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Folate deficiency has been linked to multiple health problems including neural tube defects and cancer, especially colorectal cancer. Folate is an essential dietary vitamin that is required for DNA synthesis and cellular methylation reactions. The mechanistic links between these associations is not completely understood. Folate deficiency has been shown to affect genes associated with the Wnt pathway, including β-catenin. E-cadherin is an important regulator of Wnt signaling through controlling the level of free β-catenin. It has also been shown that E-cadherin is affected by folate deficiency. The purpose of this study was to determine if folate deficiency altered the levels of E-cadherin protein and RNA, β-catenin protein, and E-cadherin and β-catenin cellular localization in two colon epithelial cell lines. Caco-2 and FHC cells were grown for 15 and 30 days in folate sufficient and deficient medium. Cellular growth rates for both cell lines were reduced in folate deficient cells, although the cells continued to grow. Cell cycle phase distribution showed increases in S phase and G_2/M phase in Caco-2 cells, whereas there was no change in FHC cells. E-cadherin and β-catenin protein levels decreased slightly in folate deficient Caco-2 cells at days 15 and 30. This was associated with a decrease in E-cadherin transcripts at day 30. FHC cells showed similar results at day 15, but a small increase in E-cadherin protein at day 30 and no change in β-catenin. Immunofluorescent staining of Caco-2 cells showed diffuse cytoplasmic staining of E-cadherin and β-catenin in folate sufficient cells for both Caco-2 and FHC cells. Folate deficient Caco-2 cells showed no change in E-cadherin, but an increase in membrane

associated β-catenin. Folate deficient FHC cells showed an increase in nuclear localization of both E-cadherin and β-catenin. The AKT pathway was also analyzed in Caco-2 cells to determine if alterations in this growth regulatory pathway was associated with folate deficiency. In Caco-2 cells, an increase in activated AKT was detected at day 30 in folate deficient cells, and other proteins in the AKT pathway (PDK1, PTEN, and c-Raf) showed slight decreases whereas a small increase was detected for GSK-3β. Finally, we confirmed published findings that Caco-2 cells were adapting to folate deficiency by upregulating the folate transporters: folate receptor 1 (FolR-1) and reduced folate carrier (RFC). In general, those results suggest that alterations in E-cadherin and β-catenin occur in folate deficient colon epithelial cells, although the cells can adapt and continue to proliferate.

THE EFFECTS OF FOLATE DEFICIENCY ON E-CADHERIN AND β-CATENIN IN COLON EPITHELIAL CELLS

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Statement of Problem

The purpose of this study is to determine if folate deficiency causes any alterations in the protein levels and localization of E-cadherin and β-catenin in two colon epithelial cell lines.

There is strong epidemiological data supporting a relationship between a diet rich in fruits and vegetables and decreased risk of cancer. However, the molecular basis for this relationship is still not well defined. Folate is an essential vitamin derived from the diet. The metabolites of the folate pathway are required for synthesis of nucleotides for DNA synthesis and methyl donors for methylation reactions. Folate deficiency has been associated with increased risk of various cancers, in particular, colorectal cancer.

The Wnt signaling pathway is highly conserved and critically important in cellular growth, proliferation, and development. As such, the Wnt pathway has been studied extensively to elucidate its role in various forms of cancer. Mutations in the Wnt signaling pathway genes, such as APC, are commonly found in colorectal cancer.

E-cadherin is a protein that is important in cell-cell adhesion and regulates cell growth. This protein is involved in the Wnt signaling pathway by its interaction with β-catenin, an important transcription factor and downstream effector molecule of the

canonical Wnt pathway. The E-cadherin protein has also been implicated in cancer, as a reduction in this protein is common in transformed cells.

The relationship between folate deficiency, Wnt signaling, and E-cadherin is the focus of this project. E-cadherin and β-catenin protein levels and cellular localization will be compared in folate sufficient and deficient normal and transformed colon epithelial cells. The hypotheses are that 1) the level of E-cadherin protein is reduced in folate deficient cells as compared to folate sufficient cells and 2) the cellular localization of E-cadherin and β-catenin will be altered in folate deficient cells in comparison to folate sufficient cells.

In addition, the AKT signaling pathway will be analyzed in cells grown under folate sufficient and deficient conditions. The AKT pathway is a major growth regulatory pathway in the cell and is likely affected by folate deficiency. Moreover, AKT has been shown to be involved in the levels of β-catenin and its cellular localization.

Folate Pathway and Metabolism

Folate is an essential dietary B-vitamin that cannot be synthesized. It is obtained through a diet that includes leafy green vegetables, broccoli, citrus fruits, dry cereals, and organ meats. It can also be obtained through dietary supplements that contain folic acid. After folate is ingested it is absorbed by the enterocytes of the small intestines and enters the blood. Folate travels through the blood plasma bound to albumin, a high affinity folate binding protein, and erythrocytes. The serum level of folate is regulated by the liver (Lucock, 2000).

Folate is transported from the plasma into cells through a variety of mechanisms. A low affinity reduced folate carrier (RFC) functions as a carrier that directly moves folate into the cell. There are also high affinity folate receptors (FR), which move folate into the cells via endocytosis (Kim, 2007). Once plasma folate has entered the cell it is converted to 7,8-dihydrofolate and then converted to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR). The THF is converted to 5,10-methyleneTHF, which through the enzymatic activity of methylenetetrahydrofolate reductase (MTHFR), is converted to 5-methylTHF. This is the main form of folate found in the plasma, which provides the methyl group for *de novo* methionine synthesis. 5,10-methyleneTHF is also converted to 10-formylTHF, which is used for purine synthesis for DNA. It is also used for conversion of deoxyuridylate to thymidylate via thymidylate synthetase (Figure 1B). This is the pathway used for generating the nucleotide precursors for DNA synthesis.

The folate pathway is also responsible for producing methyl donors for *de novo* DNA methylation reactions (Figure 1A). 5,10-methyleneTHF is converted to 5-methylTHF and this provides the methyl group necessary to convert homocysteine to methionine. The newly produced methionine is converted to S-adenosylmethionine (SAM). This is the major methyl donor for methylation reactions such as DNA methylation. SAM is converted to S-adenosylhomocysteine (SAH) after the methyl group is removed from SAM for various methylation reactions. SAH is converted back to homocysteine (Lucock, 2000; J Ma et al., 1997). Importantly, SAH is a strong inhibitor of SAM-dependent methyltransferases and is a strong predictor of

hypomethylation. Studies have shown the levels of SAH increase in folate deficient cells (James et al., 2002; Jhaveri et al., 2001).

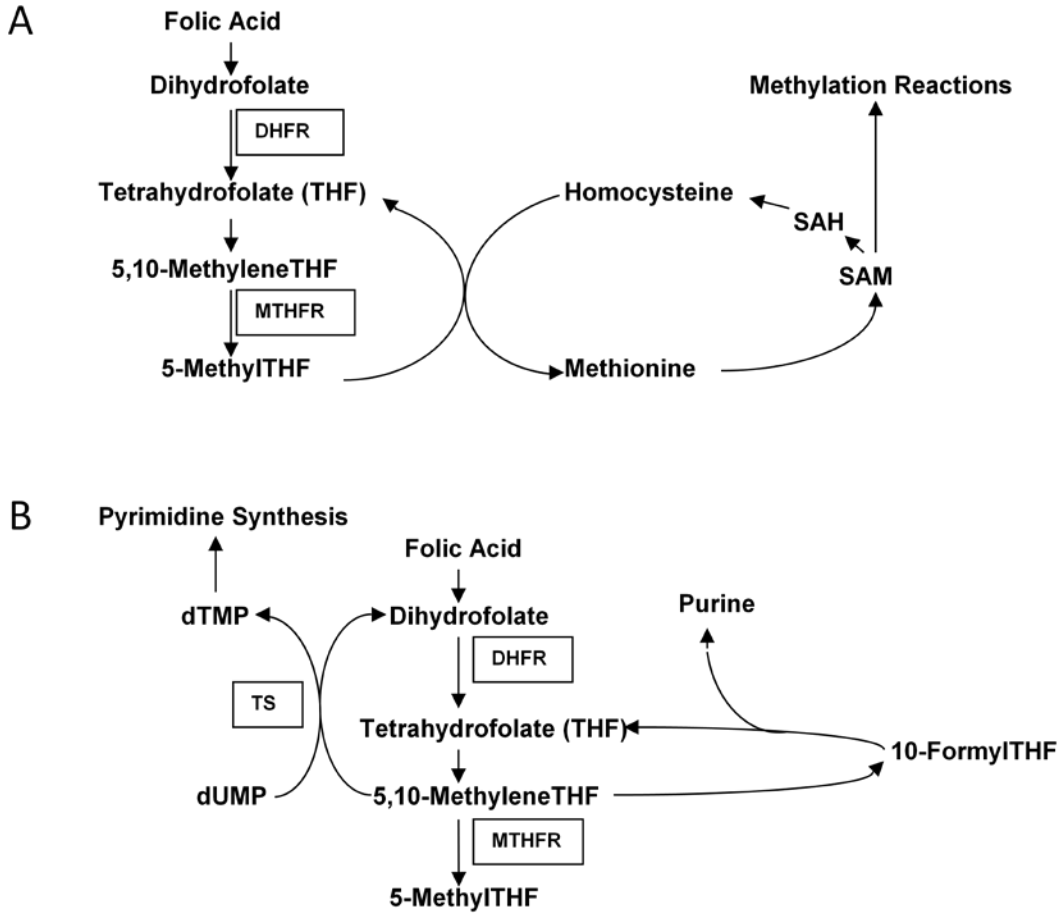


Figure 1: Folate Pathway. A) Methylation of Homocysteine to Methionine. B) Biosynthesis of Purines and Pyrimidines.

Abbreviations: DHFR, Dihydrofolate Reductase; MTHFR, Methylenetetrahydrofolate Reductase; SAM, S-adenosylmethionine, SAH, S-adenosylhomocysteine; TS, Thymidylate Synthetase.

Folate Deficiency and Cancer

Folate has been shown to be a risk factor in some types of cancer. In particular, increased risk of colorectal cancer has been strongly correlated with folate deficiency. The basis for this increased risk has not been definitively determined, but it is a reasonable assumption that it results from the multiple cellular consequences of folate deficiency. These would include decreased cell proliferation, increased DNA damage, impaired DNA repair, and altered DNA methylation patterns (Ma et al., 2005).

Folate deficiency has been found to induce chromosome breaks by causing deoxynucleotide pool imbalances. In folate deficient cells, the amount of 5,10-methyleneTHF is reduced, which is required to convert deoxyuridylate to thymidylate. This can cause an accumulation of deoxyuridylate, which becomes incorporated into new strands of DNA. The removal of these abnormal bases increases the possibilities of DNA strand breaks (Kim, 2007). In the colonic mucosa of rats it was found that exons 5-8 of p53, a tumor suppressor gene, were very susceptible to strand breaks due to folate deficiency (Novakovic et al., 2005). This loss of p53 function is common in most cancers.

Mutations in the gene for MTHFR, an enzyme in the folate pathway (see Figure 1), has also been shown to increase the risk of colorectal cancer through reduced plasma folate and increased plasma homocysteine (Ma et al., 1997). Supporting the idea that folate deficiency increases cancer risk, mutations have also been identified in the folate receptors RFC1 and Folbp1 that affected the folate levels in colonocytes. The reduced activity of Folbp1 due to mutation caused a decreased amount of folate to be reabsorbed

by the kidneys, leading to reduced folate levels in the plasma and tissue (Ma et al., 2005). These findings suggest that folate deficiency caused by reduced folate transporters increases the risk of colorectal cancer.

The Wnt Signaling Pathway

The Wnt signaling pathway was originally characterized from research on colorectal cancer (Jin et al., 2008). Since then, much more has been discovered about this important signaling pathway. There are 16 Wnt ligands that have been characterized in vertebrates. These ligands are secreted and bind to various cell surface receptors to promote cell growth, proliferation, and differentiation. The Wnt proteins are highly conserved, which is indicative of this pathway's importance.

The canonical Wnt pathway is stimulated when a secreted Wnt ligand, such as Wnt-3a, associates with Frizzled, a seven transmembrane cell surface receptor (Figure 2). These ligands will also associate with low density lipoprotein receptor-related protein (LRP). Receptor stimulation causes the promotion of a Wnt receptor complex that recruits the proteins Disheveled and Axin. This complex allows for the stabilization of β-catenin, leading to a rise in cytoplasmic levels of β-catenin. The β-catenin protein is the effector molecule of the Wnt pathway, and is an important transcription factor. With a rise in cytoplasmic levels, β-catenin localizes to the nucleus and complexes with the co-activators T-cell factor (TCF) and lymphoid enhancer factor (LEF). This complex then initiates gene transcription through binding to various promoter regions of specific genes, including cyclin D1 and Myc.

