



5500 North St. Louise Avenue
Chicago, IL 60625-4699

Office of Research Development

NIH Pilot Grant



Applicant Information

Personal Information

Full Name: Terrence Puryear Phd

Department: Biology

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E-mail: t-puryear1@neiu.edu

Project Information

Title: The Temporal Effects Of Varying Levels Of Folate S

Recommended Reviewer: Hales, Dale B Phd Email: dbhale@uic.edu

Recommended Reviewer: Wilson, Allison PhD Email: awilson@ben.edu

Recommended Reviewer: _____ Email: _____

DESCRIPTION: See instructions. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project (i.e., relevance to the **mission of the agency**). Describe concisely the research design and methods for achieving these goals. Describe the rationale and techniques you will use to pursue these goals.

In addition, in two or three sentences, describe in plain, lay language the relevance of this research to **public** health. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Maternal folate supplementation has been recognized as a method for combating neural tube defects (NTD's)- especially spina bifida for many years now. The exact mechanism by which folate supplementation works, however, has yet to be elucidated. Analysis of folate supplementation has shown that both genetic^{1 2} and environmental factors³ work separately and together as causative agents in neural tube defects.^{4 5 6 7} While folate supplementation is fairly effective in preventing the majority of NTD's^{9 10 11 12} three questions arise that are of interest to our research group. First: At what point or points in early development are proper maternal folate levels key to neural development and the prevention of NTD's and later cognitive and/or motor problems? Second: While the levels of folate supplementation that prevent gross NTD's are known, are these levels sufficient to prevent more subtle forms of neural damage that are not apparent on the gross level. This type of subtle damage might lead to later cognitive and/or motor problems. Third: Traditionally, economically disadvantaged groups, recent immigrants and teenagers either have not had access to proper or consistent nutrition or have had poor diet. What effects do varying maternal folate levels during pregnancy have on the final outcome of neural, cognitive and/or motor development?¹³ This question will be addressed in a separate proposal. Much of the current research into the mechanism(s) by which folate supplementation works to prevent NTD's fall into three categories, epidemiological studies, analysis of allele frequency and genetic knockouts in animal models to determine the roles of specific genes involved in folate uptake and metabolism. While all 3 types of studies can and do provide information on the mechanism of folate supplementation in the prevention of NTD's none are useful for addressing the types of questions of interest to our group.

Current techniques do not address the question of whether folate requirements change during key periods during development and if alterations in dietary folate during development may lead to subtle defects that might lead to later cognitive and/or motor problems, and whether the recommended levels of folate supplementation are sufficient to correct these subtle defects.^{14 15 16 17 18}

PERFORMANCE SITE(S) (organization, city, state)

Northeastern Illinois University, Chicago, IL

Principal Investigator/Program Director (Last, First, Middle): **Rueckert, Linda / Puryear, Terrence**

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator(s). List all other key personnel in alphabetical order, last name first.

Name	eRA Commons User Name	Organization	Role on Project
Puryear ,Terrence PhD		NEIU	PI
Kimble ,Mary PhD		NEIU	Senior Consultant

OTHER SIGNIFICANT CONTRIBUTORS

Name	Organization	Role on Project
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Human Embryonic Stem Cells No Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: <http://stemcells.nih.gov/registry/index.asp>. *Use continuation pages as needed.*

If a specific line cannot be referenced at this time, include a statement that one from the Registry will be used.

Cell Line

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.

**RESEARCH GRANT
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Appendix *(Five collated sets. No page numbering necessary for Appendix.)*



Check if Appendix is Included

Number of publications and manuscripts accepted for publication *(not to exceed 10)* _____

Other items (list): _____

DETAILED BUDGET FOR INITIAL BUDGET PERIOD DIRECT COSTS ONLY						FROM 07-01-08	THROUGH 06-30-09	
PERSONNEL <i>(Applicant organization only)</i>		Months Devoted to Project			INST.BASE SALARY	DOLLAR AMOUNT REQUESTED <i>(omit cents)</i>		
NAME	ROLE ON PROJECT	Cal. Mnths	Acad. Mnths	Summer Mnths		SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Puryear ,Terrence PhD	Principal Investigator		2.25	3		0	0	0
Kimble ,Mary PhD	Senior Consultant		2.25	3		0	0	0
McMurray, Laura R.	Graduate student		2.25	2		0	0	0
Przewodnikowska, Luiza	Graduate student		2.25	2		0	0	0
Mackovic, Dragan	Honors student		2.25	2		375	0	375
Brunner, Rachel	Honors student		2.25	2		375	0	375
SUBTOTALS →						750		750
CONSULTANT COSTS								0
EQUIPMENT <i>(Itemize)</i> axioskop 2 "bulb"								385
SUPPLIES <i>(Itemize by category)</i> Experimental animals and animal care Tissue fixing and staining Antibodies and IHC supplies General lab supplies								8,064
TRAVEL Cell Biology meeting								800
PATIENT CARE COSTS		INPATIENT 0						0
		OUTPATIENT 0						0
ALTERATIONS AND RENOVATIONS <i>(Itemize by category)</i>								0
OTHER EXPENSES <i>(Itemize by category)</i>								0
CONSORTIUM/CONTRACTUAL COSTS					DIRECT COSTS		0	
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD <i>(Item 7a, Face Page)</i>							\$	9,999
CONSORTIUM/CONTRACTUAL COSTS					FACILITIES AND ADMINISTRATIVE COSTS		0	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD							\$	9,999

