Effect of maternal and postweaning folic acid supplementation on mammary tumor risk in the offspring

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Precis: Findings in a rodent model raise concerns that folic acid supplementation widely employed in pregnant women may significantly increase the risk of mammary tumors in the offspring.
Abstract

Intrauterine and early life exposure to folic acid has significantly increased in North America owing to folic acid fortification, widespread supplemental use and periconceptional supplementation. We investigated the effects of maternal and postweaning folic acid supplementation on mammary tumor risk in the offspring. Female rats were placed on a control or folic acid supplemented diet prior to mating and during pregnancy and lactation. At weaning, female pups from each maternal diet group were randomized to the control or supplemented diet and mammary tumors were induced with DMBA at puberty. At necropsy, mammary tumor parameters, genomic DNA methylation, and DNA methyltransferase activity were determined in the offspring. Both maternal and postweaning folic acid supplementation significantly increased the risk of mammary adenocarcinomas in the offspring (OR=2.1, 95% CI 1.2-3.8, p=0.008 and OR=1.9, 95% CI 1.1-3.3, p=0.03, respectively). Maternal folic acid supplementation also significantly accelerated the rate of mammary adenocarcinoma appearance (p=0.002) and increased the multiplicity of mammary adenocarcinomas (p=0.008) in the offspring. Maternal, but not postweaning, folic acid supplementation significantly reduced global DNA methylation (p=0.03), whereas postweaning, but not maternal, folic acid supplementation significantly decreased DNA methyltransferase activity (p=0.05) in non-neoplastic mammary glands of the offspring. Our findings suggest that a high intrauterine and postweaning dietary exposure to folic acid may increase the risk of mammary tumors in the offspring. Further, they suggest that this tumor-promoting effect may be mediated in part by altered DNA methylation and DNMT activity.
Introduction

Dietary intake and blood levels of folate appear to be inversely related to the risk of several malignancies in humans (1). For breast cancer, however, epidemiologic evidence has not been consistent (2, 3). However, folate status seems to interact with alcohol, a well-established risk factor for breast cancer and a folate antagonist, in modifying breast cancer risk; low folate intake increases, whereas high intake decreases, breast cancer risk among women who regularly consume moderate or high amounts of alcohol, but not among women with low or no alcohol consumption (2). Alarmingly, recent epidemiologic studies have suggested that high folate intake, largely from folic acid (the synthetic form of folate that is used in supplements and fortified foods), and high plasma folate concentrations may increase breast cancer risk (4-6). The potential tumor promoting effect of high folate status has been further suggested in other organs. The Aspirin/Folate Polyp Prevention Study reported that folic acid supplementation at 1 mg/day for 6-10 years significantly increased the risk of recurrent colorectal adenomas (7) and of prostate cancer (8) in subjects with the predisposed colon and prostate that likely harbored preneoplastic foci. Combined analyses of two Norwegian cardiovascular secondary prevention trials reported that folic acid (0.8 mg/day) plus vitamin B12 significantly increased cancer incidence, cancer mortality and all-cause mortality (9). Furthermore, Animal studies have shown that folic acid supplementation prevents the development of cancer in normal tissues but promotes the progression of established (pre)neoplastic lesions (10-14). Animal studies have also suggested that supraphysiologic supplemental doses of folic acid may promote, rather than prevent, the cancer development (11). Collectively, these observations suggest folate possesses dual modulatory effects on cancer development and progression depending on the dose and the...
stage of cell transformation at the time of high folate exposure or folic acid supplementation (15, 16).