When no Wnt ligand associates with its receptors, β-catenin is rapidly degraded. This is controlled by a complex of adenomatous polyposis coli (APC), axin, and glycogen synthase kinase (GSK)-3β that associate with cytoplasmic β-catenin. This complex causes phosphorylation of β-catenin and targets it for ubiquitinization. The targeted β-catenin is then degraded by a proteosome system. This reduces the amount of available β-catenin that can move to the nucleus, and causes a reduction in gene transcription (Polakis, 2000).

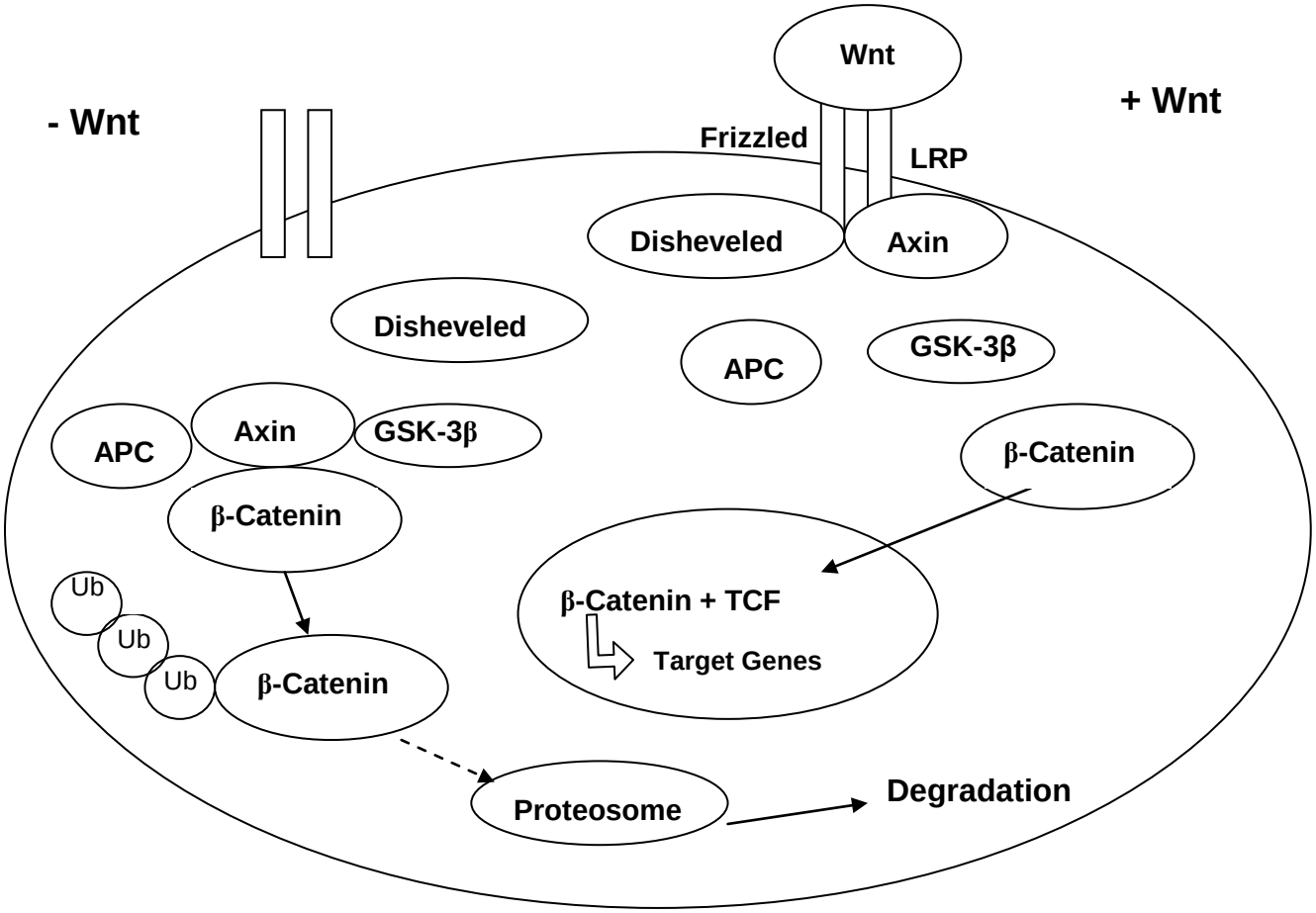


Figure 2*:* Canonical Wnt Signaling Pathway. See text for explanation.

The Wnt Pathway and Cancer

Many studies have shown that aberrant regulation of the Wnt pathway leads to various forms of cancer. It has been shown that this aberrant Wnt signaling is an early event in 90% of human colorectal cancers (Fodd et al., 2001). There are multiple mutations in the Wnt pathway that can cause cancer. Mutations in the APC gene play a major role in colorectal cancer. It is widely agreed upon that mutations in APC and reduced expression alter Wnt signaling, which is the cause of more than 85% of sporadic colorectal cancers (Liu et al., 2007). Inherited colorectal cancer patients normally have germ line mutations of the APC gene (Jin et al., 2008). These mutations reduce the levels of APC, causing an increase in the level of available β-catenin that can move to the nucleus and initiate transcription. Normal cells contain very low levels of β-catenin in the nucleus unless they are at certain stages of development or are tumor cells (Ki et al., 2008). The majority of β-catenin activity in colorectal epithelium is found in the bottom of colonic crypts where the epithelial stem cells are located. The colonic villi tips normally have low levels of β-catenin and high levels of APC (Voutsadakis, 2008). APC mutations lead to increased β-catenin levels in these cells, which increase cellular growth and formation of lesions associated with colorectal cancer.

There are other mutations in the Wnt pathway that can lead to an increased risk of cancer. The silencing of the Wnt regulator Dickkopf-1 (DKK-1) may be another factor in increased colorectal cancer risk. DKK-1 acts as a negative feedback to the Wnt pathway and causes increased degradation of β-catenin. A study has shown that DKK-1

inactivation by hypermethylation causes an increased risk of colorectal cancer due to this loss of an autoregulatory mechanism in the Wnt pathway (Gonzalez-Sancho et al., 2005).

E-cadherin and β-Catenin

E-cadherin is a transmembrane protein that contains an extracellular domain that binds with the extracellular domain of E-cadherin displayed by neighboring cells. It is primarily responsible for cell-cell adhesion in epithelial cells. E-cadherin also mediates the formation and organization of distinct cell junctions (Gottardi et al., 2001). E-cadherin controls cell growth through interactions with neighboring cells and through direct transduction of growth inhibitory signals.

The E-cadherin protein also affects cellular growth through interactions with the Wnt signaling pathway. The cytoplasmic domain of E-cadherin complexes with α- and β-catenin. E-cadherin is able to affect Wnt signaling by altering the amounts of free β-catenin in the cytoplasm (Prange et al., 2003). The loss of E-cadherin causes β-catenin to be released from the cell junctions and then allows β-catenin to translocate to the nucleus. This has been shown to cause an increase in Wnt signaling. (Kuphal el al., 2005).

Alternatively, increasing the expression of E-cadherin leads to reduced Wnt signaling. E-cadherin actively competes with TCF/LEF-1 to bind β-catenin, which causes a decrease in LEF-1/β-catenin dependent transcription (Ki et al., 2008). Studies have shown that overexpression of E-cadherin will antagonize β-catenin and prevents its nuclear signaling (Gottardi et al., 2001).

The Wnt Pathway and AKT

AKT overexpression was demonstrated to be an early event in sporadic colorectal carcinogenesis (Roy et al., 2002). The stabilization of β-catenin through APC mutations can increase transcription of AKT, a target gene of β-catenin. AKT kinases have diverse functions including the regulation of cell cycle, cell survival, and apoptosis. In normal colon cells, AKT activity is not significant, whereas there is an upregulation of AKT in colorectal cancer. AKT was shown to be regulated by β-catenin when overexpression of AKT was found to correlate with overexpression/increased nuclear localization of β-catenin. AKT affects Wnt signaling by complexing with Axin, GSK-3β, and Disheveled, which helps to increase levels of free β-catenin. Since AKT was found to affect Wnt signaling through the disruption of the complex that targets β-catenin for proteosomal degradation, it would result in a positive feedback loop that could increase Wnt signaling and increase cell survival (Dihlmann et al., 2005). The phosphorylation of AKT has also been shown to be an important factor in colorectal cancer. A study has shown that increased activation of AKT through phosphorylation suppresses apoptosis and promotes cell growth (Itoh et al., 2002).

Linking Folate Deficiency, Wnt Signaling, and E-cadherin to Cancer

As previously discussed, folate deficiency may increase risk of cancer by altered DNA methylation, increased DNA damage, and impaired DNA repair. Folate deficiency is known to cause DNA mutations in cell cycle genes, such as p53, through uracil misincorporation and other chromosome aberrations. These genes important for cell cycle regulation have been shown to be affected by folate status (Crott et al., 2008).

There is also evidence showing that folate deficiency is affecting the expression of genes involved in cell adhesion, migration, and growth.

Of particular relevance are studies indicating that folate deficiency alters Wnt signaling. Several microarray studies have shown altered expression of genes involved in Wnt signaling due to folate deficiency. One microarray study showed that in normal human fibroblasts grown for 7 days in folate deficient medium, changes in Wnt genes DKK1, WISP1, and WNT5A were detected (Katula et al., 2007). These genes are all important regulators of Wnt signaling and are important in regulating cell development and replication. A second microarray study showed that in several normal human colon epithelial cell lines grown in folate deficient medium for 30 days, there were varying changes in Wnt genes β-catenin, APC, and E-cadherin detected between the different cell lines (Crott et al., 2008). Another microarray study showed in a mouse model there was a decrease in APC expression and an increase in β-catenin expression in the mice due to folate deficient diets along with a deficiency in other B vitamins (Liu et al., 2008). More recently, a study performed in this lab has shown that folate deficiency causes increased nuclear localization and activation of β-catenin. Together these findings suggest folate deficiency leads to alterations in Wnt signaling, which may contribute to increased cancer risk.

There is evidence that folate deficiency also affects levels of E-cadherin. E-cadherin acts as a tumor suppressor protein and has been implicated in early and late stages of tumor progression (Gottardi et al., 2001). Studies have shown that levels of E-cadherin mRNA were decreased in colon epithelial cells under folate deficient conditions

(Crott et al., 2008). The deregulation of the cadherin cell adhesion system is frequently seen in aggressive and invasive cancers. The invasive fronts of colorectal tumors exhibit reduced levels of E-cadherin expression (Kuphal et al., 2005). In colorectal cancer, tumor progression is associated with accumulation of β-catenin and a loss of membranous E-cadherin. However, there is a low incidence of E-cadherin mutations related to colorectal cancer. Hypermethylation of the E-cadherin promoter may be a cause of decreased E-cadherin protein expression (Lugli et al., 2006). These changes in E-cadherin expression can alter cell signaling through affecting proteins involved in cancer progession and metastasis, such as urokinase and β-catenin (Crott et al., 2008). It has been also been shown that E-cadherin proteins lacking a β-catenin binding domain did not suppress growth (Gottardi et al., 2001). This could indicate that altered expression or possible mutations in E-cadherin would cause cancer by affecting Wnt signaling.

Based upon these findings, an additional mechanism for how folate deficiency increases the risk of colorectal cancer involves a decrease in E-cadherin protein with an accompanying increase in free β-catenin.

Project Overview

In this study the effects of folate deficiency on E-cadherin and β-catenin were analyzed in two colon epithelial cell lines (Caco-2 and FHC). E-cadherin and β-catenin protein levels, E-cadherin transcript levels, and the cellular localization of both E-cadherin and β-catenin were determined in cells grown in folate sufficient and deficient medium. The protein levels of several proteins in the AKT pathway were also determined in Caco-2 cells grown in folate sufficient and deficient medium. Finally, the

transcript levels of two folate transporters, FolR-1 and RFC, were determined in cells grown in folate sufficient and deficient medium.

This study is significant as it provides provides further understanding of the mechanism by which folate deficiency increases the risk of cancer and other health problems. Folate deficiency is one of a select few dietary components shown to be associated with many health conditions. This study also helps to show that these cells have mechanisms that allow them to adapt to folate deficiency. The mechanisms for all of these associations are still not fully understood.

