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Folate Deficiency Inhibits Development of the Mammary Gland and its Associated Lymphatics in FVB Mice

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ABSTRACT

Background: Folate is essential for DNA synthesis, DNA repair, cell proliferation, development, and morphogenesis. Folic acid (FA) is a nutritional supplement used to fortify human diets.

Objectives: We investigated the effects of dietary FA on early mammary gland (MG) development and hyperplasia.

Methods Study 1: nulliparous female FVB wild-type (WT) mice were fed control (Con; 2 mg FA/kg), deficient (Def; 0 mg FA/kg), excess (Ex; 5 mg FA/kg), or super excess (S-Ex; 20 mg FA/kg) diets for 8 wk before mating to WT or heterozygous FVB/N-Tg[mouse mammary tumor virus long terminal repeat (MMTV)-polyomavirus middle T antigen (PyVT)]634Mul/J (MMTV-PyMT$^{+/-}$) transgenic males. Dams were fed these diets until they weaned WT or MMTV-PyMT$^{+/-}$ pups, which were fed the dam's diet from postnatal day (PND) 21 to 42. Tissues were collected from female progeny at PNDs 1, 21, and 42. Study 2: Con or Def diets were fed to WT intact females and males from PND 21 to 56, or to ovariectomized females from PND 21 to 77; tissues were collected at PND 56 or 77. Growth of all offspring, development of MGs, MG hyperplasia, supramammary lymph nodes, thymus and spleen, cell proliferation, and expression of MG growth factors were measured.

Results Study 1: Ex or S-Ex did not affect postnatal MG development or hyperplasia. The rate of isometric MG growth (PND 1–21) was reduced by 69% in Def female progeny ($P < 0.0001$). Similarly, hyperplastic growth in MGs of Def MMTV-PyMT$^{+/-}$ offspring was 18% of Con ($P < 0.05$). The Def diet reduced supramammary lymph node size by 20% ($P < 0.0001$) and increased MG insulin-like growth factor 2 mRNA by 200% ($P < 0.05$) and protein by 130%–150% ($P < 0.05$). Study 2: the Def diet did not affect MG growth, but it did reduce supramammary lymph node size ($P < 0.05$), spleen weight ($P < 0.001$), and thymic medulla area ($P < 0.05$).

Conclusions: In utero and postnatal folate deficiency reduced the isometric development of the MGs and early MG hyperplasia. Postnatal folate deficiency reduced the development of lymphatic tissues. *J Nutr* 2020;150:2120–2130.

Keywords: folic acid, mammary gland, insulin-like growth factor 2, polyomavirus middle T antigen, lymphatics

Introduction

The mammary glands (MGs) begin to develop when their various components including the ductal epithelium, the future MG fat pad, and the supporting vasculature all arise in utero (1). After birth, the MG ducts of mice then undergo a short phase of isometry when they slowly elongate at the same rate as overall body growth. Leading up to puberty, the onset of estrogen secretion from the ovaries stimulates the ductal epithelium to initiate a distinct phase of allometric growth when the ducts extend rapidly to the periphery of the MG fat pad (2, 3), led by highly proliferative terminal end buds (TEBs) (4).

Like in other organs and disease states (5, 6), the MGs are sensitive to environmental factors, including deviations in maternal nutrition (7–9), that can affect the risk of tumorigenesis later in life. Among the essential nutrients required to support rapid cellular proliferation during fetal growth is the B vitamin, folate, where its deficiency in humans leads to growth retardation, low birth weight, failure to thrive, and neural tube defects (10). Folic acid (FA) fortification was mandated in the United States and Canada in the mid-1990s with the goal of reducing the incidence of neural tube defects, the incidence of which has since declined by 20%–40% (11, 12).

Ironically, pregnant women are also at risk of folate intakes above the tolerable upper intake level, where common medical practice is to prescribe 400–800 μg FA/d via supplements, in addition to that consumed from fortified foods (13). Mothers may also continue to transfer high amounts of folate to infants via breast milk (14). High amounts of maternal folate intake are associated with a greater likelihood for offspring to

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develop asthma, lower respiratory infection, and altered glucose metabolism and body composition (15–17). A recent study also implicated high maternal folate intake as a possible risk factor for autism (18). Given the role of folate in one-carbon metabolism (19), we hypothesized that early-life exposure to high and excess amounts of FA would sustain or advance normal MG development and promote tumorigenesis. With respect to folate deficiency, we hypothesized it would suppress DNA synthesis and/or methylation, leading to reduced MG development and increased mutagenesis, but decreased tumor growth. Two previous studies identified that rats exposed to excess FA in utero subsequently had altered MG development and risk of MG tumorigenesis. In 1 study where dams were fed excess dietary FA (5 mg/kg compared with controls fed 2 mg/kg) during gestation and through weaning, their female offspring had fewer TEBs and more alveolar buds (20). In a second study, female offspring exposed to the same excess of FA were more susceptible to MG tumorigenesis induced by 7,12-dimethylbenz[a]anthracene, as reflected by reduced tumor latency and increased multiplicity (21). These findings suggest that the MGs of rats are sensitive to excess FA in utero and through weaning. However, the molecular mechanisms underlying any phenotypic changes in mice and whether they occur in other models of genetically dependent breast cancer have not been established.

Herein we studied the effect of diets deficient in, or containing excess, FA on the MGs of wild-type (WT) mice and transgenic mice having MG-specific expression of the polyomavirus middle T antigen (PyMT) (22). Mice overexpressing the PyMT oncogene directed by the mouse mammary tumor virus long terminal repeat promoter (MMTV) initiate hyperplasia before the onset of allometric MG growth (23). Tumors in these transgenic mice share similarities with breast cancers resulting from the amplification of erb-b2 receptor tyrosine kinase 2, as occurs in ~25% of human breast cancers (24). Our analysis focused specifically on the pre- and peripubescent phases of isometric and allometric development that have been increasingly identified as critical windows for lifetime breast cancer risk in humans (25).

Methods

Animals and diets

Animal experiments and procedures were performed as approved by the Institutional Animal Care and Use Committee at the University of California, Davis. In study 1 (**Supplemental Figure 1**), WT FVB females at postnatal day (PND) 21 were assigned to treatment diets containing either deficient (Def; 0 mg FA/kg), control (Con; 2 mg FA/kg), excess (Ex; 5 mg FA/kg), or super excess (S-Ex; 20 mg FA/kg) concentrations of FA. Diets were formulated by Dyets, Inc. and were amino acid–defined (26), as **Table 1** details. After 8 wk on these diets, females were mated with either WT FVB males or heterozygous FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT$^{+/-}$; JAX stock #002374) transgenic males (27). Males were fed the Con diet, except during the 10 d they were group-housed with females when they shared the same diet. The treatment diet was then available to females ad libitum through pregnancy and lactation. At parturition, litters destined to be killed at PND 21 and PND 42 were normalized to $n = 6$ pups within 24 h, with as many female offspring retained per litter as possible. Offspring shared access to the same diets as their dams. Female offspring (WT and MMTV-PyMT$^{+/-}$) were weighed weekly during PND 1–42, and killed and necropsied on PND 1, 21, or 42. Male offspring (WT and MMTV-PyMT$^{+/-}$) were weighed weekly from PND 7, and all male pups in PND 21 and PND 42 litters were killed at weaning on PND 21 (Supplemental Figure 1). Estrous cyclicity of the weaned females in PND 42 litters was monitored by visual assessment of external vaginal appearance (28) from PND 37 to 41, which was accompanied by daily monitoring of vaginal cytology from PND 42 to 47 and/or until their first diestrus, which is when they were killed and necropsied. Genotyping by PCR identified 46.8% of the offspring to be FVB MMTV-PyMT$^{+/-}$. Neonates were killed by decapitation, whereas other mice were killed by carbon dioxide asphyxiation followed by cervical dislocation or cardiac puncture. Blood was collected by cardiac puncture, and serum stored at −80°C. The left #4 MG was prepared as a whole mount, whereas the right #4 MG (after removal of the supramammary lymph node) was weighed, then flash frozen in liquid nitrogen and stored at −80°C. The second and third thoracic MGs were fixed in 10% formalin and embedded in paraffin before sectioning. The liver was removed, weighed, and a portion flash frozen in liquid

