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TITLE: Molecular Modeling of Estrogen Receptor alpha Mutated Breast Cancer to Guide New Therapeutic Strategies

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14. ABSTRACT
The expression of estrogen receptor alpha (ER) is assayed in every breast cancer biopsy at the time of diagnosis with the majority of tumors (~70%) expressing ER. Hormone therapy is given to patients with ER expressing tumors for years after surgical resection to block the creation of estrogens or the activity of ER. Unfortunately, many patients develop hormone therapy resistant tumors and once metastatic disease is found in these patients, the outlook is poor with the median survival time of slightly over one year. The recent discovery of mutations in the ligand-binding domain (LBD) of ER in 10-40% of metastatic hormone therapy resistant breast tumors suggests that these mutations may be a common mechanism leading to resistance. There is a need to understand the molecular changes that arise due to ER LBD mutations and find effective treatments for patients whose tumors harbor these mutations. We have two main goals for this project. The first goal is to characterize the molecular consequences that are specific to ER LBD mutations found in metastatic breast cancer. The second goal of the project is to identify promising targeted therapies for patients with hormone therapy resistant breast tumors that harbor ER LBD mutations.

15. SUBJECT TERMS
None provided

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Table of Contents

Page

1. INTRODUCTION

The expression of estrogen receptor alpha (ER) is assayed in every breast cancer biopsy at the time of diagnosis with the majority of tumors (~70%) expressing ER and being treated with hormone therapy for years. Unfortunately, many patients develop hormone therapy resistant tumors and once metastatic disease is found in these patients, the outlook is poor with the median survival time of slightly over one year. The recent discovery of mutations in the ligand-binding domain (LBD) of ER in approximately 20% of metastatic hormone therapy resistant breast tumors suggests that these mutations are a common mechanism leading to resistance. The goals of this project are to understand the molecular changes that arise due to ER LBD mutations and find effective treatments for patients whose tumors harbor these mutations. We will accomplish these goals by undertaking two specific aims: **Aim I**, Discover the molecular and phenotypic consequences of mutations in the ligand-binding domain of estrogen receptor alpha; **Aim II**, Determine the efficacy of gene regulation targeted therapies at blocking mutant estrogen receptor alpha driven transcription and growth. The successful completion of this project will lead to a thorough appreciation for the molecular consequences of ER LBD mutations and uncover a set of drugs that specifically block mutant ER's ability to regulate transcription and promote growth, representing great candidate treatments for hormone therapy-resistant disease. It is our hope that this preclinical work will motivate clinical trials and lead to effective targeted treatment options for patients with metastatic tumors that harbor ER LBD mutations.

2. KEYWORDS

Breast cancer, Estrogen receptor alpha, hormone therapy resistance, gene regulation, genomics, CRSIPR/Cas9, epigenetic modifying drugs, anti-androgen therapy

3. ACCOMPLISHMENTS

What were the major goals of the project?

We have two main goals for this project. The first goal is to characterize the molecular consequences that are specific to ER LBD mutations found in metastatic breast cancer. The second goal of the project is to identify promising targeted therapies for patients with hormone therapy resistant breast tumors that harbor ER LBD mutations.

What was accomplished under these goals?

Below we describe accomplishments of the tasks, as outlined in the approved statement of work, along with the progress made thus far.

<u>Aim I. Discover the molecular and phenotypic consequences of mutations in the ligand binding domain of estrogen receptor α.</u>

Task 1: Creation and genomic characterization of isogenic cell line models harboring estrogen receptor mutations

Subtask 1.1: Create MCF-7 and T-47D strains that harbor mutations in the gene encoding ER. (Months 1-9, performed in the Gertz lab, 80% completed) – An essential step in all downstream aspects of this project is the creation of isogenic breast cancer cell lines that contain heterozygous ER LBD mutations and their wildtype controls. We first started this project using the standard CRISPR/Cas9 approach: design and clone guide RNAs that target near the desired mutation, construct homologous recombination donors that carry the mutation, produce a Cas9 expression construct, and transfect each component into breast cancer cell lines. We developed a Sanger sequencing approach to monitor our progress and found that the mutation rate was too low, requiring us to screen hundreds of clones just to create one mutant line. We therefore turned to an alternative strategy called CETCH-seq (Savic et al *Genome Research* 2015). CETCH-seq was developed by our colleague Dan Savic (St. Jude's Children's Research Hospital) to endogenously tag proteins of interest using Cas9 mediated homologous recombination. Because the LBD mutations are in the last exon of ER and close to the stop codon, we decided to combine genome editing and the epitope tagging approach of CETCH-seq.

