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14. ABSTRACT

Moving new breast cancer prevention approaches forward into clinical trials requires robust data supporting both mechanism and efficacy. Our Breakthrough-funded study identified early changes in the breast cancer cell-of-origin. Here, we seek to understand these changes and the unique cell sensitivities they create, as a means of revealing new and unanticipated targets for breast cancer prevention. 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.

15. SUBJECT TERMS
None listed.

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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. We have also employed CRISPR knock-in models of specific BRCA1/2 mutations in non-transformed mammary epithelia as a means to support findings from primary tissues.

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: The data described in the original proposal application and our manuscript publishes in 2020, led to our discovery that a Replication Stress Response Deficiency (RSRD) gene expression signature is increased in Luminal Progenitor (LP) epithelial cells carriers vs. non-carriers (Karaayvaz et al. Science Advances 2020, 6(5):eaay2611). This observation strongly supports data generated from our ex-vivo analyses presented in the original proposal application. To validate these findings, we generated mammary epithelial cells (MCF-10A) with CRISPR knock-in of the common loss-of-function mutation BRCA1 185delAG. We then carried out RNA sequencing of these cells compared to matched wild-type (WT) cells (Figure 1A). The major finding was that differentially expressed genes are predominantly adhesion/basal-like genes. This observation is consistent with trans-differentiation reported with BRCA1 haploinsufficiency in multiple prior papers. Consequently, the findings support this BRCA1-deficient model as a valuable and faithful means to test potential phenotypes and therapeutic vulnerabilities in the BRCA-deficient context (see below).

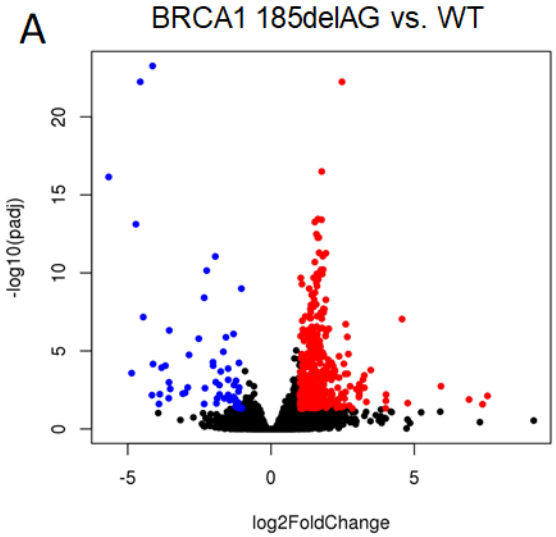

Figure 1A. Isogenic cell models highlight features of BRCA1 haploinsufficiency. The *BRCA1* 185delAG mutation was introduced into the breast epithelial cell line MCF10a using CRISPR/Cas9 gene editing. (A) Volcano plot showing differential gene expression from RNAseq, identifying genes with adjusted p-value < 0.05 and absolute log2 fold change > 1. A total of 469 genes were increased (Red) or decreased (Blue) in BRCA1-mutant compared to WT cells. These genes are predominantly adhesion/basal-like genes consistent with trans-differentiation reported with BRCA1 haploinsufficiency.

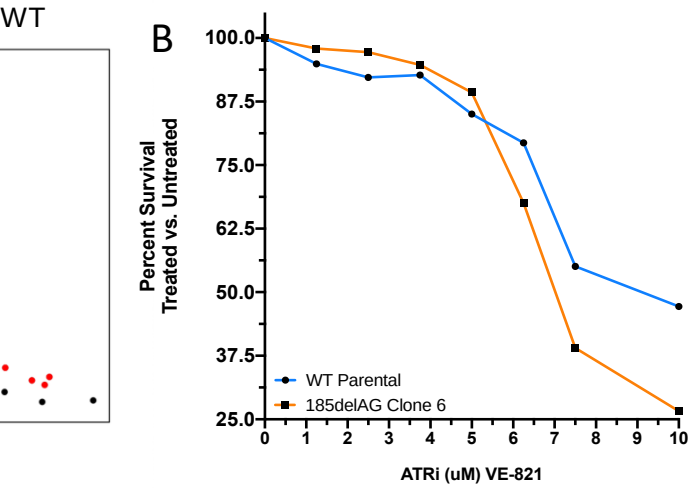

Figure 1B. Isogenic cell models of BRCA deficiency reveal therapeutic vulnerabilities related to replication stress. BRCA1 185delAG cells or wild-type (WT) parental controls were treated with ATR inhibitor and viability determined by cell-titre glow assay. We found that haploinsufficient cells are more sensitive to ATR inhibition with VE-821. These data are in keeping with replication stress deficiency we and others have reported in the BRCA1/2 heterozygous state.

