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Peri-gestational Dietary Folic Acid Deficiency Protects Against Medulloblastoma Formation in a Mouse Model of Nevroid Basal Cell Carcinoma Syndrome

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Abstract

Hereditary nevroid basal cell carcinoma syndrome (NBCCS) is caused by *PTCH1* gene mutations that result in diverse neoplasms including medulloblastoma (MB). Epidemiological studies report reduced pediatric brain tumor risks associated with maternal intake of prenatal vitamins containing folic acid (FA) and FA supplements specifically. We hypothesized that low maternal FA intake during the peri-gestational period would increase MB incidence in a transgenic NBCCS mouse model, which carries an autosomal dominant mutation in the *Ptch1* gene. Female wild-type C57BL/6 mice (n=126) were randomized to one of three diets with differing FA amounts: 0.3 mg/kg (low), 2.0 mg/kg (control), and 8.0 mg/kg (high) one month prior to mating with *Ptch1*^{+/-} C57BL/6 males. Females were maintained on the diet until pup weaning; the pups were then aged for tumor development. Compared to the control group, offspring MB incidence was significantly lower in the low FA group (Hazard Ratio (HR)=0.47; 95% confidence interval (CI) 0.27–0.80) at one year. No significant difference in incidence was observed between the control and high FA groups. Low maternal peri-gestational FA levels may decrease MB incidence in mice genetically

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predisposed to tumor development. Our results could have implications for prenatal FA intake recommendations in the presence of cancer syndromes.

Introduction

Medulloblastoma (MB) is a cerebellar tumor representing the most common malignant central nervous system (CNS) tumor in children aged 0–4 years. The peak incidence occurs between 1–4 years of age with a 1.6 fold higher incidence in males than females¹. Approximately 66% of children survive five years from diagnosis² and many long-term survivors face adverse late effects as a result of the disease and its treatment^{3–7}. These data underscore the need to understand the biology of MB and identify preventable causes.

Established risk factors for MB include prenatal radiation exposure (which accounts for very few cases currently) and certain genetic syndromes including Li-Fraumeni, tuberous sclerosis, and nevoid basal cell carcinoma syndrome (NBCCS)⁸. NBCCS, also known as Gorlin Syndrome, is an autosomal dominant disorder with near complete penetrance and extremely variable phenotype caused by a *PTCH1* mutation. NBCCS is estimated to affect one in 55,600 persons with symptom onset starting as early as age two years and a median onset of age 25 years^{9,10}. NBCCS is characterized by the presence of a variety of developmental anomalies and predisposition to several cancers especially basal cell carcinomas, rhabdomyosarcoma, and MB¹¹. The risk of an NBCCS patient developing MB in the first years of life is 5–10%^{9,11}.

Other risk factors for MB include high birth weight (increased risk), and prenatal vitamin supplementation (decreased risk), suggesting that factors related to maternal nutrition influence MB development in the child^{12,13}. In particular, prenatal vitamin supplementation has been associated with a reduced risk of childhood brain tumors in the offspring in several studies¹². Since folic acid (FA) reduces the risk of neural tube defects¹⁴, it has been hypothesized that FA may be the mediating nutrient in prenatal vitamins accounting for the protective effect observed for MB^{15,16}, with recent support for this hypothesis from an Australian study of childhood brain tumors¹⁷.

Two main mechanisms have been proposed to explain how dietary FA could impact cancer risk. The first is through its role as a cofactor in the synthesis of purines and thymidylate that are necessary for nucleic acid synthesis. Deficiency in these molecules could increase cancer risk through higher rates of uracil incorporation into DNA resulting in breakage¹⁸ that could subsequently lead to translocations and other chromosomal abnormalities¹⁹. The second mechanism is through its role as a necessary factor in the synthesis of S-adenosylmethionine (SAM), a donor of methyl groups for DNA methylation reactions^{20,21}. DNA methylation has been linked to gene expression levels^{22,23} and has been shown to be disturbed in nearly all tumors²⁴. Furthermore, aberrant methylation of the imprinted gene insulin-like growth factor-2 (IGF-2) has been linked to an increased risk of childhood embryonal tumors and colon cancer in adults²⁴.

