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14. ABSTRACT

Making a major impact on the incidence and lethality of breast cancer will require more effective approaches for breast cancer risk assessment and prevention. These goals will not be met without a detailed understanding of the earliest tissue changes that ultimately drive the process of breast cancer development. Through the support of our original Breakthrough proposal we carried out molecular analysis of tissues from breast cancer-predisposed BRCA1/2 genetic carriers. This study revealed the striking presence of breast cells that had already suffered substantial DNA damage, even when the tissue looked microscopically normal. We therefore hypothesize 1) that discovering markers defining this cell population will propel the development of new tissue-based predictors of breast cancer risk; and 2) that this damaged subpopulation is likely to have specific therapeutic vulnerabilities that could be exploited to eliminate these cells. Accordingly, our objectives are to unravel the mechanisms that give rise to the damaged cells and to reveal specific methods for eliminating them, thereby laying the groundwork for clinical trials of novel breast cancer prevention approaches.

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1. Introduction

Making a major impact on the incidence and lethality of breast cancer will require more effective approaches for breast cancer risk assessment and prevention. These goals will not be met without a detailed understanding of the earliest tissue changes that ultimately drive the process of breast cancer development. Through the support of our original Breakthrough proposal we carried out molecular analysis of tissues from breast cancer-predisposed BRCA1/2 genetic carriers. This study revealed the striking presence of breast cells that had already suffered substantial DNA damage, even when the tissue looked microscopically normal. We therefore hypothesize 1) that discovering markers defining this cell population will propel the development of new tissue-based predictors of breast cancer risk; and 2) that this damaged subpopulation is likely to have specific therapeutic vulnerabilities that could be exploited to eliminate these cells. Accordingly, our objectives are to unravel the mechanisms that give rise to the damaged cells and to reveal specific methods for eliminating them, thereby laying the groundwork for clinical trials of novel breast cancer prevention approaches.

2. Keywords

Breast cancer; BRCA1/2; cancer prevention; aneuploidy; single-cell analysis

3. Accomplishments

Major Goals:

Aim 1. Mechanism and consequences of failed DNA repair/checkpoint responses in primary human BRCA1 and BRCA2 mutant breast epithelia.

Major Task 1: Understand Checkpoint and Repair
Major Task 2: Test hypothesis-driven inhibitors

Aim 2. Reveal abnormal cell subpopulations of primary BRCA1 and BRCA2 mutant epithelia through single-cell RNAseq.

Major Task 3: Identify subpopulations through single-cell RNAseq and analysis
Major Task 4: Isolate and characterize deregulated subpopulations

Aim 3. Employ high-throughput approaches to uncover selective vulnerabilities of primary BRCA1/2-mutant epithelia.

Major Task 5: Organoid Models of Primary Mammary Tissues
Major Task 6: Therapeutic compound screening of organoids
Major Task 7: Validating effects of identified agents on malignant progression

Major Accomplishments:

We are pleased to share the accomplishments under this award to date. In order to facilitate review, accomplishments are organized and enumerated based on tasks corresponding to the approved SOW for this award. Accomplishments are supported by figures and graphs as appropriate.

***Aim 1.** Mechanism and consequences of failed DNA repair/checkpoint responses in primary human BRCA1 and BRCA2 mutant breast epithelia.*

Major Task 1: Understand Checkpoint and Repair

Activities: Ongoing studies are employing RNA and DNA sequencing of primary tissues, histological analysis of damage and checkpoint markers in primary tissues, ex-vivo short-term cultures for specific cell populations exposed to DNA damage and replication stress agents, and longer-term cultures.

Objectives: We seek to define basal replication and/or DNA damage stress and checkpoint responses in BRCA1/2 carrier tissues vs. controls. Furthermore, we aim to define the response to exogenous damage-inducing agents as a means to test repair competence in carrier vs. control cells and tissues.

Methodology and Results: Following on data described in the original proposal application, we have carried out and interrogated additional bulk RNA sequencing of flow cytometry-sorted populations from carriers and controls. Through supervised analysis, a major discovery we have made is that a Replication Stress Response Deficiency (RSRD) signature is increased in Luminal Progenitor (LP) epithelial cells carriers vs. non-carriers (Fig. 1). This observation strongly supports data generated from our ex-vivo analyses presented in the original proposal application.

