AD_____

Award Number: DAMD17-99-1-9058

TITLE: Role of the Spindle Checkpoint in Preventing Breast Cancer

PRINCIPAL INVESTIGATOR: Tomohiro Matsumoto, Ph.D.

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine
Bronx, New York 10461

REPORT DATE: July 2001

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Role of the Spindle Checkpoint in Preventing Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9058

6. AUTHOR(S)
Tomohiro Matsumoto, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Albert Einstein College of Medicine
Bronx, New York 10461

E-MAIL:
tmatsumo@aecom.yu.edu

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)*
Abnormal chromosome number is a phenotype characteristic for most of the cancer cells. Thus, it may be a direct cause of human cancer including breast cancer. In this research project, we aim to test this hypothesis by abrogating the spindle checkpoint that is a major surveillance mechanism responsible for maintenance of the normal chromosome number. p55CDC serves as a target of the checkpoint. If it is unable to bind to a spindle checkpoint protein, Mad2, it abrogates the checkpoint in a dominant manner. In Year 1 of this project, we have generated several mutants of p55CDC which are defective in binding to Mad2 in the yeast two-hybrid system. In the current year (Year 2), we have placed these mutants under an inducible promoter and integrated in the genome of Hela cells. We have confirmed that some of these mutants are indeed defective in binding to Mad2 *in vivo*. We have also demonstrated that expression of the p55CDC mutants 1) abolishes a cell cycle arrest caused by a spindle poison, nocodazole and 2) produces aneuploid cells. These results indicate that we have succeeded in generating dominant p55CDC mutants which can abolish the function of the spindle checkpoint.

14. SUBJECT TERMS
Breast Cancer, chromosome instability, spindle checkpoint, p55CDC

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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.

X 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.

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

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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

7/5, 2001

PI - Signature Date

Table of Contents

Introduction: Abnormal karyotype is a hallmark of cancer cells, including breast cancer. Therefore, it has been postulated that chromosome instability may be a direct cause of the genesis/stage progression of cancer. The spindle checkpoint, that delays the onset of sister chromatid separation until all the kinetochores capture the mitotic spindle, plays an important role for maintenance of the normal chromosome number. We suspect that loss of the checkpoint may produce aneuploids which serve as the epicenter of cancer. To test this hypothesis, we propose to abolish the checkpoint and examine the phenotypes.

The heart of the mechanism of the checkpoint is that Mad2, a component of the checkpoint, binds to its target, human p55CDC, and prevents it from promoting proteolysis prerequisite to sister chromatid separation. If Mad2 is unable to bind to p55CDC, the checkpoint is not functional. On the basis of this principle, we have proposed to mutate the Mad2-binding domain of p55CDC and generate mutants of p55CDC defective in binding to Mad2. If properly mutated, expression of such mutants would abrogate the checkpoint in a dominant manner. A similar strategy has proven successful in the yeast model study [1, 2]. In year 1, we have generated a number of mutants of p55CDC to which Mad2 may not bind in the yeast two-hybrid system. In the current year (Year 2), we have characterized these mutants *in vivo*.

Body: As reported in the previous year (Year 1), approximately 70 mutants of p55CDC which would have lost the binding activity to Mad2 have been cloned into a tetracycline-inducible expression vector and integrated in the genome of cultured cells (a derivative of Hela cell that allows the use of tet-inducible system). A modified compound, doxycyclin (Dox), which is less toxic, can be used to induce the expression.

In the current year, we have induced expression of the p55CDC mutants and examined the phenotypes individually with the following criteria;

1) Sensitivity to nocodazole: We have assumed that if the spindle checkpoint is not functional, the cell becomes hyper-sensitive to a spindle poison, nocodazole. Thus, viability of the Hela cells was determined after incubation with nocodazole. Among the 70 mutants, 3 of them, namely M9, M4 and M45, have reduced the viability significantly only after incubation with nocodazole.

2) Binding activity to Mad2: These three mutants have been tagged with EGFP (a derivative of GFP which can be recognized by an antibody to GFP) and expressed in Hela cells. Immuno-precipitation with the antibody to GFP has demonstrated that these mutants are defective in binding to Mad2 (Figures 1A, B and C).

3) Checkpoint activity: In order to demonstrate that reduction in the viability is actually due to loss of the checkpoint, we have examined the activity of the checkpoint. The active checkpoint arrests cell cycle progression at prometaphase if nocodazole interferes with the spindle formation. As a result, phosphorylated histone H3, which reaches a peak in early to mid-mitosis, accumulates in the arrested cells. We have found that there is no accumulation of phospho-H3 if expression of the M45 mutant is induced (Figure 1D).
4) DNA content: Loss of the checkpoint would result in aneuploidy. DNA content of the Hela cells expressing the M45 mutant has been measured. As shown in Figure 1D, expression of the M45 mutant has produced cells with more or less than 2N DNA content, suggesting that loss of the checkpoint causes instability in chromosome number.