CHAPTER II

MATERIALS AND METHODS

Cell Culture in Folate Sufficient and Deficient Medium

FHC cells were obtained from American Type Culture Collection (ATCC). These cells were initially plated in a T-25 flask using Delbecco's Modified Eagle's Medium (DMEM)/Ham's Nutrient Mixture F-12. DMEM/F-12 Complete Medium was prepared with and without folate. The base medium was prepared at NIEHS. The medium was then supplemented with 10% fetal bovine serum, 10 ng/mL cholera toxin, 5 µg/mL insulin, 5 µg/mL transferrin, 100 ng/mL hydrocortisone, 10% sodium bicarbonate, 0.2 M L-glutamine, 0.5 mM sodium pyruvate, and 10 mM HEPES. The cells were split once they reached 60-70% confluency. The cells were collected for replating using 0.25% Trypsin, 1mM EDTA in Hank's salt (Millipore Cat. #SM2003C). The cells were grown in a 37^{o} C and 5% carbon dioxide incubator. The medium was changed after four days and the cells were replated into new T-75 flasks and 100 mm dishes at a density of $3x10^{5}$ and $2.3x10^{5}$ cells, respectively. The cells were then grown for 30 days in folate sufficient and deficient medium. The cells were replated in 100 mm dishes for protein and RNA, 24 well plates for immunofluorescence, and 6 well plates for growth curves several days prior to collection at days 15 and 30. Cells were collected at days 15 and 30 by both scraping and trypsinization.

Caco-2 cells were initially plated in a T-75 flask using RPMI 1640 Complete Medium (Sigma Cat. #R8758) until the cells reached 80% confluency. The cells were collected for replating by trypsinization using 0.25% Trypsin, 1mM EDTA in Hank's salt. The cells were plated at a density of $7.5x10^5$ cells per T-75 flask and $5.8x10^5$ cells per 100mm dish in RPMI 1640 Complete (Sigma Cat. #R1145), with or without folate. The cells were grown in a 37^oC and 5% carbon dioxide incubator. The cells are then grown for 30 days in folate sufficient and deficient medium. The cells were replated in 100 mm dishes for protein and RNA, 24 well plates for immunofluorescence, and 6 well plates for growth curves several days prior to collection at days 15 and 30. Cells were collected at days 15 and 30 by both scraping and trypsinization.

Flow Cytometry

Caco-2 cells were collected by trypsinization in 1x PBS and pelleted. The cells were resuspended in 1x PBS and pelleted again. The cells were then fixed with 2 mL of 100% ice cold methanol while vortexing. The cells were later pelleted, resuspended in 1x PBS, and pelleted again. The cells were resuspended in PI staining solution (5 µL 10 mg/mL propidium iodide, 10 µL 10 mg/mL RNase A, 1 mL 1x PBS) and left in the dark for 30 minutes prior to analysis using a BD FACSCalibur flow cytometer.

Nuclei were isolated from FHC cells before PI staining. FHC cells were released by trypsinization and pelleted by centrifugation. The pelleted cells were washed in 1x PBS and resuspended in cold nuclear lysis buffer (320 mM Sucrose, 5 mM $MgCl_2$, 10 mM HEPES, 1% Triton X-100, at pH 7.4) and left on ice for 15 minutes. The nuclei were pelleted and resuspended in 1x PBS with 0.25% Tween-20. The nuclei were

pelleted again and resuspended in PI staining solution and left in the dark for 30 minutes prior to analysis on the flow cytometer.

Folate Assay

The intracellular concentration of folate in both Caco-2 and FHC cells was determined using a standard microtiter plate assay utilizing *Lactobacillus casei* (Tamura, 1990). Caco-2 and FHC cell pellets were resuspended in 200 µL of an extraction buffer (50 mM Tris, 1mM EDTA at pH 7.4 and 1% ascorbic acid). The extracts were spun at 10k and 4^{o}C for 10 minutes. The extracts were boiled for 5 minutes and centrifuged at 10k for 5 minutes. The supernatant was placed into a new microcentrifuge tube and placed on ice. Varying amounts (10 µL to 50 µL) of this cellular extract was added to a 96 well plate in a 0.2 M phosphate buffer and 0.1% ascorbic acid buffer. Then 10 µL of a 10% dilution of *L.casei* bacteria in 0.9% NaCl was added to each well along with 150 µL of *L. casei* medium containing no folate. A standard curve (20 pg to 800 pg) was prepared using folinic acid. The plate was allowed to incubate overnight at 37^{o}C. The plate was then read on a Bio-Tek Synergy HT microplate reader at 660 nm. Folate concentrations were obtained by utilizing the folinic acid standard curve.

Protein Quantification

Caco-2 and FHC cells used for protein quantification were collected via scraping. 500 µL of 2x SDS lysis buffer was added to each 100 mm dish along with 5 µL of 100 mM Na_3VO_4, 2.5 µL of 1 M DTT and 70 µL 7x Protease Inhibitor. The samples were sonicated and placed on ice. The Pierce 660 nm Protein Assay was used to determine the amount of protein in the samples, using a 96 well format. The plates were read on a Bio-

Tek Synergy HT microplate reader at 660 nm. The protein concentrations were obtained by utilizing a standard curve using known quantities of bovine serum albumin (BSA).

Western Blot

Caco-2 and FHC cells used for Western blot analysis were collected by scraping cells at different days of folate deficiency. Cells grown in 100 mm dishes were lysed using 2xSDS, 100 mM Na_3VO_4, 1 M DTT, and 7x Protease Inhibitor. The samples were stored at –80^{o}C. When ready for analysis, the samples were allowed to thaw, sonicated, and placed on ice. The protein content of the samples were determined as previously described with the Pierce 660 nm Protein Assay.

An equal amount of cellular protein from each folate sufficient and deficient sample was loaded into separate wells of a 6% SDS-PAGE gel. Color protein markers (Prosieve, Cat. #50550) were loaded into one lane of the gel. After electrophoresis, the protein was transferred onto nitrocellulose using standard methods. The blot was stained using Ponceau S to visualize the proteins and confirm that the samples were loaded equally. The blot was blocked in 5% milk in Tween-Tris Buffered Saline (TTBS) (0.15 M NaCl, 0.01 M Tris pH 8.0, 0.5% Tween) for one hour. For Caco-2 cells, a 1:1,000 dilution of rabbit anti-E-Cadherin antibody (Cell Signaling Technology Cat. # 4065) in 5% BSA in TTBS was added to the blots and left overnight. For FHC cells mouse anti-human E-cadherin (Takara Cat. # M106) was used. A 1:1,000 dilution of mouse anti-β-catenin antibody (Invitrogen Cat. #13-8400) in in 5% BSA in TTBS was added to the blots and left overnight. A 1:200 dilution of mouse anti-Tubulin (Developmental Studies Hybridoma Bank) in 1% milk in TTBS was added to the blots and left overnight. Also,

1:1,000 dilutions of anti-rabbit phospho-AKT (Thr 308), phospho-AKT (pan), phospho-GSK-3β, phospho-PTEN, phospho-PDK1, and phospho-c-Raf (Cell Signaling Technology Cat. # 9916) were added to the blots and left overnight with a 1:200 dilution of mouse anti-Tubulin. The following day, the nitrocellulose was washed in TTBS three times for five minutes per wash and once for 15 minutes. A 1:10,000 dilution of horse radish peroxidase in 1% milk in TTBS was placed on the nitrocellulose for one hour. The nitrocellulose was washed three times in TTBS for 5 minutes per wash and once for 15 minutes. The Supersignal West Pico-Substrate detection kit (Thermo-Scientific Cat. # 34080) was used to detect the antibody on the nitrocellulose. The imaging of the blots was performed using the BioRad Chemi Doc XRS imager using the Quantity One software.

Immunofluorescence

Caco-2 and FHC cells used for immunofluorescence were grown on round glass coverslips in 24 well plates. The cells in each well were directly fixed with 1 mL of 2% paraformaldehyde in 1x PBS for 15 minutes at room temperature. After fixation, the cells were washed three times with 500 µL PBS for five minutes per wash. The cells were stored in 1 mL 1x PBS at 4^{o} C.

The cells on each disk were permeabilized with 400 µL of 0.25% Triton X-100 in 1x PBS for 5 minutes, and then washed three times with 500 µL PBS for 5 minutes per wash. For Caco-2 cells, a combined 65 µL of primary antibodies, mouse anti-β-catenin (Invitrogen Cat. #13-8400) and rabbit anti-E-cadherin (Cell Signaling Technology Cat. # 4065) diluted 1:100 each were added directly onto each coverslip and incubated at 37^{o} C

for 90 minutes. For FHC cells, a combined 65 µL of primary antibodies, rabbit anti-β-catenin (Millipore Cat. #AB19022) and mouse anti-human E-cadherin (Takara Cat. #M106) After incubation, the cells were washed three times with 500 µL PBS for 5 minutes per wash. Then a combined 95 µL of secondary antibodies, goat anti-mouse IgG with fluoroform Alexa 488 (Invitrogen, Cat. #A11001) and donkey anti-rabbit IgG with fluoroform Alexa 594 (Invitrogen, Cat. #A21207), diluted 1:400 were then added directly to each coverslip. The cells were shielded from light and incubated at room temperature for 90 minutes. The cells were washed four times with 500 µL PBS for 5 minutes per wash. The coverslips were then mounted onto glass slides using DakoCytomation Fluorescent Mounting Medium and analyzed using confocal microscopy.

RNA Isolation and Real Time PCR

RNA was isolated from cell samples using the SV Total RNA Isolation Kit (Promega Cat. #23100). The RNA was converted to cDNA using the Omniscript RT Kit (Qiagen Cat. #205110). qRT-PCR was conducted using the Taqman Gene Expression Master Mix (Applied Biosystem Cat. # 4369016) and Applied Biosystem gene specific primers for E-cadherin (Applied Biosystem Cat. #Hs01013953_m1), FolR-1 (Applied Biosystem Cat. #Hs00360501_m1), and RFC (Applied Biosystem Cat. #Hs00953344_m1) using the Applied Biosystems StepOne real time PCR system. Three reactions were conducted for each primer. Glyceraldehyde 3-phosphate (GAPDH) (Applied Biosystem Cat. #Hs99999905_m1) was used as the internal control.

CHAPTER III

RESULTS

Cell Proliferation in Folate Sufficient and Deficient Medium

It was previously shown in this lab that folate deficiency alters cell growth rates and cell cycle phase distribution in NIH3T3 cells. Folate deficiency resulted in decreased cell proliferation rates and an increase in the percentage of cells in S phase of the cell cycle (unpublished data). As colon epithelial cell lines (Caco-2 and FHC) were used in these studies, it was necessary to investigate the effects of folate deficiency on the growth rate and the cell cycle of these cells. Published data has shown that various cell types, including intestinal epithelial cells, can adapt biochemically to folate deficiency (Crott et al., 2008; Subramanian et al., 2003; Sadasivan et al., 2002), but no data is currently available on growth rates and cell cycle for either cell lines.

Caco-2 is a transformed human adult colon epithelial cell line and FHC is a normal human fetal colon epithelial cell line. Morphologically the two cell lines appear similar, however they behave differently. FHC cells grow slower than Caco-2 cells. FHC cells also adhere to the culture plate overnight after trypsinization, whereas Caco-2 cells require one to two days to reattach.

Cell number was controlled by collecting, counting, and aliquoting equal cell numbers for both folate sufficient and deficient cells.

Caco-2 cells grown under folate sufficient and deficient conditions were replated at equal cell number on day 15 then collected and counted over the next 6 days (Figure 3A). Six days after replating a reduction in cell number was detected for cells grown under folate deficient conditions. A second growth curve for Caco-2 cells beginning at day 30 was conducted (Figure 3B). Caco-2 cells grown under folate deficient conditions for 10 days after replating at day 30 showed reduced rates of proliferation. At day 40 the cell number was $2.1x10^5$ for folate sufficient cells compared to $5.3x10^4$ for folate deficient cells. During the next 10 days (day 40 to day 50), the growth rate of folate deficient cells increased and was more similar but slightly less than the folate sufficient cells.

