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TITLE: Antibodies Targeting EMT

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14. ABSTRACT Monoclonal antibodies are drugs that can specifically bind targets present on tumor cells. The highly aggressive triple-negative subtype of breast cancer does not have specific antibody drugs like Herceptin, and there is considerable need for targeted therapeutics and diagnostic biomarkers. We have developed a new technique allowing for discovery of new antibodies that disrupt a key process in cancer progression termed "epithelial to mesenchymal transition" or EMT. This process is important in several cancers, but is particularly associated with "triple-negative" breast cancer. We have applied our technology to identify unique antibodies that inhibit EMT and are now characterizing the antibodies to determine their targets on the cell. The newly discovered antibodies will then be engineered for utility as new highly specific drugs and diagnostics in preclinical experiments. This research could provide a new class of antibody therapeutic and diagnostic for triple-negative disease, identify new drug targets or pathways in cancer cells and make a major impact on breast cancer.					
15. SUBJECT TERMS Monoclonal Antibody, Epithelial to Mesenchymal Transition, Antibody Library					
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Table of Contents

	<u>Page</u>
1. Introduction.....	1
2. Keywords.....	1
3. Accomplishments.....	1
4. Impact.....	8
5. Changes/Problems.....	9
6. Products.....	9
7. Participants & Other Collaborating Organizations.....	10
8. Special Reporting Requirements.....	11
9. Appendices.....	11

1. INTRODUCTION:

Our proposal aims to “Revolutionize treatment regimens by replacing drugs that have life-threatening toxicities with safe, effective interventions”, particularly for triple negative breast cancer which is often associated with epithelial-to-mesenchymal transition (EMT) features and has substantial need for new and effective therapeutics. Epithelial to mesenchymal transition is a process normally used during embryonic development where epithelial cells downregulate their tight junctions (and adhesive properties), degrade extracellular matrix, and effectively “invade” other tissues to migrate to new cellular locations. The EMT program involves transcription of multiple new genes and regulation of several proteins including intermediate filaments and cell-surface proteins. Not surprisingly, invasive cancer often co-opts this process and advanced cancer cells often show properties of EMT. Since EMT is an extremely important biological process in cancer progression, and since antibodies are proven therapeutic and diagnostic molecules, our goal is to use a novel EMT assay to select antibodies from newly developed lentiviral libraries that either inhibit or reverse EMT. Further, we will employ a new antibody structure derived from cows to identify novel antibodies against unique epitopes. We will determine the cellular target and effects of these antibodies, and evaluate their potential as therapeutic candidates *in vitro* and *in vivo*.

2. KEYWORDS:

Monoclonal Antibody

Lentiviral Library

Epithelial to Mesenchymal Transition

Ultralong CDR H3

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

What were the major goals of the project? The goals of the project encompass two specific aims:

Specific Aim #1: Generate unique antibodies that inhibit EMT and characterize their functional properties

Specific Aim #2: Develop anti-EMT antibodies for therapeutic use

The aims and specific tasks associated with them from the Statement of Work are described below in Table 1.