Principal Investigator/Program Director (Last, First, Middle): Rueckert, Linda / Puryear, Terrence

Budget Justification

Description	Vendor	Amount	Total animal cost	Shipping	Total cost
Timed pregnant female ICR Mice	Harlan Sprague Dawley	100	3520	128	3648
Animal care and housing	In house				460
Tissue preparation, sectioning and staining	Electron microscope supply				900
General lab supplies	Fisher Scientific				500
Kits					
Abc Hrp Staining Std	Fisher	2 ea			482
Promega* DeadEnd* Fluorometric TUNEL System	Fisher	ea			438.75
Antibodies and reagents for immuno florescence					
Cleaved Caspase-3 antibody	Cell Signaling Technologies	Ea		25	287
Rabbit Anti-Shh (H-160) Polyclonal Antibody,	Santa Cruz Biotechnology, Inc.	Ea		25	273
PAX6 Polyclonal Antibody	Santa Cruz Biotechnology, Inc.	Ea		25	273
Pax-3 (L-14) Polyclonal Antibody	Santa Cruz Biotechnology, Inc.	ea		25	273
goat anti-rabbit IgG-FITC	Santa Cruz Biotechnology, Inc.	4 each		25	265
rabbit anti-goat IgG-FITC:	Santa Cruz Biotechnology, Inc.	4 each		25	265
Equipment					
axioskop 2 "bulb"	Ziess	Ea			385
Travel 1 meeting student and PI					800
salary					750
Total requested					9999.75

Principal Investigator/Program Director (Last, First, Middle): Rueckert, Linda / Puryear, Terrence

Researcher	Primary duties	salary
Puryear ,Terrence PhD	Animal handling, injections, surgery Immunohistochemistry	0
Kimble ,Mary PhD	Immunohistochemistry	0
McMurray, Laura R.	Animal handling, injections, surgery Immunohistochemistry	0
Przewodnikowska, Luiza	Animal handling, injections, surgery Immunohistochemistry	0
Mackovic, Dragan	Animal handling, injections, surgery Immunohistochemistry	37.5hrs/\$10hr
Brunner, Rachel	Animal handling, injections, surgery Immunohistochemistry	37.5hrs/\$10hr

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Puryear, Terrence PhD		POSITION TITLE Instructor	
eRA COMMONS USER NAME			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
University of Illinois, Chicago	BS	1981	Biology
University of Illinois	PhD	1988 /1993	Physiology/ Endocrinology
NIAAA Fellowship CS Mott Center Wayne State	Post Doctoral	1993/1994	Fetal Alcohol Syndrome

NOTE: The Biographical Sketch may not exceed four pages. Follow the formats and instructions on the attached sample.

A. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

1994 Instructor in Marine Molecular Biology at MDIBL

1994-1995 Research Scientist Department of Zoology, University of Maine

1995-1997 Research Scientist Max Planck Institute

1995 – 2000 Contract work Polar programs

1997- 1998 Research and Development Scientist Life Technologies

1998- 2000 Research and Development Scientist Genelogic

2001-2002 Germanna College

2003-2004 Chicago City Colleges Instructor

2003- Present Instructor, NEIU

B. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation. None in the last 3 years

C. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and your role (e.g. PI, Co-Investigator, Consultant) in the research project. Do not list award amounts or percent effort in projects.

2004 – 2007 Provosts Grant The effects of varying levels of folate supplementation on development in the ICR mouse.

Biographical Sketch

Mary Kimble, Ph. D.

Associate Professor

Education & Training:

Arizona State University, Tempe, AZ	B.S.	1974	Biology
Arizona State University, Tempe, AZ	M.S.	1978-1981	Zoology
Indiana University, Blmgtn, IN	Ph.D.	1982-1989	Mol., Cell. & Dev. Biol.
University of Minnesota, Mnpls, MN	Postdoc	1989-1996	Cell Biology

A. Positions & Honors:

1989-1992: Postdoctoral Fellow, Dept. of Cell Biology & Neuroanatomy, U of MN, Mnpls, MN
1992-1996: Postdoctoral Research Assistant, Dept. of Cell Biology & Neuroanatomy, U of MN, Mnpls, MN
1996-2003: Assistant Professor, Dept. of Biology, University of South Florida, Tampa, FL
2003-2007: Assistant Professor, Dept. of Biology, Northeastern Illinois University, Chicago, IL
2007-present: Associate Professor, Dept. of Biology, Northeastern Illinois University, Chicago, IL

Honors and Awards:

1984-1986: NIH Predoctoral Trainee in Molecular and Cellular Biology, Indiana University
1989-1992: NIH Postdoctoral Fellow (NRSA), University of Minnesota

Professional Societies

Genetics Society of America
American Microscopical Society
American Association for the Advancement of Science

B. Peer-reviewed Publications:

Kimble, M. & K. Church (1983) Meiosis and Early Cleavage in *Drosophila melanogaster* Eggs: Effects of the Claret non-disjunctional Mutation. J. Cell Sci. 62:301-318.

Lin, H-P. P., J.G. Ault, **M. Kimble** & K. Church (1984) Meiosis in *Drosophila melanogaster*. V. Univalent behavior in $ln(1)sc^{4L}sc^{8R}/BsY$ males. Can. J. Genet. Cytol. 26:445-458.