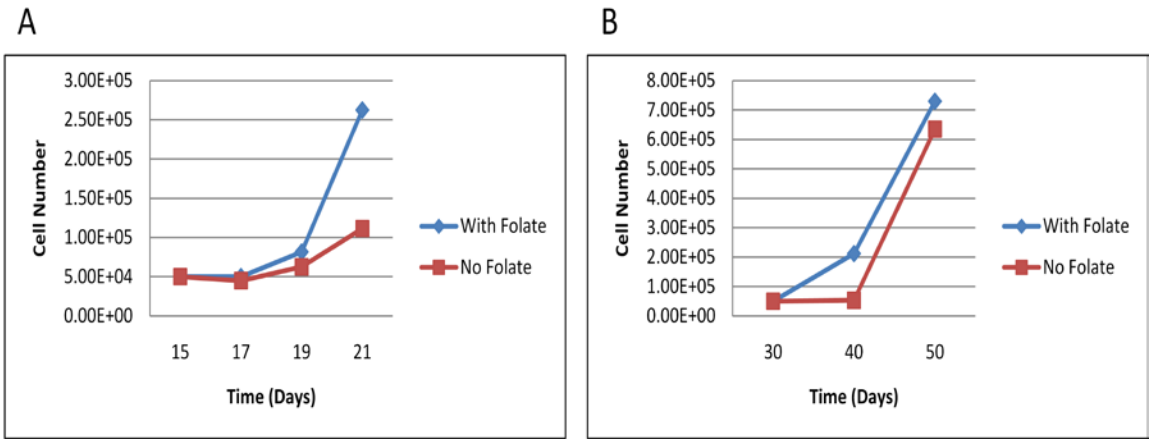


Figure 3: Growth curves for Caco-2 cells grown under folate sufficient (With) and deficient (No) conditions. A) Caco-2 cells grown for 15 days in With/No medium and replated on day 15. Cells were collected and counted at the indicated time points. B) Caco-2 cells grown for 30 days in With/No medium and replated at 30 days. Cells were collected and counted at the indicated time points.

For FHC cells grown under folate sufficient and deficient conditions, the growth curve beginning at day 15 was comparable to the Caco-2 cells (Figure 4A). Five days after replating at the same cell number at day 20 FHC cells continued to proliferate at a normal rate under folate deficient conditions, although a little slower than the folate sufficient cells. However, the proliferation rate of the folate deficient cells decreased between days 20 and 25 after replating in comparison to the folate sufficient cells. A second growth curve for FHC cells beginning at day 30 showed a similar trend to Caco-2 (Figure 4B). After the cells were allowed to grow for another 20 days in folate sufficient and deficient medium, the folate deficient cells continued to proliferate but at a lower rate than the folate sufficient cells.

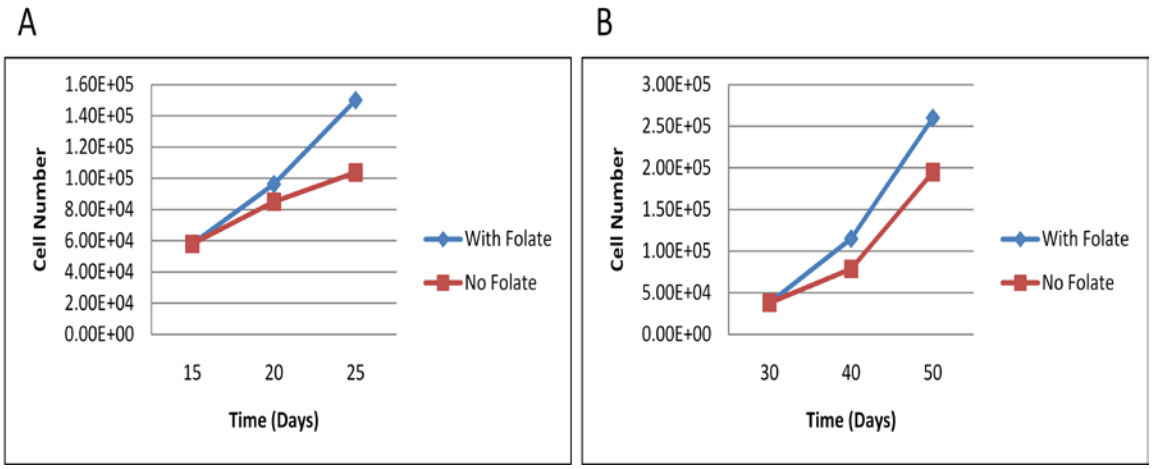


Figure 4: Growth curves for FHC cells grown under folate sufficient (With) and deficient (No) conditions. A) FHC cells grown for 15 days in With/No medium and replated on day 15. Cells were collected and counted at the indicated time points. B) FHC cells grown for 30 days in With/No medium and replated at 30 days. Cells were collected and counted at the indicated time points.

Effects of folate deficiency on the cell cycle

Cell cycle analysis was performed on both Caco-2 and FHC cells to determine the effects of folate deficiency on cell cycle phase distribution. Caco-2 cells were collected at days 15 and 30. FHC cells were collected at day 30 and 70. Whole cells for Caco-2 cells and isolated nuclei for FHC cells were analyzed for DNA content using a flow cytometer (Figure 5).

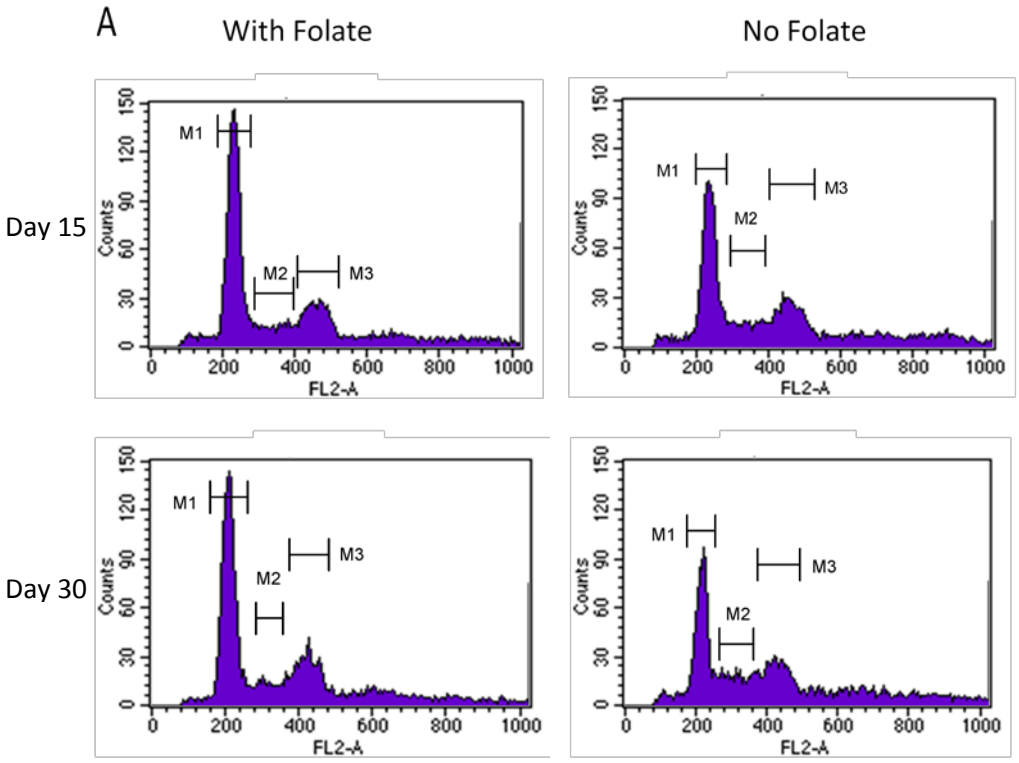


Figure 5: Cell cycle phase distribution of Caco-2 and FHC cells grown under folate sufficient (With) and deficient (No) conditions. A) Caco-2 cells grown for 15 and 30 days in With/No medium. Cells were collected, counted, fixed with 100% methanol, and stained with propidium iodide at indicated time points. B) FHC cells grown for 30 and 70 days in With/No medium. Cells were collected, nuclei were isolated, and stained with propidium iodide at the indicated days. Samples were analyzed using a BD FACSCalibur flow cytometer. M1: G_1 phase; M2: S phase; M3:G_2/M phase. (Figure continued on next page)

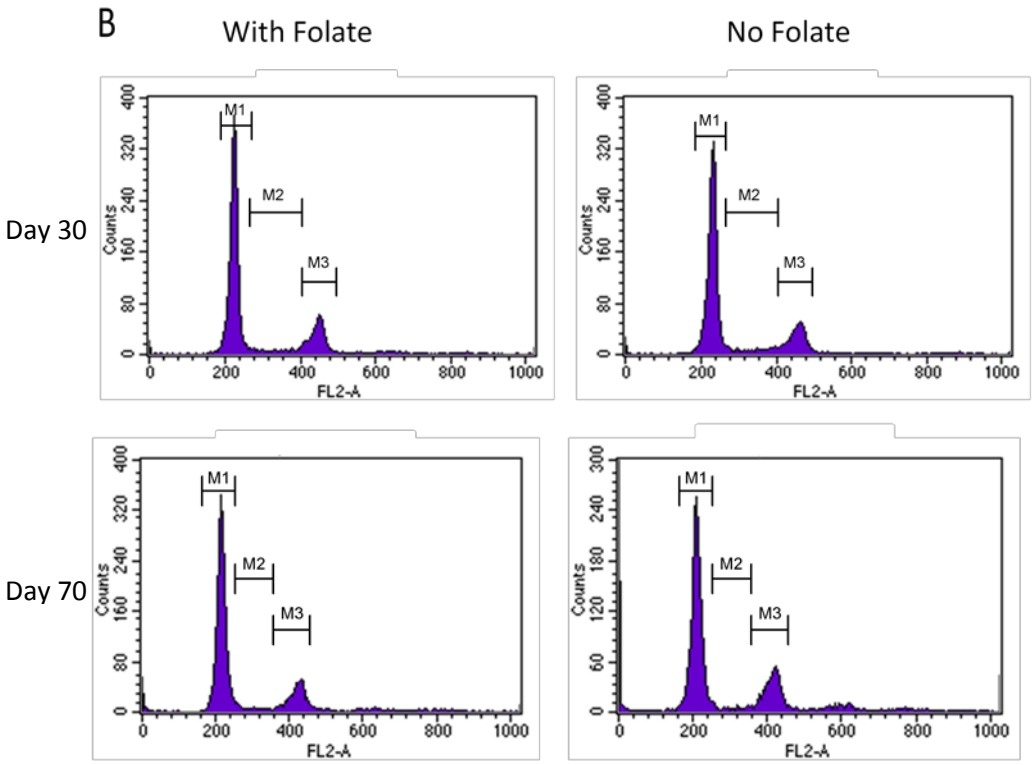


Figure 5 (continued)

Table 1: Cell cycle phase distribution of Caco-2 and FHC cells grown under folate sufficient and deficient conditions. The percentage for each phase was obtained from the values for M1, M2, and M3 as shown in Figure 5. W: with folate; N: no folate

	Caco-2 Day 15 W	Caco-2 Day 15 N	Caco-2 Day 30 W	Caco-2 Day 30 N	FHC Day 30 W	FHC Day 30 N	FHC Day 70 W	FHC Day 70 N
G_0/G_1 (M1)	65.6%	54.9%	65.4%	52.2%	70.9%	71.7%	76.9%	74.3%
S (M2)	11.3%	16.6%	9.7%	18.4%	5.8%	6.5%	3.5%	3.5%
G_2/M (M3)	23.1%	28.5%	24.9%	29.4%	18.9%	18.8%	19.6%	22.2%

At day 15 for Caco-2 cells, there was a greater percentage of cells in S phase in folate deficient cells (16.6%) as compared to folate sufficient cells (11.3%). There was also an increase in the cells in G_2/M phase in folate deficient cells (28.5%) as compared to folate sufficient cells (23.1%). At day 30 for Caco-2 cells, there was a higher percentage of cells in S phase in folate deficient cells (18.4%) than in folate sufficient cells (9.7%). The percentage of cells in G_2/M phase was also higher in folate deficient cells (29.4%) at day 30 than folate sufficient cells (24.9%) (Table 1). These results suggest that the cell cycle phase distribution of Caco-2 cells is being affected by folate deficiency after 15 days, and continues to be affected in a similar way after 30 days.

In FHC cells, after 30 days the percentage of cells in each cell cycle phase was similar for both folate sufficient and deficient cells. In an attempt to elicit a response to folate deficiency in FHC cells we cultured the cells for 70 days in folate sufficient and deficient medium. Even after this extended period of time we saw no change in cell cycle distribution. These results demonstrate that the cell cycle phase distribution of FHC cells is not affected by folate deficiency, unlike Caco-2 cells.

