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14. ABSTRACT: : **Project 1** We will determine the early molecular changes in STIC and their biological significance in developing high-grade serous carcinoma. marker selection and sample preparation will begin in the next coming months. **Project 2** We will evaluate whether the presence of a STIC is associated with different clinical manifestations and/or outcome compare to those patients in whom a STIC was not identified. Molecular profiling will be initiated after quality contorol checking. **Project 3** We will identify the early molecular changes that precede the development of STICs using gene expression analysis of morphologically normal FTE from high-risk women compared to FTE from normal control specimens and use an *in vitro* system and a mouse model to generate a molecularly defined carcinoma resembling HGSC from FTE and OSE using oncogenes expressed in ovarian carcinoma. **Project 4** We plan to if the statin drugs are effective in preventing STIC formation and suppress tumor progression in the OVGP1 mouse model that spontaneously develops STIC and neoplasms.. **Project 5** With the data and cases piling up, we will be able to address the molecular and epidemiologic profile of putative precursor lesions including STIC in the fallopian tubes and ovaries from women at high-risk for ovarian cancer.Also, a pilot study will be performed to determine the most cost-effective way to prepare the tissue sections for studies related to study early tumor development in ovarian cancer. This information will be shared with science community.

15. SUBJECT TERMS
prevention, p53 mutations, high grade serous ovarian cancer and STIC.

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Section I- Purpose and Scope of the Research Effort (all projects)

The purpose of this Ovarian Cancer Consortium is to test the overarching hypothesis that serous tubal intraepithelial carcinoma (STIC) is the precursor and not a metastasis of many, if not most, pelvic high-grade serous carcinomas (HGSCs) but we believe all the other proposed candidates should be investigated in order to determine if STIC is the precursor of all ovarian and pelvic HGSCs or that OSE and CICs harbor precursor lesions as well. Our objective is to then carefully characterize the morphologic, molecular genetic, immunohistochemical (IHC) and epidemiologic features of the precursor lesions(s) (Projects 1-5). If STIC is shown to be the precursor lesion, the data generated by our studies will provide the rationale for our long-term objective, which is the prevention of ovarian HGSC by surgical or medical approaches. Opportunities in the field of cancer prevention have never been greater and therefore our Consortium will undertake innovative studies aimed at providing the scientific underpinning for reducing the burden of ovarian cancer through prevention. Finally, it is important to note that clear cell, endometrioid and mucinous carcinomas are clinically important but they represent only 25 % of all ovarian carcinomas and account for 10% of deaths. In contrast, as noted above, HGSC represents 75% of all ovarian cancers and accounts for 90% of the deaths. Accordingly, we will focus our studies exclusively on the early events associated with HGSC, as it clearly is the most important histologic subtype in terms of frequency and mortality.

The main research efforts in this Consortium are summarized in our five projects.

Project 1: Evaluate whether STICs are precursor lesions and not metastases from a primary ovarian HGSC by analyzing STICs from women with concomitant ovarian HGSCs and determining if the ovarian tumors have acquired additional molecular alterations compared to the STICs which would confirm that STICs are precursor lesions.

Project 2: Evaluate all the proposed site of origin (FTE, OSE, CICs and peritoneum) showing that the morphologic and molecular features of tubal, ovarian and primary peritoneal HGSCs are the same and in conjunction with Project 1 confirming our hypothesis that many, if not most, HGSCs originate in the fimbria and involve the ovary secondarily.

Project 3: Identify the early molecular changes that precede the development of STICs using gene expression analysis of morphologically normal FTE from high-risk women compared to FTE from normal control specimens and use an in vitro system and a mouse model to generate a molecularly defined carcinoma resembling HGSC from FTE and OSE using oncogenes expressed in ovarian carcinoma.

Project 4: Locate and characterize precursor lesions of “ovarian” cancer in a mouse model and explore the role of ovulation and changes in the microenvironment of the ovary and tube in “ovarian” carcinogenesis using human tubal xenografts in nude mice.

Project 5: Determine the molecular and epidemiologic profile of putative precursor lesions in the fallopian tubes and ovaries from women at high-risk for ovarian cancer. In addition, Project 5 will determine if these biomarkers and associated precursor lesions are modifiable by oral contraceptives (OCPs) or anti-inflammatory agents as OCPs in particular are known to prevent ovarian cancer and impact survival.

Section II, III and IV (5 projects are reported here individually)

Before the recent progress is summarized, we would like to update the list of publications related to this consortium.

Publications supported by DoD Ovarian Cancer Consortium (OCPR: W81XWH-11-2-0230)

Title: Prevention of Ovarian High-Grade Serous Carcinoma by Elucidating Its Early Change

2011-Current

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5. Kuhn E, Kurman RJ, Sehdev AS, Shih IM: Ki-67 labeling index as an adjunct in the diagnosis of serous tubal intraepithelial carcinoma. Int J Gyn Pathol 2012, 31:416-22. PMCID: PMC3715095

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13. Kuhn E, Ayhan A, Shih Ie M, Seidman JD, Kurman RJ: Ovarian Brenner tumour: a morphologic and immunohistochemical analysis suggesting an origin from fallopian tube epithelium. Eur J Cancer 2013, 49:3839-49. PMID: 24012099

14. Vang R, Shih Ie M, Kurman RJ: Fallopian tube precursors of ovarian low- and high-grade serous neoplasms. Histopathology 2013, 62:44-58. PMID: 23240669

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16. Ardighieri L, Lonardi S, Moratto D, Facchetti F, Shih IM, Vermi W, Kurman RJ: Characterization of the immune cell repertoire in the normal fallopian tube. Int J Gyn Pathol 2014, 33:581-91. PMCID: PMC4617751.

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36. Visvanathan K, Wang TL, Shih IM. Pre-cancerous lesions of ovarian cancer- a US perspective. J Natl Can Inst, 110:djx269, 2018. PMID: 29281080

Project 1: Evaluate whether STICs are precursor lesions and not metastases from a primary ovarian HGSC by analyzing STICs from women with concomitant ovarian HGSCs and determining if the ovarian tumors have acquired additional molecular alterations compared to the STICs which would confirm that STICs are precursor lesions.
Research site: Johns Hopkins University
Project Leader: Ie-Ming Shih
Co-investigators: Doug Levine (NYU), Robert J. Kurman (JHU)

Section II. Progress to Date

Task 1. Determine the clonal relationship and tumor progression pathway from STIC to invasive high-grade serous carcinoma (HGSC).

Task 1a. Case selection and sample preparation including LCM, DNA extraction (1-20 months)
Progress: We have collected a sufficient number of cases for this task.

Task 1b. TP53 mutational analysis of the potential precursor lesions of HGSC (8-24 months).
Progress: The task has been completed and the data have been published PMID:21990067. In 2017, we also published a paper reporting the most recent results obtained from this task in Nat Comm PMID: 29042553.

Task 1c. Allelic imbalance assay by digital SNP analysis and data analysis (24-36 months)
Progress: This task has been modified and the results have been published (Mod Pathol, 29:1254-1261, 2016).

Task 2. Determine the early molecular changes associated with serous tubal intraepithelial lesions.

Task 2a. Immunohistochemistry study on ovarian cancer-associated markers on STICs and other putative precursor lesions (18-40 months)
Progress: This task has been completed. The progress is that we have identified at least 6 highly specific ovarian cancer-associated markers on STICs including LAMC1, CCNE1, topoisomerase II, RSF-1, TET1, and loss of ALDH1A1.

Task 2b. In situ hybridization and/or mRNA expression analysis on those markers that the antibodies are not available (30-40 months)
Progress: As discussed in the last report, we have been fortunate to identify good antibodies (laminin C1, ALDH1A1, p53, topoisomerase II, cyclin E1, etc.) for immunostaining purposes and there is no need for us to consider *in situ* hybridization at this moment.

Task 2c. Verification of new markers from Project 3 in precursor lesions (24-56 months)
Progress: This task has been completed and the results have been published in the following journals including J Pathol (PMID: 23378270), Am J Surg Pathol (PMID: 22892598) and Mod Pathology (PMID: 25216223).

Task 2d. Telomere FISH on STICs and other precursor lesions (36-48 months)
Progress: This part of study has been completed as previously discussed.

Task 2e. Data analysis and preparation for publications (40-60 months)
Progress: We will perform data analysis and prepare the results for another new publication using the data generated from Project 1 and the results will be discussed in the final progress report.

Section III. Problem Areas for Project 1

There are no potential areas needed to be discussed at this moment as we continue making progress as expected.

Section IV. Future Works in Project 1

This is in the no cost extension period and therefore, we are focusing on publishing all data generated in Project 1. This is the main task in the coming months before the project officially ends.

Project 2: The relationship between serous tubal intraepithelial carcinoma and invasive pelvic serous carcinoma

1. INTRODUCTION:

Ovarian cancer has traditionally been thought to develop from the OSE or cortical inclusion cysts, but recent data suggest that a majority of advanced HGSC may originate from the fallopian tube epithelium. Although highly provocative, this hypothesis requires further validation and therefore we propose to analyze a large group of pelvic, which includes ovarian, tubal and primary peritoneal, HGSCs diagnosed using traditional criteria with and without STICs in women whose fallopian tubes have been processed using the SEE-FIM technique, currently the most comprehensive method of evaluating fallopian tube epithelium. Our primary objective is to determine whether there are subsets of HGSC, which have different molecular profiles and different clinical behavior based on their presumed site of origin or whether there are no differences and that they are essentially the same irrespective of their site of origin. We will also compare the molecular profiles of normal tissues to HGSCs as a whole and HGSCs with and without STICs placing specific emphasis on the ovarian surface and distal fallopian tube epithelium.

2. KEYWORDS: Serous tubal intra-epithelial carcinoma, ovarain cancer, ovarian carcinoma, high-grade serous carcinoma, serous, STIC, in-situ cancer, SEE-FIM, ovarian surface epithelium, fallopian tube, genomic profiling, molecular, genomics.

3. ACCOMPLISHMENTS:

MAJOR GOALS:
Task 1: Determine the frequency of STICs in patients with advanced pelvic HGSC.

Task 2: Evaluate whether the presence of a STIC is associated with different clinical manifestations and /or outcome compare to those patients in whom a STIC was not identified.

Task 3: Compare the molecular features of advanced pelvic HGSCs with and without associated STIC to various normal pelvic tissues.

ACCOMPLISHEMENTS UNDER THESE GOALS (FOR THIS REPORTING PERIOD):
Task 1: Complete – nothing to report for this period.

Task 2: Complete – nothing to report for this period.

Task 3: Compare the molecular features of advanced pelvic HGSCs with and without associated STIC to various normal pelvic tissues.

Task 3a. Collect 100 HGSC specimens that had SEE-FIM processing from Consortium sites; 10-20 specimens per site with the balance contributed from MSKCC (Completed previously)

Task 3b. Collect 10 normal tissues from each of 5 anatomic sites; 50 total tissues. Tissues to be collected across Consortium sites, with each site contributing at least 2 of each normal tissue type with the balance contributed from MSKCC (Completed previously)

Task 3c. Process all specimens on various genomic platforms

All tumor specimens had been processed during previous reporting periods. We had also been able to finalize quality control (QC) on our normal tissues, which had been challenging due to small cellular quantities obtained from anatomical brushings. We developed a pooling strategy such that at least 3 pools (of 3-5 patient specimens per pool) from each normal anatomic site generated sufficient material to perform RNA sequencing. The RNA sequencing on these normal samples pools have now been completed. (All complete)

Task 3d. Analyze data on each platform according to proposal.

1. Major activity – basic analysis of tumor samples is complete for each platform.
2. Specific objectives: To better understand the molecular etiology of HGSCs, we report a multi-center integrated genomic analysis of advanced stage tumors with and without STIC lesions.
3. Significant results: All results have been published since the last reporting period. A copy of the publication is attached to this report.

Other achievements: None to report

TRAINING AND PROFESSIONAL DEVELOPMENT: Nothing to Report.
DISSEMINATION TO COMMUNITIES OF INTEREST: A manuscript draft has been published in Nature Communications as: Ducie J, Dao F, Considine M, Olvera N, Shaw PA, Kurman RJ, Shih IM, Soslow RA, Cope L, Levine DA. Molecular analysis of high-grade serous ovarian carcinoma with and without associated serous tubal intra-epithelial carcinoma. Nat Commun. 2017 Oct 17;8(1):990. doi: 10.1038/s41467-017-01217-9. PubMed PMID: 29042553; PubMed Central PMCID: PMC5645359.

PLANS DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH GOALS:
We have identified several microRNAs that appear to be differentially expressed between tumors with and without STIC lesions. There was no proposed work to validate these findings. In the no cost extension period, we propose to validate these findings with independent samples that are archival (FFPE) and not frozen, as was the case with the original set of specimens. We will use the nanostring platform to determine the gene expression of these select microRNAs in a cohort of validation specimens. In this manner, we can determine if these differentially expressed microRNAs may be associated with ovarian cancers that do or do not have identifiable STIC lesions. We have been obtaining IRB approval to identify cases for validation at NYU.

4. IMPACT:

IMPACT ON THE DEVELOPMENT OF THE PRINCIPAL DISCIPLINE: These results are expected to make an impact on the understanding of ovarian cancer tumorigenesis. The outstanding question in our principal discipline is whether or not all high-grade serous carcinomas develop from the distal fallopian tube through serous tubal intra-epithelial carcinomas. These data and results will support the notion that nearly all high-grade serous carcinomas do develop from the distal fallopian tube through serous tubal intra-epithelial carcinomas since little genomic variation has been found between tumors with and without serous tubal intra-epithelial carcinoma. These data will ultimately help to shape prevention and early detection approached for ovarian cancer.

IMPACT ON OTHER DISCIPLINES: Nothing to report.

IMPACT ON TECHNOLOGY TRANSFER: The data will affect the development of technology used for early detection of ovarian cancer considering that devices will need to be developed for interrogation of the distal fallopian tube.

IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY: The findings will help to educate the society about the origins of ovarian cancer and encourage women throughout the world to request bilateral salpingectomy (removal of both fallopian tubes) instead of bilateral tubal ligation as a measure to prevent

unwanted pregnancy. The data is also likely to result in a greater role for bilateral salpingectomy at the time of hysterectomy with ovarian preservation.

5. CHANGES/PROBLEMS:

CHANGES IN APPROACH: None noted.

ACTUAL OR ANTICIPATED PROBLEMS OR DELAYS AND ACTIONS OR PLANS TO RESOLVE THEM: We had some delays in collecting and processing the normal tissue samples due to the limited cellular contents obtained from fresh intra-operative brushings. We were able to overcome this problem through a pooling approach that is statistically sound and eliminates batch effects among the normal samples.

CHANGES THAT HAD A SIGNIFICANT IMPACT ON EXPENDITURES: There was an accounting error at MSKCC that resulted in approximately $130,000 being charged to this account during the wrong budgeting period. This was a result from a failure to properly encumber anticipated and approved charges. During prior reporting periods we have had to review all expenses from prior years and cost transfer money from other funds to cover this shortfall. During the current reporting period there have been no issues.

OTHER CHANGES: There were no other changes to any human subjects, vertebrate animals or biohazard concerns.

6. PRODUCTS:

PUBLICATIONS, CONFERENCE PAPERS, AND PRESENTATIONS: Abstract presentation at the 2015 Annual Meeting of the Society of Gynecologic Oncology.

Publication: Ducie J, Dao F, Considine M, Olvera N, Shaw PA, Kurman RJ, Shih IM, Soslow RA, Cope L, Levine DA. Molecular analysis of high-grade serous ovarian carcinoma with and without associated serous tubal intra-epithelial carcinoma. Nat Commun. 2017 Oct 17;8(1):990. doi: 10.1038/s41467-017-01217-9. PubMed PMID: 29042553; PubMed Central PMCID: PMC5645359.

OTHER PRODUCTS: All the genomic data including microRNA, RNAseq and copy number data have been deposited in the GEO database under the accession code GSE102094.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS :

INDIVIDUALS WHO HAVE WORKED ON THE PROJECT: Douglas A. Levine, Maria Bisogna, Narciso Olvera, Fanny Dao – No change.

CHANGE IN THE ACTIVE OTHER SUPPORT OF THE PD/PI(S) OR SENIOR/KEY PERSONNEL: Nothing for this reporting period.

OTHER ORGANIZATIONS INVOLVED AS PARTNERS: Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS: Nothing to report.

9. APPENDICES: None.

PROJECT 3. Identification of molecular changes preceding STICs in FTE from high-risk women using in vitro and in vivo models

Investigators: PI - Shaw, Shih

Research site: University of Toronto

1. **INTRODUCTION:**

In this project we proposed to determine the expression profiles of anatomically high risk FTE (fimbrial) from women at high genetic risk (BRCA1 mutation carriers) compared to the FTE profiles from women at low risk of High Grade Serous Carcinoma (HGSC), and we propose that these changes may play key roles in the earliest events of serous carcinogenesis. To this end, we will use a molecularly defined system to sequentially express ovarian cancer-associated genes including those identified in this project into ovarian surface epithelium (OSE) as well as fimbrial FTE to determine a) if FTE is more prone to neoplastic transformation and b) if the FTE-derived tumors more closely simulate HGSC than OSE-derived tumors.

Recently described precursors of HGSC, the p53 signature, a latent precursor, and Serous Tubal Intraepithelial Carcinoma, a pre-malignant precursor, occur most frequently at the distal and fimbriated end of the fallopian tube (FTE). We recently demonstrated that the FTE of BRCA1 mutation carriers, at genetic risk of HGSC, have altered signaling pathways compared to controls. A key question is whether the gene expression differences identified at the ampulla between BRCA1 and non- mutation carriers is similar to differences at the fimbria. This study determines the transcriptome profiles of normal fimbrial FTE and normal ampulla FTE which may lead to insight of why the distal end of the fallopian tube is preferentially predisposed to malignant transformation

Specific Aim 1. *Detect and select genes differentially expressed in morphologically normal fimbrial FTE from women at high genetic risk of HGSC.*

Specific Aim 2. *Model alterations associated with normal FTE from high-risk women and STIC in vitro and in vivo.*

2. **KEYWORDS:** *STIC, FTE, HGSC (High Grade Serous Carcinoma), BRCA, GSTA2, CEBPD, xenograft, modelling*

3. **ACCOMPLISHMENTS:**

The purpose of this Project is to identify the early molecular changes that precede the development of STICs using gene expression analysis of morphologically normal FTE from high-risk women compared to FTE from normal control specimens and use an *in vitro* system and a mouse model to generate a molecularly defined carcinoma resembling HGSC from FTE and OSE using oncogenes expressed in ovarian carcinoma. Project 3 consists of several tasks listed below:

Task 1. Establish expression profiles of fallopian tube epithelium from *BRCA1* mutation carriers and controls, and of serous cancers in mutation carriers.