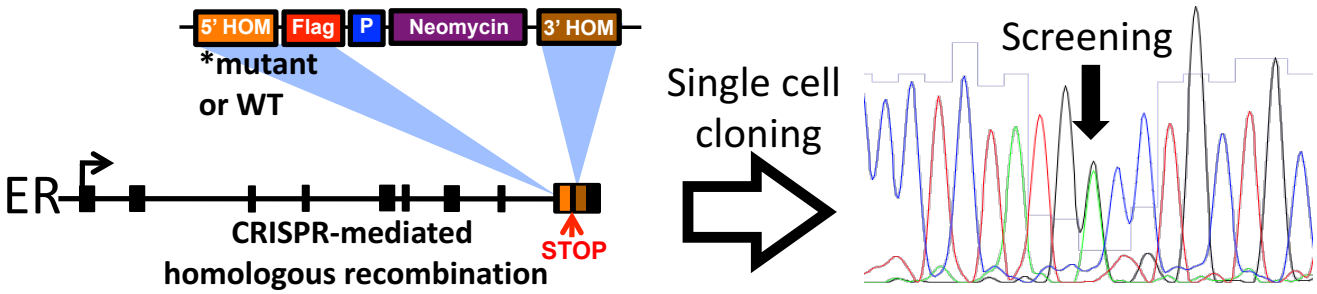

Figure 1. ER LBD mutation generation strategy. *We are using CETCH-seq to both introduce mutations and a FLAG epitope tag at the endogenous locus. Single cell cloning is then followed by Sanger sequencing to screen for mutant lines.*

Figure 1 shows the overall strategy that we've used to successfully introduce D538G and Y537S mutations into breast cancer cells. We first designed and cloned two guide RNAs that target close to ER's stop codon. We used dual vectors that simultaneously and transiently express Cas9 and the guide RNAs. Next, we constructed a homologous recombination donor vector that contained a 5' homology arm, a 3x FLAG epitope tag, a self-cleaving P2A linker, a neomycin resistance cassette, a stop codon, and a 3' homology arm. For the 5' homology arm, we either used PCR products amplified from genomic DNA for wildtype controls, or gBlocks from Integrated DNA Technologies that matched the genomic DNA except for the desired mutation (D538G or Y537S). Once each vector was verified by Sanger sequencing, we transfected the three plasmids into MCF-7 or T-47D cells: two guide RNA/Cas9 vectors and one homologous recombination donor. Cells were then treated with G418 to select for expression of the neomycin resistant gene. After selection, a portion of the cells were used to evaluate mutation rates and we found that 10-20% of alleles were mutated depending on the cell line (T-47D cells exhibited higher mutation rates). Once the presence of the desired mutation was confirmed, cells were plated at limiting dilution and colonies were picked after a few weeks. Individual colonies were analyzed by Sanger sequencing and western blot for an antibody that recognizes the FLAG tag (**Figure 2**). We also checked that the wildtype copy remained intact by PCR and sequencing. We have used this strategy to create at least six clones each of T-47D and MCF-7 cells that have heterozygous D538G mutations as well as at least six wildtype controls that are heterozygous for a FLAG tag at the C-terminal of ER. For the Y537S mutation, we have created the homologous recombination donor plasmid and transfected it into cells along with the guide RNA/Cas9 vectors. We have identified mutations in the bulk population and are in the process of isolating and screening individual clones. We believe that this subtask will be completely finished by the end of the calendar year (December 2017). The successful creation of these clones supports the rest of the tasks outlined in the statement of work and should keep us on track to complete the tasks in the proposed timeline.

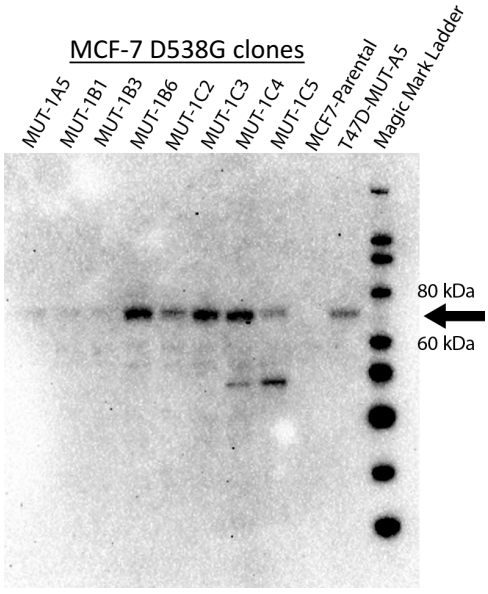

Figure 2. Western blots confirm FLAG tagged ER. *Western blots using an anti-FLAG antibody show that most clones express a FLAG tagged protein at roughly the same size as ER.*