Intracellular Detection of Folate

A standard folate assay utilizing *L. casei* bacteria was performed on Caco-2 and FHC cells grown under folate sufficient and deficient conditions (data not shown). This was done to determine if the intracellular levels of folate were reduced in Caco-2 and FHC cells grown in folate deficient medium. The levels of folate were undetectable in Caco-2 and FHC cells grown under folate deficient conditions. The level of intracellular folate was detectable in Caco-2 cells (409 pg) and FHC cells (14.5 pg) grown under folate sufficient conditions. Cell number was determined for Caco-2 cells ($4x10^6$ cells) but not for FHC cells, hence it is not possible to compare these values. Our results are what would be expected considering that no folate was added to the deficient medium during preparation. However, some folate may still be present in the dialyzed serum used in both folate sufficient and deficient medium. This could explain the continued proliferation of both Caco-2 and FHC cells even under folate deficient conditions.

E-cadherin and β-catenin Protein Levels

There is supporting evidence showing that E-cadherin and β-catenin protein levels are affected by folate deficiency (see Background). β-catenin has been shown to

associate with membrane-bound E-cadherin and is part of a complex that anchors E-cadherin to the cytoskeleton. Any alterations in E-cadherin protein could cause a change in the level of free cytoplasmic β-catenin.

A western blot analysis was performed to determine if folate deficiency leads to any change in E-cadherin and β-catenin protein levels. Caco-2 and FHC cells were grown for 15 and 30 days in folate sufficient and deficient medium. Protein lysates were prepared and analyzed by western blot. The detected bands were quantified and standardized to tubulin protein.

E-cadherin and β-catenin protein levels in Caco-2 cells grown for 15 and 30 days in folate sufficient and deficient medium was determined by western blot analysis (Figure 6).

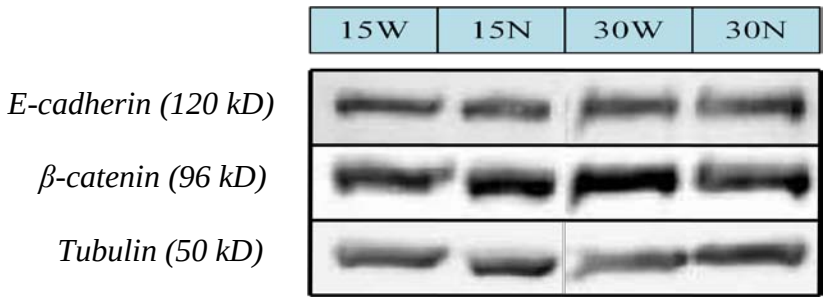


Figure 6. Western blot detection of E-cadherin, β-catenin, and Tubulin in Caco-2 cells. Caco-2 cells were grown for 15 and 30 days in folate sufficient and deficient medium. Protein lysates were prepared and 50µg of each lysate was electrophoresed on a 6% SDS-PAGE gel. W: with folate; N: no folate.

At day 15 and 30, there was a decrease in E-cadherin protein in folate deficient cells as compared to folate sufficient cells (Figure 7A). The protein levels of β-catenin decreased at both day 15 and day 30 in the folate deficient cells (Figure 7B).

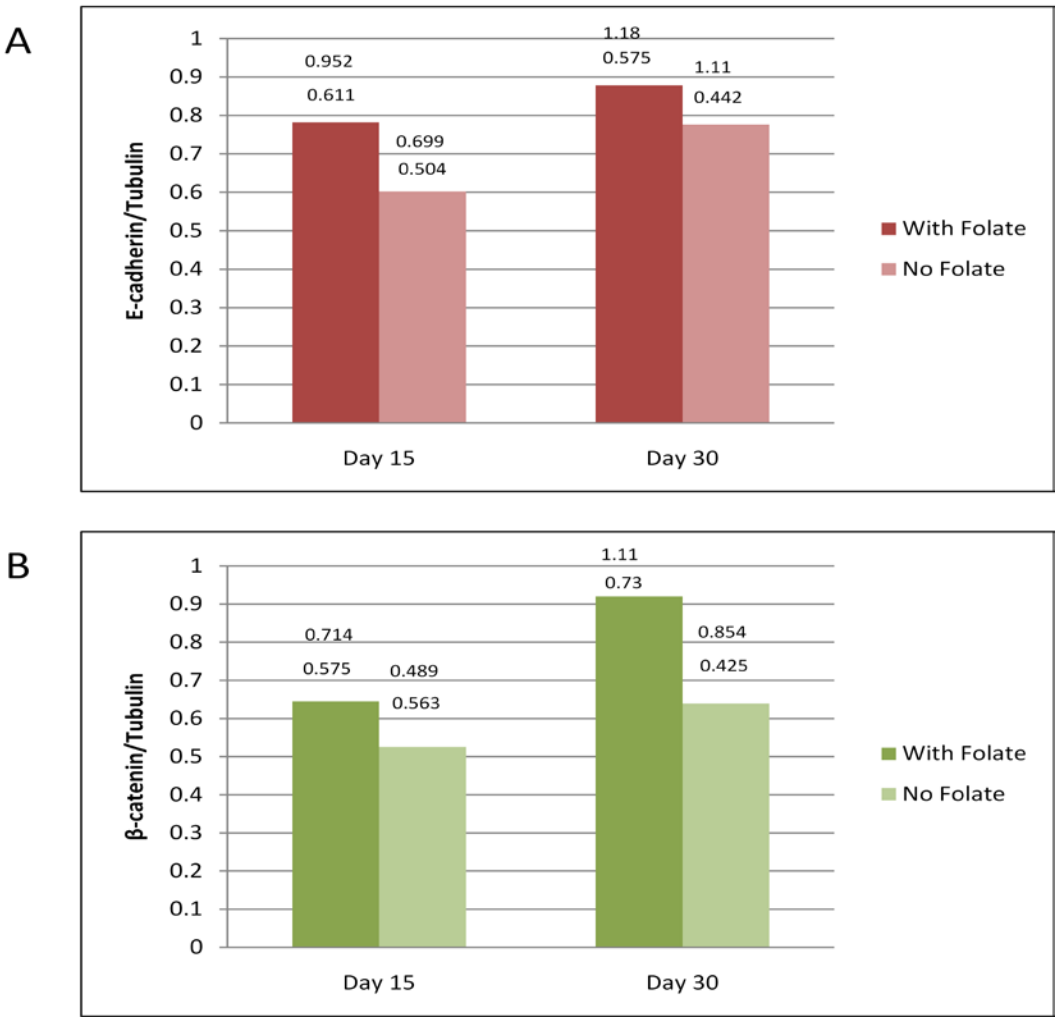


Figure 7. Relative Protein Levels of E-cadherin and β-catenin in Caco-2 cells. Relative protein levels of E-cadherin and β-catenin in Caco-2 cells grown for 15 and 30 days in folate sufficient and deficient medium. Relative protein levels of A) E-cadherin and B) β-catenin was determined by standardizing to tubulin. Values represent the average of two technical repeats. Values above bars represent values for each trial (Top: Trial 1, Bottom: Trial 2).

E-cadherin and β-catenin protein levels in FHC cells grown for 15 and 30 days in folate sufficient and deficient medium were also determined by western blot analysis (Figure 8). For FHC cells, a different E-cadherin antibody was used because the E-cadherin antibody used for Caco-2 cells did not detect FHC E-cadherin.

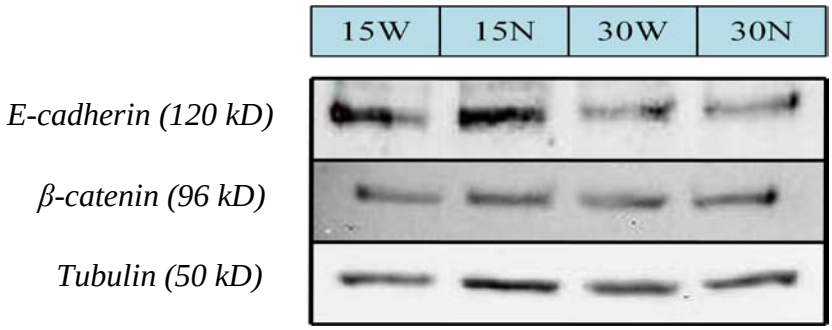


Figure 8. Western blot detection of E-cadherin, β-catenin, and Tubulin in FHC cells. FHC cells were grown for 15 and 30 days in folate sufficient and deficient medium. Protein lysates were prepared and 25μg of lysates were electrophoresed on a 6% SDS-PAGE gel. W: with folate; N: no folate.

At day 15, there was a decrease in E-cadherin protein in folate deficient cells as compared to folate sufficient cells. At day 30, there was no change or possibly a small increase in E-cadherin protein due to folate deficiency (Figure 9A). The protein level of β-catenin decreased at day 15 due to folate deficiency, but there was no change at day 30 (Figure 9B).

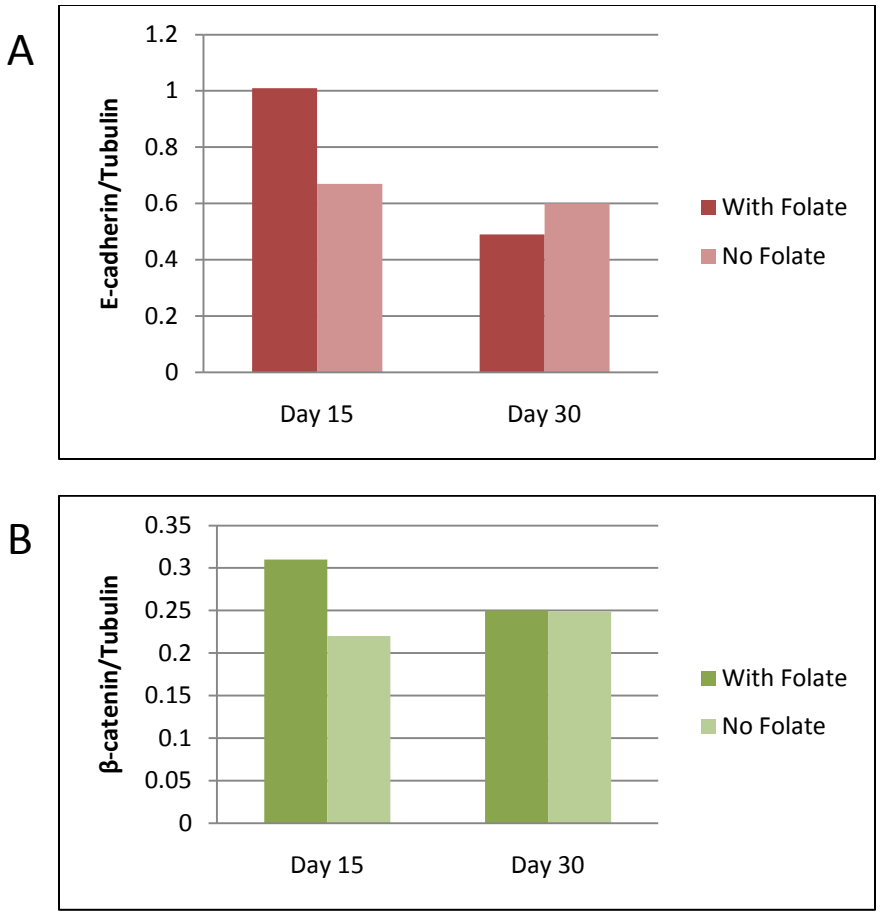


Figure 9. Relative Protein Levels of E-cadherin and β-catenin in FHC cells. Relative protein levels of E-cadherin and β-catenin in FHC cells grown for 15 and 30 days in folate sufficient and deficient medium. Relative protein levels of A) E-cadherin and B) β-catenin was determined by standardizing to tubulin.

Additional biological replicas of these western blots are needed to verify these results and determine statistical significance.

E-cadherin Transcript Levels in Folate Sufficient and Deficient Cells

Our analysis suggests that E-cadherin protein levels are slightly decreased in folate deficient cells at the earlier time points for both Caco-2 and FHC cells. To determine if folate deficiency leads to alterations in E-cadherin mRNA, qRT-PCR was performed using Caco-2 and FHC cells. Caco-2 and FHC cells were grown in folate

sufficient and deficient medium and cells were collected for RNA isolation at days 15 and 30. The RNA was converted to cDNA and analyzed using primers specific for E-cadherin and GAPDH. The E-cadherin cycle values were normalized to the cycle values for GAPDH. Then, the relative change was determined by dividing the normalized cycle values for folate deficient samples by the value for the folate sufficient samples. At day 15, the E-cadherin transcripts were unaffected by folate deficiency. At day 30, the level of E-cadherin transcripts was reduced in comparison to folate sufficient cells by approximately one half (Figure 10).

