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Award Number: W81XWH-05-1-0323

TITLE: Role of Rac GTPases in Chemokine-Stimulated Breast Carcinoma Metastasis

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REPORT DATE: July 2008

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

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
01-07-2008	Annual	1 JUL 2007 - 30 JUN 2008

4. TITLE AND SUBTITLE

Role of Rac GTPases in Chemokine-Stimulated Breast Carcinoma Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0323

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)

North Shore-LIJ Research Institute
Manhasset, NY 11030

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
CXCR4 is highly expressed in breast carcinoma cells and is essential for breast cancer metastasis to the lung. CXCR4 is the receptor for CXCL12, a chemokine that is enriched in organs that are targeted by metastatic breast cancer, such as lung and liver. The molecular mechanisms of CXCR4-mediated breast cancer metastasis however are poorly understood. In this project we aimed to test the hypothesis that Rac proteins are essential for CXCR4-mediated breast carcinoma cell proliferation and survival, thereby contributing to breast cancer metastasis. In Task 1, we have investigated the role of Rac proteins in CXCL12-regulated breast carcinoma cell proliferation and survival in vitro. We have focused our analysis on a novel splice form of Rac1 that is induced during breast cancer progression. Our findings indicate that Rac1 and Rac1b play distinct roles in cell proliferation and cellular signaling events. In Task 2, we planned to determine the contribution of Rac proteins to breast cancer metastasis in vivo, but we had to terminate this line of research due to technical difficulties.

15. SUBJECT TERMS
Cancer cell biology, metastasis models, chemokines, Rac1, Rac1b, Rac3, CXCL12, CXCR4, proliferaton, survival, RNA interference

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)
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INTRODUCTION

This project examines the roles of the small GTPases Rac1 and Rac3 in CXCR4-mediated metastasis of breast carcinoma cells. CXCR4 is highly expressed in breast carcinoma cells and is the receptor for CXCL12, a chemokine that is produced in abundance in organs that are targeted by metastatic breast cancer, such as lung, liver and bone. Rac1b is a splice form of Rac1 that is constitutively active and expressed in breast cancer tissue and therefore the specific role of this splice variant in CXCR4-mediated breast carcinoma metastasis is examined separate from the role of Rac1 itself. The first aim was to determine the specific roles of Rac1, Rac1b and Rac3 in CXCL12-stimulated carcinoma cell survival and proliferation as well as in downstream signaling events. The second aim was to examine the roles of Rac1, Rac1b and Rac3 in two animal models of breast cancer metastasis, the first using orthotopic mammary fat pad-implanted human breast carcinoma cells (spontaneous metastasis) and the second tail vein-injected cells (experimental metastasis).

Notes:
1) Tasks 1 and 2, as outlined in the Body of the report below, refer to the Tasks described in the revised Statement of Work, effective June 8, 2006.
2) The time period of the grant has been extended to 12/31/08 under a No Cost Extension (Amendment F00002, 5/23/08).

BODY

Task 1. **To determine the role of Rac proteins in CXCL12-regulated functions in breast carcinoma cells *in vitro*.**

Task 1a: Determine the contribution of Rac1 and Rac3 to CXCL12-stimulated proliferation and survival of breast carcinoma cells.
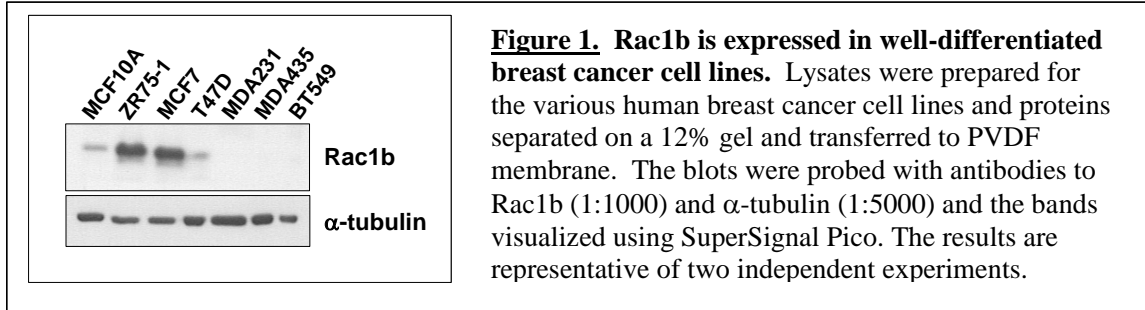
With respect to our search for breast carcinoma cell lines that are CXCL12-responsive, in last year's write-up we reported that CXCL12 caused an increase in ERK activation in BT474 cells. This increase however turned out to be very modest and inconsistent. In view of this and of the limited time remaining of the grant period, we decided to put these efforts on hold and focus on the functional characterization of Rac1b in breast carcinoma cells.

