FOLATE DEFICIENCY PROVIDES PROTECTION AGAINST COLON CARCINOGENESIS IN DNA POLYMERASE β HAPLOINSUFFICIENT MICE
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Running head: Folate deficiency, β-pol and Colon Carcinogenesis

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Aging and DNA polymerase β deficiency (β-polH/W) interact to accelerate the development of malignant lymphomas and adenocarcinoma and increase tumor bearing load in mice. Folate deficiency (FD) has been shown to induce DNA damage repaired via the base excision repair (BER) pathway. We anticipated that FD and BER deficiency would interact to accelerate aberrant crypt foci (ACF) formation and tumor development in β-pol haploinsufficient animals. FD resulted in a significant increase in ACF formation in wildtype (WT) animals exposed to 1, 2-dimethylhydrazine (DMH), a known colon and liver carcinogen; however, FD reduced development of ACF in β-pol haploinsufficient mice. Prolonged feeding of the FD diet resulted in advanced ACF formation and liver tumors in wild type mice. However, FD attenuated onset and progression of ACF and prevented liver tumorigenesis in β-pol haploinsufficient mice, i.e., FD provided protection against tumorigenesis in a BER deficient environment in all tissues where DMH exerts its damage. Here we show a distinct downregulation in DNA repair pathways, e.g., BER, NER and MMR, and decline in cell proliferation, as well as an upregulation in PARP, proapoptotic genes and apoptosis in colons of FD β-pol haploinsufficient mice.

Folate deficiency is an important public health concern because of the role folate plays in the development of many different health problems, including neural tube defects, cardiovascular disease, Alzheimer’s disease and cancer, specifically colon cancer. It has been proposed that the carcinogenic properties of folate deficiency may be related to a decrease in DNA methylation, perhaps as a function of reduced S-adenosylmethionine (SAM) levels, an increase in the uracil content of DNA or an increase in oxidative stress by alterations in thiol switches. Folate deficiency has also been shown to increase (in cells, animal models and humans) levels of single strand breaks (1, 2, 3, 4, 5), micronucleus formation (6, 7), chromosomal aberration (8,9) and mutation frequency (10, 11), all potentially downstream effects of high levels of uracil in DNA and oxidative damage to DNA.

The DNA repair pathway for removal of uracil and oxidized bases is the base excision repair (BER) pathway. The BER pathway is believed to repair small, non-helix-distorting lesions in the DNA. It has been estimated to be responsible for the repair of as many as one million nucleotides per cell per day (12), stressing its importance in the maintenance of genomic stability. It has been suggested that BER has evolved in response to in vivo exposure of DNA to ROS and endogenous alkylation, and that this pathway suppresses spontaneous mutagenesis (13). In the initial elucidation of the BER pathway the following steps were involved: i) removal of the damaged base by a DNA glycosylase; ii) incision of the phosphate backbone by an endonuclease; iii) synthesis of new DNA by a polymerase; iv) excision of the deoxyribose phosphate (dRp) moiety; and v) ligation. This remains the predominant BER pathway and is
clearly the pathway for repair of uracil. DNA polymerase β (β-pol) performs the polymerization steps in the predominant short patch BER pathway and appears to perform the rate-limiting step, by virtue of its dRp lyase activity (14). In the process of uracil removal and repair of oxidized bases, a transient formation of a DNA single strand break occurs.

Our laboratory has shown that haploinsufficiency in β-pol would result in persistence of the DNA single stand breaks, where this persistence could result in DNA double strand breaks and chromosomal aberration (15). In addition, these animals exhibit an accumulation of spontaneously arising single strand breaks and chromosomal aberrations with age. Furthermore, in response to alkylating and oxidizing agents, an even greater accumulation of single strand breaks and chromosomal aberrations is observed in these mice (15). Moreover, β-pol haploinsufficient mice display an acceleration of normal, age-related tumors, e.g., lymphomas, developing alongside an increased susceptibility to epithelial tumors, e.g., adenocarcinomas, which do not typically occur at a high incidence in C57BL/6 mice (16).

Based on the striking similarities between DNA damage induced by folate deficiency and that induced by a reduction in BER capacity, we suggest a strong association between BER and folate. We have reported previously that folate deficiency overwhelms the capacity of BER through the inhibition of upregulation of β-pol (17). It is feasible that the inability to induce β-pol when folate is deficient results in a functional BER deficiency, providing a logical explanation for the phenotype induced by folate deficiency. While a tight correlation between DNA damage and cancer exists, it is necessary to evaluate preneoplasic lesions and tumors arising in response to the interaction between β-pol loss and folate deficiency. The purpose of this study is to determine whether β-pol haploinsufficiency accelerates the development and/or aggressiveness of these lesions in colon and liver in response to the carcinogen, 1,2-dimethylhydrazine (DMH). This study has important human health implications, as polymorphisms within the human population may render individuals haploinsufficient for β-pol and increase cancer risk by reducing their DNA damage tolerance.

**EXPERIMENTAL PROCEDURES**

*Animals*- Experiments were performed in young 4 to 6 month old C57BL/6-specific pathogen free male mice and mice heterozygous for the DNA polymerase β gene (β-pol<sup>+/-</sup>) (16). All practices performed on animals were in agreement with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Mice heterozygous for the DNA polymerase β gene (β-pol<sup>+/−</sup>) were created in Rajewsky's laboratory by deletion of the promoter and the first exon of the β-pol gene (18).

Homozygous deletion of β-pol results in embryonic lethality, but the heterozygous mice survive and seem to be normal and are fertile; there is no retardation in food intake, weight gain, or growth rate. All mice were backcrossed into C57BL/6 for at least 20 generations. The genotype of the mice was determined by Southern blot analysis as described by Cabelof et al. (15). The Wayne State University Animal Investigation Committee approved the animal protocol. Mice were maintained on a 12-h light/dark cycle and given water ad libitum.