Raff, E.C., H.B. Diaz, H.D. Hoyle, J.A. Hutchens, **M. Kimble**, R.A. Raff, J.E. Rudolph & M.A. Subler (1987) Origin of Multiple Gene Families: Are there both Functional and Regulatory Constraints? In Development as an Evolutionary Process, eds R.A. Raff & E.C. Raff, pp. 203-238. Alan R. Liss, Inc. NY.

Rudolph, J.E., **M. Kimble**, H.D. Hoyle, M.A. Subler & E.C. Raff (1987) Three *Drosophila* Beta-Tubulin Sequences: a Developmentally Regulated Isoform ($\beta 3$), the Testis-Specific Isoform ($\beta 2$), and an Assembly-Defective Mutation of the Testis-Specific Isoform ($\beta 2t8$) Reveal Both an Ancient Divergence in Metazoan Isoforms and Structural Constraints for Beta-Tubulin Function. Mol. & Cell. Biol. 7:2231-2242.

Kimble, M., J.P. Incardona & E.C. Raff (1989) A Variant Beta-tubulin Isoform of *Drosophila melanogaster* ($\beta 3$) is Expressed Primarily in Tissues of Mesodermal Origin in Embryos and Pupae, and is Utilized in Populations of Transient Microtubules. Dev. Biol. 131:415-429.

Kimble, M., R.W. Dettman & E.C. Raff (1990) The $\beta 3$ Tubulin Gene of *Drosophila melanogaster* is Essential for Viability and Fertility. Genetics 126:991-1005.

Sellitto, C., **M. Kimble** & R. Kuriyama (1992) Heterogeneity of Microtubule-Organizing Center Components as Revealed by Monoclonal Antibodies to Mammalian Centrosomes and to Nucleus-Associated Bodies

Principal Investigator/Program Director (Last, First, Middle): Rueckert, Linda / Puryear, Terrence

from *Dictyostelium*. Cell Motil. Cytoskel. 22:7-24.

Kimble, M. & R. Kuriyama (1992) Functional components of microtubule organizing centers. Intl. Rev. Cytol. 136:1-50.

Kimble, M., A.L. Khodjakov & R. Kuriyama (1992) Identification of ubiquitous high molecular mass, heat stable microtubule-associated proteins (MAPs) that are related to the *Drosophila* 205-kDa MAP but are not related to the mammalian MAP4. Proc. Natl. Acad. Sci. U.S.A. 89:7693-7697.

Vassilev, A., **M. Kimble**, C. D. Silflow, M. LaVoie, & R. Kuriyama (1995) Identification of intrinsic dimer and overexpressed monomeric forms of g-tubulin in Sf9 cells infected with baculovirus containing the *Chlamydomonas* g-tubulin sequence. J. Cell Sci. 108:1083-1092.

Kuriyama, R., A. Levin, D. Nelson, J. Madl A. Frankfurter & **M. Kimble** (1995) Monoclonal anti-dipeptide antibodies crossreact with dephosphorylated and glutamylated forms of tubulins. Cell Motil. Cytoskel. 30:171-182.

Ohta, T., **M. Kimble**, R. Essner, M. Kofron & R. Kuriyama (1996) Cell cycle-dependent expression of the CHO2 Antigen, a minus-end directed kinesin-like motor in mammalian cells. Protoplasma 190:131-140.

Kimble, M., **C. Kuzmiak, K.N. McGovern**, and E.L. de Hostos (2000) Microtubule organization and the effects of GFP-tubulin expression in *Dictyostelium discoideum*. Cell Motil. & the Cytoskel., 47(1) : 48-62.

Kimble, M., Y. Coursey, **N. Ahmad**, & G.W. Hirsch (2002). Behavior of the yolk nuclei during embryogenesis, and development of the midgut diverticulum in the horseshoe crab, *Limulus polyphemus*. Invert. Biol. 121(4): 365-377.

Coursey, Y., **N. Ahmad, B.M. McGee, N. Steimel**, & M. Kimble (2003) Amebocyte production begins at stage 18 during embryogenesis in *Limulus polyphemus*, the American horseshoe crab. Biol. Bull. 204:21-27. <http://www.biolbull.org>

C. Recent Research Support:

1. **2005-2006 NEIU COR award.** Analysis of hemocyanin production in horseshoe crab embryos and larvae. \$2910.00

2. **2006-2007 NEIU COR award.** Analysis of a new eye mutation in the fruit fly, *Drosophila melanogaster*. \$2950.00.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

4 dedicated laboratories at NEIU, approximately 950 sqft in total dedicated to this project. 1 all purpose room, 1 molecular/ IHC room, 1 dedicated microscope laboratory containing standard and fluorescent microscopes and 1 dedicated tissue handling facility. These rooms are located adjacent to each other. All rooms are equipped for their specific tasks.

Clinical:

N/A

Animal:

Dedicated mouse room approximately 600 sqft located on the same floor of the building.

Computer:

General and molecular labs are supplied with computers and internet access.

Office:

2 faculty office, with computers, approximately 144 sqft each.

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each. The tissue handling facility contains equipment necessary for tissue fixation and embedding for both paraffin and resin techniques. In addition this space contains the equipment need for histological staining. The molecular /IHC facility contains the basic equipment for these techniques and the general lab is set up for solution preparation, chemical and expendable storage.