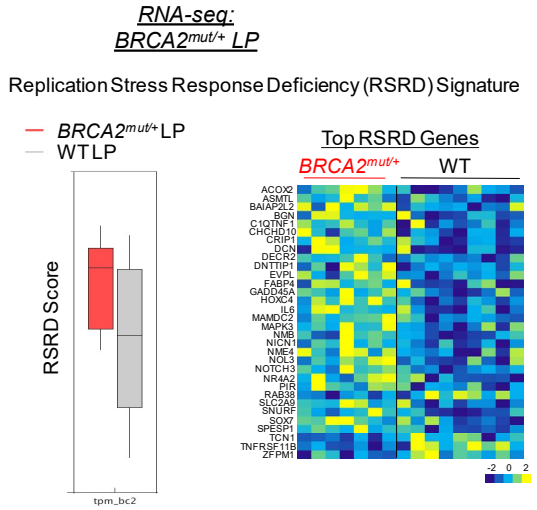

Figure 1. Replication Stress Response Deficiency (RSRD) signature in primary Luminal Progenitors (LPs) of BRCA2 carriers. Left, normalized RSRD score derived from RNAseq data in carriers (red) vs. controls (WT, gray) shows increased RSRD in carriers. Right, top differentially expressed genes between carriers and controls. Rows correspond to the labeled genes, and columns to individual subjects. RSRD genes are increased in carriers compared to controls. RSRD signature established in McGrail et al, Cell Rep. 23, 2095–2106 (2018).

Our second major finding comes from histological analysis of BRCA1/2 carrier tissues and controls for apoptosis with the classical TUNEL protocol. The proportion of TUNEL-positive cells in normal breast tissues is well established, and this finding was replicated in our analysis. In contrast, however, BRCA1/2 carrier tissues were found to have dramatically lower TUNEL-positivity (Fig. 2). This finding is in keeping with the suppressed NF-kB and checkpoint response that our work has uncovered. Importantly, these findings have been incorporated into a *manuscript published during the reporting period* (Karaayvaz et al. Science Advances 2020, 6(5):eaay2611).

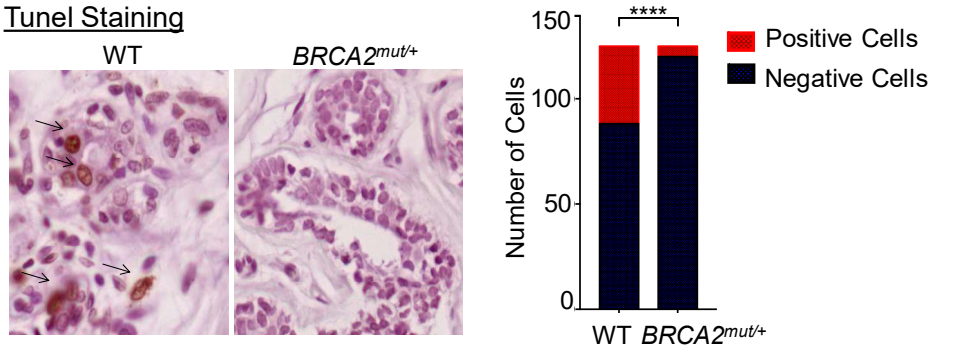

Figure 2. Deregulated apoptosis in primary tissues of BRCA2 carriers. Left, TUNEL staining (brown nuclear staining) of primary histologically normal breast tissues of controls (WT) and BRCA2 carriers. Right, Summary data obtained by counting four fields for five patients per genotype are shown. ****P < 0.0001 by Fisher's exact test.

Major Task 2: Test hypothesis-driven inhibitors

Activities: Ongoing studies are proceeding as described in the SOW, including testing specific inhibitors that may exploit the defective replication stress response (Fig. 1) in carrier tissues. Also as described, we continue to carry out single-cell Whole Genome Sequencing (WGS) analysis of BRCA1/2 primary breast epithelia to identify patterns of CNVs. Notably, these analysis have been extended to BRCA1 carriers in the current reporting period.

Objectives: We seek to identify vulnerabilities and damage response/repair defects that are selective to the BRCA1/2 carrier but no control cell types.

