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PRINCIPAL INVESTIGATOR: Jan Vijg

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Albert Einstein College of Medicine

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Jan Vijg

E-Mail:

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Albert Einstein College of Medicine
1300 Morris Park Ave
Bronx, NY. 10461

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14. ABSTRACT

Analysis of how mutations accumulate in pretumor tissue, although widely presumed to occur, has been extremely difficult to study. This is principally because, with most such mutations being unique to individual cells within a tissue, their detection is technically challenging. In this study we propose to apply "Single Cell Multiple Displacement Amplification (SCMDA)" that we recently developed for high accuracy detection of a spectrum of mutations from single nucleotide substitutions to indels and aneuploidy in individual cells within pre-tumor tissues of women who inherited mutations in the *BRCA1* or *BRCA2* genes. We hypothesize that mutations from single nucleotide substitutions to indels, large genomic rearrangements, and aneuploidy accumulating as consequence of defects in homology dependent DNA repair in mammary epithelial cells are the underlying cause of increased cancer risk in these women. We further hypothesize that estrogen, which is known to generate metabolites that directly damage DNA, mechanistically acts as a modifier of *BRCA1/2* cancer penetrance by working in concert with the *BRCA1/2* repair defects to increase the somatic mutation rate in the cells of *BRCA1/2* carriers. In **Aim 1**, we will utilize SCMDA to test if mutation frequencies are elevated in individual *BRCA1/2* heterozygous mammary epithelial cells. In **Aim 2**, we will directly test the hypothesis that estrogen increases mutation frequencies in *BRCA1/2* mutant cells.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction

Cancer is a genetic disease caused by mutations accumulating in somatic cells during aging, environmental exposure or other endogenous factors (i.e. hormone exposure). How somatic mutations in non-tumor tissue result in tumor initiation remains largely unknown, in part due to the technical difficulties of studying non-clonal casual mutations in tissues. By leveraging *in house* generated approaches including "Single Cell Multiple Displacement Amplification (SCMDA)" in this project we aim to map all forms of mutations from single nucleotide substitutions to indels, large genomic rearrangements, and aneuploidy to test the hypothesis that accumulation of these mutations underlie increased cancer risk. As a model we selected women who inherited germline mutations in *BRCA1* or *BRCA2* because of their intrinsic defect in the homologous repair (HR) pathway. We further hypothesize that estrogen (from endogenous production, contraceptives, pregnancy, hormone replacement therapy), which is known to generate metabolites that directly damage DNA, mechanistically acts as a modifier of *BRCA1/2* cancer penetrance by working in concert with the HR repair defects to increase the somatic mutation rate in the cells of *BRCA1/2* carriers. Our hypothesis is being tested along two specific aims: in Aim 1, we utilize SCMDA to test if mutation frequency in normal mammary epithelial cells is increased in *BRCA1/2* carriers relative to aged matched control women undergoing reduction mammoplasty purely for cosmetic reasons. In Aim 2 using primary organoids obtained from *BRCA1/2* carriers or controls exposed to mammary gland estrogen levels we are defining how the hormonal microenvironment of the mammary epithelium influence genomic instability and promoted transformation by acquisition of a cascade of genetic event converging to tumorigenesis.

2. Keywords

Breast cancer, BRCA1, BRCA2, germline mutations, mutations, genomic instability, transformation, estrogen, 17β estradiol, inherited cancer, DNA damage