Subtask 1.2: Perform ChIP-seq experiments to determine how protein-DNA binding is impacted by ER mutations (Months 9-18, performed in the Gertz lab, 33% completed) – The addition of a FLAG tag on the mutant copy of

ER (and wildtype copy in control cells) gives us the unique opportunity to specifically track the mutant form of ER. Since we first made the D538G mutation, we've started the ChIP-seq experiments in the T-47D D538G mutant lines. For our initial experiment, we picked two wildtype and two D538G clones based on their average expression of the FLAG tagged ER copy and treated them with 10nM 17beta-estradiol (E2) or DMSO (vehicle control) for 1 hour. ChIP-seq was performed with an anti-FLAG antibody and the libraries were sequenced on a HiSeq 2500. We are still analyzing the data, but our standard analysis shows a clear pattern where the wildtype doesn't bind DNA without E2 and the mutant binds the genome both with and without E2 (**Figure 3**). In addition, we compared the loci bound by FLAG tagged wildtype ER and untagged ER from a previous experiment using an antibody that recognizes ER. There was 90% overlap between the datasets, suggesting that the FLAG tag does not interfere with ER binding and that the single cell cloning procedure didn't dramatically alter ER genome binding. Our ChIP-seq results clearly show that mutant ER can bind the genome without E2 and further analysis will determine whether mutant ER binds to novel loci and if different mutations have different effects on genome binding. We plan to complete the same ChIP-seq experiments for MCF-7 D538G cells in the next month. Once clones are identified for the Y537S mutation, we will perform ChIP-seq on these lines as well and we anticipate completing all ChIP-seq experiments within six months.

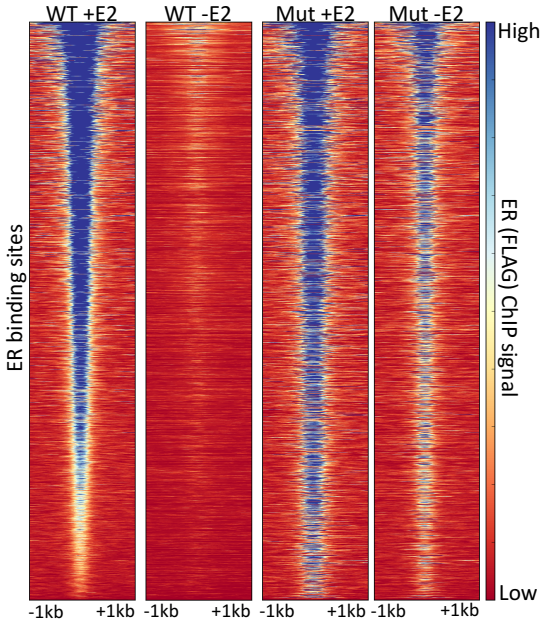

Figure 3. Mutant ER binds the genome in the absence of estrogen. *Heatmaps show the anti-FLAG ChIP-seq signal found within 1kb of ER binding sites for representative T-47D wildtype and D538G lines. There is an absence of signal in the WT vehicle treated, however the mutant vehicle treated looks similar to the E2 treated samples.*

Subtask 1.3: Perform RNA-seq experiments to determine how transcript levels are affected by ER mutations (Months 9-18, performed in the Gertz lab, 25% completed) – The isogenic cell lines provide a nicely controlled system for assessing the gene expression consequences of ER LBD mutations. As with the ChIP-seq experiments, we've started to perform these experiments on the T-47D wildtype and D538G lines. We grew two clones of each (matching the ChIP-seq clones) in hormone depleted media and induced cells with E2 or DMSO for 8 hours before harvesting RNA. Library construction was performed with standard reagents and the libraries were sequenced on a HiSeq 2500. We received this data only one weeks ago and are in the process of analyzing the data. Our initial analysis found that many estrogen responsive genes are already up- or down- regulated in the mutant in the absence of estrogen, which is expected in light of the ChIP-seq data. To our surprise, we found that many genes were specifically up- or down-regulated in the mutant lines compared to the wildtype lines regardless of E2 induction (**Figure 4**). These findings indicate that the D538G mutation is creating a unique transcriptional program that doesn't just encompass genes that are normally regulated by E2. Future experiments will uncover whether this effect is seen in MCF-7 cells and if the different mutations have different effects on gene expression. We plan to complete the same RNA-seq experiments for MCF-7 D538G cells in the next month. Once clones are identified for the Y537S mutation, we will perform RNA-seq on these lines and we anticipate completing all RNA-seq experiments within six months.

