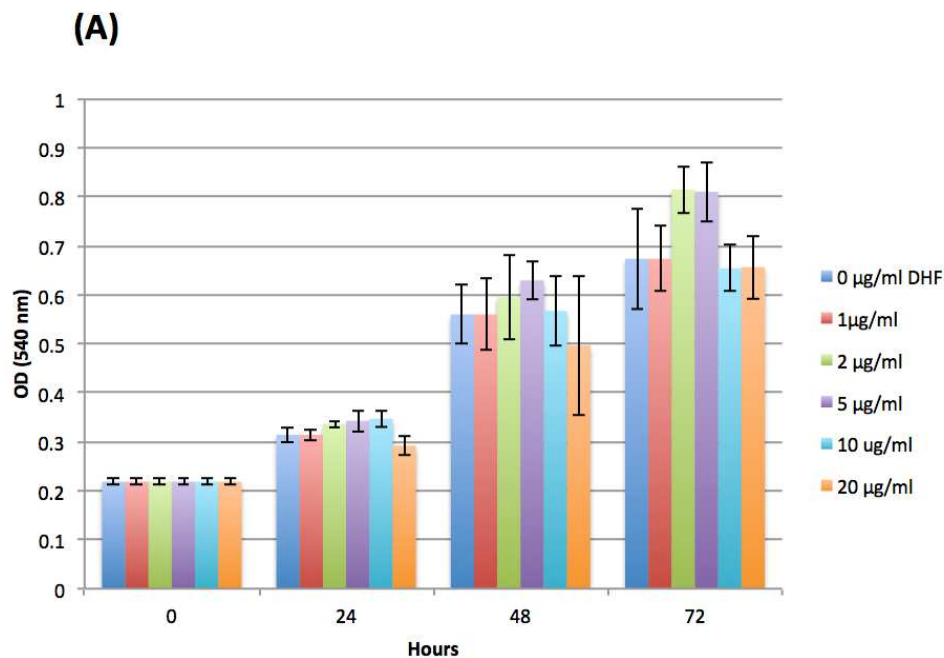


Figure 3.6 FA did not affect protein levels of ATF3 in HCT116 cells. HCT116 cells were treated with different concentrations of FA (0-20 $\mu\text{g/mL}$) in serum-free RPMI Medium and incubated for 48 hours. The cells were harvested and cell lysates were analyzed using Western Blot. Protein levels of ATF3 were measured (A) and analyzed. Values are means \pm SD, n = 3 (B).

3.7 Effect of dihydrofolic acid (DHF) on cell proliferation and cell cycle regulation in HCT116 cells

The first step in FA metabolism is the reduction of FA by dihydrofolate reductase to dihydrofolic acid (DHF) (24). It is possible that FA is partially metabolized in the small intestine and the metabolites of FA reach colon cells to influence CRC cell growth. In previous studies, DHF has been shown to inhibit cell growth in Caco-2 colon cancer cells. The treatment of colon cancer cells with metabolites of FA, such as DHF, may control cancer cell growth and development.

To understand the effect of FA metabolites on the development of CRC, HCT116 cells were treated with different levels of DHF. As shown in Fig 3.7A, cell proliferation rates did not change with different treatment amounts of DHF (0-20 $\mu\text{g}/\text{mL}$). In addition, DHF treatment (0-10 $\mu\text{g}/\text{mL}$) did not significantly affect proteins involved in cell cycle regulation, including p21 and cyclin D1 (Fig 3.7B).