Several biologically plausible mechanisms exist to explain the dual effects of folate on cancer development and progression (1). As an essential cofactor for nucleotide biosynthesis, folate plays an important role in DNA synthesis, integrity, and repair, aberrancies of which are integrally related to cancer development (1). In normal tissues, folic acid supplementation provides nucleotide precursors for DNA synthesis and replication, thereby ensuring DNA fidelity, maintenance of DNA integrity and stability, and optimal DNA repair; this would reduce the risk of neoplastic transformation (1). In contrast, folic acid supplementation promotes the progression of (pre)neoplastic lesions by providing nucleotide precursors to the rapidly replicating transformed cells, allowing accelerated proliferation (1). Folate also modulates DNA methylation of cytosine within the cytosine-guanine (CpG) sequences because its’ role in the provision of S-adenosylmethionine, the primary methyl group donor for most biological methylation reactions (17). Neoplastic cells simultaneously harbor widespread global DNA hypomethylation and more specific regional areas of hypermethylation (17). Global DNA hypomethylation contributes to cancer development through several purported mechanisms including chromosomal instability (17). In contrast, DNA methylation at promoter CpG islands silences transcription and hence inactivates the function of a wide array of tumor suppressor and critical cancer-related genes (17). Folic acid supplementation appears to be able to reverse pre-existing global DNA hypomethylation and to increase the extent of global DNA methylation above the pre-existing level (17), thereby reducing the risk of neoplastic transformation. In contrast, folic acid supplementation may cause de novo methylation of promoter CpG islands of
tumor suppressor genes with consequent gene inactivation leading to tumor development and progression (1).

Folate intake and blood levels in North America have dramatically increased over the past decade. This is due to the drastic increase in dietary folate intake from mandatory folic acid fortification (18) aimed at reducing the rate of neural tube defects and also to the consumption of supplemental folic acid by up to 30-40% of the North American population (19). In addition, women of childbearing age are routinely advised to consume folic acid supplementation (0.4-1.0 mg) for the prevention of neural tube defects (20). These facts suggest that the developing fetus in North America is likely exposed to high levels of folate and folic acid. Epigenetic and metabolic programming takes place during embryogenesis and hence the embryonic stage is highly susceptible to changes in the intrauterine environment, which may influence the risk of developing cancer in adulthood (21). In this regard, the intrauterine nutritional status and hormonal environment play an important role in mammary gland development and subsequent breast cancer risk (22). Given these considerations, we investigated the effects of maternal and postweaning folic acid supplementation on the risk of mammary tumors and on DNA methylation in the offspring.
Methods

Animals, dietary intervention and mammary tumor induction

This study was approved by the Animal Care Committee of the University of Toronto. Six week old Sprague-Dawley rats were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Female rats were housed in pairs with males when breeding, and singly during pregnancy and lactation. Female rats were randomized to receive an amino acid-defined diet (Dyets, Bethlehem, PA) containing 2 (control) or 5 (supplemented) mg folic acid/kg diet 3 weeks prior to mating and throughout pregnancy and lactation (Figure 1). Male rats used for breeding were placed on the same diet as the female breeding mate. Litters remained with the mothers until the end of the weaning period (3 weeks of age). At weaning, 30 pups from each maternal diet group were killed for baseline plasma folate and homocysteine (an accurate inverse indicator of tissue folate status) and hepatic folate measurements (Figure 1). The remaining female pups were randomized to receive either the control or folic acid supplemented diets (Figure 1).

At puberty (7 weeks of age), 12 pups from each maternal/pup diet group (total=48) were killed for folate analyses (Figure 1). All remaining pups (n=70 per each maternal/pup diet group) received a single intragastric dose of 5 mg of 7,12-dimethylbenz[a]anthracene (DMBA) (Sigma-Aldrich, St. Louis, MO) dissolved in 1.0 mL of corn oil for mammary tumor induction (23, 24) and continued to receive the experimental diets until necropsy at 28 weeks of age (Figure 1).

Amino acid-defined diets containing different levels of folic acid constitute a standard method of providing supplemental dietary folate in rodents and have been extensively used in previous studies of dietary folate and cancer in rodents (10-14) including mammary tumors (25, 26). The control diet containing 2 mg of folic acid/kg diet is the basal dietary requirement (BDR).
for rats (27) and was selected to represent the recommended dietary allowance for humans (0.4 mg/day of dietary folate equivalents). The supplemented diet containing 5 mg folic acid/kg diet (2.5x BDR) was selected to represent the likely average postfortification total folate intake of ~0.8-1.0 mg folic acid/day in North American populations taking multivitamins containing 0.4 mg folic acid (15), and the recommended dose for all women planning a pregnancy or capable of becoming pregnant (0.4-1.0 mg folic acid/day) (20). The detailed composition of the diets has been published previously (14). Diets and water were provided *ad libitum*.

