AD_____

Award Number:
W81XWH-09-1-0610

TITLE:
In Utero Exposure to Dietary Methyl Nutrients and Breast Cancer Risk in Offspring

PRINCIPAL INVESTIGATOR:
Chung S. Park, Ph.D.

CONTRACTING ORGANIZATION:

North Dakota State University

Fargo, ND 58102-3400

REPORT DATE:

September 2010

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2010	Annual	25 AUG 2009 - 24 AUG 2010

4. TITLE AND SUBTITLE
In Utero Exposure to Dietary Methyl Nutrients and Breast Cancer Risk in Offspring

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0610

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Chung Park

email: c.park@ndsu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

North Dakota State University
Fargo, ND 58102-3400

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research and Material Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Lipotropes (methionine, choline, folate, and vitamin B_{12}) are dietary methyl donors and cofactors that are involved in one-carbon metabolism providing methyl groups for all biological methylation pathways. This study assessed the effect of maternal lipotrope supplementation on breast cancer risk of the offspring using chemically-induced mammary carcinogenesis. We hypothesize that lipotrope supplementation reduces breast cancer risk of the offspring by inducing an epigenetic imprint (memory) of the expression of genes involved in development and differentiation of mammary tissue. In a series of in vivo experiments, the latency period was significantly increased in offspring with maternal lipotrope supplementation (2.17 weeks, P=0.008). Moreover, maternal lipotrope diet significantly reduced tumor volume (P=0.007) and tumor number (P=0.001) in offspring. The results suggest that maternal dietary lipotrope may reduce breast cancer risk of offspring, and warrants further investigation for development of dietary strategies for breast cancer reduction.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

1. Introduction

Lipotropes are methyl group (CH_3) containing essential nutrients (methionine, choline, folate, and vitamin B_{12}) and are important methyl donors and cofactors which play key roles in one-carbon metabolism. One-carbon metabolism provides methyl groups for all biological methylation pathways, and is highly dependent on methyl donors and cofactors (11, 17). The coenzymes necessary for DNA methylation reactions include folate, vitamin B_{12}, and riboflavin, whereas important donor compounds in this mechanism include methionine and choline (9, 10). Methyl groups needed for DNA methylation are acquired through the folate and methionine pathways, and DNA methylation patterns may be altered by changes in diet, genetic polymorphisms, and environmental chemicals (5, 20).

Maternal methyl supplements directly affect DNA methylation and gene expression which are required for cell growth, maintenance of tissue integrity, and long-term health in offspring (5). Maternal deficiency of methyl nutrients can cause fetal growth retardation and congenital abnormalities (13). For example, folic acid supplementation prevents both the occurrence and recurrence of neural tube defects (2) and significantly reduces the incidence of low birth weight (7). Maternal diets supplemented with methyl nutrients have positive effects on growth and health of the infant: this may be due to persistent epigenetic modifications in DNA methylation and gene expression in the offspring (19, 20).

DNA methylation is an essential epigenetic mechanism in maintaining cellular function and changes in the methylation pattern of specific genes are correlated with the development of autoimmune diseases and cancer (14). Epigenetic changes (i.e., DNA methylation and demethylation, methyl CpG recognition, histone modification, and chromatin remodeling) are inheritable and influenced by environmental factors, including diet (4, 16).

The term metabolic imprinting describes the process whereby cells have a biological memory for nutritional influences that can be passed on to daughter cells through mitosis (18). Nutritional status during a full-term pregnancy affects adult metabolism perhaps via epigenetic modifications of the expression of specific genes from the physiological process of pregnancy (3, 18). Maternal diet can alter the secretion of one or more hormones that regulate mammary growth and differentiation during pregnancy as growth of mammary parenchyma (secretory alveolar epithelial tissues) shift from isometric to allometric (1, 12). The most mammary gland growth occurs during pregnancy at an exponential rate (8). During this phase, mammary alveolar epithelial cells complete differentiation with gradual formation of an extensive development of the dynamic structure of the lobulo-alveolar clusters (lobules) [12, 15].

2. Body

Hypothesis: *In utero* exposure to methyl nutrients brings about a stable epigenetic imprint (memory) of the expression of genes that are involved in the development and differentiation of lobules in the mammary gland of the dam. These genomic signatures induced during the allometric growth period of the pregnancy may be concurrently passed on to female offspring; these prenatally imprinted gene signals, once set, may exert a protective effect against breast cancer development in the offspring.