To clarify whether FA exposure during pregnancy can influence MB incidence, we modeled the association between maternal dietary FA intake and MB development in offspring using a mouse model of MB with intermediate penetrance. We used mice that were heterozygous for the *Ptch1* mutation²⁵. Analogous to humans with NBCCS, mice with a mutant *Ptch1* allele have an increased risk for MB, rhabdomyosarcoma, and basal cell carcinoma. We found that low maternal dietary FA intake decreased MB incidence in offspring, suggesting that nascent tumors developing after loss of *Ptch1* function are highly sensitive to folate levels. Our results also imply that higher maternal dietary FA level may influence tumor development in genetically susceptible populations such as NBCCS, and that optimal

maternal dietary FA levels for people with cancer syndromes may differ from those of the general public.

Materials and methods

Animals and diets

The University of Minnesota Institutional Animal Care and Use Committee approved all protocols for this study. All mice were housed in plastic cages layered with shavings in a temperature and humidity-controlled room with 12-hour day/night cycles and had *ad libitum* access to food and water throughout the experiment. Our breeding colony was established with 3 heterozygous *Ptch1*^{+/-} male mice obtained from Dr. James Wascheck that had been crossed onto the C57BL/6 background for at least six generations²⁵. *Ptch1*^{+/-} mice were bred for a minimum of three additional generations prior to being assigned to the experiment to ensure genetic homogeneity of experimental mice. Six to eight week old wild-type (*Ptch1*^{+/+}) female C57BL/6 female mice were purchased from Charles River Laboratory (Wilmington, MA) and assigned randomly to one of three amino acid defined diets that varied with respect to the FA content (described below). The female mice were fed the diets for one month prior to crossing them with *Ptch1*^{+/-} male mice from the breeding colony. The dams were maintained on the diets until weaning at ~3 weeks at which time the offspring were removed from their mothers and placed on normal chow containing 4 mg/kg FA (Teklad Lab Animal Diets 2018, Harlan Laboratories, Madison, WI) for the experiment's duration. Offspring were followed for up to twelve months for tumor development (Figure 1). Mice were monitored 3 times a week for signs of morbidity and neurodegenerative problems such as loss of balance, reduced motor function, and other behavioral abnormalities that are indicative of a cerebellum tumor in this mouse model (www.jax.org)²⁶. If any of these signs were present, the mouse was euthanized by CO₂ asphyxiation followed by necropsy. The age of death was defined as the number of days between birth and euthanization.

Amino acid defined diets (TD.070056-TD.070058) were obtained from Harlan Laboratories (Madison, WI). The diets contained 0.3 (low), 2.0 (control), or 8.0 (high) mg/kg FA and 1% succinylsulfathiazole to prevent the synthesis of folate by intestinal bacteria²⁷. Diets had identical formulation with the exception of FA and contained 15.4% protein, 63.9% carbohydrate (CHO), and 8% fat by weight. The low and control FA dosages were chosen based on the minimum dosage that has been shown to be compatible with a successful pregnancy outcome by other investigators, on recommendations of the National Research Council²⁸, and on results from our preliminary studies that showed dose dependent increases in RBC folate in association with dietary FA in both the dams and their offspring.

Genotyping

Tails were clipped from pups at ~2 weeks of age and genomic DNA (gDNA) was purified using the DNeasy Blood & Tissue Kit (cat. # 69504, information available at <http://www.qiagen.com/products/genomicdnastabilizationpurification/dneasytissuesystem/dneasybloodtissuekit.aspx>) according to the manufacturer's instructions. Genotyping of animals for the *Ptch1* mutant allele was conducted with minor modifications using the Jackson Laboratory (Bar Harbor, ME) standard protocol available at <http://jaxmice.jax.org/strain/003081.html>. Briefly, PCR reactions containing 1 µl gDNA, 1X PCR Buffer (Carlsbad, CA), 0.2 mM dNTPs, 1.5 mM MgCl₂, 20 mM of each primer below, 1 unit of Taq polymerase were carried out in an Eppendorf Mastercycler Gradient thermocycler at 94°C for 1.5 minutes, 94°C for 30 seconds, 69°C for 1 minute, 72°C for 45 seconds for 35 cycles, followed by 72°C for 2 minutes. The PCR products were resolved on 1.5% agarose gels with an Invitrogen TrackIt 50 bp DNA ladder (cat # 10488-043, Carlsbad, CA) by

running them at 100V for 1 hour. The primer sequences were as follows: *Ptch* 1 Forward (5'-GCCCTGAATGAAGTGCAGGACG-3'); *Ptch* 2 Reverse (5'-CACGGGTAGCCAACGCTATGTC-3'); WT 1 Forward (5'-CTGCGGCAACTTTTGGTTG-3'); and WT 2 Reverse (5'-AGGGCTTCTCGTTGGCTACAAG-3'). In mice that are heterozygous for the *Ptch1* mutant allele, two amplicons of 479 and 200 base pair fragments are observed that are indicative of the mutant and wild-type alleles respectively.