Experimental Design

Principal Investigator/Program Director (Last, First, Middle): Rueckert, Linda / Puryear, Terrence

Inhibition of endogenous folate synthesis, Dose response to varying levels of Methotrexate(MTX) and Folic acid

Specific Aims

Our research group has developed a mouse model that allows us to address the specific problems of timing, folate levels and whether or not cognitive and/or motor problems may occur. (See preliminary results) In brief, by administering various amounts of Methotrexate (MTX) (an inhibitor of endogenous folate production) at specific times during pregnancy we can investigate at what points in development folate has its greatest effect on neural development. By supplementing sub-populations of these treated animals with various amounts a folate analog (Leucovorin) we can investigate how much folate is necessary at different points in pregnancy for normal development. In addition, by allowing randomly selected pregnancies to come to term and using standard techniques to measure cognitive and motor functions, we will be able to assess whether recommended levels of folate supplementation are sufficient to prevent all neural defects, or whether subtle defects in brain function persist. We intend to measure a series of parameters, individually or in different groupings to identify treatment and supplement regimens and specific points in development that are likely to provide us with pilot information regarding the role of folate in the developmental processes. We should also be able to begin to understand the temporal aspects of fetal response to folate supplementation. Using this model we hope to be able to investigate the effects of folate in the diet of economically disadvantaged groups, recent immigrants and teenagers who either have not had access to proper or consistent nutrition or have had poor diet. We intend to investigate if varying maternal folate levels during pregnancy have an effect on the final outcome of neural, cognitive and/or motor development. The proposed cognitive and motor studies will be funded and done separately. We intend to apply for separate funding from the university COR program to fund this section of the research. We feel that by eventually looking at putative cognitive and motor defects we can develop a model that might more closely reflect what occurs in a heterogeneous human population.

Building on the results of our preliminary studies we will now, using the model system we have developed, focus on a survey of the role of folate supplementation in development. In order to do this we propose to add an additional four time points to our preliminary work. By adding these time points we will be able to generate an overview of the role of folate supplementation over a period of time approximately equivalent to the first two trimesters of human pregnancy.

In order to develop an overview of the effects of our experimental model on development we propose to examine eight parameters. The first four parameters, H+E, Alcian Blue, Nissl Staining and Tunel staining will be used to look at overall alterations in morphology in our experimental model. The second set of parameters, Cleaved Caspase 3, Sonic hedgehog (SHH), PAX6 and PAX3, are well studied regulators of embryonic development and organogenesis.

By studying these eight parameters we hope to begin to understand the role of folate in early development and to determine when folate, either endogenous or supplemental, is most essential for normal development.

Background and Significance

Maternal folate supplementation has been recognized as a method for combating neural tube defects (NTD's)- especially spina bifida for many years now. The exact mechanism by which folate supplementation works, however, has yet to be elucidated. Analysis of folate supplementation has shown that both genetic^{19 20} and environmental factors²¹ work separately and together as causative agents in neural tube defects.^{22 23 24} While folate supplementation is fairly effective in preventing the majority of NTD's^{25 26} three questions arise that are of interest to our research group. First: At what point or points in early development are proper maternal folate levels key to neural development and the prevention of NTD's and later cognitive and/or motor problems? Second: While the levels of folate supplementation that prevent gross NTD's are known, are these levels sufficient to prevent more subtle forms of neural damage that are not apparent on the gross level. This type of subtle damage might lead to later cognitive and/or motor problems. Third: Traditionally, economically disadvantaged groups, recent immigrants and teenagers either have not had access to proper or consistent nutrition or have had poor diet. What effects do varying maternal folate levels during pregnancy have on the final outcome of neural, cognitive and/or motor development?³¹ This question will be addressed in a separate

proposal. Much of the current research into the mechanism(s) by which folate supplementation works to prevent NTD's fall into three categories, epidemiological studies, analysis of allele frequency and genetic knockouts in animal models to determine the roles of specific genes involved in folate uptake and metabolism. While all 3 types of studies can and do provide information on the mechanism of folate supplementation in the prevention of NTD's none are useful for addressing the types of questions of interest to our group.

Current techniques do not address the question of whether folate requirements change during key periods during development and if alterations in dietary folate during development may lead to subtle defects that might lead to later cognitive and/or motor problems, and whether the recommended levels of folate supplementation are sufficient to correct these subtle defects.^{32 33 34 35 36}

Preliminary results

Our research group has developed a mouse model that allows us to address the specific problems of timing, folate levels and whether or not cognitive and/or motor problems may occur. (See preliminary results)

In brief, by administering various amounts of Methotrexate (MTX) (an inhibitor of endogenous folate production) at a specific time during pregnancy we investigated we were able to induce specific developmental defects. By supplementing sub-populations of these treated animals with various amounts a folate analog (Leucovorin) we were able to examine the effect of folate supplementation on embryo survival and on the formation of specific developmental defects. In addition we have demonstrated an effect for high levels of folate supplementation on head size in normal, non Methotrexate treated embryos.

**Please refer to Figures Part 1 and Part 2 in the Appendix*

Research Design and Methods

Experimental Animals

Out bred ICR mice will be used in this study because they have excellent reproductive and maternal characteristics, a docile disposition. Also because they are robust and have an average litter size of 11.5 they are the most widely used out bred mouse strain in the area of teratology research (personal communication, Harlan Laboratories). The ICR strain is genetically heterogeneous (heterozygous at about 25% of all loci studied). This characteristic, as opposed to a genetically homogenous mouse strain, more closely resembles the human population. All ICR timed-pregnant mice used in this study will be plug date zero, will be delivered on Embryonic day 5, and will be purchased from Harlan Laboratories (Indianapolis, Indiana).