Progress: To date, we have collected and processed over 200 formalin-fixed and/or cryopreserved cases of fallopian tube and fimbriae specimens. These cases include samples from BRCA1 and BRCA2 mutation carriers undergoing prophylactic surgery, patients undergoing debulking surgery for High Grade Serous Carcinoma, and patients undergoing salpingo-oophorectomy for non-malignant reasons. An integral aspect of Project 3 *Specific Aim 1* is to determine the relationship between hormonal response and BRCA mutation status in the normal fallopian tube epithelium. As a result, a significant effort has been placed on determining the menstrual status of samples collected – this included reviewing the endometrium of corresponding samples when available. We completed histological reviews of fallopian tubes from BRCA1 and BRCA2 mutation carriers, along with matching controls and cancers and identified 84 cases that were eligible for gene expression profiling. One of the key questions within this aim is whether the gene expression differences identified at the ampulla between BRCA1 and non-BRCA1 mutation carriers is similar to differences at the distal end of the fallopian tube – the fimbria.

To best answer the ampulla versus fimbria conundrum, and perform a technically robust experiment, we used cryopreserved ampulla and fimbria from non-BRCA mutation carriers with known ovulation cycle status.

A). Micro-dissection of selected cryopreserved tissue samples

Manuscript Submitted to Nature Communications for review

Title: Integrative Transcriptome analyses of the ampulla and fimbria of the human fallopian tube

Background*:* Recently described precursors of high-grade serous carcinoma (HGSC), the p53 signature, a latent precursor, and Serous Tubal Intraepithelial Carcinoma (STIC), a pre-malignant precursor, occur most frequently at the distal and fimbriated end of the fallopian tube (FTE). In 3 previous reports, we have demonstrated that the FTE of BRCA1 mutation carriers, at genetic risk of HGSC, have altered signaling pathways compared to controls. Ovarian production of ROS is released after the LH surge to induce ovulation. Reactive oxygen species (ROS) have been implicated in serous carcinogenesis. The objective of this study is to compare the transcriptome profiles of normal fimbria (high-risk epithelia prone to transformation) FTE and normal ampulla (low-risk epithelia) FTE which may lead to understanding the distal end of the fallopian tube as the preferential anatomic location of the fallopian to tube for cellular transformation.

Methods*:* Snap-frozen matched fimbria and ampulla tissues were controlled for age and ovarian cycle status. Cases included 12 luteal phase and 12 follicular phase women at no known risk for ovarian cancer. Laser capture microscopy (LCM) was used to micro-dissect FTE cells, using 7-10 sections per case. Total RNA was isolated, RNA extracted and cDNA amplified. The expression profiles were generated using Affymetrix Human Genome HTA-2.0 Array. 5um sections of the FFPE specimen of the profiled cases were stained for Ki67, p53, CK7 and GSTA2

Results/Developments*:* Using gene level differential expression analysis with the Affymetrix Expression Console software, we performed unsupervised hierarchical clustering analysis with all 24 samples. We used a fold change of < -2 or > 2 and ANOVA p-value < 0.05 as a cut-off criteria for selecting genes. The cases clustered predominantly by ovarian cycle status rather than by their differences in anatomical origin or their matched pair. There were 427 genes differentially expressed amongst the 4 groups – Fim-Luteal, Fim-Follicular, Amp-Luteal and Amp-Follicular. Independent of ovarian cycle status, very few differences (35 genes – SALL1, SERPINA3, ANXA13, PDK4, ME1, GSTA1, GSTA2 – genes involved in metabolic pathways) were observed between the ampulla and fimbria FTE. The epithelia of the anatomically high-risk fallopian tube – the fimbria, show few differences in gene expression profiles compared to the lower risk portion – the ampulla. Expression differences predominantly are in response to the hormonal milieu, i.e. the secretory and proliferative phases of the ovarian cycle. The increased anatomic risk of the fimbria is likely due to effects of the microenvironment, such as repeated exposure to follicular fluid at ovulation, rather than intrinsic differences of the FTE in the two sites. We have validated the expression of glutathione S-transferase A2 (GSTA2) in both fimbria and ampulla using IHC. The expression array data had shown a difference of 2.1 fold increase in the fimbria. This is recapitulated in fimbria and ampulla (of the same case) with IHC (Figure 1A-C).

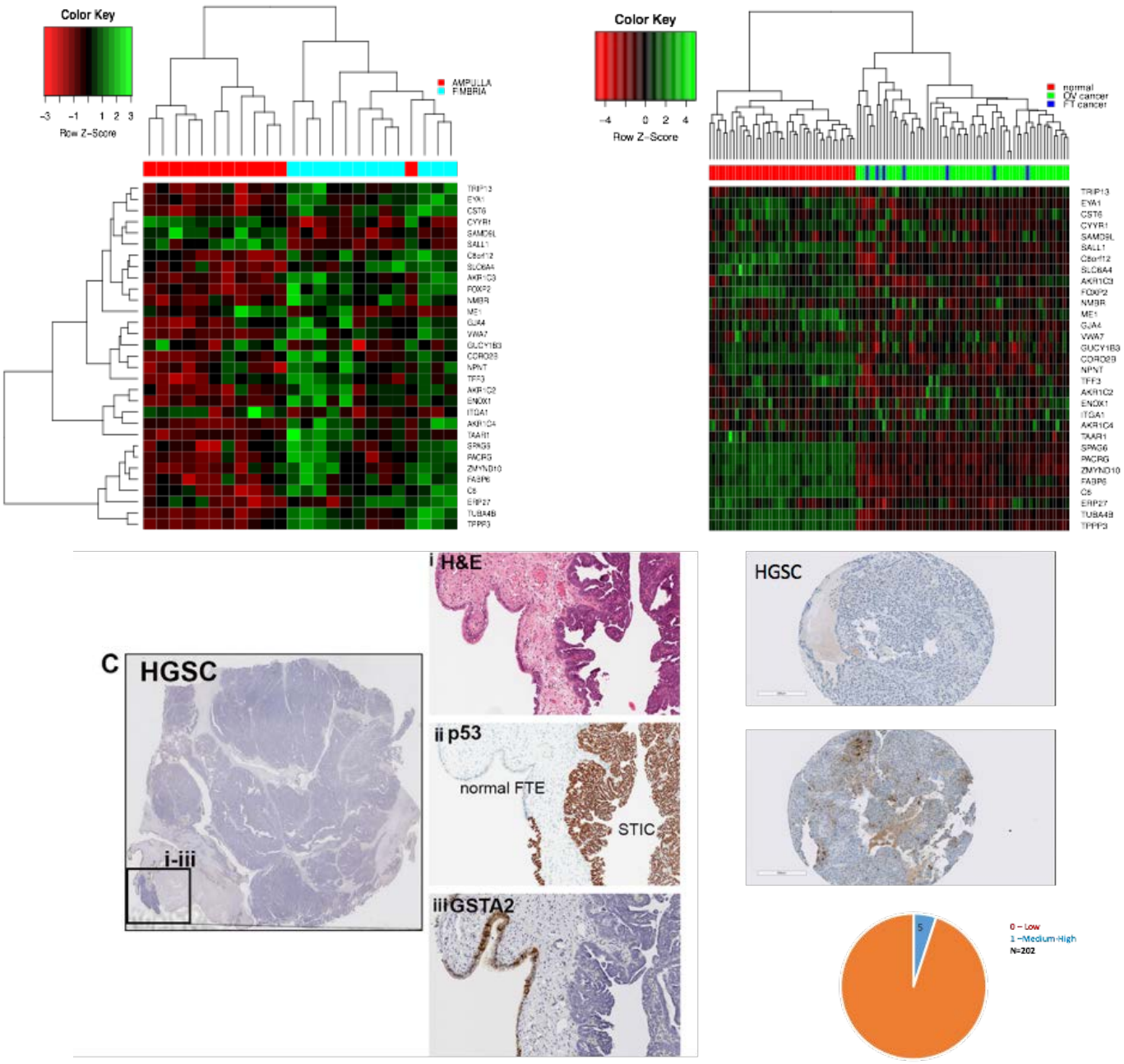


Figure 1: A-C. **A.** Differential expression of genes between the high-risk fimbria and ampulla. **B.** This gene signature can also segregate normal FTE from fallopian tube cancer and HGSC. **C.** There are more GSTA2 positive cells in the fimbria compared to the ampulla. Decrease in expression of GSTA2 in a fallopian tube epithelial cells which over-express p53. Expression of GSTA2 in high-grade serous cases is markedly reduced. 95% of HGSC cases in TMA show a decrease in this antioxidant enzyme.

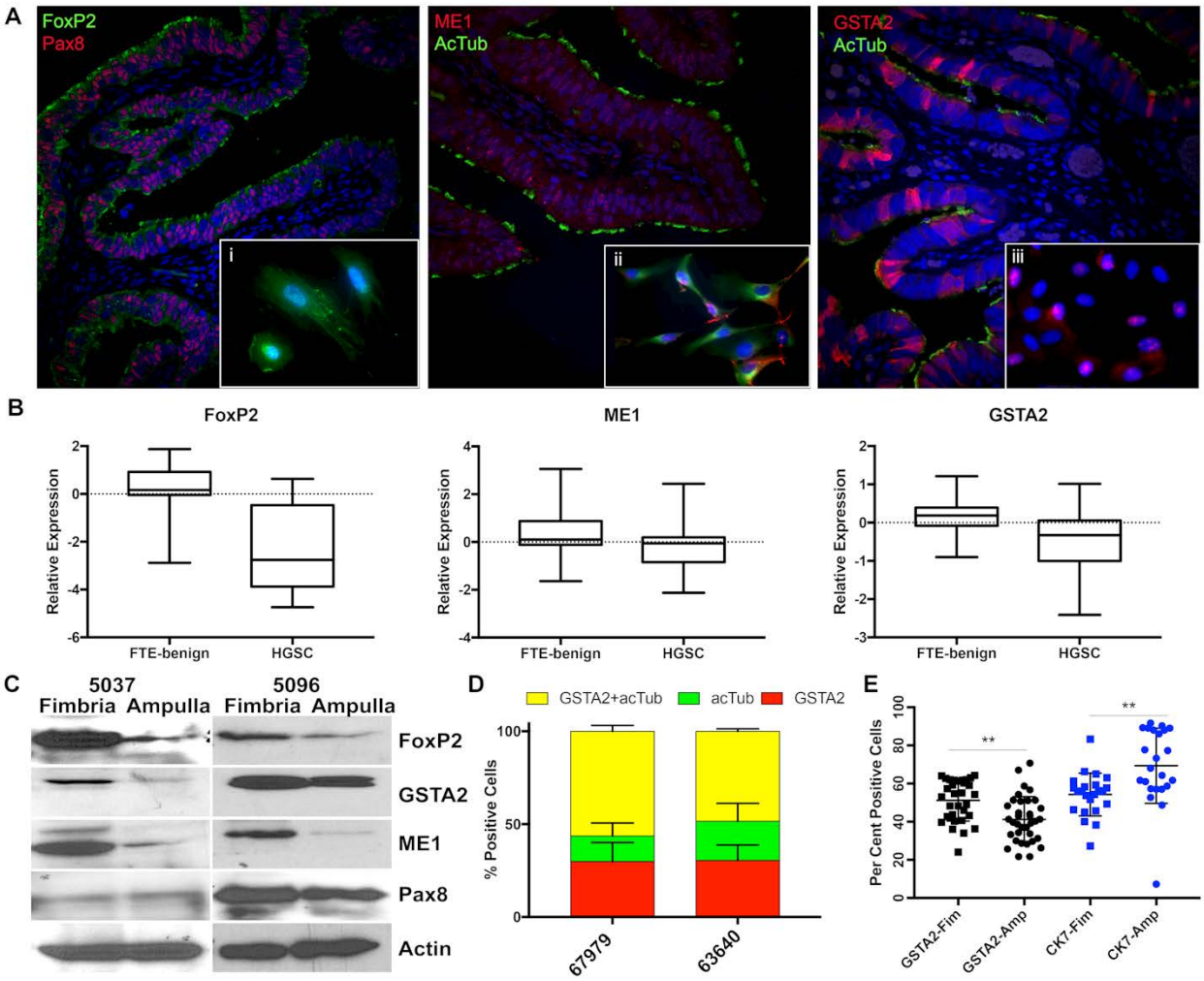


Figure 2. Differences in gene expression profile of normal FTE and cancer. **A.** Immunofluorescence (IF) of the fimbria. Pax8 marks secretory cells and acetylated-tubulin marks ciliated cells. Sections were counter stained with Foxp2, Malic enzyme 1(ME1) and GSTA2 respectively. I: Foxp2 and Pax8; II: ME1 and acetylated-tubulin and III; GSTA2 and acetylated-tubulin expression in cells from normal FTE. **B.** The normal FTE had a higher relative gene expression of Foxp2, Me1 and GSTA2 compared to HGSC. **C.** Western blots of 2 independent normal cases show that GSTA2, Foxp2 and Me1 is highly expressed in fimbria compared to the ampulla. **D.** IF was used to quantify the ratio of ciliated and secretory cells in 2 independent normal cases, 50-60% all GSTA2 positive cells were ciliated cells. **E.** Immunohistochemistry was used to quantify the GSTA2 and CK7 positive cells in the fimbria and ampulla. There were significantly more GSTA2 positive cells in the fimbria compared to the ampulla, the opposite relation was observed for CK7 positive cells.

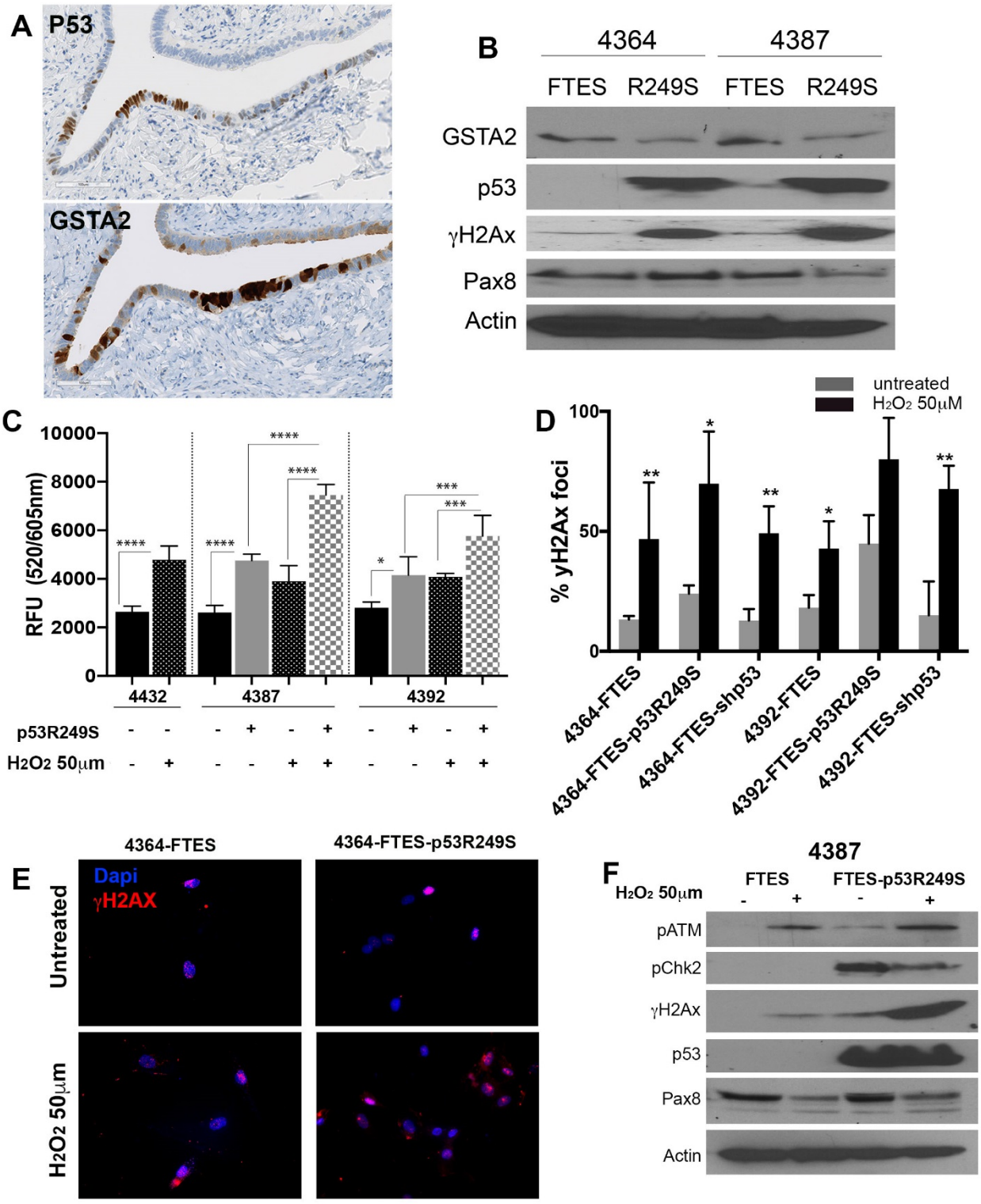


Figure 3. Effect of mutant p53 on gene expression on FTE and DNA damage response. **A.** There was an inverse relationship between GSTA2 expression and p53 accumulation in histological normal FTE identified in a non-BRCA follicular phase case (x40). **B.** Western blot comparison of pATM, pCHk2, γ-H2Ax, p53 and Pax8 in normal FTE and mutant p53-R249S in cases 4364 and 4387. There was a slight but not significant decrease in the expression of GSTA2 in normal FTE compared to mutant p53. Compared to normal FTE there was a significant increase in basal γ-H2Ax expression in mutant p53 cells. **C.** Both basal ROS and H_2O_2 induced ROS was significantly higher in FTES-p53R249S compared to FTES cells for FTE cases 4432, 4387 and 4392 **D.** γ-H2Ax foci quantification in case 4364 and 4392. In both cases, γ-H2Ax basal foci in pre- and post-treatment with H_2O_2 were higher in mutant compared to normal FTE. **E.** IF of γ-H2Ax (red) in case 4364-FTES and 4364-FTES-p53R249S untreated and treated with 50μM of H_2O_2. γ-H2Ax foci were pronounced in mutant p53 compared to normal FTE pre- and post H_2O_2 treatment. **F.** Western blots of DNA damage response genes in case 4387. pATM, a sensor for DNA double strand break was higher in FTES-p53R249S treated with H_2O_2

compared to H_2O_2 treated FTES cells or untreated cells. Similar observation was seen for γ-H2Ax. pChk2 was decreased in FTES-p53R249S after treatment with H_2O_2. Surprisingly, Pax8, a marker of secretory cells, was decreased upon treatment with H_2O_2 in both normal and mutant FTE.