Methodology and Results: Preliminary results suggest the higher prevalence of larger CNVs (e.g. whole arm) in BRCA1 compared to BRCA2 LP epithelial cells. Furthermore, our analyses have uncovered small clonal populations of cells that share a common CNV pattern (Fig. 3). Conceivably, this early clonal outgrowth may represent the first steps toward malignant progression.

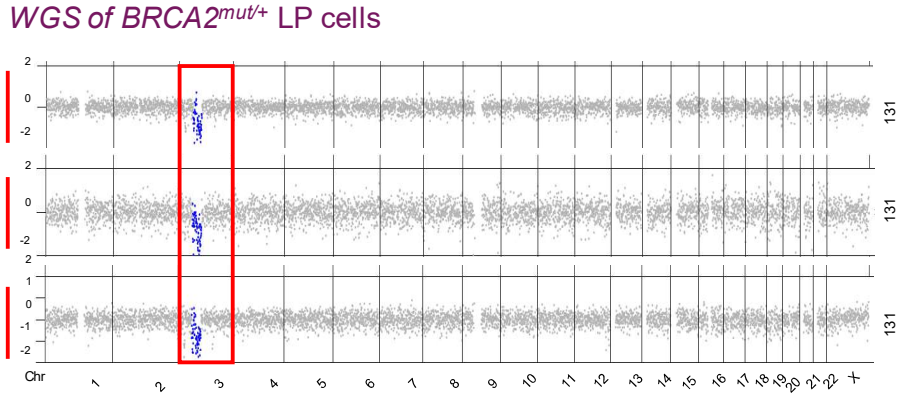

Figure 3. Shared (clonal) CNVs in a BRCA2 carrier. Whole Genome Sequencing (WGS) of 3 LP cells from a BRCA2 carrier. Each row represents one cell. Gray dots indicate genomic sequence reads (X-axis corresponds to read counts). Columns/numbers correspond to individual chromosomes. Blue dots indicate significantly decreased genomic read counts, indicating heterozygous loss.

Aim 2. *Reveal abnormal cell subpopulations of primary BRCA1 and BRCA2 mutant epithelia through single-cell RNAseq.*

Major Task 3: Identify subpopulations through single-cell RNAseq and analysis

Activities: Although collections of tissue were somewhat slowed due to the Covid pandemic and consequent cancellation of elective (cancer preventative) surgeries, we have nonetheless been able to make substantial progress toward the collection, single-cell RNA sequencing and analysis of BRCA1/2 carrier and control breast tissues.

Objectives: We aim to identify deregulated cell types, cell states, and cell subpopulations, particularly among epithelial cells in carriers versus controls.