ICR (normal out bred strain) timed-pregnant mice will be delivered on E5, housed independently, and kept under controlled conditions (room temperature (21° C), with a 12/12 hr light/ dark cycle. Mice will be given free access to Purina rat chow and tap water ad libitum.

Animal protocols:

Three groups of 30 ICR mice each will be used in separate experiments to determine the effects of varying levels of inhibition of endogenous folate synthesis by Methotrexate (MTX) and exogenous folic acid rescue over the course of pregnancy.

Proposed experimental timing:

Start day	injection	injection	injection	Embryo isolation day
Group A E6	Folate PM dose E6	Folate AM+ PM dose MTX AM injection E7	Folate AM+ PM dose MTX AM injection E8	E10
Group B E10	Folate PM dose E10	Folate AM+ PM dose MTX AM injection E11	Folate AM+ PM dose MTX AM injection E12	E14
Group C E13	Folate PM dose E13	Folate AM+ PM dose MTX AM injection E14	Folate AM+ PM dose MTX AM injection E15	E17

Experimental groups:

Within each timing group (see previous) pregnant dams will be assigned randomly to one of the following dosage treatments. Values used in this dosing regimen were established in a previous study. (see preliminary results) The number of dams assigned to each group has been based on the results of our previous studies. For the 0 mg/kg MTX groups with varying amounts of folic acid 3 dams should yield between 30 and 60 embryos (previous average of 3 trials = 51). For the 15 mg/kg MTX groups with varying amounts of folic acid 4 dams in the 15 mg/kg MTX /0 FA group have yielded between 32 and 44 embryos (previous average of 3 trials = 38). 15 mg/kg MTX plus either 12mg/kg or 48 mg/kg folic acid 3 dams should yield between 20 and 45 embryos (previous average of 3 trials = 33). 4 dams in the 20 mg/kg MTX /0 FA group have yielded between 15 and 22 embryos (previous average of 3 trials = 18.6). 20 mg/kg MTX plus either 12mg/kg or 48 mg/kg folic acid 4 dams should yield between 20 and 31 embryos (previous average of 3 trials = 24.6).

Since the mice we selected are the ICR strain which is genetically heterogeneous (heterozygous at about 25% of all loci studied). This means that each embryo, because of its heterogeneity, will react as an individual. Mice have 20 pairs of chromosomes and assuming that they are heterozygous for at least one gene on each chromosome and using the basic laws of genetics we can predict a maximum of approximately 10^{13} possible genetic combinations in the embryos. We chose these mice for this characteristic, as opposed to a genetically homogenous mouse strain, as ICR mice will more closely resemble the type heterogeneity found in the human population. Hence statistics will be performed on groups of embryo's rather than on the number of dams. With an expected yield of between 15 and 60 embryos per experimental group we should have a large enough N for initial statistics.¹⁸

	0 FA	12 mg/kg folic acid	48 mg/kg folic acid
0 mg/kg MTX	3	3	3
15 mg/kg MTX	4	3	3
20 mg/kg MTX	4	4	4

Upon completion of experimental treatments pregnant dams will be sacrificed using CO₂ on the appropriate day. All embryos will be dissected, washed in PBS, fixed overnight in 10% buffered formalin and finally placed in 70% EtOH. Within one week embryos will be photographed to document gross morphology, including any NTDs and other teratologies. Embryos will then be processed for histological analyses.

*Initial measurements:**Resorptions:*

The numbers of resorptions from all uteri will be quantified and compared between treatment groups as a measure of embryo survivability to that point in pregnancy.

Gross morphology:

Embryos will be photographed and the external morphology of each will be examined. Embryos will initially be assigned to groups based on treatment regimen and external morphology.

Head-Size Ratios:

Head measurements will be taken of all embryos as ratios between the outermost arc of the curvature of the head to the tip of the snout and the middle of the otic vesicle and the tip of the snout. Head size ratios are a useful method for selecting embryos for later sectioning for examination of brain/skull/facial abnormalities. In addition, our preliminary studies have shown that high levels of folate supplementation in pregnant dams may lead to a decreased variability in head size and shape. (see preliminary results).

Sectioning and Staining: H+E, Alcian Blue, Nissl Staining and Tunel staining

Embryos will be paraffin embedded and sectioned for histology and Immunohistochemistry. We have chosen four basic staining techniques that should give us an overview of both gross and fine changes in the development of embryos using our model system.

H+E

Selected embedded embryos will be section and sentinel sections (1 in every 5 sections) will be stained with Harris Hematoxylin and Eosin –Y. Embryo selection for sectioning and staining will be based on how well an embryo represents the morphology in question. H & E is a standard stain used to detect both the cytoplasm and the nuclei of cells in order to better analyze the shapes of the internal organs, count cells/area, and if present, detect apoptotic cells.

Alcian Blue

Since cartilage is the precursor for bone it can be used to measure patterns in presumptive bone formation during development. We will utilize Alcian Blue staining³⁷ in order to detect cartilage and pre-cartilage growth in order to determine normal vs. abnormal cartilaginous growth patterns resultant from the treatments.³⁸ This will allow us to exam differences in skull and limb formation during development in response to our experimental manipulations. Since our preliminary experiments have shown that we can alter the pattern of skull, facial and limb development Alcian Blue staining will allow us to compare patterns of skeletal development through early-mid embryogenesis in our experimental groups.

