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Award Number: DAMD17-03-1-0345

TITLE: Interactions Between C-Myc and Development in Mammary Carcinogenesis

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REPORT DATE: May 2004

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2004	Annual (1 May 2003 - 30 Apr 2004)

4. TITLE AND SUBTITLE
Interactions Between C-Myc and Development in Mammary Carcinogenesis

5. FUNDING NUMBERS
DAMD17-03-1-0345

6. AUTHOR(S)
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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)

The purpose of the research funded by this award is to identify molecular interactions between the c-MYC proto-oncogene and normal mammary developmental events. Ultimately, our goal is to identify how these interactions affect the process of mammary carcinogenesis. To this end, we seek to identify mammary development stage specific effects of aberrant c-MYC activation, to identify molecular mechanisms accounting for these effects, and to determine what role these interactions plays in mediating the biological effects of aberrant c-MYC expression within the mammary epithelium. This work has led to the finding that aberrant c-MYC activation for discrete periods during early and late pregnancy has dramatically different outcomes. Specifically, c-MYC activation during late pregnancy results in premature Stat5 activation followed by precocious lactation, whereas c-MYC activation during early pregnancy results in neither of these dramatic phenotypes. We are now in the process of determining what role c-MYC-induced activation of the Stat5 mammary developmental signaling pathway plays in mediating the effects of aberrant activation of c-proliferation, differentiation, apoptosis, and tumorigenesis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
c-MYC, Stat5, Mammary development, carcinogenesis	7
	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

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Department of Defense Annual Report 2004

Introduction:

Subsequent to the submission and approval of my pre-doctoral fellowship application the focus of my project has changed to a different but related set of studies. The central hypothesis of my proposal remains that the developmental stage of the mammary gland at the time of c-MYC expression can have a dramatic impact on the biological effects of deregulated c-MYC activity. Recently our lab has discovered that mammary epithelial expression of c-MYC during a discrete stage in pregnancy promotes precocious lactation. Rather than comparing the mammary specific effects of puberty and parity on the outcome of deregulated c-MYC expression, we have chosen to address how developmental events within pregnancy itself can modulate the effects of deregulated c-MYC expression. Through these studies, we have identified a potential role for the mammary developmental signaling molecule Stat5 in mediating the biological effects of c-MYC in the mammary gland. Subsequently, we have begun genetic studies to determine the precise role of Stat5 in c-MYC-induced mammary epithelial proliferation, differentiation, apoptosis, and tumorigenesis. These changes to my proposal are reflected in the revised specific aims and statement of work as outlined below.

Specific Aims:

I. Compare the mammary specific effects of transient deregulated c-MYC expression during early and late pregnancy.

II. Determine the role of Stat5 in c-MYC-induced mammary epithelial proliferation, apoptosis and differentiation.

III. Determine the role of Stat5 in c-MYC-induced mammary carcinogenesis.

Statement of Work:

Technical Objective I: Compare the mammary developmental effects of deregulated c-MYC expression from D6.5-D9.5 to D12.5-D15.5 of pregnancy.

Task 1: Months 1-6: Compare c-MYC mammary expression and transcriptional activation activity levels in mice induced to express c-MYC from D6.5-D9.5 or D12.5-D15.5 of pregnancy.

Task 2: Months 1-6: Compare mammary histological phenotypes of mice induced to express c-MYC from D6.5-D9.5 vs. D12.5-D15.5 of pregnancy.

Task 3: Months 6-12: Compare Stat5 activation status and expression of differentiation markers in response to c-MYC induction from D6.5-D9.5 or D12.5-D15.5 of pregnancy.

Task 4: Months 6-12: Compare mammary gene expression profiles of mice induced to express c-MYC from D6.5-D9.5 or D12.5-D15.5 of pregnancy.

Technical Objective II: Determine the role of Stat5 in c-MYC-induced mammary epithelial proliferation, apoptosis, and differentiation.

Task 1: Months 12-18: Breed MMTV-rtTA(MTB)/Tet-Op-Myc(TOM) mice to *Stat5a/b* knockout mice to generate MTB/TOM; *Stat5a/b -/-* mice.

Task 2: Months 12-18: Titrate doxycycline dose in MTB/TOM; *Stat5a/b +/+* and in MTB/TOM; *Stat5a/b -/-* mice to yield equivalent c-MYC transgene expression.

Task 3: Months 18-24: Mammary gland whole-mount and histological analysis to assess the effect of *Stat5a/b* deletion on the ability of short-term deregulated c-MYC expression to induce mammary epithelial hyperplasia.

Task 4: Months 18-24: BrdU, TUNEL and Northern blot analysis to assess the effect of *Stat5a/b* deletion on the ability of short-term deregulated c-MYC expression to induce mammary epithelial proliferation, apoptosis and differentiation.

