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CONTRACTING ORGANIZATION: Albert Einstein College of Medicine

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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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TABLE OF CONTENTS

1. INTRODUCTION:

Cancer is a genetic disease caused by mutations that accumulate in somatic cells during aging, environmental exposure or other endogenous factors. How these somatic mutations acquired by cells transform them into tumors remains largely unknown. This is due, in part, to the technical difficulties of studying non-clonal casual mutations accumulated in tissues before transformation. In this application we will test the hypothesis that mutations accrued as consequence of endogenous DNA damage caused by high estrogen levels cause increased breast cancer risk. By leveraging *in house* generated approaches including "Single Cell Multiple Displacement Amplification (SCMDA)" we aim to map all forms of mutations from single nucleotide substitutions to indels, large genomic rearrangements, and aneuploidy, using as a model women who inherited germline mutations in *BRCA1* or *BRCA2* resulting in intrinsic defects in the homologous repair (HR) pathway, which greatly increase their susceptibility to develop tumors. We hypothesized 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* mutation carriers. Our hypothesis is being tested along two specific aims: in Aim 1, we apply SCMDA to test if mutation frequency in normal mammary epithelial cells is increased in *BRCA1/2* mutation carriers relative to age-matched control women undergoing reduction mammoplasty purely for cosmetic reasons. In Aim 2 we define how the hormonal microenvironment of the mammary epithelium influence genomic instability to promote transformation by acquisition of a cascade of genetic events increasing the risk for tumor transformation. To do so we established a unique collection of primary organoids obtained from *BRCA1/2* mutation carriers or age-matched controls which we exposed to estrogen levels mimicking those found in the mammary gland.

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

3A Major goals of the project

Specific Aim 1: Test if mutation frequency in normal mammary epithelial cells is increased in *BRCA1/2* carriers.

Major Task 1: Test if mutation frequency in normal mammary epithelial cells is increased in *BRCA1/2* carriers.

We have analyzed by Single-Cell Multiple Displacement Amplification (SCMDA) a strain of primary telomerized mammary epithelial cells (human telomerase reverse transcriptase–immortalized mammary epithelial cells [hTERT-IMECs]) heterozygous for 185delAG, a pathogenic and highly penetrant BRCA1 mutation, to isogenic WT control hTERT-IMECs. The results indicated a significant increase of both SNVs and INDELs (2.3-fold increase in SNVs and 1.7-fold increase in INDELs) in hTERT-IMEC BRCA1 heterozygous mutant cells as compared with isogenic WT cells (i.e., for mutant and control cells, respectively, 4196 ± 1536 SNVs and 1825 ± 473 SNVs per cell and 397 ± 129 INDELs and 231 ± 82 INDELs per cell; P = 4.21 × 10-4 and P = 3.93 × 10-2, respectively; negative binomial generalized linear model [NBGLM]) (**Figure 1A-B**).

