AWARD NUMBER: W81XWH-21-1-0002

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

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REPORT DATE: JANUARY 2024

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;
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*Form Approved*
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| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| JANUARY 2024 | Annual | 1JAN2023 - 31DEC2023 |

**4. TITLE AND SUBTITLE:**
**Amplification Events Altering Tumor Microenvironment That Drive Metastasis in HER2+ Breast Cancer**

**5a. CONTRACT NUMBER** W81XWH-21-1-0002

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Development 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**

After several rounds of failed knockout/knockdown attempts we have now had a company build the knockdowns for the various constructs. We have knockdowns of three genes and have observed compensatory gene expression results in other related genes. We have also constructed overexpression constructs as a backup in case these knockdowns did not work and they can be used in rescue experiments. A bioinformatic screen to inhibit the pathways through alternative approaches has revealed several potential drugs that could be used to inhibit the pathways in tissue culture. Initial tissue culture results into growth rate and morphology are being repeated to confirm potential results.

**15. SUBJECT TERMS**

NONE LISTED

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

**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

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## 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

Enclosed is the overall summary of progress for calendar year 2023 for Department of Defense Congressionally Directed Medical Research Programs award number: W81XWH-21-1-0002.

After disappointing western blot results from the third complete attempt at making needed cell lines, progress in early 2023 was focused on the fourth complete attempt at creating the necessary cell lines for project completion. The parental cell lines in question were BT-474, an ER+/PR+/HER2+, p53 mutant human invasive ductal carcinoma cell line, and NDL2-5, a cell line derived from MMTV-NeuNDL2-5-IRES-Cre transgenic mice on the FVB background expressing oncogenic human *ERBB2* (neu8342, NDL2-5, neu deletion). We were attempting to create cell lines using CRISPR interference technology to downregulate genes COL1A1, CHAD, and PHB in both BT-474 and NDL2-5 cell lines. The main hypothesis of the funded proposal states that COL1A1, CHAD, and PHB play a role in regulating the migration and metastatic capacity of HER2+ breast cancer, where these cell lines would be used to directly test the effects of knocking down gene expression for these three putatively key genes.

Transfection of 293T cells at 90-95% confluency with psPAX2, pMD2.G, and CRISPRi vectors was completed in late January of 2023. Lentivirus was collected for each condition, concentrated, and then used to infect respective BT-474 and NDL2-5 cell lines in February 2023. Cell line selection was accomplished via a minimal dose to kill curve of puromycin, which the CRISPRi vectors should provide resistance to. However, supposedly lentivirus infected cells died at the same rate as the parental cell line, suggesting puromycin resistance had not been conferred. A selectable mCherry marker was also present within the CRISPRi vectors, so puromycin treatment was ended early and cells sent for flow cytometry sorting based on presence of mCherry in March of 2023. Unfortunately, no cells in either BT-474 or NDL2-5 cell lines showed detectable levels of mCherry during flow sorting. During this time and into April 2023, we began reevaluating project goals and how we were going to accomplish these goals. With permission of the program officer, we began soliciting quotes from companies for creation of COL1A1, CHAD, and PHB knockdown cell lines at this point to accomplish project goals.

The primary graduate student working on this project was out of the lab for an internship from June until early September of 2023, but the contracted company (Vector Builder) was working on creating COL1A1, CHAD, and PHB knockdown cell lines at that time.

Given that the prior four attempts at creating the needed cell lines with existing lab protocols had not worked, in addition to the Vector Builder constructs we decided to start over with brand new plasmids and reagents for creating CRISPR activation and CRISPRi. New sgRNAs were designed for COL1A1, CHAD, and PHB for both mice and in humans using the CRISpick software.

Results are detailed below.