Specific Aims: To determine the extent to which *in utero* exposure to methyl nutrients during pregnancy reduces and potentially prevents breast cancer risk of the offspring; it entails investigating a relationship between susceptibility of the offspring to mammary carcinogenesis and DNA methylation status and expression of genes.

Experimental Approach

Animal and Dietary Treatment. Fifty-four female Sprague-Dawley rats (10 weeks of age) and all experimental diets (control and methyl-supplemented AIN-93G) were purchased from Harlan (Madison, WI) and the rats were acclimated to the experimental environment of ~25^{o}C and a 12:12-hour light-dark cycle for 1 week with ad libitum access to water and a standard control AIN-93G diet. After acclimation, all rats were housed in pairs, in plastic boxes, until transferred to single housing in late gestation. After confirmation of mating, rats were housed individually in standard rat cages containing wood shavings, and randomly assigned to receive control or methyl-supplemented AIN-93G diets (Table 1). All animal procedures and techniques were approved by the Institutional Animal Care and Use Committee of North Dakota State University. The methyl-supplemented AIN-93G diet was designed to provide approximately 5 times more folic acid, choline, and vitamin B_{12} than in the control AIN-93G diet (Methionine was 1.8 times). The experimental diets were formulated to be both isocaloric and isonitrogenous. After lactation, the dams and offspring received basal control diet for the remainder of the trial.

Tumor Induction and Measurement. Female offspring from each dietary treatment group were injected intraperitoneally with a single dose of *N*-nitroso-*N*-methylurea (NMU, Sigma-Aldrich, St Louis, MO, 50 mg/kg body weight) dissolved in PBS acidified with acetic acid at 50 days of age. The rats were palpated twice weekly to detect the presence of mammary tumors. The time of appearance of the first tumor (latency period) and location of mammary tumors were recorded and tumor sizes were measured. The tumor volume was calculated using the following formula: (length X width^2)/2 (6).

Measurement of Global DNA Methylation. Mammary gland tissues were collected from dams and offspring in the later stage of lactation (on day 21 of lactation) immediately after sacrificing, and then the genomic DNA was purified by the standard method. The concentration of DNA was quantified using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Global levels of cytosine methylation in DNA samples were measured using Imprint Methylated DNA Quantification Kit (Sigma-Aldrich). DNA samples were incubated with capture and detection antibodies, and the absorbance was measured at 450 nm with a Spectra-Max Microplate Reader (Molecular Devices, Sunnyvale, CA). The global DNA methylation level was calculated and expressed based on the following formula: (treatment group absorbance / control group absorbance) X 100.

Real-time RT-PCR. Selected genes were analyzed for mammary development and differentiation (beta casein [Csn2, Qiagen catalog number QT00495047], acetyl-coenzyme A carboxylase alpha [Acaca, QT00190946], and gamma-glutamyl transferase 1 [Ggt1, QT00373072]), and beta-actin [Actb, QT00193473] was used as a control gene. Mammary gland tissues were placed in RNAlater (Ambion, Austin, TX) prior to freezing, and then homogenized. Total RNA was extracted from tissue samples using TRI-Reagent (MRC, Cincinnati, OH), and RNA concentration was quantified using NanoDrop ND-1000 (NanoDrop). One microgram of RNA sample was reverse-transcribed to cDNA with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA), in accordance with the manufacture's recommended protocol. Real-time RT-PCR was performed with QuantiTect Primers (Qiagen) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) using ABI Prism 7500 Sequence Detection System (Applied Biosystems). Normalization factors were calculated based on the geometric mean of control for changes in relative gene expression and the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis. Results are expressed as means ± SEM of independent determinations. Statistical analysis was performed using Student's *t*-test (Minitab Release 14.1, Minitab Inc., State College, PA). Differences were considered significant at $P<0.05$.

Results

This study determined the in vivo effect of *in utero* exposure to lipotropes using Sprague-Dawley rats. Breast tumors were induced in rats by intraperitoneal injection of the alkylating carcinogen NMU. There was no significant differences in average body weights ($P=0.769$) between control and lipotrope groups. However, the lipotrope-supplemented group showed decreased tumor incidence (Figure 1). The latency period was determined to be the days between NMU administration and the first day of tumor detection. As shown in Table 2, the latency period was significantly increased in offspring by maternal lipotrope diet (2.17 weeks, $P=0.008$). Moreover, the maternal lipotrope diet significantly reduced tumor volume (Figure 1B, $P=0.007$) and tumor numbers (Table 2, $P=0.001$) in offspring. However, there is no significant difference in global DNA methylation (Figure 2A) and the expression of mRNA of mammary development and differentiation related genes (Figure 3) in dams and offspring.