Task 1c: Determine the contribution of Rac1b to CXCL12-stimulated proliferation and survival of breast carcinoma cells.
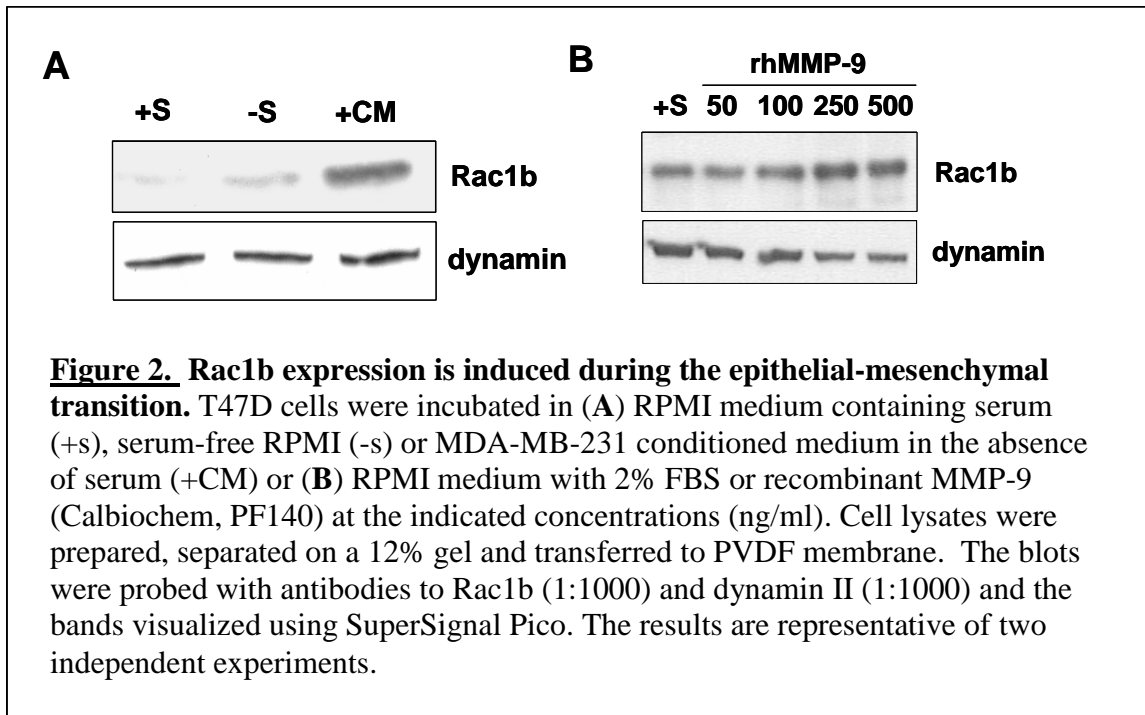
In year 2 of the project, we had demonstrated that Rac1b is important for the formation lamellipodia and the activation of the ERK and JNK MAP kinase cascades in ZR75-1 cells. Surprisingly, in these cells, simultaneous siRNA-mediated depletion of both Rac1 and Rac1b stimulated ERK and JNK, indicating that Rac1 and Rac1b play antagonistic roles in the regulation of these MAP kinase pathways. To further examine this, we set out to generate siRNA oligos that specifically target Rac1 without affecting Rac1b. To this end, we designed two oligos that spanned the sequence 5' and 3' of the Rac1b insert sequence. Although both siRNAs were able to strongly inhibit the expression of Rac1, they also decreased Rac1b expression (data not shown), making these siRNAs uninformative.

Rac1b is a constitutively active form of the Rac1 protein and therefore presumably is mainly regulated at the level of transcription. The only condition known thus far to induce Rac1b expression is the epithelial-mesenchymal transition (EMT) in murine breast carcinoma cells that is caused by matrix metalloproteinase MMP3 or MMP9 [1]. Thus, in order to study Rac1b under physiologically relevant conditions, we set out to identify human breast carcinoma cell lines that are appropriate for the functional characterization of Rac1b in conditions where Rac1b is induced during the EMT process. Interestingly, Rac1b is poorly expressed in MCF10A**,** a normal human breast epithelial cell line, and is undetectable in all the highly invasive breast carcinoma cell lines that we have tested (MDA-MB-231, MDA-MB-435 and BT549), but is highly expressed in two poorly invasive, well-differentiated cell lines (ZR75-1 and MCF7) (Figure 1). These results are consistent with the hypothesis that Rac1b plays a role in the EMT conversion of well-differentiated breast carcinoma.