Red blood cell folate measurement

Samples of 25–50 µl of peripheral blood were obtained from the dams using glass capillary tubes coated with a non-coagulant, EDTA, through retro-orbital bleeding in accordance with NIH guidelines (<http://oacu.od.nih.gov/ARAC/Bleeding.pdf>) at weaning of their pups. Diluted aliquots (1:11) of the dam's blood in 1% ascorbic acid were frozen at –80°C. RBC folate was measured using a modified version of the lactobacillus assay^{29–31} as described briefly in Supplementary Methods.

Histology

Mice were necropsied when they became moribund or at one year of age, whichever came first. Brains were removed and visually inspected for cerebellar tumors and/or hydrocephalus and then later confirmed by histopathology. Two separate board-certified pathologists (American Board of Pathology) confirmed all tumors. If the pathologist disagreed, deeper sections of the tissues were made into slides and the pathologists re-examined the sample in question together to reach a diagnosis. Brain tumor samples, spleens and any abnormal tissues were archived by fixation in 10% buffered formalin and snap frozen in liquid nitrogen. Formalin fixed-paraffin embedded sections from brain tissues were sectioned at 5 microns using a standard microtome, mounted and heat-fixed onto glass slides. Slides were processed and stained with hematoxylin-eosin (HE) using standard protocols. The pathologist used the criteria of densely packed small, anaplastic cells with scant cytoplasm, and polygonal nuclei to verify that the tumor histology resembles that of human MBs in appearance.

Statistical analysis

All statistical analyses were performed using SAS version 9.2 (Cary, NC). Significant differences in maternal RBC folate levels and offspring survival times between dietary FA groups were assessed using one-way ANOVA. Differences in the frequency of offspring *Ptch1* heterozygosity by maternal dietary group were assessed using chi-square tests. We employed Cox proportional hazards regression analysis to compute hazard ratios (HRs) and 95% confidence intervals (CIs) to examine whether the hazard of offspring MB was different in offspring exposed to the low and high maternal FA diet compared to the control FA diet. The robust sandwich covariance matrix³² was specified to control for non-independence of litter mates. The effect of FA levels on MB and overall survival was also examined using Kaplan-Meier analysis using Prism by Graph Pad Software with statistical differences in survival times between the groups calculated by the log rank test controlling for multiple comparisons through the Sidak method³³. In both Cox proportional hazards regression and Kaplan-Meier analysis, mice that had a microscopically confirmed cerebellar tumor were classified as events, while those who had no signs of tumor were classified as non-events. Survival time was calculated as the number of weeks from birth to tumor development for events and from birth to death from other cause or one year of age, whichever came first for non-events. Differences in median survival time were assessed using the log rank test. Two tailed p-values of 0.05 were considered statistically significant.

Results

The experimental design is shown in Figure 1. A total of 126 female mice were assigned to low, control, and high FA diets (42 mice/group). There were no significant differences in the proportion of females with litters ($p=0.9$), litter size ($p=0.5$) or the percentage of heterozygote offspring ($p=0.95$) between the three diet groups (Table 1). In previous work, we established that RBC folate levels in both the dams and offspring at weaning are correlated with maternal dietary FA intake (Supplementary Figure 1). In this experiment, we only measured maternal RBC folate at weaning and found that higher maternal dietary FA intake was significantly associated with RBC folate level at weaning ($p<0.001$) in a sample of dams assigned to each of the three dietary FA levels (low $n=15$; control $n=21$, high $n=17$) (Figure 2). One hundred fifty-six heterozygote offspring (low: $n=53$, control: $n=50$, high: $n=53$) that survived to weaning were observed for ~one year for MB development.