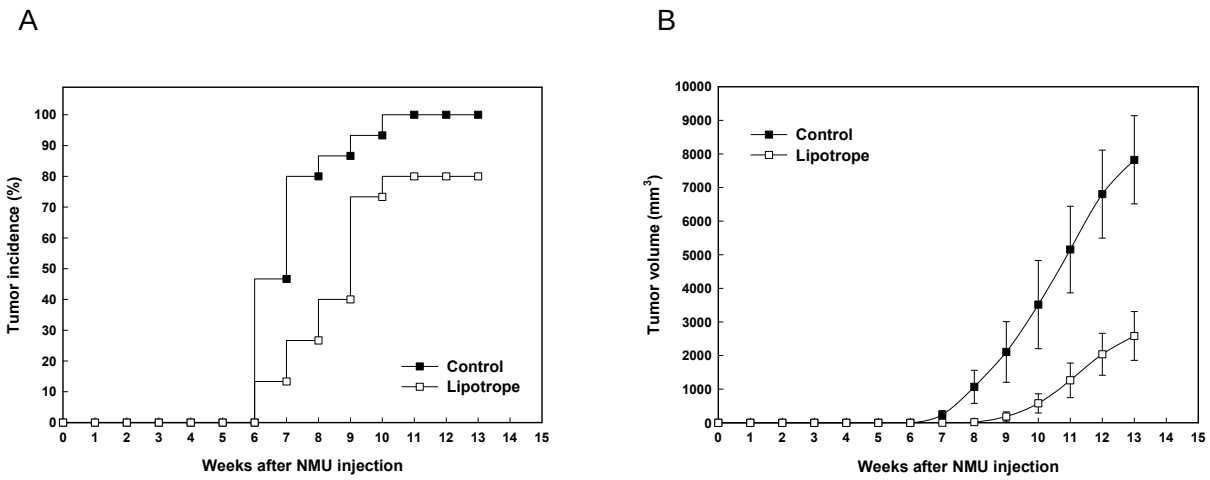

Figure 1 In vivo effects of lipotrope in offspring female rats. Data represent (A) tumor incidence, and (B) tumor volume (P=0.007) of rats exposed *in utero* to control and lipotrope diets and given a single dose of NMU carcinogen injection. Data are expressed as percentages of tumor incidence and tumor volumes (mm^3, n = 15).

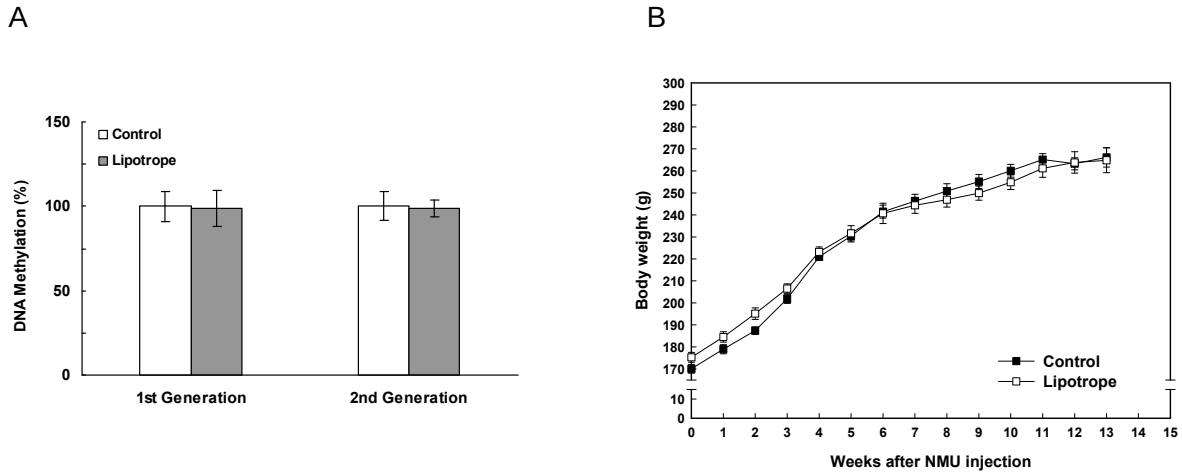

Figure 2 In vivo effects of lipotrope in female offspring. Data represent (A) Global DNA methylation of dams and offspring and (B) offspring body weight in response to a single dose of NMU injection. The global DNA methylation level was calculated and expressed based on the following formula: (treatment group absorbance / control group absorbance) X 100. Offspring body weight was expressed as means ± SEM, n = 15, P=0.769.