TABLE 1 Composition of amino acid–defined diets, without succinylsulfathiazole, fed in studies 1 and 2[1]

Ingredients	Con, g/kg	Def, g/kg	Ex, g/kg	S-Ex, g/kg
FA/sucrose premix[2]	0.4	0	1	4
Sucrose	207	207	210	207
Dextrin	397	397	393	393
Cellulose	50	50	50	50
Corn oil[3]	100	100	100	100
Choline chloride	2	2	2	2
Sodium acetate	8.1	8.1	8.1	8.1
Amino acids[4]	175.86	175.86	175.86	175.86
Vitamin mix[5]	10	10	10	10
Mineral mix[6]	50	50	50	50

[1] Con, control diet containing 2 mg folic acid/kg; Def, deficient diet containing 0 mg folic acid/kg; Ex, excess diet containing 5 mg folic acid/kg; FA, folic acid; S-Ex, super excess diet containing 20 mg folic acid/kg.
[2] 5 mg FA/g premix.
[3] Stablized with 0.15% butylated hydroxytoluene.
[4] Composition by g/kg of total diet: L-alanine, 3.5; L-arginine (free base), 11.2; L-asparagine.H_2O, 6.82; L-aspartic acid, 3.5; L-cystine, 3.5; L-glutamic acid, 35; glycine, 23.3; L-histidine (free base), 3.3; L-isoleucine, 8.2; L-leucine, 11.1; L-lysine HCl, 18; L-methionine, 8.2; L-phenylalanine, 11.6; L-proline, 3.5; L-serine, 3.5; L-threonine, 8.2; L-tryptophan, 1.74; L-tyrosine, 3.5; L-valine, 8.2.
[5] Composition by g/kg of vitamin mix: thiamin HCl, 0.6; riboflavin, 0.7; pyridoxine HCl, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; biotin, 0.02; vitamin B-12 (0.1%), 5; vitamin A palmitate (500,000 IU/g), 0.8; vitamin D_3 (400,000 IU/g), 0.25; vitamin E acetate (500 IU/g), 10; menadione sodium bisulfite, 5; sucrose finely powdered, 972.
[6] Composition by g/kg of mineral mix: calcium carbonate, 292; calcium phosphate dibasic, 3.4; sodium chloride, 247.37; potassium phosphate dibasic, 343.24; magnesium sulfate anhydrous, 49.03; manganese sulfate monohydrate, 3.64; ferric citrate USP, 12.52; zinc carbonate basic, 1.15; cupric carbonate basic, 1.08; potassium iodide, 0.005; sodium selenite, 0.0099; molybdic acid ammonium salt, 0.025; chromium potassium sulfate dodecahydrate, 0.38; sodium fluoride, 0.05; sucrose finely powdered, 46.1001.

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Supplemental Figures 1–3 and Supplemental Tables 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn.
Address correspondence to RCH (e-mail: rchovey@ucdavis.edu).
Abbreviations used: Con, control diet containing 2 mg folic acid/kg; CSF1, colony stimulating factor 1; Def, folic acid–deficient diet containing 0 mg folic acid/kg; DMR, differentially methylated region; *Egf*, epidermal growth factor; *Egfr*, epidermal growth factor receptor; Ex, excess folic acid diet containing 5 mg folic acid/kg; FA, folic acid; *Igf1*, insulin-like growth factor 1; *Igf2*, insulin-like growth factor 2; MG, mammary gland; MMTV, mouse mammary tumor virus long terminal repeat; OVX, ovariectomized; PH3, phosphorylated histone H3; PND, postnatal day; PyMT, polyomavirus middle T antigen; S-Ex, super excess folic acid diet containing 20 mg folic acid/kg; TEB, terminal end bud; WT, wild-type.

nitrogen and stored at −80°C, whereas another portion was fixed in 10% formalin and embedded in paraffin.

Given our finding in study 1 that folate deficiency inhibited isometric growth of the MGs in WT mice, we hypothesized that folate deficiency would specifically affect growth of the ductal network during other states of isometric growth, such as occurs in the absence of ovarian stimulation that normally begins around puberty. In study 2, we therefore examined the effects of postnatal folate deficiency on the MGs of prepubertal, ovary-intact mice as well as in ovariectomized (OVX) females whose MGs subsequently underwent a sustained period of isometric growth (2, 3). WT FVB males and females ($n = 7$) were assigned to either the Def (0 mg FA/kg) or Con (2 mg FA/kg) amino acid–defined diet upon weaning at PND 21. A subset of females ($n = 20$) were OVX at weaning according to our previous approach (29), then immediately started either the Con or Def diet. Ovary-intact females and all males received the diets for 5 wk, whereas OVX females were fed the diets for 8 wk. All animals were killed by carbon dioxide asphyxiation followed by cardiac puncture at either PND 56 (ovary-intact females and males) or PND 77 (OVX females). In addition to the tissues collected as outlined previously, the supramammary lymph nodes, thymus, spleen, and uterus were dissected out and weighed (except the lymph nodes), then fixed in 10% formalin and embedded in paraffin. Given that organ size is a function of body size, we expressed all tissue weights and sizes relative to metabolic body weight [(body weight)$^{2/3}$; (2, 3)]. Given the MGs of mice undergo isometric growth before allometric expansion starting just before puberty (1), we analyzed the rate of growth in each of these phases separately by expressing change in ductal area ($\log_{10}$) relative to metabolic body weight as described (3, 29).

MG whole mounts

Whole mounts were prepared from left #4 MGs as described (30). Digital images of MG whole mounts were analyzed using ImageJ (http://imagej.nih.gov/ij/). Ductal elongation was measured as the length from the teat to the end of the most distal ductal terminus (29). Ductal area was measured as the polygonal area outlining the ductal tree (29), whereas hyperplastic area was measured as the area occupied by hyperplastic lesions that had a diameter more than twice the thickness of the ducts from which they arose.

Histology and immunohistochemistry

Tissue histology (MG, supramammary lymph nodes, and thymus) was assessed in formalin-fixed paraffin-embedded tissue sections (5 μm) stained with hematoxylin and eosin. Digital images of thymus and supramammary lymph nodes were analyzed using ImageJ. Lymph node area was measured as a polygon outlining the lymph node. Thymic medulla area and total thymus area were measured as polygons outlining the respective structures, and cortex area was calculated from these 2 measurements. Immunohistochemistry for phosphorylated histone H3 (PH3) was performed on sections of MG and thymus as described (31) with the following modifications. Antigen retrieval was for 20 min in the autoclave before 4°C overnight incubation with anti-PH3 antibody (1:50, Santa Cruz Biotechnology). Immunohistochemistry on supramammary lymph nodes for CD3 (1:1000; clone SP7; Abcam; $n = 3$ mice/treatment) and B220 (1:800; Clone RA3-6B2; BD Pharmingen; $n = 3$ mice/treatment) was performed as aforementioned except that antigen retrieval was performed for 45 min and primary antibodies were incubated with sections overnight at room temperature.