We are currently examining the M4 and M9 mutants in the same way. For Year 2 (July 2000 to June 2001) of this research project, we have proposed the following tasks;
(Tasks 1 and 2 have been completed in Year 1)
Task 3: to analyze the mutants biochemically
Task 4: to test the mutants for sensitivity to a spindle poison
Task 5: to inject mouse oocytes with p55CDC mutant gene construct
Task 6: to select transgenic mice which carry the mutant p55CDC

As shown in Figure 1, Tasks 3 and 4 have been completed in Year 2. However, we have not completed Tasks 5 and 6 (to generate transgenic mice) for the following reason. In the original application, we have proposed to determine if loss of the checkpoint results in genesis/progression of breast cancer in the mouse animal model. At the time of the preparation of the application (June 1998), the mouse animal model is only the system which allows study of tumorigenesis. It has however been shown that the normal human cell lines can be transformed by ectopic expression of three defined components, a subunit of the telomerase, SV40 large T-antigen and oncogenic Ras GTPase [3]. Therefore, we feel that it is important to test our hypothesis first in the cultured human cells. This modification will let us avoid to sacrifice animals in the research project. The project will be modified as described below.

Modification: The inducible construct of p55CDC-M45 mutant has been integrated in the

genome of the BJ cell, a normal human cell line, which is able to maintain the normal chromosome number. In Year 3, we will induce the expression of p55CDC-M45 and test if it increases the rate of tumorigesis. Upon neoplasmic transformation, the BJ cell line would be able to grow in an anchorage-independent fashion on the soft agar media. In addition, we will test if loss of the checkpoint contributes to the stage progression of cancer. While some primary transformed cells are able to grow on soft agar, they are not able to invade a layer of extracellular matrix (ECM). When they progress through the stage of tumorigenesis and become more aggressive, they can invade ECM. The assay can be performed readily with materials commercially available (Becton Dickinson Labware, NJ). The two methods, soft agar assay and Matrigel invasion assay, would allow staging of the transformed cells in our study. If aneuploidy plays a role only in a later stage of tumorigenesis, (such as from primary cancer to invasive or metastatic cancer), expression of p55CDC-M45 would not shorten the period required for primary transformation (assayed by soft agar). However, it would shorten the period required for invasive or metastatic transformation (assayed by Matrigel).

Addition: Although we believe that the p55CDC-M45 mutant would be a powerful reagent to abrogate the spindle checkpoint, we also feel that the results obtained with p55CDC-M45 should be confirmed by another strategy. We have recently identified a novel Mad2-binding protein, Cmt2. Overexpression of Cmt2 results in dissociation of the Mad2-p55CDC complex and abrogates the spindle checkpoint. We like to use overexpression of Cmt2 as an additional strategy in the project. Overexpression of Cmt2 affects the spindle checkpoint by a mechanism slightly different from that of p55CDC-M45. If we observe the same results with the different reagents, it would strengthen our conclusion.

Key Research Accomplishment:

1. to examine the p55CDC mutants biochemically and physiologically.
2. to identify three mutants of p55CDC which abolish the spindle checkpoint in a dominant negative fashion.
3. to introduce the inducible construct of the p55CDC mutant in the BJ cell line.
4. to characterize a novel Mad2-binding protein, Cmt2.

Reportable Outcomes:

Habu, T., Kim, S. H. and Matsumoto, T. (2001). A Mad2-binding protein, Cmt2, is required for silencing the spindle checkpoint. (submitted on June 12, 2001)

Conclusion: We have generated the p55CDC-M45 mutant that is defective in binding to Mad2. When ectopically expressed, it abrogates the function of the spindle checkpoint. In Year 2, we have also identified and characterized a novel Mad2-binding protein, Cmt2, and found its overexpression abrogates the checkpoint. These two reagents will be used in Year 3 to test if loss of the checkpoint contributes to genesis/progression of cancer in normal human cell line, BJ.

References:

1. Kim, S. H., D. P. Lin, S. Matsumoto, A. Kitazono, and T. Matsumoto. 1998, Science 279, 1045.

2. Hwang, L.H., L.F. Lau, D.L. Smith, C.A., Mistrot, K.G., Hardwick, E.S., Hwang, A., Amon, and A.W. Murray. 1998, Science 279, 1041.

3. Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W. and Weinberg, R. A. (1999). Creation of human tumor cells with defined genetic elements. Nature 400,464-468.

Appendix: Figure 1 is attached in the next page.