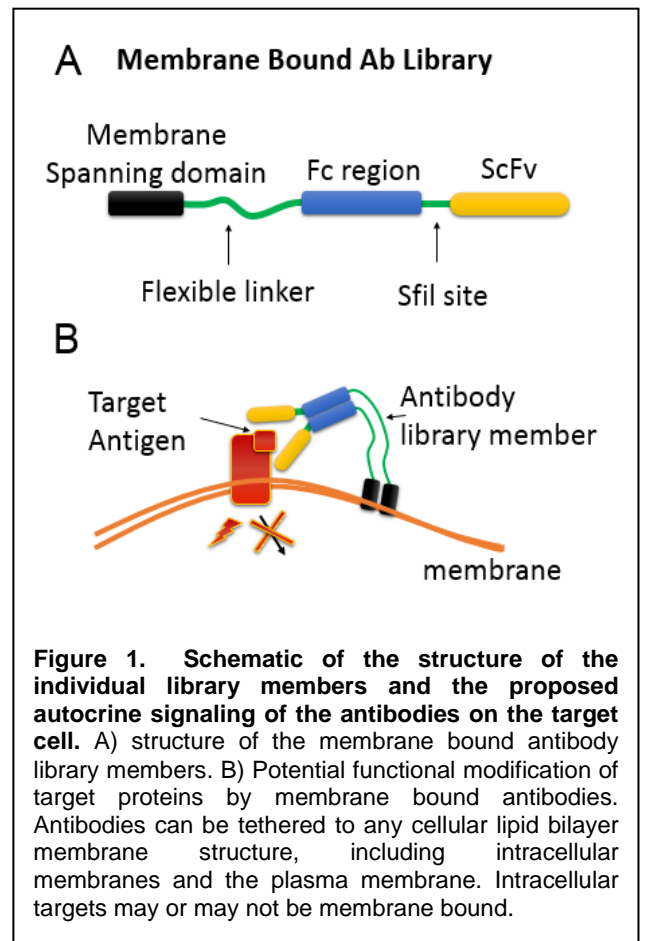
Table 1. Statement of Work goals, tasks, and progress

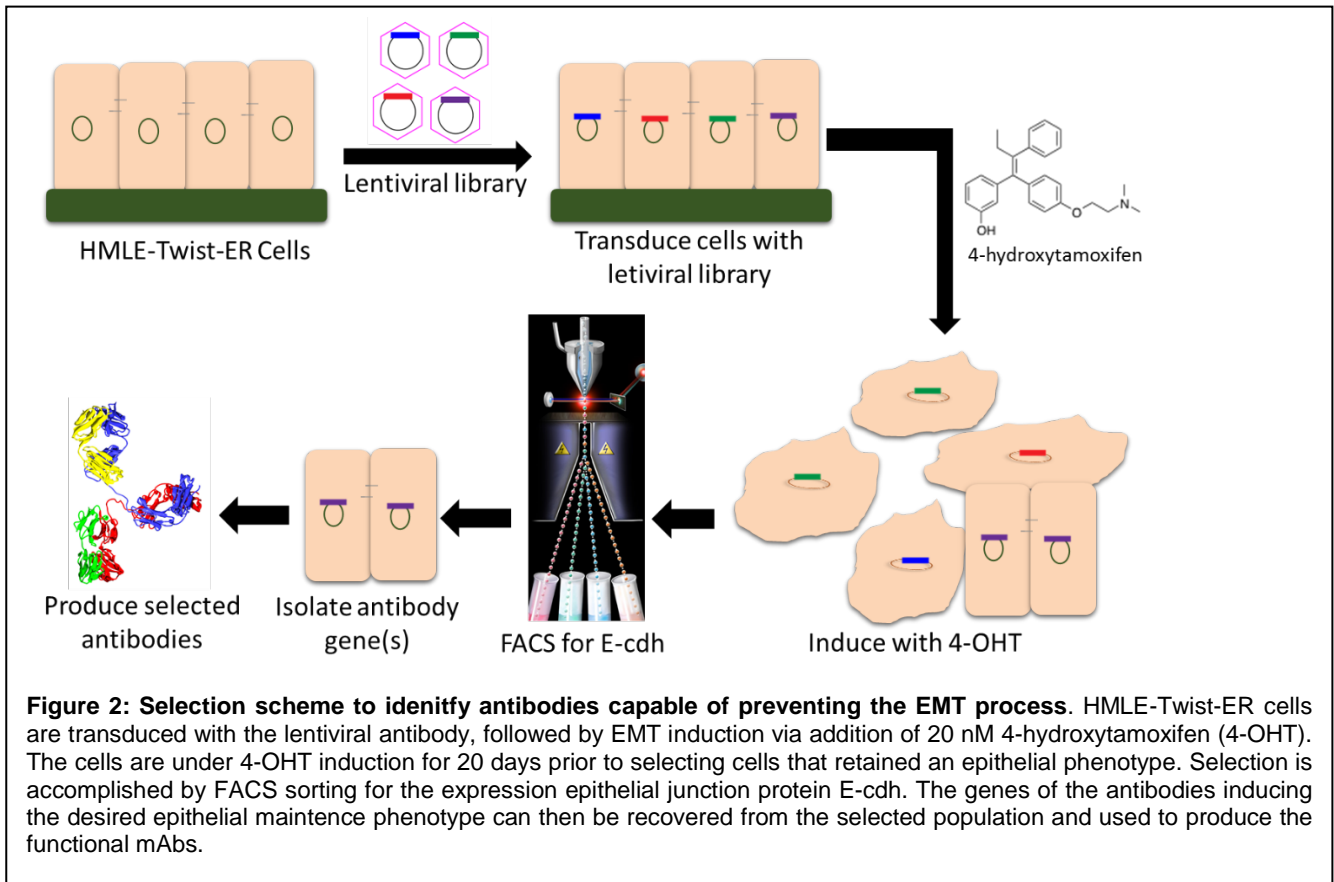
Goal	Timeline (Months)	Progress
Specific Aim #1: Generate unique antibodies that inhibit EMT and characterize their functional properties		
Major Task 1: Screen lentiviral antibody libraries		
Subtask 1: Construct new antibody libraries	1-3	(50% completed) We have constructed new vectors for bovine library insertion, and are currently completing this library. Our experiments below used a fully human scFv-Fc library.
Subtask 2: Screen libraries for EMT <u>inhibiting</u> antibodies	4-10	(75% complete) We have screened for EMT inhibiting antibodies and have identified multiple candidates that appear to inhibit EMT. We are confirming these results and attempting to identify the antigen to which they bind.
Subtask 3: Screen libraries for antibodies that induce EMT <u>reversal</u>	8-12	(20% complete) We have developed the assays for EMT reversal screening and are currently screening our libraries.
Milestone(s) Achieved: antibodies that inhibit or reverse EMT <i>in vitro</i> .	12	(75% complete). We appear to have antibodies that inhibit EMT, and are confirming these results.
Major Task 2: Identify targets of antibodies		
Subtask 1: Immunoprecipitation and MS of antigen	12-14	(25% complete) We are in the process of identifying the antigens for candidate antibodies.
Subtask 2: Confirm antigen binding	14-20	(0% complete)
Milestone(s) Achieved: Targets of anti-EMT antibodies are verified	20	(0% complete)
Specific Aim #2: Develop anti-EMT antibodies for therapeutic use		
Major Task 3: Analyze in vitro activity of antibodies		
Subtask 1: Evaluate cell growth inhibitory/killing, invasion, stem-cell self renewal and mammosphere	20-24	(0% complete)