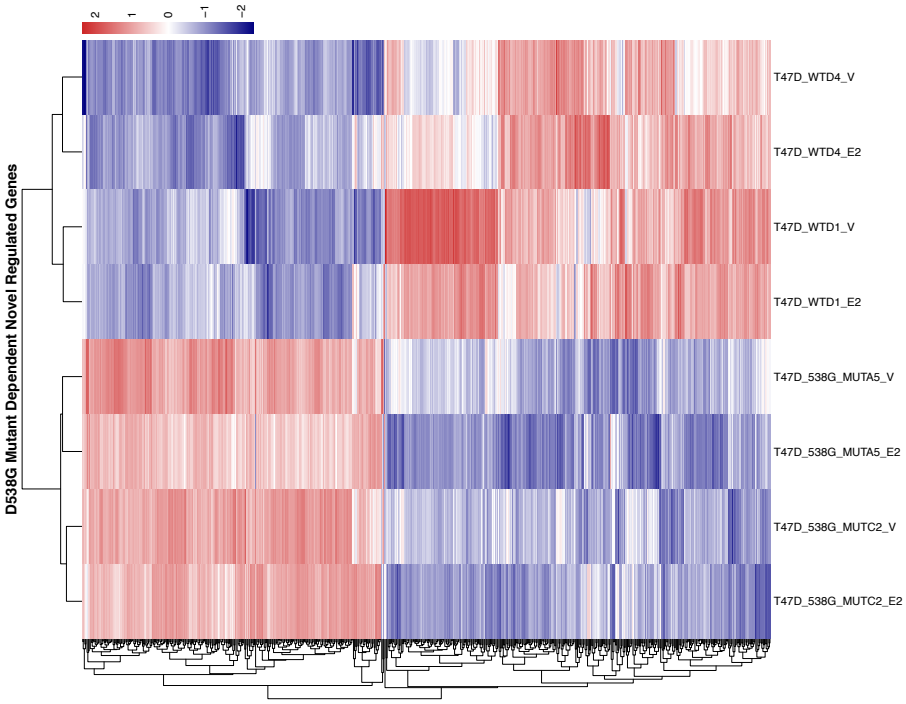

Figure 4. Hundreds of genes change expression due to the D538G mutation, independent of estrogen. *Heatmap shows the expression of genes across two T-47D D538G clones and two T-47D wildtype clones, both with and without an 8 hour E2 treatment. For these 600 genes, their expression is very different between wildtype and mutant lines regardless of E2 treatment. The values represent Z-scores.*

Task 2: Assessing the phenotypic consequences of estrogen receptor α mutations

Subtask 1.4: Evaluate the phenotypic consequences of ER mutations *in vitro* (Months 9-15, performed in the Richer lab, 25% completed) – The isogenic cell lines also provide an opportunity to directly attribute cancer associated phenotypes to ER LBD mutations. We initially looked at 2D growth on plastic and found only subtle differences in growth rates between pooled wildtype MCF-7 or T-47D cells and pooled D538G mutant cells (data not shown). We plan to perform these assays on individual clones and the Y537S mutant lines. We next tested whether the T-47D D538G lines differ from wildtype in their ability to survive in anchorage independent conditions, since that is a characteristic associated with increased metastatic potential. We used a soft agar colony formation assay to determine the mutation's effect on anchorage-independent growth under normal conditions. The bottom layer contained 0.5% agar, the middle layer contained 0.3% agar with 20,000 cells/well and the final layer was regular media. Cells were allowed to grow for three weeks with bi-weekly media changes. We saw a drastic increase in colony formation in soft agar when the D538G mutation was present (**Figure 5**). We are excited to see if these results are reproduced in MCF7 D538G lines as well as the Y537S lines, once those are established. We also plan to continue the *in vitro* phenotyping work by looking at migration, apoptosis rates, and sensitivity to SERMs.