The cumulative incidence of microscopically confirmed MB was 28% for the low FA diet, 50% for the normal diet and 45% for the high diet (Table 2). The mean latency of tumor development varied significantly between groups with means of 28.5, 19.0, and 26.1 weeks for the 0.3, 2.0, and 8.0 groups, respectively (ANOVA p -value 0.009). Some of the offspring ($n=9$) that became moribund did not have macroscopic tumors despite displaying signs similar to mice with macroscopic MB (enlarged head, ataxia). Upon histological analysis, these mice had intracranial signs indicative of hydrocephalus and did not have cerebellar neoplastic lesions that could be microscopically detected. The frequency of hydrocephalus varied non-significantly (Fisher's exact p -value=0.6) by maternal FA group with 7.5% (4/53), 2.0% (1/50) and 7.5% (4/53) in the low, control, and high FA groups (Table 2).

Cox proportional hazards regression analyses indicated a significantly reduced hazard for MB development in the offspring of mothers who were fed the low FA diet compared to those fed the control FA diet (HR=0.47, 95% CI 0.27–0.80) (Table 2). There was no statistical difference in tumor incidence between offspring of mothers fed the high vs. control FA diet (HR=0.73, 95% CI 0.43–1.23).

Kaplan-Meier survival analyses indicated a more rapid rate of overall mortality for offspring of mothers assigned to the control vs. those assigned to the low or high FA group. Although the overall difference in survival from any cause between groups was not statistically significant ($p=0.63$) (Figure 3A), there was a marginally significant overall difference in the MB survival curves between maternal dietary FA groups ($p=0.06$). The survival difference in offspring with MB at death was significant between the low and control groups ($p=0.02$) (Figure 3B). At necropsy, 64 total cerebellar tumors were grossly visible and subsequently confirmed microscopically by two pathologists. The tumors were found to recapitulate the classic subtype of MB (Figure 4).

Discussion

We examined whether maternal dietary FA level from conception through weaning affects MB tumor incidence in a genetically engineered mouse model of a cancer syndrome that incurs a strong predisposition to MB development through a germ line mutation in the *Ptch1* gene. Surprisingly, we observed a significantly lower incidence of MB at one year in the offspring of dams assigned to the low vs. control FA diet. Maternal dietary FA had no detectable influence on heterozygote frequency at weaning or overall survival. *Ptch1*^{+/-} mice are predisposed to structural defects in their ventricles, which can lead to the obstruction of proper flow of the cerebrospinal fluid and thus lead to hydrocephalus³⁴. This defect was macroscopically observed at necropsy in mice belonging to all FA diet groups. The developmental abnormalities observed in the ventricles of *Ptch1*^{+/-} brains could explain

why we did not observe the same trend in overall survival between the FA dietary groups as we observed in overall tumor burden.

MB incidence in the *Ptch1*^{+/-} heterozygote offspring of mothers fed low dietary FA was 17% lower than in those fed the two higher dietary FA doses. No significant difference in MB incidence was observed between the control and high FA groups. Lawrance et al³⁶ found a nonlinear effect of maternal dietary FA on the incidence of offspring colon adenoma in the *Apc*^{min/+} mouse model. A significantly lower adenoma incidence was observed in the offspring of dams fed a FA deficient diet (0.3 mg/kg) compared to those of mothers who were fed the control FA (2 mg/kg) or high FA (20 mg/kg) diet at 10 weeks. Also similar to our results, no significant difference in tumor incidence was observed between the control and high FA groups. The authors postulated that “any factor that limits folate availability to the intestine of the developing offspring” could impede progression of early tumors in mice that have a strong genetic predisposition to early onset adenomas. By analogy, the C57BL/6 *Ptch1*^{+/-} mouse model has a high MB incidence with reported incidences ranging from ~25–>40%^{25,37} with palpable tumors developing as early as 3 weeks of age. If FA is necessary for progression of early MB lesions, then developmental FA deficiency could block tumor growth and consequently result in a lower offspring tumor incidence.

Maternal dietary FA plays an important role in fetal brain development after neural tube closure. After a period of rapid cell division and growth during which high FA levels are important for neural tube closure in the first weeks of pregnancy, the level of FA continues to play an essential role in brain development including that of the cerebellum. Maternal FA deficiency has been shown to lead to reduced proliferation of progenitor cells and increased apoptosis in regions of the brain³⁹, providing a biological mechanism by which low FA could protect against brain tumor development. Future time course experiments that evaluate the incidence of preneoplastic lesions in offspring exposed to varying levels of FA during the peri-gestational period will help to clarify whether low maternal dietary FA hampers progression of early tumors.