*Diets and Carcinogenic Treatment*- After acclimation for 7 days, wildtype (WT) and β-pol<sup>−/−</sup> mice were randomly assigned to two dietary groups: a folate adequate (FA) or folate deficient (FD) AIN93G-purified isoenergetic diet (Dyets, Inc., Lehigh Valley, PA) as described previously (17). The FA group received a folate adequate diet containing 2 mg/kg folic acid. The FD group received a folate-deficient diet containing 0 mg/kg folic acid. Diets were stored at −20°C. 1% succinyl sulfathiazole was added to all diets. One week after commencement of food ingestion, randomly selected mice from both FA and FD were injected i.p. with 1,2-dimethylhydrazine HCL (DMH, 30 mg/kg body weight) in 10mmol/L of NaHCO<sub>3</sub> (Fisher Scientific, Fair Lawn, NJ) once a week for 6 wks (Figure2, panel A). Both food intake and body weights were checked twice weekly to monitor for signs of toxicity, e.g., weight loss, and the diets were continued for 12 weeks.
Aberrant colonic crypt (ACF) analysis-
Animals were anesthetized under CO₂ asphyxiation, the abdominal cavity was opened and the colon excised, rinsed with cold PBS, cut longitudinally, and fixed flat overnight in 10% neutral buffered formalin. The colonic crypts were stained with 2g/L of methylene blue in PBS for 5 min. The number of ACF and aberrant crypts per foci were determined by light microscopy at 10X magnification in a blinded manner.

Realtime PCR- Total RNAs were isolated from the colon mucosa of mice using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocol. cDNAs were synthesized from 1 µg RNA using random hexamer primers (Promega, Madison, WI) and purified with the QIAquick PCR Purification kit (Qiagen). The levels of cDNAs were quantified using a LightCycler real-time PCR machine (Stratagene, La Jolla, CA). PCR reactions contained 3 µL purified cDNA, 12.5 µL qPCR master mix, and 0.5 µmol/L each of sense and antisense primers (Roche) (Table 1, supplementary data 1). For all amplifications, PCR conditions consisted of an initial denaturing step of 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds, and 60°C for 30 seconds, with a melting curve analysis from 60°C to 95°C to confirm specificity. External standards were prepared by amplification of cDNAs for each gene. The amplicons were cloned into pGEM-T Easy vector, linearized with appropriate restriction enzyme, and used to prepare external standard curves. The level of each transcript was normalized to GAPDH. Results are expressed as mean values from five animals per experimental group.

Western blot analysis- Western blot analysis was performed using 200µg nuclear protein as previously described (19). Upon completion of SDS–PAGE, the region containing the protein(s) of interest was excised and prepared for Western blot analysis, whereas the remaining portion of the gel was stained with GelCode blue stain reagent (Pierce Biotechnology) to ensure equal protein loading. Western analysis was accomplished using affinity purified polyclonal antisera developed against mouse β-pol. As an internal control for protein loading, membranes were reprobed with anti-Lamin B antibody (Santa Cruz Biotechnology, Santa Cruz, CA) The bands were visualized and quantified using a ChemiImager™ System (AlphaInnotech, San Leandro, CA) after incubation in SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Data are expressed as the integrated density value (I.D.V.) of the band per µg of protein loaded.

Microarray assays- Total RNAs were isolated from the colon mucosa of mice using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocol. RNA samples were quantified with NanoDrop ND-1000 (NanoDrop Technologies, Inc, Wilmington, DE), and 260/280 ratio in the range of 2.0-2.2 was defined as acceptable. A quality check of the total RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One micro liter of total RNA sample was applied on RNA 6000 NanoChip, and the assay was run on the Bioanalyzer to determine if the 18S and 28S ribosomal bands were defined and to ensure no RNA degradation was present. Optimal concentration used to check the RNA quality is 250ng/µl.

Microarray expression profiling was conducted by Microarray & Bioinformatics Facility Core at Wayne State University (Institute of Environmental Health Sciences, Detroit, MI) according to the manufacturer’s protocol. A balanced block experimental design was used: 4 microarrays were completed for each comparison, with each microarray representing 1 randomly selected labeled mucosal sample from each experimental group paired with 1 randomly selected FAWT labeled mucosal sample, i.e., in total 4 mucosal RNA samples from FDWT cohybridized with 4 FAWT, 4 FDWT DMH treated cohybridized with 4 FAWT and 4 FD β-pol+/− DMH cohybridized with 4 FAWT treated, representing 4 microarrays for each experimental comparison. Samples on a given array were oppositely labeled with Alexa 647 and Alexa 555 dyes. The four microarrays for a given group (i.e., WT untreated, WT DMH or β-pol+/− DMH treated) represent samples from eight separate mice, providing consideration of biological variation. In total, 12 arrays representing 24 mice (8 WT, 8 WT DMH-treated, 8 β-pol+/− DMH-treated) were
completed. Dye swaps were used to account for dye bias effects such that of the four arrays in a given phenotype group, two had FD treated samples labeled with Alexa 647 co-hybridized with control samples labeled with Alexa 555 while the other two arrays within the same phenotype group had opposite dye orientations. Microarrays were scanned using the Agilent dual laser DNA microarray scanner model G2565AA, with 10 micron resolution.