Major Task 2: Test hypothesis-driven inhibitors

Activities: Ongoing activities are performing functional assays as described in our recent (2020) manuscript, most notably including targeted inhibitors of key pathways hypothesized to be important in the BRCA-deficient setting. These activities include studies in primary cells and tissues and in the isogenic BRCA-mutant cell models presented above.

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: A major finding for this Task comes from analysis of cellular vulnerabilities of BRCA-deficient cells, which were hypothesized to occur based on the Replication Stress Response Deficiency that we previously reported. The ATR kinase enforces a key checkpoint related to replication stress. Consequently, we hypothesized that cells with a deficiency in the replication response will be highly sensitive to further disabling of this checkpoint by inhibition of ATR. We subsequently tested this hypothesis in the matched isogenic BRCA1 185delAG cells or wild-type (WT) parental controls, employing the clinical ATR inhibitor VE-821 (Figure 1B). Indeed, our hypothesis was supported, as the BRCA-deficient cells were more sensitive to ATR inhibition than the matched controls. Given that VE-821 has already entered clinical trials in a number of settings, with further validation it could be a focus of future testing as a potential preventative in the setting of BRCA deficiency.

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.

ID	Genotype	Age	Menopause	Cancer history	Digestion	Comment
MGH18005	BRCA1	28	Pre		Long	
MGH19006	BRCA1	45	Pre		Long	
MGH19002	BRCA1	48	Post		Long	
MGH19028	BRCA1	31	Pre		Long	
MGH19013	BRCA1	36	Pre		Long	
MGH19017	BRCA1	43	Post	TNBC (treated with PARPi then ddAC-T	Long	
MGH20042	BRCA1	35	Pre		Short	Sorted, all
MGH21010	BRCA1	41	Pre	ER+ DCIS	Short	Sorted, E+I
MGH21016	BRCA1	35	Post	On HRT	Short	Sorted, E+I
MGH21031	BRCA1	34	Pre		Short	Sorted, E+I
MGH21012	BRCA1	30	Pre		Short	Sorted, E+I
GRBI #7	BRCA1	37	Post		Short	Sorted, E+I
MGH19012	BRCA2	31	Pre	HR+ DCIS and tumor	Long	
MGH19025	BRCA2	38	Post		Long	
MGH19007	BRCA2	40	Pre		Long	
MGH19016	BRCA2	56	Post		Long	
MGH19022	BRCA2	25	Pre		Long	
MGH19018	BRCA2	43	Post		Long	
MGH19020	BRCA2	60	Post		Long	
MGH20024	BRCA2	37	Pre		Short	Sorted, E&NE
GRBI #2	BRCA2	31	Pre		Short	Sorted, all
MGH21001	BRCA2	43	Post		Short	Sorted, all
GRBI #5	BRCA2	64	Post	ER+ (weakly) tumor	Short	Sorted, E&NE
MGH21021	BRCA2	45	Post	ER+ DCIS and tumor	Short	Sorted, E+I
MGH21023	BRCA2	48	Peri	TNBC	Short	Sorted, E+I
MGH21026	BRCA2	48	Post	ER+ DCIS	Short	Sorted, E+I
MGH21011	BRCA2	51	Pre	ER+ DCIS	Short	Sorted, E+I
GRBI #6	BRCA2	67	Pre	DCIS	Short	Sorted, E+I
MGH19004	Control	28	Pre		Long	
MGH19024	Control	44	Pre		Long	
MGH19008	Control	50	Post (or peri)		Long	
MGH19023	Control	40	Pre		Long	
MGH20017	Control	58	Post		Long	
MGH20026	Control	24	Pre		Long	
MGH21017	Control	53	Post		Short	Sorted, E+I
MGH21003	Control	31	Pre		Short	Sorted, E&I
MGH21004	Control	37	Pre		Short	Sorted, E&NE / E+I
MGH21005	Control	59	Post		Short	Sorted, E&NE / E+I

Table 1. Primary noncancerous breast tissue samples, collected, processed and analyzed by single-cell RNAseq. Genotypes and subject demo-graphics are shown. Each row represents one subject/patient. Contralateral breast cancer diagnosis (if any) is indicated. Multiple cell sorting/selection approaches were employed to enrich for populations of interest, as indicated in the Comment. Blank = all cells; E= Epithelial; NE= Non-Epithelial; I=Immune.