We next examined whether Rac1b is induced during EMT in T47D cells. To induce EMT, in addition to recombinant human MMP9 (rhMMP9), we also used MDA-MB-231 conditioned medium (that is enriched in MMP9 [2;3]) and a number of other factors that
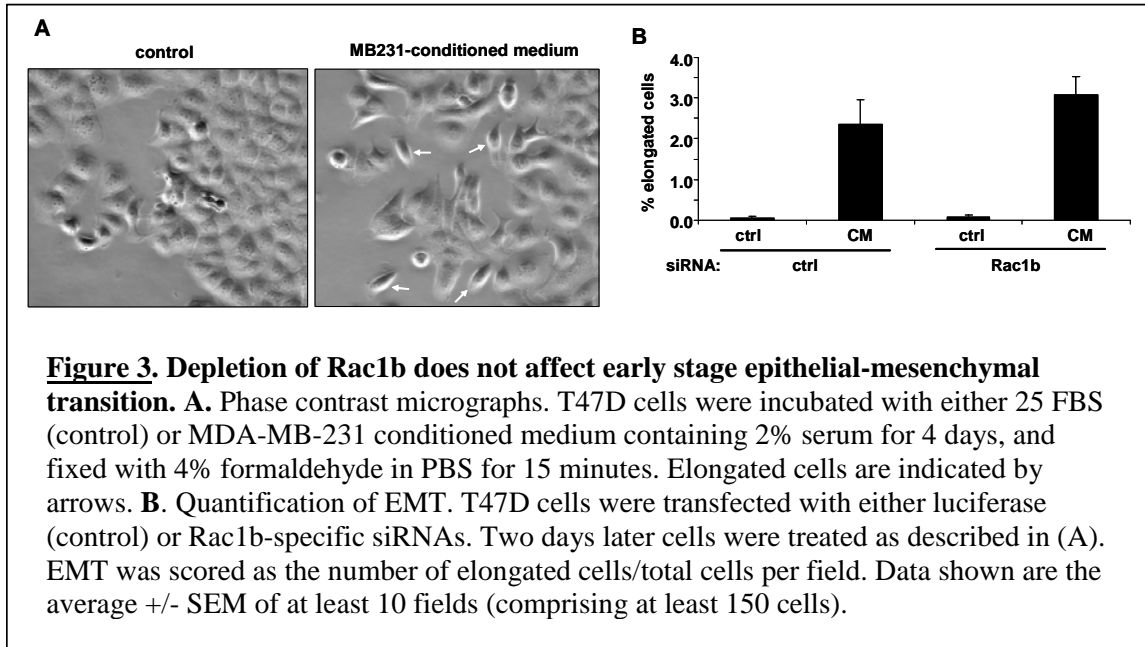
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Figure 1. Rac1b is expressed in well-differentiated breast cancer cell lines. Lysates were prepared for the various human breast cancer cell lines and proteins separated on a 12% gel and transferred to PVDF membrane. The blots were probed with antibodies to Rac1b (1:1000) and α-tubulin (1:5000) and the bands visualized using SuperSignal Pico. The results are representative of two independent experiments.

have been shown to induce EMT-like processes in other systems (HGF, EGF, TGFβ and IL-6). In addition, we inhibited adherens junction formation by application of an E-cadherin blocking antibody (Zymed, clone SHE78-7, mAb). Both MMP9 and MDA-MB-231 conditioned medium significantly upregulated Rac1b (Figure 2), whereas the other treatments did not. Notably, expression of Rac1b also is increased by serum starvation, indicating that Rac1b can be induced by stress conditions. We plan to extend these observations to other stress conditions that are clinically relevant, including hypoxia, ionizing radiation and chemotherapeutic drugs. We also examined whether rhMMP9 or MDA-MB-231 conditioned medium could stimulate Rac1b expression in ZR75-1 cells, but none of these treatments caused a further increase in Rac1b, possibly because Rac1b is expressed at a constitutively high level in these cells (data not shown).