(B)

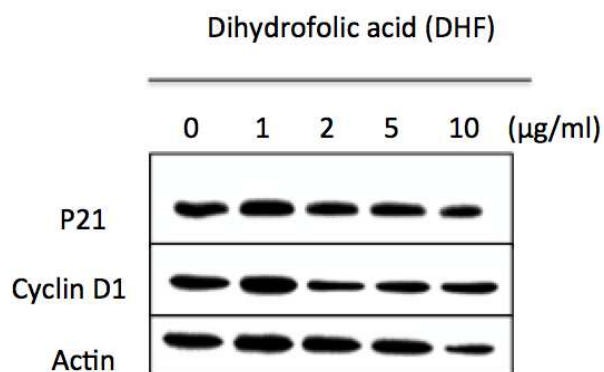


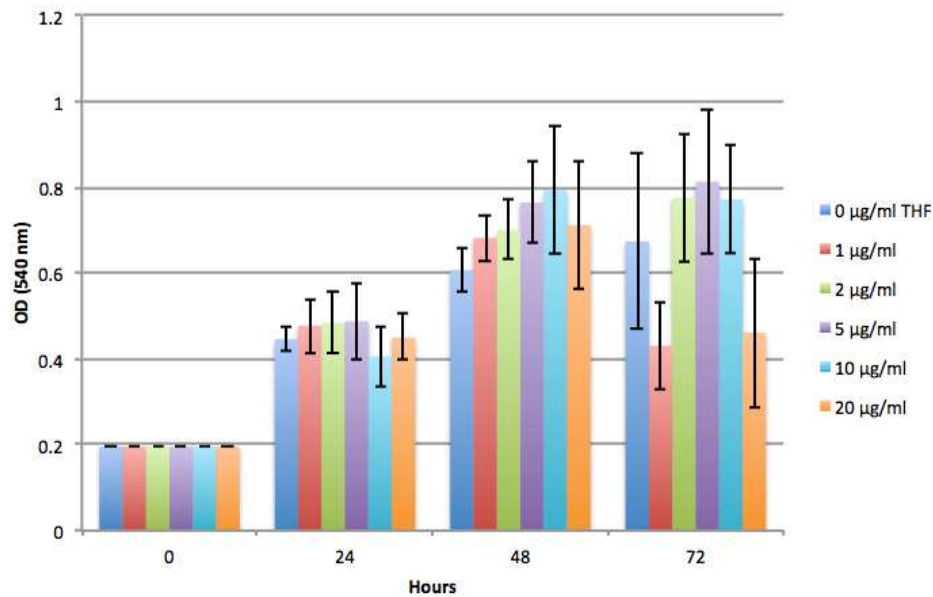
Figure 3.7 DHF did not affect HCT116 cell proliferation and expression of cell cycle-regulating genes. HCT116 cells were plated onto a 96-well plate and grown overnight. The cells were treated with different concentrations of DHF (0-20 µg/mL) in complete RPMI Medium for 24, 48 and 72 hours at 37°C under 5% CO₂. Cell proliferation was assessed by the MTT method. The optic density was recorded at 540 nm using an enzyme-linked immunosorbent assay plate reader. Values are means ± SD, n = 4 (A). HCT116 cells were treated with different concentrations of DHF (0-10 µg/mL) in serum free RPMI Medium and incubated for 48 hours. The cells were harvested and cell lysates were analyzed using Western Blot. Protein levels of p21 and cyclin D1 were measured (B).

3.8 Effect of Tetrahydrofolic acid (THF) on cell proliferation and cell cycle regulation in HCT116 cells

FA metabolism involves the breakdown of FA to THF through a series of reduction reactions. This process is essential for the use of THF in one-carbon metabolism. THF can then be used in DNA synthesis and repair through the formation of nucleotides. The compound is also essential for the metabolic reaction of homocysteine conversion to methionine. Methionine is subsequently transformed into S-adenosylmethionine (SAM), which participates in over 100 types of methylation reactions. (24). Metabolites of FA, such as THF, may influence the development of colon cancer depending on the level of the metabolite. High levels of THF may increase the ability of colon cancer cells to proliferate or progress through the cell cycle. In a study by Akoglu et al., 10 $\mu\text{g/mL}$ of THF increased colon cancer cell growth after 24 hours, but the effect was not maintained after 48 hours (48).