Task 5: Months 18-24: Oligonucleotide microarray analysis of short-term inductions comparing expression profiles of MTB/TOM; *Stat5a/b* +/+ and MTB/TOM; *Stat5a/b* -/- to identify c-MYC-induced gene expression changes that require Stat5a/b.

Technical Objective III: Determine the role of Stat5 in c-MYC-induced mammary carcinogenesis.

Task 1: Months 18-24: Generate cohorts of mice with the MTB; *Stat5a/b* +/+, MTB; *Stat5a/b* +/-, MTB; *Stat5a/b* -/-, MTB/TOM; *Stat5a/b* +/+, MTB/TOM; *Stat5a/b* +/-, and MTB/TOM; *Stat5a/b* -/- genotypes.

Task 2: Months 18-36: Chronically induce animals at doxycycline doses shown to yield equivalent transgene expression for each genotype. Determine average mammary tumor latency and multiplicity for each genotype.

Task 3: Months 24-36: Pathological analysis of tumors.

Task 4: Months 24-36: Examine lungs and livers of tumor-bearing animals for evidence of metastases.

Task 5: Months 24-36: Oligonucleotide microarray analysis of tumors arising from each genotype to identify differences in pathways activated in the presence or absence of Stat5a/b.

Accomplishments:

Task 1: Months 1-6: Compare c-MYC mammary expression and transcriptional activation activity levels in mice induced to express c-MYC from D6.5-D9.5 or D12.5-D15.5 of pregnancy.

To determine whether the ability of c-MYC to induce precocious lactation is specific to c-MYC expression from D12.5-15.5 of pregnancy, we assessed the developmental effects of c-MYC expression from D6.5-D9.5 of pregnancy. To accurately compare the effects of c-MYC transgene expression during these different developmental stages, we first assessed whether c-MYC transgene expression and activity levels during these two developmental stages are equivalent by Northern analysis. As shown in figure 1, mRNA expression of the *c-MYC* transgene is equivalent between MTB/TOM mice induced from D6.5-D9.5 and D12.5-D15.5 of pregnancy, as is the expression of the direct targets of c-MYC transcriptional activation: *cdk4*, *Fibrillarin*, and *Shmt1*.

Task 2: Months 1-6: Compare mammary histological phenotypes of mice induced to express c-MYC from D6.5-D9.5 vs. D12.5-D15.5 of pregnancy.

Histological analysis was utilized to compare the ability of c-MYC to induce precocious differentiation when induced from D6.5-D9.5 or D12.5-D15.5 of pregnancy. As shown in figure 2, mammary epithelial expression of c-MYC from D6.5-D9.5 of pregnancy in MTB/TOM mice results in no discernable increase in the number of lobuloalveolar structures within the mammary fat pad by D9.5 of pregnancy. This is in striking contrast to the precocious alveolar distension and luminal secretion observed when c-MYC is expressed from D12.5-D15.5 of pregnancy.

Task 3: Months 6-12: Compare Stat5 activation status and expression of differentiation markers in response to c-MYC induction from D6.5-D9.5 and D12.5-D15.5 of pregnancy.

To investigate the molecular mechanism accounting for the developmental stage specificity of c-MYC-induced precocious lactation, we performed Western blot analysis to assess the level of Stat5a/b tyrosine (Y695/Y699) phosphorylation in response to c-MYC induction from D6.5-D9.5 and D12.5-D15.5 of pregnancy. Induction of c-MYC in MTB/TOM mammary glands from D6.5-D7.5, D6.5-D8.5, or D6.5-D9.5 of pregnancy results in no detectable difference in Stat5 tyrosine-phosphorylation as compared to MTB controls (Fig. 3). This is in contrast to the elevated Stat5 tyrosine-phosphorylation observed when c-MYC is expressed from D12.5-D13.5, D12.5-D14.5, or D12.5-D15.5 of pregnancy (Fig. 3). Furthermore, as determined by Northern blot analysis, expression of c-MYC from D6.5-D9.5 of pregnancy does not result in precocious expression of the lactation-specific mRNAs *ε-casein* and *Pip*, as it does when c-MYC is expressed from D12.5-D15.5 of pregnancy (Fig. 1). These results provide molecular evidence that the ability of c-MYC to induce precocious lactation is dependent upon the developmental stage of the mammary gland in which c-MYC is activated. Additionally, we have demonstrated that this developmental stage specificity is highly dependent on the ability of c-MYC to promote Stat5 activation.

Task 4: Months 6-12: Compare mammary gene expression profiles of mice induced to express c-MYC from D6.5-D9.5 and D12.5-D15.5 of pregnancy.

