

AD \_\_\_\_\_

GRANT NUMBER DAMD17-94-J-4268

TITLE: Analysis of Tumor Suppressor Gene Loss in Mouse Mammary  
Models of Mammary Neoplasia

PRINCIPAL INVESTIGATOR: Robert J. Coffey, M.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center  
Nashville, Tennessee 37232-2279

REPORT DATE: July 1999

TYPE OF REPORT: Final, Phase II

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

**DTIC QUALITY INSPECTED 4**

**20001013 075**

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
<small>Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.</small>				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1999	3. REPORT TYPE AND DATES COVERED Final, Phase II (1 Jul 98 - 30 Jun 99)	
4. TITLE AND SUBTITLE Analysis of Tumor Suppressor Gene Loss in Mouse Mammary Models of Mammary Neoplasia			5. FUNDING NUMBERS DAMD17-94-J-4268	
6. AUTHOR(S) Robert J. Coffey, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, Tennessee 37232-2279			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)				
<p>The original sabbatical proposal was modified to two separate aims, both designed to acquire knowledge of genetics to be applied to mammary carcinoma. These studies were carried out at Stanford University in the laboratories of Stuart Kim, David Botstein and Pat Brown. First, in the Kim lab, a genetic screen was performed in <i>C.elegans</i> in a sensitized background (using worms mutant for Gap) to identify worms that missorted Let-23, the worm EGF receptor, in polarized vulva precursor cells. By complementation testing and STS mapping, a locus has been identified on chromosome 4 that results in missorting of Let-23 from the basolateral to apical surface. Second, microarray technology in the Botstein and Brown labs was utilized to identify sets of genes that are induced by antioxidants in mammary and colorectal carcinoma cells that culminate in p53-independent, p21-dependent apoptosis. Candidate genes have been identified and are being characterized. In addition, the latter experience enabled me to develop a microarray core lab at Vanderbilt University.</p>				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 9	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

\_\_\_\_\_ Where copyrighted material is quoted, permission has been obtained to use such material.

\_\_\_\_\_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

\_\_\_\_\_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ \_\_\_\_\_ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

\_\_\_\_\_ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ \_\_\_\_\_ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ \_\_\_\_\_ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

\_\_\_\_\_ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Wendell G. Coffey  
PI - Signature

8/31/98  
Date

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

\_\_\_\_ Where copyrighted material is quoted, permission has been obtained to use such material.

\_\_\_\_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

\_\_\_\_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

\_\_\_\_ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

\_\_\_\_ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓  
\_\_\_\_ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓  
\_\_\_\_ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

\_\_\_\_ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Wendell G. Coffey  
PI - Signature

8/31/98  
Date

## Table of Contents

	Page Number
Front Cover	1
Standard Form (SF) 298	2
Foreward	3
Table of Contents	4
Introduction	5
Body	5-6
Conclusions	6
References	7-8
Personnel List	9

Coffey, Robert J  
DAMD17-94-J-4268

Introduction: The goal of my sabbatical was to acquire a working knowledge of genetics. As has been previously discussed, logistical problems prevented the transfer of transgenic mice to Allan Balmain's laboratory at Onyx in Richmond, California. This led to a major change in direction. Two projects were conducted that built on advances in my lab but were designed to harness the power of molecular genetics to propel the work in my lab ahead.

Body: The first project was performed in the laboratory of Stuart Kim in the Department of Developmental Biology at Stanford University. My lab has been studying the sorting and processing of mammalian EGF receptor ligands in mammalian polarized epithelial cells (1-3). In mammalian cells, EGF receptor is restricted to the basolateral surface. Ligand engagement of the EGF receptor initiates a signal transduction cascade that activates Ras, Raf and MAP kinase. We have found that TGF $\alpha$  (1) and amphiregulin (3) are delivered preferentially to the basolateral surface but then are processed differently. Multiple forms of amphiregulin are released and we are studying their possibly different biological roles. EGF (2) is delivered to both the basolateral and apical surface, but is preferentially cleaved by a metalloprotease-like enzyme in the basolateral compartment.

Dr. Kim's lab has focused on signaling events in polarized vulva precursor cells that result in a fully differentiated vulva (4). Lin-3, a TGF $\alpha$  homologue, binds to Let-23, an EGF receptor homologue, in the basolateral compartment of polarized vulva precursor cells in the second larval stage of the worm (5, 6). This initiates a signal transduction cascade that activates Ras and MAP kinase which results in a fully differentiated vulva. The Kim lab has identified three mutants that result in missorting of this worm EGF receptor from the basolateral to the apical compartment and the worms are no longer able to form a vulva (7-9). Mutations of three PDZ-containing proteins (Lin-2, Lin-7 and Lin-10) are responsible for this phenotype. Mammalian homologues of these proteins have been identified, and, in at least with Lin-2, it appears to be a tumor suppressor gene.