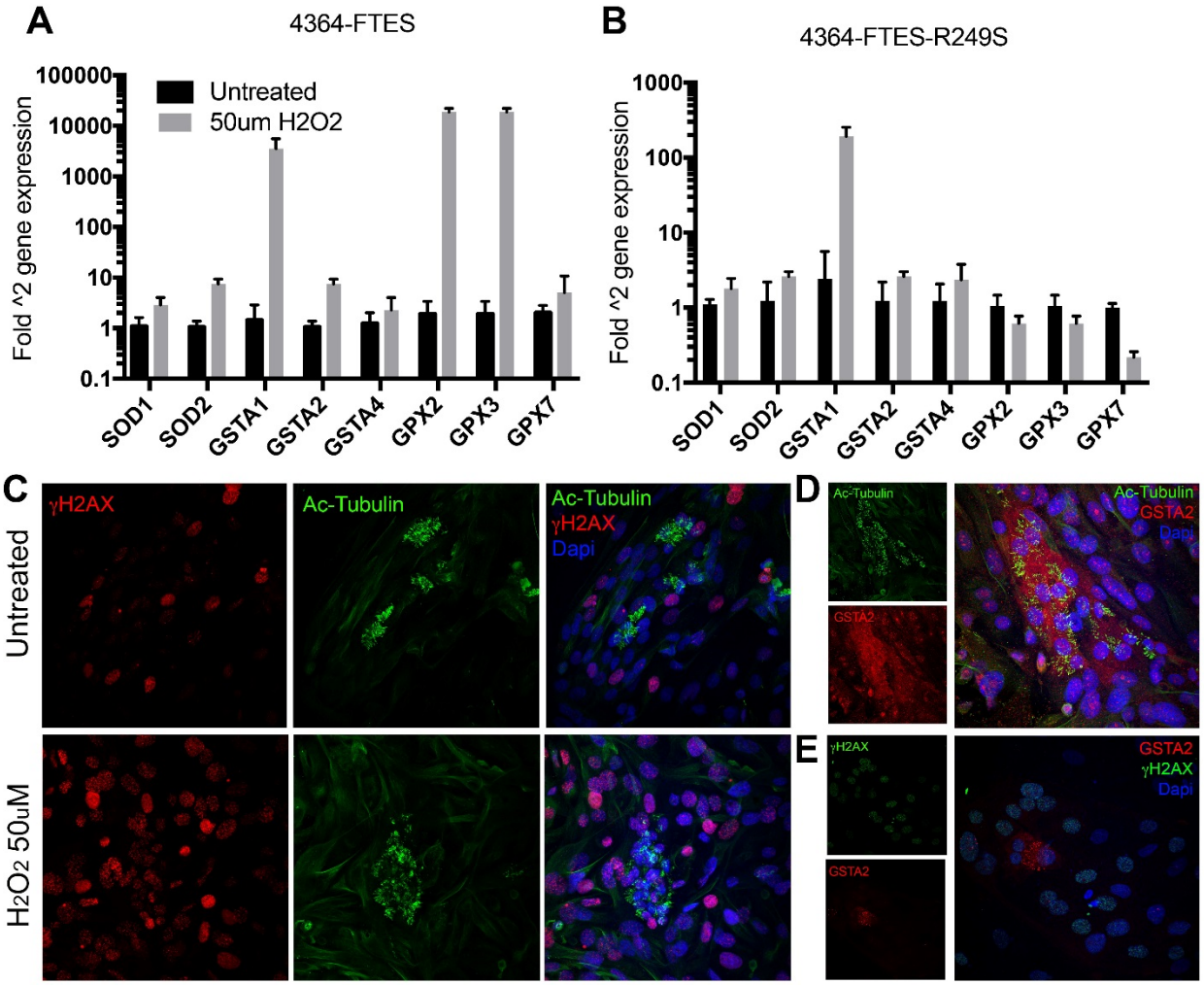


Figure 4. Effect of H_2O_2 on phase I and II antioxidant genes in FTE. **A-B** qPCR data showing fold changes in the mRNA levels SOD1, SOD2, GSTA1, GSTA2, GPX2 and GPX3 in FTES and FTES-p53R249S with and without H_2O_2 treatment. There were significant ($p<0.05$) mRNA increases of phase I and II antioxidant genes in FTES following H_2O_2 treatment, only GSTA1 showed a significant increase in FTES-p53R249S **C.** IF of γ-H2Ax and acetylated-tubulin of normal case 5217 FTE untreated and treated with 50µM of H_2O_2 on transwell filters. There were more γ-H2Ax foci after H_2O_2 treatment and non-ciliated cells had a greater number of γ-H2Ax foci compared to ciliated cells. **D.** IF of GSTA2 and acetylated-tubulin of normal case 5217 FTE treated with 50µM of H_2O_2 on transwell filters. There were higher levels of GSTA2 in ciliated cells versus un-ciliated cells. **E.** IF of GSTA2 and γ-H2Ax of normal case 5217 FTE treated with 50µM of H_2O_2 on transwell filters. There was an inverse relationship between GSTA2 and γ-H2Ax foci.

Manuscript in preparation with anticipated submission in February 2018

Title: Cebpd acts as a tumor suppressor in High Grade Serous Ovarian Cancer

Abstract

Background Previously, we showed CEBPD is up-regulated in the luteal phase of the menstrual cycle in BRCA mutation carriers. The proposed site of origin of HGSC (High Grade Serous Carcinoma) is the hormonally responsive fallopian tube epithelium (FTE). Estrogen receptor (ER) is expressed throughout the fallopian tube

and is expressed in 70-80% of HGSC. HGSC involves the transformation of normal FTE into a serous tubal intraepithelial carcinoma (STIC) lesion followed by metastatic progression to HGSC. During these steps, the FTE undergoes an epithelial to mesenchymal transition (EMT) allowing cells to metastasize and revert back to an epithelial phenotype through a modified mesenchymal to epithelial transition (MET). Our goal was to understand the role that CEBPD may play in mediating and EMT/MET under the influence of estrogen in ovarian carcinogenesis. **Methods** The study protocol for collection of tissue and clinical information for all patients was approved by the UHN REB. FTE, STICs and ovarian cancer TMAs were used to validate immunohistochemical protein expression. Fallopian tube epithelial cells were propagated as previously described. Cells were transfected with CEBPD overexpression vectors to understand the role of CEBPD in FTE. Statistical analysis was performed using ANOVA ($p<0.05$) and t-tests ($p<0.05$). **Results** CEBPD is downregulated in 60% of HGSC and corresponding STIC lesions. LGSC has higher expression than HGSC ($p<0.01$). Overexpression of CEBPD demonstrated an increase in cell proliferation ($p<0.05$) and reduced vimentin and N-cadherin expression and increased expression of CK7. Soft agar assays and migration assays demonstrated that cells with CEBPD were less likely to form colonies ($p<0.01$), but had higher migratory potential ($p<0.05$). Furthermore, cells with CEBPD had lower growth potential compared to controls ($p<0.01$). The results suggest that CEBPD modulates the function of cells by reducing the potential for transformation to a more carcinogenic phenotype. **Conclusion** Our results support the notion that CEBPD acts as a tumor suppressor and modulates the functionality of fallopian tube cells. Despite its downregulation in HGSC, CEBPD may function early on in the pathogenesis of HGSC to regulation the transformation of cells towards subsequent phenotypes.

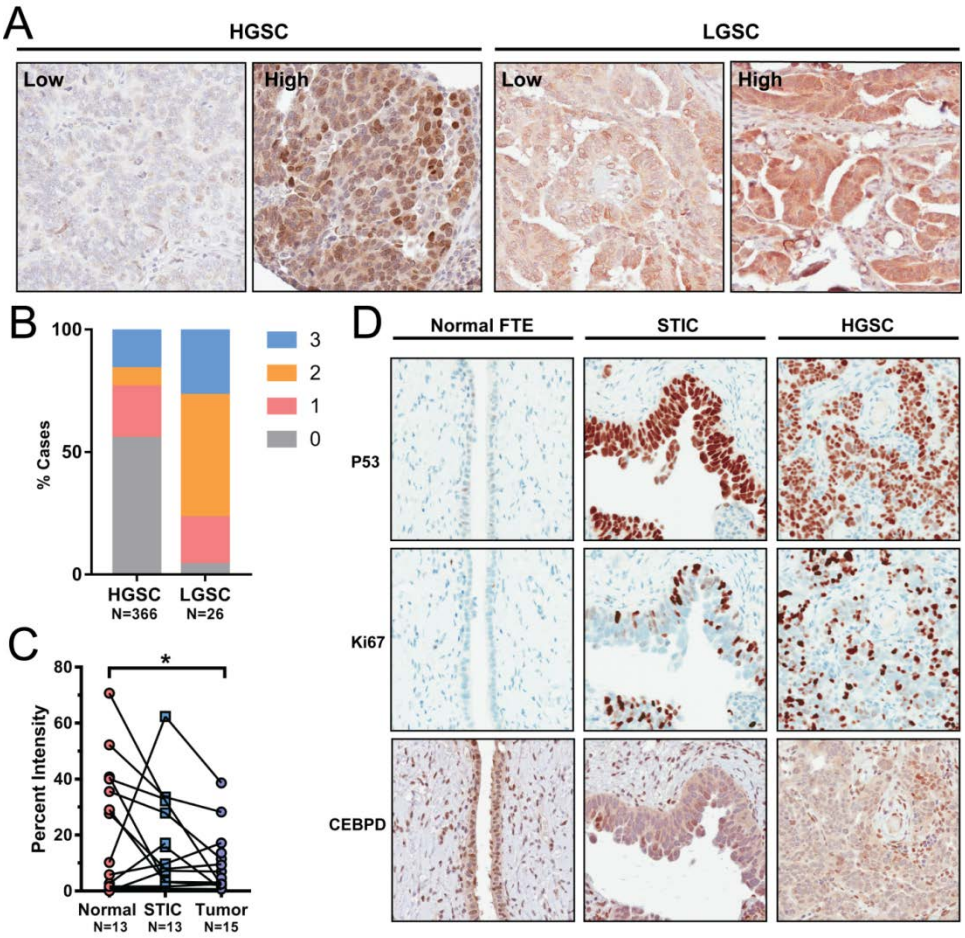


Figure 5: CEBPD is differentially expressed across histotype and decreases with development of disease. A. CEBPD is differentially regulated across HGSC and LGSC. High and low examples of CEBPD expression were identified. **B.** CEBPD expression is significantly higher in LGSC compared to HGSC, with the majority of HGSC clustering with low CEBPD intensity (10-30%). Error bars represent Min (5%) and Max (95%) values. (95% CI: 22.71 to 35.24). **C.** The development of the disease initiates when normal tissue incurs a p53 mutation which develops to STIC and to HGSC. CEBPD expression was found to decrease across this development, with tumor CEBPD expression levels significantly lower than normal tissue (95% Confidence Interval (CI):0.7632 to 27.96). **D.** Tumor cases with high proliferation (Ki67 protein expression) and deregulated p53 is correlated with lower CEBPD expression compared to normal fallopian tube. * p<0.05, ****p<0.0001

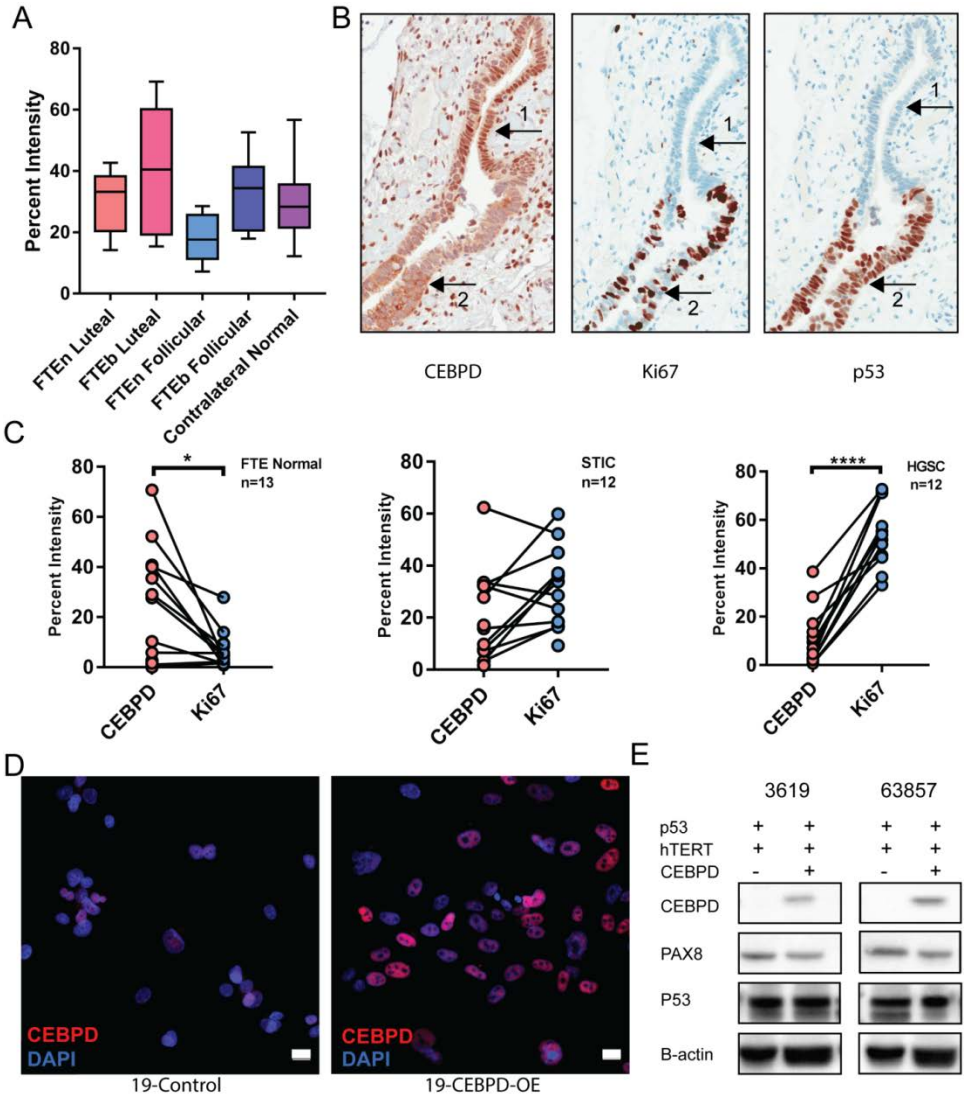


Figure 6: **CEBPD expression inversely related to proliferation (Ki67) A.** CEBPD expression is significantly higher in the BRCA mutation carriers (n=6) compared to normal cases in the follicular phase (n=5). Error bars represent Min (5%) and Max (95%). (95% CI:0.1681 to 29.44). **B.** Black arrows (1) indicate expression of CEBPD in normal epithelium and (2) lower CEBPD expression in STIC. CEBPD is expressed in epithelium where p53 and Ki67 are absent. **C.** Quantification of CEBPD and Ki67 in normal, STIC , and Cancer Cases demonstrated statistically significant inverse relationship between CEBPD and Ki67 in normal (95% CI -30.96 to -3.5) and HGSC cases (95% CI: 34.04 to 53.15). **D.** Low levels of CEBPD expression in FTE cells grown in-vitro was identified by immunofluorescence. CEBPD-OE lentiviral vector transfected into cells was primarily localized to the nucleus. **E.** Western Blot analysis demonstrated that cells in-vitro expressed CEBPD after infection with CEBPD-OE lentiviral vector. Cells also expressed PAX8 demonstrating a mullerian lineage.*p<0.05; ****p<0.0001

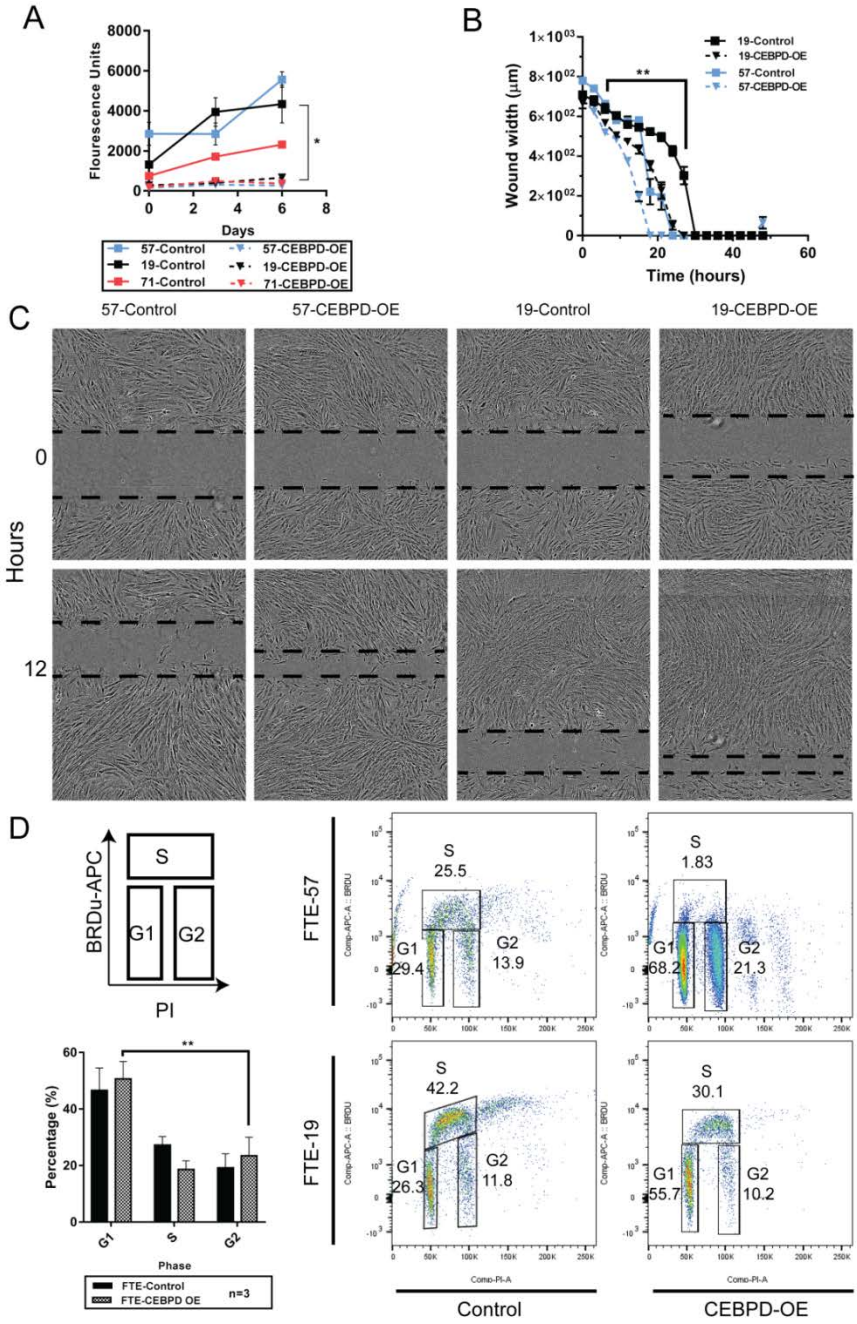


Figure 7: CEBPD decreases growth and migration of FTE cells. A. Growth assays demonstrated significant decrease in growth of FTE-CEBPD-OE cells compared to FTE-Controls (FTE-57 - 95%CI: -6013 to -989.1; FTE-19 – 95%CI: -5403 to -106.2; FTE-71 – 95%CI: -2531 to 45.6). **B.** Migration assays between FTE-Control and FTE-CEBPD-OE of cell lines 57-p53DN-hTERT and 19-p53DN-hTERT displayed decreased migration of FTE-CEBPD-OE cells compared to FTE-Control cells. The result was significant for a 12hr period ($p<0.01$) at which point cells were confluent. **C.** 57-CEBPD-OE and 19-CEBPD-OE cells displayed decreased migratory rates at 12 hrs compared to FTE-Controls. **D**. Cell cycle analysis demonstrated that FTE-CEBPD-OE increase the number of cells in the G1 phase, with a decrease in S phase. Results also demonstrate significantly more FTE-CEBPD-OE in the G1 compared to G2 phase.*$p<0.05$;**$p<0.01$.