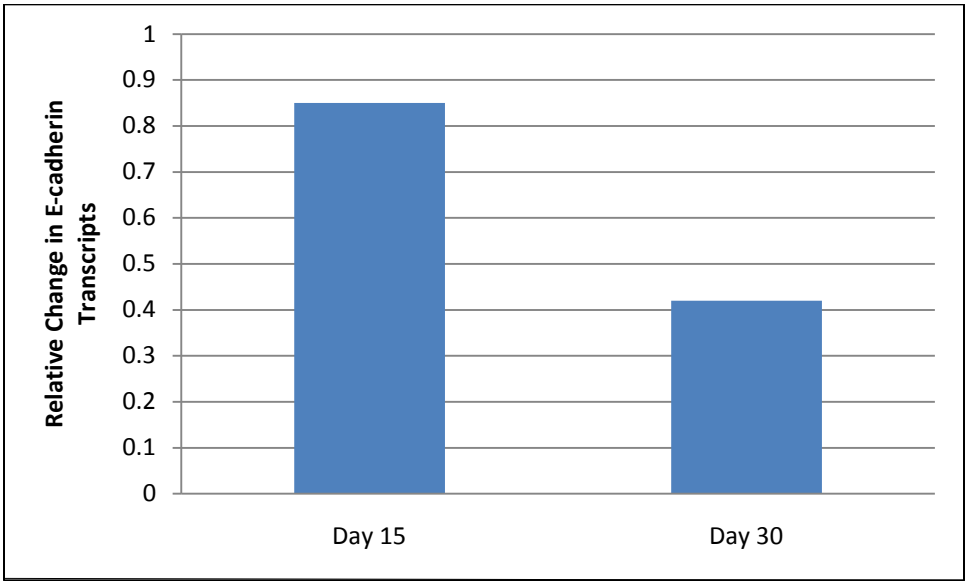


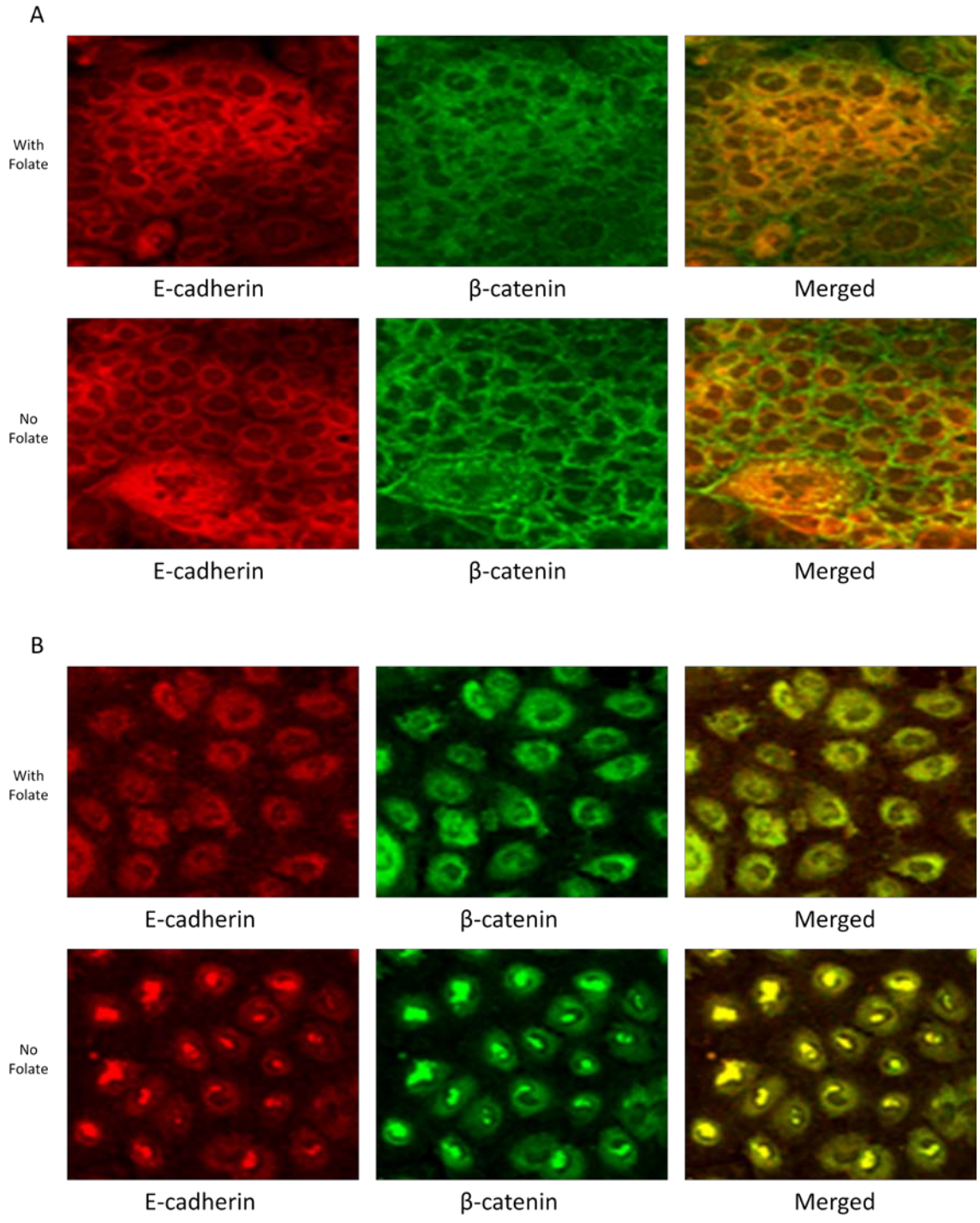
Figure 10. Relative change in E-cadherin transcripts in Caco-2 cells. E-cadherin and GAPDH primers were used to amplify the target cDNAs prepared from RNA isolated from Caco-2 cells grown for 15 and 30 days in folate sufficient and deficient medium. E-cadherin transcript levels were normalized to GAPDH and the relative change determined.

We were unable to amplify the E-cadherin transcripts using RNA isolated from FHC cells and two distinct E-cadherin primer sets. This is likely due to these cells having a distinct fetal E-cadherin transcript.

E-cadherin and β-catenin Localization

We found a slight decrease in E-cadherin and β-catenin protein levels due to folate deficiency in Caco-2 cells at days 15 and 30. We also found a decrease in both proteins in FHC cells at day 15, but relatively no change in E-cadherin protein levels at day 30 and no change in β-catenin protein levels. We then wanted to determine if the E-cadherin/β-catenin complex that anchors E-cadherin to the cytoskeleton was affected by folate deficiency. A decrease in E-cadherin would be expected to lead to a reduced membrane association of β-catenin. Cellular localization of both proteins was examined using immunofluorescence.

Caco-2 and FHC cells were grown in folate sufficient and deficient medium for 15 days. The cells were plated onto coverslips, and fixed 5 days later (day 20). This was done to allow the Caco-2 cells ample time to attach. The cells were immunostained for E-cadherin and β-catenin with each having its own distinct fluorophore. The cells were then analyzed using a confocal microscope (Figure 11).



(Figure Legend on next page)

Figure 11: Immunofluorescent staining for E-cadherin and β-catenin in Caco-2 and FHC cells. A) Immunofluorescent staining of Caco-2 cells grown for 20 days in folate sufficient (With) and deficient (No) medium. B) FHC cells grown for 20 days in folate sufficient (With) and deficient (No) medium. Cells were immunostained for E-cadherin (red) and β-catenin (green). Overlap of both proteins is shown in yellow.

In Caco-2 cells, at day 20 we see that E-cadherin and β-catenin are diffuse in folate sufficient cells. The merged image shows an overlap of these two signals due to the yellow color produced by the overlap of the red and green fluoroforms, which demonstrates that these two proteins are found in the same areas throughout the cells. In the folate deficient cells at day 20 we see a similar staining pattern for E-cadherin when compared to the folate sufficient cells. We do, however, see a difference in β-catenin in the folate deficient cells. There is increased staining of β-catenin at the cell membrane in the folate deficient cells, but it also remains diffuse throughout the cells. When the images are merged the red (E-cadherin) and green (β-catenin) are distinct and display less overlap in the folate deficient cells when compared to the folate sufficient cells. There is an increase in β-catenin localization at the cell membrane shown by the increase in the green color at the membrane which is associated with β-catenin, whereas the localization of E-cadherin remains unchanged (Figure 11A).

In FHC cells, at day 20 we see that both E-cadherin and β-catenin are diffuse throughout the cells under folate sufficient conditions. The merged image shows an even overlap of these proteins. In the folate deficient cells we see that both E-cadherin and β-catenin remain diffuse throughout the cells. We also see that there is an increase in nuclear staining for both proteins. The merged image shows a nearly perfect overlap

between the two colors in both folate sufficient and deficient cells. This holds true for the signals for both proteins found in the nuclei of the folate deficient cells (Figure 11B).

AKT Pathway Protein Levels in Folate Sufficient and Deficient Cells

In an effort to understand why folate deficient cells continued to cycle we looked to see if the AKT pathway may be affected. Since the AKT pathway is important for cell survival during stress and cell growth regulation, this pathway seemed a likely candidate to help explain how the cells were able to seemingly adapt to folate deficiency.

Caco-2 cells were used to test if the AKT pathway was affected by folate deficiency. The cells were grown for 15 and 30 days in folate sufficient and deficient medium. The cells were collected and cell lysates prepared for Western Blot analysis. The most commonly activated form of AKT, phospho-AKT (Thr 308), was analyzed via western blot and compared to the total amount of AKT protein present (Figure 12).

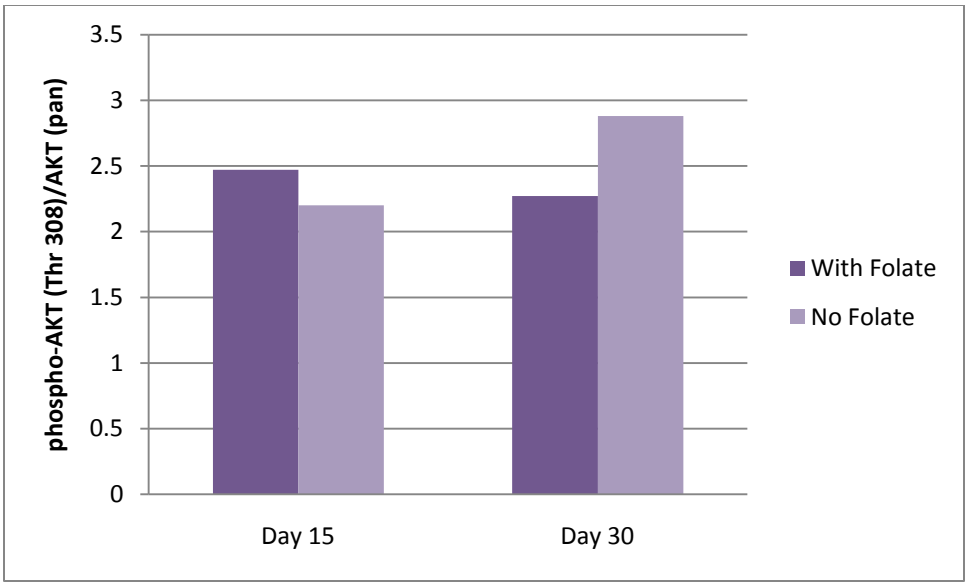


Figure 12: Relative phospho-AKT (Thr 308) protein levels in Caco-2 cells. Relative protein levels of phospho-AKT (Thr 308) in Caco-2 cells grown for 30 days in folate sufficient and deficient medium. Relative protein levels determined by standardizing to total AKT protein.

The level of phosphorylated AKT was decreased slightly due to folate deficient conditions at day 15. There was a minor increase in phosphorylated AKT after the cells were grown for 30 days in folate deficient medium. These results demonstrate that there is no major increase in the activation of AKT due to folate deficiency, although the increase at day 30 cannot be discounted. Additional biological replicas will need to be carried out to determine if these small changes may be significant.

To gain further insight into any possible effect of folate deficiency on the AKT pathway, we examined whether other proteins in the AKT pathway were altered due to folate deficiency. Caco-2 cells grown for 30 days in folate sufficient and deficient medium were analyzed via western blot to detect the levels of phospho-GSK-3β,

phospho-c-RAF, phospho-PDK1, phospho-PTEN (Figure 13). All of these proteins are important downstream effectors of the AKT pathway.