Microarray gene profile analysis
Microarray image analysis was performed with Agilent Feature Extraction software, version A.5.1.1. Hierarchal Clustering analysis was performed using GeneSpringGX V10 (Agilent Technologies) software, and the parameters were set for centroid linkage which calculates the euclidean distance between the respective centroids of two clusters. False discovery rate for FDWT was 5.1%, 1.7% for FDWT DMH treated and 0.39% for FD β-pol⁺/⁻ DMH-treated experimental groups. Heatmaps were created by inputting accession numbers of differentially expressed genes, at p<0.001, for FDWT and FD β-pol⁺/⁻ DMH-treated experimental groups through DAVID functional annotation. Outputs revealed gene ontologies of differentially expressed genes, which were then compared to created differentially expressed gene lists of DAVID biological processes, such as apoptosis and DNA repair. Data sets were then combined for FDWT and FD β-pol⁺/⁻ DMH-treated to 1 large data set in order to create heatmaps for each biological function. Gene ontology analysis was performed using Gene Ontology Tree Machine (GOTM), (Bioinformatics,Vanderbilt University), applying differentially expressed genes as depicted in the heatmaps. We chose a single gene set analysis, where GOTM compares the distribution of single gene sets in each GO category to those in an existing reference gene list from the mouse genome, identifying GO categories with statistically significant enriched gene numbers as determined by the hypergeometric test (p<0.01) (20, 21). Real time quantitative RT-PCR was used to confirm the data obtained for selected genes in DNA repair pathways as described above.

Terminal deoxynucleotidyl transferase-mediated nick-end labeling of apoptotic cells in situ- Colon tissues were dissected, opened longitudinally, fixed in 10% neutral buffered formalin, embedded in paraffin wax then cut into 5 μm-thick sections. Sections were put on slides for the in situ terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) (CHEMICON International Inc., Temecula, CA) as per manufacturer’s protocol. Cells with positive staining (brown staining) were considered apoptotic cells and the number of apoptotic cells was determined as the percentage per crypt.

5-Bromo-2-deoxyuridine (BrdU)-staining of proliferating cells in situ- Two hours prior to CO₂ asphyxiation, mice were injected i.p. with a BrdU solution (10 mol/L, 1 mL per 100 g body weight) (5-Bromo-2'-deoxy-uridine Labeling and Detection Kit II, Roche Diagnostic, Mannheim, Germany) to immunostain proliferating cells, following manufacturers instructions for paraffin-embedded tissues. Briefly, after CO₂ asphyxiation, as mentioned above, colons were excised and fixed in formalin. The tissue was then embedded in paraffin and sections of 3 – 5 μm were cut longitudinally the full length of the colon. Tissues were incubated in 10 μmol anti-BrdU monoclonal antibody for 1 hr at 37°C followed by incubation with anti-mouse-Ig alkaline phosphatase. The labeling index was used to quantify cell proliferation, defined as the percentage of BrdU-positive cells per crypt.

Tumor Analysis- All animals were sacrificed at 40 weeks after the last dose of DMH, by asphyxiation with carbon dioxide, and all organs and tissues were examined for grossly visible lesions. Liver and colon tissues, including gross abnormalities, were fixed in 10% neutral buffered formalin, trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, stained with H&E, and examined microscopically. Preparation of slides for histopathology evaluation was performed by pathologists at the Department of Pathology, Wayne State University.

Statistical analysis- Statistical significance between means was determined using ANOVA, followed by the Fisher’s least significant difference test where appropriate (22). P-values less than 0.05 were considered statistically significant.
RESULTS

Induction of aberrant crypt foci (ACF) formation in DNA polymerase β haploinsufficient mice in response to dimethyl hydrazine (DMH)-
The purpose of this study was to determine the impact of BER deficiency on colon carcinogenesis in an animal model. In the first series of experiments, we determined the impact of β-pol heterozygosity on the development and progression of ACF in DNA polymerase β heterozygous knockout mice of C57BL/6 background, characterized previously by our laboratory (23). We have previously observed a deficiency in β-pol gene expression in various tissues of β-pol−/− mice (brain, liver, spleen and testes) with a concurrent decline in BER capacity (23). As shown in Figure 1A, in this study we have confirmed a similar decline in expression of β-pol gene in the colon mucosa of the β-pol−/− mice suggesting a parallel decline in BER activity in the colon of these mice.

It is well established that carcinogen-induced ACF are early indicators of initiation of colon cancer in animal models (24, 25, 26). In this study, we utilized 1,2-dimethylhydrazine (DMH), an established colon and liver carcinogen. It is proposed that DMH converts to the active metabolites azoxymethane and methyloxazymethanol in the liver, which are then transported to the colon via blood and bile (27). DMH in these tissues extends its damage to DNA by induction of alkylation damage (O6-meG and N7-meG) as well as oxidative damage. We have previously demonstrated that the β-pol−/− mouse is not sensitive to the O6-meG lesion (removed by direct reversal), but is sensitive to the N7-meG lesion processed by BER pathway (28). Additionally, we have observed increased accumulation of DNA single strand breaks in the β-pol−/− mouse in response to oxidative stress, as compared to its wildtype counterpart (15). Thus, we postulated that β-pol haploinsufficiency may predispose animals to increased colon carcinogenicity and induce development of ACF in response to DMH. At the outset of the current study, mice were injected with 30 mg/kg body weight DMH for 6 weeks to induce ACF. Six weeks after the final injection, the mice were sacrificed and the level of ACF in the colon was determined. As shown in Fig. 1B, wildtype and β-pol haploinsufficient untreated mice did not display any ACF in their colon. Thus, β-pol haploinsufficiency and subsequent deficiency in BER capacity is not enough to induce ACF in mice, suggesting that β-pol is a low-penetrance gene, requiring a high penetrance environmental insult, e.g., DMH or nutritional deficiency, for the damage to accumulate. Furthermore, as shown in Fig. 1B, β-pol−/− DMH-treated mice exhibited a significantly higher level of ACF formation (67% higher) as compared to wildtype counterparts (15.4±1.8 versus 29.7±1.4, for wildtype and β-pol−/− mice, respectively, p<0.01). These findings indicate that β-pol haploinsufficient mice are more sensitive to DMH, i.e., β-pol−/− animals show an inability to respond to oxidative stress and alkylation damage as compared to their wildtype littermates. Interestingly, no significant differences in ACF size and aberrant crypts per focus were observed in these animals. Having confirmed these findings, we were interested in the effect of folate deficiency in a BER deficient environment on the response to DMH.