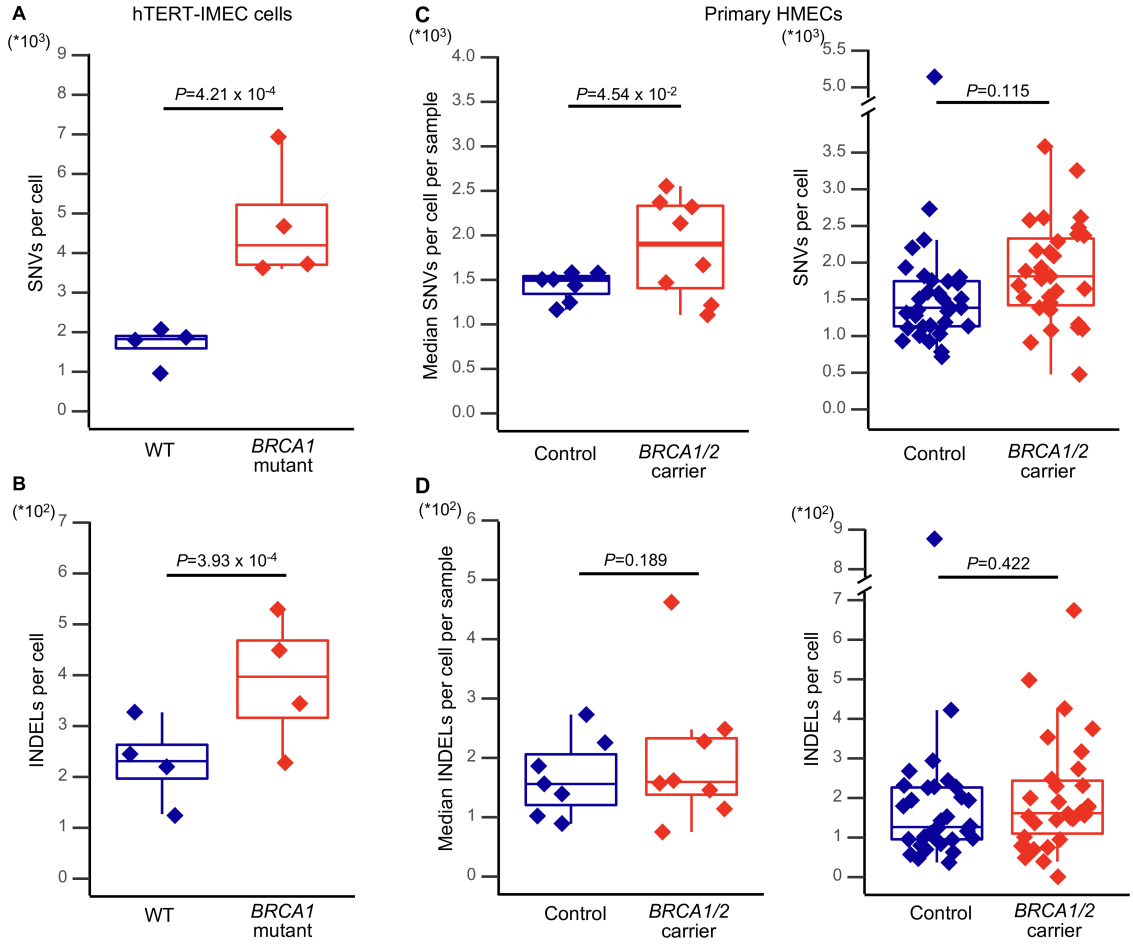


Figure 1. Mutation levels in human mammary epithelial cells.
(A) SNV and (B) INDEL levels in hTERT-IMEC wt (blue) and BRCA1 mutant (red) cells (n=4 for each type). (C) SNV and (D) INDEL levels in primary HMECs. Left panels in (C) and (D) depict the median mutations per sample in both groups (control: blue, n=7; carrier: red, n=8; negative binomial generalized linear model), while right panel depicts the distributions of single HMECs in control (blue; n=32) and BRCA1/2 mutant carrier (red; n=31) groups (negative binomial generalized linear mixed-effect model).

To establish *in vivo* relevance of these results, we isolated non tumor mammary epithelial cells from women diagnosed with a *BRCA1/2* germline mutation (8 individuals for a total of 31 cells) as well as from age-matched women undergoing reduction mammoplasty purely for cosmetic reasons used as controls (7 individuals for a total of 33 cells) and performed SCMDA for 2–8 single primary from each individual alongside genomic DNA obtained from bulk mammary gland tissue of the same individuals to correct for germline variants.

