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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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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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 woman who inherited germline mutations in *BRCA1* or *BRCA2* because of their intrinsic defect 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 event 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: to collect and isolate mammary epithelial cells from *BRCA1/2* mutation carriers and controls During this funding period we completed the collection of tissues from women undergoing prophylactic risk-reducing mastectomy in women diagnosed with pathogenic germline mutations in *BRCA1* or *BRCA2* for the work proposed in Specific Aim 1 (**Table 1**).

Table 1: Task 1 - Clinical characteristics of study subjects (Aim 1)

ID	Type	Age	Race	BMI	Co morbidities	Prior cancer history	Path report	parity
M05	*BRCA1+*	39	W	unk	BRCA1+	no	Benign breast	n=3 fT
M08		42	W	unk	BRCA1+	no	Benign breast	n=2 fT
M21		42	W	29.8	BRCA1+	no	No gross lesions	unk
M23		37	A	unk	BRCA1+		unk	unk
M24		45	B	39.8	HTN, obesity, BRCA1+	Uterine cancer	L/benign fibroadipose tissue	unk
M27		44	W	unk	BRCA1+	no	Benign breast	n=1 fT
M07	*BRCA2+*	38	W	unk	Personal history of cancer	yes	DCIS	0
M28		42	W	unk	Personal history of cancer	yes	unk	n=3 n=2 ft n=1 pre T

M01	Control	28	W	25.8	no	no	No gross lesions	unk
M10		46	W	25.6	no	no	No gross lesions	unk
M20		43	W	52.1	HTN, obesity, GERD	no	No gross lesions	unk
M22		35	B	32.8	Obesity	no	No gross lesions	unk
M25		33	W	28.1	no	no	No gross lesions	unk
M26		47	W	32.6	obesity	no	R/ focal papillary apocrine metaplasia; negative for atypical ductal hyperplasia or malignancy. L/mammary parenchyma with dilated benign ducts/cysts, papillary apocrine metaplasia; negative for atypical ductal hyperplasia or malignancy.	unk
M31		48	W	25.5	no	no	No gross lesions	unk
BRCA1+		41.5+/-3	W=4 B=1 A=1	34.8+/-7	no=0 yes=6	no=4 yes=1		
BRCA2+		41+/-2	W=2	unk	no=0 yes=2	no=0 yes=2		
Controls		40+/-7.9	W=5 B=1	31.79+/-9.5	no=4 yes=3	no=7 yes=0		
		n.s.	**n.s.**	**n.s.**		**n.s.**		

Major Task 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 relative to age matched controls

We enrich for luminal or basal mammary epithelial cells after staining single cells with antibodies against CD49f and CD326 (EpCAM) as well as lineage specific markers CD31, CD45, CD235a, CD140b followed by sorting to separate basal epithelial cells (Lin- CD49f+/high EpCAM-/low) and luminal epithelial cells (Lin- CD49f-/low EpCAM+/high) into single cells. Globally we successfully amplified and sequenced 64 single cells in total from 15 individuals of which 31 cells were obtained from 8 women diagnosed with a *BRCA1/2* germline mutation. Libraries from each single cell were sequenced along patient matched bulk DNA used as a reference genome.

Major Task 3: to run analytical pipelines and biostatistical analyses to evaluate genomic instability index across all experimental groups

The raw sequencing reads were trimmed to remove adapter and low-quality nucleotides, aligned to the human reference genome (GRCh37 with decoy) using BWA. To correct mapping errors made by genome aligners, the known indels and SNPs were collected from the 1000 Genomes Project (phase I) and dbSNP (build 144). Then indels realignment and base quality score recalibration were performed based on known indels and SNPs via Genome Analysis Toolkit (GATK, version 3.5.0). Somatic mutations between each single cell and the corresponding bulk were identified by SCcaller (version 2.0.0) developed by Dr. Vijg's laboratory. The frequency of somatic SNVs per cell was estimated after normalizing genomic coverage and calling sensitivity: frequency of somatic SNVs per cell =(# somatic SNVs)/((surveyed genome)/(total size of genome)*sensitivity). To identify mutation signature, we used non-negative matrix factorization (NMF) and confirmed the results with hierarchical Dirichlet process (hdp).