I carried out a mutant screen in worms that were mutant in Gap, a gene important in inactivation of active Ras (10). These worms had no observable phenotype. In this sensitized background, I identified 10 mutants that resulted in a multivulva. By immunohistochemistry, the worm EGF receptor appeared to be misdirected to the apical compartment. Complementation tests revealed that this mutant was not due to any of the previously characterized PDZ-containing proteins. STS mapping was carried out and I found that the locus mapped to chromosome 4 (11). More refined mapping has narrowed the region on chromosome 4 to 1.5 map units between stP51 and stP35. I intend to carry out Yac injections and deficiency mapping to identify the gene responsible for this phenotype. The mammalian homologue then will be identified and its role in mammary carcinoma will be studied.

The second project was a follow-up of an important clinical observation that we have made recently. That is, that antioxidants enhance the anti-tumor efficacy of cytotoxic chemotherapy in mammary and colorectal cancer cells *in vitro* and *in vivo* (12). Furthermore, we have elucidated a molecular mechanism by which one of these antioxidants, PDTC, acts (13). This involves activation of protein kinase A which

phosphorylates serine<sup>299</sup> in C/EBP $\beta$  that translocates to the nucleus, binds to a p53-independent site in the p21 promoter to induce apoptosis. I was exposed to microarray technology in the labs of David Botstein and Pat Brown who pioneered this technology (14-16) at Stanford University and employed it to identify genes that are expressed following administration of PDTC. We have examined the effect of PDTC on the expression of 5,000 genes in mammary and colorectal cancer cells. These studies should allow us to identify possible additional targets of the action of PDTC. We are now setting up a microarray at Vanderbilt University and intend to utilize it to identify molecular events in rodent and human mammary carcinoma. It is anticipated that microarray technology, coupled with laser capture microdissection, will be utilized to identify genetic events in this model and the results will provide insights into the molecular pathogenesis of human mammary carcinoma.

Conclusions: In summary, I accomplished my goal for the sabbatical, which was to acquire a working knowledge of genetics. Stanford University provided an ideal environment for this work. This knowledge will be applied to elucidating molecular events underlying the pathogenesis of mammary carcinoma, as well as to formulate new therapeutic approaches to mammary and colorectal cancer. For example, we have observed that there is accelerated mammary tumor formation in mice bigenic for TGF $\alpha$  and c-neu (17) and, more recently, that an EGF receptor tyrosine kinase inhibitor blocks tumor formation in these bigenic mice (18). We have developed a novel treatment for mammary and colon cancer by blocking the EGFR and inhibiting the enzyme that cleaves cell surface TGF $\alpha$  to release the mature soluble growth factor (19). A recent manuscript from our group demonstrates that inhibition of the Ras pathway with farnesyltransferase inhibitors may be useful in the treatment of mammary cancer due to sustained overexpression of TGF $\alpha$  (20). Finally, the lessons that I learned during my sabbatical enabled me to compete successfully for the Mouse Models of Human Cancer Consortium; a major focus of the proposal is to develop mouse models of mammary cancer by downregulating or eliminating the type II TGF $\beta$  receptor in the mouse mammary gland.