The median SNV frequency per individual was significantly elevated (1.3-fold) in the *BRCA1/2* germline mutation carrier group as compared with the control group ($P = 4.54 \times 10^{-2}$, NBGLM) (i.e., 1902 ± 561 SNVs and 1506 ± 163 SNVs, respectively (**Figure 1C**). In contrast to the increase in hTERT-IMEC *BRCA1* heterozygous mutant cells, there were no statistically significant differences in the frequencies of INDELs between the mutant and

control groups in either median INDEL frequency per individual (i.e., 160 ± 120 INDELs and 156 ± 66 INDELs, respectively) or the average number per cell (i.e., 162 ± 143 INDELs and 126 ± 155 INDELs, respectively) (P = 0.189 and P = 0.422, NBGLM and NBGLMM, respectively (**Figure 1D**).

Specific Aim 1 accomplishments

Specific Aim 1 is completed and a manuscript describing the findings "*Single-cell analysis on somatic mutation burden in mammary cells of pathogenic BRCA1/2 mutation carriers*" was published in the Journal of Clinical Investigation (PMID: 35025760).

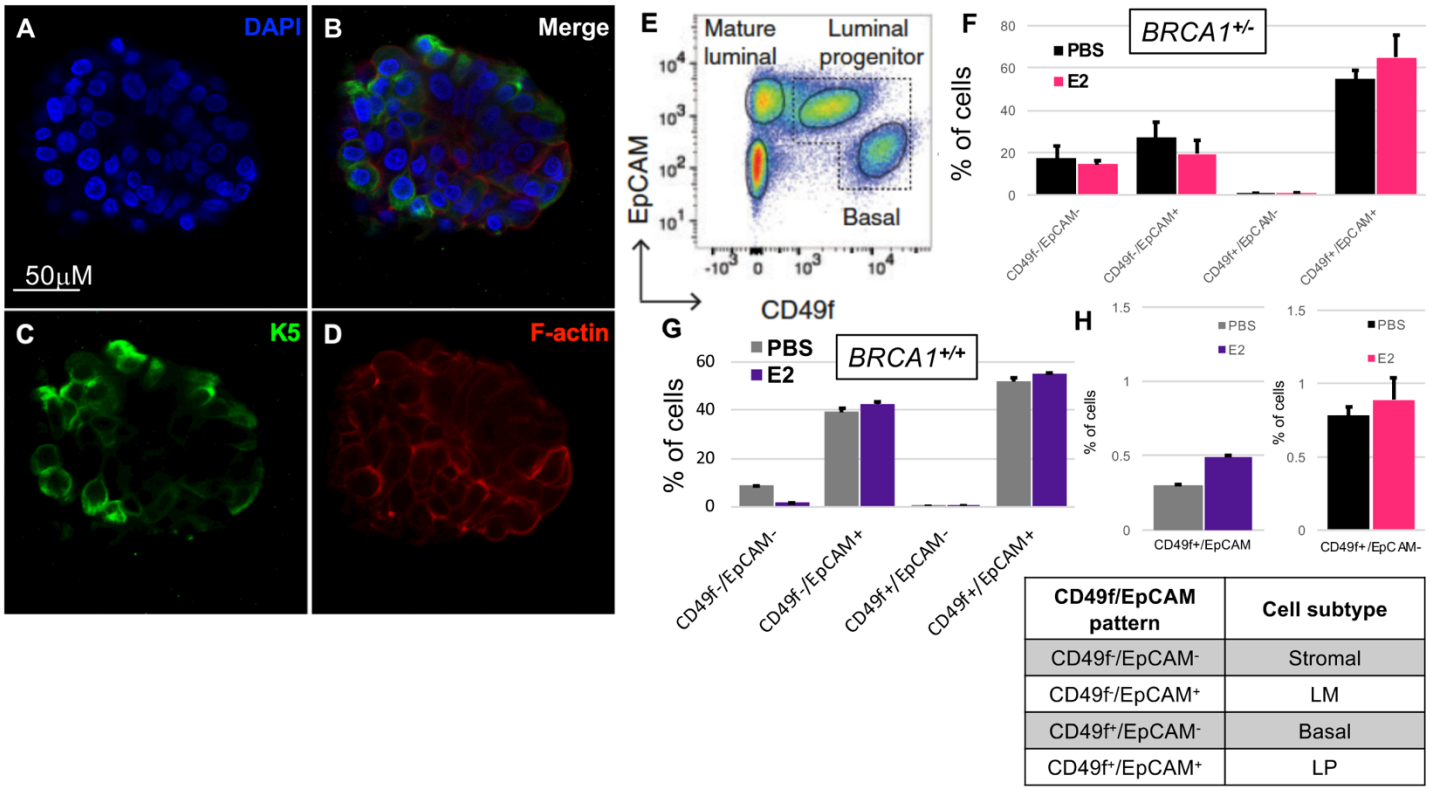
Table 1: Summary of samples used to establish mammary organoids

