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GRANT NO: DAMD17-94-J-4154

TITLE:

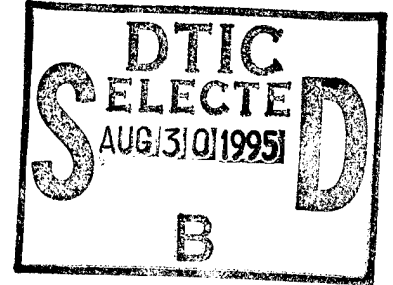
Genetic Construction and Molecular Characterization of
Breast Cancer Precursor Cells

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REPORT DATE:

June 30, 1995

TYPE OF REPORT:

Annual

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

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 30, 1995	3. REPORT TYPE AND DATES COVERED Annual 31 May 94 - 30 May 95	
4. TITLE AND SUBTITLE Genetic Construction and Molecular Characterization of Breast Cancer Precursor Cells		5. FUNDING NUMBERS DAMD17-94-J-4154	
6. AUTHOR(S) Kimberly M. Spancake		8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Utah, Graduate School Salt Lake City, Utah 84112		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		11. SUPPLEMENTARY NOTES	
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Mutations in genes involved in cell-cycle and growth regulation, such as the retinoblastoma gene (RB), are expected to have a significant role in the early stages of breast cancer. RB-deficient cells are being created by two distinct methods. First, targeting vectors, currently being constructed, will be transfected into human mammary epithelial cell lines (HMEC) in order to knock out both RB alleles via homologous recombination. Second, a human papilloma virus (HPV) E6/E7 fusion construct, previously shown to specifically target the retinoblastoma protein (pRb) for degradation, will be transfected into HMEC. The E6/E7 gene fusion will be introduced into the HMEC under the control of a regulatable promoter, allowing for direct regulation of protein expression. Breast cells made deficient for pRb by these two methods will be examined for cellular and molecular alterations, such as changes in morphology and behavior, and their ability to form duct-like structures when grown on or within an extracellular matrix. Additionally, since the normal RB product has been implicated in transcriptional regulation, the effect on mRNA transcription in the RB-deficient breast cells will be assayed by Northern blot analysis and differential display.			
14. SUBJECT TERMS Breast Cancer; Mammary Epithelial Cells; Retinoblastoma; Homologous Recombination; E6/E7 fusion; Differential Display			15. NUMBER OF PAGES 12
17. SECURITY CLASSIFICATION OF REPORT Unclassified			16. PRICE CODE
18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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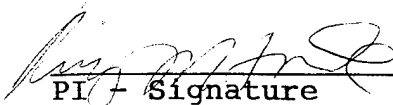
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INTRODUCTION

Genetic Construction and Molecular Characterization of Breast Cancer Precursor Cells

The retinoblastoma gene has been shown to be inactivated in 20% of human breast tumors and 25% of human breast carcinoma cell lines by conventional loss of heterozygosity (LOH) studies^{1,2}. More recently, LOH of *RB* was demonstrated in the majority of breast carcinomas (59%) after tumor cell enrichment by cell sorting³. Researchers have also found an increase in the incidence of breast cancer in a family with a constitutional *RB* mutation⁴. The observations of *RB* LOH in breast tumors and the increase of breast cancer incidence in an *RB*-family suggest an important role for *RB* in breast carcinogenesis. Additional studies have shown that restoration of pRb expression in breast carcinoma cell lines with *RB* mutations causes a reduction in the cells' ability to grow in soft agar and form tumors in nude mice⁵. This data provides functional evidence for *RB* playing a role in breast carcinogenesis.

I am currently investigating the role of *RB* in breast carcinogenesis. Specifically, my hypothesis is that elimination of functional pRb from HMEC will promote cellular changes that mimic an early stage in the progression of breast carcinogenesis. Removing pRb will cause discernible cellular and molecular alterations resulting from changes in the transcription of pRb-related genes.

