RANJANA, RASHMI, M.S. Possible Involvement of NF-κB in the Response of Cells to Folate Deficiency. (2009)
Directed by Dr. Karen S. Katula. 45 pp.

Folate, a water soluble B vitamin, serves as a methyl donor for the synthesis of nucleotides and methionine. Folate deficiency has been linked to a variety of health problems that include developmental defects, cancer and neurological diseases. The relation between folate deficiency and human health problems has not been fully explained. Our preliminary results and limited published data have shown that the activity of NF-κ B increases in folate deficient conditions. NF-κB is a cytosolic dimeric transcription factor that responds to a variety of stimuli by regulating genes involved in immune response, cell survival and cell growth. The purpose of the study was to determine if folate deficiency leads to altered NF-κB activity, protein levels and nuclear localization, and to determine the possible mechanisms behind this alteration. NIH3T3 cells containing a NF-κB reporter construct (NF-κB-LUC-3T3 cells) were grown for 7 and 11 days in folate sufficient and folate deficient DMEM medium either with or without TNF-α (stimulator of NF-κB). NF-κB activity was higher in folate deficient medium at days 7 and 11 in both unstimulated and stimulated cells, as measured by luciferase assay per unit DNA. NF-κB protein levels analyzed by Western Blot showed no difference between folate sufficient and deficient cells. Immunofluorescence by confocal microscopy showed that there was no significant change in the nuclear localization of NF-κB in folate deficient conditions even when stimulated with TNF-α. Analysis of reactive oxygen species (ROS) using a fluorescent assay with flow cytometry revealed that there was more fluorescence per cell in folate deficient cells at day 11. This

increase in ROS could be one of the mechanisms behind the increase in NF-κB activity in folate deficient cells.

POSSIBLE INVOLVEMENT OF NF-κB IN THE RESPONSE OF CELLS TO FOLATE DEFICIENCY

By

Rashmi Ranjana

A Thesis Submitted to
The Faculty of The Graduate School at
The University of North Carolina at Greensboro
In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

Greensboro
2009

Approved by

_____
Committee Chair

To my husband Ramesh Jha, my coming baby, my parents, and my family

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

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Date of Acceptance by Committee

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Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to express my deep sense of gratitude and sincere thanks to my advisor, Dr. Karen S. Katula, Biology Department, UNCG, for her valuable guidance and encouragement during my thesis work. During my thesis work she was always ready to help me with my experiments and provide me with bright ideas. She was a true mentor without whose support this work would have been not possible.

I am also indebted to Matt, Amber and Alison for all the help and co-operation they extended to me during my work. Their patience while listening to me and their promptness while helping me are commendable.

I would also like to thank Maurice, a previous member of my lab, for helping me with experimental techniques and my colleague Avik, for his support.

Last but not least I would like to convey my special thanks to my committee members Dr. John Tomkiel and Dr. Yashomati Patel for their support and advices during my work to make it a successful thesis.

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CHAPTER I

INTRODUCTION

Vitamins and minerals are indispensable components of the human diet. Most vitamins are not sufficiently synthesized by humans and must be obtained from the diet. They have diverse biochemical functions ranging from enzyme co-factors, antioxidants, hormones, and regulators of cell and tissue growth and differentiation.

Folate is a water soluble B vitamin that serves as a methyl donor in the synthesis of nucleotides and methionine. Folate deficiency has been associated with numerous health problems, including developmental defects, neurological diseases and cancer. The relationship between folate deficiency and human disease has not been thoroughly defined.

Our preliminary results and limited published data indicate that NF-κB activity increases in folate deficient cells. NF-kappa B (NF-κB) is a cytosolic dimeric transcription factor that responds to a variety of stimuli. It regulates expression of genes involved in immune response, cell survival, and cell growth. An increase in NF-κB activity may contribute to the health defects associated with folate deficiency. However, the relationship between folate deficiency and NF-κB expression has not been fully

explored. The purpose of this study was to provide an analysis of NF-κB activity in folate deficient cells and elucidate the mechanism leading to the alteration in NF-κB activity.

Project Overview

In this study, the effects of folate deficiency on NF-κB were analyzed in a fibroblast (NIH3T3) and a normal human colon epithelial (FHC) cell lines. NF-κB protein levels, activity, and cellular localization were determined in cells grown in folate deficient and sufficient medium, stimulated with and without TNF- α, a NF-κB inducer. The levels of homocysteine and ROS were determined in the same cell lines grown in folate deficient and sufficient medium. The mRNA levels of NF-κB target genes, Cyclin D1, were analyzed in a fibroblast cells grown in folate deficient and sufficient medium.

This study is significant as it provides further understanding of the mechanism by which folate deficiency increases the risk of cancer and possibly other human health conditions. Folate deficiency is one of the few dietary components proven to be associated with numerous health conditions, yet the mechanism of this relationship is still not completely known.

CHAPTER II

BACKGROUND

Folate pathway

Folate is a water soluble vitamin B that can only be obtained from diet. It is found in green leafy vegetables, yeast extracts, citrus fruit, fortified cereal, wheat germ, bread and potatoes (Lucock, 2000). Folate is essential in maintaining normal cell division, as it functions as a coenzyme in *de novo* biosynthesis of purines and thymidylate. As such, it is required for DNA replication and DNA repair (Shanebeck et al, 1995). It is also required for the synthesis of certain amino acids, most importantly, methionine. Dietary folate predominantly exists as 5-methyl tetrahydrofolate. The folate is transported across the enterocytes brush border membrane by specific carriers involving an anion exchange, and enters into the plasma. After absorption the dietary folate is released into portal circulation. The liver plays a central role in maintaining serum folate levels. Plasma folate is bound to several low affinity binding proteins, mainly albumin, and enters blood circulation. Serum plasma folate is transported into each cell by the cell surface transporter FBP (Folate binding protein) and FcoT (Folate co-transporter).

Fig. 1 shows the intracellular folic acid metabolic pathway. In brief, intracellular folate is converted into 7,8-dihydrofolate (DHF), which is converted into tetrahydrofolate

(THF) by dihydrofolate reductase (DHFR). THF is converted into 5,10-methylenetetrahydrofolate and is then converted into 5-methyl-tetrahydrofolate by 5,10-methylenetetrahydrofolate reductase (MTHFR). The metabolite 5,10-methylenetetrahydrofolate is used in the conversion of deoxyuridylate monophosphate (dUMP) into deoxythymidylate monophosphate (dTMP), an essential nucleotide required in DNA synthesis. The metabolite 5-methyl-tetrahydrofolate is required for the conversion of homocysteine into methionine, which in turn is converted into S-adenosylmethionine (SAM), the only methyl donor for all the intracellular methylation reactions, including, methylation reactions of DNA and protein (Ulrich, 2005).

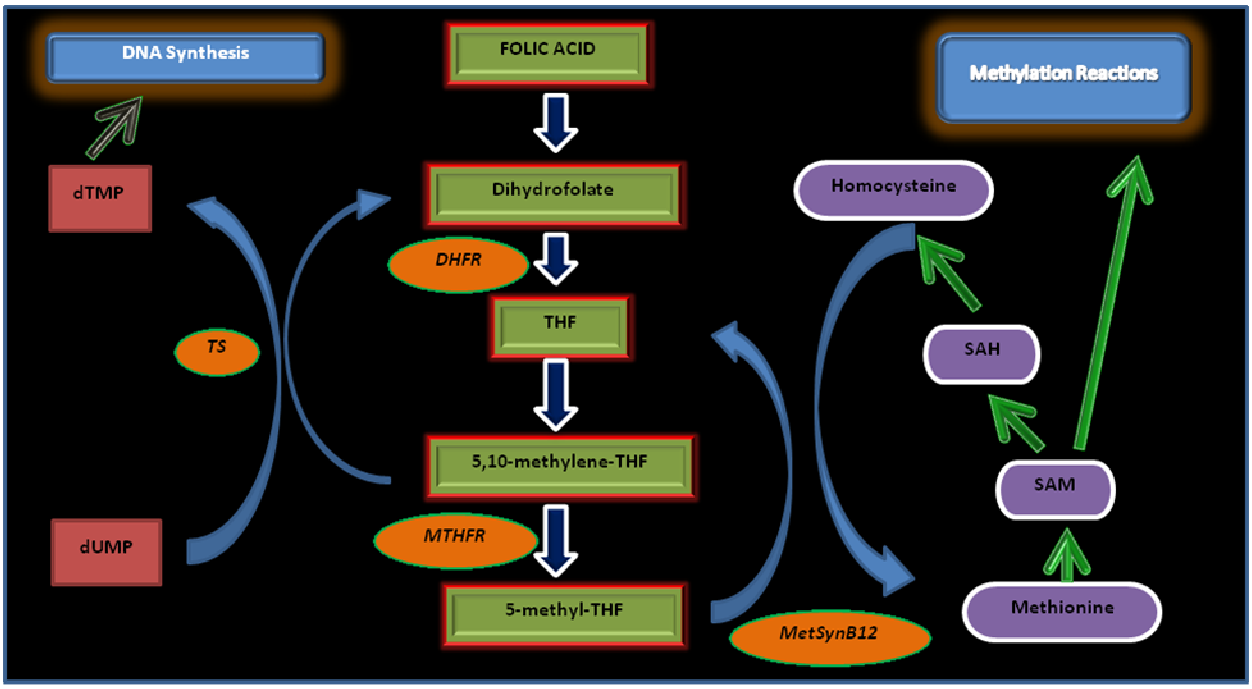


Fig. 1. Major folic acid pathway (Modified form of Stokstad, 1967). TNF- Tetrahydrofolate, DHFR- Dihydrofolate reductase, MTHFR- 5,10-methylenetetrahydrofolate reductase, TMP- Thymidylate monophosphate, dUMP- deoxyuridylate monophosphate, TS- Thymidylate synthase, MetSynB12- Methionine synthase, SAM- S-adenosylmethionine, SAH- S-adenosylhomocysteine.