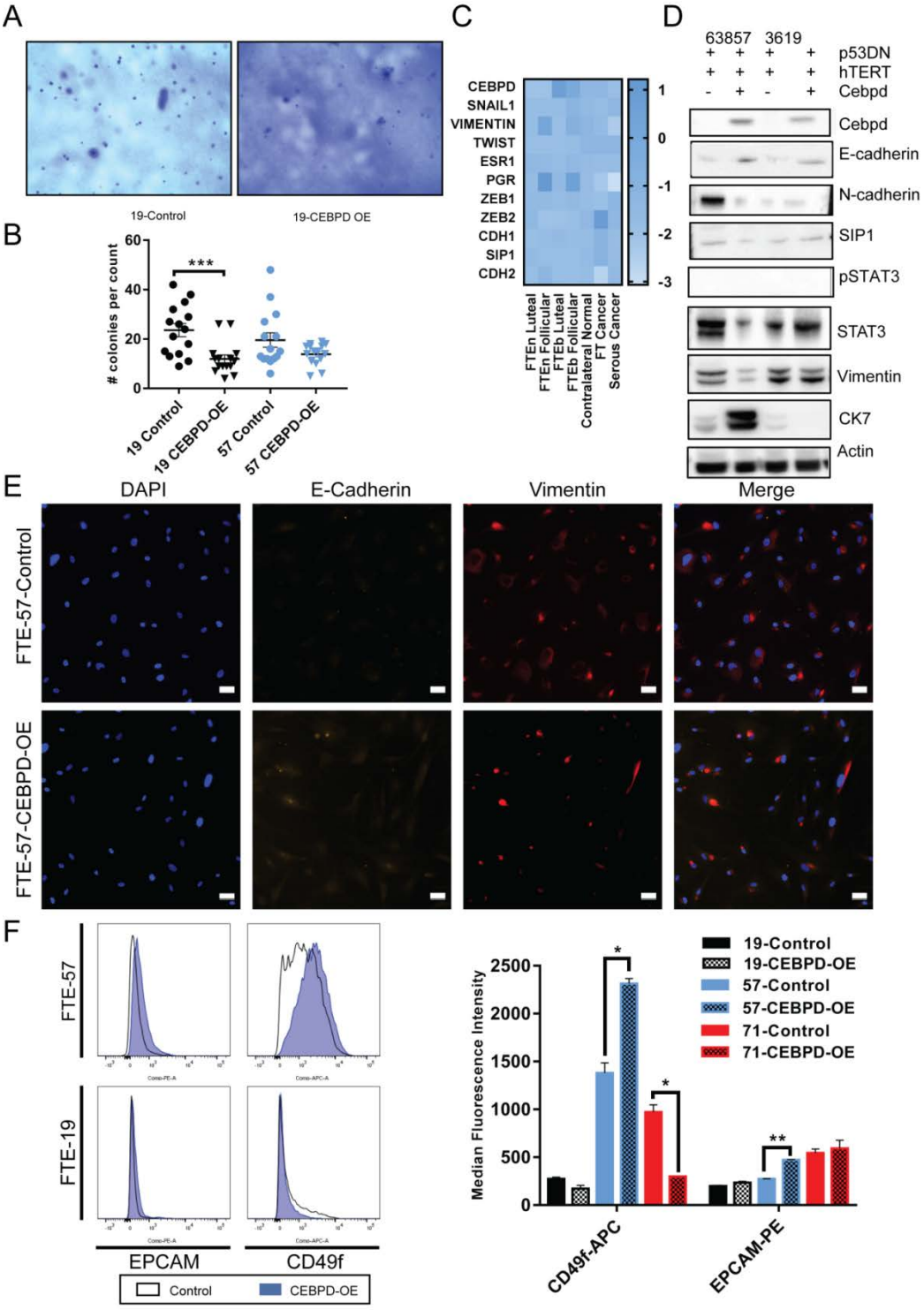


Figure 8: CEBPD increases the expression of epithelial markers in-vitro. (A-B) Soft agar assays performed on FTE-Control and FTE-CEBPD-OE cells grown for 20 days. 19-CEBPD-OE demonstrated significantly fewer colonies compared to 19-Control cells (95%CI -18.08 to -5.256). 57-CEBPD-OE cells displayed fewer colonies, but this result did not reach statistical significance (95% CI: -12.19 to 0.7278). **C.** Microarray analysis demonstrated the differential expression of multiple EMT markers across several tissue subtypes. **D**. Western Blot analysis demonstrated two mesenchymal markers, vimentin and n-cadherin, were decreased in FTE-CEBPD-OE cells. A slight decrease in expression of STAT3 was identified. E-cadherin expression in 57-CEBPD-OE and 19-CEBPD-OE was slightly increased compared to controls. CK7 was increased in 57-CEBPD-OE compared to controls. **E**. Immunofluorescence performed on FTE-Control and FTE-CEBPD-OE demonstrates increased E-Cadherin expression, localized at the cytoplasm of FTE-CEBPD-OE cells compared to controls. A slight decrease of vimentin was found in FTE-CEBPD-OE cells but expression is still present. **F.** Cell surface epithelial and mesenchymal markers were analyzed by Fluorescence Activated Cell Sorting. All cell lines demonstrated

increased levels of epithelial marker, EPCAM in FTE-CEBPD-OE cells. Increased mesenchymal marker, CD49f, was found in 1/3 cell lines. *** p<0.001; ****p<0.0001.

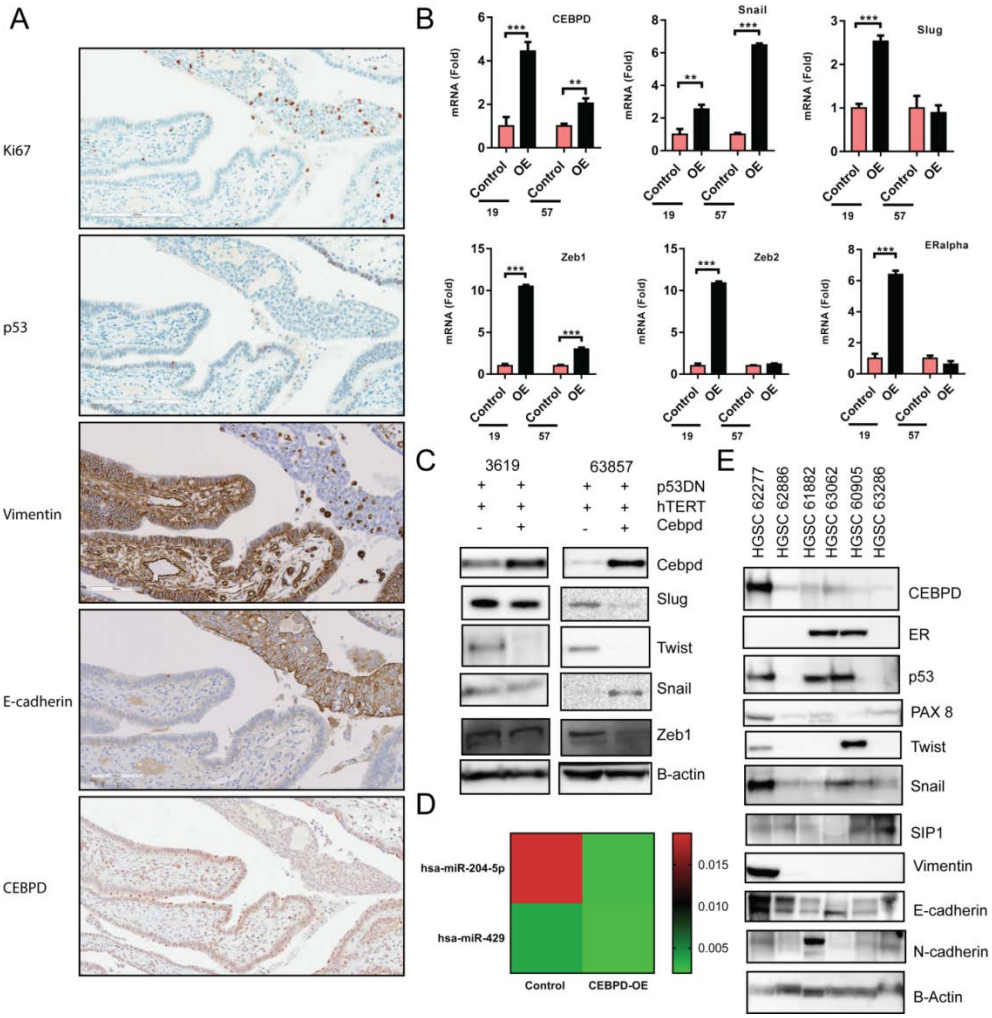


Figure 9: CEBPD regulates EMT/MET markers and induces a partial mesenchymal to epithelial transition. A. Immunohistochemistry, performed on sections of fallopian tube tissue with a STIC lesion demonstrates differences in the level of expression of vimentin, e-cadherin and CEBPD. Inverse expression was found between CEBPD and e-cadherin and vimentin. **B.** RT-PCR, performed on two FTE cells lines demonstrates that Snail was increased in FTE-CEBPD-OE relative to controls. FTE-19-CEBPD-OE Slug expression was increased relative to controls and in both cell lines; ZEB1 was also increased relative to controls. **C.** Twist and Slug were downregulated in FTE-CEBPD-OE cells. Snail was upregulated in FTE-57-CEBPD-OE. ZEB2, a target of miR-429 was downregulated in FTE-57-CEBPD-OE compared to controls. A slight decrease of ZEB1 was found in FTE-19-CEBPD-OE. **D.** Micro-RNA expression analysis performed on FTE-Control and FTE-CEBPD-OE by PCR Array demonstrated two miRNA (miR-429 and miR-204-5p) that were significantly decreased in FTE-CEBPD-OE compared to FTE-Controls. **E.** EMT/MET markers analyzed in six HGSC express e-cadherin and few express vimentin. 5/6 cases have low CEBPD expression.***p<0.001.

B). Microdissection of selected paraffin tissue samples

LCM of samples completed, Bioinformatic Results Completed (100%)

Summary/Developments: We have completed laser capture micro-dissection (LCM) on fimbriae from formalin-fixed paraffin embedded (FFPE, processed by SEE-FIM protocol) out of the proposed 68 cases; we have performed LCM on 68 cases which includes 9 HGSC (germline BRCA1 mutation) cases. We have RNA-sequenced 68/68 cases and completed the bioinformatics analysis of all cases. We have submitted the data to our colleague at John Hopkins University for subsequent gene expression analysis. Below is a summary of FFPE cases that have had LCM performed and sequenced. Refer to Appendix 1 for a complete list of case

Methods: FFPE tissue samples were sectioned at 10um and placed onto Pen-membrane slides (Leica). Sections were then cut using a Laser Capture Microdissection microscope (Leica). Approximately 6-12 sections were cut per case depending on surface area of epithelium available. Sections were stored in lysis buffer, snap-frozen and stored at –80 degrees until RNA extraction. RNA was extracted using the Roche High Pure FFPE Micro Kit (Roche). Samples were stored at –80 and submitted to the sequencing facility for RNA quantification and RNA sequencing. These samples are prepared by using Illumina Tru-Seq Stranded Total RNA kit with Ribo Goldready for the Illumina Hi-seq 2000 V3.

We are in the process of analyzing RNA sequencing data and will validate genes when results are available

Task 2. Validate mRNA and protein expression of selected classifier genes

Progress: In preparation for targets from the genomic profiling of fimbrial gene expression arrays, we are in the process of creating one additional cancer TMA containing a set of 300 HGSC samples with known family history of breast/ovarian cancer and patient clinical history including debulking status, treatment, recurrence and overall survival. This TMA will be useful in assessing the alteration of chosen targets within a larger set of cancers with different family history of cancer, as well as assessing the impact of such targets on clinical outcome. Pathological examination of each HGSC case is also being performed prior to building the TMA. All micro-dissected FFPE RNA has been analyzed.

We have acquired results from the FFPE micro-dissection project and completed the construction of the HGSC TMA.

We used genes that were differentially expressed between normal FTE and high-grade serous cancer (LKB1/STK11- published Oncogene 2015), ampulla derived differences between BRCA1 mutation carriers and non-carriers (Rb, p16, CD3, CD8, CD68 and CEBPD) and ampulla versus fimbria derived gene expression differences (GSTA2 and other antioxidant enzymes) to study and validate classifier genes. In addition, we studied the hormonal induced gene signatures between and amongst these groups of experiments. We have subsequently validated and characterized the role of these genes in normal fallopian tube tissue and STIC cases. Our results suggest novel roles of these genes in the context of the development of ovarian cancer.

To study the expression profile of these different genes, we constructed 2 fallopian tube tissue microarrays and 3 high grade serous TMAs (PT2, HGSC2011, HGSC4). In addition, we recently completed an additional high grade serous TMA –(all cases were selected, organized and reviewed prior to TMA construction).

Task 3. Model alterations associated with high-risk tubal epithelium and tubal intraepithelial carcinomas, in vitro.

We have established and propagated FTE cells and characterized it using western blots analysis, growth curves and immunocytochemistry staining. Transformed FTE and OSE cell lines were injected into mice in 2015/2016. Over the past year we have generated multiple tumors and are in the process of characterizing each to determine if candidate alterations modulate in-vivo malignant phenotypes and if there are differences between malignant phenotypes of FTE and OSE, and FTE-BRCA1/2 and OSE-BRCA1/2 origins. Copy number alterations and gene expression arrays will be performed on these tumors. Histological staining will require that we provide antibodies and utilize core pathological services to carry out stains.

Manuscript in preparation with anticipated submission in the next few months

Title: Characterization of FTE and OSE xenograft models

Summary - Immortalized and transformed cell lines from both FTE and OSE, cells derived from both BRCA1 mutation carriers and non-carriers. In particular, 16 OSE cell lines have been created –3 from BRCA1 carriers – iOSE390F, iOSE267F, iOSE592F (F=familial ovarian cancer) and non-carriers – iOSE120 and iOSE523. Cell lines were transfected with hTERT (ht) and SV40, and either vectors over-expressing cMYC, hRASV12 or PIK3CA-H1047R.

In vitro assays were performed to compare proliferation, anchorage-independent growth and invasion between 2 BRCA1 (iOSE 267F and iOSE 592F) and 2 non-BRCA cell line over expressing hTERT and SV40 and either cMYC or hRASV12. All 12 lines showed proliferation and anchorage-independent growth. 8 of these lines with cMYC or hRASV12 were then injected in NSG mice (NOD.Cg-Prkdcscid). All injections with these cell lines were done in the mammary fat pad of 6 week old female mice. All mice have been sacrificed and tumors have been analyzed.

Similarly, 16 FTE lines were generated from BRCA1 carriers - FTE3793 and FTE3798, BRCA2 carrier - FTE3313 and non-BRCA carriers – FTE3437, FTE3619 and FTE63857 over-expressing hTERT and SV40 and either cMYC or hRASV12. As with iOSE lines, 8 of the FTE lines have been injected in the mammary fat pad of NSG mice and we have completed analysis of tumors derived from these mice. In addition to FTE cells with SV40 hTERT, FTE cell lines – FTE3793, FTE3313, FTE3319 and FTE63857 were generated with 3F (TP53-R175H, E7, and hTERT).

The parental immortal lines of 4 cases were karyotyped: FTE-3437 and FTE-3619 (controls); FTE-3313 (BRCA2 mutant) and FTE-3798 (BRCA1 mutant). The FTE BRCA mutant cell lines demonstrated varying types of tetraploidy whilst the control (BRCA wild type) demonstrated had fewer tetraploid chromosomes. It is hypothesized that SV40 alone is sufficient to stimulate genomic instability in the FTE cells.

These lines were subsequently transfected with either PIK3CA-H1047R, HRASV12, cMYC and/or CCNE1 and injected into NSG mice at the mammary fat pad.

Methods – Cell lines were generated and grown in tissue culture. At approximately 80% confluency, cells were trypsinized, counted and suspended in matrigel. Cells were injected within the mammary fat pad and the intraperitoneal region of female 6 week old NSG mice. Mice are monitored twice a week for signs of tumor development and are sacked when tumor has reached 1.5 cm. Mice injected intraperitoneally are monitored twice a week along with a weight check performed at the end of each week. Mice are sacked if the abdomen is distended or mice exhibit signs of lethargy and malaise (indicated by hunched posture and raised fur). Mouse dissection involves removing the tumor, liver, spleen, lungs and intestines which are then embedded in paraffin for histological analysis. Tumor sections are also snap-frozen for subsequent genomic analysis.

Results/Developments - To date, tumors have developed from mice injected with ovarian epithelial surface cells and fallopian tube epithelium cells at the mammary fat pad and the intraperitoneal cavity. FTE cells injected intraperitoneally have produced ascites in conjunction with tumors that line the abdominal cavity and major organs. Multiple tumor nodules have been found around the uterine horn and within the mesocolon.