**Observation parameters**

Body weights were recorded weekly. All rats were palpated for mammary tumors once a week beginning at 4 weeks post-DMBA administration. The number, size, and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All rats were monitored daily for clinical evidence of illness or morbidity and those approaching a pre-defined moribund state (25, 26) were promptly killed.

**Sample collection and analysis of mammary tumors**

Rats were killed by carbon dioxide inhalation followed by cervical dislocation. At necropsy, blood was collected and plasma was stored at -80°C with 0.5% ascorbic acid for plasma folate determination and without ascorbic acid for plasma homocysteine assay. The liver and normal mammary tissue from each rat were excised, snap-frozen, and stored at -80°C for determination of hepatic and mammary folate concentrations and mammary global DNA methylation and DNA methyltransferase (DNMT) activity.

All macroscopic mammary tumors were counted, excised and weighed, and two longest diameters of each tumor were measured using a digital caliper for final tumor size computation (area calculated using the formula of length/2 x width/2 x π). All macroscopic mammary tumors
were fixed and processed in a standard manner for H&E staining and independently analyzed histologically by 2 study pathologists (R.R. and A.M.) blinded to the study group.

**Folate and homocysteine concentrations**

Plasma and tissue folate concentrations were determined by a standard microbiological microtiter plate assay (25, 26). Total plasma homocysteine concentrations were determined using the Axis™ Homocysteine EIA kit (Abbott Laboratories, Mississauga, ON, Canada) (12).

**Global DNA methylation analysis**

DNA from non-neoplastic mammary tissue was extracted by a standard technique using proteinase K followed by organic extraction (25, 26). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. The final preparations had a ratio of A_{260} to A_{280} between 1.8 and 2.0 and were free of RNA and protein contamination. The concentration of each DNA sample was determined as the mean of three independent spectrophotometric readings.

The methylation status of CpG sites in mammary genomic DNA was determined by in vitro methyl acceptance capacity of DNA using ³H-methyl-S-adenosylmethionine (New England Nuclear, Boston, MA) as a methyl donor and a prokaryotic CpG DNMT, Sss1 (New England Biolabs, Beverly, MA) (25, 26). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous ³H-methyl incorporation. All samples were run in duplicate and assays performed twice. Both intra- and inter-assay CV of this assay were 5%.

**DNMT activity assay**

Total cellular DNMT activity was measured by incubating cell lysates from non-neoplastic mammary tissue containing 10 μg of protein with 0.5 μg of poly[d(I-C)].d(I-C)]
template (Sigma-Aldrich, St. Louis, MO) and 3 μCi $^3$H-methyl-S-adenosylmethionine (New England Nuclear, Boston, MA) for 2 h at 37°C (28). Each reaction was performed in triplicate.

**Statistical analysis**

Continuous variables were log-transformed due to departure from normality. Characteristics measured at the time of weaning were analyzed using univariate one-way ANOVA, and those measured after weaning were analyzed using univariate two-way ANOVA. Tumor incidence, a binary variable, was analyzed using logistic regression. Kaplan-Meier and Cox proportional hazards survival analysis were used to compare time until tumor appearance (latency) among the four groups. To correct for any maternally inherited artifacts, tumor incidence was re-analyzed using the general estimating equation with a binary response variable to take the repeated measures nature of the data into account. Rat body weights over time, another repeated measures data, were also analyzed using the generalized estimating equation. Ordinal variables (litter size, tumor multiplicity) were assessed using the Kruskal-Wallis and Mann-Whitney non-parametric tests. With the exception of the analyses of characteristics measured at the time of weaning, all analyses included an interaction term between maternal and pup diets in order to test whether the effect of the maternal diet depended on the pup diet and vice versa. Significant interactions found in the two-way ANOVAs were followed by Tukey’s Honestly Significant Difference post-hoc analyses to determine the nature of the interaction. Correlations between continuous dependent variables were evaluated using the Spearman’s rank correlation test. All significance tests were two-sided and were considered significant at $p<0.05$. Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS 11.0 for Macintosh (SPSS, Chicago, IL).
Results

Body weight

At weaning, the mean body weight of the pups from the folic acid supplemented dams was significantly higher, by 13% (95% CI 1-27%), than that of the pups from the dams fed the control diet (p=0.04). The initial difference in pup weights due to the maternal folic acid supplementation remained significant but decreased over time (p-interaction for maternal diet*time <0.001), and disappeared by week 10. The interaction between maternal and postweaning diet was not significant and postweaning folic acid supplementation did not affect the weight of pups.