Folate deficiency and cellular consequences

Folate deficiency has been associated with DNA strand breaks, chromosomal gaps and breakages, increased level of dUMP, which in turn leads to increased uracil misincorporation into DNA, impaired DNA repair and increased mutations (Blount et al, 1997). The consequence of these alterations leads to the loss of DNA integrity and stability (Blount et al, 1992).

Folate is essential for the transfer of one-carbon units in the de-novo synthesis of nucleotides (Blount et al, 1997). Folate deficiency leads to low levels of cytosolic N^5,N^{10}-methylene tetrahydrofolate (folate cofactor for thymidylate synthase) resulting in decreased synthesis of dTMP and an increase in the cellular dUMP/dTMP ratio. The consequence of which will be reduced incorporation of dTMP into DNA and increased dUTP misincorporation into DNA (Goulian et al, 1980; Das and Herbert 1989; Wickramasinghe and Fida 1994). Normally, DNA repair mechanisms remove the misincorporated uracil, but due to the large amount of uracil a catastrophic repair cycle is established. The uracil removed from DNA leaves behind transient-single strand breaks. These nicks can ultimately induce the formation of double strand breaks (Dianov et al, 1991), which are more difficult to repair and therefore more hazardous to the cell.

Folate deficiency also leads to a reduction in remethylation of homocysteine to methionine leading to reduced SAM, the only methyl donor for all the cellular methylation reactions. Consequently, there is decreased methylation. Reduced SAM due to folate deficiency has been found to lead to genome wide DNA hypomethylation and in some studies cause activation of oncogenes and malignant transformation (Duthie et al, 2004). For example, rats fed on a diet devoid of folate displayed increased global hypomethylation especially in the liver (Pogribny et al, 2004). Global hypomethylation, particularly of repetitive elements, is a common epigenetic event during early stages of carcinogenesis (Ehrlich, 2005; Yamada et al, 2005). A decreased SAM/SAH ratio also

results in hypermethylation of CpG islands of certain tumor suppressor genes (Kim 2004; Park et al, 2005).

NF-κB pathway

NF-κB is a dimeric transcription factor. It is a membrane of protein family sharing a highly conserved N-terminal dimerization domain and a DNA binding domain (Rel homology domain) (Gilmore, 1990). Functionally, NF-κB is widely conserved from fruit fly to human and also exits in Cnidarian and Porifera (Gilmore, 2006). Mammalian NF-κB has five members: RelA, RelB, c-Rel, p50/p105, p52/p100 (Gilmore, 2006). NF-κB is activated by a wide variety of stimuli such as stress, cytokines, free radicals, UV radiation and antigens. NF-κB regulates the expression of genes involved in immune response, cell growth control and cell survival regulation (Hayden and Ghosh, 2004). Such genes include cytokines, chemokines, Cyclin D1, Cyclin E, CDK2, c-MYC, antiapoptotic proteins, Bcl-2, IL-1/6, VEGF, metalloproteinases, MMP2/13, MMP2/9, TNF-α, IL-8 and MCP-1.

NF-κB normally resides as a cytosolic protein bound to the inhibitor IκB, a protein kinase having ankyrin repeats. There are several IκB proteins. These proteins interact with the NF-κB nuclear localization signal stopping transport of NF-κB into the nucleus. In response to different stimuli there are two pathways that may lead to NF-κB activation. The canonical and non-canonical pathway. The canonical pathway is the most common. In the canonical pathway the stimuli leads to the activation of the IKK

complex, containing catalytic subunits IKKα / IKKβ and scaffold regulator sensing protein NEMO (IKKγ). Generally, the stimulating ligand binds to the cell surface receptor, in this case either TNFR or TRAF. These then recruit the IKK complex. This activated IKK complex then phosphorylates IκB at two specific serine residues, leading to its ubiquitination and proteosomal degradation. NF-κB becomes free and is translocated to the nucleus and as a dimer activates different target genes. NF-κB is the regulator of its own inhibition in that it activates the revival of IκB (Hoffmann and Baltimore, 2006).

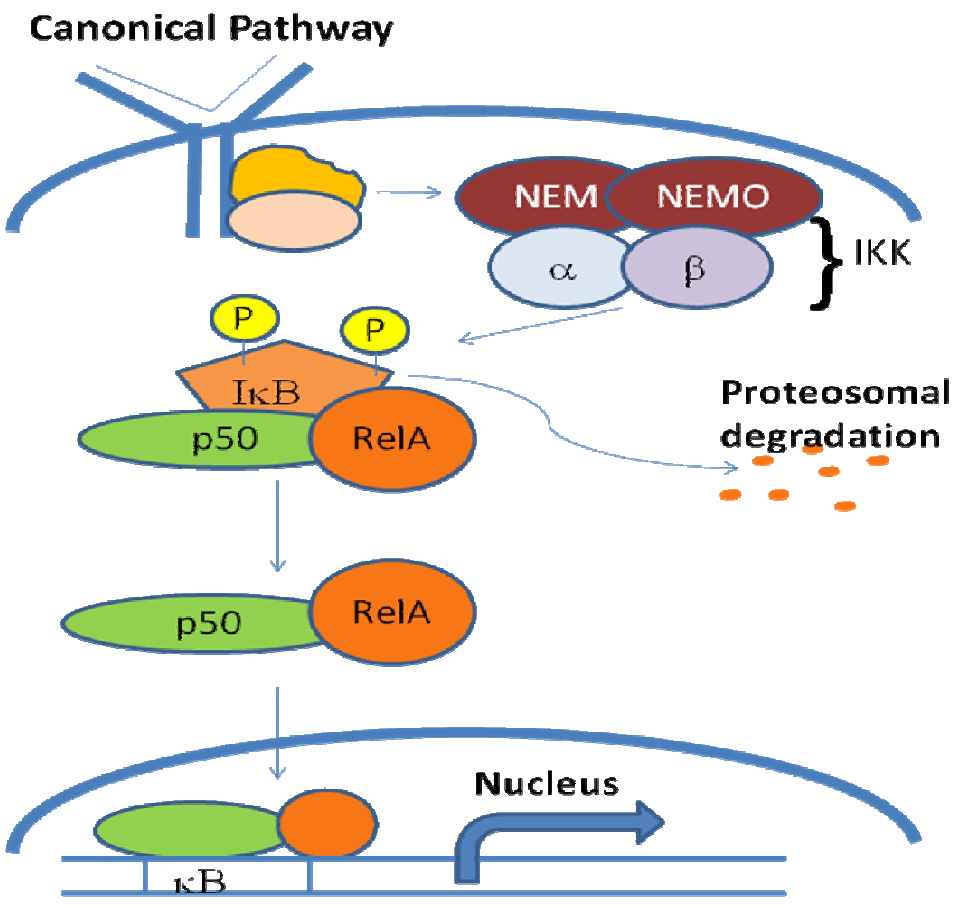


Fig. 2. NF-κB signal transduction pathway (Modified from Gilmore, 2006)

Misregulation of NF-κB plays a role in promoting oncogenesis (Baldwin, 2001). The NF-κB pathway remains constitutively activated in human colorectal carcinoma tissues (Ahmed et al, 2006). NF-κB has been found to be upregulated in various cancers such as T-cell lymphomas, chronic lymphocytic leukemias, myelomas and B-cell lymphomas (Courtis and Gilmore, 2006), breast cancer (Ahmed et al, 2006), and prostate cancer (Huang et al, 2001). It has also been associated with Huntington's disease

(Khoshnan et al, 2004) and Alzheimer's disease (Collister and Albensi 2005). Misregulation of NF-κB is associated with autoimmune disease, inflammatory diseases and improper immune development (Albensi and Mattson 2000).

Reactive oxygen species, homocysteine and NF-κB activation

Oxidative stress occurs due to lack of balance between reactive oxygen species (ROS) production and the ability of biologically system to detoxify or repair the reactive intermediates produced. ROS are oxygen centered free molecules that include oxygen ions, peroxides and hydroxyl free radicals and often are described as free radicals or non-radicals. ROS are the outcome of byproducts of normal oxygen metabolism and most of the time play an important role in cell signaling. Certain circumstances like stress caused by ultraviolet rays, hypoxia, X-rays and heavy metals cause higher levels of ROS that results in significant cellular damages.

Homocysteine (Hcy) is a sulfhydryl compound and an intermediate in the folate pathway (Fig 1). It is capable of acting catalytically with transition metal ions to form hydrogen peroxide, oxygen radicals and homocysteinyl radicals (Starkebaum et al, 1986; Olszewski et al, 1993; Tapiero et al, 2001).

NF-κB is a redox sensitive transcription factor. It has been demonstrated that ROS works as a secondary messenger for NF-κB activation (Bowie and O'Neill, 2000). Some groups have reported that hydrogen peroxide can activate the IKK complex in human bronchial epithelial cells and HeLa cells (Jaspers et al, 2001; Kamata et al, 2002).

In fact, in HeLa cells treatment with hydrogen peroxide resulted in phosphorylation of Ser 180 and Ser181 of IKKα, hence activation of the IKK complex and release of NF-κB (Kamata et al, 2002). Chen and Green (2004) in their review also mentioned that several RelA phosphorylating kinases can be directly activated by ROS, which can result in enhanced NF-κB activity. Other studies have shown that oxidative stress can promote translocation of NF-κB dimers in the nucleus either by promoting phosphorylation and subsequent degradation of IκBα (Karin, 1999) or by phosphorylation of IκBα at a tyrosine residue which facilitates the dissociation of IκBα from NF-κB without proteosomal degradation (Imbert et al, 1996; Koong et al, 1994; Takada et al, 2003).