Tunel staining

A major question in development and pattern formation involves the roll of apoptosis or programmed cell death in different processes. Apoptosis is a critical feature of limb, head and face development. Since in our preliminary work we demonstrated effects of methotrexate exposure on limb and head development, we will evaluate the effect of the treatments on the distribution and level of apoptosis using the TUNEL assay.³⁹ TUNEL staining is a method of detecting apoptosis at different stages on histological sections. The most commonly used method is Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling. One of the characteristics of apoptosis is the degradation of DNA after the activation of Ca/Mg dependent endonucleases. This DNA cleavage leads to strand breaks within the DNA creating new free ends. The TUNEL method identifies apoptotic cells in situ by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the ends of cleaved DNA.

Nissl Staining

This method is used for the detection of Nissl bodies in the cytoplasm of neurons of formalin-fixed, paraffin embedded tissue sections. The Nissl body will be stained purple-blue. This stain is commonly used for identifying the basic neuronal structure in brain or spinal cord tissue. We propose that, in our model system, while folate supplementation can prevent gross morphological defects in the embryo that this supplementation may not always be enough to prevent less obvious neuronal damage. Using Nissl staining on selected sections in conjunction with our other staining protocols will allow us to assess whether or not we have fine structural malformations.

Immunohistochemistry: Cleaved Caspase 3, sonic hedge hog (SHH), PAX6 and PAX3

After staining of selected sections adjacent sections will be selected used for Immunohistochemistry. By examining the distribution of known proteins involved in Apoptosis and developmental morphogens we can further elucidate the mechanisms by which folate is functioning at various points in development.

Cleaved Caspase 3

Caspase-3 (CPP-32, Apoptain, Yama, SCA-1) is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins within the cell. In conjunction with H+E and TUNEL staining we should be able to begin to determine the role of folate in apoptosis and development. Since apoptosis is important in many areas of development an understanding of its relationship to folate levels is key to understanding the temporal role of folate in development.⁴⁰

Neuronal cell death in the embryonic brain was first recognized almost a century ago. Its significance for normal nervous system development and function has been a major focus of neuroscientific investigation ever since.⁴¹ Targeted gene disruption demonstrates the significance of neural precursor cell death and immature neuron death in nervous system development. Pathological activation of apoptotic death pathways may lead to neuroanatomic abnormalities and possibly to developmental disabilities.⁴² The use of H+E, TUNEL staining and Cleaved Caspase 3 can help in understanding this developmental process.

Sonic Hedgehog (Shh)

The morphogen Hedgehog is an autoprolytic secreted protein that activates an essential cellular pathway (Hedgehog Signaling Pathway) required during the development of many species. The pathway is involved in cell fate determination, pattern formation, proliferation, and differentiation in multiple tissue types. The size and shape of brain structures can be controlled by the molecule Sonic Hedgehog (*Shh*).^{43,44} During development, the brain becomes organized into highly specialized groups of neurons, called brain nuclei, each expressing its own set of genes and participating in very specific neural functions. Researchers have shown that this process can be coordinated by the secretion of *Shh*, which operates as a 'positional signal'.^{45,46}

Shh has been found to have critical roles in development, acting as a morphogen involved in patterning many systems, including the limb and midline structures in the brain and spinal cord and the thalamus.

⁴⁷Mutations in the human sonic hedgehog gene, HuSHH, cause holoprosencephaly type 3 (HPE3) as a result of the loss of the ventral midline.⁴⁸ Sonic hedgehog is secreted by the zone of polarizing activity (ZPA), which is located on posterior side of limb buds in embryos. More recently, *Shh* has also been shown to act as an axonal guidance cue. It has been demonstrated that *Shh* attracts commissural axons at the ventral midline of the developing spinal cord. Specifically, *Shh* attracts retinal ganglion cell (RGC) axons at low concentrations and repels them at higher concentrations.⁴⁹

By examining the pattern of *Shh* localization in sections of our experimental embryos we can investigate the temporal role of folate supplementation on a major determinant of differentiation.

PAX6

PAX6, a member of the paired box gene family, encodes a transcriptional regulator involved in oculo-genesis and other developmental processes. Along with SHH PAX6 plays a major role in eye, mid-facial and nasal development.⁵⁰ Since our preliminary results point to a major role for folate supplementation in the development of the mid-facial region an understanding of the pattern and timing of expression of PAX6 and SHH genes will be key.

Central Nervous System Development: PAX6 also appears to play a major role in Central Nervous System Development. PAX6 has a role in arealization of the neocortex (formation of connections between the cortex and thalamus). PAX6 regulates Neurog2 expression in the spinal cord by controlling distinct Neurog2 enhancer elements that are active at different positions along the dorsoventral axis leading to proper spinal topography.