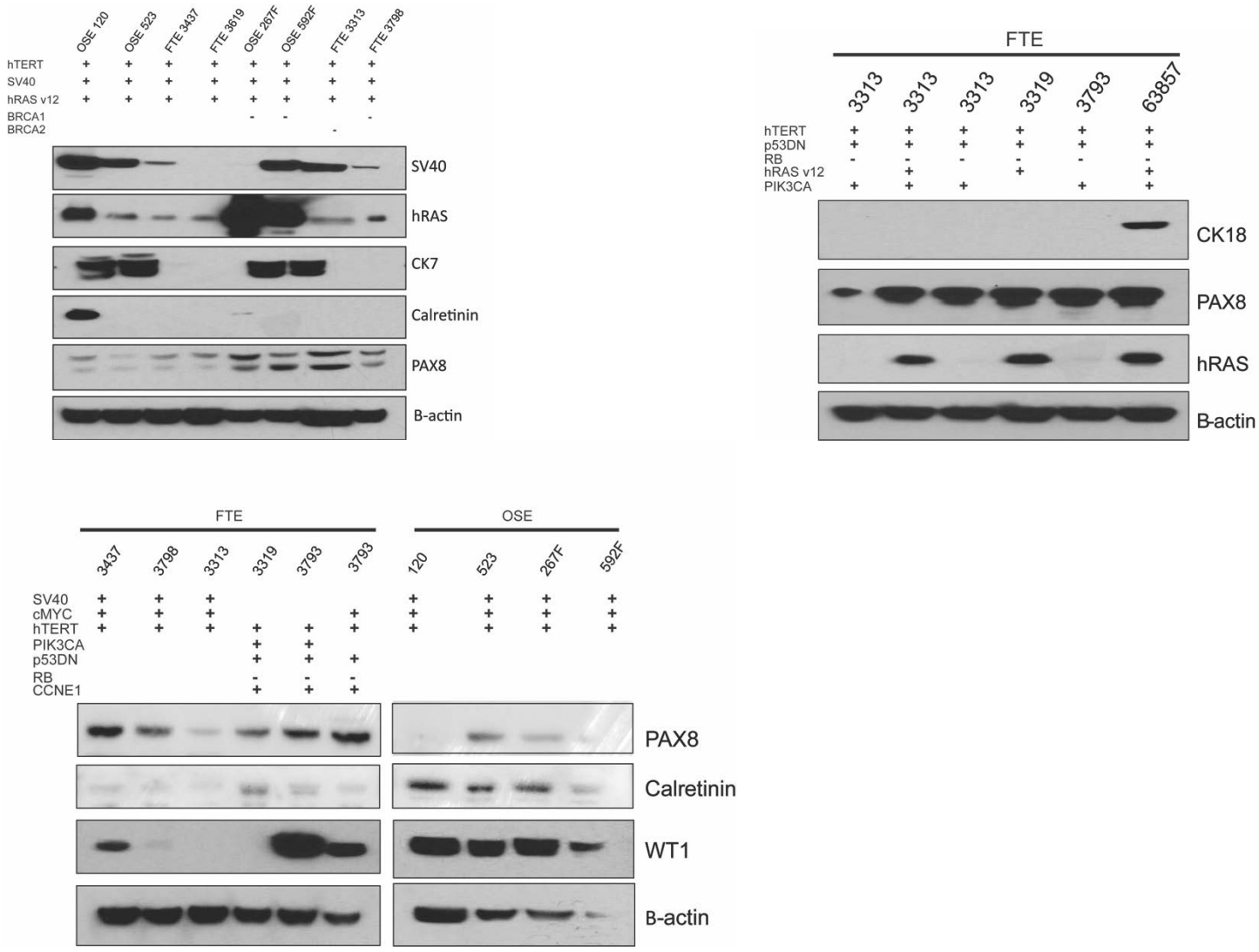


Figure 10. Western Blot analysis was performed on multiple cell lines prior to mice injection. Results demonstrate that FTE cell lines are secretory and retain epithelial characteristics. OSE cell lines retain calretinin, a marker associated with the mesothelium.

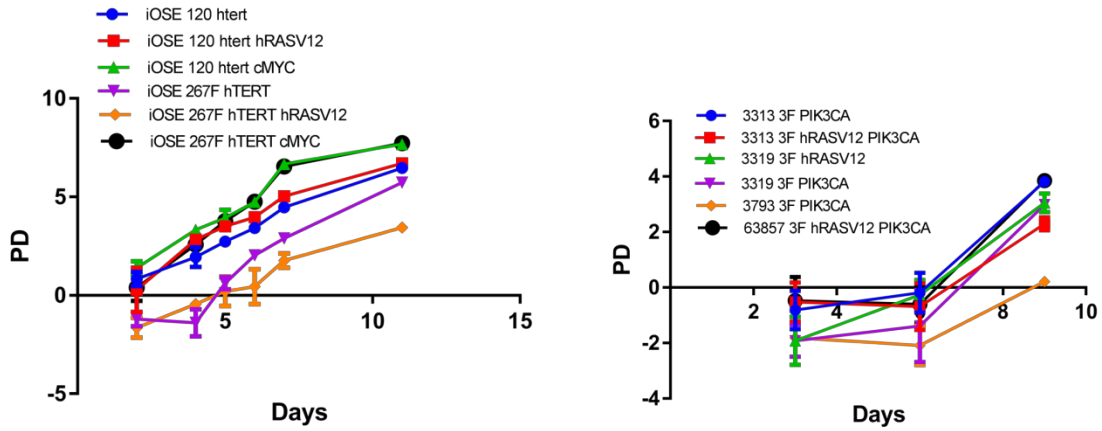


Figure 11. Growth curve of FTE cells in-vitro. Cells were transformed with mutant p53-R175H, E7 and hTERT along with hRAS-v12 or PIK3CA.

Figure 12. Karyotype analysis was performed on FTE cell lines. Cell lines with SV40 mutation demonstrate tetraploidy in comparison to 3F (hTERT,p53DN, Rb loss) cells. Mice were dissected and organs sent to histology for sectioning and staining. Tumors are identified by black arrows.

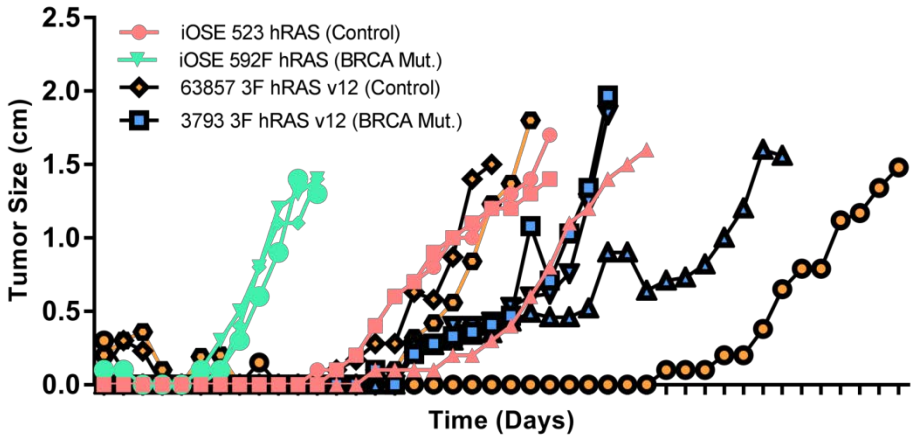


Figure 5. Xenograft tumors were tracked twice a week. In FTE, control and BRCA1 mutants xenografts grew at similar rates. OSE lines demonstrated that BRCA1 mutants grew much quicker.

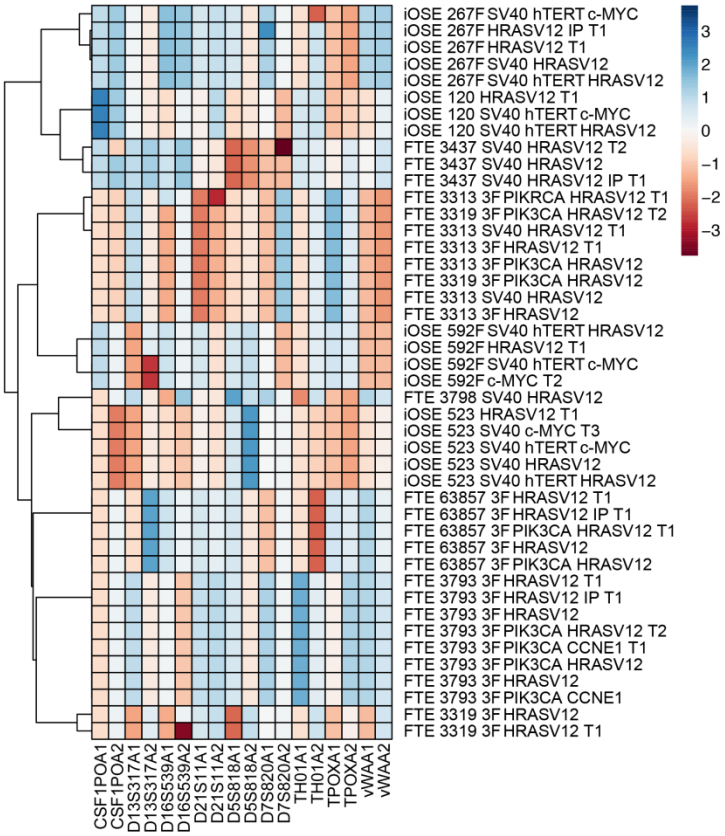


Figure 13. STR analysis was performed on cell lines and xenograft tumors. The heat map above demonstrates that cell lines and xenograft tumors of the same origin clustered together

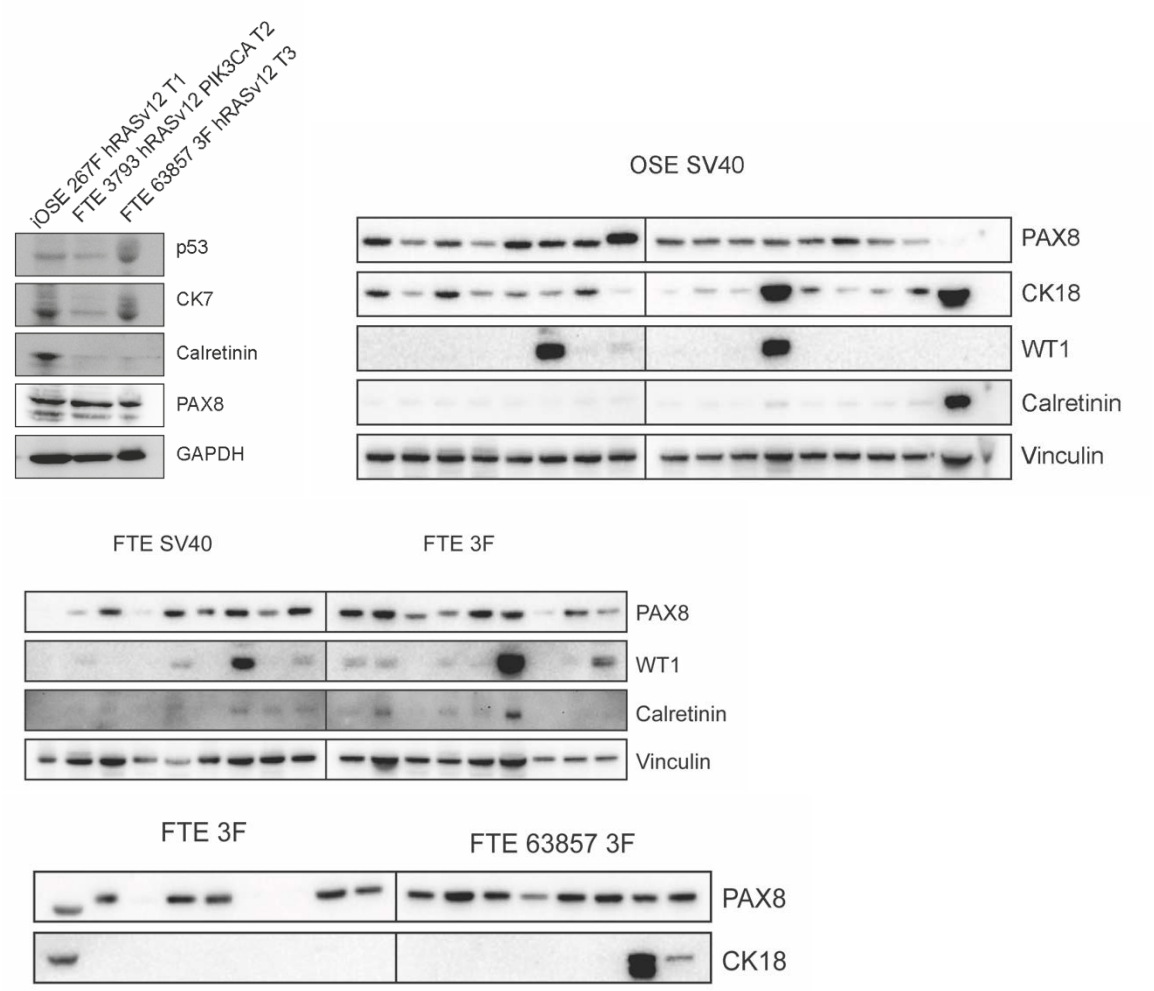


Figure 14. Xenograft tumors were lysed and protein extracted. Protein was run on westerns and probed with several immunohistochemical markers. Western blots reveal that FTE xenografts retain PAX8 and WT1. Interestingly, OSE tumors also expressed PAX8, however minimal WT1 was expressed.

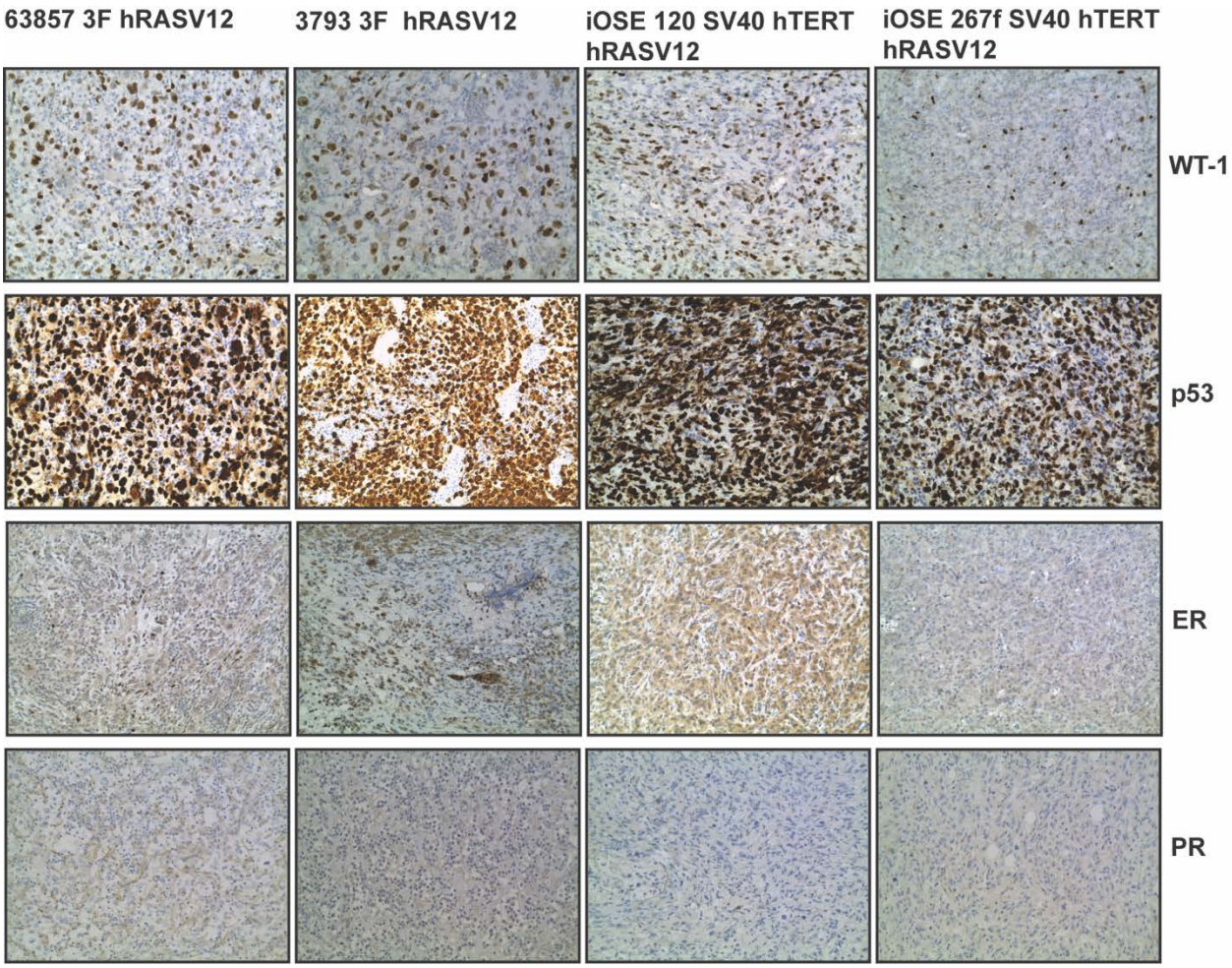


Figure 15. Xenografts derived from FTE and OSE injected mice were characterized by immunohistochemistry (IHC). Tumors expressed p53, WT1 and ER. Low to absent levels of PR were detected in HGSC.

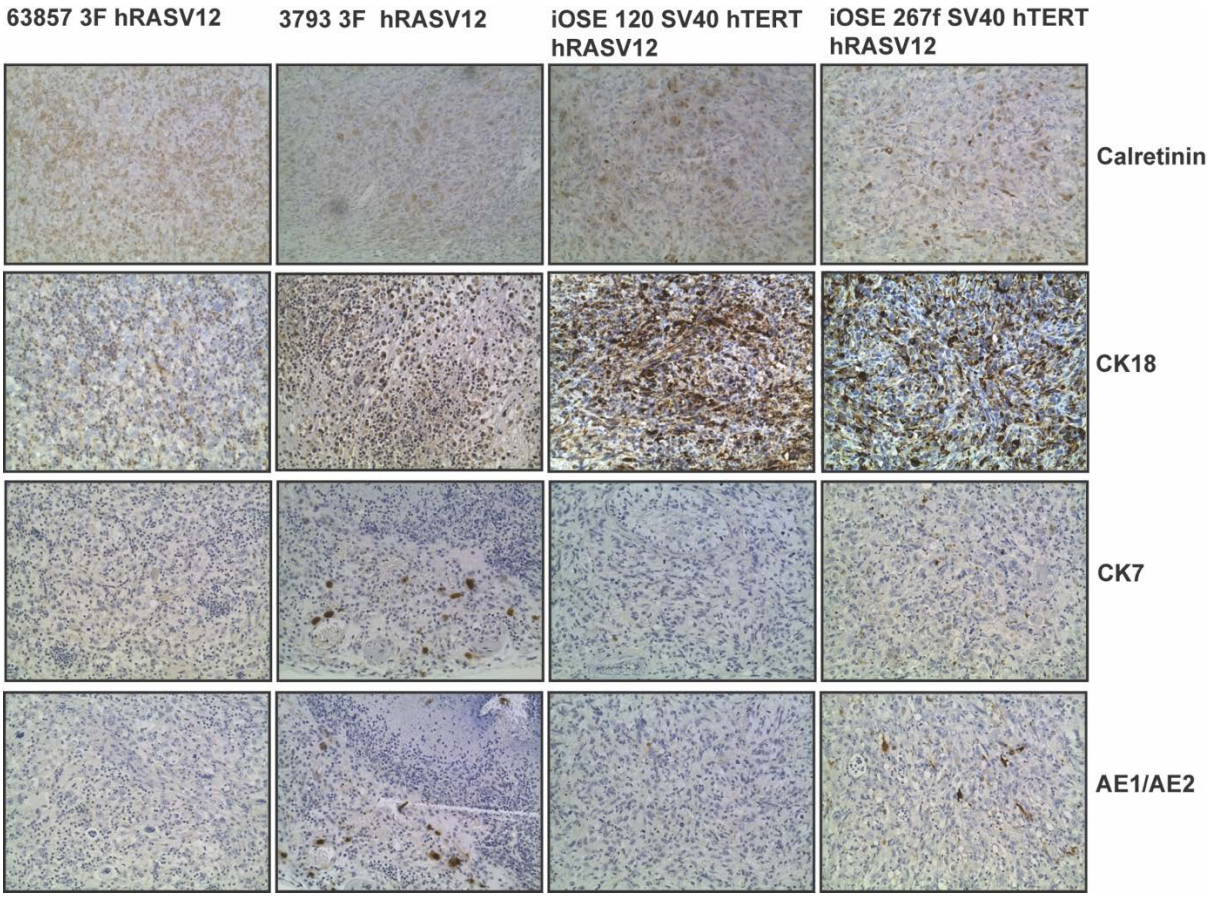


Figure 16 : Cyotkertin markers, CK18 and CK7, was expressed in tumors. Higher levels of calretinin was found in OSE tumors compared to FTE. Low levels of AE1/AE2 were found in both FTE and OSE cells.