ID	Type	Age	Race	Co morbidities	Prior cancer history
O_M01	WT	23	W	Obesity	no
O_M02	WT	42	W	no	no
O_M03	WT	37	B	no	no
O_M04	WT	28	W	no	no
O_M05	WT	50	B	no	no
O_M06	WT	22	W	no	no
O_M07	WT	28	B	no	no
O_M08	WT	49	B	Obesity	no
O_M09	BRCA1	37	W	no	no
O_M10	BRCA1	35	W	Obesity	no
O_M11	BRCA1	38	B	no	no
O_M12	BRCA1	28	W	no	no
O_M13	BRCA1	39	W	no	no
O_M14	BRCA1	27	W	no	no
O_M15	BRCA1	36	W	no	no
O_M16	BRCA1	35	W	no	no
O_M17	BRCA1	44	B	no	no
O_M18	BRCA2	34	W	no	no
O_M19	BRCA2	30	W	Obesity	no
O_M20	BRCA2	48	?	no	no
O_M21	BRCA2	33	B	no	no
O_M22	BRCA2	57	W	no	no
O_M23	BRCA2	31	B	no	no
O_M24	BRCA2	37	B	no	no
O_M25	BRCA2	46	W	no	no
WT		34.88+/-11.24	W=4, B=4		
BRCA1		35.44+/-5.27	W=7, B=2		
BRCA2		39.5+/-9.72	W=4, B=3, Unk=1		
		n.s.	n.s.		

Specific Aim 2: Determine the effects of estrogen (E2) exposure on mutation rate in *BRCA1/2* mutant cells.

Major Task 1: to validate in culture model of 3D mammary organoids exposed to 17β estradiol

In the previous funding period we reported the establishment and banking of 12 mammary organoids from both *BRCA1*-wt or *BRCA1/2*-mutation carriers (highlighted in blue in **Table 1**). The samples set has been expanded to 25 patients, from all of which we established mammary organoids (white in **Table 1**). As expected, 3D organoids established from experimental and control groups express basal and luminal markers (**Figure 2A-D**). The mammary organoids can be expanded for several passages (we limit the culture to 4-months as this period of time is sufficient for us to obtain a sufficient number of cells for the analyses proposed). The organoids maintain their viability in culture which allows for long term hormonal exposure to study the effect of 17β estradiol (E2) on genomic instability. To mimic the exposure of mammary epithelial cells to estrogen levels present in the breast tissue we exposed the organoids derived from *BRCA1*-wt or *BRCA1/2*-mutation carriers to 20nM estradiol for 21 to 50 days. Circulating estrogen levels in premenopausal women are: 69.75 - 528pM in the follicular phase, 238pM - 1.9 nM mid cycle, 205 – 785pM in the luteal phase. In pregnant women E2 circulating levels increase to 690 pM – 9.1 nM in the first trimester, 4.6 nM – 26.4 nM in the second trimester and 22.5 nM – 49.4nM in the third trimester. Because the levels of E2 in the breast tissue have been reported to be higher than the circulating levels (PMID: 19591931 and 21286801), we established a regiment of hormonal treatment of 20nM E2 for 21 to 50 days.