SPECIFIC AIMS

Specific Aim 1. Construct HMEC deficient for pRb.

Breast epithelial cells are currently being made deficient for pRb by two independent methods. First, targeting vectors, specific for *RB*, will be transfected into HMEC and *RB* knockouts selected by drug resistance. Second, the viral transforming protein HPV E6/E7 gene fusion construct will be expressed in HMEC, functionally deleting *RB* by inactivating pRb.

Specific Aim 2. Characterize the RB-deficient cell lines

RB-deficient cells created in specific aim one will be characterized by several criteria. Cells will be assayed for changes in morphology, growth rate, transformation properties, and ability to form duct-like structures on an extracellular matrix. Since pRb plays an active role in transcription regulation, changes in mRNA transcription also will be examined.

BODY OF REPORT

Specific aim number one has served as the focus of my most recent research, including the May 1994 to May 1995 time period.

Approach #1: Construct HMEC containing homozygous deletions of *RB*

As mentioned previously, I am constructing RB-deficient cells by two separate methods. HMEC *RB* knockouts will be created via homologous recombination using replacement vectors, currently under construction, to completely delete both *RB* alleles. The targeting vectors are pUC9 derivatives that contain three essential components: a positive selectable marker (neomycin or hygromycin), a negative selectable marker (Herpes simplex virus thymidine kinase gene, *HSV-tk*), and 6 kb each of DNA from both the 5' and 3' regions of *RB* (Figure 1).

An 81-nucleotide DNA oligomer and its complementary strand were synthesized on an ABI 394 DNA/RNA synthesizer. The 81-mers, when annealed, serve as a polylinker containing all the restriction endonuclease sites necessary for the subsequent cloning steps. The 81-mers were PCR amplified, digested with *EcoRI* and *Hind III*, and cloned into pUC9. The neomycin drug resistance gene was then cloned into the corresponding region of the polylinker. The 3' and 5' *RB* clones and the two *HSV-tk* genes are currently being cloned into this specialized pUC9 plasmid. These knockout vectors will be linearized with the restriction endonuclease *AatII* and transfected into HMEC by lipofection; cells will be screened for successful integration by culturing in media containing G418/hygromycin and gancyclovir.

Approach #2: Construct HMEC with conditionally-deficient pRB

Interactions between the tumor suppressor proteins pRb and p53 and transforming proteins of various DNA tumor viruses are thought to be essential components of the transformation process. Two Human Papillomavirus (HPV) early genes, E6 and E7, bind p53 and pRb, respectively^{6,7}. Binding of HPV type-16 (HPV-16) E6 to p53 enhances degradation of the p53 protein⁸. Binding of HPV-16 E7 to pRb inactivates pRb by mimicking the hyperphosphorylated state, disrupting pRb interactions with other proteins, and thus effectively deleting pRb without actually knocking out both *RB* alleles. A fusion protein consisting of the full-length HPV-11 E6 protein and the HPV-16 E7 pRb-binding domain induces the *in vitro* degradation of pRb without effecting p53 degradation⁹. Expression of this E6/E7 construct in HMEC should cause degradation of pRb, creating an RB-deficient cell line.

Since the metabolic consequences of this experiment are not known, using the E6/E7 construct in a conditional expression system will allow for better control of the experiment. Two different regulatable systems are being used. A sheep metallothionein promoter construct was recently obtained by our laboratory and will be used for inducible regulation of E6/E7 expression¹⁰. The E6/E7 fusion construct will be cloned into the MT-CB6+ plasmid, transfected into HMEC, and expression levels regulated by varying the concentration of $ZnSO_4$ in the growth media.