Specific Aim 1 accomplishments

Specific Aim 1 is nearly completed and a manuscript describing the findings has been submitted "*Single-cell analysis on somatic mutation burden in mammary cells of pathogenic BRCA1/2 mutation carriers*" (Journal of Clinical Investigation #148113-JCI-CC-1).

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

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

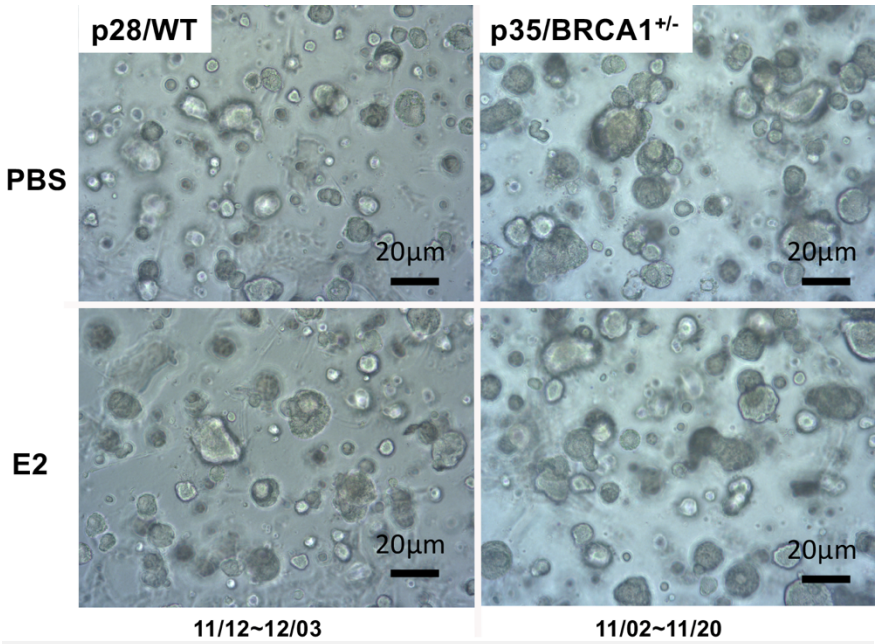


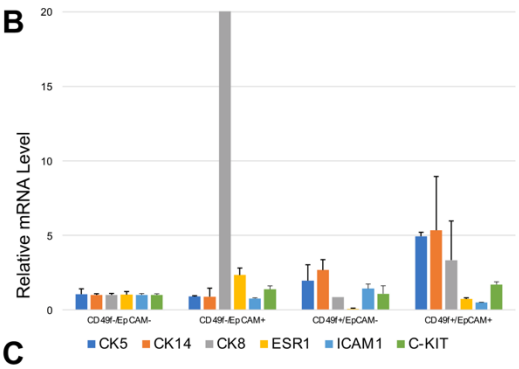
Figure 1: In culture E2 exposure of primary mammary organoids. Representative images of primary mammary organoids established from control women undergoing reduction mammoplasty for cosmetic reasons (left) or from prophylactic risk-reducing mastectomy because of a pathogenic germline mutation in *BRCA1* (right). Top panels depict organoids grown in control culture conditions; bottom panel depict organoids exposed to 20mM E2 for two weeks.

For establishing mammary organoids, we tested two 3D 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. We opted to implement mammary spheroids for aim 2 because these cultures are established from a defined mixture of enriched mammary luminal and basal cells (ratio of 1:1) which greatly limits confounding factors due to patient-to-patient variability in cell type composition. In addition, we are able to culture the mammary spheroids over several passages and maintain their viability in culture for over 1 month, which allows for long term hormonal exposure this be needed to study the effect of 17β estradiol (E2) on genomic instability and transformation of mammary epithelial cells of *BRCA1/2* mutation carriers.

Mammary spheroids are being exposed to 20mM E2 reflecting the local levels estimated in the mammary tissue; this concentration remains within the physiological range detected in the blood of women in the third trimester of pregnancy (**Figure 1**).