Relationship between folate deficiency, NF-κB and Reactive Oxygen Species (ROS)

In one study, folate deficiency was shown to cause a drastic increase in Hcy production in Hep G2 cells (Chern et al, 2001). A high level of Hcy could be suppressed by addition of exogenous folate. Fig 1 shows the interconnection between demethylation of 5-methyl-THF to THF and remethylation of homocysteine to methionine. A deficiency in folate will cause reduced methylation of homocysteine to methionine, resulting in accumulation of homocysteine. Folate deficiency has been shown to increase the level of serum homocysteine leading to occlusive vascular disease (Frosst et al, 1995). Clinical studies for the Alzheimer's disease (AD) also suggest that folate deprivation causes an increase in ROS and an increase in homocysteine generation (Ho et al, 2003). Another study on human intestinal epithelial line, Caco-2 cell line showed that folate deficiency leads to reduction in homocysteine remethylation and an increase in intracellular

homocysteine (Townsend et al, 2004). As previously discussed, accumulated homocysteine has been shown to stimulate NF-κB activity. In addition, the effects of homocysteine on NF-κB activity were shown to be nullified by addition of folic acid and apocynin (Au-Yeung et al, 2006). In this study, pretreatment of macrophages with folic acid (200ng/ml) abolished the Hcy-induced IκBα phosphorylation and NF-κB activation.

One mechanism by which folate deficiency and increased homocysteine may be affecting NF-κB is by increasing reactive oxygen species (ROS). ROS has been shown to increase in cells with higher than normal levels of homocysteine (Zhang et al, 2006). In another study, ROS levels were almost 8-fold higher in folate deprived neurons (Ho et al, 2003). Based on these findings, a possible mechanism by which folate deficiency alters cell function is increased homocysteine, leading to increased ROS and activation of NF-κB. Activated NF-κB would be expected to lead to altered gene expression, having a profound effect on cell function.

CHAPTER III

MATERIALS AND METHODS

Cell Lines

NIH3T3, a mouse fibroblast cell line, was originally obtained from the American Type Culture Collection (ATCC). NIH3T3 cells stably transfected with the plasmid pNF-κB-TA-LUC (Clontech) was previously constructed in the lab. This plasmid contains four tandem copies of the NF-κB consensus sequence upstream of a minimal TA promoter P_{TA}, composed of the TATA box derived from Herpes simplex virus thymidine kinase (HSV-TK) promoter. Downstream from the P_{TA} is the coding region of the luciferase firefly gene.

Preparation of Custom Medium

Delbecco's Modified Eagle Medium (DMEM) required for growing NIH 3T3 cells under folate deficient and folate sufficient conditions was prepared using the formulation listed in Appendix A minus folic acid. The components were added to and mixed in 500 ml of distilled water for three to four hours. The volume was adjusted to 2 liters and the medium was divided equally into two portions. To one portion folic acid (4 mg/L) was added. To the other portion folic acid was not added. The medium was filter sterilized with a 0.20 μm filter. To prepare complete medium, 50 ml of dialyzed calf serum and 5 ml of Penicillin/Streptomycin (5,000 IU/ml and 5,000 μg/ml) was added to

450 ml of DMEM. This prepared medium was referred to as DMEM Complete. The medium with folic acid was referred to as DMEM Complete WITH (WITH) and the medium without folic acid was DMEM Complete NO (NO).

The DMEM medium for the ROS assay was prepared in the same way as explained above with the exception that phenol red was not added.

Cell Culture

NF-κB-LUC-3T3 cells were grown in a T-75 flask with DMEM Complete medium until there were enough cells for replating. The cells were maintained at a subconfluent density. The cells were plated at a density of 5.6×10^5 in T-75 flask with a total volume of 12 ml DMEM Complete. 10 μl of G418 (200 mg/ml) was added to the flask. Cells were grown in a 37^0C incubator with 5% carbon dioxide and 95% air. When the cells were approximately 80-90% confluent, they were collected and counted. The cells were washed once with 5 ml Hank's, trypsinized with 4 ml of 1X Trypsin-EDTA (0.05% Trypsin, 0.053 mM EDTA) for 1-2 minutes. 4 ml of DMEM Complete medium was added to the flask. The solution was transferred to a 15 ml Falcon tube and centrifuged in a clinical centrifuge at setting #3 for 5 minutes. The cell pellet was resuspended 5 ml DMEM Complete NO medium and an aliquot was removed for counting using a haemocytometer. The cells were again replated in two T-75 flasks at a density of 5.6×10^5 in either DMEM Complete WITH folate or DMEM Complete NO folate and incubated in the cell culture incubator. This day was counted as day 0. For

ROS studies, cells were grown in DMEM Complete WITH and NO, phenol red free medium.

For the day 7 time point reactive oxygen species (ROS) studies, the cells were collected and replated on day 6 in a 6-well plate with 4 X 10^5 cells per well in either DMEM Complete WITH medium, no phenol red or DMEM NO medium, no phenol red. For NF-κB activity, cells were plated at a density of 3 X 10^4 cells per well in 48-well plates in either DMEM Complete WITH medium or DMEM Complete NO medium. The remaining cells were replated in separate T-75 flasks containing either DMEM Complete NO medium, no phenol red or DMEM Complete WITH medium, no phenol red with a cell density of 5.6 X 10^5 brought to a final volume of 12 ml with 10 µl of G418 (200 mg/ml) added. For the day 11 time point, the cells were collected, counted and replated on day 10 as previously described for day 7.

Western Blot Analysis

Cells for Western Blot were treated with and without TNF-α (1.25 ng/ml) for 4 hours and then washed with phosphate buffered saline (PBS). 200 µl of 2X SDS lysis buffer (4% SDS, 20% glycerol, 100 mM Tris-HCl, pH 6.8, 5 mM DTT) with Protease Inhibitor Cocktail (Complete Mini, Roche, Inc.) was added to the cells and left on the rocker for 15 minutes. The cells were scraped off the plate, transferred to microfuge tubes and centrifuged at 10K for 10 minutes at 4^0C. Supernatants were transferred to clean microcentrifuge tubes, labeled, and all samples were stored at -80^0C. Protein content of the samples were determined by using the Pierce 660 nm Protein Assay kit.

An equal amount of cellular protein from each sample was loaded into separate wells of an acrylamide gel (10%). Prestained molecular weight markers (Prosieve, Catalogue. #50550) were loaded into one lane of the gel. Electrophoresis was performed at a constant 21 mA for 3 hours. After electrophoresis, the protein was transferred to a nitrocellulose membrane using a standard protocol. The blot was stained in Ponceau S (1% Ponceau S in 1% acetic acid diluted 1:10 with ddH_20) to visualize protein and confirm transfer and equal loading of the samples. The blot was incubated for an hour in 5% dry milk dissolved in Tween-Tris Buffered Saline (TTBS) (0.15M NaCl, 0.01M Tris pH 8.0, 0.05% Tween 20). A 1:500 dilution of mouse anti-NF-κB (Sigma-Aldrich, catalogue # 046K4852) antibodies in 1% dry milk in TTBS was added to the blots and left for an hour. The nitrocellulose membrane was then washed in TTBS 3X for 5 minutes each wash and once for 15 minutes. A 1:10,000 dilution of goat anti-mouse horseradish peroxidase (HRP) (Sigma-Aldrich, catalogue #A3673) in 1% dry milk contained in TTBS was added to the blot for 1 hour. The blot was washed again 3X in TTBS for 5 minutes each wash and one time for 15 minutes. The HRP on the nitrocellulose membrane was detected using the Pierce Supersignal West Pico Chemiluminescent detection kit. The BioRad Chemi Boc DOCX RS imager and Quality One software was used for blots imaging and quantification.

ROS Assay

Cells were collected by trypsinization, counted, and replated in 6-well plates in phenol red free medium at a density of 4 X 10^5 cells. Next day, the medium was changed in appropriate wells and replaced with DMEM Complete NO or WITH medium (without phenol red). The cells were treated with and without 100 μM H_2O_2 by adding the appropriate amount of 100 mM H_2O_2. The cells were incubated for an hour at 37^0C. After treatment, the cells were washed once with 2 ml PBS and once with 2 ml Hank's buffer. After washing, the cells were trypsinized and collected by centrifugation. The pellets were resuspended in either PBS containing 10 μM C400 ROS compound (Invitrogen) or just PBS. After ten minutes cell are analyzed by a BD FACSCalibur flow cytometer (settings: P1-E00, P2-310, P3-425, P4-375, P5-650, FL1H-15). Quantification of the resulting data was carried out by using the Summit 4.x software.