The second system to control E6/E7 expression is regulated by tetracycline-responsive promoters

and will be used to control E6/E7 expression¹¹. Briefly, the plasmid pUHD15.1 contains a transactivator (tTA) which, when expressed, binds to a tetracycline operator and minimal promoter sequence on a second plasmid, pUHC13.3 (Figure 2). This binding of the tTA stimulates transcription of luciferase or a gene of interest and transcription can be regulated by culturing the cells with varying concentrations of tetracycline. HMEC will be made conditionally deficient for pRb by transfection of the HPV E6/E7 fusion construct under the control of tetracycline-inducible system. The tTA on the pUHD15.1 (neo^R) plasmid will be transfected into early-passage 184 and 184A1 cells. Cells that have been successfully, stably transfected with pUHD15.1 will be selected by culturing in 200 µg/ml GENETICIN^R (G418). Responsiveness to tetracycline will be tested by transient transfection with the pUHC13.3 luciferase⁺ construct into the G418-resistant 184 and 184A1 cells. The pUHC13.3 (hygro^R) vector will be modified by replacing the luciferase gene with the E6/E7 construct, placing E6/E7 under the control of the tetracycline promoter. This construct will be transfected into previously established G418-resistant 184(tTA) and 184A1(tTA) cells. Successful transfection of the E6/E7-modified pUHC13.3 vector will be selected by culturing in media containing hygromycin and subsequently tested for tetracycline-induced regulation of E6/E7. Levels of E6/E7 fusion protein and pRb will be determined using immunoprecipitation with the appropriate antibodies.

Preliminary experiments with the tetracycline-repressible system indicate that it is functional in the 184A1 cell line. pUHD15.1 and pUHC13.3 constructs were transiently expressed in the 184A1 cell line to assay the degree of regulation that could be obtained with this tetracycline system in these HMEC. 184A1 cells were grown in 60 mm dishes until they reached 80 - 85 % confluency at which time they were transfected using lipofection (DOTAP, Boehringer Mannheim). Nine different conditions were assayed: (1) no DNA or DOTAP, (2) DOTAP (1 & 2 served as negative controls), (3) a luciferase construct driven by an SV40 promoter (positive control), (4) pUHD15.1, (5) pUHC13.3, (6) pUHC13.3 plus 1.0 µg/ml tetracycline, (7) pUHD15.1 and pUHC13.3, (8) pUHD15.1 and pUHC13.3 plus 0.1 µg/ml tetracycline, and (9) pUHD15.1 and pUHC13.3 plus 1.0 µg/ml tetracycline. DNA and DOTAP were mixed, added to the cells, and incubated for 6 hours. The DNA/DOTAP containing media was then aspirated and replaced with fresh media (containing tetracycline, where indicated). Cells were grown an additional 48 hours before lysis. Lysates were assayed for luciferase activity using the Luciferase Assay System (Promega), and quantitated using a luminometer (Table 1). These results show that in 184A1 cells, tetracycline inhibits the luciferase expression driven by the transactivator by 20 - 30 fold (comparing luminometer readings from pUHD15.1 and pUHC13.3 versus both vectors in the presence of tetracycline). The results also indicate that this inducible system is functional in the 184A1 cell line and, as such, is encouraging for future stable transfection experiments.

CONCLUSIONS

1. The *RB* targeting vector being constructed is half-way to completion and, upon completion, will be transfected into different HMEC. The RB-deficient cells will be identified by growth selection and further characterized by the criteria described in the original report.
2. Transient transfection of the tetracycline-repressible system indicates that these constructs are functional in the 184A1 cell line and expression can be regulated 20-30 fold. The E6/E7 fusion construct will be expressed in different HMEC using this system as well as the inducible, sheep metallothionein construct. RB-deficient cells created using these systems will also be characterized as described previously.