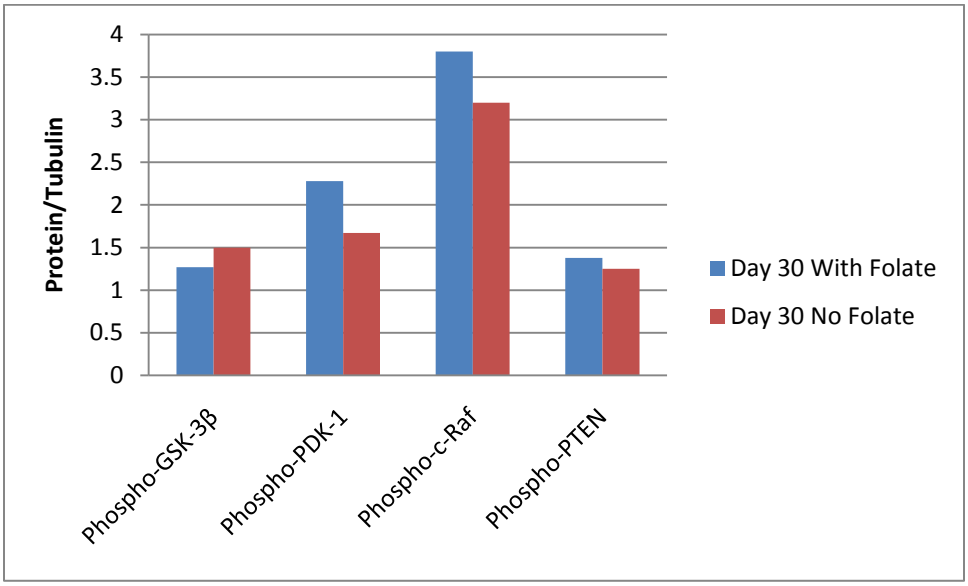


Figure 13: Relative Protein Levels of phospho-GSK-3β, phospho-PDK1, phospho-c-RAF, and phospho-PTEN in Caco-2 cells. Relative protein levels of phospho-GSK-3β, phospho-PDK-1, phospho-c-Raf, and phospho-PTEN in Caco-2 cells grown for 30 days in folate sufficient and deficient medium. Relative protein levels determined by standardizing to Tubulin.

The results of this experiment show that after the Caco-2 cells are grown for 30 days in folate deficient medium there is a slight decrease in the phosphorylated protein levels of phospho-PDK-1 and phospho-c-Raf, and likely no change in phospho-PTEN phospho-GSK-3β. Again, it has not been demonstrated that these small changes are significant.

Transcripts Levels of Folate Transporters

We found that both Caco-2 and FHC cells continued to grow under folate deficient conditions. Upon review of the literature (see Discussion), we found published studies supporting our observation that colon epithelial cells can adapt to folate deficiency. Moreover, there is data indicating that the molecular basis for this adaptation is an increase in various folate transporters. To determine if similar molecular changes are occurring in our two cells lines, the transcript levels of two folate transporters were quantified.

A common folate receptor found in both cell lines was the Reduced Folate Carrier (RFC). We used quantitative real time PCR to determine if there was a change in the transcript levels of RFC in Caco-2 and FHC cells due to folate deficiency. Caco-2 cells were grown for 15 and 30 days in folate sufficient and deficient medium. FHC cells were grown for 15, 30, and 70 days in folate sufficient and deficient medium. The RNA was isolated, converted to cDNA, and analyzed using primers specific for RFC and GAPDH. The RFC values were normalized to GAPDH and the relative change was determined by dividing the normalized folate deficient samples by the folate sufficient samples.

In Caco-2 cells there was essentially no change in RFC transcripts due to folate deficiency at day 15. However, at day 30 there was greater than a 2.5-fold increase in RFC transcripts in folate deficient cells when compared to folate sufficient cells (Figure 14A).

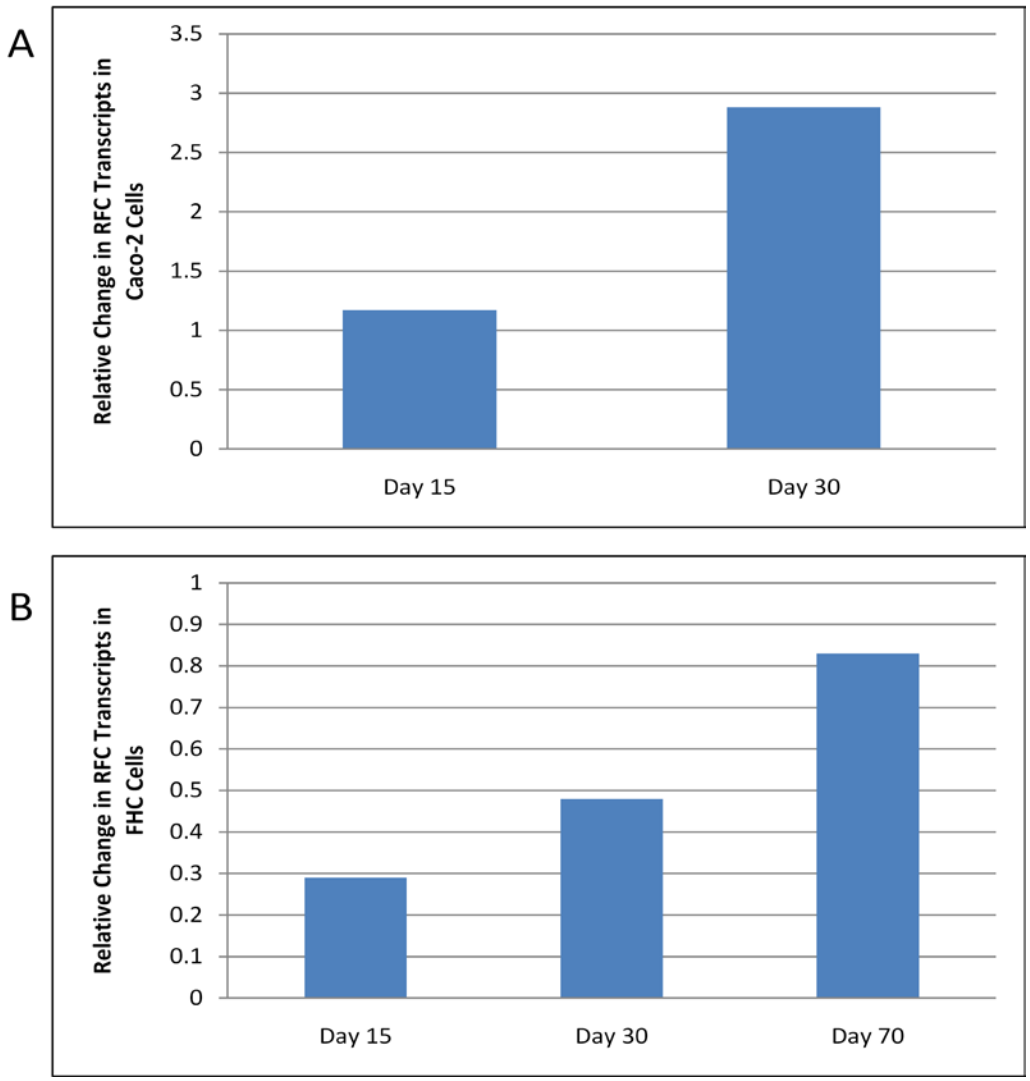


Figure 14: Relative change in RFC transcripts in Caco-2 and FHC cells. A) RFC and GAPDH primers were used to amplify the target cDNAs prepared from RNA isolated from Caco-2 cells grown for 15 and 30 days in folate sufficient and deficient medium. B) RFC and GAPDH primers were used to amplify the target cDNAs prepared from RNA isolated from FHC cells grown for 15, 30, and 70 days in folate sufficient and deficient medium. RFC transcript levels were normalized to GAPDH and the relative change determined.

In FHC cells, it was shown that there was a decrease in the relative transcripts of RFC due to folate deficiency at day 15. Then an incremental increase in RFC transcript

levels followed at days 30 and 70 in folate deficient cells when compared to the folate sufficient cells (Figure 14B). However, the transcript levels for all time points are lower in folate deficient cells than folate sufficient cells.

Another folate receptor, FolR-1, was analyzed using real time PCR to determine if it was affected by folate deficiency. In Caco-2 cells there was an almost 2.5-fold increase in the relative transcript levels at day 15 in folate deficient cells when compared to folate sufficient cells. Then at day 30 the transcript level was only slightly higher in the folate deficient cells when compared to the folate sufficient cells (Figure 15). Again, the significance of these changes have not been determined.

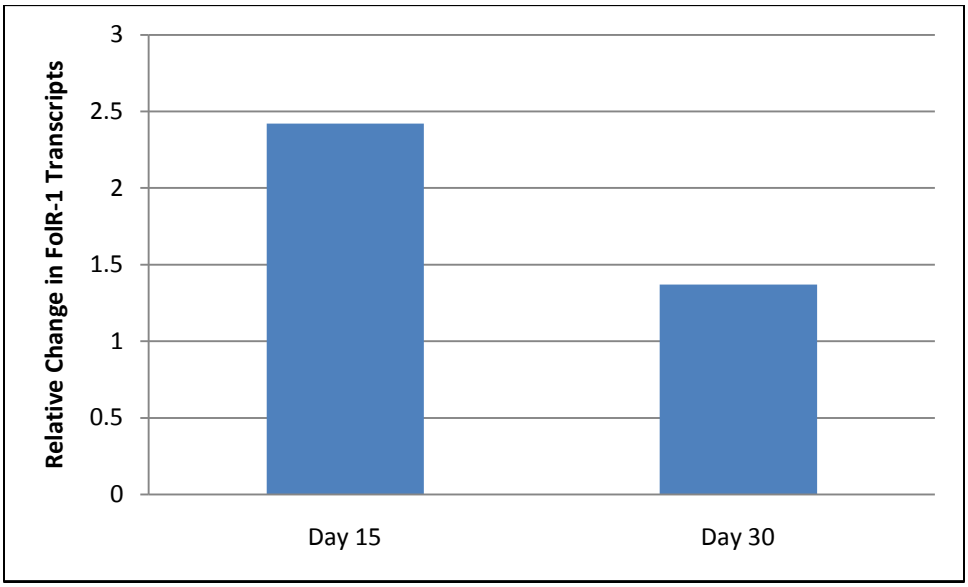


Figure 15: Relative change in FolR-1 transcripts in Caco-2 cells. FolR-1 and GAPDH primers were used to amplify the target cDNAs prepared from RNA isolated from Caco-2 cells grown for 15 and 30 days in folate sufficient and deficient medium. FolR-1 transcript levels were normalized to GAPDH and the relative change determined.

We were unable to amplify the FolR-1 transcripts using RNA isolated from FHC cells. This is likely due to these cells having a distinct fetal FolR-1 or this receptor is not utilized in these cells. We also used a primer for the FolR-2 folate receptor, which is the reported fetal form for this class of receptors. Again, no amplification was detected.

CHAPTER IV

DISCUSSION

Many studies have shown a correlation between folate status and cancer risk. The risk of colorectal cancer, in particular, has been linked to folate status (Kim, 2004). Aberrations in the Wnt signaling pathway have been shown to be involved in 90% of human colorectal cancers (Fodd et al., 2001) and Wnt mutations are involved in sporadically occurring and familial forms of colorectal cancer (Liu et al., 2007; Jin et al., 2008). E-cadherin can regulate Wnt signaling due to the fact that the intracellular domain of E-cadherin complexes with β-catenin at the cell membrane, and thus helps to control free cytoplasmic β-catenin (Prange et al., 2003). The deregulation of the cell adhesion system is seen in invasive cancers, and a reduction in E-cadherin has been found in the invasive fronts of colorectal tumors (Kuphal et al, 2005). Thus, one mechanism by which folate deficiency may increase the risk of colorectal cancer is by affecting E-cadherin levels and Wnt signaling. Support for such a mechanism are studies showing alterations in E-cadherin, β-catenin, and Wnt pathway gene expression in folate deficient cells (Katula et al., 2007; Liu et al., 2007; Crott et al., 2008).

The purpose of this study was to determine if folate deficiency altered the levels of E-cadherin and the co-localization of both E-cadherin and β-catenin in two colon epithelial cell lines (Caco-2 and FHC).

Altered cell growth and adaptation of Caco-2 and FHC cells in folate deficient medium

The first phase of our study was to characterize the effect of folate deficiency on cell proliferation and the cell cycle in both Caco-2 and FHC cells. It was expected that cell proliferation would decrease under folate deficient conditions and that there would be an increase in the number of cells halted in S phase of the cell cycle. Our results showed that both Caco-2 and FHC cells grew at a different rate under folate deficient conditions, but they continued to grow even after 70 days of folate deficiency for FHC cells. This was unexpected as previous studies in this lab showed that fibroblastic cell lines essentially stop growing in folate deficient medium after 10 days.

