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Award Number: DAMD17-03-1-0327

TITLE: The Role of RAs in Myc-Induced Mammary Tumorigenesis

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REPORT DATE: May 2005

TYPE OF REPORT: Annual Summary

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-05-2005	Annual Summary	1 May 2004 - 30 Apr 2005

4. TITLE AND SUBTITLE
The Role of RAs in Myc-Induced Mammary Tumorigenesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0327

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Pennsylvania
Philadelphia, PA 19104-6205

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. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have previously demonstrated that induced *MYC* oncogene expression in the murine mammary epithelium results in the formation of mammary tumors. MYC-induced tumors known to have *Kras2* mutations all fail to regress after MYC deinduction while most tumors without the mutations do regress.
We have attempted to build a second transgenic mouse model to regulate *Kras2* expression independently of MYC in the mammary gland. After testing and characterizing the transgenic mice from this system, we have concluded that this system cannot be successfully used to regulate Kras2 in the mammary gland. As an alternative method, we are currently attempting to retrovirally infect MYC-induced tumors cells with virus expressing active Kras2.

15. SUBJECT TERMS
c-MYC, transgenic animals, Ras, mammary, carcinogenesis, tumorigenesis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	7	

Table of Contents

INTRODUCTION:

Amplification of the c-*MYC* oncogene occurs in 10-20% of all human breast cancers. Our lab has previously established a mouse model of breast cancer that inducibly expresses c-*MYC* in the mammary gland. Induction of c-*MYC* with doxycycline results in mammary carcinogenesis with an average tumor latency of 22 weeks. With the cessation of c-*MYC* expression in the mammary gland, 50% fully regress to a non-palpable state. The remaining 50% either regress slightly and then resume growth, or continue growth as if c-*MYC* expression had not been stopped. In addition, 50% of c-*MYC*-induced mammary tumors harbor activating *Nras* and *Kras2* mutations. Strikingly, activating *Kras2* mutation correlates with incomplete regression of the mammary tumor. To study the role of *Kras2* in the progression of mammary tumorigenesis, we have been generating a second inducible system in mice to regulate *Kras2* independently of c-*MYC*. By combining our two different inducible systems, we will be able to investigate the requirements for mammary tumor induction and maintenance. Finally, the second inducible system will be used to regulate *Kras2* mutants that activate specific *Ras* effector pathways. Combining these inducible *Kras2* mutants with c-*MYC* induction will allow us to study the components of *Ras* signaling required for c-*MYC*-induced tumorigenesis and tumor maintenance.

Specific Aims:

I. **Develop a mifepristone-inducible transgenic mouse model for breast cancer.**
II. **Determine the ability of activated Ras to prevent regression of *MYC*-induced tumors after Myc deinduction.**
III. **Determine the *Ras* effector pathways required for inhibiting regression of *MYC*-induced tumors.**

Statement of Work:

Task 1: Months 1-12: Isolate cDNA clones for *Kras2*.

This task was completed on schedule. RNA was isolated from a c-*MYC*-induced mammary tumor with wild-type *Kras2* and used to generate a cDNA library. *Kras2* was cloned by PCR amplification.

Task 2: Months 1-12: Generate cDNA clones with an activating point mutation in *Kras2* as well as the mutants *Kras2*(12V, 35S), *Kras2*(12V, 37G), and *Kras2*(12V, 40C).

This task was completed on schedule. Site-directed mutagenesis by PCR amplification of the cDNA clone from Task 1 was successfully used to generate an activating point mutation in *Kras2*, which changes codon 12 from glycine to valine. The same method was used to introduce additional point mutations to make the mutants *Kras2*(12V, 35S), *Kras2*(12V, 37G), and *Kras2*(12V, 40C).

Task 3: Months 1-12: Generate expression constructs with GLp65 under the control of the MMTV promoter.

This task was completed on schedule. Constructs for transgenic injection were generated by cloning the activator GLp65 after the MMTV promoter. An internal ribosomal entry sequence (IRES) followed by the coding sequence for a nuclear LacZ (NLS-LacZ) were cloned downstream of GLp65 for use as a reporter of GLp65 expression. Additionally, an MMTV-

GLp65 transgenic mouse (the "MG" mouse line) that has already been characterized was obtained from Dr. Sophie Tsai at Baylor University.

Task 4: Months 6-18: Generate GAL4UAS expression constructs for *Kras2*(12V), *Kras2*(12V, 35S), *Kras2*(12V, 37G), and *Kras2*(12V, 40C).

This task was completed on schedule. Point mutants from Task 2 were cloned after a GAL4 upstream activating sequence (GAL4UAS). An IRES followed by the coding sequence for renilla luciferase were cloned downstream of the *Kras2* point mutant to act as a reporter of transgene expression.

Task 5: Months 6-18: Inject expression constructs for GLp65, *Kras2*(12V), *Kras2*(12V, 35S), *Kras2*(12V, 37G), and *Kras2*(12V, 40C) into fertilized mouse oocytes.

This task was completed on schedule. The constructs MMTV-GLp65-IRES-NLS-LacZ, G-*Kras2*(12V), G-*Kras2*(12V, 35S), G-*Kras2*(12V, 37G), and G-*Kras2*(12V, 40C) were successfully injected into fertilized oocytes from FVB mice and implanted into pseudo-pregnant mice. Several attempts were made for some constructs in order to increase the number of possible founders.