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APPENDIX

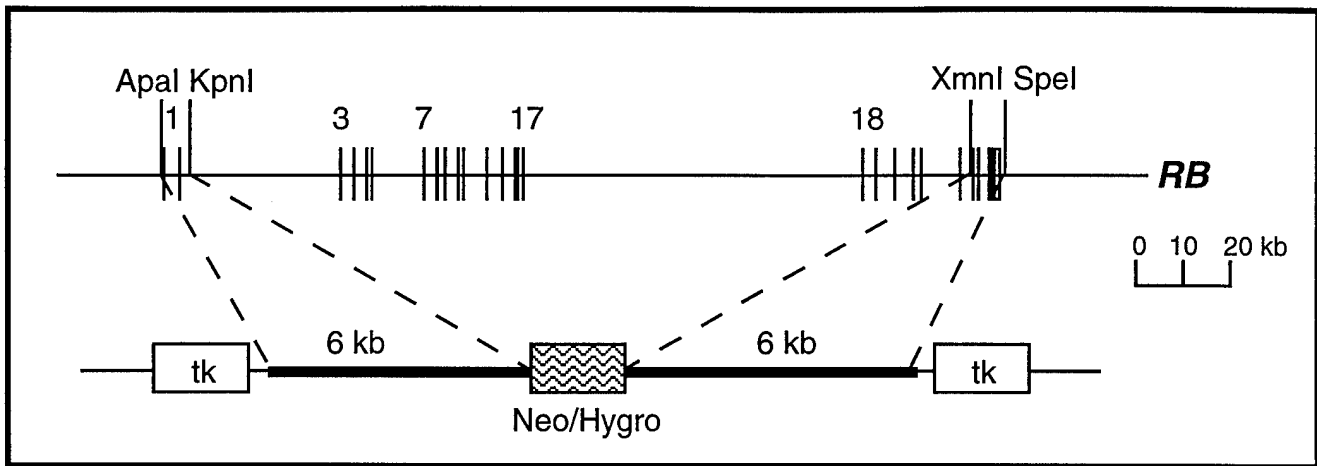


Figure 1. Schematic representation of the *RB* targeting vector. The *RB* targeting vector contains a positive selectable marker (neomycin or hygromycin) flanked by the 5' and 3' most *RB* sequence. Additionally, the vector contains two negative selectable markers, HSV-*tk* genes. This construct will be used to knock out *RB* in HMEC.

	Expected Results	Experiment #1	Experiment #2
Control (No DNA or DOTAP)	-	0.128	0.175
DOTAP	-	0.123	0.191
SV40-luciferase	+++	ND	37.350
pUHD15.1	-	0.136	0.179
pUHC13.3	-	160.700	10.910
pUHC13.3 & 1.0 μ g/ml tet.	-	ND	3.734
pUHD15.1 + pUHC13.3	+++	22.890	60.650
pUHD15.1 + pUHC13.3 & 0.1 μ g/ml tet.	+(+)	0.755	3.230
pUHD15.1 + pUHC13.3 & 1.0 μ g/ml tet.	-	0.815	2.025

all dishes, 20 μ g total DNA (pUHD15.1 and pUHC13.3, 5:1)

+/- indicates relative levels of luciferase expression

ND not determined

tet. tetracycline

Table 1. Expression levels of luciferase using the tetracycline inducible system transiently transfected into 184A1 cells.