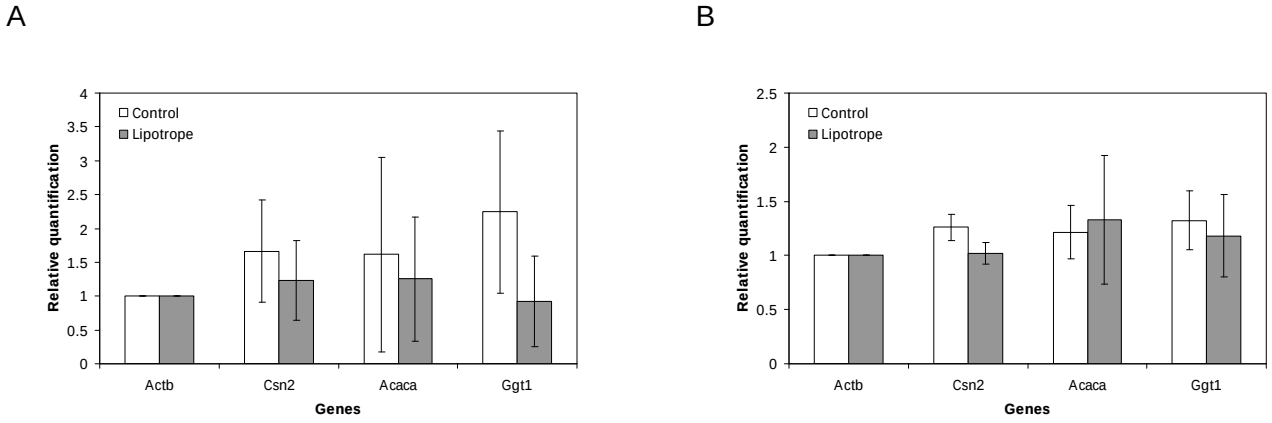

Figure 3. Effects of lipotrope in dams and offspring. Data represent expression of mRNA in (A) dams and (B) offspring of mammary tissues. Beta casein [Csn2], acetyl-coenzyme A carboxylase alpha [Acaca], and gamma-glutamyl transferase 1 [Ggt1]) were used for mammary development and differentiation, and beta-actin [Actb] was used as a control gene. Data are expressed as means ± SEM, $n = 5$. Asterisks indicate the difference between control and treatment ($P < 0.05$). The $2^{-\Delta\Delta Ct}$ method was used.

Table 1. Composition of the experimental diets.

Ingredients	Diets (per 100g)	
	Control	Lipotrope
Corn starch	39.8	38.4
Casein	20.0	20.0
Maltodextrin	13.2	13.2
Sucrose	10.0	10.0
Soybean oil	7.0	7.0
Cellulose	5.0	5.0
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
L-Cystine	0.3	0.3
Choline Bitartrate	0.25	1.25
TBHQ (antioxidant, mg)	1.4	1.4
L-Methionine		0.37
Folic Acid (mg)		0.8
Vitamin B12		0.01

Table 2. Comparison of the tumor incidence, latency period, and number of tumors, between control and lipotrope groups[1].

Treatments	Tumor incidence[2]	Latency period (weeks)	Tumor numbers	Tumor free rats (%)[3]
Control	15/15	7.93±0.32	2.93±0.20	0
Lipotrope	12/15	10.07±0.61	1.33±0.21	20
P-value		0.008	0.001	

[1]Values are means ± SEM.
[2,3]Values were determined at 13 weeks after NMU injection.

3. Key Research Accomplishments

- Global DNA methylation
- mRNA analysis of dam and offspring mammary tissue

- Tumor measurement of offspring
- Increased latency period seen in offspring whose dams received lipotrope supplemented diet
- Reduction in tumor volume and number observed in offspring whose dams received lipotrope supplemented diet

4. Reportable Outcomes

None.