To examine the effect of THF on the proliferation of colon cancer cells, HCT116 cells were treated with increasing levels of THF. Different levels of THF (0-20 $\mu\text{g/mL}$) did not lead to a dose-dependent trend in the proliferation of colon cancer cells (Fig 3.8A). As shown in Fig 3.8B, proteins involved in cell cycle regulation (p21 and cyclin D1) did not change with increasing levels of THF (0-10 $\mu\text{g/mL}$).

(A)



(B)

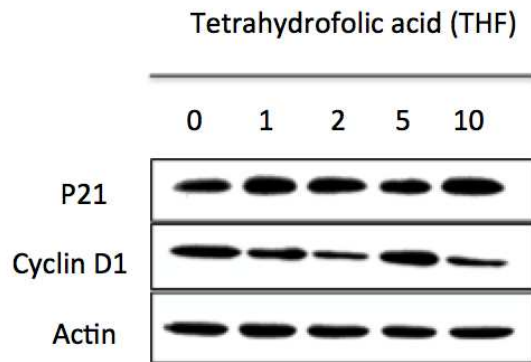


Figure 3.8 THF did not affect HCT116 cell proliferation or cell cycle regulation. HCT116 cells were plated onto a 96-well plate and grown overnight. The cells were treated with different concentrations of THF (0-20 µg/mL) in complete RPMI Medium for 24, 48 and 72 hours at 37°C under 5% CO₂. Cell proliferation was assessed by the MTT method. The optic density was recorded at 540 nm using an enzyme-linked immunosorbent assay plate reader. Values are means ± SD, n = 4 (A). HCT116 cells were treated with different concentrations of THF (0-10 µg/mL) in serum-free RPMI Medium and incubated for 48 hours. The cells were harvested and cell lysates were analyzed using Western Blot. Protein levels of p21 and cyclin D1 were measured (B).

Chapter 4: Discussion, Conclusion, and Future Perspectives

4.1 Discussion

Folic acid (FA), a major dietary methyl donor, transfers one-carbon units to amino acids, nucleotides, and other molecules. The main function of one-carbon metabolism is the support of DNA synthesis and methylation reactions (24). Folate deficiency can lead to DNA strand breaks, uracil misincorporation, chromosomal breakage, and impaired DNA repair. When the integrity of the DNA is compromised, cancer risk increases, particularly colorectal cancer (CRC) (20). Therefore, adequate FA levels are necessary to allow for the proper functioning of one-carbon metabolism.

In our study, we wanted to investigate the influence of FA and its metabolites on human CRC cell development. First, we observed cell proliferation in five different colon cancer cell lines. The cancer cells used represent cancer at the adenocarcinoma, or invasive stage of development. A range of FA concentrations were used to represent deficient, adequate, and excessive levels. A treatment level of 0 $\mu\text{g/mL}$ FA represented a deficiency, 1 $\mu\text{g/mL}$ FA represented an adequate amount, and levels above 1 $\mu\text{g/mL}$ FA represented an excess amount. The optimal level of FA was determined because the RPMI medium contains 1mg/L FA to support the colon cancer cells in their ideal environment. In all five colon cancer cell lines spanning a variety of potential cancer mutations, there was no significant influence of FA on cell proliferation (Fig 3.1A-E). Therefore, FA may not be able to influence cancer cells once they have reached the more advanced stages of development.

Next, we observed the effect of FA on colon cancer cell apoptosis. Apoptosis is an essential process through which cancer cells in the body are killed in order to prevent further proliferation (37). An important step in the process of apoptosis is the cleavage of PARP by caspases (38). The Apoptosis Assay demonstrated no effect of FA on colon cancer cell death in HCT116 cells (Fig 3.2A). PARP protein levels demonstrated no cleavage of PARP, and no change in the amount of full length PARP with different treatments of FA (Fig 3.2B). We then analyzed the effect of FA on cell cycle regulation. There was a significant decrease in p21 protein levels at 5 $\mu\text{g/mL}$ compared to 0 $\mu\text{g/mL}$ FA ($p=0.03$) (Fig 3.3A-B). This change was not observed at any other FA treatment level. Additionally, there was no significant dose-dependent response. Although there was a significant decrease in p21, the result was not consistent when measuring cyclin D1 levels (Fig 3.3C).