Impact of folate deficiency and β-pol haploinsufficiency on ACF formation in colon-
Folate deficiency has been suggested to impact on SAM/SAH ratio (28), increase uracil incorporation into DNA (a substrate for BER pathway) and induce oxidative stress in animals. Subsequently, to elicit the role of folate deficiency on ACF formation in wildtype and β-pol−/− animals, animals were fed either a folate adequate (FA, 2 mg/kg folic acid) or folate deficient (FD, 0 mg/kg folic acid) diet as outlined in Figure 2A. To induce severe folate deficiency, 1% succinyl sulfathiazole was added to the diet deficient in folic acid to prevent synthesis of folic acid in the gut by bacteria. The animals’ food intake and body weight were monitored weekly. Folate deficiency for 8 weeks did not affect body weight while it reduced the plasma folate level by 90% as determined by a SimulTRAC-SNB radioassay kit for vitamin B12 (57Co) and folate (125I) per the manufacturer’s protocol (ICN Diagnostics, Orangeburg, NY) as described previously (17). In addition, under these conditions we observed an approximate 40% decrease in colon folate concentration when folate was deficient, a finding
treatment. After sacrifice, colons were macroscopically examined for the development of ACF and the number of aberrant crypts per focus (Figure 4A). As shown in Figure 4B, as has been reported elsewhere, there was a lack of further increase in aberrant crypt focus numbers, but rather a regression of ACF as well as a further development to larger ACF with increased aberrant crypts per focus in these mice. Although we observed no significant change in crypt multiplicity at 6 weeks post treatment for any group studied, the number of crypts per focus significantly increased in FD mice as compared to their FA counterparts after 40 weeks in wildtype mice (7.63±0.4 verses 6.33±0.4, for FD and FA mice, respectively, p<0.05). Moreover, ACF in FD mice displayed a well defined elevation above the surrounding mucosa compared to its FA counterpart which appeared flat (Fig. 4A, Panels IV). Thus based on the notion that the size of ACF and the number of aberrant crypts per focus are better indicators of tumor formation, folate deficiency does not only increase the number of ACF in response to DMH early on, but its adverse impact persists resulting in further development of aberrant foci and formation of microscopic adenoma, while this phenomenon could be hampered where β-pol is deficient. Accordingly, it was important to evaluate the experimental groups for tumor analysis. Determining tumor incidents in these mice is important as these data potentially shed light on the impact of BER deficiency on colon tumors in a folate deficient environment, and determine potential continuity between ACF formation and development and metastasis of tumors.

Analysis of pathology in β-pol haploinsufficiency and wildtype counterparts in response to folate deficiency and DMH treatment- Having established the DMH-induction of ACF in colon of β-pol+/− mice and their wildtype counterparts fed a FA and/or FD diet, we studied the impact of folate deficiency and DMH treatment on tumor formation in these animals, based on the known impact of DMH on colon and liver tissues. In this study, we followed animals for 40 weeks after the last DMH treatment, to determine the incidence of tumor formation as outlined in Figure 5A. Analysis of formalin-fixed and methylene blue stained colons provided evidence of advanced
ACF in the colon of wildtype FD (Figure 4, IV) mice treated with DMH as compared to FA mice (Figure 4, III). In other words, while a more defined elevation above the surrounding mucosa was observed in ACF of FD fed mice, the FA mice displayed less developed ACF. Upon dissection of animals, we perceived gross changes in the pathology of the liver in FDMH-treated mice (50% tumor formation) and FADMH-treated β-pol<sup>−/−</sup> (100% tumor formation). As shown in Figure 5B, panels II and III, the architecture of the liver tissue in all the FDMH and β-pol<sup>−/−</sup> fed a FA diet demonstrated an atypical morphology depicting progression of tumors. In contrast, there were no visible changes in the liver for wildtype mice consuming a FA diet and β-pol<sup>−/−</sup> animals fed a FD diet. Taken together, FDMH and β-pol<sup>−/−</sup> fed a FA diet with DMH treatment show more developed ACF and tumor formation than FAWT DMH-treated and FD β-pol<sup>−/−</sup> animals. This is suggestive, yet again, of haploinsufficiency conferring protection when exposed to oxidative/alkylation stress induced by DMH treatment in a folate deficient environment. Based on these data, it is inviting to suggest that β-pol haploinsufficiency in combination with folate deficiency might impact on cell cycle arrest and apoptosis in response to DMH, thus impacting on ACF development and consequent tumor formation. Accordingly, we wanted to determine the impact of β-pol haploinsufficiency and folate deficiency on gene expression in mucosal tissue of the colon.

**Effects of folate deficiency on gene expression in colon mucosal cells**- Having seen a difference in colon pathology, as aforementioned, we wanted to determine differential expression of genes in colon tissues of β-pol<sup>−/−</sup> mice subjected to DMH in a folate deficient environment. In order to ascertain possible mechanisms of reduced ACF and tumor formation, we decided to conduct a microarray analyses on FD β-pol<sup>−/−</sup> DMH-treated and FDMH-treated colon mucosa, relative to FAWT. As depicted in the scatter plots of differentially expressed genes, there was a marked difference in expression of many genes in DMH treated β-pol haploinsufficient mice subject to a folate deficient environment (4621 upregulated, 5757 downregulated in FD β-pol<sup>−/−</sup> DMH-treated versus 528 upregulated, 557 downregulated in FD WT mice) (Figure 6A). In other words, β-pol haploinsufficiency and DMH-treatment resulted in more extensive differential expression of genes as compared to wildtype untreated mice. To authenticate the outcome of microarray analysis, we performed quantitative RT-PCR using FullVelocity™ SYBR® Green QRT-PCR Reagents (Stratagene) on select genes depicted as differentially expressed through microarray analysis. The selected gene products studied included UNG, MPG, β-pol, XRCC1, ligase 3 and RAD51li. qRT-PCR results for the 6 genes selected were consistent with microarray data. All were significantly downregulated in the FD β-pol<sup>−/−</sup> DMH-treated mice (p<0.01) (Supplemental Table IV). Having confirmed the validity of microarray findings, we wanted to identify the inter-relationships that existed between the abundance of differentially expressed genes.