5. Conclusion

Our study represents the first demonstration that high levels of maternal dietary intake of methyl nutrients may be beneficial in reducing breast cancer in the offspring. The prevention of cancer worldwide is one of the most pressing challenges facing scientists and public health policy-makers, among others. We hope that results from our studies will be useful in dietary counseling in breast cancer prevention and therapy. Data on the effects of methyl nutrient supplementation during pregnancy and susceptibility to mammary cancer of offspring may have clinical implications for the development of meaningful prenatal maternal dietary strategies that could prevent breast cancer development in offspring of women who have risk factors for breast cancer.

6. References

1. Akers, R. M. Lactation and the Mammary Gland, 1st ed., Iowa State University Press, Ames, IA, 2002.

2. Bailey, L.B., G.C. Rampersaud, and G.P.A. Kauwell. 2003. Folic acid supplements and fortification affect the risk for neural tube defects, vascular disease and cancer: evolving science. J. Nutr. 133(Suppl.):1961S-1968S.

3. Balogh, G.A., R. Heulings, D.A. Mailo, P.A. Russo, F. Sheriff, I.H. Russo, R. Moral, and J. Russo. 2006. Genomic signature induced by pregnancy in the human breast. Int. J. Oncol. 28:399-410.

4. Bird, A. 2002. DNA methylation patterns and epigenetic memory. Genes and Development 16(1):6-21.

5. Cooney, C.A., A.A. Dave, and G.L. Wolff. 2002. Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J. Nutr. 132(Suppl. 8):2393S-2400S.

6. Corbett, T., F. Valeriote, P. LoRusso, L. Polin, C. Panchapor, S. Pugh, K. White, J. Knight, L. Demchik, J. Jones, L. Jones, and L. Lisow. 1997. *In vivo* methods for screening and preclinical testing. In: Teicher BA. Anticancer drug development guide. Humana Press 75-99.

7. de Onis, M., J. Villar, and M. Gulmezoglu. 1998. Nutritional interventions to prevent intrauterine growth retardation: evidence from randomized controlled trials. Eur. J. Clin. Nutr. 52(Suppl. 1):S83-S93.

8. Dijkstra, J., J. France, M.S. Dhanoa, J.A. Maas, M.D. Hanigan, A.J. Rook, and D.E. Beever. 1997. A model to describe growth patterns of the mammary gland during pregnancy and lactation. J. Dairy Sci. 80:2340-2354.

9. Kutzbach, C., and E.L. Stokstad. 1997. Mammalian methylenetetrahydrofolate reductase. Partial purification, properties, and inhibition by S-adenosylmethionine. Biochimica et Biophysica Acta 250:459-477.

10. Mason, J.B. 2003. Biomarkers of nutrient exposure and status in one-carbon (methyl) metabolism. J. Nutr. 133(Suppl. 3):941S-947S.

11. Newberne, P.M., and A.E. Rogers. 1986. Labile methyl groups and the promotion of cancer. Annu. Rev. Nutr. 6:407-432.

12. Park, C.S., and G.L. Lindberg. 2004. Mammary Gland and Lactation. Pages 720-741 in Dukes' Physiology of Domestic Animals. 12 ed., W.O. Reece, ed. Cornell University Press, Ithaca, NY.

13. Picciano, M.F. 2003. Pregnancy and lactation: physiological adjustments, nutritional requirements and the role of dietary supplements. J. Nutr. 133(Suppl.):1997S-2002S.

14. Ross, S.A. 2003. Diet and DNA methylation interactions in cancer prevention. Ann. NY Acad. Sci. 983:197-207.

15. Russo, J., and I.H. Russo. 2004. Development of the human breast. Maturitas 49:2-15.

16. Sutherland, J.E., and M. Costa. 2003. Epigenetics and the environment. Annals of the New York Academy of Sciences 983:151-160.

17. Van den Veyver, I.B. 2002. Genetic effects of methylation diets. Annu. Rev. Nutr. 22:255-282.

18. Waterland, R.A., and C. Garza. 1999. Potential mechanisms of metabolic imprinting that lead to chronic disease. Am. J. Clin. Nutr. 69:179-197.

19. Waterland, R.A., and R.L. Jirtle. 2004. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 20:63-68.

20. Wolff, G.L., R.L. Kodell, S.R. Moore, and C.A. Cooney. 1998. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy / a mice. FASEB J. 12:949-957.

7. Appendices

None.