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TITLE: Cell Communication in Antiestrogen Resistance

PRINCIPAL INVESTIGATOR: Robert Clarke, PhD, DSc

CONTRACTING ORGANIZATION: Georgetown University

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14. ABSTRACT
More women die from the estrogen receptor positive (ER+) breast cancer subtype than from any other. The proportion of early ER+ recurrences (=5 years since diagnosis) approaches that for all triple-negative breast cancers alone. Late recurrences (>5 years after diagnosis), the result of dormancy, are most common in ER+ disease and can arise decades after the initial diagnosis. Since recurrent breast cancers have escaped the effects of endocrine therapies, and are lethal, we will study endocrine resistance (Tamoxifen; Fulvestrant). Our primary objective is to identify what drives breast cancer growth and determine how to stop it. We will learn about why some breast cancers are aggressive and others are indolent, and why/how some breast cancers lay dormant for years and then re-emerge.

15. SUBJECT TERMS
Breast cancer, drug resistance, admixing, ecology, multiscale modeling

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1. Introduction

~70% of newly diagnosed breast cancers are ER+ [1]. Many of these women die because metastatic ER+ disease becomes treatment resistant. Resistance is multiscale, i.e., evident at many levels, with genetic, cellular, and phenotypic features (including intratumor heterogeneity; ITH), all are molecularly manifested, and functionally realized, as networked changes in the transcriptome and proteome. We will take a systems biology approach to portray the proteome and transcriptome topology of treatment-induced adaptive remodeling of cell admixtures in vitro and in vivo. Overarching goals are to understand the principles of this remodeling and uncover the mechanisms that confer endocrine resistance in breast cancer, leading to new treatment strategies.

2. Keywords

Drug resistance, admixing, ecology, multiscale modeling

3. Accomplishments

A) Major goals (and related subtasks) of the project from approved SOW:

SPECIFIC AIM 1 (specified in proposal)	Timeline	Site 1	Site 2	Percent complete	Date completed (if 100%)
Major Task 1 (Aim 1a)	Months				
Subtask 1: Determine prevalence of **R** and/or **P** cells in **S** populations	1-6	Dr. Clarke Dr. Sengupta			
Subtask 2: Determine the effects of different **S**:**R** ratios on response to TAM and ICI in MCF-7, LCC, T47D, and ZR-75-1 **S** and **R** matched cell models *in vitro*	1-18	Dr. Clarke Dr. Sengupta			
Milestone(s) Achieved: Identified effects of **S**:**R** ratio on responsiveness to TAM and ICI *in vitro* in multiple breast cancer cell models and identified optimal admix ratios for *in vivo* studies					
Local IACUC approval (annual renewal required only – approval for studies already in place)	1	Dr. Clarke		100%	8/31/19
Local IRB approval (add this award as an exemption for use of existing data – no new clinical data will be generated in this BT#2)	1	Dr. Clarke		Pending	
Subtask 3: Determine the effects of different **S**:**R** ratios on response to TAM and ICI in **S**:**R** matched cell models *in vivo* (models and admixes guided by the optimal* results in Major Task 1/Subtask 2) It is difficult to provide direct numbers until the *in vitro* work is completed. A standard design for a single would include the following (n=15/group as in application): R cells alone ± ICI (15+15=30) S cells alone ± ICI (15+15=30) R+S cells at a single ration ± ICI (15+15=30) Total = 90/experiment We may do 3 such experiments over the 18-month period for 270 mice.	6-24	Dr. Clarke Dr. Sengupta		10%	
Milestone(s) Achieved: Identified effects of **S**:**R** ratio on responsiveness to TAM and ICI *in vivo*					
Major Task 2 (Aim 1b)					
Subtask 1: Determine the role of GJIC in the ability of **R** to make **S** cells resistant to TAM and ICI in MCF-7, LCC, T47D and ZR-75-1 matched cell models *in vitro* (guided by the optimal experimental conditions from Aim 1a)	6-18	Dr. Clarke Dr. Sengupta		20%	
Subtask 2: Determine the role of microvesicles and protein secretion	6-18	Dr. Clarke Dr. Sengupta			