CD49f/EpCAM pattern	Cell subtype
CD49f⁻/EpCAM⁻	Stromal
CD49f⁻/EpCAM⁺	LM
CD49f⁺/EpCAM⁻	Basal
CD49f⁺/EpCAM⁺	LP

Figure 2: Establishment of 3D mammary organoids and E2 regimen.
(A-D) Representative image of a 3D organoid established from a BRCA1-mut patient. A= DAPI, B=merged channels, C= IF staining with an anti-keratin 5 (K5) antibody which labels cells of the basal layer, D= IF staining with phalloidin which stains luminal epithelial cells. Images of merged z-stack images acquired with a LeicaSP8 confocal microscope. (E) Representative FACS analysis showing the gating to measure the percentage of cells in one organoid after exposure to the E2 20nM regiment. (F-H) Plots depicting the percentage of cells from the 4 cell lineages listed in the table as observed in organoids from BRCA1-wt or BRCA1-mut patients. On the bottom summary table of the cell lineages analyzed and the markers use for FACS analysis.

To evaluate the impact of E2 exposure on lineage differentiation during mammary organoids culture we performed FACS analysis to define the percentage of mature luminal and progenitor cells, basal cells and stroma cells based on the expression of well-established mammary lineage markers (Cd49f and EpCAM, **Figure 2A-E**).
We observed that 20nM E2 promotes the expansion of luminal progenitor cells, with a trend for higher levels in organoids obtained from *BRCA1*-mut carriers (**Figure 2 F-H**). This is consistent with the knowledge that inactivation of *BRCA1* leads to the expansion of luminal progenitor cells and promotes triple negative breast cancer tumors in *BRCA1* germline mutation carriers.
Globally these results validate the 3D organoids models established during this funding period.

Major Task 2: Sequencing and QC of 3D organoids exposed to 17 β estradiol established from *BRCA1/2* carriers versus control
We have now collected and validated the organoids from *BRCA1*-wt controls and *BRCA1/2* mutation carriers. The E2 regimen to carry on specific aim 2 has been established. We performed preliminary SCMDA analysis on single cells isolated from 1 organoid established from one *BRCA1*-wt and BRCA1-mut patient (n=3 and n=18 cells respectively) (**Figure 3**). We confirmed the increased mutation frequency in *BRCA1*-mut carriers in the organoids as we observed in primary cells obtained from patient's prior tissue culture, which further validates the mammary organoids model (**Figure 3A)**. We observed no major differences in mutation frequency in response to E2 exposure in these two preliminary patients. Efforts are ongoing to expand the dataset. We performed preliminary analyses on mutation signatures (**Figure 3B**). We extracted signatures from cell levels and observed that E2-treated cells have more SBS18/SBS36 signatures (related with ROS and/or cancer).

Major Task 3: LOH analysis to establish clonal evolution pattern of mutations in cells with wt and deleted *BRCA1* or *BRCA2* alleles.