Cell proliferation per unit area in supramammary lymph nodes was determined by counting the number of PH3-positive cells in the entire supramammary lymph node, and measuring the area of the lymph node. Average cell proliferation per unit area in thymus was determined by counting the number of PH3-positive cells in 2 or 3 regions of cortex and 2 regions of medulla, and measuring the respective structure areas. Images for B220 and CD3 immunohistochemistry were graded using the Aperio ImageScope system, whereby slide images were assessed for the intensity of 3,3'-diaminobenzidine and the corresponding hematoxylin. Staining intensity was scored as 0 for no staining, +1 for weak intensity, +2 for moderate intensity, and +3 for strong intensity. Cells scored as +2 and +3 were considered positive.

Determination of folate status

Liver portions were weighed, homogenized in PBS with a glass homogenizer, sonicated, then centrifuged (2350 × *g*) for 15 min at 4°C. Samples (100 μL supernatant) were assayed in duplicate using a mouse FA ELISA kit (MyBiosource) according to the manufacturer's instructions. The concentration of folate was determined from a 4-parameter logistic standard curve. Total plasma homocysteine was measured by HPLC with postcolumn fluorescence detection (32).

Gene expression

Total RNA was extracted from the MG, DNase treated, and 1 μg was reverse transcribed as previously described (33). qPCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems), and relative gene expression was normalized to 18S rRNA expression. **Supplemental Table 1** lists the primer and probe sequences and cycling conditions. Expression of murine insulin-like growth factor 1 (*Igf1*; mouse liver cDNA standard curve) and *Tnf* (lactating mouse MG cDNA standard curve) was analyzed with TaqMan Gene Expression Master Mix (Applied Biosystems) as described (34). Murine insulin-like growth factor 2 (*Igf2*; mouse liver cDNA standard curve), epidermal growth factor receptor (*Egfr*; mouse kidney cDNA standard curve), and epidermal growth factor (*Egf*; mouse kidney cDNA standard curve) were analyzed with Fast SYBR Green Master Mix (Applied Biosystems) as described (31).

IGF2 ELISA

Female PND 42 MGs were homogenized in PBS containing 50 mM EDTA and 1× HALT Protease Inhibitor Cocktail (Thermo Scientific). An equal volume of 1× RIPA buffer (with Triton X-100; Alfa Aesar), 50 mM EDTA, and 1× HALT Protease Inhibitor Cocktail was added to the homogenate and proteins were solubilized at room temperature for 30 min. After centrifugation (10 min, 20,000 × *g*, 4°C), protein concentrations of the supernatants were measured using Bradford reagent (Thermo Scientific) (35). All samples were diluted in homogenization buffer (0.5× RIPA in PBS) to the same protein concentration. Homogenates were assayed for mouse IGF2 in triplicate using the IGF2 DuoSet ELISA kit and DuoSet ELISA ancillary reagent kit 3 (R&D Systems Inc, Minnesota) against IGF2 standard curves prepared in homogenization buffer.

DNA methylation

Bisulfite conversion of genomic DNA (2 μg) was achieved using the EpiTect Fast DNA Bisulfite Kit (Qiagen). Bisulfite-treated DNA (1 μL) was then amplified using the PyroMark PCR kit (Qiagen) using primers listed in Supplemental Table 1. Amplicons were purified using streptavidin-sepharose high-performance beads (GE Healthcare) and annealed to sequencing primers (Supplemental Table 1) using the PyroMark Q24 pyrosequencing system (Qiagen). Percentage methylation was measured in triplicate for 5 CpGs within 3 differentially methylated regions (DMRs) (DMR0, DMR2, and H19 DMR) in the *Igf2* gene.

Statistical analysis

Values are presented as means ± SDs. Where necessary, data were transformed to achieve homogeneity of variances or normal distribution, and/or a Greenhouse–Geisser correction was performed. Data from studies 1 and 2 were analyzed for main effects of diet and age (study 1), or diet and sex (studies 1 and 2), and their interactions, using a 2-factor ANOVA (PROC GLM) in SAS version 9.3 (SAS Institute Inc.) followed by a post hoc Tukey test while controlling for multiple testing, or a 2-factor ANOVA in Prism 8 (Graphpad Software) followed by a post hoc Bonferroni or Tukey test while controlling for multiple testing. When a significant interaction occurred between diet and sex or diet and age, we performed multiple comparisons for the effects of individual diets on each sex or time point individually to parse out the exact dietary effects. Concentrations of plasma homocysteine and liver folate, hyperplasia at PNDs 21 and 42, and body weights at PNDs

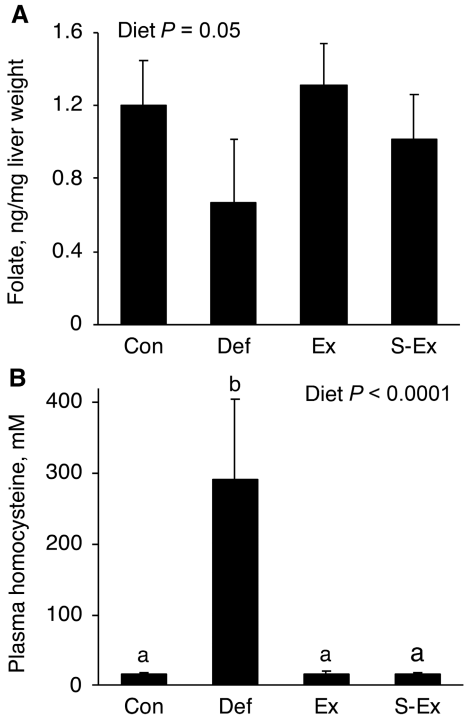

FIGURE 1 Liver folate (A) and plasma homocysteine (B) concentrations at postnatal day 42 in female mice exposed to (via dam nutrition), and then fed, diets containing different concentrations of folic acid (study 1). Values are means ± SDs. (A) $n = 3$ or 4 or (B) $n = 5$–7. Labeled means without a common letter differ, $P < 0.0001$. Con, control diet containing 2 mg folic acid/kg; Def, folic acid–deficient diet containing 0 mg folic acid/kg; Ex, excess folic acid diet containing 5 mg folic acid/kg; S-Ex, super excess folic acid diet containing 20 mg folic acid/kg.

28, 35, and 42 (study 1) were analyzed for main effects of diet using a 1-factor ANOVA in Prism 8 or SAS 9.3, followed by a post hoc Tukey test or a post hoc Dunnett test (liver folate) while controlling for multiple testing. Body weights of study 2 OVX mice during PND 63–77 were analyzed using Student's t test in Prism 8 for an effect of diet. The MG and hyperplasia growth rates within a dietary group were calculated and compared by linear regression analysis using Prism 8. Numbers of mice in some analyses varied owing to reproductive effects of different FA diets on fertility and litter sizes, the randomness of the sex and genotype of pups in each litter, deficient pups failing to thrive and needing euthanasia before their assigned day ($n = 3$), and other experimental factors; for example, we occasionally had to remove statistically significant outliers (using the ROUT method with $Q = 1\%$ in GraphPad Prism 8) if the data could not be transformed to normality with the outliers included. Significance was declared at $P \leq 0.05$.

Results

Study 1: offspring folate status and growth

As expected, there was an effect of diet on hepatic folate concentrations at PND 42 (Figure 1A). Mice fed the Def diet had a tendency toward lower concentrations of hepatic folate than the Con animals ($P = 0.06$) (Figure 1A). Total plasma homocysteine was measured as a functional indicator of folate status. Mice fed the Def diet had markedly elevated total plasma homocysteine compared with those fed the Con, Ex, and S-Ex FA diets (Figure 1B).