Task 6: Months 9-24: Identify transgenic founder lines for GLp65, G-*Kras2*(12V), G-*Kras2*(12V, 35S), G-*Kras2*(12V, 37G), and G-*Kras2*(12V, 40C).

This task was completed on schedule. Tail DNA from founder mice was screened by PCR to determine the presence of a transgene. Five founders that showed germline transmission were identified from the G-*Kras2*(12V) transgene. In addition, one founder was identified from the G-*Kras2*(12V, 35S) transgene, and three founders from the G-*Kras2*(12V, 40C) transgene.

Task 7: Months 12-24: Determine induced and uninduced expression characteristics of transgenic found lines.

This task was completed on schedule. All founders for the G-*Kras2*(12V) transgene were bred to the MMTV-GLp65 transgenic line to test for expression of *Kras2*. Female bitransgenic mice that had both the MMTV-GLp65 transgene as well as the G-*Kras2*(12V) transgene were either given daily intra-peritoneal injections of mifepristone for three weeks or fed chow containing mifepristone for three weeks. Mammary gland tissue was harvested from these bitransgenic mice as well as single transgenic mice induced with mifepristone in the same manner. The tissue was examined by luciferase assay for expression of the *Kras2* transgene. The same testing was completed for the G-*Kras2*(12V, 35S) and G-*Kras2*(12V, 40C) founders.

One G-*Kras2*(12V) founder was found to have expression of Kras2 even without induction with mifepristone, rendering that founder unusable. All other founders were found to have no expression of *Kras2* upon mifepristone induction. Thus the mifepristone-inducible system does not seem to be a viable method of determining whether or not activated *Kras2* is sufficient for Myc-independent tumor growth.

Task 8: Months 12-18: Generate viral constructs with activated Kras2.

This task was completed on schedule. Because transgenic studies were not proceeding as expected, an alternative method to introduce activated *Kras2* into Myc-induced tumors cells was desired. To do this, we planned to retrovirally infect Myc-induced tumor cells in vitro, inject the

cells into nude mice on doxycycline, wait for tumors to grow, and then deinduce the mice to assess the ability of the cells to grow independently of doxycycline.

Kras(12V) was cloned into two retroviral expression vectors. One construct is MIGR1-Kras12V, which has *Kras*(12V) under the control of the 5'LTR from MSCV. It also contains GFP as an expression marker for infection under the control of an IRES element. The second construct is pK1-Kras12V, which also has *Kras*(12V) under the control of the 5'LTR from MSCV. In contrast, this plasmid has a puromycin selection marker under the control of an IRES element so that infected cells can be selected out with puromycin.

Task 9: Months 18-36: Generate virus that expresses activated Kras2 to infect Myc-induced tumor cells in vitro.

This task was completed on schedule. MIGR1-Kras12V and pK1-Kras12V retrovirus was made in 293T cells using the constructs described above as well as additional viral production vectors. Myc-induced tumors were harvested, collagenased, and plated onto plastic petri dishes. It was difficult to make cell lines from Myc-induced tumors. Tumor cells did not grow well *in vitro*. They did not divide well and eventually progressed to senescence with additional passages. Attempts to infect them with MIGR1-Kras12V and pK1-Kras12V were largely unsuccessful.

Task 10: Months 18-36: Generate cells that produce Kras2-expressing virus to co-inject with Myc-induced tumor cells.

This task is in progress as scheduled. MIGR1-Kras12V-producing 293T cells will be co-injected with Myc-induced tumor cells that have been harvested and digested only three hours before injection. The cells will be grown on doxycycline until tumors are detected. The infection efficiency in these tumors will be assessed by GFP expression, and the ability of the tumors to grow without Myc expression will be tested by withdrawing doxycycline.

Key Research Accomplishments

- *Kras2* was cloned from *MYC*-induced mammary tumors.
- *Kras2* point mutants *Kras2*(12V), *Kras2*(12V, 35S), *Kras2*(12V, 37G), and *Kras2*(12V, 40C) were generated.
- The construct MMTV-GLp65-IRES-NLS-LacZ was cloned.
- The constructs MMTV-GLp65-IRES-NLS-LacZ, GAL4UAS-*Kras2*(12V), GAL4UAS-*Kras2*(12V, 35S), GAL4UAS-*Kras2*(12V, 37G) and GAL4UAS-*Kras2*(12V, 40C) were injected into fertilized oocytes to generate transgenic mice.
- Using the mifepristone-inducible system to express activated Kras2 was found to be unfeasible.
- *Kras2* was cloned into retroviral production vectors MIGR1 and pK1 and retroviral supernatants were produced.

Reportable Outcomes

- Poster presentation - 2003 Gordon Conference of Mammary Gland Biology.
- Poster presentation – 2005 Era of Hope DOD Breast Cancer Research Program Meeting.

Conclusions

We have been trying to introduce an activated *Kras2* allele into Myc-induced tumor cells. We have concluded that the mifepristone-inducible system cannot be used successfully to express activated Kras2 in the murine mammary gland. As an alternative method, we are currently using retroviruses to infect and express activated Kras2 in Myc-induced tumor cells. These experiments allow us to further investigate the mechanisms of mammary tumorigenesis and tumor maintenance.