Our results differ from those of human epidemiological studies of sporadic childhood brain tumors. Most previous studies, including those of primitive neural ectodermal tumors and MB specifically, have reported protective effects in association with prenatal vitamin or folic acid supplementation with odds ratios ranging from 0.5–0.9^{12,15–17,40,41}. No association was found for dietary FA specifically in one case-control study⁴², which could be due to the difficulty of accurately measuring past dietary FA intake by survey^{43,44}. If prenatal FA protects against childhood brain tumors, we would expect to see a decreasing incidence trend following mandatory FA fortification of enriched grains and cereal products between 1996–1998 in the United States; however a recent large population-based ecological study reported no change in MB incidence following FA fortification⁴⁵, making it now seem unlikely that maternal FA exposure has a strong protective effect on childhood brain tumors. However, the unexpected finding from our study in a mouse model of a cancer syndrome suggests the possibility that the association between FA and childhood brain tumors may differ between high risk populations and the general population.

In conclusion, our study provides evidence that low maternal FA during the peri-gestational period decreases MB incidence in a genetically engineered mouse model of a human cancer syndrome. These results raise questions about FA supplementation in pregnant women in the presence of cancer syndromes. Further research is needed from other mouse models and in human epidemiological studies to determine whether there is any adverse influence of higher levels of FA during the peri-gestational period in individuals with a strong genetic predisposition to childhood cancer.

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References

1. Surveillance, Epidemiology, and End Results (SEER) Program. SEER*Stat Database: Incidence - SEER 13 Regs Research Data, Nov 2010 Sub (1973–2008) <Single Ages to 85+, Katrina/Rita Population Adjustment> - Linked To County Attributes - Total U.S., 1969–2009 Counties. National Cancer Institute, DCCPS, Surveillance Research Program, Cancer Statistics Branch; (www.seer.cancer.gov)released April 2012, based on the November 2011 submission
2. Linabery AM, Ross JA. Childhood and adolescent cancer survival in the US by race and ethnicity for the diagnostic period 1975–1999. *Cancer*. 2008; 113(9):2575–96. [PubMed: 18837040]
3. Bowers DC, Liu Y, Leisenring W, McNeil E, Stovall M, Gurney JG, Robison LL, Packer RJ, Oeffinger KC. Late-occurring stroke among long-term survivors of childhood leukemia and brain tumors: a report from the Childhood Cancer Survivor Study. *J Clin Oncol*. 2006; 24(33):5277–82. [PubMed: 17088567]
4. Neglia JP, Robison LL, Stovall M, Liu Y, Packer RJ, Hammond S, Yasui Y, Kasper CE, Mertens AC, Donaldson SS, Meadows AT, Inskip PD. New primary neoplasms of the central nervous system in survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *J Natl Cancer Inst*. 2006; 98(21):1528–37. [PubMed: 17077355]
5. Ness KK, Mertens AC, Hudson MM, Wall MM, Leisenring WM, Oeffinger KC, Sklar CA, Robison LL, Gurney JG. Limitations on physical performance and daily activities among long-term survivors of childhood cancer. *Ann Intern Med*. 2005; 143(9):639–47. [PubMed: 16263886]
6. Pang JW, Friedman DL, Whitton JA, Stovall M, Mertens AC, Robison LL, Weiss NS. Employment status among adult survivors in the Childhood Cancer Survivor Study. *Pediatr Blood Cancer*. 2008; 50(1):104–10. [PubMed: 17554791]
7. Park ER, Li FP, Liu Y, Emmons KM, Ablin A, Robison LL, Mertens AC. Health insurance coverage in survivors of childhood cancer: the Childhood Cancer Survivor Study. *J Clin Oncol*. 2005; 23(36):9187–97. [PubMed: 16361621]
8. Pizzo, PA.; Poplack, DG. Principles and practice of pediatric oncology. 5. Philadelphia: Lippincott Williams & Wilkins; 2006.
9. Lo Muzio L. Nevroid basal cell carcinoma syndrome (Gorlin syndrome). *Orphanet J Rare Dis*. 2008; 3:32. [PubMed: 19032739]
10. Jones EA, Sajid MI, Shenton A, Evans DG. Basal cell carcinomas in gorlin syndrome: a review of 202 patients. *J Skin Cancer*. 2011; 2011:217378. [PubMed: 21152126]
11. Shah M, Mavers M, Bree A, Fosko S, Lents NH. Quality of life and depression assessment in nevroid basal cell carcinoma syndrome. *Int J Dermatol*. 2011; 50(3):268–76. [PubMed: 21342158]
12. Goh YI, Koren G. Prenatal supplementation with multivitamins and the incidence of pediatric cancers: clinical and methodological considerations. *Pediatr Blood Cancer*. 2008; 50(2 Suppl): 487–9. discussion 498. [PubMed: 18064661]
13. Harder T, Plagemann A, Harder A. Birth weight and subsequent risk of childhood primary brain tumors: a meta-analysis. *Am J Epidemiol*. 2008; 168(4):366–73. [PubMed: 18579539]
14. Eichholzer M, Tonz O, Zimmermann R. Folic acid: a public-health challenge. *Lancet*. 2006; 367(9519):1352–61. [PubMed: 16631914]
15. Bunin GR, Gallagher PR, Rorke-Adams LB, Robison LL, Cnaan A. Maternal supplement, micronutrient, and cured meat intake during pregnancy and risk of medulloblastoma during childhood: a children's oncology group study. *Cancer Epidemiol Biomarkers Prev*. 2006; 15(9): 1660–7. [PubMed: 16985028]