Based on our assessment of a possible role of DNA repair and apoptosis we used DAVID biological function to determine the changes in individual gene expression related to these two pathways. Once identified, we generated a series of hierarchal clustered heatmaps based on intensity of gene expression from raw data files (Figure 6B). Hierarchal clustering assisted in exploring the relationships that exist among the statistical data identified in the microarray analysis. Here we show that upon conducting a clustered analysis, all the data demonstrate a thrust toward a reduction in DNA repair and an upregulation in apoptotic related gene activity in the FD β-pol<sup>−/−</sup> DMH-treated mucosal tissue. In contrast, WT-untreated counterparts had a propensity toward either no difference in or slight upregulation in DNA repair and a downregulation in proapoptotic gene expression. Furthermore, FDMH treated groups showed either no difference or an upregulation in DNA repair capacity and an upregulation in apoptosis.

To further characterize the differences in gene expression, we input differentially expressed genes, from the raw data files, into Directed acyclic Graphs (DAG) view of Gene ontology to acquire clusters of statistically (p<0.01) enriched differentially expressed genes according to their gene ontology. In this analysis, all gene names
from raw data files were input into GOTM without reference to significance or intensity levels. Here, again, even with limited data input, we confirmed enrichment in DNA repair response (blue boxes) and apoptotic activity (red boxes) in FD β-pol<sup>+/−</sup> DMH-treated. As shown in Figure 7, the ontology groups enriched at program cell death and apoptosis, as well as in BER, NER and recombination repair, all converging on DNA damage response, signal transduction and induction of apoptosis.

Analyzing expression data with DAVID biological processes highlighted several genes related to apoptosis and DNA repair activity (Supplemental Table II, III, IV and V). Interestingly, we observed a distinct decline in overall DNA repair activity, including but not exclusive to BER (UNG, Apex, and β-pol), mismatch repair (MSH2 and MSH3), and nucleotide excision repair (ERCC2 and XPC). In contrast, there was an enhanced expression of several proapoptotic genes, including genes involved in intrinsic/extrinsic apoptotic pathways (CASP4 and CASP8), TNF signaling (Tnfsf12 and Tnfrsf26), as well as GAS1 and Trp63. All of these data suggest enhanced programmed cell death, either based on enhanced apoptotic activity, forgoing DNA repair, or lack of response of DNA repair pathways to cellular damage, increasing the sensitivity of apoptotic related pathways. Interestingly, the differential expression of genes in the WT FD DMH-treated colonic mucosa was not differentially expressed or upregulated for DNA repair, with the exception of β-pol and upregulated in apoptosis, apart from a downregulation in CASP3. In addition, FD WT colonic mucosa were either not differentially expressed or for the most part, slightly upregulated for DNA repair and showed reduction in apoptotic activity suggesting that in addition to folate deficiency, a compromised BER pathway is required to trigger apoptosis in DMH-treated mice. In view of these findings, we wanted to further confirm these results through a series of immunohistological experiments.

**Evaluation of apoptotic and proliferative activity in colonic mucosal cells** - Having determined the impact of β-pol haploinsufficiency on colon and liver tissue, we wanted to establish why BER insufficiency attenuates development of lesions in the face of deleterious surroundings of DMH-induced carcinogenesis and folate deficiency. We, therefore, conducted both apoptotic and proliferative assays because maintenance of mucosal integrity is reliant on the regulation of these two entities, i.e., development of ACF may arise if this integrity becomes compromised. To determine apoptotic activity in our experimental groups, we measured apoptosis in the colon using the TUNEL assay. Firstly, folate deficiency increased apoptotic bodies in wildtype animals (data not shown). Interestingly, folate deficiency induced a greater level of apoptotic activity in β-pol haploinsufficient mice as compared to wildtype counterparts (Figure 8A, IV). This confers with our previous data showing a significant increase in caspase 3 activity in liver of 2-nitropropane treated FD BER deficient mice as compared to FA counterparts, as measured by the Enztech CASP3 Assay (19). Herein, we show a significant increase (20%) in CASP3 activity in FD β-pol<sup>+/−</sup> DMH-treated animals relative to FAWT DMH-treatment (data not shown). These findings are indicative of increased apoptotic activity when dietary folate is deficient and BER is compromised. In addition, we ascertained a 35% increase in SSBs in FD β-pol<sup>+/−</sup> with DMH treatment using SSB assay in comparison to FAWT DMH-treated mice using a Fast Micromethod DNA Single Strand Break assay (data not shown). Next, to characterize the effect of β-pol heterozygosity and folate deficiency on cell proliferation in response to DMH treatment, we examined the proliferative activity of colon tissues by examining BrdU incorporation. As shown in Figure 8D, I, FA β-pol<sup>+/−</sup> mice showed significantly more proliferation (BrdU incorporation) as compared to FD β-pol<sup>+/−</sup> mice. Thus, the TUNEL and BrdU assays confirmed the differential expression observed in microarray analysis, suggesting that folate deficiency provide protection against tumorigenesis in DMH-treated β-pol<sup>+/−</sup> mice by altering the balance between DNA repair and apoptotic pathway favoring apoptosis.

**DISCUSSION**

In this study we examined the impact of BER deficiency on colon carcinogenesis in a
mouse model. BER processes endogenous damage; as such, our interest has been in developing model systems to test the processing of endogenous damage and determine the impact of BER deficiency on tumorigenesis in vivo, specifically in the colon. Firstly we display a significant decline in $\beta$-pol expression in the colon of the knockout mice as compared to their wildtype littermates, suggesting a BER deficiency in the colon of these mice. In addition, the $\beta$-pol$^{-/-}$ mice treated with DMH show a significant increase in ACF formation relative to wildtype, substantiating the need for an intact BER process. We confirm that the $\beta$-pol heterozygous mice are predisposed to preneoplastic lesions and cancer development, primarily by reducing tolerance to the DNA damage in response to the DNA damaging agent, DMH. As such, these manipulations gave rise to an animal model of colon carcinogenesis for future study.