Figure 2. Rac1b expression is induced during the epithelial-mesenchymal transition. T47D cells were incubated in (**A**) RPMI medium containing serum (+s), serum-free RPMI (-s) or MDA-MB-231 conditioned medium in the absence of serum (+CM) or (**B**) RPMI medium with 2% FBS or recombinant MMP-9 (Calbiochem, PF140) at the indicated concentrations (ng/ml). Cell lysates were prepared, separated on a 12% gel and transferred to PVDF membrane. The blots were probed with antibodies to Rac1b (1:1000) and dynamin II (1:1000) and the bands visualized using SuperSignal Pico. The results are representative of two independent experiments.

To examine the role of Rac1b in MMP-induced EMT, we first determined whether siRNA-mediated depletion of Rac1b could interfere with the marked morphological changes that characterize the EMT process, i.e. cell-cell junction breakdown and cell elongation. However, using this morphological phenotype as a read out, our preliminary

experiments did not detect a significant effect of Rac1b depletion on MMP-induced EMT in T47D cells (Figure 3). Thus, Rac1b may play a role at a later stage of EMT, or subsequent events, such as migration and invasion. Alternatively, Rac1b may not be important for the EMT process itself, but protects the cell against the stress that is caused by the EMT process.

Figure 3. Depletion of Rac1b does not affect early stage epithelial-mesenchymal transition. A. Phase contrast micrographs. T47D cells were incubated with either 25 FBS (control) or MDA-MB-231 conditioned medium containing 2% serum for 4 days, and fixed with 4% formaldehyde in PBS for 15 minutes. Elongated cells are indicated by arrows. **B**. Quantification of EMT. T47D cells were transfected with either luciferase (control) or Rac1b-specific siRNAs. Two days later cells were treated as described in (A). EMT was scored as the number of elongated cells/total cells per field. Data shown are the average +/- SEM of at least 10 fields (comprising at least 150 cells).

EMT is thought to occur at early stages of breast cancer progression, i.e. DCIS-Stage I [4]. Thus, it would be very informative to determine Rac1b expression levels in breast tumor tissue corresponding to various stages of tumor progression. To this end, we first examined a reverse phase protein microarray (Protein Biotechnologies). This microarray is still under development and comprises tumor lysates from different stages of breast cancer, spotted onto a nitrocellulose coated slide. We could not detect a significant Rac1b signal from the tumor material however. Since the Rac1b antibody has been validated for use in immunohistochemistry (Matt Slater, Millipore, personal communication), we next plan to utilize a tumor tissue microarray to examine Rac1b expression levels *in situ*.

Task 2. To determine the contribution of Rac1, Rac1b and Rac3 to CXCL12/CXCR4-mediated breast cancer metastasis.

Task 2a: Construction and production of viruses for retroviral transfection of shRNA.

As mentioned in the report of year two, we did not succeed in making stable breast carcinoma cell lines with decreased levels of expression of Rac1, likely because cells with lower levels of Rac1 are selected against, since they proliferate significantly slower. We have considered engineering cells that express Rac1-directed shRNA together with GFP, with the idea to sort out Rac1-depleted cells, but reasoned that this only would constitute temporary improvement and that Rac1-depleted cells also would be selected

against in the tumor setting, making it impossible to test the hypothesis that Rac1 contributes to the metastatic process. Another alternative that we considered was to engineer cells that express Rac1-directed shRNA from an inducible promoter. However, we finally decided to terminate this line of research due to the considerable technical challenges and time constraints and to rather focus on the functional analysis of Rac1b, which is more likely to yield publishable results during the time frame of the grant.

KEY RESEARCH ACCOMPLISHMENTS

We found that Rac1b is preferentially expressed in well-differentiated breast carcinoma cell lines and can be induced under stress conditions. Our results also indicate that Rac1b is not necessary for early stages of epithelial-mesenchymal transition in breast carcinoma cells.

REPORTABLE OUTCOMES
None

CONCLUSION

The poor responsiveness of the breast carcinoma cell lines that we have examined to CXCL12 *in vitro* have made it exceedingly difficult to examine the role of Rac proteins in CXCL12-stimulated functions in breast carcinoma, which constituted a large part of Task 1. Technical difficulties with establishing stable Rac1-depleted cell lines also have prevented us from accomplishing Task 2. Over the past year, we therefore have continued the functional characterization of Rac1b in breast carcinoma cells. We found that Rac1b is preferentially expressed in well-differentiated breast carcinoma cell lines and plan to examine Rac1b expression levels in tumor tissues of the respective stages of breast carcinoma progression. We also found that Rac1b can be induced by serum starvation and will extend these studies to additional clinically relevant stress conditions. Our results also indicate that Rac1b is not necessary for early stages of epithelial-mesenchymal transition in breast carcinoma cells.

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APPENDICES
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