APPENDIX

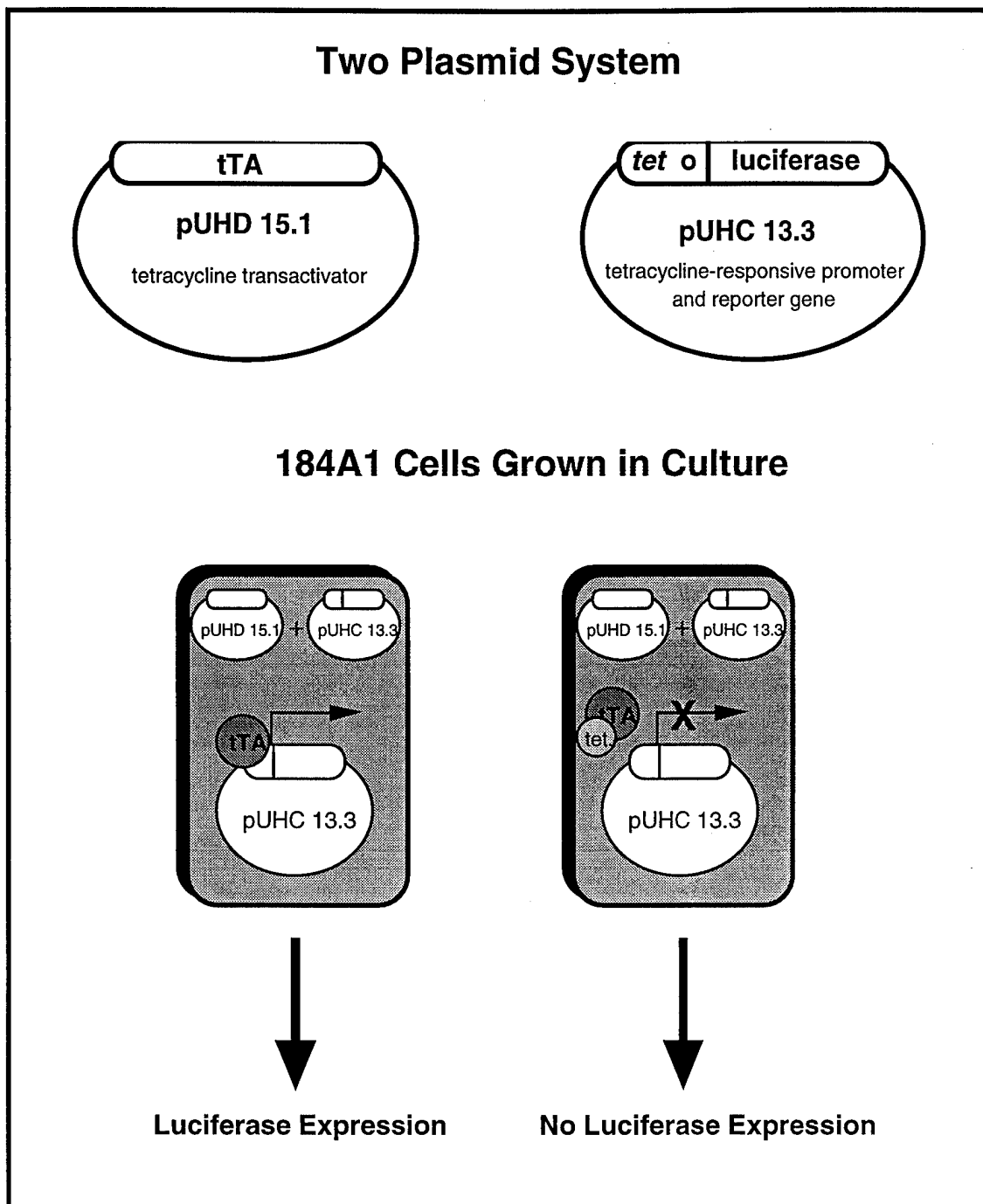


Figure 2. Tetracycline-regulated system¹¹. A chimeric tetracycline-controlled transactivator (tTA) was constructed by fusing a bacterial tetracycline repressor with the herpes simplex virus VP16 activation domain. This transactivator stimulates transcription of a gene of interest, such as the E6/E7 fusion, from a construct containing tetracycline operator sequences and a minimal promoter sequence derived from human cytomegalovirus (CMV). Tetracycline, added to the cell culture media, blocks the transactivator thus decreasing the level of transcription from the gene of interest. This control system allows an individual gene to be regulated over up to five orders of magnitude in HeLa cells.

APPENDIX
ADDITIONAL INFORMATION

July 19, 1994 -- Poster presentation. Construction and Molecular Characterization of Breast Cells
Containing Early Genetic Alterations.

American Association for Cancer Research. Histopathobiology of Neoplasia Workshop.
Keystone, Colorado.

March 1, 1995 -- Passed qualifying examination for the department of human genetics.