There was no main effect of sex on body weight (Figure 2A, B). At PND 1 there was no main effect of diet on body weight

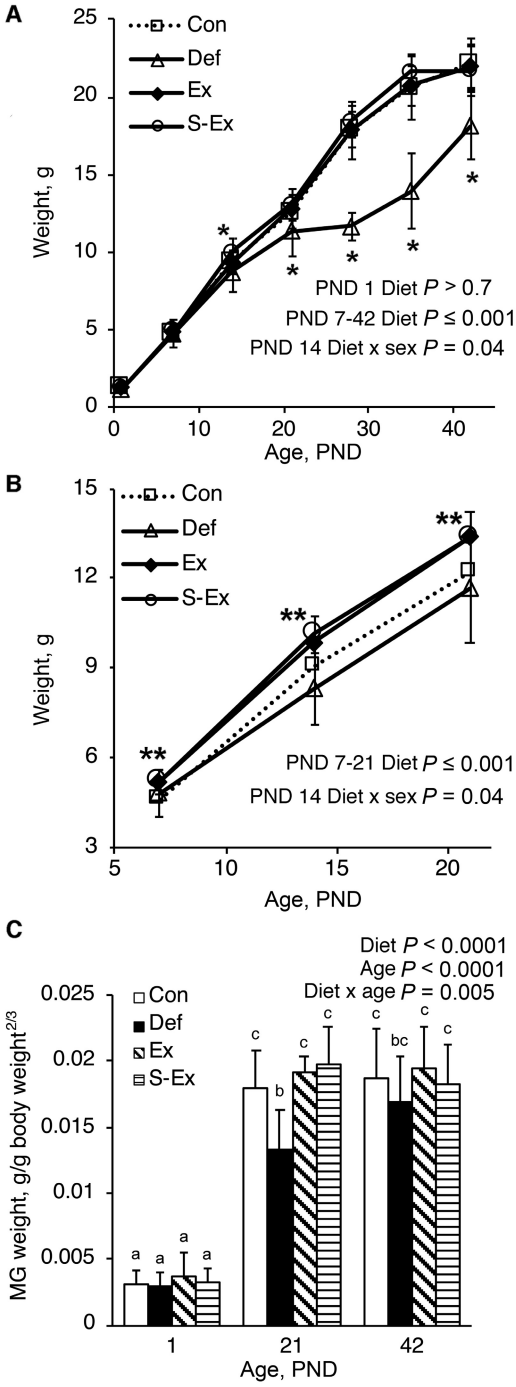

FIGURE 2 Body weights of female (PND 1–42) (A) and male (PND 7–21) (B) mice and MG weights relative to metabolic body weight in female mice at PNDs 1, 21, and 42 (C) exposed to (via dam nutrition), and then fed, diets containing different folic acid concentrations (study 1). Values are means ± SDs. (A) Con, $n = 19$–21 (PND 1, 28–42) or 41 (PND 7–21); Def, $n = 11$–12 (PND 1, 28–42) or 19–24 (PND 7–21); Ex, $n = 21$ (PND 1), 51 (PND 7–21), or 29 (PND 28–42); and S-Ex, $n = 17$ (PND 1), 37–42 (PND 7–21), or 25 (PND 28–42); (B) Con, $n = 29$; Def, $n = 18$–19; Ex, $n = 16$; and S-Ex, $n = 17$–18; (C) Con, $n = 16$–17; Def, $n = 11$–12; Ex, $n = 21$ (PND 1), 15 (PND 21), or 29 (PND 42); and S-Ex, $n = 16$–17 (PND 1, 21) or 25 (PND 42). ∗Different from all other treatments, $P < 0.05$; ∗∗Different from Con, $P < 0.02$. Labeled means without a common letter differ, $P < 0.002$. Con, control diet containing 2 mg folic acid/kg; Def, folic acid–deficient diet containing 0 mg folic acid/kg; Ex, excess folic acid diet containing 5 mg folic acid/kg; MG, mammary gland; PND, postnatal day; S-Ex, super excess folic acid diet containing 20 mg folic acid/kg.