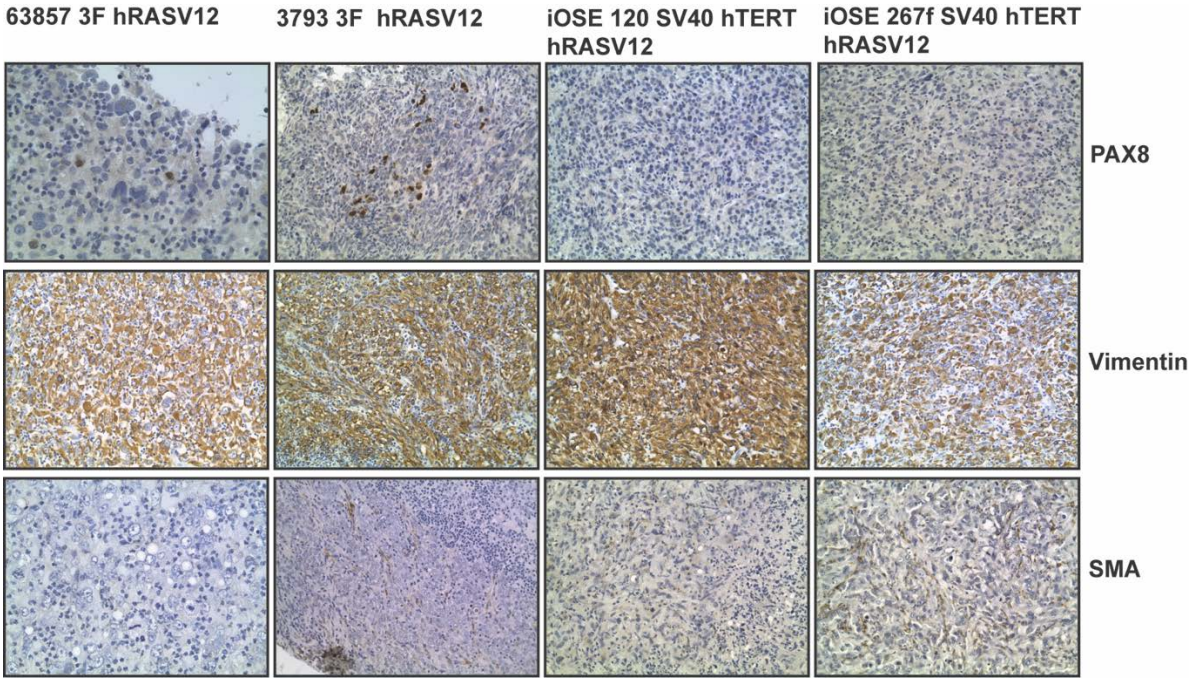


Figure 17. IHC on xenograft derived tumors staining for PAX8, Vimentin and Smooth Muscle Actin (SMA).

Future directions – We are in the process of drafting a manuscript for this project and will submit to a peer-reviewed journal within the next few months.

Summary of Task 1, Task 2 and Task 3

Task 1

1. Completed histological reviews of fallopian tubes from BRCA1/2 mutation carriers, along with matching controls and cancers.
2. Completed selection of a minimum of 32 fallopian tubes from *BRCA1* carriers, 32 from normal controls, 16 STICs, and 16 serous cancers from BRCA1 mutation carriers
3. Completed microdissection of selected tissue samples.
4. Gene expression profile of cellular samples.
   1) Completed gene expression array on ampulla versus fimbria from cryo-preserved tissue **–Manuscript submitted to peer-reviewed journal – awaiting reviewer comments**
   2) Completed RNA-Sequencing on FFPE fimbria from BRCA1/2 mutation carriers and non-carriers.
   3) Analysis of expression profiles with focus on genes differentially expressed in *BRCA1* mutation carriers **– Currently analyzing results**

Task 2

1. Completed creation of: 2 histologically normal FTE (FTEb, FTEn, luteal and follicular designations) TMAs; 3 HGSC TMAs.
2. Completed validation of selected genes by quantitative RT-PCR: LKB1 **(Oncogene 2016)**, GSTA2, CEBPD ER, PR, DKK3, SOX11, TKTL1 **(Manuscript submitted, Nature Communications)**.
3. Completed selection of antibodies for validation of protein expression in normal FTE, STIC and HGSC: CD3, CD8, CD68 **(Clinical Cancer Research 2012)**, p16/Rb, CCNE1, CCND1 **(Modern Pathology 2014)**, LKB1 **(Oncogene 2016)**, GSTA2, ME1, CK7 **(Completed)**, CEBPD **(Manuscript 95% completed, submission due for February 2018),** Estrogen and Progesterone receptors, DKK3, SOX11, TKTL1 **(Manuscript 90% complete, submission due within the next few months).**

Task 3

1. Completed isolation and propagation of primary FTE (n=15 cell) cell lines, including characterization and growth kinetics of the established cell lines.
2. Completed generation of pre-malignant (immortalized/non-tumorigenic) FTE cell lines (FTE-p53 R175H, FTE- p53 R175H +hTert, FTE-E7+hTert.
3. Definition of phenotypes associated with pre-malignant FTE-BRCA vs FTE-nonBRCA is an ongoing process that is dependent on the genes understudy (for example, immune infiltrates, CEBPD and GSTA2)
4. We chose to focus on in vitro cell culture assays, rather than CAM assays. This work is in progress and some of this work has been published (Oncogene 2015) and manuscripts are in preparation.
5. We have generated xenograft tumors in vivo. We have completed characterizing the xenograft derived-tumors (3F = (p53 DN R175H, E7, hTERT)). The tumors generated from transformed OSE and FTE, in NSG assay, has undergone molecular characterization – molecular pathology, karyotyping and western blot analysis. We characterized 28 independent xenograft derived tumors. In particular, tumors were screened for expression of: vimentin, p53, smooth muscle actin, CK7, PAX8, reticulin, EPCAM, CK18, calretinin, caldesmin and WT1. Molecular genomic analysis using the U1333 Affymetrix gene expression array and Affymetrix 6.0 SNP assay. The molecular profiles will be compared with existing HGSC expression profiles.

Training and professional development has the project provided?

- One lab member attended the 8th Canadian Conference on Ovarian Cancer Research held in Niagara Falls, Ontario. In addition, this lab member has submitted numerous abstracts and is currently writing several manuscripts. Furthermore, he has presented two projects at the Terry Fox Research Symposium in 2016 and 2017. By writing manuscripts, attending conferences and working with his mentor′s, he has broadened his knowledge of ovarian cancer and the relevant research being conducted in the field.

- Lab members have attended numerous conferences and presented abstracts (please refer to conference section below for list of conferences attended). Our oral presentation, knowledge of field of study, critical thinking skills have developed as a result of these activities.
- One lab member is now a professor at the University of Miami focusing on the early events of ovarian cancer. She recently attained a young investigator DOD research award to continue elucidating development of the disease.

Dissemination to communities of interest

- Nothing to Report

Plans for the next reporting period to accomplish the goals

To successfully complete the project we plan on fulfilling the goals outlined in the statement of work. This will be achieved by continuing research activities for Tasks 2 and 3 which includes:

- **Finalizing and submitting prepared manuscripts**
- Analyzing data relevant to LCM FFPE project

4. **IMPACT:**

Our project focuses on supporting the notion that the fallopian tube and not the Ovarian Surface Epithelium is the tissue origin of High Grade Serous Carcinoma. To this end we have investigated the fallopian tube epithelium to a great extent and have identified several findings relevant to the advancement of knowledge in the field of ovarian cancer. We have identified that a difference in gene expression exists between patients with BRCA1 mutation status and a non-mutated status. We have gone further to study these differentially expressed genes that include LKB1, GSTA2 and CEBPD. Our results have demonstrated that a loss of LKB1 in conjunction with a mutated p53 gene results in a loss of apical to basal polarity and a loss of ciliated cells in fallopian tube cells suggesting that a loss of LKB1 disrupts the fallopian tube epithelium. With our microarray results we identified that a loss of LKB1 is frequently observed in tubal cancer precursor lesions suggesting that the loss of this gene may be an early event in disease progression. These results indicate that LKB1 may be a therapeutic target for disease control.

Furthermore, we determined that that several genes involved in metabolism and glutathione mediated oxygen and xenobiotic antioxidant response were up-regulated in fimbrial epithelial cells compared to the ampulla, including, AKR1C2/C3/C4, NMBR, NMNT, ME1 and GSTA2. These genes encode enzymes of reactive oxygen species, xenobiotic and arachidonic metabolism. HGSC cells down-regulate or differentially express many of these genes, indicating both a susceptibility to tolerate distinct cytotoxic stressors due to the hormonally rich environment. This data provides insight into disease susceptibility as fimbria FTE express a cadre of genes to protect their genome from cytotoxic stressors found in the microenvironment and follicular fluid released from the ovary.

Additionally, we also determined a role of CEBPD in HGSC. Our results demonstrate that CEBPD is lost early and promotes a partial MET in serous ovarian carcinogenesis. While CEBPD presented characteristics conducive to an EMT/MET, a phenomenon associated with tumor metastasis, it presented as a tumor-suppressor indicating its complex role in the pathogenesis of the disease. These findings suggest a target for the regulation of EMT/MET in the development of HGSC.

Our research has focused on demonstrating a significant difference in the percentage of leukocytes in serous tubal intraepithelial lesions and HGSC cases compared to normal cases. Our results suggest that leukocytes may play a role in the progression or inhibition of the disease early. Additionally, these results were independent of ovarian cycle. We demonstrated no inherent difference in proliferation between normal and BRCA1/2 mutation

carriers at the distal fallopian tube epithelium. However, a proliferative difference is present at later stages of disease progression when an early lesion is identifiable suggesting that molecular changes to the tubal epithelium occur later in the development the disease.

We have also demonstrated that a deregulated Rb1 pathway is correlated with clinical outcomes and may play a role in the development of early serous tumor development. Furthermore RB1 may be a target for chemotherapeutic and novel therapeutic strategies. In addition to this gene, by comparing

Together our results along with manuscripts under preparation have broadened our understanding of ovarian cancer by providing an early events context to the disease.

- **What was the impact on other disciplines?**

Immunotherapy has emerged in recent years as a successful therapeutic modality that is capable of effecting durable long-term remissions. In particular, recent advances in understanding tumor mechanisms of immunosuppression have led to new treatments focused on immune checkpoint blockade, which have proven effective in a subset of patients with malignancies such as melanoma, lung cancer, and colorectal cancer. Individually, however, immune checkpoint inhibitors have shown activity in only a modest percentage of patients with these types of cancer. While a clinical trial to evaluate immune checkpoint inhibitor therapy in

ovarian cancer has been initiated, it has been reported that only 10-20% of patients will benefit from checkpoint blockade as a monotherapy in ovarian cancer. Accordingly, additional strategies are needed to prospectively identify the subset of patients who will most likely benefit from this approach, and to further enhance therapeutic efficacy. Understanding ovarian cancer immunobiology in relation to genetic defects in DNA repair pathways, which may generate neo-antigens that could serve as effective targets for immunotherapy, but which may also affect infiltrating immunocytes through changes in exosome-mediated paracrine signaling, will enable us to develop methods for prospectively identifying ovarian cancer patients who will show significant responses to immunotherapy.

- **What was the impact on technology transfer?**

"Nothing to Report."

- **What was the impact on society beyond science and technology?**

"Nothing to Report."

- **CHANGES/PROBLEMS:**

"Nothing to Report."

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

"Nothing to Report."

PRODUCTS:

Omar Nelson, **Ramlogan Sowamber**, Anca Milea, Michael Considine, Leah Dodds, Noor Salman, Victoria De Castro, Brian Slomovitz, Leslie Cope, **Patricia A Shaw and Sophia HL George**. Integrative Transcriptome analyses of the ampulla and fimbria of the human fallopian tube. ***Under Review***

Sophia HL George, Ramlogan Sowamber, Anca Milea, Noor Salman and Patricia Shaw. The role of estrogen and progesterone in HGSC. ***In preparation***

Ramlogan Sowamber, Rania Chehade, Noor Salman, Mahmoud Bitar, Patricia Shaw, Sophia HL George. **Cebpd acts as a tumor suppressor in High Grade Serous Ovarian Cancer.** ***In preparation***

Sophia HL George, Ramlogan Sowamber, Mauricio Medrano, Mahmoud Bitar, Patricia Shaw. Characterization of FTE and OSE xenograft models. ***In Preparation.***

Patricia A. Shaw and Blaise A. Clarke. Prophylactic Gynecologic Specimens from Hereditary Cancer Carriers. Surgical Pathology Clinics. June 2016 doi:10.1016/j.path.2016.02.002

Sophia HL George, Anca Milea, Ramlogan Sowamber, Alicia Tone, Rania Chehade and Patricia Shaw. Loss of LKB1 Protein Expression is Frequent in Serous Carcinoma. *Oncogene, 23 March 2015* doi: 10.1038/onc.2015.62

Sophia HL George and Patricia Shaw. BRCA and the fallopian tube epithelium. *Frontiers in Oncology, 2014 Jan 23*

Milea, A., S. H. George, D. Matevski, H. Jiang, M. Madunic, H. K. Berman, M. L. Gauthier, B. Gallie and P. A. Shaw (2013). "Retinoblastoma pathway deregulatory mechanismsdetermine clinical outcome in high-grade serous ovarian carcinoma." Mod Pathology 27, 991-1001.

- **Books or other non-periodical, one-time publications.**

Patricia Shaw, Blaise Clarke and Sophia HL George. Precancerous lesions of high-grade pelvic serous carcinoma, Precancerous lesions of the gynecologic tract: diagnostic and molecular pathology. Springer 2015

- **Other publications, conference papers, and presentations.**

Abstracts

Ramlogan Sowamber *, Mauricio Medrano, Mahmoud Bitar, Patricia Shaw, Sophia HL George.* Characterization of FTE and OSE xenograft models. Submitted to Terry Fox Research Symposium, 2017.

SH George, O Nelson, R Sowamber, A Milea, X Xu, M Huang, MP Schlumbrecht, JM Pearson, P Shaw, B Slomovitz. Antioxidant gene expression program is deregulated early in serous ovarian cancer. Gynecologic Oncology 145, 130

Sophia George, Anca Milea, Sowamber Ramlogan, Michael Considine, Leslie Cope, Noor Salman, Patricia Shaw. Abstract A27: Transcriptome analyses of human ampulla and fimbriae highlight similarities and differences. Clinical Cancer Research 22 (2 Supplement), A27-A27

SHL George, R Sowamber, P Shaw. Abstract POSTER-BIOL-1312: The role of BRCA and CEBPD in serous ovarian cancer carcinogenesis. Clinical Cancer Research 21 (16 Supplement), POSTER-BIOL-1312-POSTER-BIOL-1312,2015

SH George, A Milea, NH Salman, PA Shaw. Differential transcription profile of epithelia in fimbria versus the ampulla of the fallopian tube. Cancer Research 75 (15 Supplement), 2080-2080,2015

S George, A Milea, N Salman and P Shaw. Fimbria and Ampulla Tubal Epithelium Have Similar Transcriptome Profiles. *Lab Invest. 95, 286A-286A 2015*

Sophia HL George, Anca Milea, Ramlogan Sowamber, Danielle Toccalino and Patricia Shaw. BRCA and early events in the development of serous ovarian cancer. *Current Oncology 21:e377 (2) April 2014*

Sophia HL George, Anca Milea, Ramlogan Sowamber, Danielle Toccalino and Patricia Shaw. The role of estrogen receptor signaling in serous ovarian cancer. *Cancer Research August 14, 2013 73:4765; doi:10.1158/1538-7445.AM2013-4765*

Kolin, D., George, S., Milea, A., Narod, S., Clarke, B., & Shaw, P. (2015, February). The Familial Ovarian Tumor Study: A Morphological and Immunohistochemical Review. In *LABORATORY INVESTIGATION* (Vol. 95, pp. 293A-293A). 75 VARICK ST, 9TH FLR, NEW YORK, NY 10013-1917 USA: NATURE PUBLISHING GROUP.

Peerani, R., George, S., Sowamber, R., Siu, A., Shao, T., Milea, A., ... & Shaw, P. (2015, February). Tumor Infiltrating Lymphocytes (TILs) as a Function of Histological Subtype and Genetic Background of Ovarian Epithelial Carcinomas. In *LABORATORY INVESTIGATION* (Vol. 95, pp. 301A-301A). 75 VARICK ST, 9TH FLR, NEW YORK, NY 10013-1917 USA: NATURE PUBLISHING GROUP.