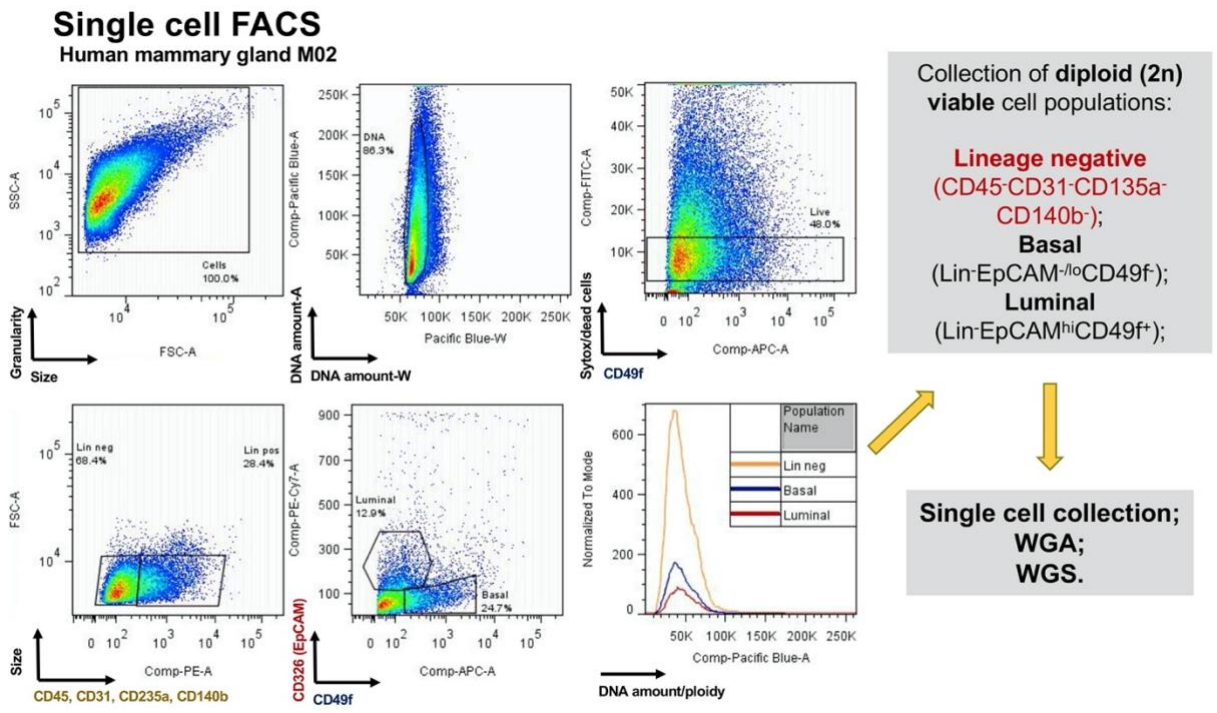
3. Accomplishments

Major goals of the project and accomplishments:

Goal 1: to collect and isolate mammary epithelial cells. Tissue for this study is being obtained from the Cooperative Human Tissue Network (CHTN). During this funding period we obtained six tissue samples for the control group from women of 28-42 y.o. undergoing plastic surgery reduction mammoplasty and indicating no previous family history of breast cancer. Six tissue samples considered as early onset cancer risk group were also collected from cancer-free female patients carrying a pathogenic mutation in *BRCA1* gene and, hence, undergoing preventive care mastectomy. Normal tissue samples were also collected upon oncology surgery from two patients with early onset breast cancer (<45 y.o.), carrying pathogenic mutation in *BRCA 1* or *BRCA 2* gene (2 samples). All tissues were collected fresh at the time of surgery from the eastern division of the Cooperating Human Tissue Network (CTHN) and shipped over night at room temperature in transportation media. Mammary gland tissues, 0.5-2 g, were dissected into smaller pieces of ~3-4 mm and digested with Collagenase III and hyaluronidase to obtain mammary organoids for subsequent digestion into single cells. To enrich for luminal or basal mammary epithelial cells, single cells were stained with and antibody against CD49f and CD326 (EpCAM) as well as lineage specific markers CD31, CD45, CD235a, CD140b before sorting to separate basal epithelial cells (Lin- CD49f+/high EpCAM-/low) and luminal epithelial cells (Lin- CD49f-/low EpCAM+/high) as shown in **Figure 1**. Single cells obtained from both populations were deposited into Eppendorf tubes and stored at -80^{0}C for further analyses.

Goal 2: to perform whole genome single cell sequencing with the goal to establish if mammary epithelial cells of *BRCA1/2* carriers have increased genomic instability. After enrichment single primary mammary epithelial cells subjected to whole genome amplification (WGA) using our advanced single cell multiple displacement amplification method (SCMDA) as described in the application. To control the quality of amplified single cell MDA products we routinely use the 8-target locus-dropout tests. Qualified samples (4 single cell MDA products per each individual) are next subjected to library preparation and whole-genome sequencing using protocols described in the application and as reported by us [PMID: 28319112]

Figure 1: Schematic of FACS enrichment of luminal and basal cells. Mammary tissue from donor M02 was processed as described in Goal 1 to enrich for luminal and basal mammary epithelial cells. Top row= removal of doubles and cell viability analysis by Sytox green nucleic acid stain; bottom row = lineage negative selection (Lin-) followed by enrichment of luminal and basal cells. On the bottom right yield of cell populations.



Goal 3: to run analytical pipelines and biostatistical analyses to evaluate genomic instability index across experimental groups. As sequencing data become available single cell WGS and its matching reference genome are analyzed using our custom in house generated pipelines described in [PMID: 28319112; PMID: 32064334; PMID: 30992375]. Currently, analysis has been completed for 14 samples. As new samples are collected they will flow their sequencing results into the analytical pipeline.