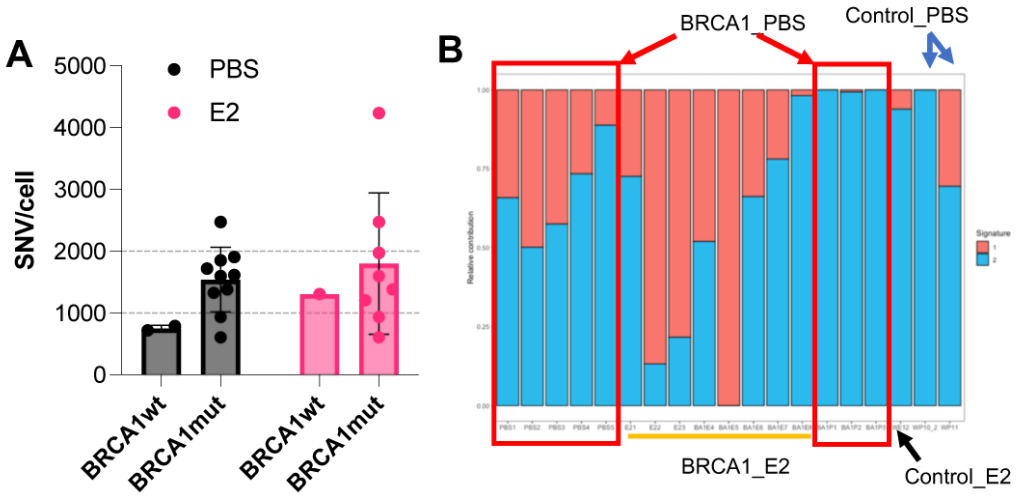


Figure 3: Preliminary mutation levels in human mammary epithelial cells *(A) SNV levels in BRCA1- wt and BRCA1-mutant cells mock treated with PBS (grey) or exposed to 20nM E2 for three weeks (magenta). (B) Two mutational signatures (M1, M2) were de novo identified in cells shown in A. The contributions of M1, and M2 signatures to all SNVs in these 4 groups using the nonnegative matrix factorization method is shown.*

In order to study loss of heterozygosity (LOH) of the wt allele in *BRCA1* heterozygous mutant cells under estrogen exposure parental and mutant isogenic clones have been exposed to 20mM E2 for 7 weeks. We prepared cells from dissociated organoids for Spectral Karyotyping (SKY) analysis and Fluorescent *in situ* hybridization (FISH) using custom probes mapping to BRCA1/2, *c-MYC*, *TERC* and *TP53* (**Figure 4**). SKY analysis revealed a small but consistent deletion mapping to the distal region of the p-arm of chromosome 12 (**Figure 4A-C**). Based on the SKY and G-banding analysis we analyzed 500 cells isolated from the organoids of the same *BRCA2*-mutation carrier exposed to PBS only or grown in the presence of 20nM E2 for 7 weeks to validate potential deletions mapping to 12p13.2 based on the presence of copies of two locus specific probes mapping to ETV6 (ETS Variant Transcription Factor 6), a known tumor suppressor gene in leukemias and previously reported as one of 11 genes enriched for indels/structural variant breakpoints in *BRCA1/2*-mutated tumors (PMID: 31182087) (**Figure 4D**). Preliminary results are consistent with the SKY findings and suggest that a small clone of cells containing cells with chromosome 12p deletion may arise in *BRCA2*-mut cells in response to E2 treatment. Additional validation to establish if E2 exposure induce insertion/deletions in *BRCA1/2*-mut organoids is ongoing.

Locus specific probes mapping to *BRCA1/2*, *ATM*, *MYC* and *TP53* have been generated. In addition, to establish if 20nM E2 treatment in *BRCA1/2*-mut organoids promotes the acquisition of point mutations and Copy Number Alterations (CNAs) in well-established oncogenes or tumor suppressor genes frequently mutated in breast cancer we generated DNA sequencing libraries from *BRCA1/2*-wt and *BRCA1/2*-mut organoids cultured in PBS or in the presence of 20nM E2 for 5 weeks using a targeted sequencing panel (NUPROBE). The target panel enables the detection of hotspot mutations and 18 genes and detect CNAs in 14 genes including *BRCA1/2.* The assay is sensitive (variant allele frequency-VAF 0.2% and CNAs detected and quantitated at ≥2.04 and ≤1.97 copies. Libraries have been submitted for sequencing.