Immunofluorescence

NF-κB-LUC-3T3 cells were grown on circular coverslips in 24-well plates for performing immunofluorescence. The cells were replated at a density of 5 X 10^4 cells per well a day before the time point (day 11) and incubated overnight. The medium on the cells were replaced with DMEM Complete NO and WITH medium, containing either TNF-α or no TNF-α at 1.25 ng/ml. Cells were incubated for 24 hours. The cells were washed 1X in PBS and fixed directly in the 24-well plate with 1 ml 2% formaldehyde in phosphate buffered saline (PBS) for 15 minutes at room temperature. After fixing, cells were washed two times with 500 μl PBS for five minutes per wash. Cells are then stored

in 1 ml PBS at 4° C. Cells were permeabilized with 400 µl 0.25% Triton X-100 diluted in PBS for 5 minutes. After permeabilization, cells were washed three times with 500 µl PBS. 65 µl of anti-NF-kB antibody (Sigma-Aldrich, catalogue # 046K4852) was added directly onto the cells and incubated at 37° C for 1 hour. Following incubation, cells were washed three times with PBS for 5 minutes per wash. 95 µl of a secondary antibody, goat anti-mouse IgG with fluoroform Alexa 488 (Invitrogen, catalogue# A11001) was added and the samples incubated at room temperature for one hour in the dark. After incubation the cells were washed three times with 500 µl PBS for five minutes per wash. Nuclei counterstaining was performed with 300 µl of a 1:100,000 dilution of DAPI for 5 minutes at room temperature in the dark. Cells were then washed twice with 500 µl PBS for 5 minutes. Coverslips were mounted on glass slides using Dako Cytomation Fluorescent Mounting Medium and allowed to sit in the dark overnight. Cells were then imaged by using confocal microscopy (Olympus FV500) and the Fluoview imaging software (Olympus). Quantification of imunofluorescent signal was obtained using Image J software. Average nuclear signal per unit area was measured.

NF-κB activity and Luciferase Assay

Cells were collected by trypsinization, counted, and replated in 48-well plates at a density of 3 X 10^4 cells. The next day, DMEM Complete NO and WITH medium, with and without 1.25 µg/ml TNF-α, was added to the respected labeled wells and incubated for 4 hours in the cell culture incubator. After incubation, the medium was removed and cells were washed once with PBS. 100 µl of 1X Cell Lysis Buffer (Promega, Inc.) was

added and incubated at 37^0C for 15 minutes. The cells were stored at -80^0C. After collection of all samples at days 7 and 11, luciferase was measured using the Promega Luciferase Assay System. 30 µl of cell lysate was pipeted into luminometer tubes and placed in a Berthold/B950/luminometer. Samples were read for 10 seconds and relative light units (RLU) recorded. Variation in cell number was accounted for by measuring DNA content and standardizing luciferase results to DNA.

Quantification of DNA

Cells were collected and prepared as described in luciferase assay. 10 µl of each sample was transferred into 96-well plate. 50 µl of a DNA binding dye (Invitrogen, CyQuant NF Cell Proliferation Assay) diluted 1:750 in 1X HBSS is added to each well. All samples were then incubated at 37^0 C for 15 minutes and covered with aluminium foil. The sample DNA content (fluorescence) was measured on BIO-TEK Synergy HT microplate reader at excitation 485 nm and emission 520 nm using the KC4 software.

RT-PCR

Cells for RT-PCR were collected by scraping the cells into PBS. The cells were pelleted by centrifugation and washed 1X in PBS and quick frozen in liquid nitrogen. Cell pellets were stored at -80^0C. RNA was isolated using the SU Total RNA Isolation System (Promega). The RNA was converted to cDNA using the Quanti Tect Reverse Transcription kit (Qiagen, Inc.). Mouse cyclin D1 and GAPDh, primers were obtained from Applied Biosystem. RT-PCR was conducted using Taqman and a Applied Biosystems Stepone Real-Time PCR system.

CHAPTER IV

RESULTS

NF-κB activity in folate deficient and sufficient cells

Based on the previous findings (Au-Yeung, Yip et al. 2006) and our preliminary results (Katula lab) showing altered NF-κB activity due to folate deficiency, we decided to compare NF-κB activity in folate sufficient and deficient cells. It was expected that the NF-κB activity would increase.

NIH3T3 cells containing a NF-κB reporter construct (NF-κB-LUC-3T3 cells) were used as an experimental cell line to assay for NF-κB activity. The cells contain the plasmid pNF-κB-TA-LUC (Clontech), which contains four tandem copies of the NF-κB consensus sequence upstream of a minimal TA promoter P_{TA}, composed of the TATA box derived from Herpes simplex virus thymidine kinase (HSV-TK) promoter. Downstream from the P_{TA} is the coding region of the luciferase firefly gene. This plasmid responds to increased NF-κB activity by producing more luciferase mRNA.

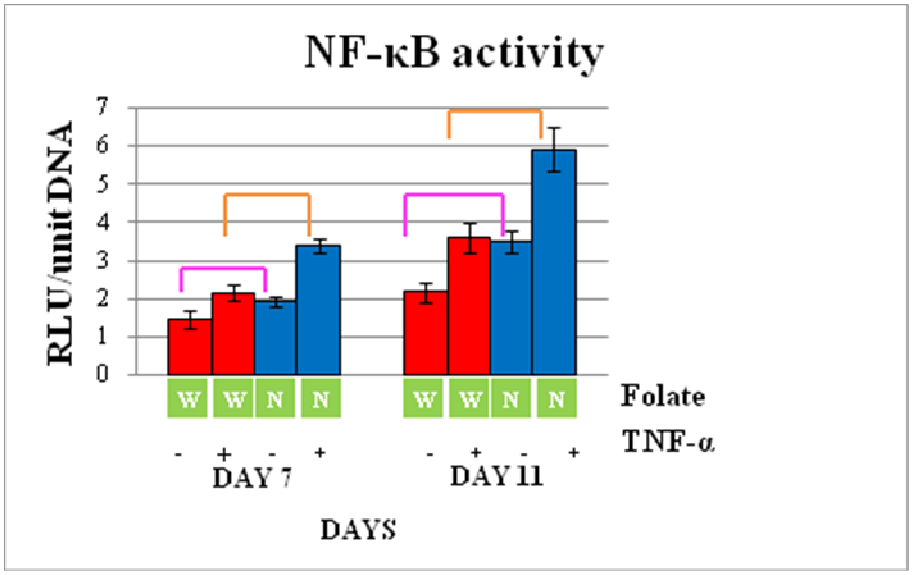
The NF-κB-LUC-3T3 cells were grown for 7 and 11 days in folate deficient and sufficient medium to appropriate number. At each time point, cells were treated with and without TNF-α (inducer of NF-κB), collected and assayed for luciferase activity. DNA content was determined to control for cell number.

Three different trials are shown in Fig. 3. At day 7, two of the trials (1 and 2) indicate a higher level of NF-κB activity in the folate deficient cells, with and without TNF-α. In contrast, trial (3), indicated no difference between the folate sufficient and deficient cells. At day 11, there was greater NF-κB activity in the folate deficient cells in comparison to folate sufficient cells for all three trials and with and without TNF-α stimulation. This difference was significant for all three trials (Fig. 3). The fold change was calculated for each trial, comparing within group changes in NF-κB activity, with and without TNF-α. As shown in Table 1, the fold change is less than 2 fold and in general, there is no major difference between the folate deficient and sufficient cells.

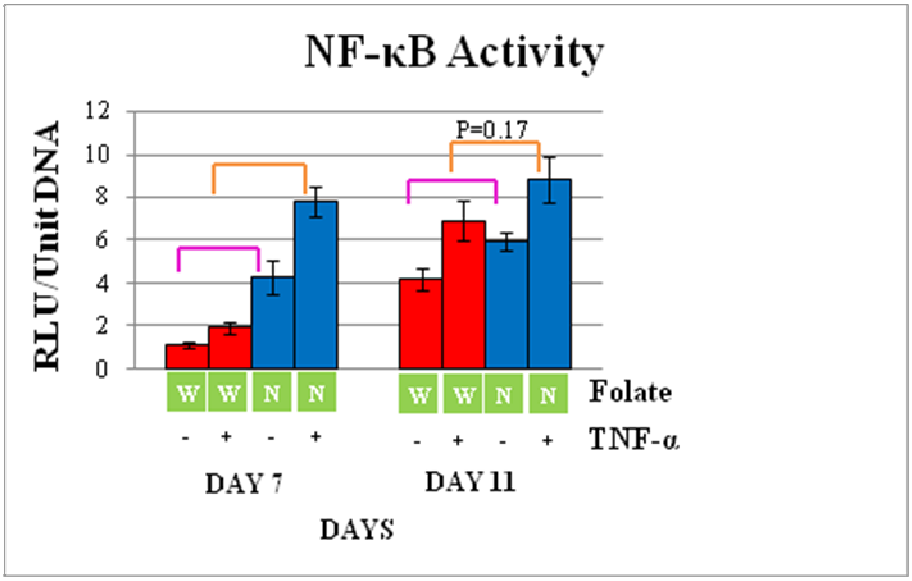
Table 1. Fold change in NF-κB activity in folate deficient and sufficient cells, stimulated with TNF-α.