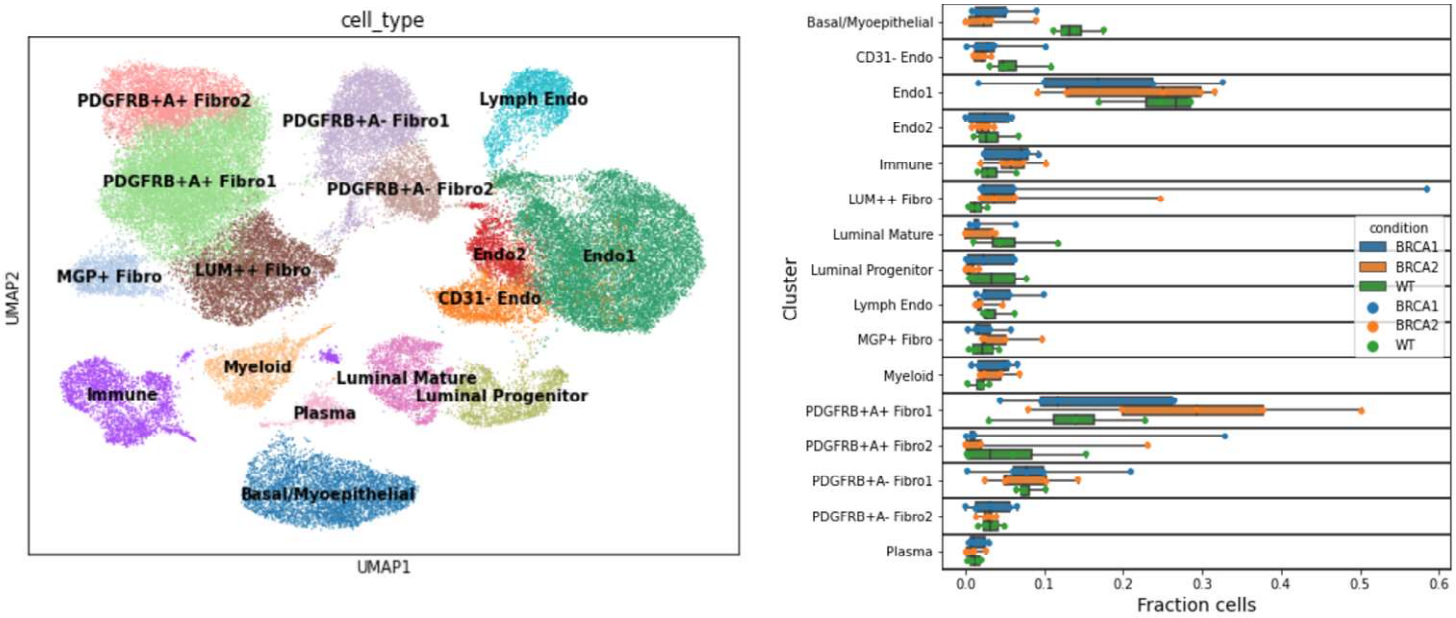

Figure 4. Summary of single-cell RNAseq analysis from BRCA1/2 carrier and control breast tissues. Left, TSNA plot of assigned cell types (color-coded) among approximately 56,000 cells from tissues of 5 BRCA1, 5 BRCA2 and 5 age-matched controls. Right, overall proportion of each cell type (rows) by genotype. Genotypes are designated by color code, and range and standard deviation (boxes) for the proportion of each cell type are indicated. A trend is observed towards increased luminal vs basal cells in carriers, as well as increased immune cells in carriers.

Methodology and Results: Our initial analysis involved single-cell RNA sequencing of freshly-collected, unselected primary breast tissues from 5 BRCA1, 5 BRCA2 and 5 age-matched control tissues, employing the 10X Chromium Single-cell library preparation. We analyzed 3,000-5,000 cells/sample as anticipated. An overview of the initial analysis is shown in Figure 4. We found the anticipated distribution of cell types in these breast tissues. Comparison of the proportion of individual cell populations in carriers vs. controls shows trends towards increased luminal vs basal cells, and increased immune cells in carriers. These trends will be further validated as the number of specimens analyzed increases.

Major Task 4: Isolate and characterize deregulated subpopulations

Activities: We are currently carrying out more detailed analysis of the data shown in Fig. 4, together with single-cell analysis of additional tissues as described above.

Objectives: We seek to define specific subpopulations and transcriptional states that are selective for carriers vs. controls. These findings will set the stage for more detailed analyses of these subpopulations and their relationship to the cancer predisposed state of BRCA1/2 carriers.

Methodology and Results: We are employing both supervised and unsupervised transcriptome analyses in these cells and subpopulations, to be followed by functional analyses of defined subpopulations.

Aim 3. *Employ high-throughput approaches to uncover selective vulnerabilities of primary BRCA1/2-mutant epithelia.*

Major Task 5: Organoid Models of Primary Mammary Tissues
Major Task 6: Therapeutic compound screening of organoids
Major Task 7: Validating effects of identified agents on malignant progression

Activities: We are currently carrying out a variety of culture methodologies for propagation of BRCA1/2 carrier and control tissues.

Objectives: Cultures will be tested in chemical screens as functional probes to elucidate deregulated pathways and unanticipated vulnerabilities that are selective to the BRCA1/2 carrier state. These data will inform future pre-clinical and ultimately clinical approaches to breast cancer prevention in this and other contexts.

Methodology and Results: We are currently defining the extent to which different culture conditions select for different subpopulations of cells, focusing on breast epithelial cells. We have noted that standard organoid cultures select for the basal epithelial-derived subpopulation, suggesting that modified conditions (e.g. low adherence culture) may be required for a focused analysis of luminal cell types.