3B **<u>Opportunities for training and professional development</u>**

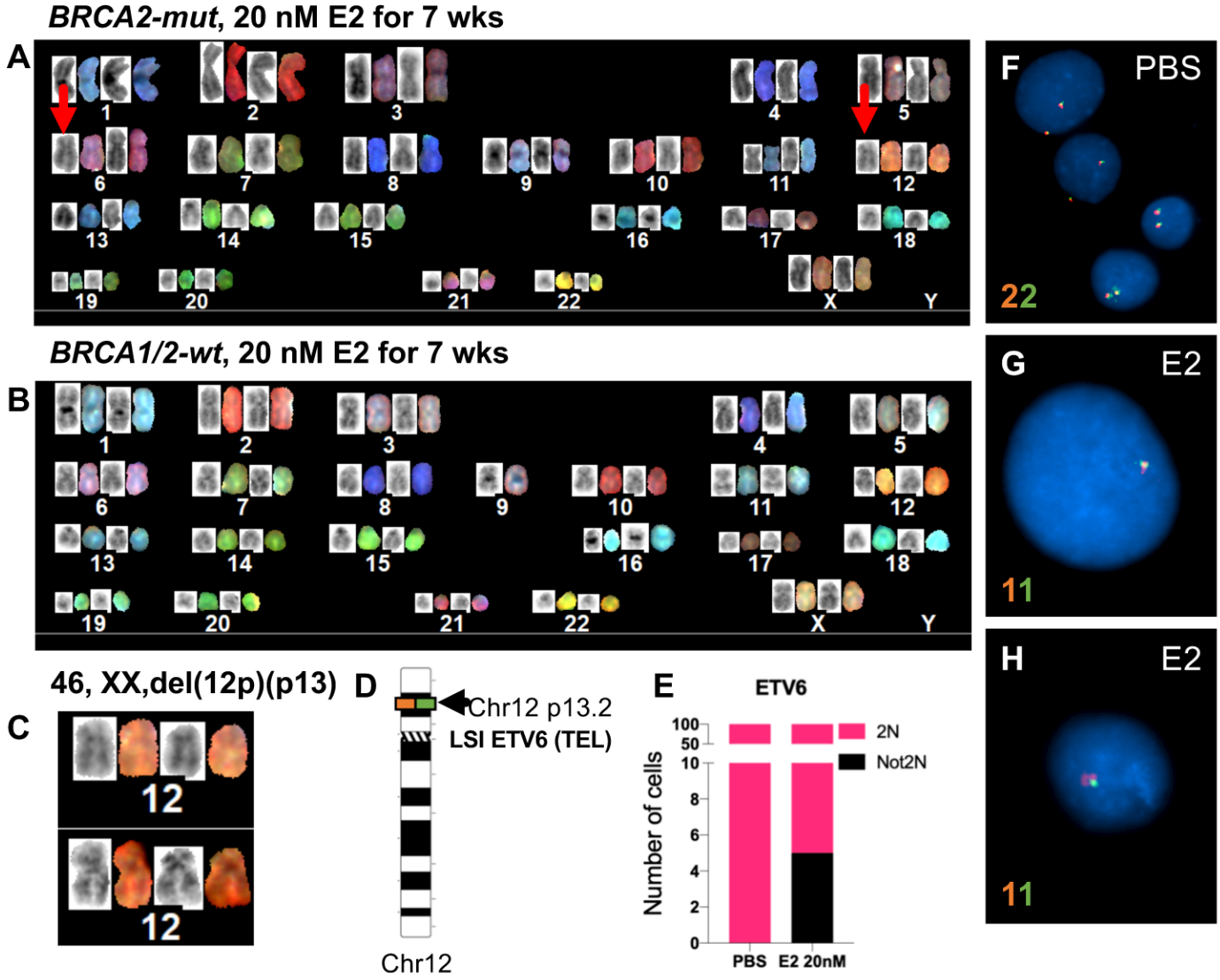
The analysis of the samples collected to complete specific aim 1 provided a new dataset to apply and develop analytical tools for students and postdoctoral trainees in the Dr. Vijg laboratory. Collection of primary mammary tissues provided postdoctoral trainees in Dr. Montagna's laboratory opportunities to master new protocols for 3D grow of primary mammary epithelial cells.