Experiment	**DAYS**	**WITH +/-**	**NO +/-**
1(4 hours)	*7*	1.49	1.76
	11	1.65	1.70
2(4 hours)	*7*	1.74	1.81
	11	1.65	1.49
3(4 hours)	*7*	1.46	1.43
	11	1.87	1.42

1)


NF-κB activity
RLU/unit DNA
7
6
5
4
3
2
1
0
W W N N W W N N
Folate
- + - + - + - +
TNF-α
DAY 7
DAY 11
DAYS

2)


NF-κB Activity
RLU/Unit DNA
12
10
8
6
4
2
0
P=0.17
W W N N W W N N
Folate
- + - + - + - +
TNF-α
DAY 7
DAY 11
DAYS

3)

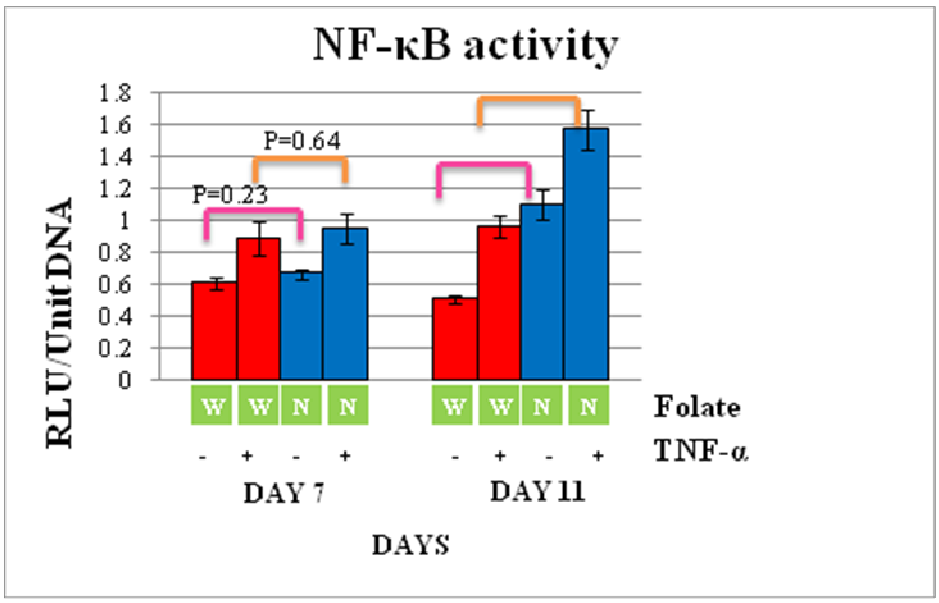


Fig. 3. Alteration in NF-κB activity in folate deficient and sufficient cells. NF-κB activity reported as RLU per unit DNA in NIH3T3 cells grown under folate sufficient and deficient conditions and either stimulated or unstimulated with TNF-α (1.25 ng/ml; 4 hours). Error Bar represents standard error (n=12). Brackets represent P value comparison. P value less than 0.05 not added. W= With folate; N= no folate.

An increase in NF-κB activity should lead to changes in the expression of NF-κB target genes. This would provide further support for our finding that NF-κB activity increases in folate deficient cells. In a preliminary study, the level of the NF-κB target gene, cyclin D1, was examined by qRT-PCR. In this experiment, cells were grown in folate sufficient (WITH) and folate deficient (NO) medium for 7 and 11 days and treated with and without TNF-α. RNA was isolated from the collected cells, converted to cDNA, and analyzed using primers specific to cyclin D1 and GAPDH. The cyclin D1 values were normalized to GAPDH. As shown in Fig. 4 folate deficient cells display a greater

increase in cyclin D1 transcript when stimulated with TNF-α than in folate sufficient cells. This increase is greater at day 7 than day 11. The folate sufficient cells display only a slight increase in cyclin D1 transcripts at day 7 and no increase at day 11.

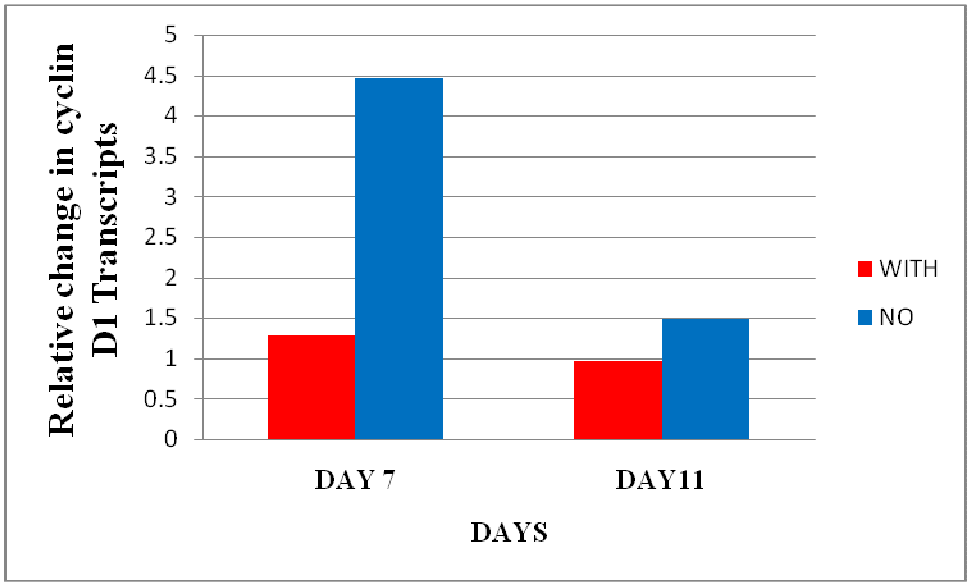


Fig. 4. Relative change in cyclin D1 transcripts. Cyclin D1 and GAPDH primers were used to amplify the target cDNAs prepared from RNA isolated from folate sufficient and folate deficient cells, treated with and without TNF-α (4 ng/mL) for 4 hrs, at days 7 and 11. The cyclin D1 transcript levels were normalized to GAPDH and fold-change comparing without TNF-α to with TNF-α determined.

No difference in NF-κB protein levels in folate deficient and sufficient cells

The cells were grown in NO and WITH folate medium for 7 and 11 days. On day 11 cells were also treated with TNF-α (stimulator of NF-κB). Western Blot was performed to determine the overall NF-κB protein level. Western Blot analysis (Fig. 5) revealed that there was no significant change in NF-κB protein levels on both day 7 and

day 11 for cells grown in folate deficient (NO) and folate sufficient (WITH) conditions. Also, the protein levels for cells treated with TNF-α (stimulator of NF-κB) showed no significant change in either folate sufficient or deficient cells.

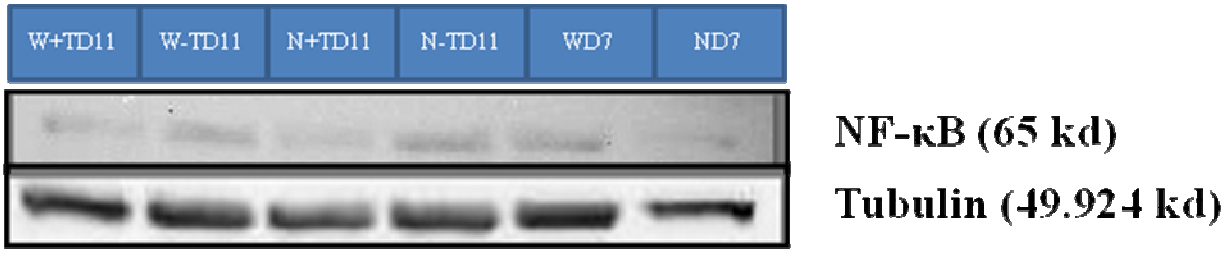


Fig. 5. Western blot of NF-κB and tubulin. Cells were grown for 7 and 11 days in folate sufficient (WITH) and folate deficient (NO) medium. For the day 11 cells, cells were treated with and without TNF-α (stimulator of NF-κB) at 1.25 ng/ml for 4 hours. Protein samples were prepared and 40 µl per lane analyzed by Western blot for NF-κB protein. W= WITH folate; N= NO folate; +T= with TNF-α; -T= No TNF-α; D= day.

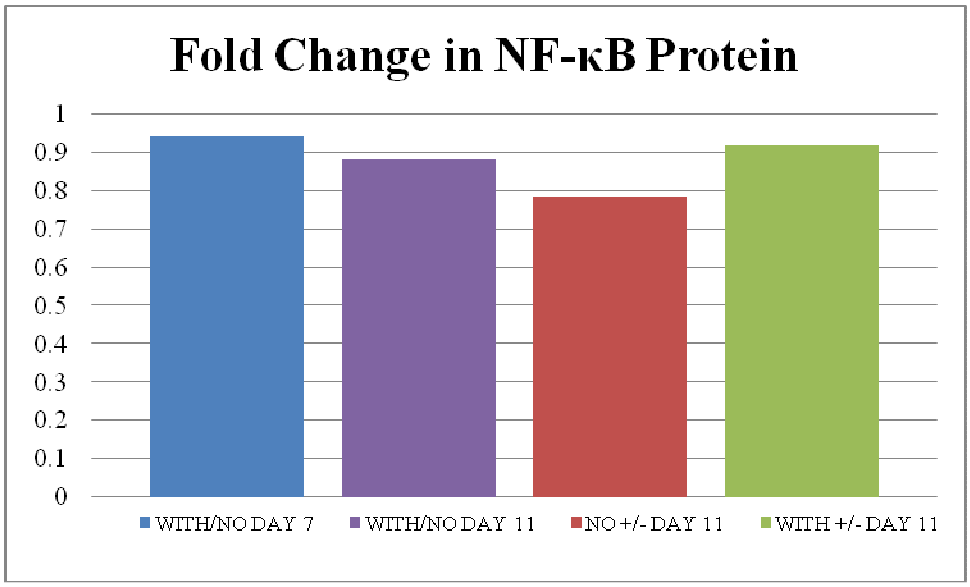


Fig. 6. Fold change in NF-κB protein level. The fold change was determined for Day 7 and 11 WITH and NO samples and +/- TNF-α of NO and WITH for Day 11.

No difference in NF-κB Localization in folate deficient and sufficient cells

We found that NF-κB activity increases in folate deficient cells but there is no significant change in NF-κB protein levels. One explanation for this result is that more NF-κB is localizing to the nucleus in the folate deficient cells. NF-κB cellular localization was determined by immunofluorescence. Cells were grown under folate deficient and sufficient conditions for 11 days, immunostained with NF-κB, and analyzed by confocal microscopy (Fig. 7). Nuclear signal per unit area was quantified.