Systemic and tissue folate status

At weaning, plasma folate and homocysteine and hepatic folate concentrations of the offspring reflected maternal folic acid supplementation (p<0.001; Table 1).

At 7 and 28 weeks of age, plasma, hepatic, and mammary gland folate (p<0.001) and plasma homocysteine (p=0.03) concentrations of the offspring reflected postweaning folic acid supplementation (Table 1). Maternal folic acid supplementation was reflected in plasma and hepatic folate concentrations of the offspring at both time points (p<0.001) but not in mammary gland folate or plasma homocysteine concentrations at either time point (Table 1). A significant interaction between the maternal and postweaning diets was observed only for increasing hepatic folate concentrations of the offspring at 7 weeks such that supplementing the postweaning diet had a larger effect if the maternal diet was not supplemented (p<0.001; Table 1) but not for other folate indices at either time point.

Effect on DMBA-induced mammary tumorigenesis
All rats were killed at 21 weeks post-DMBA administration (28 weeks of age) for mammary tumor analysis. No rats died prematurely. The proportion of rats euthanized for large and/or ulcerating tumors before the scheduled necropsy was similar among the four groups (range 6-10%). Consistent with previous observations (23, 24), >80% of macroscopic mammary lesions were identified histologically as adenomas (15%), adenocarcinomas (84%), or sarcomas (1%).

Maternal folic acid supplementation significantly accelerated the rate of mammary adenocarcinoma appearance in the offspring (RR=1.6, 95% CI 1.2-2.0; p=0.002) (Figure 2A; Table 2). A non-significant trend towards an accelerated rate of mammary adenocarcinoma appearance was also observed for postweaning folic acid supplementation (RR=1.3, 95% CI 1.0-1.7; p=0.06) (Figure 2A; Table 2). Maternal folic acid supplementation significantly increased the odds of mammary adenocarcinomas in the pups compared with the control maternal diet (OR=2.1, 95% CI 1.2-3.8; p=0.008; Figure 2A; Table 2). Postweaning folic acid supplementation also significantly increased the risk of developing mammary adenocarcinomas in the offspring compared with the control postweaning diet (OR=1.9, 95% CI 1.1-3.3; p=0.03; Figure 2A; Table 2). To control for any maternally inherited artifacts, tumor incidence was corrected for mother using a logistic regression analysis; both maternal and postweaning folic acid supplementation remained significant predictors for increased tumor incidence (OR 2.3, 95% CI 1.1-4.8; p=0.022 and OR 2.0, 95% CI 1.2-3.5; p=0.008, respectively).

Maternal folic acid supplementation also significantly increased the multiplicity of mammary adenocarcinomas in the offspring (p=0.008; Table 2), while a non-significant trend toward an increase was observed for postweaning folic acid supplementation (p=0.09; Table 2). The mean weight of mammary adenocarcinomas was significantly lower in the pups from the
folic acid supplemented dams than in the pups from the dams fed the control diet (p=0.03) while postweaning folic acid supplementation had no significant effect (Table 2). Maternal and postweaning folic acid supplementation had no significant effect on the mean size of mammary adenocarcinomas in the offspring (Table 2). No significant interaction between the maternal and postweaning diets was observed for any of the above tumor parameters (Table 2).

A similar pattern of the effect of maternal and postweaning folic acid supplementation on the combination of all mammary tumors (adenocarcinomas+adenomas+sarcomas) in the offspring was also observed. Both maternal and postweaning folic acid supplementation significantly increased the incidence of all mammary tumors in the offspring compared with the corresponding control diets (OR=1.9, 95% CI 1.0-3.6; p=0.05 and OR=1.9, 95% CI 1.0-3.6; p=0.04, respectively). Also, maternal, but not postweaning, folic acid supplementation significantly accelerated the rate of mammary tumor appearance in the offspring (RR=1.4, 95% CI 1.0-1.8; p=0.02) (Figure 2B).