formation properties of antibodies (standard cell lines, no identifiable information available to investigators)		
Subtask 2: Analyse mRNA pathways impacted by the antibodies by deep sequencing	20-24	(0% complete)
Milestone(s) Achieved: Cellular and molecular properties of inhibitory antibodies identified <i>in vitro</i>	24	(0% complete)
Major Task 4: Demonstrate anti-tumor activity <i>in vivo</i>		
Subtask 1: Evaluate efficacy in tumor xenograft models (3 groups of 8 mice)	24-36	(0% complete)
Milestone(s) Achieved: Antibodies identified that specifically inhibit tumor progression <i>in vivo</i>	36	(0% complete).
Local IRB/IACUC Approval	24	(100% complete)

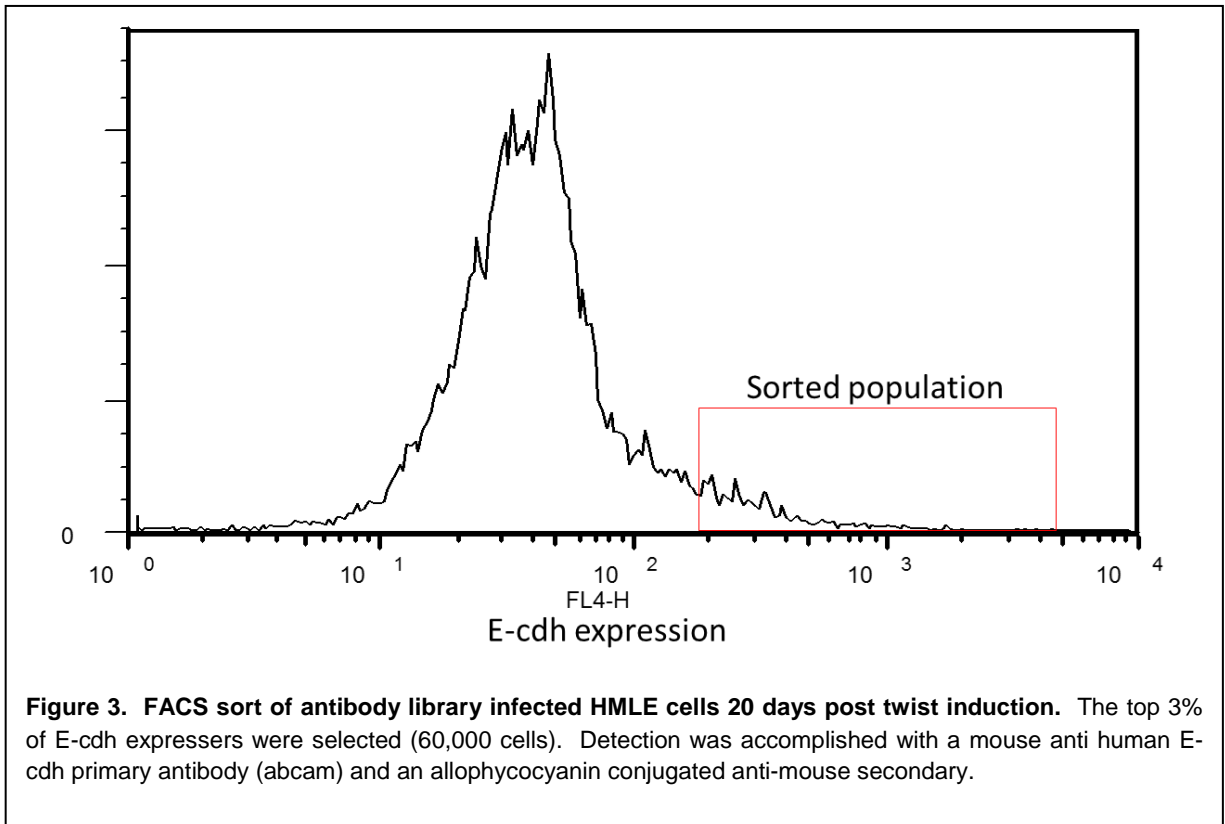
What was accomplished under these goals?

We have summarized our progress in **Table 1** above, in association with the tasks in the Statement of Work. Specifically, we were able to screen novel lentiviral antibody libraries to identify antibodies with EMT inhibitory properties, and are now characterizing these antibodies. To identify antibodies that can interfere with this process, we used the selection scheme outlined in **Figures 1 & 2**. Briefly, HMLE-Twist-ER cells were transduced with the lentiviral antibody library (or a control virus) and allowed to express the antibody library 117 for two days. Next, library transduced (and control) HMLE-TwER cells were induced to





undergo EMT with 20 nM 4-hydroxytamoxifen (4-OHT). The cells were under 4-OHT induction for 20 days prior to selecting cells that had retained an epithelial phenotype. By 12 days, the control population had fully undergone EMT. From the induced population, cells were selected that maintained expression of epithelial junction protein E-cadherin and an epithelial phenotype by FACS. During the 20 day Twist induction period, the cells were monitored for E-cadherin (E-cdh) expression by FACS, and inspected visually for maintenance of an epithelial phenotype. At 5 days post induction, E-cdh expression between the library containing population and the controls was notably different. In particular, in the library population, one can see two distinct populations, an E-cdh high and E-cdh low population. This segregation is not seen in the control cell population. Additionally, 10 days into the twist induction, the library transduced population of HMLE-TwER cells contained distinct colonies of cells with epithelial phenotype, and such colonies persisted for the