Major Task 2: Sequencing and QC of 3D organoids exposed to 17 β estradiol (E2) established from *BRCA1/2* carriers versus control

Our pipeline for establishing the 3D organoid model of E2 exposure is now fully established and we have collected 12 samples for specific aim 2 (**Table 2**); efforts are continuing to expand the cohort. We were able to establish viable organoids form all the samples and those are currently at different stages of E2 treatment, which we maintain for 2 weeks with change of media every 48hrs. We demonstrated the ability to enrich for basal or luminal subtypes at the end of the E2 regimen and isolate single cells for SCMDA which is performed as described for specific aim 1 (**Figure 2**).



C

CD49f/EpCAM pattern	Cell subtype	Marker genes
CD49f⁻/EpCAM⁻	Stromal	N/A
CD49f⁻/EpCAM⁺	LM	Ck8, ESR1
CD49f⁺/EpCAM⁻	Basal	CK5, CK14
CD49f⁺/EpCAM⁺	LP	ICAM1, c-kit

Figure 2: QC of mammary epithelial cells obtained from mammospheres post E2 exposure. A) Single cells isolated from mammospheres plated on a cell raft retain viability. **B-C)** Expression levels from bulk RNA of markers specific to basal, luminal or stromal cells indicates that during the E2 regimen the mammospheres retain a composition of cells with a diverse lineage similar to what observed at plating.

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

Table 2: Task 1 - Clinical characteristics of study subjects (Aim 2)

ID	Type	Age	Race	Co morbidities	Prior cancer history	Path report
O_M01	*BRCA1*	35	W	BRCA1+	no	Benign mammary parenchyma
O_M02		38	B	BRCA1+	no	
O_M03		37	B	BRCA1+	no	
O_M04	*BRCA2*	26	W	BRCA2+	no	
O_M05		39	W	BRCA2+	no	
O_M06	Control	23	W	Obesity	no	
O_M07		42	W	no	no	
O_M08		37	B	no	no	
O_M09		28	W	no	no	
O_M10		50	B	no	no	
O_M11		22	W	no	no	
O_M12		28	B	no	no	
BRCA1		36.6+/-1.5	W=1 B=2	no=0 yes=3		
BRCA2		32.5+/-5	W=2 B=0	no=0 yes=3		
Controls		32.8+/-10.4	W=4 B=3	no=6 yes=1		
		n.s.	**n.s.**			

We performed preliminary analysis of MCF10A and htert-immortalized mammary epithelial cells (htert-IMEC) parental lines and isogenic clones containing the founder *BRCA1* pathogenic mutation 185delAG mapping to exon 2. We established that introduction of 185delAG increase the mutation frequency as measured by SCMDA. In order to study 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 three weeks and prepared for Fluorescent *in situ* Hybridization (FISH) analysis using custom probes mapping to *BRCA1*, *c-MYC*, *TERC* and *TP53*.

3B Opportunities for training and professional development

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 Dissemination of results of interest

Nothing to report.

3D Goals for next reporting period

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

The goal is to publish and disseminate to the scientific community the findings obtained from specific aim 1.

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

The primary mammary spheroids cultures are at different stages of the E2 regimen. For some cultures (n=4) we have already collected single cells from both mock treated and E2 exposed culture and library construction by SCMDA is underway. We anticipate submitting the libraries for sequencing in the next 2-4 months. We continue the collection of tissues from new patients with the goal to expand the cohort. Data analysis will follow as described for aim 1.

Evolution of clones with copy number alterations (CNAs) in candidate oncogenes or tumor suppressor genes (*BRCA1*, *c-MYC*, *TERC* and *TP53)* will be evaluated by FISH.

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.
In the previous reporting period, we encountered substantially delays as consequence of the pandemic caused by SARS-CoV-2. We were unable to collect primary tissues as originally proposed and therefore we were restricted in performing sequencing as planned. As elective surgeries have resumed across the United States, we have been able to complete the collection of samples proposed in Specific Aim 1; all libraries were prepared and submitted for sequencing. Likewise, tissue collection resumed for preparing organoids as proposed in Aim 2. Culture of organoids is now underway, and we do not anticipate major delays.

6. PRODUCTS:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name: Cristina Montagna
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-2343-5851
Nearest person month worked: 2.4
Contribution to Project: Dr. Montagna supervised the project and directed the daily operations; she was responsible for preparing the Institutional Review Board (IRB) application and co ordinating the primary tissue collection.