3C **<u>Dissemination of results of interest</u>**

Publication of *Single-cell analysis on somatic mutation burden in mammary cells of pathogenic BRCA1/2 mutation carriers*" was published in the Journal of Clinical Investigation (PMID: 35025760).

3D **<u>Goals for next reporting period</u>**

Due to Dr. Montagna relocation to the Rutgers Cancer Institute of New Jersey work on this project was suspended between May1st 2021 and April 30th 2022. This was due to the relocation of Dr. Montagna's laboratory and to allow for the award to be transferred to Rutgers. Work has fully resumed and in the next period of time we plan on completing the goals of the project as originally proposed under a no cost extension agreement.



4. IMPACT

- **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

5. CHANGES/PROBLEMS:

There were no major changes to the experimental approach.

Figure 4: Preliminary analysis of Copy Number Alterations (CNAs) in 3D mammary organoids exposed to E2. *(A-B) Representative Spectral Karyotyping analysis (SKY) of BRCA2-mutant organoids (A) and BRCA1- wt organoids (B) exposed to 20nM E2 for 7 weeks. The images depict the RGB colors of the spectral chromosome image with the inverted DAPI staining. (C) Enlarged representative images showing a deletion mapping to the p arm of chromosome 12 identified in E2 treated organoids. (D) ideogram of human chromosome 12 depicting the locus specific probes (orange or green) corresponding to ETV6, a known oncogene mapping to 12p13.2 and frequently deleted in leukemia. (E) Plot of preliminary quantification of the percentage of cells with diploid (2N) copies of ETV6 (magenta) or with ETV6 aneuploidy (black). (F-H) representative images depicting three nuclei isolated from a BRCA2-mut patient grown in control media (F) or in the presence of 20nM E2 (G-H).*

There was a delay due to Dr. Montagna relocating to the Rutgers Cancer Institute (CINJ) of New Jersey in April of 2022. Dr. Montagna laboratory suspended all experimental procedures in January 2022 to allow for equipment and reagents to be transferred to New Brunswick (NJ) where CINJ is located. The laboratory resumed the experimental procedures in the middle of June 2021.

6. PRODUCTS:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
  - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

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: 2.64
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: Shixiang Sun
Project Role: Post Doc
Researcher Identifier (e.g. ORCID ID): 0000-0001-5499-2963
Nearest person month worked: 3.48
Contribution to Project: Dr. Sun contributes to the projects his expertise in bioinformatics and data analysis. He was responsible for the analysis of single cell whole genome sequencing data to generate mutation frequency for each cell analyzed and to perform statistical comparisons of the experimental groups to establish the contribution of BRCA1/2 and estrogen to mutations.

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?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

Dr. Montagna relocated at the Rutgers Cancer Institute of New Jersey on April 1st, 2021. As result the activities proposed under this award were suspended to allow for her laboratory to transfer the equipment and the reagents. New IRB have been established at Rutgers to carry on the work as originally proposed and the work has now resumed. As consequence there was a delay on the project.

- *If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*
  Nothing to report.

- **What other organizations were involved as partners?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed. Provide the following information for each partnership:*
    - **Organization Name: Albert Einstein College of Medicine**
    - **Location of Organization:** *(if foreign location list country) 1300 Morris Park Avenue. 10461 Bronx, NY*
    - **Partner's contribution to the projec**t *(identify one or more)*
      1. **Financial support;**
      2. **In-kind support** *(e.g., partner makes software, computers, equipment, etc., available to project staff)*;
      3. **Facilities** *(e.g., project staff use the partner's facilities for project activities)*;
      4. **Collaboration** *(e.g., partner's staff work with project staff on the project)*; Dr. Jan Vijg is a partner on this application; the partner PI and his staff contributed to the project their expertise in single cell genomics including data analysis.
      5. **Personnel exchanges** *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*; and
      6. **Other.**

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** *N/A*
   - **QUAD CHARTS:** *N/A*
9. **APPENDICES:** *N/A*