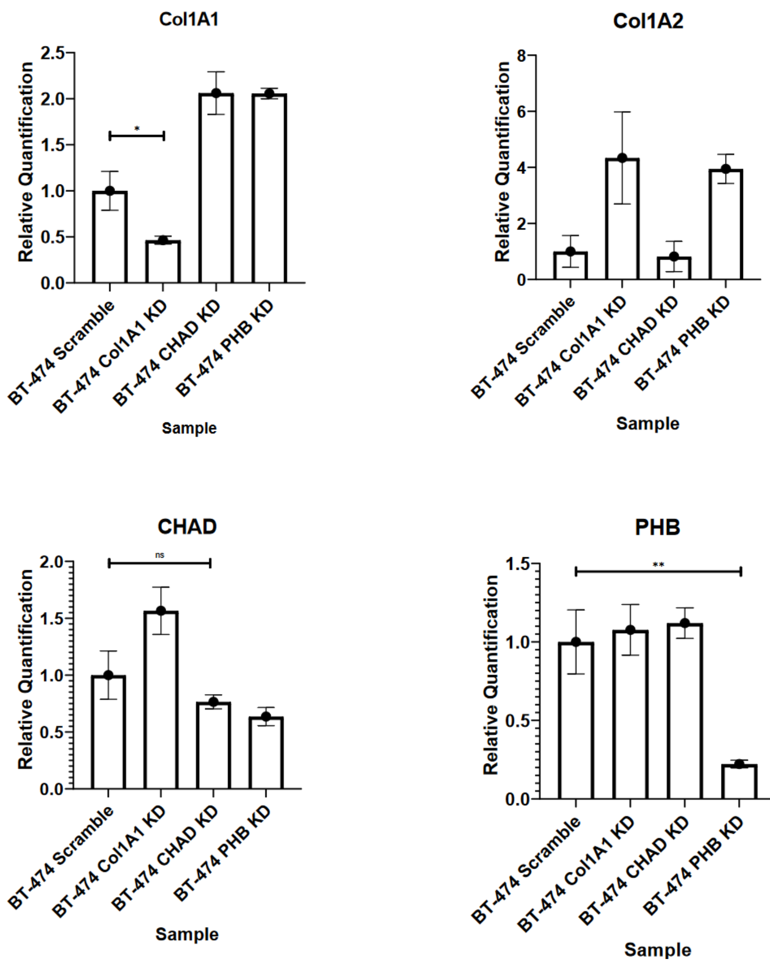
To resolve the prior issues, partially noted above, we have sought approval from our PO (Jessica Brusgard) and have been approved to contract with a company (Vector Builder) to provide these knockdown cell lines. This has been completed. This resulted in knockdowns of each of the three genes at the following levels:

CHAD: pools with 42, 34 and 36% of wild type levels.
COL1A1: pools with 33 and 38% of wild type levels, pool 3 was unsuccessful. (110% of wild type)
PHB: pools with 12, 14 and 36% of wild type levels.

Morphology differences noted for PHB knockdown, cells noted to be significantly reduced in size and morphology altered.

For each, a scramble control was included.

We have now grown these lines and tested expression by QRT-PCR and verified the knockdown levels.