Name: Tao Wang
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID): 0000-0003-0581-1251
Nearest person month worked: 0.6
Contribution to Project: Dr. Wang provided statistical support with the study design and preliminary data analysis including QC and evaluation of sample size based on the preliminary data obtained.

Name: Susan Klugman
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID): 0000-00001-8775-008X
Nearest person month worked: 0.24
Contribution to Project: Dr. Klugman was instrumental for the selection of patients to be included in our study; her expertise in genetic counseling and functional consequences of *BRCA1* or *BRCA2* mutation on genome instability provides the project insight into effect of germline mutations on tumor risk.

Name: Yi Zhang
Project Role: Post Doc
Researcher Identifier (e.g. ORCID ID): K-4058-2014
Nearest person month worked: 5
Contribution to Project: Dr. Zhang processed the samples for organoids and established the 3D in culture model to study the effect of estrogen on mammary epithelial cells as described in specific aim 2.

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

- **What other organizations were involved as partners?**
  - *Nothing to report*

8. SPECIAL REPORTING REQUIREMENTS

9. APPENDICES

OTHER SUPPORT

MONTAGNA, CRISTINA

ACTIVE

2P01AG017242-25S1 (Vijg) 8/1/20 – 4/30/21 0.6 calendar months
NIH 5%
Chromosome instability of glial cells in aging and Alzheimer's disease brain
The goals of this project are to coordinate interaction between five well-established and well-funded research groups with complementary backgrounds, to focus upon the role of genome stability mechanisms in longevity and aging as possible sources of intervention.
Role: Co-I

2P01AG017242-25 (J. Vijg) 5/1/19-4/30/24 0.36 calendar months
NIH 3%
DNA Repair, Mutations & Aging
The goals of this project are to coordinate interaction between five well-established and well-funded research groups with complementary backgrounds, to focus upon the role of genome stability mechanisms in longevity and aging as possible sources of intervention.
Role: Co-I

Breakthrough Level 2 (Montagna, Vijg) 05/01/19-04/30/22 2.4 calendar months
DOD - W81XWH-19-1-0104 20%
Somatic mutation rate as determinant of breast cancer penetrance in BRCA1/2 familial cases.
This application seeks to establish the molecular mechanisms by which estrogen modifies penetrance and increase breast cancer risk in BRCA1/2 mutation carriers.
Role: PI (Partnering PI grant)

U01CA238726 (Montagna) 07/01/19-06/30/21 0.96 calendar months
NIH 8%
Age associated genomic instability and brain tumor risk.
This project aims to test the hypothesis that age associated genomic instability of non-neuronal cells of the cortex contribute to increase tumor risk at old age.
Role: PI

R21CA240580 (C. Montagna/ J. Lenz) 06/01/19-05/31/22 1.32 calendar months
NIH 11%
Development of a high-resolution mapping platform for HPV DNA integration in premalignant lesions.
The major goals of this project are to develop a high throughput assay to identify and map HPV integrations in the human genome at the single nucleotide resolution.
Role: mPI

R21CA240580-02S1 (C. Montagna/ J. Lenz)9/1/20 – 8/31/21 No salary support
NIH
Development of a high-resolution mapping platform for HPV DNA integration in premalignant lesions.
The major goals of this project are to develop a high throughput assay to identify and map HPV integrations in the human genome at the single nucleotide resolution.
Role: mPI

Ovarian Cancer Research Program (OCRP) (Montagna)09/1/20-1/01/22 1.2 calendar months
DOD - Pilot Award- W81XWH2010247 10%
Somatic mutation rate, genomic instability and clonal evolution in BRCA1/2 carriers undergoing prophylactic risk-reducing surgery to establish serous tubular intra-epithelial lesions (STIL) and carcin.
The goal of this project is to define how increased genomic instability in BRCA1/2 germline mutation carriers contributes to transformation of ovarian cells.
Role: PI

RF1AG068908 (C. Montagna/J. Campisi) 9/30/20 – 8/31/24 1.1 calendar months
NIH 9.17%
Genomic Instability-induced Senescence in Brain Aging and Alzheimer's Disease.
The goal of this project is to study genomic instability in non-neuronal cells of the cerebral cortex to understand how age-related accumulation of genomic instability contributes to Alzheimer's Disease.
Role: mPI

PENDING

None

OVERLAP

None