## References

1. PJ Dempsey and RJ Coffey: Basolateral Targeting and Efficient Consumption of Transforming Growth Factor- $\alpha$  in Transfected Madin-Darby Canine Kidney Cells. *J Biol Chem* 269: 16878-16889, 1994.
2. PJ Dempsey, KS Meise and RJ Coffey: Human EGF Precursor is not Sorted in Polarized MDCK Cells but Accumulates at the Apical Membrane due to Increased Basolateral Proteolytic Activity. *J Cell Biol* 138:1-12, 1997.
3. CL Brown, KS Meise, GD Plowman, RJ Coffey and PJ Dempsey. Cell Surface Ectodomain Cleavage of Human Amphiregulin Precursor is Sensitive to a Metalloprotease Inhibitor. *J Biol Chem* 273:17258-17268, 1998.
4. JS Simske, SM Kaech, SA harp, and SK Kim. LET-23 Receptor Localization by the Cell Junctions Protein LIN-7 During *C. Elegans* Vulval Induction. *Cell* 85:195-204, 1996.
5. RV Aroian, PW Sternberg. Multiple Functions of let-23, a *Caenorhabditis Elegans* Receptor Tyrosine Kinase Gene Required for Vulval Induction. *Genetics* 128: 251-67, 1991.
6. RJ Hill, PW Sternberg. The Gene LIN-3 Encodes an Inductive Signal for Vulval Development in *C. Elegans*. *Nature* 358:470-476, 1992.
7. JS Simske, and SK Kim. Sequential Signaling During *Caenorhabditis Elegans* Vulval Induction. *Nature* 375: 142-6, 1995.
8. SM Kaech, CW Whitfield, and SK Kim. Basolateral Membrane Localization of the Receptor Tyrosine Kinase LET-23 is Mediated by the LIN-2/LIN-7/LIN-10 Protein Complex in the Vulval Precursor Cells of *C. Elegans*. *Cell*; in press, 1998.
9. CW Whitfield, C Benard, T Barnes, S Hekimi and SK Kim. Basolateral Localization of the *C. Elegans* EGF Receptor in Epithelial Cells by the X11/mint Homolog LIN-10, Submitted, 1998.
10. A Hajnal, CW Whitfield, SK Kim. Inhibition of *Caenorhabditis Elegans* Vulval Induction by Gap-1 and by LET-23 Receptor Tyrosine Kinase. *Genes Dev* 11:2715 - 2728, 1997.
11. BD Williams, et al. A Genetic Mapping System in *Caenorhabditis Elegans* Based on Polymorphic Sequence-Tagged Sites. *Genet* 131:609-624, 1992.
12. R Chinery, J Brockman, MO Peeler, RD Beauchamp, RJ Coffey. Antioxidants Enhance the Cytotoxicity of Chemotherapeutic Agents in Colorectal Cancer: A p53-independent Induction of p21WAF1/CIP1 via C/EBP $\beta$ . *Nature Medicine* 3:1233-1241, 1997.
13. R Chinery, JA Brockman, DT Dransfield, RJ Coffey: Antioxidant-induced nuclear translocation of CCAAT/enhancer binding protein  $\beta$ : A Critical role for protein kinase A-mediated phosphorylation of Ser299. *J Biol Chem* 272:30356-30361, 1997.
14. D Shalon, S Smith, PO Brown. A DNA Microarray System for Analyzing Complex DNA Samples Using Two-Color Fluorescent Probe Hybridization. *Genome Res* 6: 639-645, 1996.

15. J DeRisi, et.al. Use of cDNA Microarray to Analyze Gene Expression Patterns in Human Cancer. *Nat Genet* 14:457-460, 1996.
16. JL DeRisi, VR Iyer, and PO Brown. Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale. *Science* 278:680-686, 1997.
17. WJ Muller, CL Arteaga, SK Muthuswamy, MA Webster, RD Cardiff, F Li, KS Meise, SA Halter and RJ Coffey: Synergistic Interaction of the Neu Proto-oncogene and TGF $\alpha$  in the Development of Mammary Neoplasia in Transgenic Mice. *Mol Cell Biol* 16:5726-5736, 1996.
18. Lenferink AEG, Simpson JF, Shawver LK, Bogatcheva G, Pagano M, Coffey RJ, Forbes JT, and Arteaga CL. Blockade of the EGF Receptor Tyrosine Kinase Suppresses Tumorigenicity in MMTV/neu+TGF $\alpha$  Transgenic Mice. In preparation.
19. J Dong, LK Opresko, PJ Dempsey, DA Lauffenburger, RJ Coffey, and HS Wiley: Metalloprotease mediated Ligand Release Regulates Autocrine Signaling Through the Epidermal Growth Factor Receptor. *Proc Natl Acad Sci USA* 96:6235-40, 1999.
20. P Norgaard, B Law, H Joseph, DL Page, Y Shyr, D Mays, JA Pietenpol, NE Kohl, A Oliff, RJ Coffey, H Skovagaard Poulsen, and HL Moses. Treatment with Farnesyl-protein Transferase Inhibitor Induces Regression of Mammary Tumors in Transforming Growth Factor (TGF)  $\alpha$  and TGF $\alpha$ /neu Transgenic Mice by Inhibition of Mitogenic Activity and Induction of Apoptosis. *Clin Cancer Res* 5:35-42, 1999.

Personnel receiving pay from this effort:

Robert J. Coffey  
Kenta Yoshiura