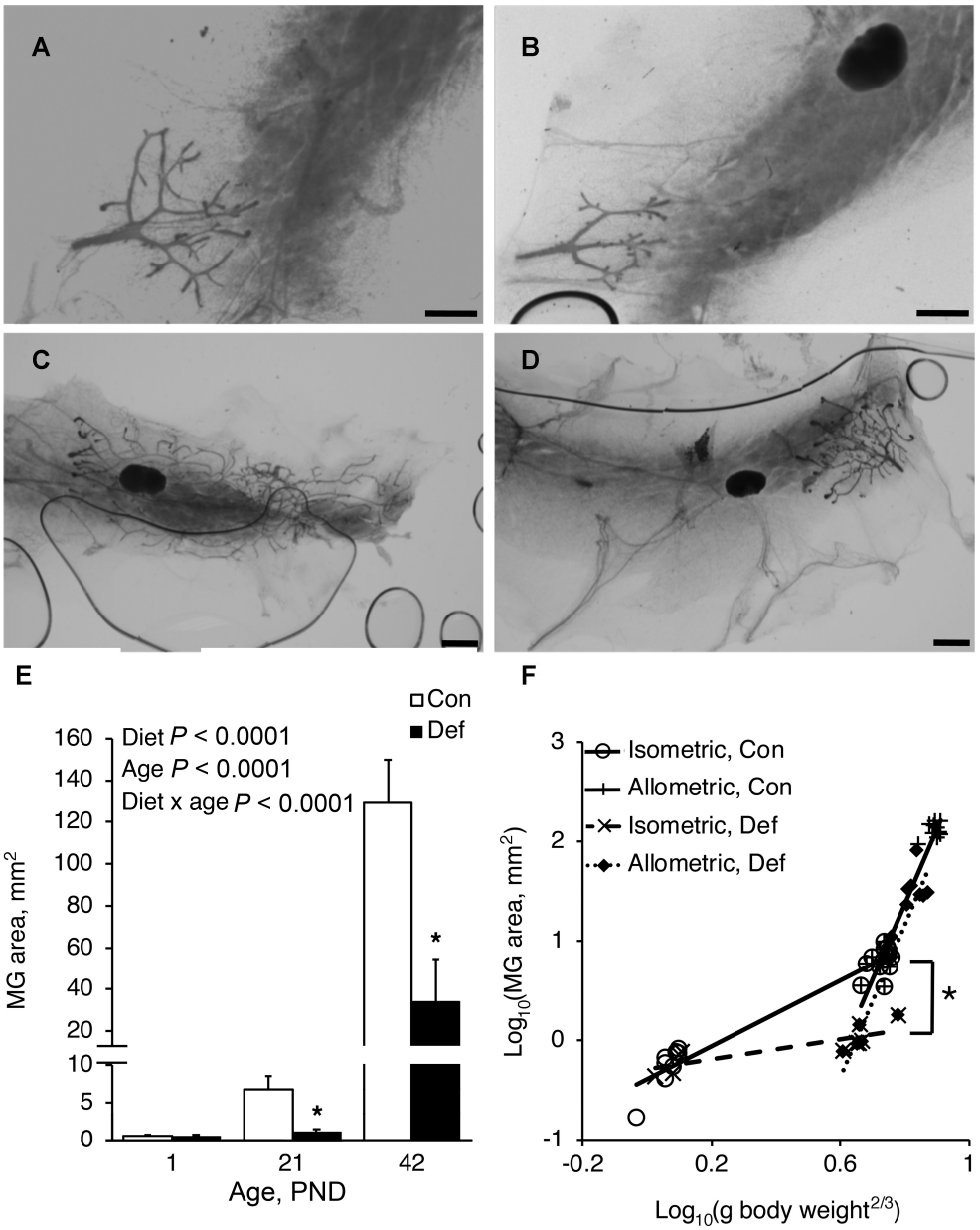

FIGURE 3 Whole mounts of MGs from wild-type females at PND 21 [Con] (A), PND 21 [Def] (B), PND 42 [Con] (C), and PND 42 [Def] (D), quantification of MG ductal network at PNDs 1, 21 and 42 (E), and changes in the area of the MG ductal network as a function of metabolic body weight from individual mice at PNDs 1, 21, and 42 (F) exposed to (via dam nutrition), and then fed, Con or Def diets (study 1). Values are means ± SDs. Con, $n = 8$ (PND 1), 15 (PND 21), or 11 (PND 42); Def, $n = 4$ (PND 1) or 6–8 (PNDs 21 and 42). ∗Different from control, $P < 0.001$. Scale bar = 1 mm. Con, control diet containing 2 mg folic acid/kg; Def, folic acid–deficient diet containing 0 mg folic acid/kg; MG, mammary gland; PND, postnatal day.

(Figure 2A). At every subsequent time point there was a main effect of diet on body weights and at PND 14 there was an interaction between diet and sex on pup weight (Figure 2A, B). Females at PND 14 that had been exposed to the S-Ex diet were heavier than all other females (Figure 2A). At PNDs 21, 28, 35, and 42, females exposed to and consuming Def diets weighed less than those raised on all other diets (Figure 2A). At PNDs 7, 14, and 21, males reared by dams fed the S-Ex FA diet were heavier than males fed the Con diet (Figure 2B).

Study 1: MG development

Regarding MG wet weight, there was a significant interaction between the effects of diet and age, where females exposed to the Def diet until PND 21 had lighter MGs than those from all other groups (Figure 2C). At PNDs 1 and 42 the wet weights of the MGs across the groups did not differ.

Area of the ductal network in the MGs increased with age, was affected by diet, and demonstrated an interaction between age and diet (**Figure 3**E). At all ages, area of the ductal network in females fed the Ex or S-Ex diets was not different from that in Con females ($P > 0.29$; data not shown). At PND 21, females exposed to the Def diet had ~15% of the ductal development found in animals exposed to the Con, Ex, and S-Ex diets (Figure 3A, B, E) (data not shown for Ex and S-Ex). By PND 42, females exposed to and fed the Def diet had ~27% of the ductal development in Con, Ex, and S-Ex mice (Figure 3C–E) (data not shown for Ex and S-Ex). During the isometric phase (PND 1–21), MGs from females in the Def

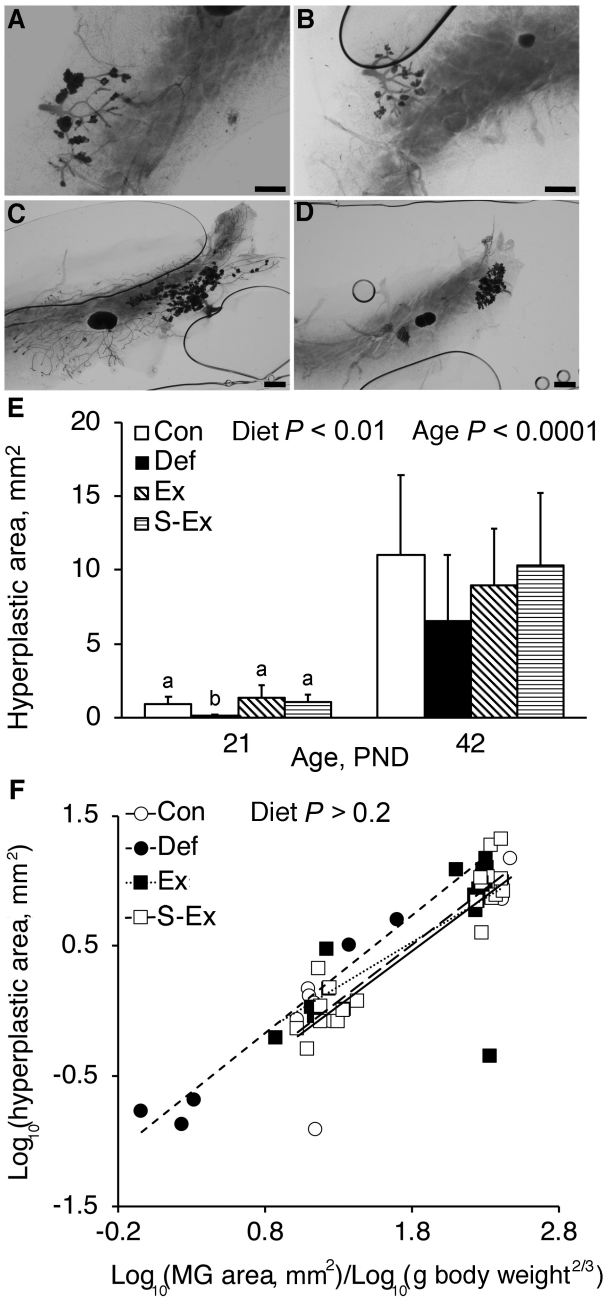

FIGURE 4 Whole mounts of MGs from mouse mammary tumor virus long terminal repeat-polyomavirus middle T antigen$^{+/-}$ females at PND 21 [Con] (A), PND 21 [Def] (B), PND 42 [Con] (C), and PND 42 [Def] (D), quantification of total hyperplastic area at PNDs 21 and 42 (E), and hyperplasic area relative to MG size corrected for metabolic body weight from individual mice at PNDs 21 and 42 (F) exposed to (via dam nutrition), and then fed, diets containing different concentrations of folic acid (study 1). Values are means ± SDs. Con, $n = 6$ (PND 21) or 2 (PND 42); Def, $n = 3$; Ex, $n = 6$ (PND 21) or 11–12 (PND 42); and S-Ex, $n = 9$ (PND 21) or 12 (PND 42). Labeled means without a common letter differ, $P < 0.05$. Scale bar = 1 mm. Con, control diet containing 2 mg folic acid/kg; Def, folic acid–deficient diet containing 0 mg folic acid/kg; Ex, excess folic acid diet containing 5 mg folic acid/kg; MG, mammary gland; PND, postnatal day; S-Ex, super excess folic acid diet containing 20 mg folic acid/kg.

TABLE 2 The rate of hyperplastic growth between postnatal days 21 and 42 in the mammary glands of mouse mammary tumor virus long terminal repeat-polyomavirus middle T antigen$^{+/-}$ females exposed to (via dam nutrition), and then fed, diets containing different folic acid concentrations (study 1)[1]

Diet	*n*	Growth rate,[2] mm^2/g
Con	8	0.81 (0.23, 1.39)
Def	6	1.06 (0.78, 1.35)
Ex	18	0.64 (0.33, 0.95)
S-Ex	22	0.81 (0.68, 0.95)

[1] Values are slopes (95% CIs). Con, control diet containing 2 mg folic acid/kg; Def, folic acid–deficient diet containing 0 mg folic acid/kg; Ex, excess folic acid diet containing 5 mg folic acid/kg; S-Ex, super excess folic acid diet containing 20 mg folic acid/kg.
[2] Growth rates (slopes) were calculated by linear regression analysis of data in Figure 4F.

group had a rate of growth that was 69% lower than in Con mice (Def slope = 0.50 mm^2/g; 95% CI: 0.23, 0.78 mm^2/g; Con slope = 1.65 mm^2/g; 95% CI: 1.47, 1.83 mm^2/g) (Figure 3F). Notably, there was no effect of any diet on the rate of allometric growth during the subsequent phase of MG development (PND 21–42) (Figure 3F).