Sophia HL George, Ramlogan Sowamber and Patricia Shaw. The role of BRCA and CEBPD in Serous Ovarian Cancer. Clin Cancer Res August 15 2015 (21) (16 Supplement) POSTER-BIOL-1312; **DOI:**10.1158/1557-3265.OVCASYMP14-POSTER-BIOL-1312

Presentations

2017	Terry Fox Research Symposium, Toronto, ON
2017	108th AACR Annual Meeting, Washington, DC
2016	Terry Fox Research Symposium, Toronto, ON
2016	8th Canadian conference on ovarian cancer research (attendance)
2015	AACR Advances in Ovarian Cancer Meeting, Orlando, FL
2015	8th AACR The Science of Cancer Health Disparities, Atlanta, GA
2015	106th AACR Annual Meeting, Philadelphia, PA
2014	Masha Rivkin Ovarian Cancer Conference, Seattle, WA
2014	Dalla Lana School of Public Health Research and Practice Day presentation
2014	5th International Symposium on Hereditary Breast and Ovarian Cancer, QC

- **Website(s) or other Internet site(s)**

  Nothing to Report
- **Technologies or techniques**
  Nothing to Report
- **Inventions, patent applications, and/or licenses**
  Nothing to Report

- **Other Products**

  Nothing to Report

5. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **Individuals who have worked on the project**

Name:	*Patricia Shaw*
Project Role:	*Principle Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*90*
Contribution to Project:	*Dr. Shaw has conceptualized and executed the objectives of this project, wrote manuscripts and presented findings.*
Funding Support:	

Name:	*Sophia HL George*
Project Role:	*Scientific Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*90*
Contribution to Project:	*Dr. George has conceptualized and performed experiments, wrote manuscripts and presented findings.*
Funding Support:	

Name:	*Ramlogan Sowamber*
Project Role:	*Research Staff*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*65*
Contribution to Project:	*Ramlogan has carried out experiments to generate results for the project.*

Funding Support:	

Name:	*Mahmoud Bitar*
Project Role:	*Research Staff*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*40*
Contribution to Project:	*Mahmoud helped carry out experiments for the CEBPD project.*
Funding Support:	

Name:	*Nick Chauvin*
Project Role:	*Research Staff*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*55*
Contribution to Project:	*Nick organized and helped with shipping DOD Project 5 slides.*
Funding Support:	

Name:	*Noor Salman*
Project Role:	*Research Staff*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*18*
Contribution to Project:	*Noor has carried out experiments to meet objectives of the project.*

Funding Support:	

Name:	*Zahra Maamir*
Project Role:	*Research Staff*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*18*
Contribution to Project:	*Zahra carried out experiments to meet objectives of the project, which included western blots*
Funding Support:	

Name:	*Gillian Geddie*
Project Role:	*Research Staff*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*18*
Contribution to Project:	*Gillian assisted with organize tissue, slides and blocks for numerous projects.*
Funding Support:	

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

  Dr. George was recently appointed to the position of Research Assistant Professor in the Department of Obstetrics and Gynecology at the University of Miami in Miami, Florida
- **What other organizations were involved as partners?**

- **Organization Name: Princess Margaret Cancer Centre**
- **Location of Organization:** *Toronto, Ontario, Canada*
- **Partner's contribution to the projec**t *(identify one or more)*
- **Financial support - Nothing to report**
  - **In-kind support** - Desktop computer
  - **Facilities** – *Facilities and lab space, lab supplies*

- **Collaboration** - Nothing to report
- **Personnel exchanges** - Nothing to report
- **Other.**

9. APPENDICES:

LCM tables

	# of cases	# cases with RNA extracted	# cases with RNA analyzed	#cases with sequence complete-October 2016
BRCA Luteal	12	12	12	12
BRCA Follicular	23	23	23	23
Normal Luteal	11	11	11	11
Normal Follicular	13	13	13	13
Cancers	9	9	9	9
Post-Menopausal	2	2	2	0
Total	70	65	19	68

Sample ID	Mutation Status	Cycle	Tissue Site	Qubit conc ng/ul	RNA Sequenced
FFPE 1	BRCA1 185delAG	Luteal	Fimbria	19.2	Yes
FFPE 2	BRCA1_3825del8	Follicular	Fimbria	24.4	Yes
FFPE 3	no mutation	Follicular	Fimbria	32	Yes
FFPE 4	BRCA1 1806C-T	Follicular	Fimbria	57	Yes
FFPE 5	no mutation	Luteal	Fimbria	4.4	Yes
FFPE 6	BRCA1	Luteal	Fimbria	64.6	Yes
FFPE 7	BRCA1	Follicular	Fimbria	8.78	Yes
FFPE 8	BRCA1 unknown mutation	Follicular	Fimbria	12.5	Yes

FFPE 9	BRCA1-1294del40 2434T-C variant	Follicular	Fimbria	64.2	Yes
FFPE 10	BRCA1 185delAG	Luteal	Fimbria	29.8	Yes
FFPE 11	BRCA1	HGSC	Tumor	71.6	Yes
FFPE 12	BRCA1	HGSC	Tumor	20.6	Yes
FFPE 13	BRCA1	HGSC	Tumor	94.8	Yes
FFPE 14	BRCA1	HGSC	Tumor	54.4	Yes
FFPE 15	BRCA1	HGSC	Tumor	102	Yes
FFPE 16	BRCA1	HGSC	Tumor	20.8	Yes
FFPE 17	BRCA1	HGSC	Tumor	39.8	Yes
FFPE 18	BRCA1	HGSC	Tumor	60.6	Yes
FFPE 19	BRCA1	HGSC	Tumor	84.2	Yes
FFPE20	no mutation	Follicular	Fimbria	13.1	Yes
FFPE21	BRCA1 185delAG	Luteal	Fimbria	20.6	Yes
FFPE22	BRCA1 4239 delAG	Follicular	Fimbria	10.9	Yes
FFPE23	no mutation	Follicular	Fimbria	21.8	Yes
FFPE24	BRCA1_1479delAG	Follicular	Fimbria	19.5	Yes
FFPE25	BRCA2	Follicular	Fimbria	11	Yes
FFPE26	BRCA2_6174delT	post-menopausal	Fimbria	6.9	Yes
FFPE27	BRCA1 5382insC	Follicular	Fimbria	4.2	Yes
FFPE28	BRCA1-185delAG	Luteal	Fimbria	4.2	Yes
FFPE30	FOC/BRCA2	Luteal	Fimbria	12.5	Yes
FFPE31	BRCA1_5382insC	Follicular	Fimbria	9.08	Yes
FFPE32	no mutation	Follicular	Fimbria	12.2	Yes
FFPE33	BRCA1 3533 INSG mutation	Follicular	Fimbria	8.74	Yes
FFPE34	BRCA1 185delAG	Luteal	Fimbria	16.1	Yes
FFPE35	BRCA1	Follicular	Fimbria	17.2	Yes
FFPE36	BRCA1 185delAG	Follicular	Fimbria	20.8	Yes
FFPE37	BRCA1 g. 56490A>G	Follicular	Fimbria	15.3	Yes
FFPE38	BRCA1 185delAG	Follicular	Fimbria	27.2	Yes

FFPE39	no mutation	Luteal	Fimbria	12.5	Yes
FFPE40	BRCA1 unknown mutation	Luteal	Fimbria	12.8	Yes
FFPE41	BRCA1 917delTT	Follicular	Fimbria	29.6	Yes
FFPE42	BRCA1 185delAG	Luteal	Fimbria	30.8	Yes
FFPE43	no mutation	Follicular	Fimbria	23.6	Yes
FFPE44	no mutation	Follicular	Fimbria	52.2	Yes
FFPE 45	BRCA1_101delGT	Follicular	Fimbria	9.12	Yes
FFPE 46	BRCA1_4603G-T	peri/post menopausal	Fimbria	26	Yes
FFPE 47	BRCA1_129del40	Luteal	Fimbria	25	Yes
FFPE 48	BRCA1_3450delCAAG	Follicular	Fimbria	35	Yes
FFPE 49	BRCA2	Follicular	Fimbria	40.8	Yes
FFPE 50	no mutation	Follicular	Fimbria	10.2	Yes
FFPE 51	no mutation	Follicular	Fimbria	21.2	Yes
FFPE 52	no mutation	Follicular	Fimbria	99.6	Yes
FFPE 53	no mutation	Follicular	Fimbria	29.2	Yes
FFPE 54	no mutation	Follicular	Fimbria	80.6	Yes
FFPE 55	no mutation	Luteal	Fimbria	*	Yes
FFPE 56	no mutation	Luteal	Fimbria	*	Yes
FFPE 57	no mutation	Luteal	Fimbria	9.8	Yes
FFPE 58	no mutation	Luteal	Fimbria	6.52	Yes
FFPE 59	no mutation	Luteal	Fimbria	*	Yes
FFPE 60	no mutation	Luteal	Fimbria	*	Yes
FFPE 61	no mutation	Follicular	Fimbria	*	Yes
FFPE 62	BRCA1	Follicular	Fimbria	16.6	Yes
FFPE 63	BRCA1	Luteal	Fimbria	86.6	Yes
FFPE 64	BRCA1	Follicular	Fimbria	15.4	Yes
FFPE 65	BRCA1	Follicular	Fimbria	11.6	Yes
FFPE 66	BRCA1 185delAG	Luteal	Fimbria	*	Yes
FFPE 67	no mutation	Follicular	Fimbria	*	Yes

FFPE 69	BRCA2_5783delT	Follicular	Fimbria	14	Yes
FFPE 70	no mutation	Luteal	Fimbria	*	Yes
FFPE 71	no mutation	Luteal	Fimbria	*	Yes
FFPE 72	no mutation	Luteal	Fimbria	*	Yes
	* Awaiting values from sequencing department				

Table2: The above cases are for the LCM FFPE project. All cases have had RNA quantified and RNA sequenced.

Cell Line	**BRCA mutation status**
iOSE 120 SV40, hTERT hRAS v12	control
iOSE 120 SV40 hTERT c-MYC	control
iOSE 267F SV40 hTERT hRAS v12	BRCA1 mut
iOSE 267F SV40 hTERT c-MYC	BRCA1 mut
iOSE 523 SV40 hTERT c-MYC	control
iOSE 592F SV40 hTERT c-MYC	BRCA1 mut
iOSE 523 SV40 hTERT hRAS v12	control
iOSE 592F SV40 hTERT hRAS v12	BRCA1 mut
FTE 3437 SV40 hTERT hRAS v12	control, 85yrs
FTE 3437 SV40 hTERT c-MYC	control, 85yrs
FTE 3313 SV40 hTERT c-MYC	BRCA2 mut, post
FTE 3798 SV40 hTERT hRAS v12	BRCA1 mut, 45yr
FTE 3798 SV40 hTERT c-MYC	BRCA1 mut, 45yr
FTE 3313 SV40 hTERT hRAS v12	BRCA2 mut, post
FTE 3619 SV40 hTERT hRAS v12	control, 48yrs
FTE 3793 3F CCNE1 c-MYC	BRCA1 mut, 47yrs
FTE 3793 3F PIK3CA-H1047R c-MYC	BRCA1 mut, 47yrs
FTE 3319 3F PIK3CA-H1047R CCNE1	control, 56yrs
FTE 3319 3F PIK3CA-H1047R hRAS v12	control, 56yrs
FTE 3793 3F hRAS v12	BRCA1 mut, 47yrs

FTE 3313 3F c-MYC	BRCA2 mut, post
FTE 3313 3F hRAS v12	BRCA2 mut, post
FTE 3793 3F PIK3CA-H1047R CCNE1	BRCA1 mut, 47yrs
FTE 3319 3F PIK3CA-H1047R c-MYC	control, 56yrs
FTE 3619 SV40 hTERT c-MYC	control, 56yrs
FTE 3793 3F PIK3CA-H1047R hRAS v12	BRCA1 mut, 47yrs
FTE 63857 3F PIK3CA-H1047R	control, 29yrs
FTE 63857 3F CCNE1	control, 29yrs
FTE 63857 3F hRAS v12	control, 29yrs
FTE 3313 3F PIK3CA-H1047R hRASv12	BRCA2 mut, post
FTE 63857 3F PIK3CA- H1047R hRASv12	control, 29yrs
FTE 3793 3F PIK3CA - H1047R	BRCA1 mut, 47yrs
FTE 3313 3F PIK3CA-H1047R	BRCA2 mut, post
FTE 3319 3F hRASv12	control, 56yrs
FTE 3319 3F PIK3CC-H1047R	control, 56yrs
FTE 3437 SV40 hTERT hRas v12 -IP	control, 85yrs
FTE 63857 3F hRasv12 -IP	control, 29yrs
iOSE 523 SV40 hTERT hRasv12 -IP	control
iOSE 3793 hRas v12 -IP	BRCA1 mut, 47yrs
iOSE 267F SV40 hTERT hRas v12 -IP	BRCA1 mut
FTE 3798 SV40 hTERT hRas v12 -IP	BRCA1 mut, 45yr
3F - (p53 DN R175H, E7, hTERT)	
CCNE1 - Cyclin E	
IP- intraperitoneal injections	

Table 3: Summary of injections to date. Intraperitoneal injections were performed in addition to mammary fat pad injections in NSG mice.

Project 4. Locate and characterize precursor lesions of "ovarian" cancer in a mouse model and explore the role of ovulation and changes in the microenvironment of the ovary and tube in "ovarian" carcinogenesis using human tubal xenografts in nude mice.
Research site: Johns Hopkins University
Project leader: Tian-Li Wang
Co-investigators: Ie-Ming Shih (JHU)

Section II. Progress to Date

Task 1. Locate the anatomic site and characterize the precursor lesions of "ovarian" cancer in the $TP53^{-/-}/Rb^{-/-}$ mouse model.

Task 1a. Mouse breeding and genotyping (1-18 months)
Progress: This task has been finished.

Task 1b. TP53/Rb KO mice study to locate the anatomic site of precursor lesions (1-15 months).
Progress: As discussed in the previous progress reports, this task has been modified and the final result has been published: J Pathol. 2014 Jul;233(3):228-37. This paper is highly cited as there are more than 59 citations since (based on Google Scholar).

Task 1c. Characterize the mouse precursor lesions using a variety of proposed methods (10-33 months).
Progress: This task has been completed and the data has been published and presented in the last progress report and will not be re-iterated in the current report. *J Pathol, 2014. PMID:24652535*

Task 2. Assess the biological effects of ovarian follicular fluid on human fallopian tube epithelium (FTE) and ovarian surface epithelium in a xenograft model.

Task 2a. Establish the human fallopian tube xenograft model (18-30 months).
Progress: We have finished exploring this model for the purpose of ovarian cancer research in mice and the results were shown in the previous progress report (with a figure illustration). So, the results will not be reiterated herein.

Task 2b. Collect human follicular fluid and primary characterization of the fluid (15-25 months).
Progress: We have collected at least 5 follicular fluids from women undergoing oocyte harvesting. We found that some of the fluid samples when incubated with human epithelial cells are associated with increased DNA damage as reflected by gH2AX immunofluorescence. We are still investigating the discrepancy among different samples.

Task 2c. Assess the biological effects of ovarian follicular fluids on FTE and OSE in a xenograft model (30-48 months).
Progress: As described in the previous reports, we have collected human follicular fluids and human tubal fluids and tested their DNA damaging effects on FT cultures. As described in the last progress report, we have performed the comet assay and gH2A staining and Western blot analyses to quantitate the damages in double strand DNA. Application of follicular fluid to the mouse xenograft model (Task a) appears challenging as the fluid may not penetrate into the grafted fallopian tube epithelium which is embedded by fibrotic tissues. In addition, we found that some of the fluid samples when incubated with human epithelial cells are associated with

increased DNA damage as reflected by gH2AX immunofluorescence. We are still investigating the discrepancy among different samples. Alternatively, we are considering an alternative study to expose the fresh human fallopian tube to the fluid and analyze DNA damage levels after exposure at different time points.

Task 3. Determine whether oral contraceptives (OCPs) and NSAIDs reduce the morphologic and molecular changes that are associated with early "ovarian" carcinogenesis.

Task 3a. To assess whether OCPs decrease the frequency of precursor lesions and/or delay tumor development (24-48 months)
Progress: We have finished this task and found that there was no difference in the experimental and control groups after exposure of OCPs.

Task 3b. To determine the effects of aspirin on reducing oxidative stress-induced molecular changes on human fallopian tube and/or on OSE (24-60 months).
Progress: This task has been modified as described in the previous progress reports. The results have been published in Clin Cancer Res 2015 Oct 15;21(20):4652-62. We demonstrate that lovastatin significantly reduced the development of STICs in mogp-TAg mice and inhibited ovarian tumor growth in the mouse xenograft model. Knockdown of prenylation enzymes in the mevalonate pathway recapitulated the lovastatin-induced antiproliferative phenotype. Transcriptome analysis indicated that lovastatin affected the expression of genes associated with DNA replication, Rho/PLC signaling, glycolysis, and cholesterol biosynthesis pathways, suggesting that statins have pleiotropic effects on tumor cells. The above results suggest that repurposing statin drugs for ovarian cancer may provide a promising strategy to prevent and manage this devastating disease.

Task 3c. Data analysis and preparation for publication (24-60 months).
Progress: Our next publication target will focus on reporting that biosphosphonate can reduce the STIC and HGSC formation in the OVGP1-statin mouse model.

Section III. Problem Areas for Project 4

There are no major problem areas noted for Project 4 as alternative approaches are proposed to address the same questions as originally proposed.

Section IV. Future Work in Project 4

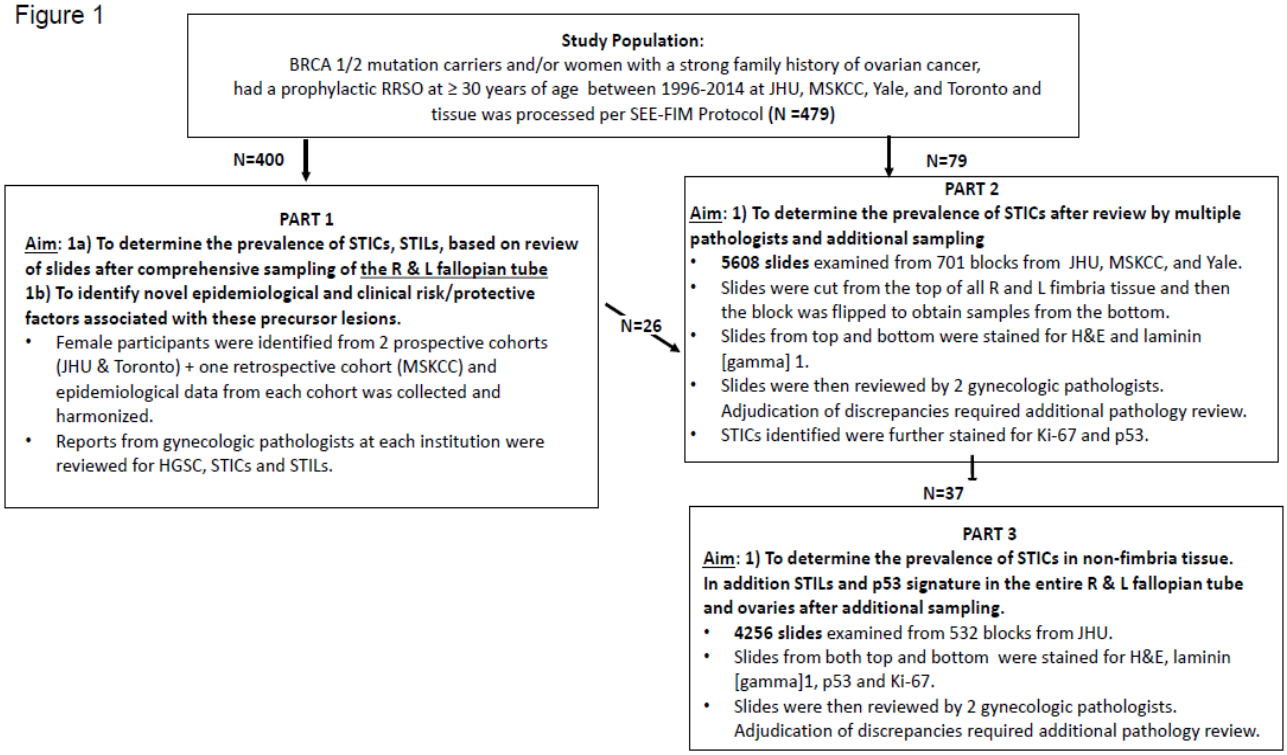
Project 4 has made substantial progress toward completing the proposed main tasks. However, Task 2 needs to be finished in order to meet the overall objective of the Consortium program. As originally proposed, Task 2 will assess the biological effects of ovarian follicular fluid on human fallopian tube epithelium (FTE) and ovarian surface epithelium in a xenograft model (Task 2a), collect human follicular fluid and primary characterization of the fluid (Task 2b) and assess the biological effects of ovarian follicular fluids on FTE and OSE in a xenograft model (Task 2c). During the no cost extension period, we are transplanting human fallopian tube segments directly to the mouse peritoneal wall. This experiment was delayed because till now we have accumulated sufficient knowledge and experience in establishing this novel surgical model. After repeated testing, we are not confident that this approach is feasible at this moment in providing a model to study the effects of follicular fluids or other chemoprevention compounds in this human/mouse hybrid model. We will also share with the science community about our protocol after publication.