(transwell) in the ability of R to make S cells resistant to TAM and ICI in MCF-7, LCC, T47D and ZR-75-1 matched cell models *in vitro* (informed by the optimal experimental conditions identified in Aim 1a)					
Milestone(s) Achieved: Identified role GJIC, microvesicles and protein secretion (transwell) in the ability of **R** to make **S** cells resistant to drug and how this is affected by different **S**:**R** ratios. Identified conditions to allow design and execution of *in vivo* studies with guggulsterone and/or GW4869 (experiments will be done if supported by data and if time permits)					
Subtask 3: Collect and store materials (*e.g.,* cell lysates) from optimal conditions for omics studies in Aim 2	1-24	Dr. Clarke Dr. Sengupta			
SPECIFIC AIM 2 (specified in proposal)	**Timeline**	**Site 1**	**Site 2**		
Major Task 3 (Aim 2a)					
Subtask 1: Collect RNA and protein from the materials stored from Aim 1a (this will be done as the optimal experiments are identified above)	1-24	Dr. Clarke Dr. Sengupta	Dr. Wang	50% Site 2	
Subtask 2: Perform array and proteome data collection, processing of raw data from Major Task 3/Subtask 1 (above), and *initial* analyses (*e.g.,* CAM, kDDN)	1-24	Dr. Clarke Dr. Sengupta	Dr. Wang	50% Site 2	
Milestone(s) Achieved: Create initial signaling maps of what is communicated by **R** to **S** to confer resistance and how this is affected by different **S**:**R** ratios					
Major Task 4 (Aim 2b)					
Subtask 1: Build initial mathematical models of cell population remodeling dynamics (*in vitro* and *in vivo* data)	4-24	Dr. Bansal		50%	
Subtask 2: Build final mathematical models of cell population remodeling dynamics (*in vitro* and *in vivo* data)	24-36	Dr. Bansal			
Milestone(s) Achieved: Identified how endocrine therapies and the starting ratios of **S**:**R** cells affects population responses to treatment					
Major Task 5 (Aim 2c)					
Subtask 1: Use the data from Aims 1 and 2 to design and execute novel drug combination and scheduling studies *in silico* (mathematical modeling), *e.g.,* ICI+DNMTi	18-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	
Subtask 2: Design and execute novel drug combination and scheduling studies *in vitro* using the predictions in Major Task 5/Subtask 1	18-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	
Milestone(s) Achieved: Identified novel optimized (activity vs. toxicity) combination regimens *in vitro*.					
Subtask 3: A small number of predictions from the *in vitro* modeling in Major Task 5/Subtask 2 will be	18-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	

tested *in vivo* (we anticipate completing ~5 such animal studies) It is difficult to provide direct numbers until the *in vitro* work is completed. A standard design for a single would include the following (n=15/group as in application): R cells alone + Vehicle (15) S cells alone + Vehicle (15) R cells alone + Drug A and + Drug B (15+15=30) S cells alone + Drug A and + Drug B (15+15=30) R+S cells at a single ratio with Vehicle, + Drug A and + Drug B (15+15+15=45) Total = 135/experiment We may do 4 such experiments over the funding period (n=540 maximum number mice).					
Milestone(s) Achieved: Identified novel optimized (activity vs. toxicity) combination regimens *in vivo*.					
SPECIFIC AIM 3 (specified in proposal)	**Timeline**	**Site 1**	**Site 2**		
Major Task 6 (Aim 3a)					
Subtask 1: Initial CAM and kDDN modeling of microarray data from human tumors (public and in-house datasets); data will be fed back to Aim 2 to increase clinical relevance	1-12		Dr. Wang	100% Site 2	8/31/19
Subtask 2: Update models using outcomes from Aim 2 and study if candidate molecules from Aim 2 are associated with clinical outcome (univariate and multivariate)	12-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	
Milestone(s) Achieved: Identified clinically relevant molecules associated with ITH and endocrine resistance					
Subtask 4: A small number of predictions from the *in vitro* modeling in Major Task 5/Subtask 3 will be tested *in vivo* (~5 such experiments will be done) A small number of predictions from the in vitro modeling in Major Task 5/Subtask 2 will be tested in vivo (we anticipate completing ~5 such animal studies) It is difficult to provide direct numbers until the in vitro work is completed. A standard design for a single would include the following (n=15/group as in application): R cells alone ± Drug A (15+15=30) S cells alone ± Drug A (15+15=30)	18-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	

