

AWARD NUMBER: W81XWH-21-1-0002

TITLE: Amplification Events Altering Tumor Microenvironment That Drive Metastasis in HER2+ Breast Cancer

PRINCIPAL INVESTIGATOR: Eran Andrechek

CONTRACTING ORGANIZATION: Michigan State University, East Lansing, MI

REPORT DATE: January 2023

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

<b>1. REPORT DATE</b> January 2023		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 01Jan2022-31Dec2022	
<b>4. TITLE AND SUBTITLE</b>  Amplification Events Altering Tumor Microenvironment That Drive Metastasis in HER2+ Breast Cancer				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-21-1-0002	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  Eran Andrechek  E-Mail: andrech1@msu.edu				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  MICHIGAN STATE UNIVERSITY 426 AUDITORIUM RD RM 2 EAST LANSING MI 48824-2600				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  In the past year a new trainee has been added to the project and we have proceeded through rounds of selection to generate the CRISPR constructs. In the generation of the knockdowns, significant issues were identified with 5 rounds of troubleshooting to eventually identify the fact that our lentiviral system was not effectively packaging virus (after switching to a newer generation lentivirus due to flow sorting facility requirements). We are now able to generate lentivirus and have generated knockdowns of the target genes (Coll1A1, CHAD and PHB). While selecting for knockdowns, assays have been optimized to characterize metastatic progression and the steps at which metastasis may be blocked, from in vitro migration assays to culture of single cells from blood to examination of metastasis of parental lines in lungs as well as the lymphatic system.					
<b>15. SUBJECT TERMS</b> None listed.					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>  Unclassified	<b>18. NUMBER OF PAGES</b>  6	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRDC
<b>a. REPORT</b>  Unclassified	<b>b. ABSTRACT</b>  Unclassified	<b>c. THIS PAGE</b>  Unclassified			<b>19b. TELEPHONE NUMBER (include area code)</b>

## TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	5
5. Changes/Problems	5
6. Products	6
7. Participants & Other Collaborating Organizations	6
8. Special Reporting Requirements	NA
9. Appendices	NA

## Introduction

Preliminary work from our laboratory on whole genome sequencing of transgenic mice overexpressing Neu (HER2, erbb2) revealed an amplification event composed of ~40 genes. Surprisingly, in HER2+ve breast cancer the same amplification event is also present and is distinct from the HER2 amplification event. This region was strongly associated with metastatic progression of the HER2+ breast cancers and we hypothesized that there were genes within the amplification event that impacted metastasis. To test this hypothesis, this proposal seeks to knockout several of the key genes and test for the impact on metastatic progression.

## Keywords

HER2 / Neu / ErbB2  
Mouse model  
Col1A1 (collagen 1A1)  
CHAD (Chondroadherin)  
PHB2 (prohibitin2)  
MMTV

## Accomplishments

### Task 1 Subtask 1

NDL2-5 knockout has been completed.

BT474 knockouts have not been completed for PHB, Col1A1 or Chad (Col1A1 and Chad were previously completed but lost during a LN2 freezer meltdown). Cells have repeatedly died during selection after transfection (attempted numerous times with numerous reagents with many variable changes). GFP was used as a transfection control and we were unable to flow sort any GFP positive cells. Switching to a lentiviral based system, we then could not produce lentivirus and once that issue was solved (defect in the packaging cell line), the lentiviral infection resulted in dead BT474 cells with both CRISPR knockout and knockdown for each of the genes. We are now using optimized conditions and procedures to improve lentivirus production, including a brand new stock of 293T cells, quantitating lentivirus via qRT-PCR, concentrating lentivirus, and optimizing transduction conditions and are still unable to generate knockdown or knockouts.

To resolve this issue, we have sought approval from our PO (Jessica Brusgard) and have been approved to contract with a company (Vector Builder) to provide these knockdown cell lines. This is project to take ~3 months to complete.

Tasks 2-5 have not been initiated as they were dependent upon the alteration of PHB, Col1A1 and CHAD.

### Task 2 Subtask 1

Expression studies of the three genes using IHC. This has been initiated to validate antibodies even though we are lacking the knockout controls from Task 1 Aim 1. Previous antibodies that were profiled and working have had lot number switches and are now generating too much background to give scientific confidence in the results. Antibodies listed in the original manuscript for preliminary data have since been discontinued by the manufacturer, and alternative antibodies tested via western blot have yielded mixed results. Western blots have been conducted for both human tumors grown in mice as well as mouse model tumors. While we have a good PHB antibody (and have purchased a frozen stock), we are still seeking suitable antibodies for Col1A1 and CHAD. We are actively exploring various sources and options to acquire appropriate antibodies for our research.

### Subtask 2-4

Have not been initiated as we are waiting for the knockout controls, now contracted out to Vector Builder.

### Major Task 3

Not initiated but planned in months 24-30. We anticipate that this will be slightly delayed but are committed and excited about this aim of the proposal. It may be necessary to request a no-cost extension but this will become more clear in the coming months. In an attempt to get the proposal back on timeline, we are recruiting another individual to the project.

## **REJECTED PRIOR SUBMISSION (2023)**

In the first year we described the loss of the previously described cell lines with Col1A1 and CHAD knockout due to a new student overfilling the liquid nitrogen freezer, resulting in cryovials with the frozen cell lines shattering. The backup lines at -80C did not survive the freeze/thaw cycle and we have had to work on regeneration of these lines. This has been hampered by supply chain issues with a lack of tissue culture plates, tips and tubes, indeed a recent order for basic supplies was quoted at a 4-5 month delivery.

At a personnel level, the lab has been quarantined two times in the past year due to positive COVID cases in the personnel working on the funded work.