Opportunities for Training and Professional Development:

While this proposal is not specifically a training grant, Mihriban Karaayvaz, PhD has providing training to Varunika Vivekanandan, a research assistant in the laboratory who has developed professional skills in preparation for graduate school. Consequently, Varunika is leaving the lab and will be replaced by Nsan Melkonjan, an highly intelligent research assistant and recent college graduate.

Dr. Karaayvaz herself was able to attend a bioinformatics course, as well as multiple conferences (now remote due to the Covid pandemic) concerning topics related to the area of the proposal.

Dissemination of Results to Communities of Interest:

1. Presentation at the Harvard Cancer Center Breast/Ovarian Cancer Retreat 4/2020.
2. Presentation at the DFHCC Connect:Science Seminar in 10/2020.
3. A **major manuscript** describing these findings was published in January of this year (Karaayvaz et al. Science Advances 2020, 6(5):eaay2611).

Plans During the Next Reporting Period:

We plan to collect additional fresh specimens for analysis through single cell methodologies including RNA-Seq, whole-genome sequencing, and other techniques. We will subsequently analyze selected primary uncultured subpopulations. We will also continue to refine culture methodologies for select subpopulations of interest. These cultured cells will be tested with select chemical probes, and ultimately in chemical screens in order to reveal deregulated DNA damage response and repair that are present selectively in the cancer-predisposed carrier tissues but not controls.

4. Impact

A. Impact on the development of the principal discipline
Our recently published papers has received attention and has already been cited by others. We believe this work will ultimately re-define our understanding of the progression of breast cancer.

B. Impact on other disciplines
Our single-cell analysis work has illuminated how a complex disease process such as cancer can be dissected through this type of technology. This finding has broad implications for the study of disease biology.

C. Impact on technology transfer
None to date, but we anticipate potential biomarkers and therapeutic approaches may be enabled by this research, with significant technology transfer/intellectual property implications.

D. Impact on society beyond science and technology
By redefining our understanding of cancer progression, we may impact the philosophical perception of cancer by both health care providers and the lay public. We intend to keep our patient advocates engaged in this process.

5. Changes/Problems

No significant problems or changes in approach or concept. The Covid pandemic temporarily impeded our ability to collect fresh tissues as noted. Fortunately, this has proved to be temporary, and we do not anticipate a long-term impact on the overall number of specimens we will be able to collect and analyze. Secondly, our collaborator Dr. Cyril Benes has recently left for another faculty position elsewhere. Fortunately, our Center for Molecular Therapeutics previously led by Dr. Benes has the full support of our institution and will continue with all capabilities relevant to this proposal. Thus, Dr. Benes' departure will have no impact on our ability to carry out the proposed studies.

6. Products

The major product in this reporting period is the major manuscript published with our findings (federal support is acknowledged):

Karaayvaz M, Silberman RE, Langenbucher A, Saladi SV, Ross KN, Zarcaro E, Desmond A, Yildirim M, Vivekanandan V, Ravichandran H, Mylavagnanam R, Specht MC, Ramaswamy S, Lawrence M, Amon A, **Ellisen LW**. Aneuploidy and a deregulated DNA damage response suggest haploinsufficiency in breast tissues of BRCA2 mutation carriers. Science Advances 2020;6(5):eaay2611.

7. Participants & Other Collaborating Organizations

Name:	Ellisen, Leif
Project Role:	PD/PI
Researcher Identifier (e.g.	

ORCID ID):	
Nearest person month worked:	0.6
Contribution to Project:	Dr. Ellisen will be involved in all aspects of the study, including designing and interpreting experiments, and communicating and publishing results. He will assume overall responsibility of the administration and conduct of this research at MGH.
Funding Support:	

Name:	Karaayvaz Yildirim, Mihriban
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Working with the Dr. Ellisen and supervising the Research Assistant, she will oversee all aspects of the proposed studies to understand response and repair defects. She will also be involved in cell-based studies emanating from single-cell RNA-seq analysis.
Funding Support:	

Name:	Melkonjan, Nsan
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	He will carry out in vitro assays and develop organoid cultures. He will also be involved in carrying out drug screens of these cultures as described in the SOW.
Funding Support:	

8. Special Reporting Requirements

None

9. Appendices

Manuscript: Karaayvaz et al. Science Advances 2020, 6(5):eaay2611