R cells alone ± Drug B (15+15=30) S cells alone ± Drug B (15+15=30) R+S cells at a single ratio + Drug A + Drug B (15+15=30) Total = 150/experiment We may do 3-5 such experiments over the funding period (n=750 maximum number mice).					
Milestone(s) Achieved: Identified novel therapeutic strategies for ER+ breast cancer to prevent, delay or reverse resistance, and do so within minimized toxicity. These insights could be used to design clinical trials to be done outside this research program.					
Major Task 7 (Aim 3b)					
Subtask 1: Test candidate molecules from the model predictions in Aims 2 and 3a. For example, as described in the narrative section, genes upregulated in resistant cells relative to sensitive cells will be overexpressed (cDNA; regulable and/or constitutive promoters) in sensitive cells and knocked down in resistant (RNAi) if their mRNA or protein is still present in sensitive cells. The gene will be knocked out (CRISPR) in resistant cells if the gene is known to be lost or expression is undetectable in sensitive cells. The reverse experiments will be done where a gene is down regulated or lost in resistant cells relative to its expression/presence in sensitive cells.	12-36 months	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	
Milestone(s) Achieved: Identified mechanistically relevant molecules associated with ITH and endocrine resistance					

B) What was accomplished under these goals?

Aim 1. Summary

To determine the effects of different S:R ratios in response to 4-hydroxy tamoxifen (TAM) and fulvestrant (ICI) in different estrogen receptor positive (ER+) breast cancer cells, we have developed fluorescently labelled sensitive (S) and resistant (R) variants for MCF7-derived LCC1 (S) and LCC9 (R) cells. When S and R cells were grown as co-cultures, cells either were found to be in direct cell-cell contact (**Figure 1**, white arrows) or spatially separated. We are in the process of developing fluorescently labelled matched pairs of S and R variants of T47D and ZR75 cells. Fluorescently labelled cells will be counted using an algorithm developed by our collaborator Dr. Shweta Bansal as mentioned in Aim 2B summary of this report.