While the mechanism by which folate deficiency increases cancer risk is not clear, folate deficiency has been shown to induce damage repaired via the BER pathway (17). Branda et al. (10) have demonstrated that folate deficient animals are less able to repair DNA damage induced by alkylating agents. For example, ENU was found to be more mutagenic within the context of folate deficiency. Interestingly, folate deficiency did not enhance the mutagenic effect of cyclophosphamide, suggesting a lack of sensitivity to lesions repaired by the nucleotide excision repair pathway. Duthie et al. (4) have further shown that human colon epithelial cells grown in the absence of folate are poorly able to repair damages induced by MMS and hydrogen peroxide. Growth in a low folate medium has also been shown to impair excision repair capacity of colonocytes (32). More oxidative damage, which is repaired by BER, accumulates in response to amyloid $\beta$-peptide in neuronal cells depleted of folate (33). Based on our previous findings and these aforementioned studies, we were interested in determining what impact folate deficiency would have on a BER deficient model. In this study, we confirm that a folate deficient diet heightens reduced DNA damage response when mice are subject to a carcinogenic environment resultant in an increase in the number of ACF. We also show an increased incidence of microscopic adenoma and liver tumor formation in wildtype mice upon DMH treatment when folate is deficient. These data indicate, in conjunction with our previous studies (17), that the pathway responsible for repairing these damages may be ineffective when folate is limiting. Based on the above findings, it is inviting to suggest that folate deficiency mimics BER deficiency perhaps by overwhelming the capacity of BER through inhibition of its rate determining enzyme, DNA polymerase $\beta$.

Herein, folate deficiency in $\beta$-pol haploinsufficient mice demonstrated reduced development of ACF. In addition, prolonged feeding of the folate deficient diet resulted in advanced ACF formation, microscopic adenoma and liver tumors in wildtype mice, while $\beta$-pol haploinsufficiency attenuated onset and progression of ACF to microscopic adenoma and prevented liver tumorgenesis. These findings are interesting because folate deficiency appears to provide protection against tumorigenesis in a BER deficient environment in all tissues where DMH exerts its damage. Microarray data show (classification by gene ontology) a distinct downregulation in DNA repair capacity including, but not restricted to, BER, NER and MMR, in FD $\beta$-pol$^{+/+}$ with DMH treatment.

Observing the lack of response of FD $\beta$-pol$^{+/+}$ in DNA repair, we studied the expression of apoptotic genes on our microarrays to determine if cell death may be responsible for eliminating cells that had extensive damage thus reducing the level of ACF and tumor formation. Notably, there was an upregulation of 75 differentially expressed genes related to apoptotic activity. There was an upregulation in intrinsic/extrinsic apoptotic, and TNF signaling, as well as cell cycle arrest in $\beta$-pol haploinsufficient mice. Based on the results obtained through microarray and TUNEL analysis, we would suggest that colon cells in DMH treated $\beta$-pol haploinsufficient mice in a folate deficient environment would prefer to undergo cell death, rather than attempt repair. Interestingly, a distinct upregulation in PARP expression in FD DMH treated $\beta$-pol haploinsufficient colonic mucosa is observed. In addition to mediating BER by recruiting BER intermediates like $\beta$-pol, PARPs are the key regulators of cell survival and cell
death (34). Huang et al. (35) in their study utilizing Bax\(^{-}\)Bak\(^{-}\) mouse embryonic fibroblasts, show depletion in ATP secondary to PARP-1 activation, which in turn inhibits FRAP1, a mediator of cellular response to DNA damage. We suggest that down regulation in expression of key enzymes in BER pathway renders available PARPs futile and are thus cleaved by CASP3 triggering apoptosis. Interestingly, while FDWT DMH treated animals show a significant decline in the level of CASP3, a key “executioner” of apoptosis (36), it is upregulated in β-pol haploinsufficiency. These findings provide further evidence of defense by BER deficiency in the multi-step process of colon/liver cancer, when folate is deficient.

Our proposal that β-pol deficiency in a folate deficient environment results in increased accumulation of DNA repair intermediates opting for cell death versus survival, reducing the onset and progression of preneoplastic lesions, is in agreement with findings by others. Ochs et al (37) demonstrated that BER deficiency results in the accumulation of repair intermediates, along with increased DSB in β-pol null fibroblasts exposed to the alkylating agent, MMS. Accumulation of DNA damage in these cells resulted in decline in Bcl-2 and increased apoptotic activity. Taverna et al. (38) have also shown that BER inhibition by methoxyamine increases cytotoxicity of the methylating agent temozolomide through its binding to abasic sites, hampering BER, resulting in accumulation of repair intermediates and ultimately, DNA DSBs. These conditions generate a perfect environment for apoptosis to take place. We find the results of these studies intriguing, for the aforementioned were conducted within cell lines, whereas our in vivo data demonstrate protection against actual onset and progression of tumors, via similar mechanisms.