16. Bunin GR, Kuijten RR, Buckley JD, Rorke LB, Meadows AT. Relation between maternal diet and subsequent primitive neuroectodermal brain tumors in young children. *N Engl J Med*. 1993; 329(8):536–41. [PubMed: 8336753]
17. Milne E, Greenop KR, Bower C, Miller M, van Bockxmeer FM, Scott RJ, de Klerk NH, Ashton LJ, Gottardo NG, Armstrong BK. Maternal use of folic acid and other supplements and risk of childhood brain tumors. *Cancer Epidemiol Biomarkers Prev*. 2012; 21(11):1933–41. [PubMed: 22941336]
18. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, Wickramasinghe SN, Everson RB, Ames BN. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A*. 1997; 94(7):3290–5. [PubMed: 9096386]
19. Povirk LF. Biochemical mechanisms of chromosomal translocations resulting from DNA double-strand breaks. *DNA Repair (Amst)*. 2006; 5(9–10):1199–212. [PubMed: 16822725]
20. Stover PJ. Physiology of folate and vitamin B12 in health and disease. *Nutr Rev*. 2004; 62(6 Pt 2):S3–12. discussion S13. [PubMed: 15298442]
21. Van den Veyver IB. Genetic effects of methylation diets. *Annu Rev Nutr*. 2002; 22:255–82. [PubMed: 12055346]
22. Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition*. 2004; 20(1):63–8. [PubMed: 14698016]
23. Weksberg R, Shuman C, Smith AC. Beckwith-Wiedemann syndrome. *Am J Med Genet C Semin Med Genet*. 2005; 137C(1):12–23. [PubMed: 16010676]
24. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet*. 2006; 7(1):21–33. [PubMed: 16369569]
25. Lelievre V, Seksenyan A, Nobuta H, Yong WH, Chhith S, Niewiadomski P, Cohen JR, Dong H, Flores A, Liao LM, Kornblum HI, Scott MP, Waschek JA. Disruption of the PACAP gene promotes medulloblastoma in *ptc1* mutant mice. *Dev Biol*. 2008; 313(1):359–70. [PubMed: 18036580]
26. Hahn H, Wojnowski L, Miller G, Zimmer A. The patched signaling pathway in tumorigenesis and development: lessons from animal models. *J Mol Med (Berl)*. 1999; 77(6):459–68. [PubMed: 10475061]
27. Sepehr E, Peace RW, Storey KB, Jee P, Lampi BJ, Brooks SP. Folate derived from cecal bacterial fermentation does not increase liver folate stores in 28-d folate-depleted male Sprague-Dawley rats. *J Nutr*. 2003; 133(5):1347–54. [PubMed: 12730421]
28. National Research Council (U.S.). Subcommittee on Laboratory Animal Nutrition. Nutrient requirements of laboratory animals. Nutrient requirements of domestic animals. 4. Washington, D.C: National Academy Press; 1995. rev. ed
29. Pfeiffer CM, Zhang M, Lacher DA, Molloy AM, Tamura T, Yetley EA, Picciano MF, Johnson CL. Comparison of serum and red blood cell folate microbiologic assays for national population surveys. *J Nutr*. 2011; 141(7):1402–9. [PubMed: 21613453]
30. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. *Methods Enzymol*. 1997; 281:43–53. [PubMed: 9250965]
31. O’Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. *J Clin Pathol*. 1992; 45(4):344–7. [PubMed: 1577973]
32. Lee, EWL. Amato D Cox-Type Regression Analysis for Large Numbers of Small Groups of Correlated Failure Time Observations. Netherlands: Kluwer Academic Publishers; 1992. p. 237–247.
33. Allison, PD. Institute S. Survival analysis using the SAS system : a practical guide. Cary, NC: SAS Institute; 1995.
34. Svard J, Rozell B, Toftgard R, Teglund S. Tumor suppressor gene co-operativity in compound Patched1 and suppressor of fused heterozygous mutant mice. *Mol Carcinog*. 2009; 48(5):408–19. [PubMed: 18781608]