PI3K and Wnt are significant signaling pathways in the progression of colon cancer. In addition, APC is a colon-specific tumor suppressor gene that is influenced by these signals and inhibits tumorigenesis at the early stages of transformation. In fact, 90% and 50% of colon cancer patients possess APC truncation and PI3K mutations, respectively (35). GSK3 β is a major mediator of PI3K and Wnt pathways, and directly influences phosphorylation and proteasomal degradation of β -catenin (26). Therefore, we measured the phosphorylation of GSK3 β at the serine 9 residue, which is an inactive phosphorylation site that leads to β -catenin expression (40). Overall, different levels of FA did not affect phospho-GSK3- β or β -catenin protein levels (Fig 3.4A-B).

Several studies have demonstrated that high doses of FA may enhance the progression of cancer in advanced stages (4, 12, 20). To test this hypothesis, we studied FA's influence on the ability of cells to undergo epithelial-mesenchymal transition (EMT) and become metastatic. EMT allows polarized epithelial cells to lose cell-cell adhesion and migrate to distant areas. An important pathway in EMT is TGF- β signaling that causes decreased expression of epithelial markers, such as E-cadherin, and increased expression of mesenchymal markers, such as N-cadherin (34). In our study, we chose the SW480 human colon cancer cell line, which is a commonly used metastatic model *in vitro* and *in vivo*. Microscopic observation indicated that TGF- β did not lead to EMT (Fig 3.5A). This is likely due to a mutation in the type II receptor of TGF- β , which is common in colon cancer patients. Therefore, we decided to select a small cell lung cancer model (A549 and H358), because they are a well-established cell line for observing EMT. Our results indicated that pretreatment of FA decreased TGF- β -induced phosphorylation of SMAD3 (Fig 3.5B), but the same observation was not made in the H358 cells (Fig 3.5C). To test the result shown in the A549 cells, other markers for EMT were measured including Snail, E-cadherin and N-cadherin. A significant change was observed in N-cadherin, but not in other markers (Fig 3.5D). Therefore, further studies including cell migration and wound healing assays are required to address the inconsistent effect by FA.

Activating transcription factor 3 (ATF3) is another protein we chose to analyze due to its dual roles in cancer development. ATF3 is a stress inducible gene that regulates homeostasis of cell proliferation, differentiation and immunity. Recently, Jiang et al. found that ATF3 induced apoptosis and collective cell migration in the same cell line (39). It has also been shown that this gene may prevent cancer cell survival at early stages

and promote metastasis at late stages of cancer (41). Because the expression pattern of this gene is very similar to the effect of FA on cancer development in terms of stage specificity, we explored whether different doses of FA may influence expression of this gene. The results indicated that FA did not influence ATF3 expression in human colon cancer cells (Fig3.6 A-B).

Due to the observation that FA did not affect CRC cell growth or progression, we hypothesized that the effect of FA is mediated by its metabolites in the body. Thus, we analyzed the effects of two FA metabolites, dihydrofolic acid (DHF) and tetrahydrofolic acid (THF). The first step in FA metabolism is the reduction of FA by dihydrofolate reductase to the intermediate DHF, or completely to THF. THF can then be used in DNA synthesis and repair reactions. It is also needed for the conversion of homocysteine to methionine. Normal levels of FA and its metabolites are fundamental for DNA replication and repair, methionine synthesis, and methylation reactions (24). It is possible that FA is partially broken down in the small intestine and the metabolites of FA reach colonic cells to influence CRC cell growth. Additionally, previous studies using Caco-2 cells showed that DHF and THF decrease and increase CRC cell proliferation, respectively (48). To examine this relationship, we first treated HCT116 cells with DHF and observed cell proliferation and cell cycle progression. The results from the MTT assay demonstrated no dose-dependent change in cell proliferation with increased levels of DHF (Fig 3.7A). In addition, increased levels of DHF did not affect proteins involved in cell cycle regulation (Fig 3.7B). We subsequently conducted a similar experiment, but used THF as the treatment compound. The MTT assay showed no significant effect of

THF on cell proliferation in HCT116 cells (Fig 3.8A). To support this result, we found that THF did not influence protein levels of cyclin D1 or p21 (Fig 3.8B).