Study 1: MG hyperplasia

The area of hyperplasia within the MGs increased with age and was also affected by FA diet (**Figure 4**E). At PND 21 the area of hyperplasia in MMTV-PyMT$^{+/-}$ females exposed to the Def diet was ~18% of that in Con females, and was lower than in all other diet groups (Figures 4A, B, E). However, by PND 42 there was no effect of Def, Ex, or S-Ex diets on the area of MG hyperplasia ($P = 0.6$) (Figures 4C–E). Given that the extent of hyperplastic growth mirrored overall ductal area, we assessed the change in hyperplastic area relative to MG ductal area (corrected for metabolic body weight) from PND 21 to PND 42 (Figure 4F). After this normalization, the rate of hyperplastic growth was similar in all 4 diets (**Table 2**), highlighting that the extent of hyperplasia was likely a direct function of the number of precursor ductal epithelial cells within the glands.

Study 1: supramammary lymph node size

Our assessment of MG development within whole mounts also revealed differences in the area of the supramammary lymph nodes due to dietary treatment. Both diet and age affected lymph node area as a function of metabolic body weight, where there also was an interaction between these 2 factors (**Table 3**). At PNDs 1 and 21, supramammary lymph node area was independent of FA intake (Table 3). However, by PND 42 the lymph nodes from Def animals were >20% smaller than those in females fed the Con, Ex, or S-Ex diets (Table 3).

Study 1: MG growth factor expression

Expression of *Tnf* and *Egf* in the MGs was unaffected by age or dietary group (**Figure 5**A, E). Expression of *Igf1* and *Egfr* mRNA increased with the transition from prepuberty to puberty (Figure 5B, C), but was unaffected by dietary treatment. By contrast, the expression of *Igf2* mRNA in the MGs was affected by diet where Def animals had 200% higher *Igf2* expression than Con mice (Figure 5D). We confirmed that the expression of IGF2 protein in the MGs of Def animals

TABLE 3 Areas of supramammary lymph nodes corrected for metabolic body weight from female mice at PNDs 1, 21, and 42 exposed to (via dam nutrition), and then fed, diets containing different folic acid concentrations (study 1)[1]

	PND 1		PND 21		PND 42		*P* value		
Diet	$mm^2/g^{0.66}$	*n*	$mm^2/g^{0.66}$	*n*	$mm^2/g^{0.66}$	*n*	Diet	Age	Diet × age
							<0.0001	<0.0001	<0.0001
Con	0.051 ± 0.02	10	0.099 ± 0.01	17	0.452 ± 0.04 [a]	11			
Def	0.066 ± 0.01	7	0.084 ± 0.03	7	0.350 ± 0.03 [b]	8			
Ex	0.053 ± 0.02	13	0.088 ± 0.02	22	0.479 ± 0.05 [a]	25			
S-Ex	0.056 ± 0.01	12	0.097 ± 0.01	17	0.477 ± 0.06 [a]	23			

[1]Values are means ± SDs. Labeled means in a column without a common superscript letter differ, $P < 0.0001$. Con, control diet containing 2 mg folic acid/kg; Def, folic acid–deficient diet containing 0 mg folic acid/kg; Ex, excess folic acid diet containing 5 mg folic acid/kg; PND, postnatal day; S-Ex, super excess folic acid diet containing 20 mg folic acid/kg.

was increased by 150% at PND 21 and 130% at PND 42 (Figure 5F), where MG IGF2 amounts were higher at PND 42 than at PND 21 in Con and Def mice.

Given this altered expression of *Igf2* mRNA and protein within the MGs, we examined the extent of DNA methylation at CpG islands within 3 regulatory regions of the *Igf2* promoter, namely DMR0, DMR2, and H19 DMR. There was no difference in the extent of methylation for these regions between the MGs of Def and Con animals (**Supplemental Table 2**).

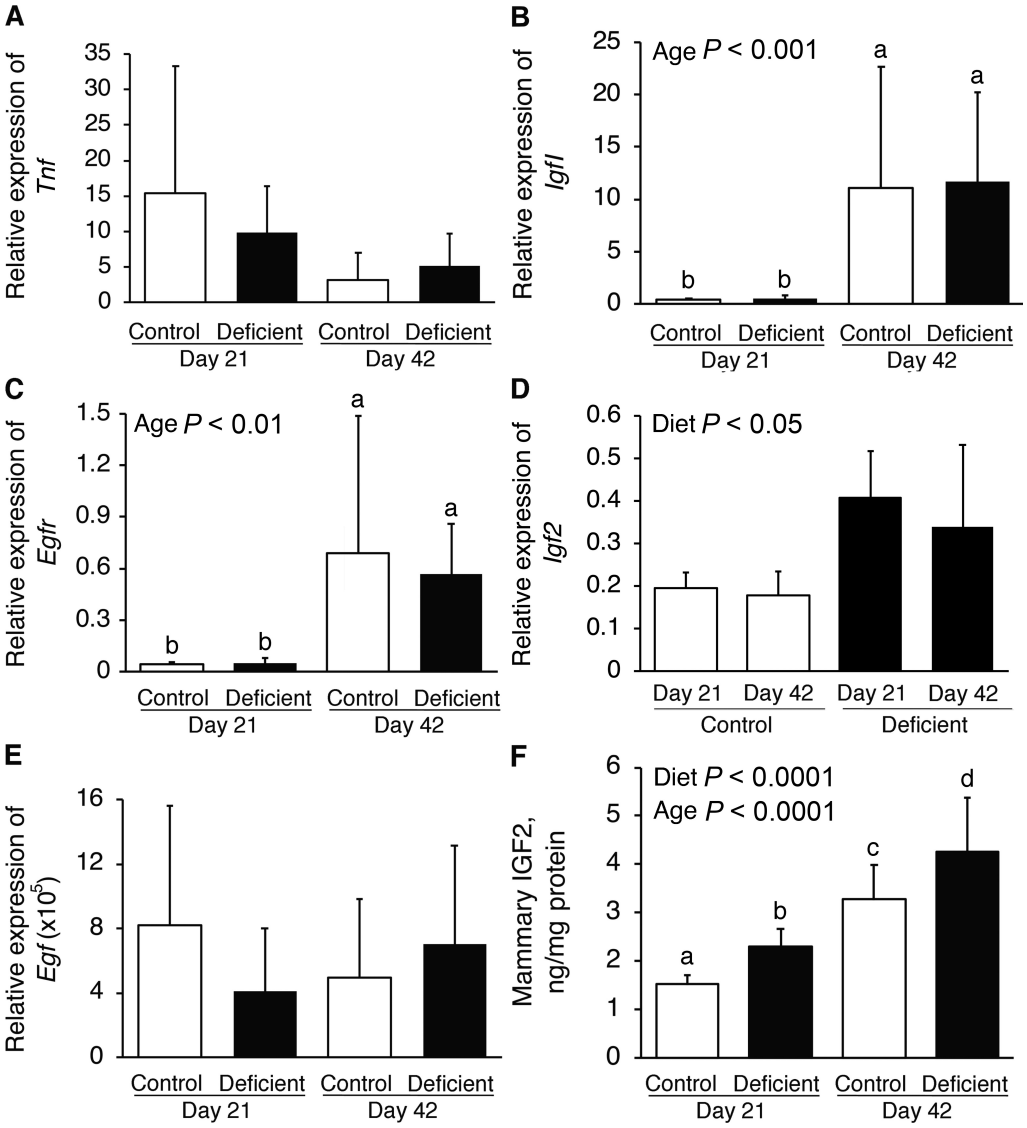

FIGURE 5 qPCR for *Tnf* (A), *Igf1* (B), *Egfr* (C), *Igf2* (D), and *Egf* mRNA (E) and ELISA for IGF2 (F) in mammary glands on PND 42 from females exposed to (via dam nutrition), and then fed, control or folic acid–deficient diets (study 1). Values are means ± SDs. (A–E) $n = 3$, (F) $n = 8$. Labeled means without a common letter differ, $P < 0.05$. *Egf*, epidermal growth factor; *Egfr*, epidermal growth factor receptor; *Igf1*, insulin-like growth factor 1; *Igf2*, insulin-like growth factor 2.