The major goals of the project during the reporting period were as follows:

- 1) Generate PHB knockdown / knockout in BT-474 and NDL2-5 cell lines.
- 2) 4 subtasks examining metastatic spread in mice (angiogenesis, intravasation, extravasation and colonization) using knockouts of Col1A1, CHAD and PHB in comparison to parental controls.

In pursuit of the goals, Col1A1 and CHAD lentiviruses were generated, introduced to NDL2-5 cell lines, selected and were flow sorted for GFP. Confirmation of knockout was done initially by RT-PCR and was attempted by western blot shortly.

These lines failed to have significant knockout in the first several attempts. Troubleshooting eventually revealed (after five rounds of generating lentivirus) that the switch to a newer generation lentiviral system resulted in an inability to package sufficient lentivirus and that the flow sorted cell lines were not actually GFP positive. This lentiviral switch to a newer generation line was done for safety at the request of the flow sorting facility. Ultimately, we found that the packaging cell line we were using was at fault. Using a new packaging cell line we have produced virus and have infected and sorted COL1A1, CHAD and PHB knockout lines (CRISPRi knockdown for PHB). The initial results were encouraging with high levels of GFP demonstrating good infection of all sorted cells. Isolation and characterization of knockout and knockdown clones is underway and is being carried out by two students.

Once all transductions are complete and flow sorted, confirmation of knockout / knockdown will be confirmed by Western blot and DNA sequencing or through RT-PCR / target alteration.

For Aim 2, we have not directly initiated any mouse work as we are still optimizing the lines. However, all mouse work has been approved by IACUC and the approved protocol was submitted to the DoD. Training and optimization of various experiments has been undertaken so that all experiments are ready to feed in the CRISPR knockouts. This includes optimization of scratch assays, culture of single cell metastases from blood and detection / characterization of metastatic lesions in the lungs. Interestingly, in our injections into mice (using general lab funds until CDMRP fully approves the animal work), we were surprised to note lymphatic metastases. While these were not previously described in mouse experiments, we have characterized this so that a comparison of the COL1A1 / CHAD / PHB lines can readily be undertaken once knockouts are generated.

With the difficulty in accomplishing goals and troubleshooting of the CRISPR experiments, we have used the time to fully train the student in bioinformatics in preparation for RNAseq in subsequent years. The student has completed several courses in R studio, Linux, optimization of High performing computer cluster analysis and in RNAseq analysis. This has also been expanded to whole genome sequence analysis so that he can integrate the RNAseq data with whole genome sequence results. Using a practice dataset previously generated and analyzed in the lab, he has actually uncovered new results during his training as new modules have been released since the initial generation of the data. For instance, while the new callers for single base variants (mutations) and copy number variants (amplifications) reinforced the Col1A1 / CHAD finding, it also uncovered conserved mutation patterns and integration of the data with RNAseq allowed for identification of subtypes. Further, the translocation caller actually identified the PGK-Neo construct that was used in the genetically engineered mice as well as several conserved events. These results were fully unexpected but a side benefit of the training in new modules for RNAseq and WGS data analysis. These results will be used to generate a computational manuscript.

## **Impact**

Nothing significant to report at this time but we anticipate writing a manuscript within the next month for submission to bioRxiv and then to journals for publication.

## **Changes / Problems**

During training for bioinformatics, the student used a practice dataset that was previously analyzed and published. The new bioinformatic modules and methods have uncovered new findings which will be detailed in a manuscript this year. We anticipate having a final draft within the next several weeks for submission. In addition to the manuscript, the analysis pipeline is now in place and only needs the RNAseq data from the knockout to be entered to allow for rapid analysis.

As described above, we faced numerous supply chain and COVID related issues, including several positive tests that resulted in quarantines for the students and myself.

Changes that impacted expenditures include not beginning the mouse experiments, resulting in much less spending that anticipated. This will be transferred to the next period of the proposal where the mice will be generated / purchased and costs will be higher.

Lentiviral production was changed to the latest generation of lentivirus. We then encountered issues with packaging, ultimately found to be in the packaging cell line. This has been replaced and we are now generating virus and are able to infect cells. The three targets are being selected again for knockout / knockdown.

## Products

My attendance at the GRC Meeting on Mammary gland biology had to be cancelled when I developed COVID several days before the meeting, so there is no product to report at this point. The student and myself both plan to attend the GRC Mammary meeting this spring to describe the various aspects of the ongoing project.

## Participants & Other Collaborating Organizations

Name: Carson Broeker

Project Role: Graduate Student

Project months worked: 12

Contribution to project: Mr. Broeker has completed all of the experimental work.

Funding support: Partially from this award and partially from an internal cancer studentship (Aitch Foundation award).

Name: Eran Andrechek

Project Role: PI

Project months worked: 3

Contributions to project: Dr. Andrechek has spent significant time mentoring and training Mr. Broeker in cell culture, CRISPR and the bioinformatic requirements of the project

Funding Support: 1 month summer from this project, 9 month annual salary appointment.

Name: James Lord

Project Role: Graduate Student (rotation)

Project months worked: 2 months

Contribution to project: Mr. Lord has been directly assisting Mr. Broeker complete the tissue culture and sequencing for this project.

Name: Caroline Downes

Project Role: Undergraduate volunteer

Project months worked: 10 hours/week x 8 months

Contribution to project: Ms. Downes has been directly assisting Mr. Broeker complete the tissue culture and sequencing for this project.

Name: Ashlee Perry

Project Role: Undergraduate volunteer

Project months worked: 10 hours/week x 8 months

Contribution to project: Ms. Perry has been directly assisting Mr. Broeker complete the tissue culture and sequencing for this project.

Active support changes: nothing to report.

Other organizations: nothing to report.