The present study suggests that FA and its metabolites do not affect CRC cell growth, apoptosis, or metastasis in the *in vitro* culture model. In support of our data, Qin et al. (2014) conducted a meta-analysis of randomized controlled trials. The researchers analyzed eight articles from PubMed and Embase databases and found that FA treatment was not associated with CRC risk. The results were consistent even after conducting subgroup analyses stratified by certain confounding variables (43). Additionally, Otani et al. (2007) conducted a case-control study of colorectal cancer patients. They measured plasma folate levels in over 38,000 patients and found no association with the risk of CRC in humans (47).

Conflicting evidence shows that a high folate intake is believed to increase cancer risk. After the mandatory FA fortification in the US, plasma levels of FA increased along with the rate of colon cancer. Mason et al. (2007) hypothesized that the relationship between increased FA and colon cancer rates may be due to the role FA plays in DNA synthesis and repair. Their observations cannot prove a causal association between FA and increased risk of CRC because it is only observational (4). Although this relationship was observed, the evidence is still inconsistent and warrants further investigation.

In contrast, Attias et al. (2006) determined that FA could decrease malignant transformation of colon cancer cells. They hypothesized that a FA deficiency could increase tumorigenic activities by altering the expression of genes related to cell cycle control and cell death. The researchers examined the gene expression of insulin-like

growth factor-I receptor (IGF-IR), which is thought to play a critical role in tumorigenesis. They found that FA downregulated IGF-I signaling transduction, potentially allowing FA to act as a chemopreventive agent (22). Although the experimental design was similar to our experiment, they did not confirm their data in other cell lines. The results must be confirmed in additional cancer cell lines, as well as in an *in vivo* model, in order to support their conclusion.

A variety of studies have demonstrated that high FA intake does not influence the risk of CRC. A study conducted by the American Cancer Society analyzed the relationship between high levels of FA intake and CRC risk. The Cancer Prevention Study II Nutrition Cohort contained about 99,000 participants, with over 1,000 patients diagnosed with CRC during the 8-year period. The findings were consistent with other major studies that folate can prevent against colon cancer. In addition, the researchers found that there is no evidence that FA fortification increases CRC risk. This relationship was sustained in the group taking in the highest amount of FA at 660 µg/d (42). Additionally, a study conducted by Cho et al. (2015) investigated unmetabolized FA as a potential cause of the observed increase in CRC incidence after fortification. They evaluated pre-diagnostic plasma levels of unmetabolized FA in case-control studies within the Nurses' Health Study. They concluded that unmetabolized FA was not associated with the increased risk of CRC (14).