**Effect on global DNA methylation and DNMT activity in non-neoplastic mammary glands**

Maternal folic acid supplementation significantly decreased mammary global DNA methylation in the offspring by 7% (95% CI 1.2-11.6%; p=0.03; Figure 3A) compared with the control maternal diet, whereas postweaning folic acid supplementation had no effect (Figure 3A). A significant interaction between the maternal diet and the presence of at least one mammary adenocarcinoma was observed (p<0.001); maternal folic acid supplementation significantly decreased mammary global DNA methylation by 18.5% (95% CI 8.5-27.3%; p<0.001) only in the tumor-free pups, whereas it had no effect in those bearing at least one mammary adenocarcinoma (Figure 3B). Regardless of folic acid supplementation, mammary global DNA
methylation was significantly lower, by 6.7% (95% CI 1.3-11.8), in the offspring harboring at least one mammary adenocarcinoma than in the tumor-free pups (Figure 3B; p=0.02).

Maternal folic acid supplementation did not modulate DNMT activity in the offspring. In contrast, postweaning folic acid supplementation significantly decreased DNMT activity by 31% (95% CI 0-52%) in the offspring (p=0.05; Figure 3C). No interactions between the maternal or postweaning diet and adenocarcinoma status were observed. Among animals bearing at least one mammary adenocarcinoma, a non-significant trend towards a decrease, by 30% (95% CI 0-51%), in mammary DNMT activity was observed (p=0.06; Figure 3D), compared with the tumor-free pups.
Discussion

In the present study, maternal and postweaning folic acid supplementation at the level equivalent to the average postfortification total folate intake in North America significantly increased the incidence of mammary adenocarcinomas in the offspring. Furthermore, maternal folic acid supplementation significantly accelerated the development of mammary adenocarcinomas and increased the multiplicity of mammary adenocarcinomas in the offspring, whereas a non-significant trend towards the same effects was observed for postweaning folic acid supplementation. No significant interaction between maternal and postweaning folic acid supplementation was observed for any of the tumor endpoints in the offspring. The mammary tumor promoting effect was more consistent and greater with maternal folic acid supplementation than with postweaning supplementation.

We posited that maternal and postweaning folic acid supplementation would increase global DNA methylation in the offspring, thereby reducing the risk of developing mammary tumors. In the present study, however, maternal folic acid supplementation significantly decreased mammary global DNA methylation in the offspring. Although this finding may appear paradoxical, this observation may be partly explained by the preferential shuttling of the flux of one-carbon units to the nucleotide synthesis pathway over the methylation pathway in response to folic acid supplementation. Although folic acid is an inhibitor of dihydrofolate reductase (29), it could also upregulate dihydrofolate reductase in certain situations (30). This upregulation may increase thymidylate synthase activity because the transcription of these genes is co-regulated by several transcriptional factors (31) and this would increase thymidylate production, thereby increasing cellular proliferation, at the expense of methylation reactions (32). In contrast, postweaning folic acid supplementation had no effect on mammary global DNA methylation in
the offspring. This observation is consistent with previous animal studies in which postweaning folic acid supplementation (4-20x BDR) provided continually for up to 27 weeks did not affect global DNA methylation in adult rat liver (33), colon (11), and mammary glands (25, 26). These observations collectively suggest that the intrauterine and early postnatal periods are highly susceptible to the epigenetic modifying effect of maternal folic acid supplementation. Indeed, a new DNA methylation pattern is established during embryogenesis soon after implantation (34) and DNA methylation of the developing fetus appears to be highly susceptible to environmental modifiers including maternal supplementation of methyl group donors (35, 36). Our data demonstrate for the first time that maternal folic acid supplementation at the level equivalent to the average postfortification total folate intake in North America can significantly decrease mammary global DNA methylation in the offspring.