Pituitary Development: PAX6 is involved in the development of the Rathke's pouch and the early anterior pituitary gland, and its expression controls the established boundaries of somatotrope, lactotrope, and thyrotrope cell types in mice. The absence of Pax6 led to a marked increase of the thyrotrope cell lineage, whereas the somatotrope and lactotrope cell lineage changes were much diminished. Kioussi et al. (1999) suggested that the transient dorsal expression of PAX6 is essential for establishing a sharp boundary between dorsal and ventral cell types, based on the inhibition of Shh ventral signals.^{51,52}

Pancreatic Development

PAX6 has also been shown to be necessary for organogenesis of the pancreas. The Pax6 gene is expressed during the early stages of pancreatic development and in mature endocrine cells. The pancreas of Pax6 homozygous mutant mice lacked glucagon-producing cells, suggesting to the authors that PAX6 is essential for the differentiation of alpha-cells.⁵³

PAX3

PAX3 plays a critical role in the formation of tissues and organs during embryonic development.⁵⁴ The PAX gene family is also important for maintaining the normal function of certain cells after birth. During embryonic development the PAX3 gene is active in neural crest cells which migrate from the developing spinal cord to specific regions in the embryo. PAX3 gene directs the activity of other genes (such as MITF) that signal neural crest cells to differentiate and form specialized tissues or cell types such as limb muscles, bones in the face and skull (craniofacial bones), some nerve tissue, and pigment-producing cells called melanocytes.⁵⁵ PAX3 mutation has been identified in individuals with a condition called craniofacial-deafness-hand syndrome. This condition is characterized by distinct facial features such as widely spaced eyes (hypertelorism) and a small nose, profound hearing loss, and hand abnormalities that affect the fingers and wrist.

Since in our preliminary results we have demonstrated the dose dependant affects of folate on craniofacial development this marker, along with SHH and PAX6, will be a useful measure of folate's action on known regulators of specific developmental processes.

Vertebrate Animals

Out bred ICR mice will be used in this study because they have excellent reproductive and maternal characteristics, a docile disposition. Also because they are robust and have an average litter size of 11.5 they are the most widely used out bred mouse strain in the area of teratology research (personal communication, Harlan Laboratories). The ICR strain is genetically heterogeneous (heterozygous at about 25% of all loci studied). This characteristic, as opposed to a genetically homogenous mouse strain, more closely resembles the human population. All ICR timed-pregnant mice used in this study will be plug date zero, will be delivered on Embryonic day 5, and will be purchased from Harlan Laboratories (Indianapolis, Indiana).

ICR (normal out bred strain) timed-pregnant mice will be delivered on E5, housed independently, and kept under controlled conditions (room temperature (21° C), with a 12/12 hr light/ dark cycle. Mice will be given free access to Purina rat chow and tap water ad libitum.

We estimate that a maximum of 100 ICR timed-pregnant mice will be used in the course of this study. The animal facility is monitored by an outside veterinarian and inspected three times a year.

Animals will be injected I.P. twice a day for three days with the appropriate comes dissolved in sterile saline solution and monitored for pain and discomfort during the injections and then sacrificed 2 day later with carbon dioxide as recommended by Panel on Euthanasia of the American Veterinary Medical Association and IACUC protocols.

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Appendix

Figures Part 1 Preliminary results

Our research group has developed a mouse model that allows us to address the specific problems of timing, folate levels and whether or not cognitive and/or motor problems may occur. (See preliminary results)

In brief, by administering various amounts of Methotrexate (MTX) (an inhibitor of endogenous folate production) at a specific time during pregnancy we investigated we were able to induce specific developmental defects. By supplementing sub-populations of these treated animals with various amounts a folate analog (Leucovorin) we were able to examine the effect of folate supplementation on embryo survival and on the formation of specific developmental defects. In addition we have demonstrated an effect for high levels of folate supplementation on head size in normal, non Methotrexate treated embryos.

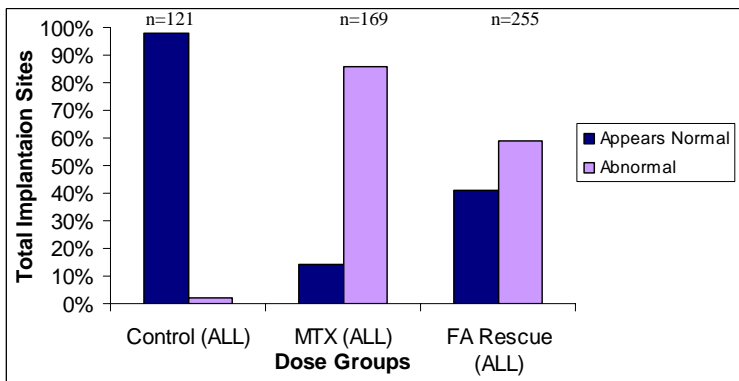


Figure 1. Significant differences in frequencies of abnormal embryos among treatments groups. (p < .001)

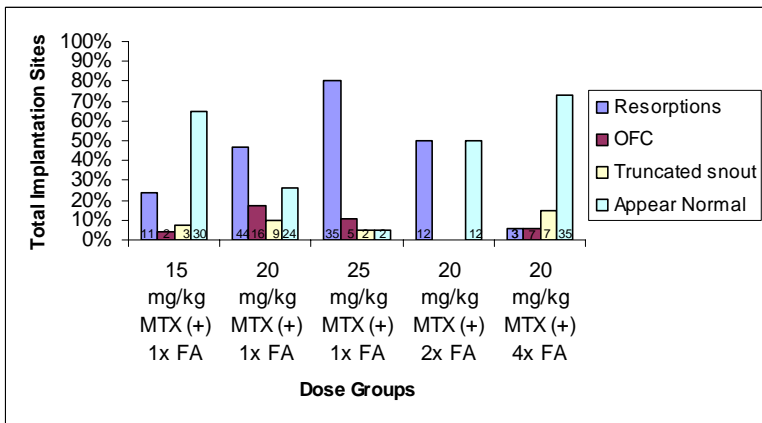


Figure 3 Effects of differing does of MTX and folic acid on resorptions and selected defects as a percentage of total implantation sites. (p<0.001) (1xFA = 12 mg/kg folinic acid)

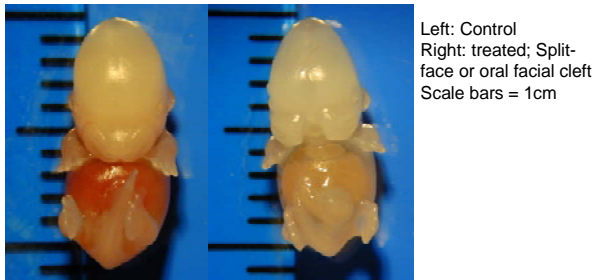


Figure 4 Typical gross morphologies of control embryos and split face embryos.