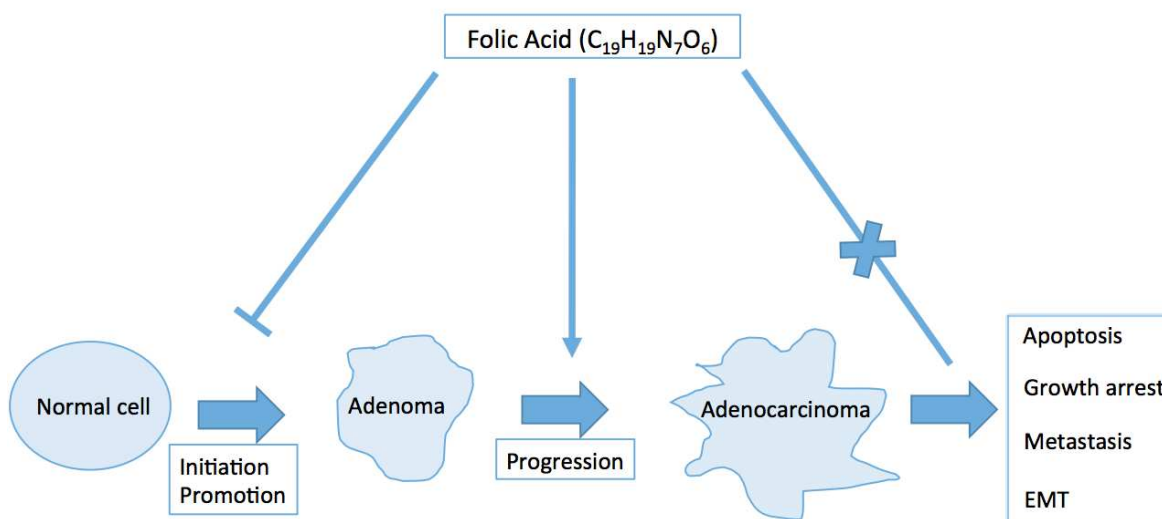


Figure 4.1.1 Proposed summary of the effect of FA on CRC. FA can have different effects on CRC depending on the stage of cancer development.

After reviewing the literature, we proposed a summary of the relationship between FA and CRC. It was previously hypothesized that FA can have dual effects on CRC depending on the stage of development. In normal colon cells, FA is known to prevent the transformation of normal cells into adenomas (23). In adenoma cells, FA has been shown to promote progression to later stages of development (4, 62, 65). In our study, we analyzed the effect of FA on apoptosis, cell growth arrest, metastasis and EMT, and discovered that FA did not influence cancer phenotypes in the advanced (adenocarcinoma) stages. We hypothesize that advanced adenocarcinoma cells with numerous mutations in cancer-associated genes overcome any beneficial effect of FA.

In conclusion, our study demonstrated that FA and its metabolites have no effect on CRC cells in the adenocarcinoma stage. This was indicated in a variety of colon cancer cell lines. There is a potential for FA to influence EMT in lung cancer cells, but

the relationship must be explored through future research. Our data may have future implications on whether other countries will adopt a mandatory FA fortification program to prevent certain health complications. In addition, the use of anti-folate medications may not be beneficial for colon cancer patients with adenocarcinomas.

4.2 Conclusion and Future Perspectives

More research should be conducted to confirm the conclusion that FA supplementation does not affect CRC risk. In our study, we analyzed the effect of FA *in vitro* by treating adenocarcinoma colon cancer cells. Unfortunately, *in vitro* studies are not conducted in the same environment as the intact organism and may produce different results. Therefore, an *in vivo* study in mice should be directed to further analyze the effect of FA on CRC. This study could be conducted in healthy mice, and in mice with current colon neoplasms. A study should also analyze the effect of FA in healthy human colon cells. They could conduct a similar *in vitro* study, but treat healthy cells with different levels of FA and observe any morphological changes that occur. This could give further insight into the proper RDA of FA that would optimally reduce colon cancer risk in the future.

In addition, studies should examine the effect of FA on cells in the early stages of cancer development. Unfortunately, colon cell lines in the early (adenoma) stages of cancer development are not available. Therefore, a similar study could be conducted in breast cancer cells. Normal breast cells, adenoma cells, and adenocarcinoma cells could be treated with different levels of FA and cell proliferation could be measured. This would be a sufficient model to observe the potential dual effects of FA depending on the stage of cancer development.

Further research should investigate the effect of FA and its metabolites on EMT and cancer cell metastasis. In our study, we analyzed EMT through TGF- β signaling in lung cancer cells. A potential experiment to further this data would be to analyze

pathways involved with tumor necrosis factor-alpha (TNF- α) in colon cancer cells. TNF- α is a pro-inflammatory cytokine known to enhance metastatic properties in colon cancer cells (44). Analyzing the relationship between FA and metastatic induction via TNF- α could provide promising new research. These efforts will continue to expand the scientific community's understanding of factors related to the reduction of cancer metastasis, which is associated with 90% of cancer mortality (25).

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