In line with our findings, Lawrance et al. (42) showed that folate deficiency in mthfr deficient Apc\(^{min/+}\) mice results in an elevation in dUTP/dTTP ratio in DNA, increasing apoptotic activity with a reduction in adenoma formation in colon tissue. Interestingly, APC has been shown to interact with β-pol (43). Furthermore, haploinsufficiency in reduced-folate carrier gene (Rfc\(^{-}\)\(^{-}\)) which creates a folate deficiency phenotype resulted in decreased adenomas and tumor load in Apc\(^{min/+}\) mice. In addition, Branda et al (44) showed folate deficiency reduced mutation frequency in 3-methyladenine glycosylase null mice exposed to methyl methanesulfonate. The aforementioned demonstrate much the same as our investigation in that modulating BER repair, through β-pol and exposure to folate deficiency, results in reduced repair activity while influencing apoptosis. Additional research conducted by Duthie et al. (45) demonstrated that NCM460 immortalized cells in a folate deficient media exposed to hydrogen peroxide exhibit increased apoptotic activity, as well as decreased downstream of glycosylase resulted in an accumulation of DNA repair intermediates, and increased DNA DSBs, triggering apoptosis. Furthermore, Trivedi et al. (41) showed that elevated MPG expression in addition to β-pol knockdown would result in increased sensitivity of human breast cancer cells to temozolomide. Additionally, they also (41) showed that overexpression of β-pol with mutation in its polymerase site, while maintaining its dRP lyase activity, resulted in restoration of resistance to temozolomide in β-pol knockdown human breast cancer cell lines. The in vitro studies outlined above utilize specific glycosylases for the creation and persistence of abasic sites, while methoxyamine potentiates the cytotoxicity of temozolomide through its binding to abasic sites, hampering BER, in addition to inhibition of the BER inhibitor methoxyamine. These mechanisms provide further evidence of defense by BER deficiency in the multi-step process of colon/liver cancer, when folate is deficient.
proliferation. Furthermore, Crott et al. (46) showed an inverse correlation between expression of genes involved in cell cycle checkpoint and media folate levels (25 to 100nM) in NCM460, HCEC and NCM356 cell lines derived from human colon, irrespective of oxidative stress. These studies, much like our own, have significantly reduced levels of folate, rather than a complete folate depletion. In our experiments, despite use of a folate deficient diet and use of succinyl sulfathiazole, an 80 – 90% reduction in serum folate levels is observed relative to folate adequate diets. It is at this reduced level that we see protective effects. This study has great therapeutic importance and human relevance as recent findings indicate BER inhibitors in combination with the DNA methylating agent temozolomide, function as an effective chemopreventive agent in colon cancer (47). In addition, these findings have relevant translational implications, since variants/polymorphisms in BER have been associated with increased cancer risk, and an alteration in micronutrients could potentially provide protection under the right conditions.

REFERENCES

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The on-line version of this article (available at http://www.jbc.org) contains supplemental spreadsheets of differentially expressed genes at P<0.05. The excel sheet of the raw data are also available at:

http://sun.science.wayne.edu/~aheydari/microarray data

The abbreviations used are: β-pol, DNA polymerase β; BER, base excision repair; FD, folate deficiency; FA, folate adequate; WT, wildtype; DMH, 1,2-dimethylhydrazine; ACF, aberrant crypt foci.

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**FIGURE LEGENDS**

**Fig 1.** Impact of β-pol heterozygosity on expression of β-pol in colon mucosa and ACF formation in colon of mice treated with DMH. (A) Analysis of β-pol expression, mRNA and Protein levels, in mucosa from wildtype and β-pol haploinsufficient mice. Expression of β-pol gene was determined using a real time PCR technique and the level was normalized base on GAPDH expression. Protein level was quantified using a western blot analysis and normalized based on the Lamin b protein level. (B) Wildtype (WT) mice and β-pol haploinsufficient mice received either no treatment (controls) or i.p. treatment with DMH for 6 weeks at 30 mg/kg body weight (DMH treated). Colons were processed after CO2 asphyxiation of mice as described in materials and methods. Colons were analyzed under light microscopy to visualize the number of ACF per mouse colon (ACF/mouse). Bars, SE, *, P<0.01.

**Fig 2.** Panel A: Experimental Design: WT and β-pol+/− mice were fed either a folate adequate (2 mg/kg, FA) or a folate deficient (0 mg/kg, FD) diet for 12 weeks. After one week of ingestion of respective diets, mice were injected with 30 mg/kg body weight DMH for 6 weeks. Six weeks after final injection, animals were sacrificed by CO2 asphyxiation. Panel B and C: ACF formation and crypt multiplicity in colon of β-pol+/− mice consuming a folate deficient diet. WT and β-pol+/− received either a FA diet or FD diet and subjected to either no treatment (control) or i.p. treatment with DMH for 6 weeks at 30 mg/kg body weight (DMH treated). After sacrifice colons were processed as per materials and methods. Colons were analyzed under light microscopy to visualize the number of ACF per mouse colon (ACF/mouse) (B) and the number of crypts per focus (C). Bars with different letters indicate significant differences at P < 0.05.

**Fig 3.** Impact of folate deficiency and DMH treatment on expression of β-pol in colon mucosa of β-pol+/− mice. (A) Analysis of β-pol mRNA levels in mucosa of wildtype and β-pol haploinsufficient mice treated with DMH. Expression of β-pol gene was determined using a real
time PCR technique and the level was normalized base on GAPDH expression as described in materials and methods. (B) Analysis of β-pol protein levels in mucosa of wildtype and β-pol haploinsufficient mice treated with DMH. Protein level was quantified using a western blot analysis. Bars with different letters indicate significant differences at p < 0.01.

Fig 4. Impact of long term feeding on ACF formation and Crypts Multiplicity. Comparison of ACF formation and crypt multiplicity in mice fed either a FA or FD diet at 6 weeks versus 40 weeks post DMH treatment. Panel A: (I) Normal colonic crypts, (II) ACF formation in FD environment 6 weeks post DMH, (III) ACF formation in mice fed FA diet 40 weeks post DMH, and (IV) number of ACF of mice fed a FD diet 40 weeks post DMH treatment. Arrows depict area of aberrant crypt formation. Panel B: Relative number of ACF/mouse in mice fed either a FA or FD diet 6 weeks or 40 weeks post DMH treatment, respectively. Panel C: Number of aberrant crypts per focus in mice fed either a FA or FD diet 6 weeks or 40 weeks post DMH treatment, respectively. Bars with different letters indicate significant differences at P < 0.05.