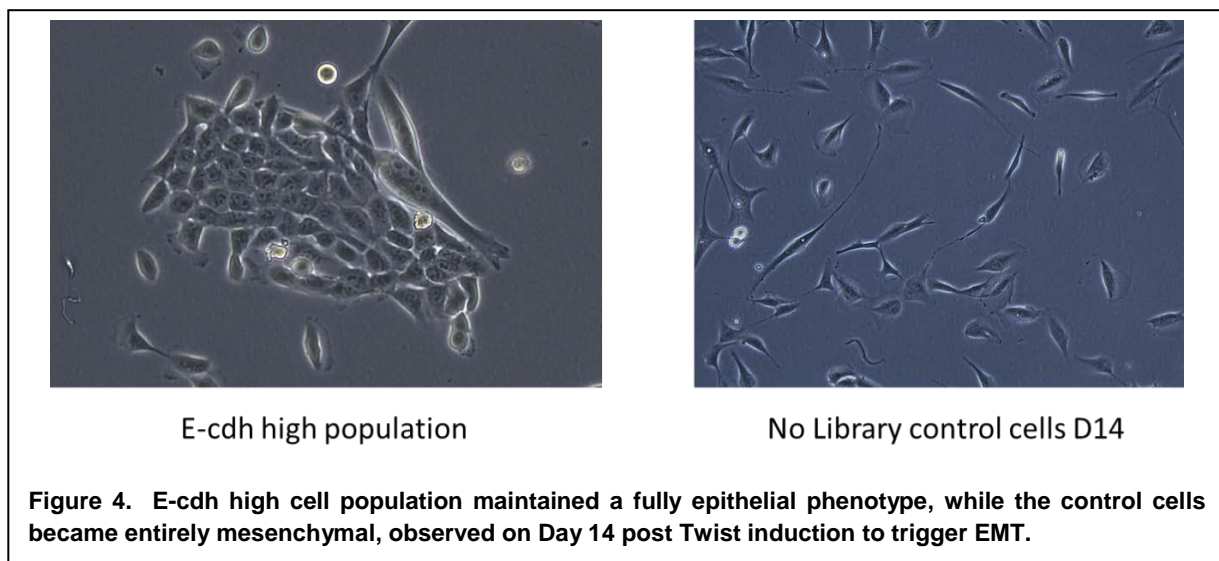


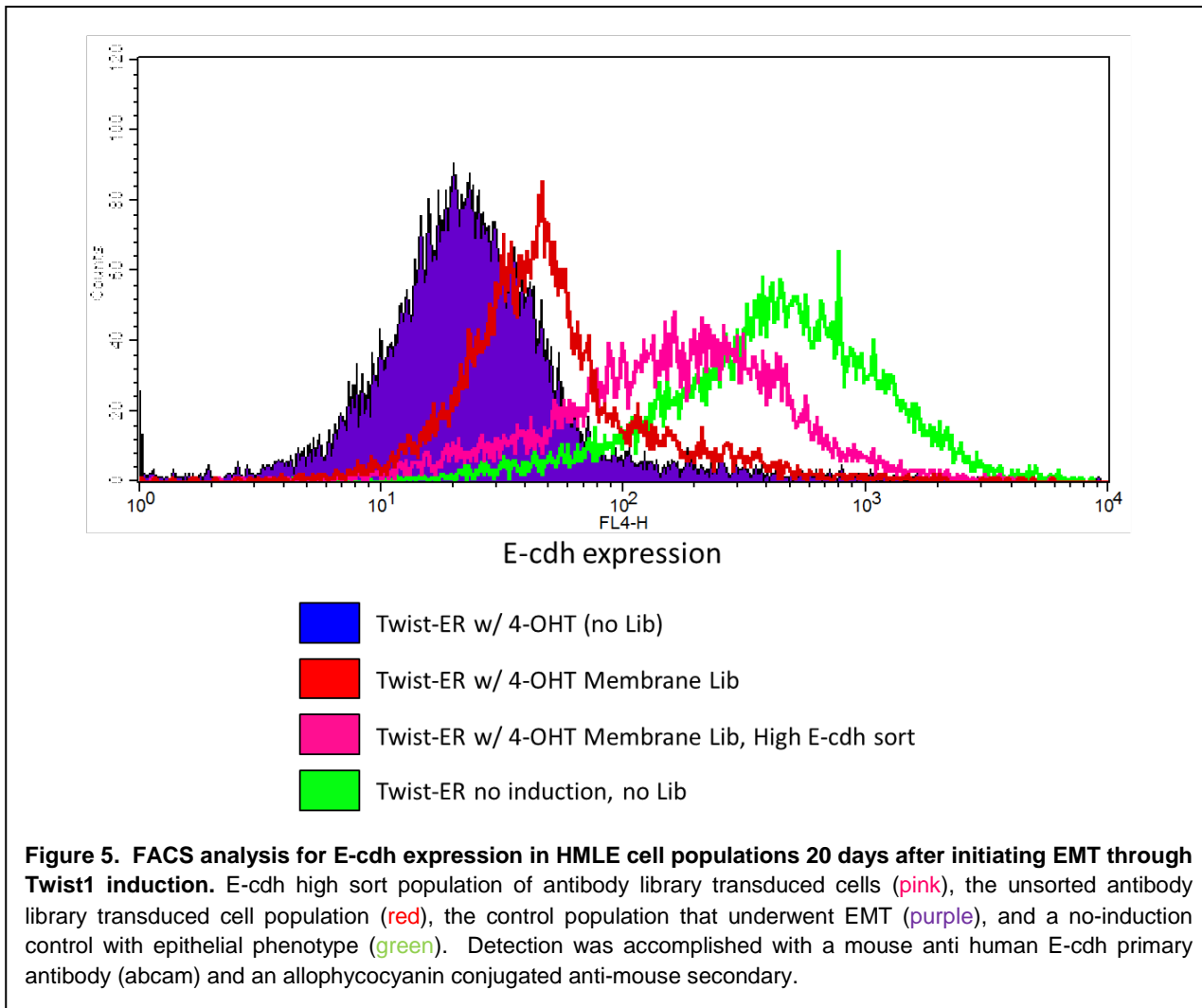
remainder of the experiment. Colonies such as these were not observed in the control population, which consistently and entirely transitioned to a mesenchymal phenotype (Figure 6.06).

Flow cytometry sorting for E-cadherin (E-cdh) positive cells allowed for selection of cells expressing antibodies that support maintenance of this key epithelial junction protein despite twist induction as a definitive driver of EMT in this model. A membrane bound lentiviral antibody library (**Figure 1**) was used so that expressed antibody would act primarily on the cell that expressed that particular antibody sequence. This allows reasonable confidence that the antibody sequences contained in cells with retained epithelial phenotype are responsible for the phenotype maintenance. At 20 days post EMT induction, the lentivirally infected cell population was sorted for E-cdh expression. The top 3% of E-cdh expressers were selected from the library-transduced cell population (**Figure 3**). The top 3% of E-cdh expressers (which totaled about 60,000 cells) were then plated into 10 cm culture plates and allowed to proliferate. The selected cell population maintained a phenotype identical to that of epithelial HMLE cells, both in morphological appearance and E-cdh expression

level, whereas the control population had an appearance and E-cdh expression levels consistent with mesenchymal cells (**Figure 4**). The selected cell population was subsequently grown under 4-OHT induction as well as blasticidine selection (the selection marker associated with the TwER gene) in order to confirm that the selected cells were indeed still expressing the protein that induces the EMT process (Twist), while being resistant to EMT indicated by retained E-cdh.