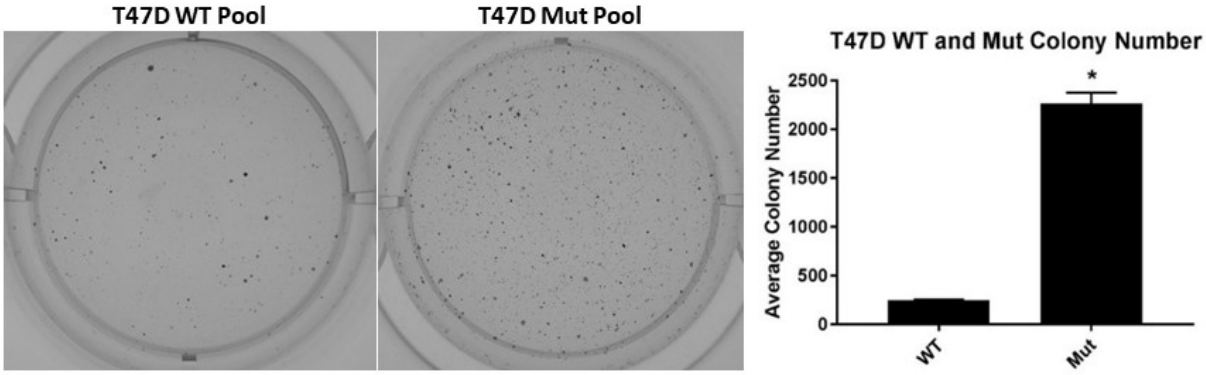

Figure 5. T47D cells with D538G mutant ER demonstrate a growth advantage in anchorage-independent conditions. *Soft agar colony formation assay shows that D538G T-47D cells exhibit increased anchorage independent growth. Representative images of T47D WT and Mut pool anchorage-independent growth are shown on the left. Average colony number and size were quantified using Image J software and the results are shown on the right. Average ± SEM, *p<0.05.*

Subtask 1.5: Evaluate estrogen-independent tumor growth and SERM sensitivity *in vivo* (Months 9-21, to be performed in the Richer lab, 0% completed) – While we have not yet started the *in vivo* work, the animal protocol has been approved (see appendix) and we have received approval to use the cell lines and xenograft model, HCI-013EI.

Aim II. Determine the efficacy of gene regulation targeted therapies at blocking ER driven transcription and growth. As only the first year of a three-year award has taken place, we have only begun work on subtask 2.1 of specific aim II. All tasks and subtasks for specific aim II are outlined below for completeness and to identify the lab(s) responsible for each aspect of the aim.

Task 1: Measure the efficacy of anti-androgens and bromodomain inhibitors in blocking the growth of estrogen receptor mutant breast cancer cells

Subtask 2.1: Use the isogenic cell line models to evaluate response to anti-androgens and bromodomain inhibitors *in vitro* (Months 18-24, to be performed in both labs, 25% complete) – The Richer lab has performed initial experiments to characterize the response to JQ1. JQ1 is a bromodomain, BET protein inhibitor that targets BRD4, a protein thought to be important in estrogen driven growth. We first measured proliferation rates in wildtype T-47D and MCF-7 cells in the presence or absence of E2 with escalating doses of JQ1 and found that the BET inhibitor significantly abrogates baseline and estrogen-induced proliferation in a dose-dependent manner (**Figure 6**). The 1000 nM JQ1 concentration is effective in the presence or absence of E2. We next tested the effect of JQ1 on proliferation in the parental T-47D and MCF-7 cells compared to the pooled wildtype and pooled D538G mutant populations and found that all increase with estrogen (except for MCF-7 D538G), all are significantly inhibited by JQ1 and that the mutant ER lines respond the best to JQ1 (**Figure 7**). These results are promising and indicate that ER mutant cells are sensitive to BET inhibitors. Once the Y537S mutant lines are established, we will analyze their sensitivity to JQ1 as well. We also plan to perform a similar analysis for anti-androgens.