Analysis of immunofluorescent images at day 11 showed that in both the folate sufficient and deficient cells, nuclear NF-κB signal is higher in the TNF-α treated cells. This is what would be expected. However, there was no significant difference between

the WITH folate and NO folate cells after TNF-α stimulation ($P<0.01$). However, there was a slight significant difference between the samples in the unstimulated cells; NF-κB was lower in the NO folate cells.

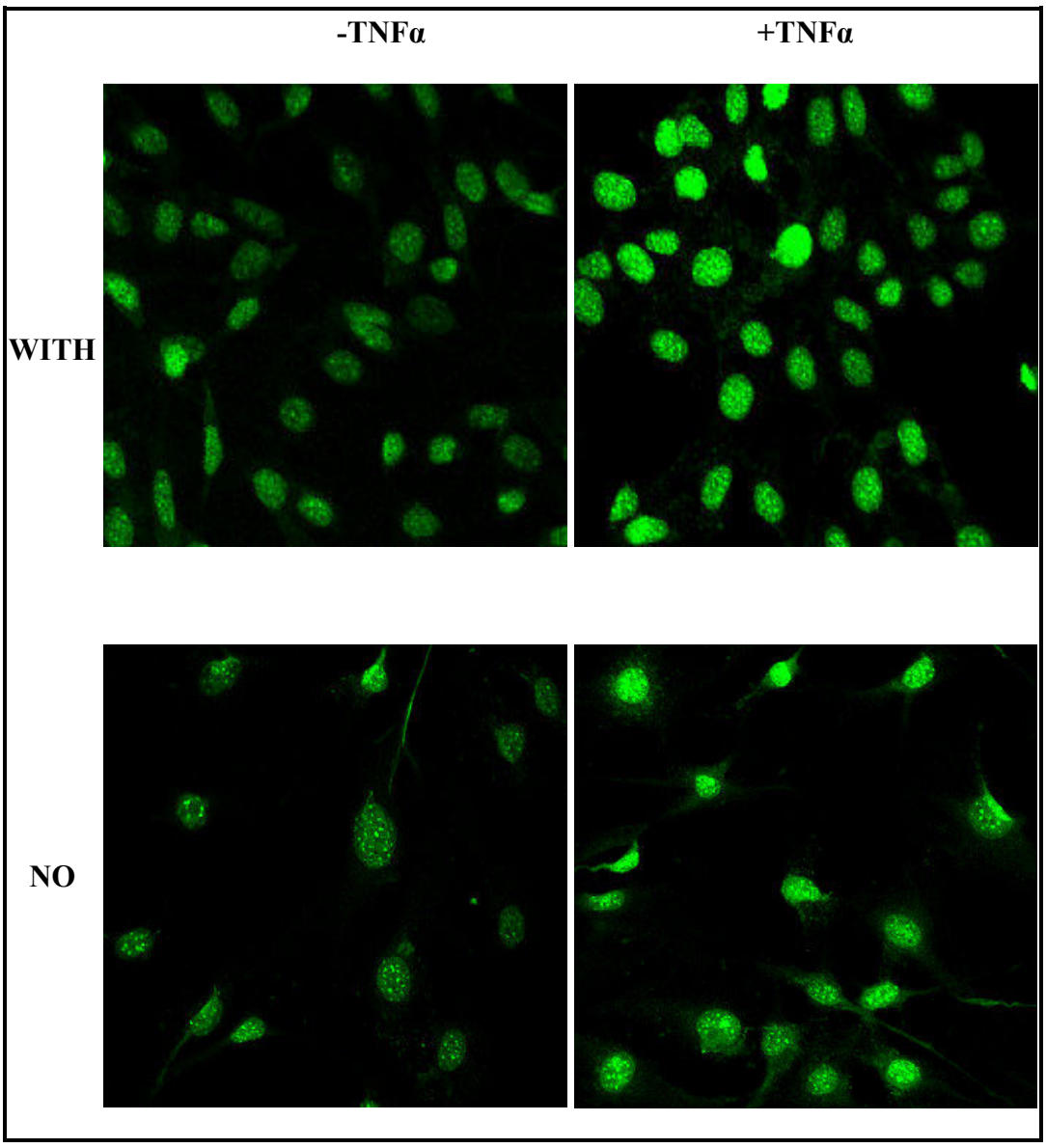


Fig. 7. Immunofluorescence staining for NF-κB. Immunofluorescent staining of cells grown for 11 days under folate sufficient (WITH) and deficient (NO) conditions and either unstimulated or stimulated with TNF-α (1.25 ng/ml) for 4 hours.

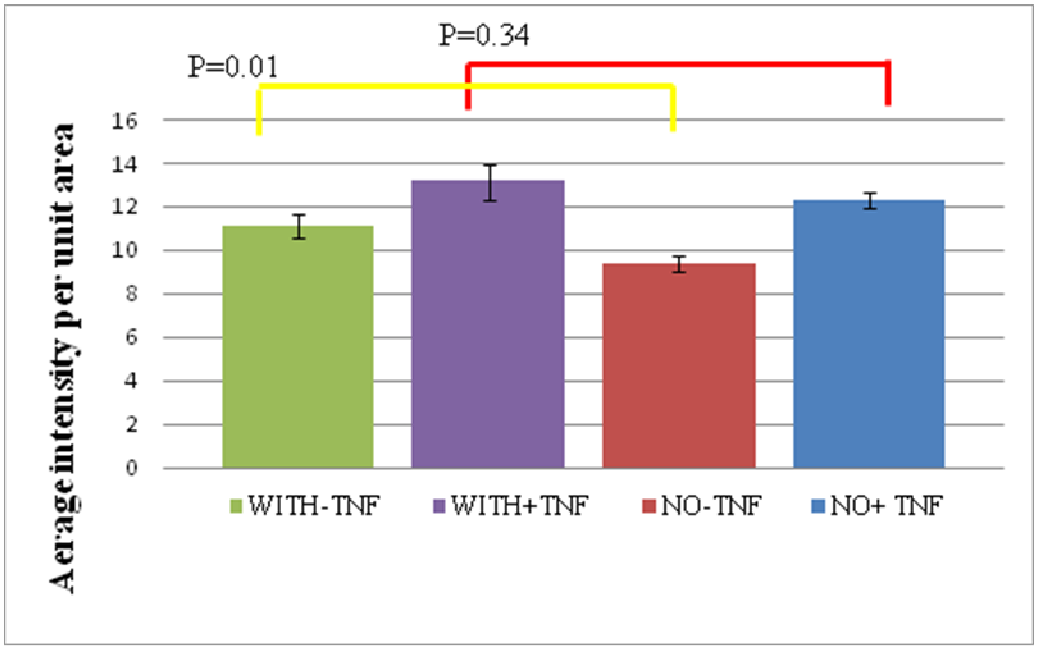


Fig. 8. Nuclear localization of NF-κB. NIH3T3 cells grown for 11 days under folate sufficient and folate deficient conditions. Nuclear localization of NF-κB reported as nuclear signal per unit area was determined from images as shown in Fig. 7. and treated with and without TNF-α (1.25 ng/ml for 4 hours). Error Bar represents standard error with P values (n=32). WITH= with folate; NO= no folate; TNF (-) and (+)= with and without TNF-α.

Folate deficient cells have higher levels of ROS

We found that there was an increase in NF-κB activity in folate deficient cells. It had been shown that under folate deficiency there is an increase in homocysteine levels and reactive oxygen species (ROS) levels (Ho et al, 2003). Another study by Zhang et al, 2006 showed that homocysteine leads to an increase in ROS resulting in an increase in NF-κB activity. Based on these findings one possible mechanism behind the increase in NF-κB activity is greater reactive oxygen species. To explore this possibility NIH3T3

cells were grown for 7 and 11 day under folate sufficient and deficient conditions without phenol red (see Materials and Methods). At each time point, cells were collected, treated with and without H_2O_2 and analyzed for ROS using the ROS sensitive compound C400.

Controls were cells (WITH and NO folate) treated with and without H_2O_2 but without addition of C400 compound. As shown (Fig. 9) the level of fluorescence is low for both WITH and NO folate cells. It is not clear why the DAY 7 controls were higher than the DAY 11, as there was no obvious peak in the 10^2 region for these cells.

At day 7, there was increase in ROS expressed as average fluorescence per cell in folate deficient NO cells treated with H_2O_2 with C400 when compared to folate sufficient cells (Fig. 9). There was an unexpected result in which increase in ROS was greater when cells were not pre-incubated with H_2O_2. The reason behind the result was not clear. However, the folate deficient NO cells had higher levels of ROS in comparison to the WITH cells (Fig. 9).

At day 11, there was an increase in ROS expressed as average fluorescence per cell in folate deficient cells treated with H_2O_2 with C400. The increase in ROS was more on day 11 as compared to day 7 (Fig. 9). The higher levels of ROS in the cells when treated without H_2O_2 with ROS at day 11 were also greater. Again, the explanation for the higher ROS in cells not preincubated with H_2O_2 is unclear.