Study 2: growth and MG development

For study 2 we investigated changes in the MGs due to dietary FA deficiency after PND 21. When the dietary treatments commenced there was no difference in body weight between males, ovary-intact females, and OVX females. There was an effect of sex on body weights during PND 28–56, and an interaction between sex and diet at PND 28 (**Supplemental Figure 2**A). Although diet did not affect body or MG weights of males or ovary-intact females up to PND 56, males were heavier than females during PND 35–56; in addition, OVX females weighed significantly more than ovary-intact females at PND 49–56 (Supplemental Figure 2A). At PND 77, OVX females fed the Def diet weighed significantly more than OVX females fed the Con diet (Supplemental Figure 2A). Irrespective of diet, MGs of OVX females at PND 77 were heavier than those from ovary-intact females at PND 56, after adjustment for metabolic body weight (Supplemental Figure 2B).

As anticipated, the MG ductal network area (data not shown) and ductal elongation were smaller in OVX females at PND 77 than in ovary-intact females at PND 56, and was even smaller in males at PND 56 (**Supplemental Figure 3**). There was no effect of diet on ductal network area ($P > 0.1$; data not shown) or on ductal elongation in either OVX or ovary-intact females, or in males (Supplemental Figure 3).

Study 2: supramammary lymph node size

We found significant effects of sex and diet, and an interaction between the 2 factors, on supramammary lymph node area (corrected for metabolic body weight) (**Figure 6**A). Lymph nodes were smaller in males than in females, and were also smaller in Def-fed than in Con-fed females in both OVX and ovary-intact groups (Figure 6A), but not males. We also assessed whether paracortical expansion within the supramammary lymph nodes was inhibited in folate-deficient mice as a result of reduced cell proliferation. Quantification of PH3-positive cells revealed no effect of either diet or OVX on cell proliferation rate within the supramammary lymph nodes at necropsy (Figure 6B). Immunohistochemistry for B220 and CD3 also revealed no effect of folate deficiency on the numbers of B-cells (0.4%–2.1% B220+) or T-cells (47%–55% CD3+), respectively.

Study 2: lymphatic organ size and development

Weight of the thymus was unchanged by diet (**Figure 7**A), whereas the spleen in Def-fed males and ovary-intact females weighed less than that from Con animals (Figure 7A). The spleen and thymus from males weighed significantly less than those from both ovary-intact and OVX females (Figure 7A). Histological analysis of the thymus revealed a main effect of diet on the size of the thymic medulla such that it was 42% smaller in Def animals (Figure 7B). There was no difference in the number of cells that were PH3-positive in the thymic cortex or medulla between animals fed the Con and Def diets (Figure 7C).

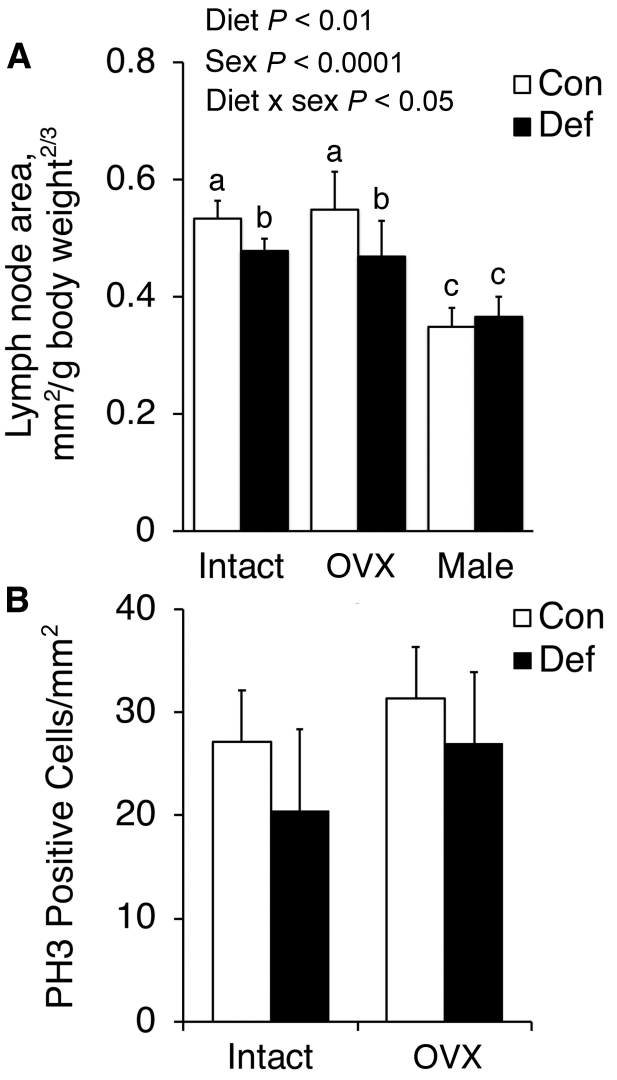

FIGURE 6 Supramammary lymph node size adjusted for metabolic body weight (A) and frequency of PH3-positive cells in lymph nodes (B) from male and female mice fed Con or Def diets from PND 21 to either PND 56 (intact and male) or PND 77 (OVX) (study 2). Values are means ± SDs. (A) $n = 7$ (intact, male) or 10 (OVX), (B) $n = 5$–6. Labeled means without a common letter differ, $P < 0.05$. Con, control diet containing 2 mg folic acid/kg; Def, folic acid–deficient diet containing 0 mg folic acid/kg; intact, ovary-intact females; OVX, ovariectomized; PH3, phosphorylated histone H3; PND, postnatal day.

Discussion

An increasing body of evidence points to changes in MG development during early life stages, such as in utero and before the onset of puberty, as having a lasting impact on lifetime breast cancer risk (36). Our data indicate that early-life folate deficiency, but not excess, joins a range of other disruptors that can alter the course of normal MG growth and tumorigenesis in mice. Maternal folate deficiency in utero and during lactation led to a specific, marked reduction in ductal elongation in female offspring between PND 1 and 21. Notably, the ductal network was unaffected up to PND 1, implying that the retardation we observed was manifest postnatally, albeit there may have also been carryover effects from in utero exposure. Folate-deficient females were smaller at weaning, consistent with the known requirement for folate during development (37, 38); however, the negative effect on MG growth was outstanding even after correcting for metabolic body weight (2, 3).