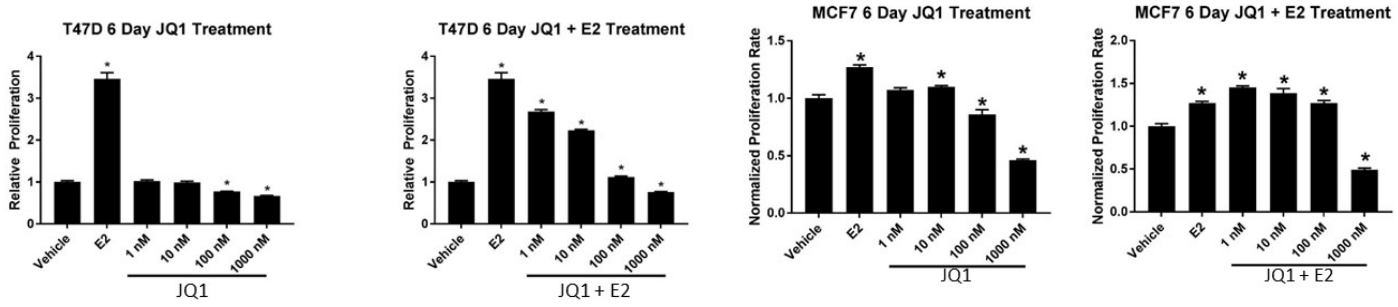

Figure 6. Dose-response of JQ1 treatment in the presence or absence of estrogen in parental MCF-7 and T-47D cells. *Parental cells were plated at 6000 cells/well in a 96-well plate for 24 hours in phenol-red free media supplemented with 10% CSS and 1% Pen/Strep. The next day, cells were treated with Vehicle or 1 nM E2, and varying concentrations of JQ1 (1-1000nM). Proliferation proceeded for 6 days before cells were fixed and stained with crystal violet, de-stained with sodium citrate, and quantified on the plate reader at 540nm. Average ± SEM, *p < 0.05.*

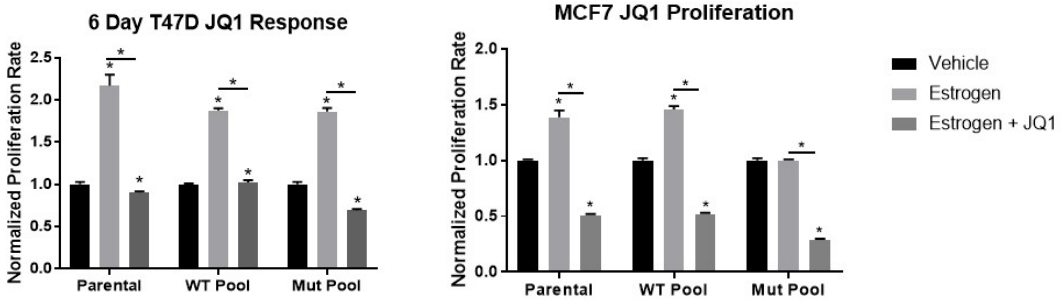

Figure 7. JQ1 inhibits proliferation in ESR1 wild type and mutant T-47D and MCF-7 cells. *Parental, wildtype (WT) pool, and mutant (Mut) D538G pool cells were plated at 6000 cells/well in a 96-well plate for 24 hours with phenol-red free media supplemented*

*with 10% CSS and 1% Pen/Strep. The next day, cells were treated with Vehicle, 1 nM E2, or 1000 nM JQ1 + 1 nM E2. Proliferation proceeded for 6 days before cells were fixed and stained with crystal violet, de-stained with sodium citrate, and quantified on the plate reader at 540nm. Average ± SEM, *p < 0.05.*

Subtask 2.2: Evaluate response to anti-androgens and bromodomain inhibitors *in vivo* (Months 18-27, to be performed in the Richer lab, 0% complete)

Task 2: Identify epigenetic modifying drugs that alter gene regulation that is driven by mutant estrogen receptor

Subtask 2.3: Use ER mutant cells to perform a screen against 140 epigenetic modifying drugs (Months 18-30, to be performed in the Gertz lab, 0% complete)

Subtask 2.4: Evaluate *in vivo* growth inhibitory properties of top epigenetic modifying drugs (Months 24-36, to be performed in the Richer lab, 0% complete)

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

Two graduate students in the Gertz lab, Zannel Blanchard and Spencer Arnesen, and a postdoc in the Richer lab, Jordan Reese, have been working on this project.

Zannel Blanchard is a Molecular Biology PhD candidate in her 3rd year (2nd year in the Gertz lab). She initially started this project before it was funded by the DOD. She has been in charge of the molecular biology work needed to introduce the ER LBD mutations. She has also expanded her work into endometrial cancer. While the endometrial cancer research is not directly supported by the DOD, and DOD funding hasn't been used to support the project, her endometrial cancer is synergistic with this project and helps to inform the experimental design with breast cancer cells. She recently attended a Gordon Research Conference on Hormone Dependent Cancers and presented a poster (the abstract is below). Her poster was well received and she ended up winning a $250 poster prize. The conference was a great training experience for Zannel and allowed her to make some great contacts with other scientists. As she is an international student, she hasn't had the opportunity to apply for fellowships, but we have worked on her writing skills together through her thesis proposal and a manuscript draft that we hope to submit within the next several months.

Spencer Arnesen is a Molecular Biology PhD candidate in his 2nd year (1st year in the Gertz lab). Spencer joined the Gertz lab in May and has devoted all of his effort on this project. He has been responsible for the RNA-seq and ChIP-seq experiments as well as screening for Y537S clones. Spencer is also performing computational analysis of the next generation sequencing experiments. A few weeks ago, Spencer applied for a National Science Foundation Graduate Research Fellowship. The grant application gave us the opportunity to work together on scientific writing and grant applications. Spencer plans to create and present at least one poster during the next year that describes his RNA-seq and ChIP-seq findings.