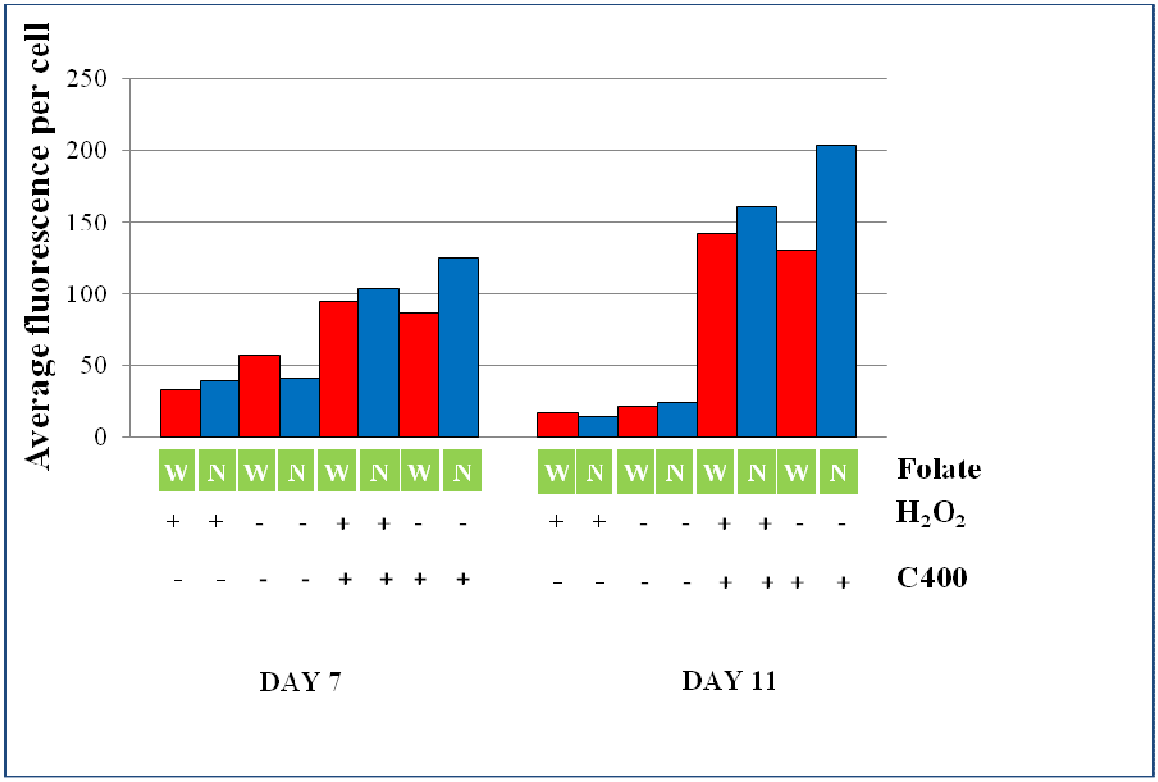
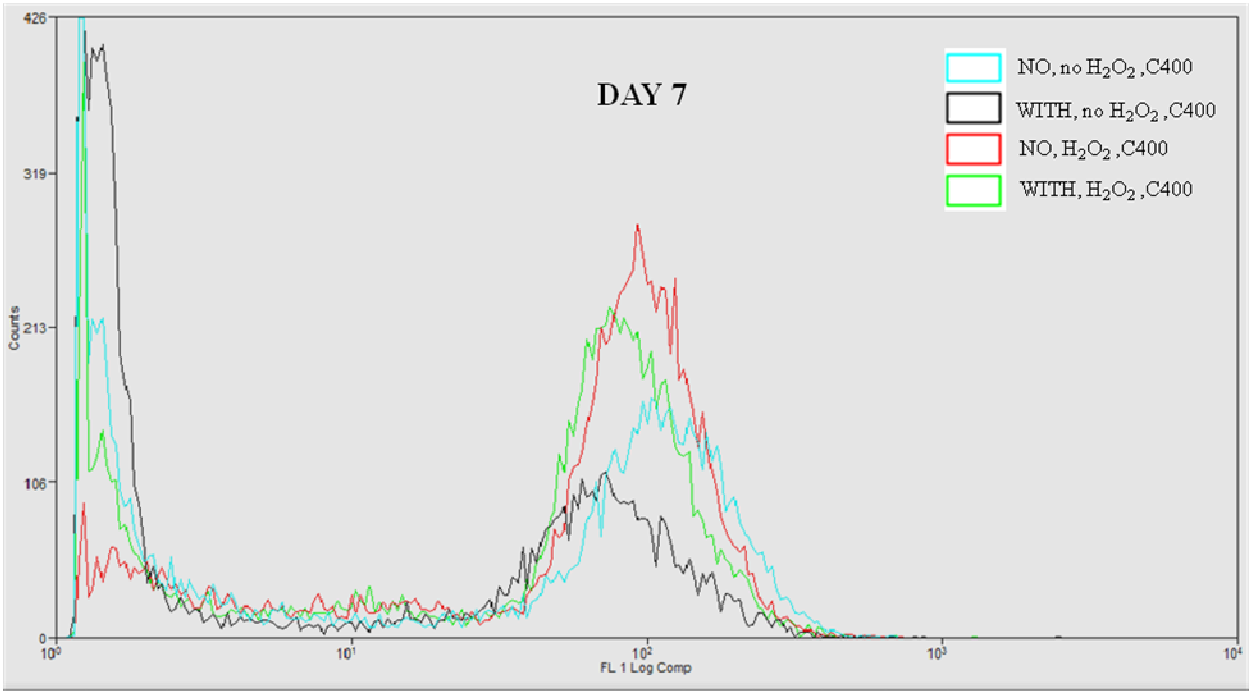


Fig. 9. ROS assay of NIH3T3 cells grown in folate deficient and sufficient medium for 11 days. Cells were grown in folate sufficient and deficient medium without phenol red as described in Materials and Methods. The cells were treated with and without H_2O_2 (100 µM) for 1 hour and then incubated with 10 µM of C400 compound. Fluorescence was measured in 10,000 cells by flow cytometry. W= With folate; N= no folate.

The overlay of histogram plots from flow cytometric analysis for days 7 and 11 support the above result. It was expected that the peak of the histogram graph would move to the right in case of increase in ROS. The peak for the cells grown under folate deficient condition treated with H_2O_2 and with C400 moved a little further to the right when compared to the cells grown under folate sufficient conditions (Fig. 10). The shift to the right was greater on day 11 (Fig. 10).

A)


DAY 7
NO, no H_2O_2, C400
WITH, no H_2O_2, C400
NO, H_2O_2, C400
WITH, H_2O_2, C400
Counts
426
319
213
106
0
10^0
10^1
10^2
10^3
10^4
FL 1 Log Comp

B)

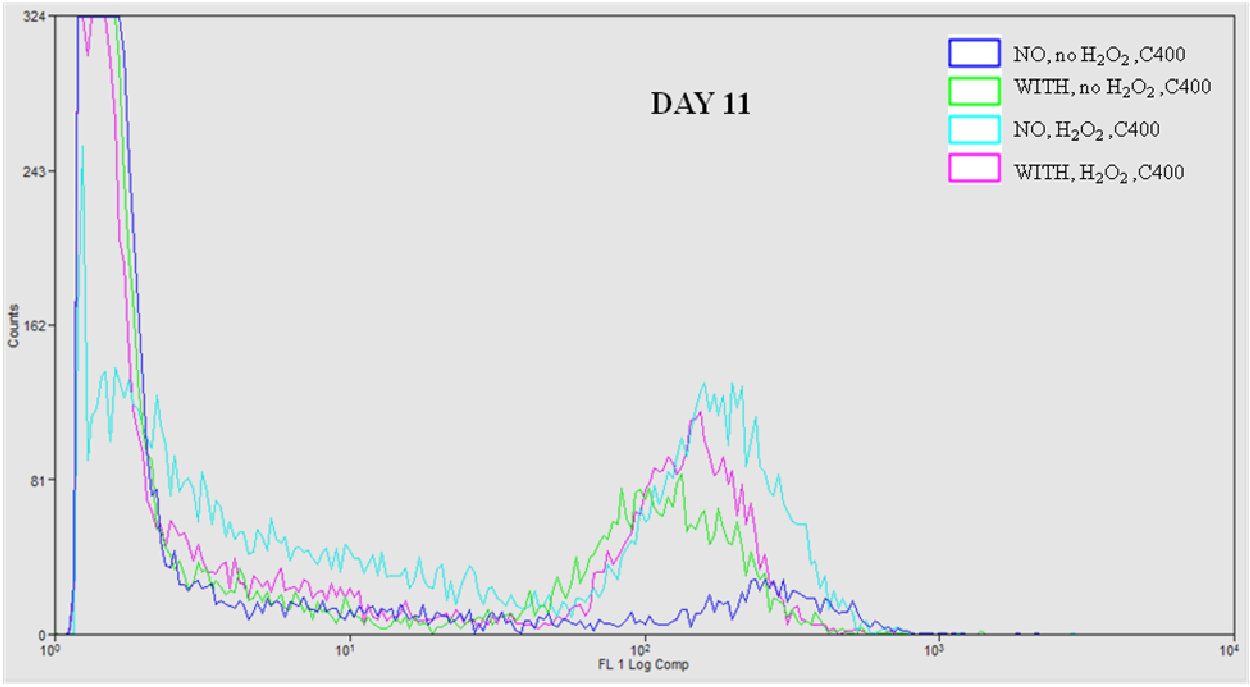


Fig. 10. Overlay of histogram plots of ROS assay for the NIH3T3 cells at day 7 (A) and day 11 (B).

CHAPTER V

DISCUSSION

Folate deficiency has been associated with increased risk of various cancers (Solomon et al, 1991), but particularly colorectal cancer (Kim, 2004). NF-κB misregulation has been reported to play an important role in promoting oncogenesis (Baldwin, 2001) and has been reported to be constitutively activated in colorectal carcinoma (Ahmed et al, 2006). There is evidence that folate deficiency results in hyperhomocysteinemia (Townsend et al, 2004). Also, accumulated homocysteine due to folate deficiency has been shown to stimulate NF-κB activity (Zhang et al, 2006). These findings suggest a relationship between folate deficiency and alterations in NF-κB activity. In this study, we provide further evidence that NF-κB activity increases in folate deficient cells and that this increase is associated with greater levels of ROS in folate deficient cells.