35. Hahn ME, Karkowski L, Weinreb L, Henry A, Schanz N, Hahn EM. Genetic and developmental influences on infant mouse ultrasonic calling. II. Developmental patterns in the calls of mice 2–12 days of age. *Behav Genet.* 1998; 28(4):315–25. [PubMed: 9803024]
36. Lawrance AK, Deng L, Rozen R. Methylenetetrahydrofolate reductase deficiency and low dietary folate reduce tumorigenesis in *Apc min/+* mice. *Gut.* 2009; 58(6):805–11. [PubMed: 19174418]
37. Tanori M, Santone M, Mancuso M, Pasquali E, Leonardi S, Di Majo V, Rebessi S, Saran A, Pazzaglia S. Developmental and oncogenic effects of insulin-like growth factor-I in *Ptc1+/-* mouse cerebellum. *Mol Cancer.* 2010; 9:53. [PubMed: 20214787]
38. Tanori M, Pasquali E, Leonardi S, Giardullo P, Di Majo V, Taccioli G, Essers J, Kanaar R, Mullenders LH, Atkinson MJ, Mancuso M, Saran A, Pazzaglia S. Opposite modifying effects of HR and NHEJ deficiency on cancer risk in *Ptc1* heterozygous mouse cerebellum. *Oncogene.* 2011; 30(47):4740–9. [PubMed: 21602895]
39. Craciunescu CN, Johnson AR, Zeisel SH. Dietary choline reverses some, but not all, effects of folate deficiency on neurogenesis and apoptosis in fetal mouse brain. *J Nutr.* 2010; 140(6):1162–6. [PubMed: 20392884]
40. Stalberg K, Haglund B, Stromberg B, Kieler H. Prenatal exposure to medicines and the risk of childhood brain tumor. *Cancer Epidemiol.* 2010; 34(4):400–4. [PubMed: 20510665]
41. Preston-Martin S, Pogoda JM, Mueller BA, Lubin F, Modan B, Holly EA, Filippini G, Cordier S, Peris-Bonet R, Choi W, Little J, Arslan A. Results from an international case-control study of childhood brain tumors: the role of prenatal vitamin supplementation. *Environ Health Perspect.* 1998; 106 (Suppl 3):887–92. [PubMed: 9646053]
42. Bunin GR, Kushi LH, Gallagher PR, Rorke-Adams LB, McBride ML, Cnaan A. Maternal diet during pregnancy and its association with medulloblastoma in children: a children's oncology group study (United States). *Cancer Causes Control.* 2005; 16(7):877–91. [PubMed: 16132798]
43. Fayet F, Flood V, Petocz P, Samman S. Relative and biomarker-based validity of a food frequency questionnaire that measures the intakes of vitamin B(12), folate, iron, and zinc in young women. *Nutr Res.* 2011; 31(1):14–20. [PubMed: 21310301]
44. Bunin GR, Gyllstrom ME, Brown JE, Kahn EB, Kushi LH. Recall of diet during a past pregnancy. *Am J Epidemiol.* 2001; 154(12):1136–42. [PubMed: 11744519]
45. Linabery AM, Johnson KJ, Ross JA. Childhood cancer incidence trends in association with US folic acid fortification (1986–2008). *Pediatrics.* 2012; 129(6):1125–33. [PubMed: 22614769]

Appendix

See separate file for supplementary material

Figure 1. Experimental design

One hundred and twenty-six female mice were assigned in equal numbers to one of three maternal dietary FA groups (0.3 mg/kg (low), 2.0 mg/kg (control), or 8 mg/kg FA) one month prior to mating with *Ptch1*^{+/-} heterozygote male mice. Dams were kept on their respective diets until weaning of their litters at three weeks of age. Offspring were genotyped at 2 weeks of age and RBC folate measurements were performed on blood samples from dams taken at the time of weaning of their offspring. Upon weaning, offspring were switched to the standard mouse chow and followed for tumor development for 12 months.