The functional ramifications of this modest decrease in global DNA methylation associated with maternal folic acid supplementation as well as its mechanistic link to the increased mammary tumorigenesis in the offspring were not interrogated in the present study. Global DNA hypomethylation is an early, and consistent, event in carcinogenesis (17) including human breast (37) and rat mammary (25, 26) cancers. In the present study, regardless of folic acid supplementation, global DNA methylation and DNMT activity in the non-neoplastic mammary glands were lower in the offspring harboring at least one mammary adenocarcinoma than in the tumor-free pups. This suggests that global DNA hypomethylation in the mammary glands might have been a predisposing epigenetic milieu for mammary tumor development in the offspring. The facts that the mammary tumor promoting effect was more consistent and greater with maternal folic acid supplementation than with postweaning supplementation and that maternal, but not postweaning, folic acid supplementation was associated with a significant
reduction in global DNA methylation suggest that perturbed intrauterine and early postnatal epigenetic programming might have contributed to the initiation and promotion of mammary tumorigenesis in the offspring. Future studies are warranted to investigate several downstream effects of global DNA hypomethylation including chromosomal instability to establish a mechanistic link between global DNA hypomethylation and increased mammary tumorigenesis in the offspring associated with maternal folic acid supplementation. Maternal and postweaning folic acid supplementation might have promoted mammary tumor development in the offspring through de novo methylation of promoter CpG islands of tumor suppressor genes, thereby silencing these genes (1, 17). However, we did not investigate the effect of folic acid supplementation on promoter CpG islands DNA methylation because most promoters that are subject to DNA methylation are not conserved between human and rats, and specific promoters that are presumably epigenetically regulated in rats have not been extensively characterized nor have they been implicated in rat mammary tumorigenesis.

Maternal folic acid supplementation had no effect on DNMT activity whereas postweaning folic acid supplementation significantly reduced DNMT activity in non-neoplastic mammary glands in the offspring. The DNMT data are not consistent with the global DNA methylation data and this cannot readily be explained. Decreased or inactivated DNMT has been shown to protect against intestinal tumors but increase the risk of lymphomas and sarcomas in animal studies (1). Although it is possible that decreased DNMT might have played a role in the observed increased mammary tumorigenesis in the offspring associated with postweaning folic acid supplementation, further studies are required to elucidate a mechanistic link between DNMT and mammary tumorigenesis.
There are other potential mechanisms by which maternal and postweaning folic acid supplementation may increase mammary tumorigenesis in the offspring. For example, maternal and postweaning folic acid supplementation may accelerate proliferation and promote uncontrolled cell growth of rapidly replicating undifferentiated cells in the mammary glands of the offspring by providing nucleotide precursors (1). We determined epithelial proliferation and apoptosis in a subset of mammary adenocarcinomas from the present study to test this hypothesis; neither maternal nor postweaning folic acid supplementation significantly affected epithelial proliferation or apoptosis in mammary adenocarcinomas (supplemental Table 1). However, future studies are warranted to test this potential tumor promoting mechanism by studying the effect of maternal and postweaning folic acid supplementation on these endpoints in normal mammary glands during embryonic and different postnatal developmental stages.

Previous animal studies conducted in another chemical carcinogen rat model collectively suggest that mild dietary folate deficiency inhibits, whereas folic acid supplementation (4-20x BDR) does not modulate, the development and progression of mammary tumors (25, 26, 38). In these studies, dietary folic acid supplementation was initiated only at weaning (25, 26, 38), suggesting that in utero supplementation has a drastically different effect on mammary tumor risk. In contrast to the present study, we have previously reported that maternal folic acid supplementation provided in utero and during lactation significantly reduced the number of terminal end buds of the mammary glands at puberty, a purported biomarker of mammary tumor risk in rodents (23, 24), in the offspring (39). One important distinction between the previous (39) and the present studies is the difference in the models used: the former being spontaneous and the current being chemically-induced. Maternal folic acid supplementation may provide a protective environment for the development of (pre)neoplastic mammary lesions in the offspring.
In the presence of a strong carcinogenic milieu (in this case, DMBA), however, any preventive effect of maternal folic acid supplementation on the initiation of mammary tumorigenesis might have likely been overwhelmed. Regardless of folic acid supplementation, DMBA induced and established (pre)neoplastic mammary lesions in the offspring and folic acid supplementation promoted the progression of these DMBA-induced (pre)neoplastic mammary lesions, similar to the tumor promoting effect of folic acid supplementation on established (pre)neoplastic colonic lesions (11-14). Another potential explanation for the contradictory finding between these studies is that terminal end bud density at puberty may not be a consistent predictor of mammary tumor risk in rodents as suggested by recent studies (40, 41).