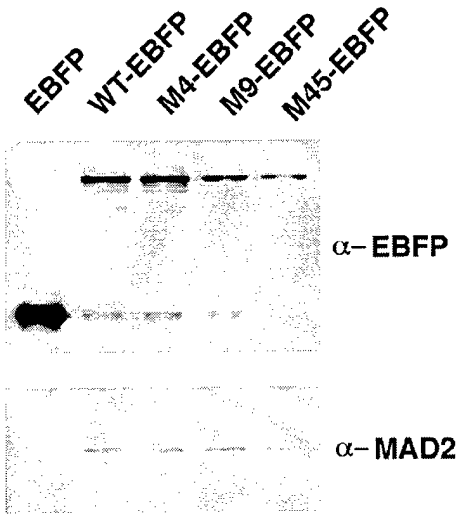

Figure 1A

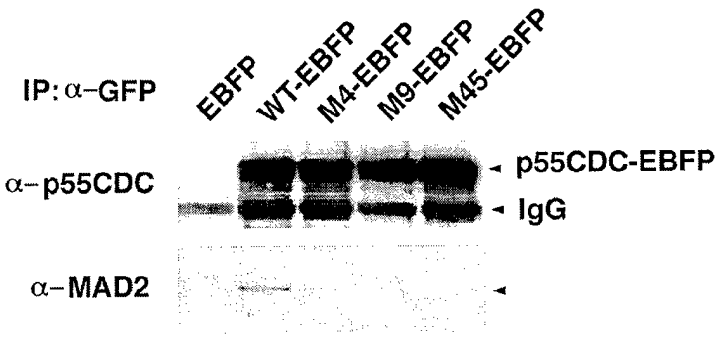

Figure 1B

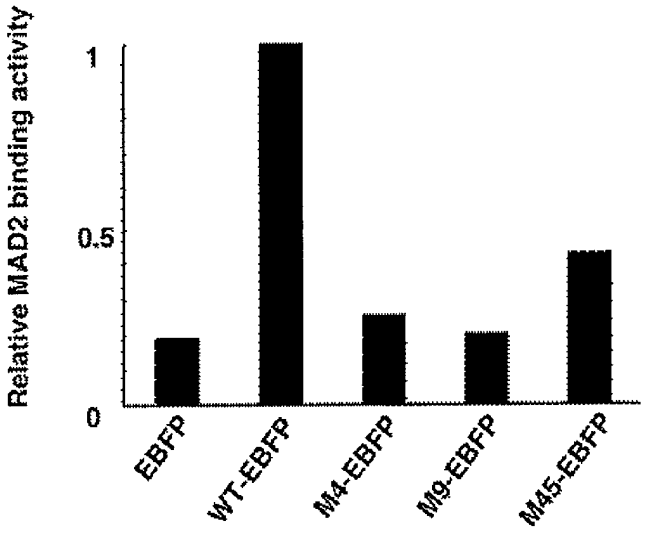

Figure 1C

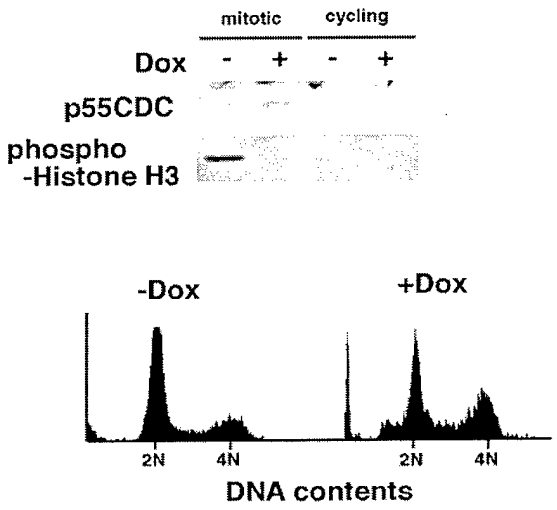

Figure 1D

Figure 1. Dominant negative mutants of p55CDC. A. The wild type p55CDC and mutants of p55CDC (M4, M9 and M45) were tagged with EBFP respectively and transiently expressed in Hela cells. EBFP alone was also expressed (negative control). Western blot was performed to measure the levels of EBFP and p55CDCs tagged with EBFP (top panel) and Mad2 (bottom panel). **B.** The same extracts shown in A were used for immuno-precipitation with the antibody to GFP, which can recognize EBFP. The precipitates were examined for the presence of p55CDC (top panel) and Mad2 (bottom panel) by western blot. **C.** The bar graph indicates the quantitative analysis of the level of Mad2 associated with each p55CDC. The intensity of the bands shown in B (bottom panel) was measure by NIH-image. **D.** (mitotic, left panel); Hela cells that express p55CDC-M45 in a Dox-dependent manner were arrested by Double thymidine block and released into the media containing nocodazole in the presence (+) or absence (-) of Dox. Twelve hours after the release, the samples were taken and processed for western blot. (cycling, right panel); Asynchronously growing Hela cells that express p55CDC-M45 in a Dox-dependent manner were cultured for 48 hours either in the presence (+) or absence (-) of Dox, and processed for western blot. Hela cells that induce the mutant of p55CDC-M45 were cultured asynchronously for 24 hours in presence (+) or absence (-) of Dox and prepared for FACS for measurement of DNA content (bottom panel).