To identify potential molecular mechanisms by which c-MYC specifically promotes Stat5 activation when expressed from D12.5-D15.5 of pregnancy, but not D6.5-D9.5 of pregnancy we performed oligonucleotide microarray analysis. Total RNA from mammary tissue was harvested and utilized to generate biotinylated cRNAs that were used to probe Affymetrix genechips. Genes whose expression is up regulated or down regulated in a developmental stage-specific manner in response to c-MYC were identified. Among the genes whose expression is down regulated specifically when c-MYC is induced from D12.5-D15.5 of pregnancy but not D6.5-D9.5 of

pregnancy is *Caveolin-1*, which has been identified as a negative regulator of the Prlr/Jak2/Stat5 signaling pathway. Western blot analysis of Caveolin-1 expression in mammary protein lysates from MTB and MTB/TOM mice induced from D6.5-D8.5 or D12.5-D14.5 of pregnancy confirms that Caveolin-1 protein expression is down regulated to late pregnancy levels when c-MYC is expressed from D12.5-D14.5, but not D6.5-D8.5 of pregnancy (Fig. 4). Down regulation of Caveolin-1 expression is expected to result in increased Jak2 kinase activity and subsequent activation of Stat5. This represents a plausible mechanism by which c-MYC induces precocious lactation in a developmental stage specific manner.

Key Accomplishments:

- Determined expression and activity level of c-MYC transgene during early and late pregnancy.
- Identified Stat5 activation and induction of precocious lactation as mammary developmental stage specific effects of c-MYC.
- Identified down regulation of Caveolin-1 as a potential mechanism accounting for developmental stage specificity.
- Began genetic studies to determine the role of Stat5 in mediating c-MYC-induced mammary epithelial proliferation, differentiation, apoptosis and tumorigenesis.

Reportable Outcomes:

- Poster presentation – 2003 Gordon Conference on Mammary Gland Biology

Conclusions:

We have demonstrated that mammary epithelial expression of the c-MYC proto-oncogene for discrete periods during early and late pregnancy results in dramatically different biological outcomes within the mouse mammary gland. Mammary epithelial expression of c-MYC from D12.5-D15.5 of pregnancy results in down regulation of Caveolin-1 expression, activation of Stat5, and precocious lactation. Meanwhile, expression of c-MYC from D6.5-D9.5 of pregnancy results in none of these dramatic molecular and morphological changes in the mammary gland. These results identify the first known mammary developmental stage specific effect of c-MYC, indicating that the developmental stage of the mammary gland at the time of oncogene exposure can have a dramatic impact on the outcome of aberrant oncogene activation.

Additionally, we have identified a novel interaction between c-MYC and the Stat5 signaling pathway. As stated in objectives II and III, we are in the process of determining the precise role this mammary developmental signaling pathway plays in mediating the effects of aberrant c-MYC activation within the mammary gland. These effects include the ability of c-MYC to induce differentiation, proliferation, apoptosis and tumorigenesis.

Appendix 1 – Figures

Fig.1

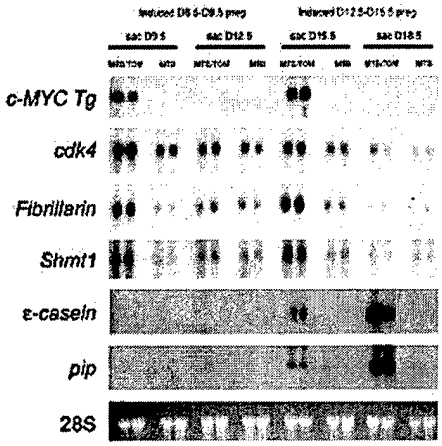

Fig. 2

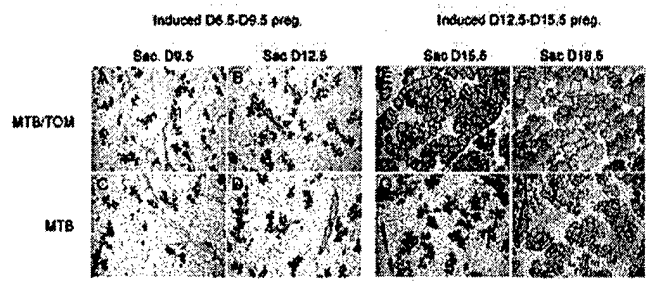

Fig. 3

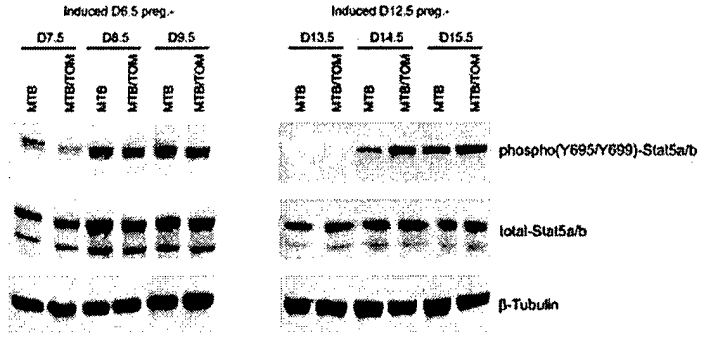

Fig. 4