In Caco-2 cells it was found that the percentage of cells in S and G_2/M phases increased due to folate deficiency, but the cells continued to cycle. FHC cells showed no noticeable change in cell cycle phase distribution under folate deficient conditions. The cells seemingly continued to cycle normally and showed no changes in the percentage of cells in any phase of the cell cycle. This was an unexpected result. Since Caco-2 cells are cancerous and FHC cells are normal the opposite results would be expected. There may be some uncharacterized aspect of FHC cells, which are fetal in origin. Alternately, the preparation of Caco-2 and FHC cells for DNA content analysis was different. Although propidium iodide was used to label the DNA, whole cells were used for Caco-2 cells and nuclei were isolated for FHC cells. FHC cells tend to clump after trypsinization, making it necessary to obtain more cells for flow cytometry, as the clumps of cells cannot be analyzed . Consequently, the alternative approach of isolating nuclei

was tried with superior results. To confirm that Caco-2 cell cycle is, indeed, affected by folate deficiency it will be necessary to repeat the flow cytometry using isolate nuclei.

The finding that both Caco-2 and FHC cells continued to cycle suggested that the cells had a sufficient source of folate. The fetal bovine serum used in the culture medium must be dialyzed to remove folate, although it is not possible to reduce the folate level to zero. As such, we were concerned that the folate deficient medium had a higher than expected level of folate. A folate determination assay on cells grown in folate sufficient and deficient medium confirmed that cells grown under folate deficient conditions had no detectable levels of folate, supporting the conclusion that both Caco-2 and FHC cells can continue to divide with low levels of folate.

The continued growth of Caco-2 and FHC cells in the folate deficient medium was unexpected. A literature search revealed that it has been known that intestinal and colon epithelial cells can adapt to folate deficiency. One study showed that in several normal colonic cell lines there was an upregulation of the reduced folate carrier (RFC) and folate receptor 1 (FolR-1) due to folate deficiency (Crott et al., 2008). This is more than likely due to the fact that one of the key physiological roles for the intestinal track is absorption of nutrients. Folate is mostly absorbed in the enterocytes of the small intestine. Several other studies on folate adaptation have shown similar results even in non-intestinal cells lines. These studies have shown that there is an upregulation of folate receptors and an increase in transcript half-life (Sadasivan et al., 2002; Zhu et al., 2001; Subramanian et al., 2003). To confirm these findings in Caco-2 and FHC cells, the transcript levels of various folate transporters were determined by qRT-PCR. Our results

showed that folate deficient Caco-2 cells upregulated RFC and FolR-1. RFC showed a 2.5-fold increase in transcript levels in folate deficient cells at day 30. FolR-1 showed about a 2.5-fold increase in transcript levels at day 15 in folate deficient cells. However, the transcript levels for FolR-1 returned to normal (folate sufficient) levels at day 30.

We found that in FHC cells there was an overall decrease in the transcript levels of RFC. However, with continued days in folate deficient conditions there was an increase in transcript levels until the levels were comparable to that measured in folate sufficient cells. This could be due to a delayed attempt by FHC cells to adapt to folate deficiency through maintaining normal transcript levels or increasing transcript half-life. It could be a combination of the two. However, a decrease in RFC is opposite of what would be expected. One issue is the internal control, GAPDH, which may not be appropriate for these cells due to the possibility of folate deficiency affecting glycolysis. The use of additional internal controls such as Actin and/or Tubulin is warranted.

Overall, our results suggest that Caco-2 cells are adapting to folate deficiency. This would be the likely explanation for why Caco-2 (and perhaps FHC) cells continued to proliferate and cycle in folate deficient medium. In a study using gonadotroph α3T-1 cells an increase in FRα (FolR-1) protein was found to be associated with increased cell proliferation and an increased percentage of cells in S phase (Yao et al., 2009). This correlates with the results we found for Caco-2 cells in that there was an increased percentage of cells in S phase due to folate deficiency. The cells continued to grow as well, but at a slower rate than folate sufficient cells. FHC cells showed no change in cell

cycle phase distribution due to folate deficiency, and also continued to grow. We were unable to confirm that folate transporters increased in these cells.

Decrease in E-cadherin and β-catenin in folate deficient Caco-2 and FHC cells

Since E-cadherin can regulate the levels of free cytoplasmic β-catenin, we wanted to determine if folate deficiency is associated with a reduction in E-cadherin protein, which could alter Wnt signaling. Western blot analyses were performed to determine if folate deficiency led to any changes in E-cadherin and β-catenin protein levels. In Caco-2 cells we found that there was a decrease in E-cadherin and β-catenin protein levels at days 15 and 30 in folate deficient cells when compared to folate sufficient cells. Similarly, we found that E-cadherin and β-catenin protein levels decreased at day 15 in folate deficient FHC cells. However, at day 30 in FHC cells there was an increase in E-cadherin protein in folate deficient cells, whereas β-catenin protein levels did not change. These findings suggest that folate deficiency is having some effect on E-cadherin protein levels, and β-catenin protein levels are being affected as well.

Various studies have shown that E-cadherin affects Wnt signaling through its ability to regulate the levels of free cytosolic β-catenin (Gottardi et al., 2001; Kuphal et al., 2005; Ki et al., 2008). An inverse relationship between E-cadherin protein levels and nuclear signaling of β-catenin was found. We found that β-catenin followed a similar trend as E-cadherin in overall cellular protein levels; both proteins decreased in folate deficient cells. Wnt signaling may be affected, but it seems more likely that in Caco-2 and FHC cells β-catenin levels are tightly regulated. This could be due to the fact that as β-catenin protein is released from the E-cadherin adhesion complex it is immediately

targeted and degraded by a normally functioning degradation complex. This would not give the released β-catenin an opportunity to localize to the nucleus, which could prevent an onset of aberrant Wnt signaling. Moreover, the observation that β-catenin protein levels are slightly reduced would further indicate that the degradation process is enhanced. One major issue with these data is that only one technical replica was performed. Since the changes in protein were subtle, at least three biological replicas are necessary to determine if these changes are significant. These replicas are currently being completed.

To determine if there were any changes in E-cadherin transcripts due to folate deficiency we performed qRT-PCR using both Caco-2 and FHC cells. In one study showed that folate deficiency was found to affect E-cadherin expression in colon epithelial cells lines (Crott et al., 2008). For two of the three cell lines a small decrease in E-cadherin was detected, whereas a slight increase was detected in the third cell line. We found that there was no effect on transcript levels after day 15 in Caco-2 cells, but there was a decrease in transcripts at day 30. This result suggests that folate deficiency is affecting E-cadherin gene expression, which could lead to an overall decrease in E-cadherin protein. Indeed, our western blot data showed a slight decrease in protein levels. One possible explanation for this result is alterations in DNA methylation, causing the E-cadherin gene to be turned off. One study showed that E-cadherin expression could be altered by hypermethylation of the E-cadherin promoter (Lugli et al., 2007). This is counter intuitive as it would be expected that decreases in promoter methylation would occur in folate deficient cells due to reduced SAM. However, various

studies have found both increases and decreases in gene-specific methylation patterns due to folate deficiency (Esteller et al., 2001; James et al., 2002). Our attempt to amplify E-cadherin transcripts in FHC cells was unsuccessful. It is likely that FHC cells express a distinct E-cadherin transcript.

Localization of E-cadherin and β-catenin is altered in folate deficient cells

We used immunofluorescence to analyze the cellular location of E-cadherin and β-catenin to determine if folate deficiency was causing any effect on the E-cadherin/ β-catenin adhesion complex. In folate sufficient Caco-2 cells at day 20, E-cadherin and β-catenin were diffuse throughout the cell. In folate deficient cells the pattern of E-cadherin staining did not change, but an increase in membrane localization of β-catenin was seen. There was no apparent increase in the nuclear localization of β-catenin. This was a surprising result, as any increase in β-catenin membrane localization would be expected to be associated with membrane-bound E-cadherin. Studies have shown that E-cadherin is a key regulator of cytoplasmic β-catenin available for nuclear localization, and affects Wnt signaling through this mechanism (Orsulic et al., 1999). However, an increase in membrane-bound E-cadherin was not detected. There may be some other regulatory mechanism governing β-catenin accumulation at the plasma membrane that is independent of E-cadherin association. One study showed that β-catenin accumulated at the membrane due to activation of the Wnt pathway (Hendriksen et al., 2008). Although there was no increase in nuclear β-catenin, activation of other Wnt pathway proteins may have occurred. This increase in membrane associated β-catenin could be through interactions with members of β-catenin degradation complex such as APC and axin.

We found strikingly different results in FHC cells. In folate sufficient FHC cells, we saw a similar localization pattern as found in Caco-2 cells. Both E-cadherin and β-catenin were diffuse throughout the cell and localized in the same areas. However, in the folate deficient cells we saw an increase in nuclear localization of both proteins. The nuclear localization of β-catenin was expected but not with E-cadherin. This finding could be due to some artifact in preparing the slides, but we had also prepared slides for a day 30 time point and found the same results (data not shown). There is also the possibility that both antibodies (anti-β-catenin and anti-human E-cadherin) are nonspecific and the signal detected could be some other cellular protein. Why both would detect a nuclear protein and give identical staining patterns is puzzling.

Nuclear localization of E-cadherin was unexpected. However, there are published studies showing that E-cadherin may localize to the nucleus. In one study, E-cadherin localized to the nucleus in most cases of Merkel cell carcinoma cells but the mechanism leading to this change was not determined (Han et al., 2000). Associated with the nuclear localization of E-cadherin was an increase in β-catenin nuclear localization. Since this is a common occurrence in this kind of cancer no correlation between E-cadherin and β-catenin was made. In another study increased nuclear localization of E-cadherin was also detected, but only when using antibodies for the cytoplasmic domain of E-cadherin (Chetty et al., 2008). Since this domain of E-cadherin is what binds with β-catenin the authors speculated that it is possible that the cytoplasmic domain of E-cadherin is cleaved from the extracellular domain and "piggy-back" with β-catenin into the nucleus. Although FHC is a normal cell line and not cancerous, our findings support this

mechanism. We found that nuclear localization of E-cadherin coincided directly with β-catenin localization. In fact, they overlapped perfectly. The antibody that we used to detect E-cadherin was for the cytoplasmic domain, and it may be possible that this domain remains attached to β-catenin as it moves into the nucleus. Further study will be needed to verify this conclusion.

AKT Pathway in folate deficient cells

We also conducted a western blot analysis for proteins in the AKT pathway. The AKT pathway is an important regulator of cell growth and survival (Itoh et al., 2002). It was surmised that this could be a potential cause for why the cells continued to grow even under folate deficient conditions. We performed a western blot analysis on Caco-2 cells. At day 15 there was a decrease in activated AKT protein in folate deficient cells as compared to folate sufficient cells. At day 30 there was an increase in activated AKT protein in the folate deficient cells. This would be the expected result. However, these changes were small and may not be significant. Other proteins involved in the AKT pathway were also analyzed to determine if there was increased activity in the AKT pathway due to folate deficiency. At day 30 we found a slight decrease in PDK-1and c-Raf in folate deficient cells and even less of a decrease in PTEN. The reduction in PDK-1 and PTEN could be some form of negative feedback due to increased activation of AKT. PDK-1 phosphorylates AKT which activates the protein so that it can, in turn, phosphorylate target proteins such as c-Raf. PTEN is also a regulator of AKT through dephosphorylating lipids involved in the activation of AKT (Paez et al., 2003). There was a slight increase in GSK-3β, which again must be confirmed through biological

replicas. One study has shown that AKT affects β-catenin through disrupting the complex that targets it for degradation. This would help to increase the available β-catenin that can localize to the nucleus, and increase cell growth and survival (Dihlmann et al., 2005). This mechanism could potentially be proved due to the increase found in phospho-GSK3β. An increase in phosphorylated GSK-3β would help prevent the formation of the degradation complex and stabilize the pool of free β-catenin. This could induce Wnt signaling at some level, or prevent the further degradation of β-catenin if there was an upregulation of the degradation complex due to increased amounts of β-catenin being released from the E-cadherin adhesion complex. If there is indeed some Wnt stimulation this could also help to explain the increased membrane recruitment of β-catenin without increasing E-cadherin.

Summary

In summary, we found that folate deficiency caused slower but continual cell proliferation, and that both cell lines continued to cycle. We also determined that the mRNA levels of two folate transporters increased in folate deficient Caco-2 cells. These results indicate that the cells are adapting to folate deficiency. We also found a reduction in E-cadherin and β-catenin protein levels, and that there was a change in the localization of both proteins due to folate deficiency. The AKT pathway showed minor changes due to folate deficiency, but further study needs to be conducted to see if these changes are significant. We also determined that the transcription levels of two folate uptake transporters were affected by folate deficiency. This leads us to conclude that both cell lines may be adapting to folate deficiency.

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