Goal 4: to establish *in vitro* model of 3D mammary organoids exposed to 17 β estradiol. For studying the consequences of 17 β estradiol (E2) on genomic instability and transformation of mammary epithelial cells of *BRCA1/2* mutation carriers and controls we explored two models: mammary organoids in which partially digested mammary epithelial tree tissue is embedded in extracellular matrix hydrogel (Matrigel) and spheroids organized from patients derived dissociated single cells grown in Matrigel (**Figure 2**). Both 3D models have been cultured in basal control media or in the presence of serial dilutions of E2 ranging from 0.02 ng/ml to 20 ng/ml, with the highest concentration (20μM) reflecting the local E2 levels estimated in the mammary tissue; all E2 concentrations remain within the physiological range detected in the blood of women in the third trimester of pregnancy. While both models preserve similar cell viability in culture up to 7 days, partially digested organoids had lower yield and showed limited response to 20μM E2 exposure as measured by growth and branching (**Figure 2A-D**). On the contrary spheroids obtained from digested cells could be cultured for up to 24 days (**Figure 2B-E**) and were highly responsive to E2 exposure at 20μM concentration as measured by the number of organoids per field of view and their size (size distribution is preliminary observation). Phosphorylated gamma-H2AX staining to measure DNA damage indicates increased frequency of cells with foci of DNA repair in 20μM E2 exposed cultures relative to untreated controls. Based on these results, at the end of year one, we have established a robust model of culture of primary human mammary epithelial cells that retain response to E2 treatment. Thus, we will base our future studies on the mammary spheroid model. In addition, we also demonstrated the ability to dissociate the organoids at the end of the E2 treatment and isolate viable single cells for future SCMDA analysis.

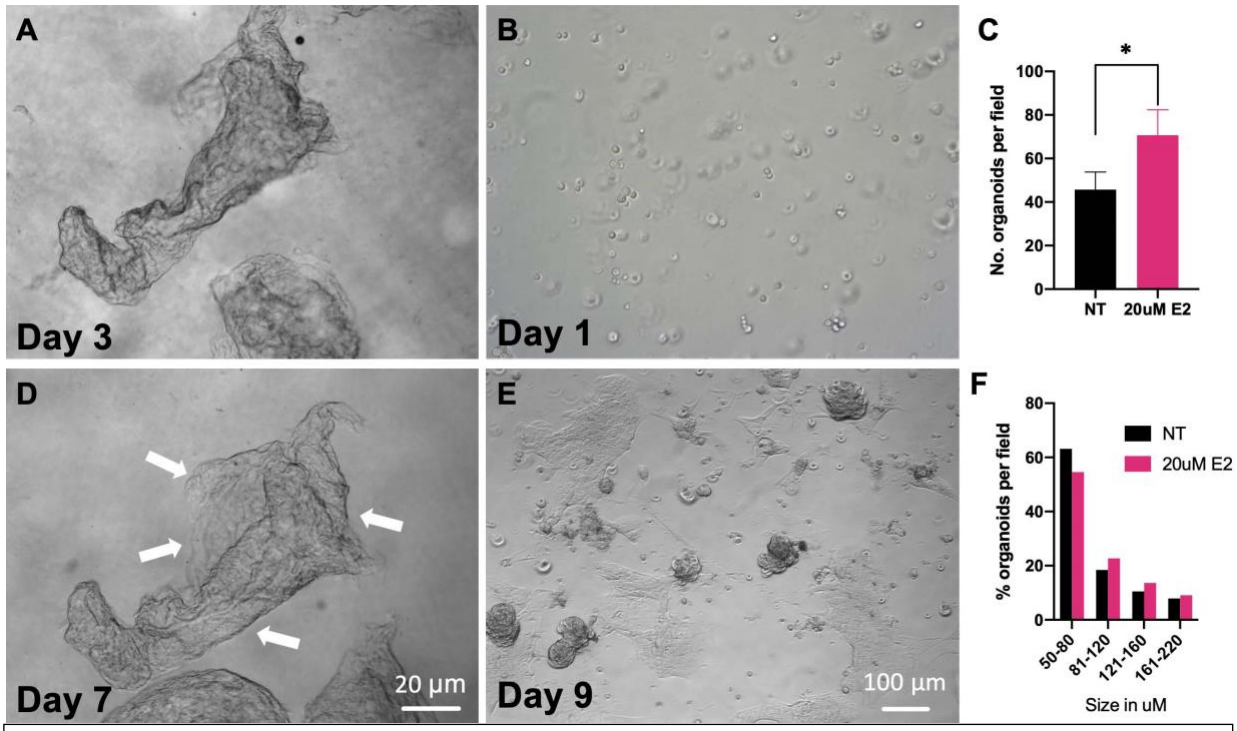


Figure 2: Human primary mammary organoids as a model to study E2 exposure. Mammary tissue from a 23 y.o. donor undergoing reduction mammoplasty for cosmetic reasons was processed following two protocols for the establishment of a *in culture* model to study the effect of E2 exposure on transformation. **A-D** Mammary organoids from partially digested tissue were embedded in Matrigel and cultured for 7 days into 3D structures. Partial ramification of epithelial tree was observed after 7 days of culture (white arrows in D). **B-E** Mammary tissue was digested into single cells; $6x10_4$ cells were plated into a single 24 well and allowed to grow for 9 days. **C-F** Quantification of number of organoids (C) and their size (F) after 9 days of culture in control media or media containing 20uM E2. NT=non-treated; * $p<0.05$ t-test.