Figure 5 Morphology of a typical control embryo vs. a typical truncated snout embryo.(20mg/kg and 48 mg/kg folic acid).

Principal Investigator/Program Director (Last, First, Middle): Rueckert, Linda / Puryear, Terrence

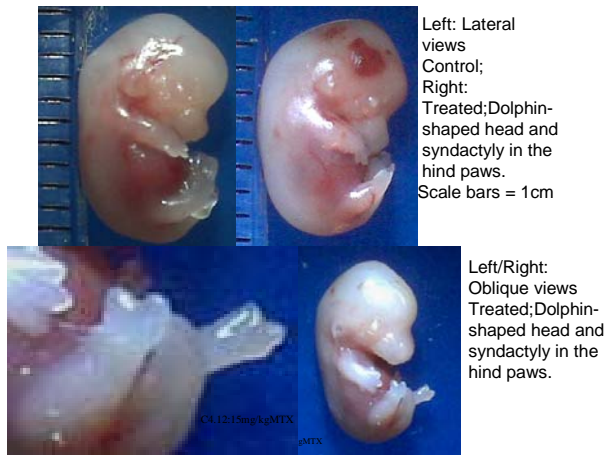


Figure 6 Comparison of a control embryo with an embryo from 15mg/kg MTX treatment group. Note the dolphin shape of the head and the syndactyly of the hind paws in the 15mg/kg MTX embryo

Principal Investigator/Program Director (Last, First, Middle): Rueckert, Linda / Puryear, Terrence

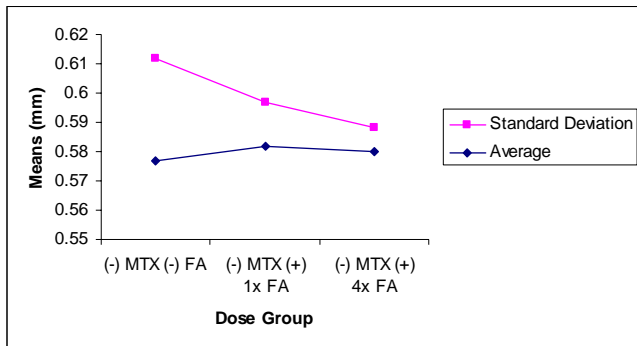


Figure 7 Increasing Folate supplementation leads to increased uniformity of head size . (1x FA 12 mg/kg folic acid , 4x FA 48 mg/kg folic acid)

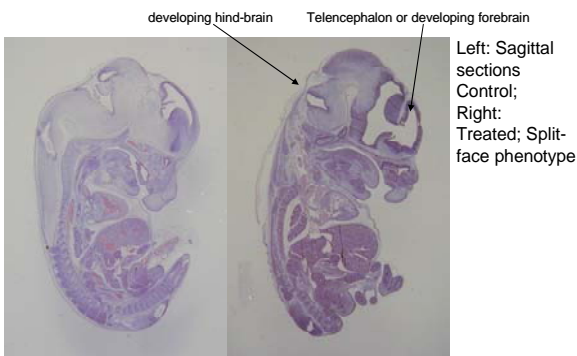


Figure 8 Sagittal sections (H+E) of a control embryo and an embryo treated with 20mg/kg of MTX and folate supplemented embryo (12 mg/kg folic acid) with a split face. Note the alterations in the developing hindbrain and the telencephalon.

Figures Part 2

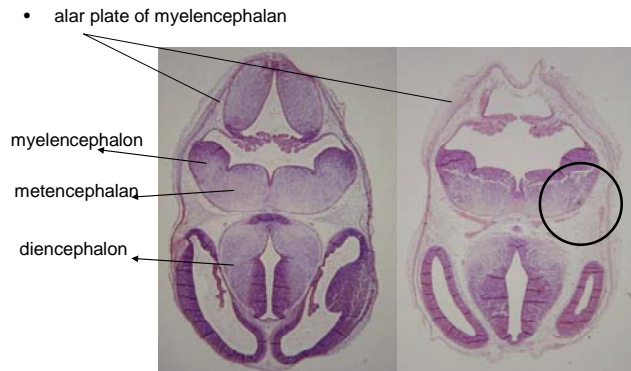


Figure 9 Cross sections (H+E) of a control embryo and an embryo treated with 20mg/kg of MTX and supplemented with folate(12 mg/kg folic acid). Note the alterations in structure of the alar plate. Microscopic analysis also shows fewer, loosely packed cells in the treated embryo. (see circled area) this finding is consistent thru the treated embryos that received low and medium folate supplementation



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OFFICE OF SPONSORED PROGRAMS

April 2, 2008

Dear Sir or Madam;

The project 'The Temporal Effects of Varying Levels of Folate', proposed by Dr. Terry Puryear has been approved by the IACUC.

Dr. Sue Mungre
Chair, IACUC