Increase in NF-κB activity in folate deficient cells

We found that NF-κB activity was higher in both TNF-α stimulated and unstimulated folate deficient cell at days 7 and 11 in comparison to folate sufficient cells. However, the fold change was not significantly different. This result indicates that TNF-α has a similar stimulatory effect on both folate deficient and sufficient cells but the

activated NF-κB level is higher in both TNF–α stimulated and unstimulated folate deficient cells. NF-κB activity can be increased by external factors such as TNF-α, which binds to cell surface receptors and activate the signaling pathway leading to inhibition of IKK binding to NF-κB and release of NF-κB. Our results suggest that folate deficient and sufficient cells have similar levels of TNF-α receptor, since the fold-change in activity is similar. NF-κB can also be activated by internal factors. Some groups have reported that hydrogen peroxide can activate IKK complex in human bronchial epithelial cells and HeLa cells (Jaspers et al, 2001; Kamata et al, 2002). In fact, treatment of HeLa cells with hydrogen peroxide resulted in phosphorylation of Ser 180 and Ser181 of IKKα and activation of the complex, which then activated NF-κB (Kamata et al, 2002). Chen and Green (2004) in their review also mentioned that several RelA phosphorylating kinases can be directly activated by ROS, which can result in enhanced NF-κB activity. Our results indicate that the increased NF-κB activity is likely due to an internal factor, based on the finding that unstimulated cells have a higher NF-κB activity. A possible experiment would be to determine if there is an increased level of ROS and if IKK is phosphorylated to a greater extent in folate deficient cells in comparison to folate sufficient cells.

Several published works can be correlated to hypothesize the role of folate deficiency and NF-κB activation. A group has shown that folate deficiency caused a drastic increase in Hcy production in Hep G2 cells (Chern et al, 2001). These high levels of Hcy could be suppressed by addition of exogenous folate. Other groups have proposed

that Hcy is a sulfhydryl compound which acts catalytically with transition metal ions to generate hydrogen peroxide (Starkebaum et al, 1986; Olszewski et al, 1993; Tapiero et al, 2001). Since NF-κB is a redox sensitive transcription factor and ROS works as a secondary messenger for NF-κB activation (Bowie and O'Neill, 2000), it is possible that folate deficiency can cause similar effects on NF-κB activation. Apart from that, as discussed earlier, in the presence of hydrogen peroxide, NF-κB can be activated through IKK phosphorylation (Kamata et al, 2002). These findings are consistent with our result.

Our preliminary data showing that folate deficient cells show a greater increase in cyclin D1 transcript levels when treated with TNF-α, supports our finding that NF-κB activity increases in folate deficient cells. However, it is not clear why the folate sufficient cells did not show a greater increase in cyclin D1 expression when stimulated with TNF-α. It will be necessary to repeat this analysis with a biological replica.

No change in levels of NF-κB protein or nuclear localization in folate deficient cells

Western blot analysis of NF-κB protein in folate sufficient and deficient cells both in the presence and absence of stimulator did not show any appreciable difference. This is consistent with the known pathway of NF-κB activation. Upregulation in protein activity and not protein level is the consequence of NF-kB activation.

Although NF-κB protein levels in folate sufficient and deficient cells were not expected to increase, a change in NF-κB phosphorylation would be expected as the p65 unit of NF-κB gets phosphorylated at serine 276 as a step towards activation (Zhong et al,

1998). An additional approach to confirm our finding that NF-κB is activated would be to detect the level of phosphorylated and unphosphorylated NF-κB using suitable antibodies in western blot experiments.

Immunofluorescence imaging was done to show the localization of activated NF-κB in the nucleus. In the TNF-α stimulated cells (folate sufficient and deficient cells) a higher NF-κB localization was detected than in unstimulated cells. This was expected based on the known model of NF-κB activation. In comparing nuclear signal per unit area of sufficient to deficient cells, we did not find any significant difference. This finding was not expected based on our data indicating greater NF-κB activity in folate deficiency. One possible explanation for this result could be that the increase in NF-κB activity is too small to be detected by immunofluorescence. In fact, the difference in NF-κB activity between folate sufficient and deficient cells is less than 2-fold. Also, the data was calculated per unit area, hence any difference in nuclear size would contribute to the per unit area value. In fact, we have observed that folate deficient cells tend to have larger nuclei, which could possibly decrease the per unit area value.

In order to confirm our findings, we would have to determine the size difference in the nuclei of the folate deficient and sufficient cells and take that value into account. Also, the total nuclear signal of NF-κB should be determined. Another approach would be to determine the total level of nuclear NF-κB protein by flow cytometry. This would be done by isolating nuclei and detecting the NF-κB by immunolabeling with a primary antibody and secondary fluorescent antibody, which can be detected by flow cytometry.

Increase in ROS in folate deficient cells

Using the oxidation sensitive C400 dye we detected the level of ROS in folate sufficient and deficient cells. Folate deficient and sufficient controls (without C400) show low levels of fluorescence. Folate deficient cells treated with H_2O_2 and with C400 showed that there was higher level of ROS than folate sufficient cells. This is what would be expected. Our finding is consistent with previous works. In one study, the authors show increased level of ROS in folate deprived cortical neurons and SH-SY-S5 neuroblastoma cells (Ho et al, 2003). Using a ROS sensitive fluorescent dye DFCD, the authors found that there was as much as 8 fold increase in ROS level in cortical neurons, when the cells were deprived of folate.

An unexpected result of this study was the detection of ROS in both folate deficient and sufficient cells that were not stimulated with H_2O_2. This finding suggests that the NIH 3T3 cells in culture are under oxidative stress. One possibility is that the experimental procedure, itself, generates ROS in the cells, since the cells are not fixed. Regardless, the higher level of ROS in the folate deficient cells suggests a mechanism for the higher NF-κB activity in the folate deficient cells.

In order to more clearly define a mechanism, we need to determine the homocysteine and hydrogen peroxide levels in folate sufficient and deficient cells. The experiment can be repeated in different cell lines to confirm the data. A rigorous proteomics assay or micro array assay and RT-PCR can be also done to find all the genes or proteins upregulated in the absence of folate. Correlating the mRNA or protein level

based on the literature will give more insight in the relationship between folate deficiency and NF-κB.

Possible mechanism for increase NF-κB activity in folate deficient cells

We were unable to determine homocysteine in the folate sufficient and deficient cells, therefore a role of homocysteine in altering NF-κB activity cannot be negated. Previous works have linked homocysteine to increased ROS which activates NF-κB (Zhang et al, 2006; Leclerc et al, 2008). We showed that there was an increase in ROS level in case of folate deficiency and we also found that NF-κB activity was higher in case of folate deficient cells.

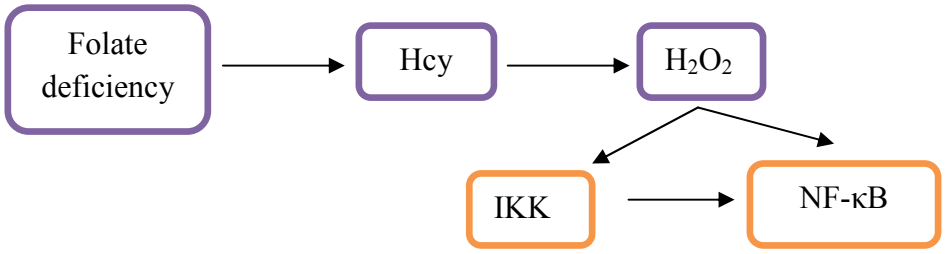


Fig. 11. Possible relationship between folate deficiency and NF-κB activation.

Based on these findings and published data, a possible model for how folate deficiency leads to NF-κB activation is presented (Fig. 11). To confirm this model it will be critical to determine levels of homocysteine and H_2O_2 in a number of different cells. Also, the level of phosphosphorylated IKK can be determined by Western blot. Further verification of our finding that NF-κB is activated to a greater level in folate deficiency

cells is required. This will include repeating the RT-PCR analysis for cyclin D1 and possibly other NF-κB target genes. In addition, as previously mentioned, the level of phosphorylated NF-κB can be determined.

In summary, we have shown that NF-κB activity increases both in TNF-α unstimulated and stimulated folate deficient cells and ROS levels increase in folate deficiency. These findings are important in that they suggest that folate deficiency is altering cell function and increasing the risk of cancer by affecting the activity of the NF-κB.

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APPENDIX A. DMEM CUSTOM MEDIUM

The following ingredients are combined in water to a volume of 4 liters. The pH of the medium is then adjusted to between 7.2 and 7.6 using 1N HCl. Medium is then filter sterilized using a 0.2 μm filter.

Inorganic Salts	**mg**
$CaCl_2$ $2H_2O$	1059.68
Fe $(NO_3)_3$ 9 H_2O	0.4
KCl	1600
$MgSO_4$ 7 H_2O (dried)	308.04
NaCl	25,600
NaH_2PO_4 H_2O	500
Amino Acids	
L-Arginine HCl	336
L-Cystine	192
L-Glutamine	2336
Glycine	120
L-Histidine HCl H_2O	168
L-Isoleucine	420
L-Leucine	420
L-Lysine HCl	584

L-Methionine	120
L-Phenylalanine	264
L-Serine	168
L-Threonine	380
L-Tryptophan	64
L-Tyrosine	288
L-Valine	376
Vitamins	
Choline Chloride	16
Folic Acid	8
Myo-Inositol	28.8
Nicotinamide	16
Pantothenate Calcium	16
Pyridoxal HCl	16
Riboflavin	1.6
Thiamine HCl	16
Other components	
D-Glucose	18000
Phenol Red	60
Sodium Pyruvate	440
Sodium Bicarbonate	6000