Major goals for the next reporting period:

Goal 1: to collect and isolate mammary epithelial cells. We will continue the collection of additional samples for analysis of mutations to reach the sample size proposed (n=20).

Goal 2: to perform whole genome single cell sequencing with the goal to establish if mammary epithelial cells of *BRCA1/2* carriers have increased genomic instability. Our preliminary analyses support the hypothesis that mammary epithelial cells obtained from *BRAC1/2* mutation carriers have increased level of somatic mutations compared to age matched non-carrier controls; we will continue these analyses and apply appropriate statistical tests.

Goal 3: to run analytical pipelines and biostatistical analyses to evaluate genomic instability index across experimental groups. During the next period of funding we will finalize our preliminary observations by completing the analysis of human primary mammary epithelial cells isolated from *BRCA1/2* carriers and controls to meet the proposed sample size. We will perform appropriate statistical comparisons to determine if significant differences between *BRCA1/2* mutation carriers and control exist. We will also begin the SCMDA assay and analysis of

Goal 4: to establish *in vitro* model of 3D mammary organoids exposed to 17 β estradiol. We will continue perfecting the 3D mammary organoids model and ensure its robustness to evaluate E2 treatment response. Primary basal and progenitor cells will be tested for their effective re organization into 3D structure when grown in Matrigel and compared to pooled cells to ultimately study their response to E2 treatment. In parallel we will begin isolating single cells from 3D organoids established from *BRCA1/2* carriers and controls exposed to E2 or not in order to carry on genomic studies. A portion of the organoids culture will formalin fixed and paraffin embedded to study the genomic changes occurring in mammary epithelial cells in response to E2 exposure.

4. Impact

Nothing to report

5. Changes/Problems

We had difficulties in obtaining samples with sufficient material for FACS enrichment of epithelial subtypes, especially from *BRCA1/2* mutation carriers. In order to obtain a sufficient number of mammary epithelial cells for efficient enrichment of subtypes by FACS we require a minimum of 20mg of tissue. To overcome limitations in obtaining sufficient amount of tissue for processing by the Cooperative Human Tissue Network (CHTN) we requested to broaden the tissue search from the eastern division to other collection sites part of the Mid-Atlantic, Southern and Western division.
Work on this project was substantially affected by the pandemic caused by SARS-CoV-2. Following NY State regulations from the second week of March until mid-June, the Albert Einstein College of Medicine was allowed to grant laboratory access only to essential personnel or individuals working in SARS-CoV-2 laboratories. Now that these state requirements have resulted in highly successful reductions in SARS-CoV-2 infections and COVID-19 hospitalizations and deaths, NY State is in the process of gradually reducing restrictions, with New York City, in which we are located, following the slowest course. Our institution is still operating with limited and staggered shift personnel to maintain safe physical spacing at the time of submission of this progress report (July 25). As a result, we were unable to perform sequencing as planned; samples have been collected and we have libraries prepared ready for sequencing; currently we are in the process of submitting these samples. Likewise, organoids experiments proposed in Aim 2 were delayed. Culture of organoids is now underway. To overcome potential further delays in case of future shut down caused by the pandemic we are exploring cryopreservation of organoids that can be cultured 3D at later time.

6. Products

Nothing to report

7. Participants & Other Collaborating Organizations

Name: Jan Vijg
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0002-8457-9595
Nearest person month worked: 1.2
Contribution to Project: Dr. Vijg supervised the single cell experiments studies and closely interacted with Dr. Montagna to ensure the timely progress of the project. Dr. Vijg oversees the development and application of analytical tools for whole genome data analysis.

Name: Yujue Wang
Project Role: Computational Analyst
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6.0
Contribution to Project: Dr. Wang is responsible for running the analytical pipelines for whole genome sequencing data and SCMDA analysis and perform QC on the data generated as part of aims 1 and 2.

Name: Moonsook Lee
Project Role: Research Tech
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6.0
Contribution to Project: Mrs. Lee is responsible for isolation of single cells for SCMDA as well as whole genome amplification and QC to ensure that the DNA used for analysis retains unbiased amplification. She is also responsible for coordinating the shipment of samples for sequencing and ensure that the delivery of the sequencing data is timely.

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

  No

- **What other organizations were involved as partner?**

  “Nothing to Report”

8. Special Reporting Requirements

9. Appendices