We reported that maternal, but not postweaning, folic acid supplementation significantly reduced the incidence of colorectal adenocarcinomas by 64% in the offspring in the azoxymethane rat model (42). In contrast, a study using $Apc^{min/+}$ mice has shown that postweaning folic acid supplementation significantly increased the number of small intestinal adenomas in the offspring (43). Interestingly, folic acid supplementation provided in utero and lactation and continued through postweaning period did not increase the number of adenomas in the offspring (43). In contrast, folic acid deficiency induced in utero and continued through lactation and postweaning period significantly reduced the number of adenomas in the offspring (43). This suggests that the effect of maternal folic acid supplementation on cancer risk in the offspring may be organ-specific and may depend on the experimental model.

Epidemiologic studies have reported a protective effect of periconceptional maternal folic acid supplementation on several pediatric cancers in the offspring including neuroblastoma and other brain tumors, acute lymphocytic leukemia, and non-Hodgkin’s lymphoma (44-47). However, some studies have not confirmed this purported protective effect (48, 49) and has even
reported an increased risk (46). Furthermore, most of these epidemiologic studies could not
delineate the effect on cancer risk specific to folic acid from other vitamins in the supplements
(50). At present, no information concerning the effect of maternal folic acid supplementation on
breast cancer risk in the offspring exists in humans.

DMBA-induced mammary tumorigenesis in rats is different from human breast cancer in
several important aspects: (i) the use of the genotoxic chemical carcinogen and (ii) molecular
 genetic differences (lack of p53 and Brca mutations) (23, 24). Nonetheless, the DMBA rat model
is widely used to determine the effects of dietary factors on mammary tumorigenesis for the
following reasons: (i) histological similarities of adenocarcinoma to human breast cancer; (ii)
molecular genetic similarities (Erb2/HER2, TGFβ, cyclin D1); (iii) a clear operational distinction
between initiation and promotion stages; (iv) hormonally dependent mammary tumorigenesis;
and (vi) expression of estrogen and progesterone receptors (23, 24).

In summary, our data suggest that maternal and postweaning folic acid supplementation
at the level equivalent to the average postfortification total folate intake in North America and to
that recommended to women at reproductive age significantly enhances the risk of mammary
tumors in the offspring. Our data also suggest that reduced global DNA methylation and
decreased DNMT activity associated with maternal and postweaning folic acid supplementation,
respectively, may be responsible for the observed increased mammary tumor risk in the
offspring. Given the drastically increased intrauterine and early life exposure to folic acid and
folate in North America (18-20), future studies are warranted to clarify the effects of maternal
and early life folic acid supplementation on breast cancer risk in the offspring and associated
mechanisms.
References


Figure legends

**Figure 1:** Study design

**Figure 2:** Effect of maternal and postweaning folic acid supplementation on (A) the rate of appearance of mammary adenocarcinomas and (B) the rate of appearance of all mammary tumors (adenocarcinomas+adenomas+sarcomas) in the offspring by the Kaplan-Meier and Cox proportional hazards survival analysis. Lines with different letters differ significantly at $P<0.05$.

**Figure 3:** Effect of maternal and postweaning folic acid supplementation on (A) global DNA methylation and (B) DNMT activity and effect of tumor status (defined as presence of at least one adenocarcinoma) on (C) global DNA methylation and (D) DNMT activity in non-neoplastic mammary tissue in the offspring. For global DNA methylation, the assay produces a reciprocal relationship between endogenous DNA methylation status and exogenous $^3$H-methyl incorporation into DNA. For DNMT activity, the assay produces a positive relationship between endogenous enzyme activity and exogenous $^3$H-methyl incorporation into DNA. Values are mean ± SEM. Bars with different letters differ significantly at $P<0.05$. 
<table>
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<th>Puberty (7 wks)</th>
<th>Endpoint (28 wks)</th>
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<td>2 mg FA</td>
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<td>2 mg FA</td>
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<td>Postweaning diet group</td>
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<td>Plasma folate (ng/mL)</td>
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<td>Plasma Hcy (μmol/L)</td>
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<td>Liver folate (μg/g tissue)</td>
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<td>Mammary folate (ng/g tissue)</td>
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**Table 1.** Folate and homocysteine concentrations of the offspring at baseline, puberty, and endpoint.