From the antibody library transduced sorted cell population, 27 single clones were isolated either by limiting dilution or physical selection from plated epithelial colonies. The single clones were grown under 4-OHT induction as well as blasticidine selection as mentioned above. The genomic DNA was extracted from each of the 27 clones, and we attempted to recover the antibody genes via PCR amplification using gene specific primers, followed by cloning of the genes back into the lentiviral vector and sequencing single clones. Optimization of the isolation of the genomic DNA, optimization of primers, and optimization of many of the parameters of the PCR reaction were necessary before we could reliably produce the desired amplicons. We identified 6 antibody sequences from 6 of the aforementioned 27 isolated single colonies: 1C6-1, 2C7-3, 3E1-4, 3F8-3, 1E3-4, and 2F5-1. Interestingly, two of the isolated sequences (1C6-1 and 2C7-3) yielded the same sequence and contained the following CDRH3: ERQRGDNYGTKYFD. Notably, being a rare sequence





within CDRH3s, the RGD motif could be indicative of ligand mimetic integrin binding properties of these antibodies. Further confirmation of the activity of these sequences, as well as the other 21 sequences will be accomplished in the upcoming year.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

We will soon be testing monoclonal viruses from each clone for their ability to recapitulate the EMT inhibitory phenotype. To analyze if each of the identified antibodies are able to recapitulate the EMT-prevention phenotype, we will transduce HMLE-TwER cells with the individual antibodies before inducing EMT. Using identical conditions as in the initial selection experiment, each of the distinct mAb sequences, a randomly selected library member control, and no virus control will be tested to see if they could induce the desired epithelial maintenance phenotype. Once again, each of the HMLE-TwER variants will be placed under twist induction (which induces the EMT process) for twelve days before being analyzed for E-cdh expression by FACS. We verified that the 12-day period is sufficient for the control cell population to fully undergo EMT).

Following confirmation of the monoclonal virus activity, we will produce recombinant IgG of the candidate antibodies, and verify that they also inhibit the EMT process. Then, as described in our original proposal, we will perform orthotopic animal models to evaluate the therapeutic potential of each candidate antibody.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

Currently there is nothing to report, however, we anticipate that the antibodies discovered will impact cancer treatment as well as provide unique reagenets to study the EMT process. This will require verification in the studies of the next reporting period.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report. However, we anticipate that antibodies that show inhibitory activity in EMT animal models will be licenseable to a biotechnology or pharmaceutical company for drug development.

What was the impact on society beyond science and technology?

Nothing to report.

5. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

We have had a delay in constructing new antibody libraries, which was mainly due to hiring delays, and not for any scientific or technical reason. We currently have new post-doctoral fellows skilled in the molecular biology techniques of library construction, and these tasks are now moving forward rapidly.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Nothing to report.

Publications, conference papers, and presentations

Nothing to report.

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Vaughn V. Smider, M.D., Ph.D.
Project Role:	P.I. (unchanged)
Name:	Brunhilde Felding-Habermann, Ph.D.
Project Role:	Co-P.I. (unchanged)
Name:	Erik Wold
Project Role:	Graduate student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Produced lentiviral library, developed EMT screening assay, identified "lead" antibody sequences
Funding Support:	

Name:	Wenyong Tong, Ph.D.
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Tong is expressing antibody sequences, and culturing cells.
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The following grant was awarded and provides additional support for both Drs. Smider and Felding:

National Institutes of Health

Smider (Co-PI)

07/01/2015 – 06/30/2017

“Antibody Fingerprinting of Triple Negative Breast Cancer by High Throughput FACS”

The goal of this research is to use a novel addressed human antibody library to “profile” the surface of breast cancer cells, uncovering new targets and antibodies simultaneously.

8. SPECIAL REPORTING REQUIREMENTS

Drs. Smider and Felding will be submitting separate versions of this report as co-PIs

What other organizations were involved as partners?

Nothing to report.

9. APPENDICES

Not applicable.