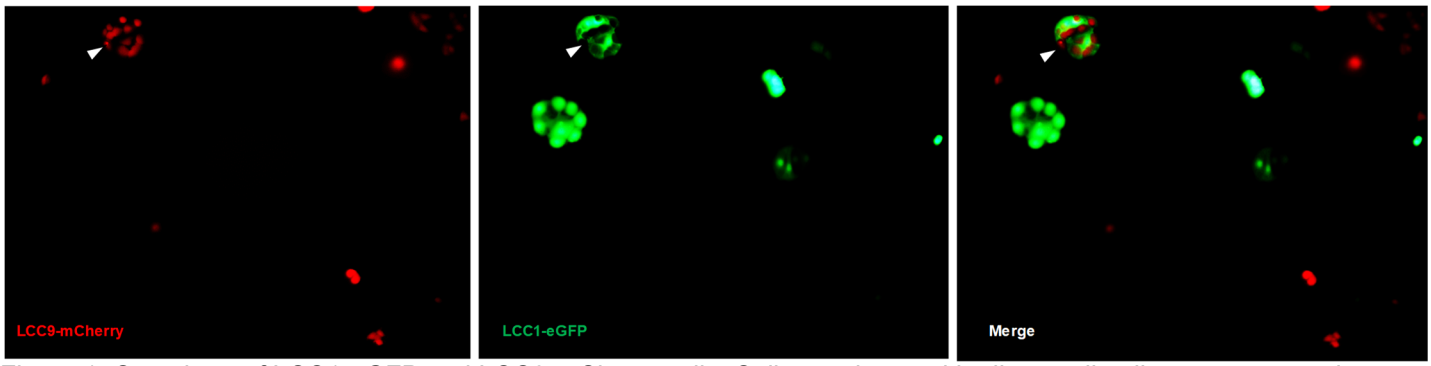

Figure 1: Co-culture of LCC1-eGFP and LCC9-mCherry cells. Cells are detected in direct cell-cell contact or growing spatially separated.

Measuring cell cycle phases in co-culture experiments:

To measure the cell cycle stages of LCC1-eGFP (S) and LCC9-mCherry (R) cells in either monoculture or co-culture conditions, we cultured the cells individually and performed flow cytometric analysis of cell cycle phase distributions. We used a live cell dye (Cyto-Phase violet dye, Biolegend) that is a cell permeant DNA binding dye and can be used in live cells. **Figure 2** shows that eGFP labelled LCC1 and mCherry labelled LCC9 cells can be assessed for their cell cycle phases using cyto-phase dye in monoculture conditions. **Figure 3** shows that when both cell populations are present (*e.g.,* co-culture conditions) we can gate the cells by flow cytometry on the basis of their fluorescent label and subsequently assess cell cycle phases for each cell type (S or R). Using this technique, we will determine the effect of endocrine

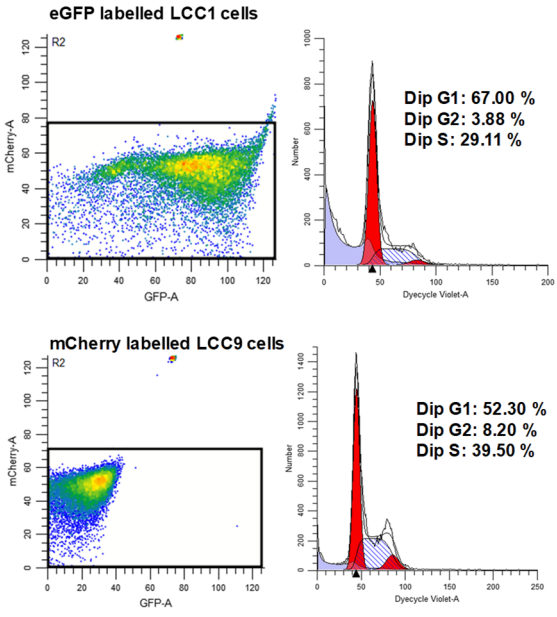

Fig 2. Assessment of cell cycle phases in monoculture

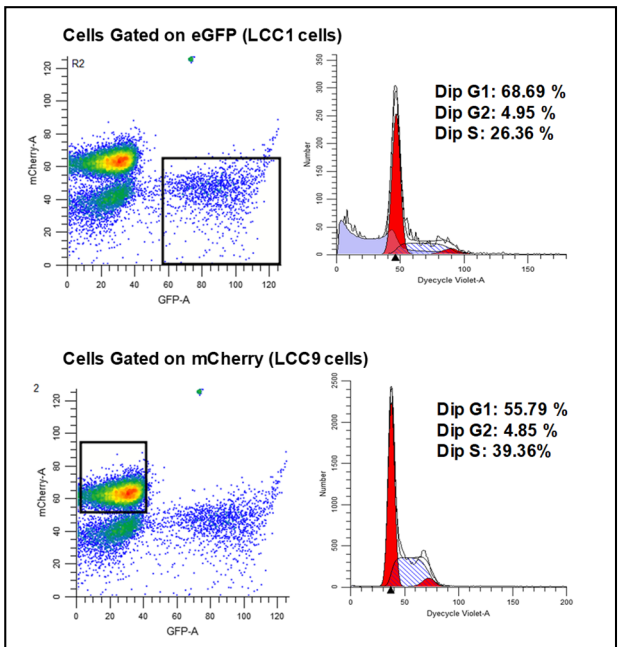

Fig 3. Assessment of cell cycle phases in mixed cell population.

therapies (4-hydroxytamoxifen (TAM), fulvestrant (ICI)) on LCC1 and LCC9 monocultures and using co-culture conditions.