Diet groups 2 mg FA and 5 mg FA represent 2 mg and 5 mg folic acid/kg diet, respectively. Results are expressed as mean ± SEM. At each time point, * denotes a significant main effect due to maternal diet at $P<0.05$, § denotes a significant main effect due to postweaning diet at $P<0.05$. Results marked with letters indicate a significant interaction between maternal and postweaning diet ($P$-interaction=0.03).
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<td>Adenocarcinoma incidence (%)</td>
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<td>Median adenocarcinoma latency (weeks post-DMBA)</td>
<td>20 ± 2</td>
<td>18 ± 1</td>
<td>16 ± 1</td>
<td>14 ± 1</td>
<td>1.55 (1.17 to 2.04)*</td>
<td>0.002*</td>
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<td>Mean adenocarcinoma multiplicity</td>
<td>1.98 ± 0.35</td>
<td>2.65 ± 0.42</td>
<td>3.01 ± 0.41</td>
<td>3.63 ± 0.45</td>
<td>0.008*</td>
<td>0.093§</td>
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<tr>
<td>Mean adenocarcinoma weight (g)†</td>
<td>0.47 ± 0.39</td>
<td>0.53 ± 0.29</td>
<td>0.45 ± 0.34</td>
<td>0.35 ± 0.28</td>
<td>0.025*</td>
<td>0.322§</td>
<td>0.086‡</td>
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<tr>
<td>Mean adenocarcinoma size (mm²)</td>
<td>201 ± 18</td>
<td>191 ± 18</td>
<td>201 ± 21</td>
<td>166 ± 12</td>
<td>0.210*</td>
<td>0.370§</td>
<td>0.610‡</td>
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Table 2. Summary effects of maternal and postweaning folic acid supplementation on the incidence, latency, multiplicity, weight, and size of mammary adenocarcinomas in the offspring. Diet groups 2 mg FA and 5 mg FA represent 2 mg and 5 mg folic acid/kg diet, respectively. Results are expressed as mean ± SEM. * represents main effect due to maternal diet; § represents main effect due to pup diet; ‡ represent an interaction effect between maternal and pup diet; and † represents geometric mean.

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Dams Pups

Wk 0  Wk 3  Wk 7  Wk 28

3 wks  3 wks gestation

Breeding  Weaning Baseline  DMBA gavage  Plasma & tissue folate

Mammary tumor analysis
Tumor proliferation & apoptosis
DNA methylation status
DNMT enzyme activity
Plasma & tissue folate

Diet randomization:

Maternal diet

| 2 mg | 5 mg |

Pup diet

| 2 mg | 5 mg |
| 2 mg | 5 mg |
| 5 mg | 5 mg |

Figure 1
Figure 2

Total cumulative tumor incidence (%)

Cumulative adenocarcinoma incidence (%)

Weeks post-DMBA

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Figure 3

A. [3H-methyl] incorporation into DNA (dpm) for 2 mg pup diet and 5 mg pup diet across different maternal diet (mg/kg diet) levels. Significant differences are indicated by different letters (a, b).

B. [3H-methyl] incorporation into DNA (dpm) for 2 mg pup diet and 5 mg pup diet. Significant differences are indicated by different letters (a, b).

C. [3H-methyl] incorporation into DNA (dpm) for 2 mg mom diet and 5 mg mom diet across different Adenocarcinoma conditions (No, Yes). Significant differences are indicated by different letters (a, b, c).

D. [3H-methyl] incorporation into DNA (dpm) for 2 mg pup diet and 5 mg pup diet across different Adenocarcinoma conditions (No, Yes).
# Effect of maternal and postweaning folic acid supplementation on mammary tumor risk in the offspring

Anna Ly, Hanna Lee, Jianmin Chen, et al.

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