Methodology and Results: Our initial analysis involved single-cell RNA sequencing of freshly-collected, unselected primary breast tissues from BRCA1, BRCA2 and 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 specimens collected processed, and analyzed by single-cell RNAseq to date is listed in Table 1. Notably, we also explored different tissue processing protocols involving short vs. long enzymatic digestion to preserve faithful representation of the in vivo transcriptome. We found the anticipated distribution of cell types in these breast tissues. Comparison of BRCA-mutant and controls reveals distinct gene expression phenotypes and cell clusters in mutant epithelial cells within these tissues (see Task 4).

Major Task 4: Isolate and characterize deregulated subpopulations

Activities: We are currently carrying out more detailed analysis of the samples shown in Table 1 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. Initial analysis reveals that specific cell subpopulations known as “clusters”, identified through unsupervised analysis of single-cell transcriptomes, appear restricted to mutant tissues (Figure 2). These findings are now being explored more deeply through additional analysis and validation through collateral approaches. Specifically, we are identifying the specific phenotypes of these BRCA-specific cell populations, which can then be validated and mechanistically explored in our primary culture and other cell-based models.

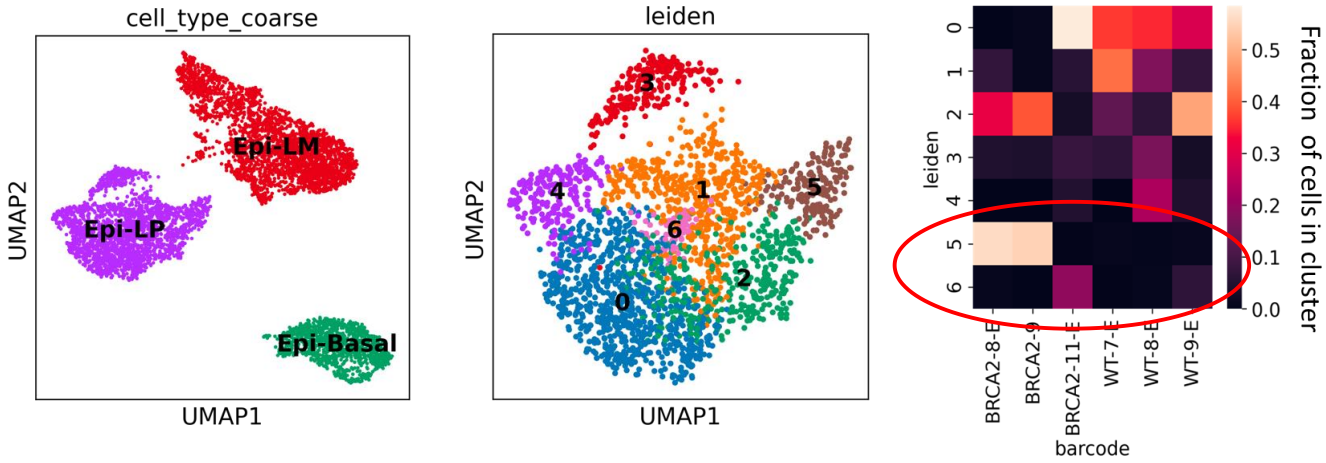

Figure 2. Single-cell RNAseq analysis reveals BRCA-specific cell clusters. Left, UMAP plot of epithelial (Epi) cells from BRCA-mutant and control primary, noncancerous breast tissues. Unsupervised analysis identifies three major cell types as Basal, Luminal Progenitor (LP) and Luminal Mature (LM). Middle, UMAP plot of LP cells in which transcriptionally distinct cell subpopulations (“clusters”) are color-coded. Seven distinct clusters of LP cells (0-6) were identified in this analysis. Right, representation of LP clusters by genotype. Red ellipse highlights that clusters 5 and 6 are present exclusively in BRCA-mutant but not control (WT) tissues.

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 advancing our studies of culture methodologies for propagation of BRCA1/2 carrier and control tissues.

Objectives: As noted in the original application, 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 continue to define 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 may be required for a focused analysis of luminal cell types. In the past year we have optimized low adherence cultures as a means to propagate the luminal progenitor subpopulation.

Opportunities for Training and Professional Development:

While this proposal is not specifically a training grant, Mihriban Karaayvaz, PhD has received extensive training in all aspects required for academic advancement. For example, 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. She has subsequently transitioned to an independent, tenure-track academic faculty position.

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. Presentation at the Science Monday seminar series in fall, 2021.
4. A **major manuscript** describing these findings was published in January 2020 (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 left for another faculty position elsewhere in 2020. 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 of this award to date 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):	0000000304447910
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):	N/A
Nearest person month worked:	3
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):	N/A
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

None