Jordan Reese is a postdoctoral fellow that recently joined the Richer lab. She is helping with this project by conducting experiments phenotypically characterizing the ESR1 mutant lines and their response to anti-androgens and BET inhibitors both *in vitro* and *in vivo*. Working on this collaborative project between the Richer and Gertz labs has provided her with the opportunity to present preliminary data as a poster at the annual Cancer Biology Graduate Program retreat. A component of this project involves ChIP-seq analysis and comparing the ChIP-seq to RNA-seq data. She is a novice at analyzing this type of data and therefore, will be attending The Genome Atlas Course at Cold Spring Harbor Laboratory in early November. This course covers gene and protein resources; genome browsers and how to utilize them effectively, analysis of high-throughput sequence data including RNA-seq and ChIP-seq, comparative genome analysis, and gene set enrichment analysis. Having a greater understanding of the resources available and insight into how the analysis is performed will add a degree of rigor to her science. Her goals for attending this course will be: 1) to utilize the information learned to analyze ChIP-Seq and RNA-Seq data generated in the Richer lab and 2) to effectively communicate the key concepts of this course to fellow lab members. Jordan is also completing the University of Colorado Responsible Conduct of Research course, attending weekly seminars in Cancer Biology and Pathology, as well as enrolling in the Biostatistics 6606 course.

How were the results disseminated to communities of interest?

Since the project is still in the early phase, we haven't had many opportunities to present our results to the research community. Zannel Blanchard presented a poster on generating ER LBD mutations and analysis of endometrial cancer at Gordon Research Conference on Hormone Dependent Cancers. The poster abstract is

included in the appendices section. Jennifer Richer will present some of these results at the San Antonio Breast Cancer Conference on Dec 5th, 2017.

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

The plans described in our statement of work will continue to be followed. We expect to complete model generation in the next couple of months. Once all of the necessary models are created, we will finish the RNA-seq and ChIP-seq experiments outlined in Aim 1, Task 1 above. We also plan to continue and complete the *in vitro* phenotyping work described in Aim 1, Task 2 above. This will include measurements of proliferation rates, migration/invasion, apoptosis, and colony formation. The *in vivo* work from Aim 1, Task 2 will begin shortly and should also be completed or near completion at the time of the next annual report. We will begin the research described in specific aim 2 as outlined in the statement of work; however, most of these studies will not be completed at the time of the next annual report.

4. IMPACT

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

This research confirms that the D538G ER LBD mutation endows ER with estrogen-independent activity and this activity at least partially overlaps wildtype ER activity. While this has been shown at some genes and some loci (usually with ectopically expressed mutant ER), our results represent the first report of ligand-independent genome-wide binding and gene regulation of an endogenously mutated ER. The preliminary results showing the effectiveness of BET inhibitors could also have a big impact on how we treat hormone therapy resistant patients and we are excited to transition to in vivo evaluation of BET inhibitors.

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?

Three trainees were given opportunities to present their work and develop important skills for their careers.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to Report

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

Nothing to Report

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

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS

Publications, conference papers, and presentations

Our mutation generation strategy and results were presented as a poster by Zannel Blanchard at the Gordon Research Conference on Hormone Dependent Cancers in August 2017. The abstract can be found in the appendices. Jordan Reese presented a poster at the Cancer Biology Graduate Program Retreat in September and Jennifer Richer will speak about this work at the San Antonio Breast Cancer Symposium in December 2017.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

We have developed a protocol for creating ER LBD mutations that is applicable to any transfectable cell. The protocol will be shared on the Gertz lab website.

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

We have created cell lines harboring ER LBD mutations where the mutant allele contains a FLAG tag. We anticipate that these cell lines will be of interest to the research community after publication and we will disseminate them upon request. We have also constructed plasmids that can be used to create ER LBD mutations in any transfectable cell. We plan to deposit these plasmids in Addgene upon publication.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Table 1. Gertz lab project participants

Name	Project Role	Identifier (eRNA commons)	Months	Contribution	Funding support
Jay Gertz	Initiating PI	JASONGERTZ	2.4	Supervises molecular biology and gene regulation aspects of the project.	NIH: R01HG008974, R21CA196455, R01DE023414
Zannel Blanchard	Grad. student	ZBLANCHARD	12	Responsible for the molecular biology, CRISPR/Cas9 work.	None
Spencer Arnesen	Grad. student	SARNESEN	5	Responsible for the genomics methods and analysis.	None

Table 2. Richer lab project participants

Name	Project Role	Identifier (eRNA commons)	Months	Contribution	Funding support
Jennifer Richer	Partnering PI	Jennifer.Richer	0.92	Supervises phenotyping aspects of the project.	DOD: W81XWH-13-1-0090, W81XWH-15-1-0039 NIH: R01CA187733, R01CA201011
Jordan Reese	Postdoc	JREESE1	1.83	Responsible for the phenotyping work.	R01CA187733

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

Not applicable.