Figure 2. Mean RBC folate measurements in dams at weaning

RBC folate was measured in dams at the weaning of their pups. RBC folate concentration increased significantly with increasing dietary FA dose ($p < 0.0001$). The number of mice included in the data for each bar was 15 (0.3), 21 (2), and 17 (8).

Figure 3. Kaplan-Meier Survival curves comparing survival of *Ptch1*^{+/-} offspring of mothers assigned to three different doses of folic acid prior to conception through weaning
A. Overall survival. The difference in survival between offspring of different diet groups is not statistically significant ($p=0.63$). **B. MB survival.** The difference in MB survival between offspring of the low and control FA groups was statistically significant ($p=0.02$).

Figure 4. Pathologic analyses of tumor tissue

Photomicrographs of hematoxylin and eosin stained representative tissue showing *Ptch1*^{+/-} brain sectioned in half (mid- or parasagittal sections). A) Representative section of brain with dilated lateral ventricle (highlighted by double-headed arrow). H – adjacent hippocampus. Image captured using 2X objective. B) Representative section of brain with medulloblastoma (denoted by asterisk) adjacent to uninvolvement cerebellum (CB). Image captured using 2X objective. C) Higher magnification of medulloblastoma represented in B. The neoplasm is characterized by relatively monomorphous sheets of small undifferentiated cells. Image captured using 20X objective.

Table 1

Reproductive characteristics of dams and offspring assigned to low, control, and high FA diets.

Folic acid diet (mg/kg)	Number of assigned dams	Number of litters (%)	Number of weanling offspring	Mean litter size (sd)	Number of <i>Ptch1</i> ^{+/-} offspring (%)
0.3 (low)	42	23 (55)	133	6.0 (1.6)	53 (40)
2.0 (control)	42	23 (55)	129	5.7 (2.0)	52 (40) ^a
8.0 (high)	42	21 (50)	132	6.3 (1.7)	55 (42) ^a
Total	126	67 (53)	394	6.0 (1.8)	160 (41)

^aIncludes four pups from the control and high FA diets that were genotyped at ~two weeks but were subsequently missing from the cage at weaning at three weeks (presumably due to maternal cannibalization).

Table 2

MB and hydrocephalus outcomes in *Ptch1*^{+/-} offspring.

	No. of <i>Ptch1</i> ^{+/-} offspring	MB all			MB only			MB + Hydrocephalus		Hydrocephalus only			No MB or Hydrocephalus No. (%)
		No. (%)	Median age (weeks)	Mean age (sd) (weeks)	No. (%)	Median age (weeks)	Mean age (sd) (weeks)	No. (%)	Age (weeks)	No. (%)	Median age (weeks)	Mean Age (sd) (weeks)	
Folic acid diet (mg/kg)													
0.3	53	15 (28.3)	29.7	28.5 (11.4)	14 (26.4)	30.2	30.2 (9.9)	1	5.6	4 (7.5)	5.0	5.4 (1.4)	34 (64.5)
2.0	50	25 (50.0)	16.4	19.0 (8.3)	25 (50)	16.4	19.0 (8.3)	0	-	1 (2.0)	19.6	19.6 (NA)	24 (48.0)
8.0	53	24 (45.2)	27.3	26.1 (10.9)	23 (43.4)	27.3	25.6 (10.9)	1	36.9	4 (7.5)	6.7	6.1 (3.4)	25 (47.2)
Total	156	64 (41.0)	21.4	23.9 (10.7)	62 (39.7)	21.4	24.0 (10.5)	2 (1.3)	21.2	9 (5.8)	5.9	7.5 (5.1)	83 (53.2)

Table 3
Cox proportional hazards regression analysis of offspring medulloblastoma development by dietary FA group
Cox proportional hazards regression models for the association between maternal FA diet and MB incidence.

Diet (mg/kg)	Hazard Ratio	95% CI	p-value ^a
Low (0.3)	0.47	0.27–0.80	0.006
Control (2.0)	1.0	Ref.	-
High (8.0)	0.73	0.43–1.23	0.24

^a p-value for statistical difference between test and control groups.