Aim 2B Summary:
Aim 2B of our project focuses on the ecology of cell population dynamics. From an ecological perspective, populations of susceptible **(S)** and resistant **(R)** cells are populations of different "species", while GLC, GLN, and O_2, are "resources" necessary for the two populations to grow. Our focus here is to study how resistant (R) and sensitive (S) cells interact to alter the response of S+R populations to treatment.

Aim 2B Progress:
We have made significant progress on this aim in the first year:

- We obtained *in vitro* population dynamics data from mono- and co-cultures of fluorescence-tagged LCC1 and LCC9 cells.
    - LCC1 cells were tagged with eGFP (enhanced green fluorescent protein) and LCC9 cells were tagged with mCherry (a red fluorescent protein).
- We have developed a new image processing algorithm for automated cell counting.
    - This algorithm is implemented in Python and is open-source.
    - We have validated our results of the automated counts with manual counts done in the lab, and our results are very positive. (See Figure 4).
- We have developed two mathematical models to capture the population dynamics of single and interacting populations based on the Generalized Lotka-Volterra interaction model.
- We have developed a statistical Bayesian inference framework for this population dynamics mathematical model to infer the growth and interaction parameters from the *in vitro* data of the two populations.
    - This framework is based on a hierarchical Bayesian model implemented in Python using the *Stan* platform.
- Using the Bayesian inference framework, we have identified intrinsic growth and capacity parameters from the mono-culture data. (See Figure 5).
- Using the Bayesian inference framework, we have identified interaction parameters from the co-culture data without drug. (See Figure 6).