Human breast cancer risk can be influenced positively or negatively during embryonic and/or early postnatal life, including through exposure to factors such as an obesogenic diet, radiation, or environmental chemicals (25, 39). The only factors known to alter this early isometric/prepubertal phase of ductal development are inappropriate exposure to environmental chemicals (40, 41) and estrogenic compounds (42). We previously found that 1 isomer of conjugated linoleic

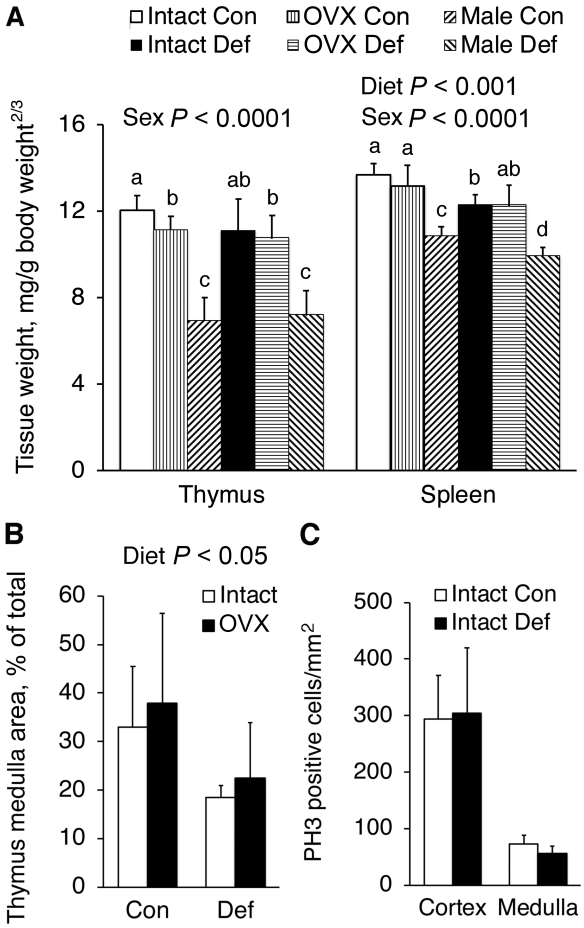

FIGURE 7 Lymphatic tissue weights adjusted for metabolic body weight (A), area of thymic medulla as a percentage of total thymus area (B), and cellular proliferation in thymus by subcompartment (C) in male and female mice fed Con or Def diets from PND 21 to either PND 56 (intact and male) or PND 77 (OVX) (study 2). Values are means ± SDs. (A) n = 6–7 (intact, male) or 10 (OVX), (B–C) n = 5 (intact) or 3 (OVX). Labeled means without a common letter differ, $P < 0.05$. Con, control diet containing 2 mg folic acid/kg; Def, folic acid–deficient diet containing 0 mg folic acid/kg; intact, ovary-intact females; OVX, ovariectomized females; PND, postnatal day.

acid induced growth of the quiescent gland from an isometric state, which we ascribed to an increased inflammatory response in the local microenvironment. Whereas folate deficiency did not affect the expression of inflammatory mediators such as TNF or EGFR (29), it was associated with increased local expression of IGF2. IGF2 expression in the MG is highest in the neonate and during prepuberty, and is primarily expressed in the MG fat pad (43). It is possible that folate deficiency affected adipose tissue function (44), which then affected IGF2 expression. Although IGF2 is regulated by DMRs in response to folate concentrations (45), there are both tissue-specific (46) and sex-specific (45, 47) factors involved. Here the increased expression of IGF2 in the MG due to folate deficiency was not regulated by DNA methylation. What remains unclear is whether increased IGF2 served any role in the suppression of ductal elongation before PND 21, given that IGF2 has been described as mitogenic for the MG epithelium (43) and is associated with increased risk of human breast cancer (48).

The suppression of epithelial growth between PND 1 and PND 21 was associated with a parallel reduction in the area of the supramammary lymph nodes. Folate deficiency negatively affects the lymphoid system in both humans and animal models, leading to impaired cell-mediated immunity (49–53). When isometrically growing MG ducts start allometric growth in response to dietary fat, there is concurrent enlargement of the supramammary lymph nodes (29), suggesting a relation between supramammary lymph node size and epithelial growth. The immune system is intrinsically linked to MG ductal development, particularly through the actions of leukocytes within the local microenvironment. Macrophages are recruited to, and stimulate the formation of, TEBs, as mediated by the actions of colony stimulating factor 1 (CSF1), where deletion of CSF1 impairs ductal elongation (54). In a similar way, eosinophils are closely apposed to TEBs and are also required for normal ductal outgrowth (55). Although we did not quantify lymphocyte abundance within the MG of folate-deficient mice, we posit that a reduction in their recruitment to the MG ducts would accompany the reduction in ductal outgrowth. The complete relation between immune function, the supramammary lymph node, ductal growth, and IGF2 requires further exploration.

Considering that folate deficiency in an OVX-induced model of isometric growth from PND 21 to 77 (study 2) had no effect on the ductal epithelium, and that the supramammary lymph nodes and thymic medulla were smaller in both studies, the deleterious effects of folate deficiency on the lymphoid system are apparently not stage-specific, in contrast to the MG phenotype. Whereas others found that dietary folate deficiency for 4 wk led to deficiency (56), the MGs of Def females in study 2 may not have reached and sustained a deficient state early enough in MG development. Also, our model of folate deficiency was through feeding a diet devoid of FA, whereas a more severe folate deficiency can be achieved by combining an FA-devoid diet with use of succinylsulfathiazole to inhibit microbial production of folate in the gut (57). We did not use that approach in these studies because we induced folate deficiency before and throughout pregnancy, whereas female mice exposed to both Def and succinylsulfathiazole have poor reproductive outcomes (37).

Retarded ductal outgrowth induced by folate deficiency before weaning in study 1 was fully reversed to an allometric state after the onset of puberty. Similarly, the area of epithelial hyperplasia at PND 21 was reduced by folate deficiency and restored by PND 42. This transient, stage-specific, and reversible phenotype is noteworthy and intriguing given that most studies find ductal outgrowth during puberty is most sensitive to growth suppression. We conclude that the stunted epithelial rudiment in deficient female progeny retained full responsiveness to known mitogenic factors such as estrogen, growth hormone, and IGF1 (1). The PyMT oncogene also stimulates both themitogen-activated protein kinase and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha pathways (58), and the latter is activated by IGF2. These increased sources of mitogenic stimulation may have masked any long-term effects of folate deficiency on hyperplastic growth. Alternatively, estrogen increases expression of phosphatidylethanolamine methyltransferase, which synthesizes phosphatidylcholine, a primary source of choline that provides one-carbon units (59) as an alternative pathway for methylation reactions, such as DNA methylation and biosynthesis, independently of folate requirements (60).

In our hands there were no significant effects of Ex or S-Ex diets on either MG development or hyperplastic growth. It is possible that folate and one-carbon metabolism were

already optimal in response to the FA concentration in the Con diet, and that liver folate concentrations were replete, so that retention of excess FA was diminished in the Ex and S-Ex groups. This outcome contrasts with data from previous studies, where in utero and postnatal excess dietary FA decreased the ratio of TEBs to alveolar buds and increased the incidence of 7,12dimethylbenz[a]anthracene-induced MG tumors (20, 21). Another study found that excess methionine, choline, folate, and vitamin B-12 in utero decreased the incidence of *N*-nitroso-*N*-methylurea–induced tumors (61). These inconsistencies may lie in differences between the study designs, e.g., using chemical carcinogens as opposed to our transgenic expression of PyMT, which may have overwhelmed the effects, if any, of Ex or S-Ex diets. Furthermore, all other studies have been performed using rats, which have notable differences in the histomorphological development of their MGs compared with mice (1, 62).

In conclusion, our findings highlight the potential importance of adequate dietary FA during preweaning development of the MG. Given that the functional framework of the MG is established during this period, there may be lifelong implications from folate deficiency in the MG that could set the stage for subsequent development of tumorigenesis.

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