Fig 5. Impact of β-pol+/− and folate deficiency on induction of tumors in DMH-treated mice. A. Feeding study was conducted as depicted in Figure 2. The mice were sacrificed 40 weeks after last treatment by CO2 asphyxiation and incidence of tumor progression was assessed. B. Exemplary H&E micrographs showing tumor formation in liver sections from (I) WT FA, (II) WT FD, (III) FA β-pol+/− and (IV) FD β-pol+/− mice treated with DMH. The % value represents the percent of mice with visible liver tumor formation. All wildtype animals fed the FD diet showed liver tumors through H&E analysis.

Fig 6. Scatter plot of differentially expressed genes and heat map representation of microarray data for proapoptotic and DNA repair differentially expressed genes. A. Scatter plot of differentially expressed genes from colon mucosa from FD β-pol+/− relative to FA β-pol+/− tissues and WT FD relative to WT FA tissues included in the Agilent Whole Mouse Genome oligonucleotide micorarray containing probes for over 41,000 well characterized genes. B. Hierarchal Clustering analysis was performed using GeneSpringGX V10 (Agilent Technologies) software, and the parameters were set for centroid linkage which calculates the euclidean distance between the respective centroids of two clusters. A heat map in quadruple (A, B, C, and D) for each condition representing proapoptotic and DNA repair genes are shown.

Fig 7. DAG view of gene ontology analysis, of colonic mucosal tissue in FD β-pol+/− versus FA WT. Blue boxes indicate enrichment of expression of genes related to DNA repair. Red boxes indicate enrichment of expression of genes related to apoptosis.

Fig 8. Impact of β-pol+/− and folate deficiency on apoptotic activity and induction of proliferation in colonocytes. A. Representative photomicrographs showing TUNEL-positive staining in cells (brown) of colonic mucosa. (I) WT FA, (II) WT FD, (III) FA β-pol+/− and (IV) FD β-pol+/− mice treated with DMH. B. Tally of the TUNEL-positive apoptotic cells. WT and β-pol+/− were fed either a FA or FD diet and subjected to DMH treatment. Colon tissue was processed, TUNEL assay conducted and percent apoptotic cells were calculated as described in methods. Means without a common letter differ, P < 0.01. C. Representative photomicrographs showing H&E of colonic mucosa. (I) FA β-pol+/− and (II) FD β-pol+/− mice treated with DMH. Representative micrographs showing BrdU staining (brown) of colonic mucosa. (III) FA β-pol+/− and (IV) FD β-pol+/− mice treated with DMH. Proliferation Analysis: β-pol haploinsufficient mice were treated as described in materials and methods. Briefly, 2 hours prior to sacrifice mice were injected i.p. with BrdU (2.16mg/kg body weight). Colon segments were fixed in 10% formalin and embedded in paraffin. BrdU incorporation was detected by immunostaining as described in materials and methods.
methods. D. Enumeration of positive proliferation. Percent cell proliferation was conducted as detailed in materials and methods. * indicate differences at P<0.01.
Figure 1

A

WT β-pol+/--

0 10 20 30 40

Level of β-pol Protein (I.D.V per μg protein)

WT β-pol+/--

0 1

β-pol mRNA Levels (normalized / GAPDH)

WT β-pol+/--

* WT β-pol+/--

B

ACF per Mouse

0 10 20 30 40

WT β-pol+/--

Control DMH Treated

*
Figure 2

A

Time (Wk)

DMH Treatment
(30 mg/kg)

0 1 6 12

Genotype (no.)

WT (n=6)  
WT (n=6)  
β-pol+/- (n=6)  
β-pol+/- (n=6)

Sacrifice for analysis of ACF

Folate (2 mg/kg)

Folate (0 mg/kg)

Folate (2 mg/kg)

Folate (0 mg/kg)

B

ACF/mouse

WT  WT  WT  WT
β-pol+/-  β-pol+/-  β-pol+/-  β-pol+/-
FA  FA  FA  FA
FD  FD  FD  FD

Control  DMH Treated

C

Aberrant crypts/focus

WT  WT  WT  WT
β-pol+/-  β-pol+/-  β-pol+/-  β-pol+/-
FA  FA  FA  FA
FD  FD  FD  FD

Control  DMH Treated
Figure 3

A

β-pol mRNA Levels

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<th></th>
<th>WT</th>
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<td>FA</td>
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<td>b</td>
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<td>b</td>
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<tr>
<td>DMH Treated</td>
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<tr>
<td>FD</td>
<td>a</td>
<td>b</td>
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B

Level of β-pol Protein (I.D.V per μg protein)

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<tr>
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<td>FD (DMH)</td>
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Figure 4

A

II

III

FA FD

6 weeks post treatment

40 weeks post treatment

Aberrant crypts/focus

B

ACF/Mouse

6 weeks post treatment

40 weeks post treatment

C

Aberrant crypts/focus

6 weeks post treatment

40 weeks post treatment
Figure 5

A

DMH Treatment (30 mg/kg)

Sacrifice for analysis of ACF and cancer

Time (Wk) 0 1 6 46

Groups

WT

WT

β-pol+/

β-pol−/

Folate (2 mg/kg)

Folate (0 mg/kg)

Folate (2 mg/kg)

Folate (0 mg/kg)

B

I II

III IV

FA WT DMH, 0% visible tumor

FD WT DMH, 50% visible tumor

FA β-pol− DMH, 100% visible tumor

FD β-pol− DMH, 0% visible tumor
Figure 8

A

FA WT DMH

FD WT DMH

FA β-pol⁻/⁻ DMH

FD β-pol⁻/⁻ DMH

B

Apoptotic body per crypt

WT  β-pol⁻/⁻  DMH Treated

FA  FD

D

Percent Cell Proliferation

FA β-pol⁻/⁻  FD β-pol⁻/⁻  DMH Treated

*
Folate deficiency provides protection against colon carcinogenesis in DNA polymerase β haploinsufficient mice

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