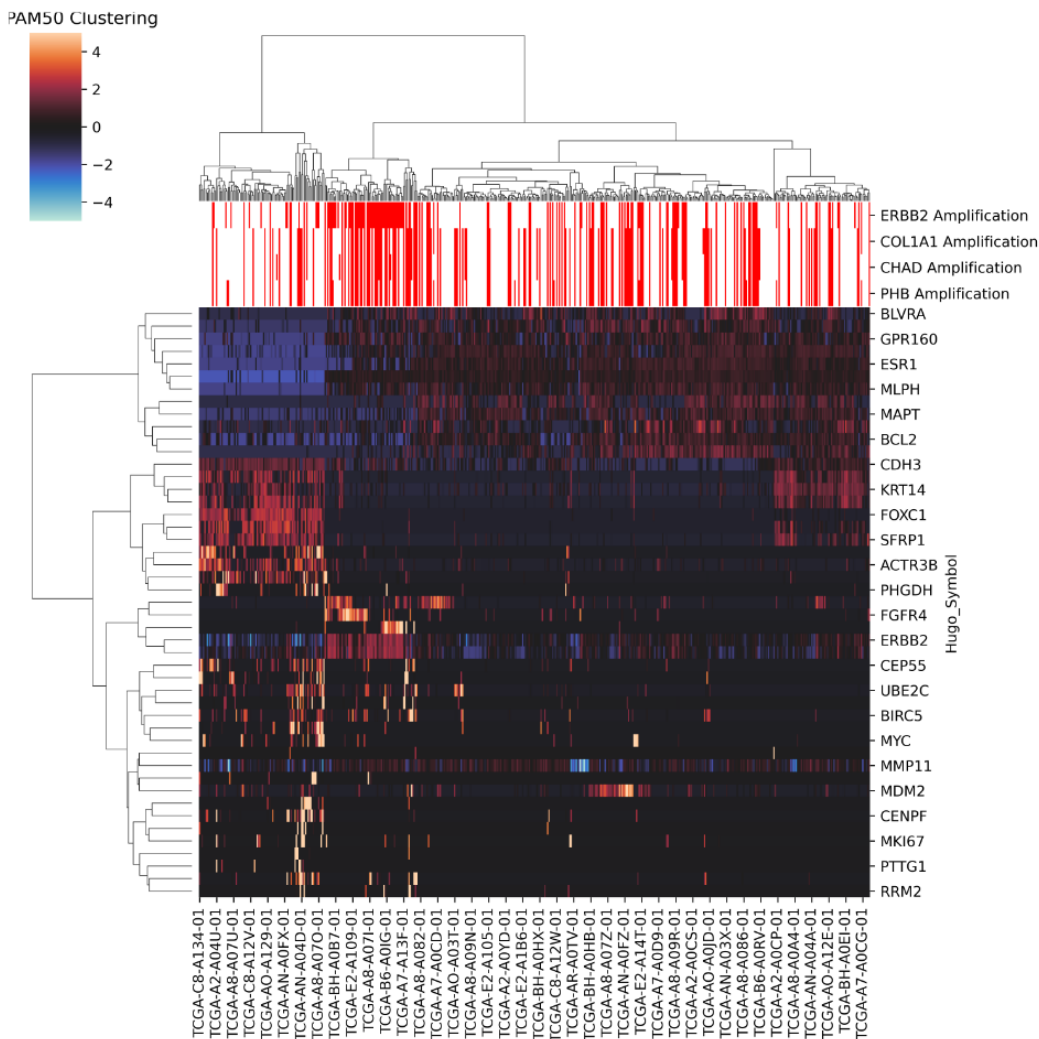



These results demonstrate significant knockdown for Col1A1 and PHB but a not quite significant knockdown of CHAD. This is being retested from an early freeze of the cell lines and we have initiated sorting so that we can generate clonal lines that may have higher levels of knockdown. Clones will be pooled so that we can avoid clonal effects.

In addition, this QRT-PCR data demonstrates several surprising findings. First, in the COL1A1 experiment, we see a 2 fold increase in Col1A1 in both the CHAD and PHB knockdown lines (top left). Next, we see a 4 fold increase in COL1A2 in the COL1A1 and PHB knockdown lines (top right). We observed a 1.5 fold increase in CHAD in the COL1A1 knockdown line and a 60% reduction of CHAD in the PHB line (bottom left). No alterations in PHB levels were noted except in the PHB knockdown (bottom right).

Careful consideration of these compensatory effects will be needed in the analysis of the cell lines for growth curves (being repeated now with PHB appearing to be slowed in preliminary data) and migration assays as well as the metastasis work for the coming year.

To ensure that we were not dependent on these knockdowns, we have also generated overexpression and CRISPRi constructs. This cloning is complete and cell lines are currently being generated for each of the three genes.




Unsupervised hierarchical clustering of TCGA Breast Invasive Carcinoma (Firehose Legacy) transcriptional data limited to PAM50 genes. Distances between clusters was computed using the Ward variance minimization algorithm. Transcriptional values used for clustering were z-scored prior to clustering. A red bar under x-axis cluster designations indicate the presence of at least a +1 copy number gain. Gene names are displayed on the y-axis, and tumor sample names are displayed along the x-axis.

In an additional step to avoid knockout issues, we have initiated a search for compounds targeting these lines. Using the above heatmap data, we have observed HER2+ve and HER2-ve tumors with amplification of ErbB2 and a subset (~25%) have amplification of the other three genes of interest. Using only the HER2+ve samples, we completed a CLUEio analysis, the successor to the C-Map software that predicted drug targets. This revealed a compound A-443654 that targeted PHB. This compound is readily available and will be used on several PHB amplified HER2+ve cell lines relative to PHB low lines.

Tasks 2 and 3 are being prepared for analysis with the cell lines that are being expanded and characterized now.

**Impact**

Nothing significant to report at this time but we are encouraged by actually having knockdowns to work with.

During training for bioinformatics, the student used a practice dataset that was previously analyzed and was unpublished. The new bioinformatic modules and methods have uncovered new findings which were detailed in a manuscript:
https://breast-cancer-research.biomedcentral.com/articles/10.1186/s13058-023-01723-3#Fun

**Changes / Problems**

None beyond those discussed with the PO.

## Participants & Other Collaborating Organizations

Name: Carson Broeker
Project Role: Graduate Student
Project months worked: 9
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) and recently (starting Oct 1) from F31.

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 from MSU.

Name: Alyssa Variola
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.

Active support changes: nothing to report.
Other organizations: nothing to report.