What other organizations were involved as partners?

This is a partnering PI award between University of Utah and University of Colorado Denver.

8. SPECIAL REPORTING REQUIREMENTS

Because this is a partnering PI award, both PIs have submitted nearly identical progress reports.

9. APPENDICES

This abstract was presented as a poster at the Gordon Research Conference on Hormone Dependent Cancers:

Molecular characterization of the D538G estrogen receptor alpha ligand binding domain mutation in endometrial cancer

Zannel Blanchard, Jeffery Vahrenkamp, Kristofer Berrett, Jay Gertz

Estrogen signaling plays a critical role in the development of endometrial cancer as shown by epidemiological studies and *in vivo* modeling. Estrogen receptor alpha (ER) is expressed in 95% of type 1 endometrial cancers and induces a pro-growth transcriptional program. Mutations in the ligand binding domain (LBD) of ER have recently been identified in 2% of primary endometrial cancers and these mutations are the same changes found in metastatic breast cancers. Functional studies undertaken in breast cancer models indicate the mutations' ability to promote estrogen-independent ER signaling, which drives ER genomic binding and proliferation in the absence of estrogens, while also conferring endocrine therapy resistance. Despite the critical roles that estrogen signaling plays in endometrial cancer, the consequences of ER LBD mutations have not been functionally explored in this disease. To fill this critical gap in knowledge, we have utilized a CRISPR-mediated epitope tagging strategy to create an isogenic model of the D538G ER LBD mutation in Ishikawa cells, where the mutant allele (or wild-type allele in control cells) has a 3' FLAG tag incorporated at the endogenous locus. Using chromatin immunoprecipitation sequencing (ChIP-seq) with an anti-FLAG antibody, we have established that mutant ER exhibits ligand-independent genomic binding. RNA-sequencing (RNA-seq) experiments indicate mutation-specific gene expression effects, with expression changes of both estradiol-regulated genes and unexpected estradiol-independent genes. We are using ATAC-seq to elucidate the regulatory regions and potentially the transcription factors that are responsible for estradiol-independent gene expression changes caused by the mutation. Together, our results indicate that the D538G mutation causes ligand-independent activity in endometrial cancer cells with some unanticipated gene expression consequences. These studies provide a foundation for studying the role of ER LBD mutations in the progression of a subset of endometrial cancers and provide functional models for further mechanistic and phenotypic investigation.

The following document is an approval of the Richer lab's animal protocol for this project.

DEPARTMENT OF THE ARMY

HEADQUARTERS, US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000

REPLY TO
ATTENTION OF

July 10, 2017

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number BC151357P1, Award Number W81XWH-16-1-0422 entitled, "Molecular Modeling of Estrogen Receptor alpha Mutated Breast Cancer to Guide New Therapeutic Strategies"

Principal Investigator Jennifer K. Richer
University of Colorado, Denver
Denver, CO

Dear Dr. Richer:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, protocol BC151357P1 entitled, "Molecular Modeling of Estrogen Receptor Alpha-Mutated Breast Cancer to Guide New Therapeutic Strategies," IACUC protocol number 83617(04)1E, Protocol Principal Investigator Jennifer Richer, is approved by the USAMRMC Animal Care and Use Review Office (ACURO) as of 30-JUN-2017 for the use of mice and will remain so until its modification, expiration or cancellation. This protocol was approved by the University of Colorado, Denver IACUC on 10-APR-2017.

Required Actions: When updates or changes occur, documentation of the following action or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)

- IACUC actions involving this protocol regarding
  a. any noncompliance;
  b. any deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
  c. any suspension of this activity by the IACUC

- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program

- AAALAC, International status change

Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used
- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

Original Signed

Bryan K. Ketzenberger, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

Copies Furnished:
Ms. Susan L. Dodd, US Army Medical Research Acquisition Activity (USAMRAA)
Dr. Henry (Hank) J. Nothnagel, Congressionally Directed Medical Research Program (CDMRP)
Dr. Mark A. Douse, University of Colorado, Denver
Mr. Ryan Holland, University of Colorado, Denver
Ms. Julia M. Huiberts, Congressionally Directed Medical Research Program (CDMRP)