Project 5: Determine the molecular and epidemiologic profile of putative precursor lesions in the fallopian tubes and ovaries from women at high-risk for ovarian cancer. In addition, Project 5 will determine if these biomarkers and associated precursor lesions are modifiable by oral contraceptives (OCPs) or anti-inflammatory agents, as OCPs in particular are known to prevent ovarian cancer and impact survival.
Research Site: Johns Hopkins University
Principal Investigator: Kala Visvanathan, MD, MHS
Collaborating Sites: University of Toronto (Steven Narod, MD and Patricia Shaw, MD: Co-investigators), Memorial Sloan Kettering Cancer Center (Douglas Levine, MD and Robert Soslow, MD: Co-investigator), Yale University (Vinita Parkash, MD, CGC: Co-Investigators)

Summary:
During the course of this project we had to make modifications to our original aims as previously reported in past progress reports due to limitations regarding feasibility. As a result we expanded Aim 1 and discontinued Aim 3. Described below is a summary of our study results that provide important contributions to the understanding of the etiology of STICs, their role in the development of ovarian cancer, and will inform future studies. This is followed our specific tasks.

Aim 1: This aim was expanded and divided into three sub-aims: Aim1a was a pilot study to evaluate prevalence of STICs, Aim 1b involved the creation of a large multicenter dataset to evaluate risk factors for STICs, and Aim 1c involved the creation of a large multicenter dataset to evaluate risk factors for ovarian cysts. *Figure 1* below provides a schematic for this aim.

Figure 1



All sub aims (1a-c) were completed. This project is the first and largest multicenter study to examine for risk factors associated with invasive cancer/STIC/STILs including lifestyle, reproductive factors, surgical procedures, and medication use in 400 high-risk women that had underwent bilateral risk reduction salpingo-oophorectomy plus comprehensive tissue processing of their entire tubes and ovaries. Surprisingly no risk or protective factors were associated with STICs/STILs after a thorough evaluation of epidemiological factors. Older age was

associated with having invasive HGSC but not STIC. Our findings were in contrast to the only study that examined for risk factors associated with STICs in a single institution. It is plausible that known risk/protective factors are associated with progression of STICs to HGSC and not the incidence of STICs. Another possibility is that the development of STICs are related to random mutations occurring by chance that target cancer drivers including *TP53*. Lastly the very large protective effect of observed between breast-feeding, oral contraceptive and ovarian cancer incidence among *BRCA1/2* mutation carriers may not be as strong for STICs/STILs. Details of the lesions are shown in table 1 below.

Table 1. Summary of lesions identified in the right and left fallopian tubes of 400 high-risk women.

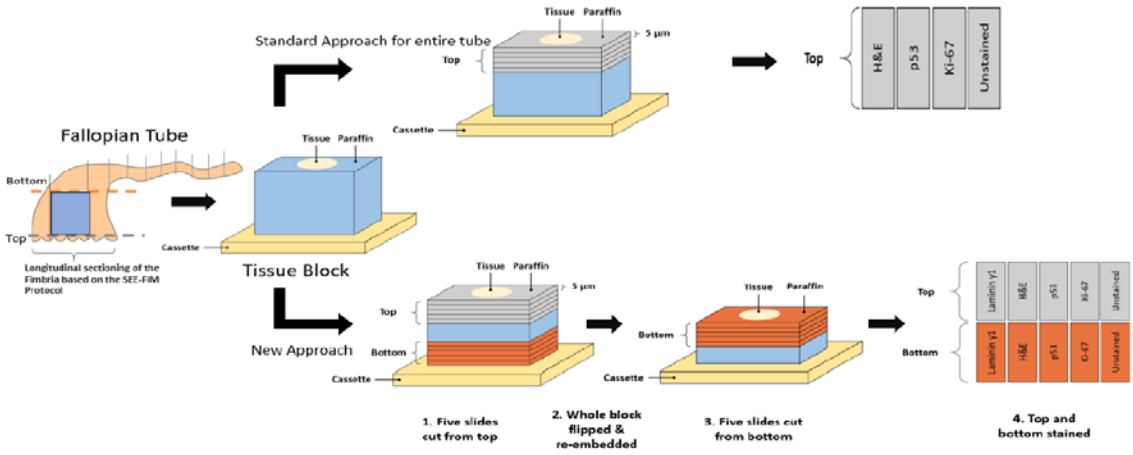
Lesion	STIC alone (n=12)	STIC + Invasive Carcinoma (n=2)	Invasive Carcinoma alone (n=4)	STIL (n=5)	No Lesion (n=355)	Total (n=400)
Mean Age at time of RRSO years, (SD)	50.9 (9.3)	60.4 (22.9) *	55.5 (9.7) *	48.1 (3.1)	47.9 (8.3)	48.3 (8.5)
Mean year of RRSO, (SD)	2004 (3.0)	2007 (4.9)	2004 (4.3)	2005 (2.4)	2007 (3.0)	2007 (3.0)
BRCA Status, n (%)						
BRCA 1 Positive	7 (58.3)	1 (50.0)	1 (25.0)	2 (40.0)	165 (46.5)	188 (47.0)
BRCA 2 Positive	5 (41.7)	1 (50.0)	3 (75.0)	3 (60.0)	157 (44.2)	178 (44.5)
BRCA 1/2 Negative	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	18 (5.1)	19 (4.8)
BRCA status unknown	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	15 (4.2)	15 (3.7)
Site, n (%)						
JHU	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	70 (19.8)	71 (17.7)
MSKCC	3 (25.0)	1 (50.0)	0 (0.0)	0 (0.0)	128 (36.3)	134 (33.5)
Toronto	9 (75.0)	1 (50.0)	4 (100.0)	5 (100)	155 (43.9)	195 (48.8)

Abbreviations: STIC, serous tubal intraepithelial carcinoma, STIL, serous tubal intraepithelial lesion, SD, standard deviation; RRSO, risk reduction bilateral salpingo-oophorectomy; BRCA, breast cancer susceptibility gene; JHU, Johns Hopkins University; MSKCC, Memorial Sloan Kettering Cancer Center; Toronto, University of Toronto; STIC, serous tubal intraepithelial carcinoma; STIL, serous tubal intraepithelial lesion

* Significant difference between mean age of STIC + Invasive Cancers versus No lesion ($p = 0.04$) and mean age of Invasive Cancer versus No lesion = ($p = 0.009$) but no difference in mean age of STIC versus No lesion ($p = 0.07$).

To gain a better understanding of the prevalence and location of p53 signatures, STICs, STILs and invasive cancers we performed more extensive tissue sampling (by flipping the tumor blocks as shown in *Supplemental 1: Diagram* below) and pathology review by multiple pathologists on almost 10,000 slides (with and without lesions) in a subset of 105 high-risk women. Extensive sampling and pathology review of this kind has not been previously done in tissue from cancer free women. By incorporating a review by multiple pathologists we were able to ensure diagnostic reliability of all the lesions. This also has not been done before.

Supplemental 1: Diagram describing the approach used for additional sampling of tissue blocks in Part 2 and Part 3 of the study.



The results from additional sampling are described below in table 2. A number of novel observations were made. We observed that STICs/STILs occur at equal prevalence in the fimbria and non-fimbriated part of the tube which raises the question of whether all STICs have the same propensity to translocate to the ovary or peritoneum and multiple lesions in 50% of cases. We also found that multiple lesions were present in 50% of cases suggesting that women with multiple STICs/STILs may be at greater risk for STIC migration to occur to the ovary or peritoneum and therefore the development of HGSC when compared to a woman with a single lesion. Women with p53 signatures in the fimbria compared to the remaining tube were significantly older suggesting that all p53 signatures may not necessarily lead to HGSC. Further studies are now needed with incorporating extensive sampling of the tube to build on our findings. In addition, we did not identify any p53signatures, STICs or STILs in the ovaries but did observe 15 inclusion cysts. They were not associated with any risk factors.

Table 2. Description of lesions in the fallopian tube after additional sampling (n = 105)

STICs in the right and left fimbria

Case ID	Lesion	Year of RRSO	Age	BRCA status	Site	Position	Location	Laminin γ1	p53	Ki-67	Fimbria Blocks #	Tube Blocks #	Path Report	Second Reviewer Agreement
1	STIC STIC & Cancer	2011	76	BRCA2	MSKCC	Top Bottom	Right Fimbria Right Fimbria	Positive Positive	Diffuse staining Diffuse staining	<10% <10%	2	0	STIC; p53 diffuse & Ki-67 ≥10%	Yes
2	STIC	2011	49	BRCA1	MSKCC	Top	Right Fimbria	Positive	Absence/ non-	≥10%	2	0	No histologic	No

Abbreviations: STIC, serous tubal intraepithelial carcinoma; RRSO, risk reduction salpingo-oophorectomy; BRCA, breast cancer susceptibility gene; MSKCC, Memorial Sloan Kettering Cancer Center; STIL, serous tubal intraepithelial lesion; JHU, Johns Hopkins University

<u>Aim 2:</u> Molecular and Epidemiological Characterization of STICs and p53 signatures

This aim was originally to be completed based on STIC samples collected in Aim 1 but given the low prevalence of STICs in Aim 1 this was not feasible. Of note prior studies at the time of the grant submission had reported much higher prevalence of these lesions. This has now been found to not be the case.
We still believe it is important to validate the molecular findings from project in a larger sample size as originally proposed. However, to do this we have had to reach out to more than 20 sites across the country to obtain 50 STICs, 50 adjacent normal tissue,50 matched controls and 50 p53 signatures. Each control was carefully matched on age (+/- 2 years), date of procedure (+/- 2 years) to the case and study site. We have

executed MTAs with all sites and obtained tissue and de-identified clinical information for the majority. Due to project funds running out, we were unable to stain and evaluate the markers as planned. We are in process of seeking additional funding that will allow us to complete this aspect of the project. Our potential list has been refined based on the results from project 1 includes γH2AX, pCHK2, 8-OHdG, telomere length, CD45+ lymphocytes, Ki-67 index, protein expression of p53, p21, cyclin E1, Rsf-1, fatty acid synthase, laminin C1 and progesterone receptor A and B.

Specific Tasks:

Task 1. Obtain approval for the addition of questions for prospective collection from all site IRBs (Aim 1) and also for the transfer of existing epidemiological and clinical data to JHH de-identified for the retrospective study (Aim 1 and 2). Obtain approval from USAMRAA for human subject's research for entire protocol (Months 1-6).

Progress: This task was **completed**.

Task 2. Identification of study population for retrospective study based on eligibility criteria from all sites (Months 1-6).

Progress: This task was **completed**. All epidemiological data and pathology reports on 400 cases was identified, collected, entered, cleaned and analyzed.

Task 3. Comprehensive pathology review of samples. A comprehensive master dataset that includes epidemiological and pathological data from approximately 550 women to be used for cross-sectional and case control study designs will be created (Months 6-18).

Progress: Task **completed**. We compiled an epidemiological and pathological database of 400 high-risk women due to the low prevalence of STICs we decided not add further to this database.

Task 4. Analyses for Aims 1a and b will be completed to determine the prevalence, location and frequency of the specific lesions in the FTEs (STICs, STILs, and p53 signatures) and OSE, as well as CICs in the ovaries, overall and by BRCA mutation status. Correlations between each of the different types of lesions will be determined. Manuscript preparation Aims 1a and b (Months 6-18).

Progress: These have been **completed** and a manuscript has been submitted and is under review. This is described above and in the attached manuscript.

Task 5. Analyses for Aim 1c the association between exposures (both risk and protective factors for ovarian cancer) and each type of prevalent lesion will be examined through cross-sectional studies. Manuscript preparation for Aim 1c (Months 12-24).

Progress: This task was **completed**. See attached manuscript.

Task 6. As described above, merging of epidemiological and pathological data for aims 1 and 2 will be performed and a comprehensive master dataset to be used from which matched case-control studies will be identified for Aim 2 (Months 6-18).

Progress: This task has been **completed**. See attached manuscript.

Task 7. Case-control sets for molecular analyses of the FTE and ovary samples from Aim 1 will be used to assess a panel of markers. Cases will be defined by the lesion/region of interest (i.e. STICs, STILs, p53 signatures, CICs, and/or morphological changes in OSE). Controls will vary depending on the analysis: marker expression within a specific lesion will be compared to (1) adjacent normal tissue from the same case and (2) normal tissue from women with no identifiable lesion. For the latter comparison, 2 controls will be matched to

each case from the same research site, within +/- 2 years of age at the time of surgery. To conserve this valuable tissue, and maximize efficiency, the same controls will be reused for subsequent case-control analyses where possible (Months 18-24).

Progress: This task has been **completed**. We reached out to more than 20 sites across the country to obtain 50 STICs, 50 adjacent normal tissue,50 matched controls and 50 p53 signatures. Each control was carefully matched on age (+/- 2 years), date of procedure (+/- 2 years) to the case and study site. We have executed MTAs with all sites and obtained tissue and de-identified clinical information for the majority.

Task 8. Molecular analyses to determine molecular profile of each lesion type will be performed on case control sets. The resulting laboratory results will be merged with existing epidemiological data and analyses will be performed (Months 24-48).

Progress: This has not been completed and will require additional funding as the funds were used to acquire the tissue.

Task 9. Statistical analyses of case-control sets will be completed for each lesion type and panel of markers. Multiple manuscripts will be generated from Aim 2 based on the different lesions and also markers. These will be prepared and submitted (Months 24-48).

Progress: This will also occur once we have more funding.

Task 10-13. These tasks are not relevant anymore based on the modification of our original aims.

Task 10. High-risk women considering BSO in the next 2 years, and meeting the same eligibility criteria used in Aims 1 and 2, will be prospectively enrolled at each site. Information on NSAIDs, OCP and Vitamin E use as well as other ovarian cancer risk/protective factors will be collected through questionnaires completed within 2 years of surgery (N ~ 300-400) (Months 6-48).

Task 11. Ongoing comprehensive pathological review and merging of epidemiological and pathological data will occur (Months 6-50).
Task 12. Complete merging and data management of Master data set (Months 48–50).

Task 13. Analysis of prospective data with respect to NSAIDs and OCP use will be completed: a) Associations between OCP/NSAID/Vitamin E use and prevalence of lesions will be evaluated, overall and stratified by BRCA mutation status as in Aim 1, and b) associations between use of these substances and molecular markers identified in Aim 2. Manuscripts for Aim 3 will be prepared and submitted. This aim will be informed from data generated in Aims 1 and 2 (Months 48-60).

Administration (Admin) Core, Biostatistics/Bioinformatics/Epidemiology (BBE) core, and Pathology (Path) Core

In the past years, the three cores have been integrated to each other; therefore, we combine their progress in this section to avoid reiteration. Because the tasks related to the three cores are relatively generic and are applicable for the entire research period, so we will rather report the specific progress related to the cores.

Section II. Progress to Date:

The Administration Core led by Drs. Wang (Dr. Kurman retired in June 30, 2017) and Shih continues providing all the administrative support to all 5 research projects. The progress in this Core has been described in prior progress report. The new activity during the no cost extension period is that we convened the last DoD Ovarian Cancer Consortium Conference in New York City (please see the conference brochure for details in the appendix).

The BBE core provides excellent support to the current study design and assists statistical analysis for all the data generated in the past 6 months. The BBE core is currently working with investigators to prepare for manuscripts to be submitted to journal publications in the future.

The Path Core led by Drs. Visvanathan, Soslow, Kurman and Shih continues to serve as our central collection resource for the various projects. Highlights of Path Core progress this past period include:

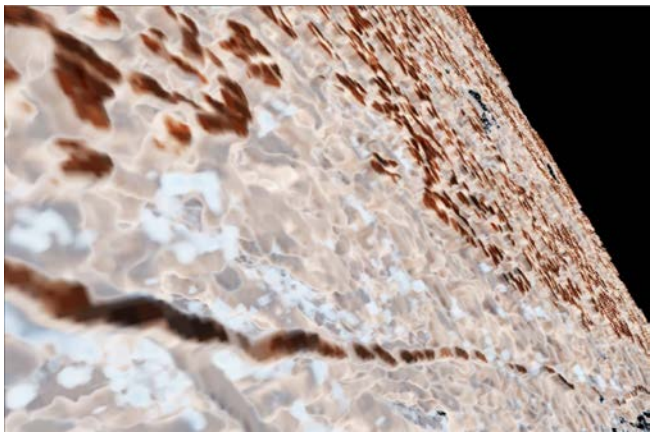
Section III. Problem Areas for the Cores

So far, we do not expect major problems for the three cores.

Section IV. Future Works in Cores

In the no cost extension period, there will be no future works pending.

Resource Sharing Plan. Per the DoD OCRP guideline, the Administrative Core and Pathology Core will work together for resource sharing from those generated by the five projects through this consortium grant in the past 6 years. The resource to be shared with science community includes data, reagents, animal models and cell lines/primary cultures. The administrative core and the Biostatistics Core will be responsible to deposit the RNAseq and other related raw data, and the Pathology Core will gather and centralize a copy of aliquots of reagents and cell lines/primary cultures in our bank located at the main consortium site, the Johns Hopkins Medical Institutions.



Section V- Administrative Comments (Optional)

Dr. Kurman retired from the overall PI in this constortium and Professor Tian-Li Wang from the Department of Pathology has become the PI upon approval by the Johns Hopkins Medical Institutions and OCRP, DoD.