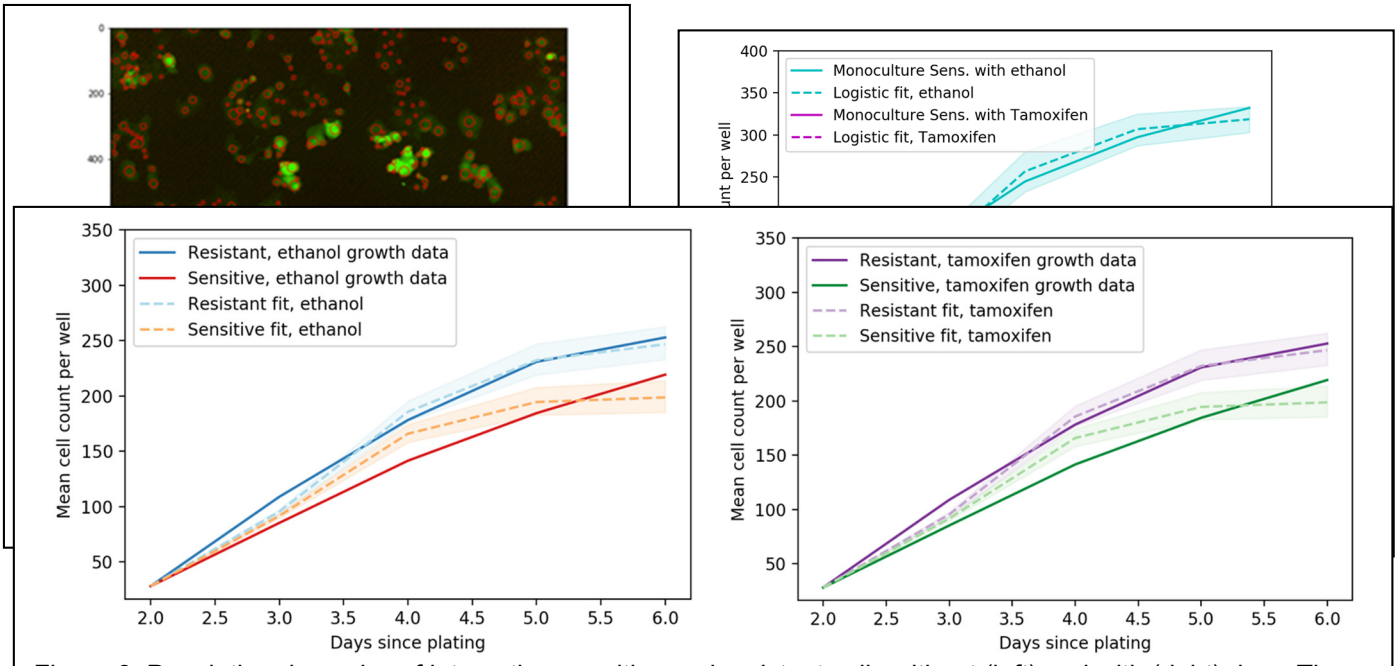

Figure 6: Population dynamics of interacting sensitive and resistant cells without (left) and with (right) drug. The dotted lines show the inferred population dynamics from the Bayesian inference framework

C) What opportunities for training and professional development has the project provided?
Nothing to report (this project was not intended to provide training and professional development opportunities).

D) How were the results disseminated to communities of interest?
Nothing to report.

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

Aim 2B Next Steps:

- Generate *in vitro* population dynamics data from additional replicates to decrease the uncertainty in inferred parameters.
- Confirm the inferred carrying capacity parameters with additional *in vitro* data.
- Use the Bayesian inference framework to identify interaction parameters from the co-culture data with drug.

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?
Nothing to report.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
Nothing to report.

5. Changes/Problems

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."

Publications, conference papers, and presentations
Report only the major publication(s) resulting from the work under this award.
Journal publications.

Robert Clarke, John J Tyson, Ming Tan, William T Baumann, Lu Jin, Jianhua Xuan, and Yue Wang, "Systems biology: perspectives on multiscale modeling in research on endocrine-related cancers," ***Endocrine-Related Cancer***, 2019. (accepted)

Books or other non-periodical, one-time publications.
Other publications, conference papers, and presentations.

Website(s) or other Internet site(s)
Technologies or techniques
Inventions, patent applications, and/or licenses
Other Products

7. Participants & Other Collaborating Organizations

Name: Robert Clarke
Project Role: Principal Investigator
Research Identifier (ORCID ID): 0000-0002-9802-8241
Nearest person months worked: 2
Contribution to project: Dr. Clarke has served as Initiating PI, performing tasks as listed in SOW

Name: Surojeet Sengupta
Project Role: Co-Investigator
Nearest person months worked: 4
Contribution to project: Dr. Clarke has served as Co-Investigator, performing tasks at Georgetown University site as detailed in the project SOW

Name: Alan Zwart
Project Role: Technician
Nearest person months worked: 11
Contribution to project: Mr. Zwart assisted Drs. Clarke and Sengupta with laboratory and animal work at Georgetown during Year 01 of the project.

Name: Lu Jin
Project Role: Bioinformatician
Nearest person months worked: 2
Contribution to project: Mr. Jin works as project data analyst and Bioinformatician, and collects the laboratory data as it is generated to prepare it for work to be done by the mathematical modelers at Virginia Tech.

Name: Shweta Bansal
Project Role: Co-Investigator
Nearest person months worked: 1
Contribution to project: Dr. Bansal is a mathematical biologist who assists Dr. Clarke with experimental design and data analysis, primarily related to Specific Aims 2 and 3 as detailed in the SOW.

Has there been a change in the active other support of the PI or senior personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report (aside from Partnering PI institution, Virginia Tech University).

8. Special Reporting Requirements

The Partnering PI (Yue Wang, PhD) has submitted an independent annual report for this period.

9. Appendices