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14. ABSTRACT The proposal was designed to address three issues. First could we identify stem cells from the fallopian tube, including from patients with high grade serous cancer (HGSC). Second, could we link the molecular abnormalities in cancer associated stem cells and validate them in pathologic material, specifically in what we feel are stem cell outgrowths or SCOUTs and third could we identify molecular alterations that would place the oviduct or the patient at risk for HGSC. In essence we wished to drill down to the cell of origin and link it to cancer risk, identifying an assay that could predict the presence of cancer by analyzing lower genital tract fluids or other samples. All of these aims have been addressed and our studies have reinforced the likelihood that novel pathways to ovarian cancer exist, but evidence points to more than one mechanism and site of origin.					
15. SUBJECT TERMS HGSC = high grade serous cancer; Fallopian tube; BRCA; Tp53, SCOUT = stem cell outgrowth; STIC = serous tubal intraepithelial carcinoma; STIN = serous tubal intraepithelial neoplasia.					
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1. INTRODUCTION

The proposal was designed to address three issues. First could we identify stem cells from the fallopian tube, including from patients with high grade serous cancer (HGSC). Second, could we link the molecular abnormalities in cancer associated stem cells and validate them in pathologic material, specifically in what we feel are stem cell outgrowths or SCOUTs and third could we identify molecular alterations that would place the oviduct or the patient at risk for HGSC. In essence we wished to drill down to the cell of origin and link it to cancer risk, identifying an assay that could predict the presence of cancer by analyzing lower genital tract fluids or other samples. All of these aims have been addressed and our studies have reinforced the likelihood that novel pathways to ovarian cancer exist, but evidence points to more than one mechanism and site of origin.

2. KEY WORDS

Ovarian Cancer
Fallopian tube
High grade serous carcinoma
Stem cell
Serous tubal intraepithelial carcinoma
Secretory cell outgrowth
Serous tubal intraepithelial lesion

3. ACCOMPLISHMENTS

Major goals.

AIM 1: To isolate, grow in culture, and compare stem cells from the fallopian tubes of patients with and without malignancy.

AIM 2: To link and validate the molecular disturbances observed in cancer associated stem cells in pathologic material, specifically in an entity we have described called the stem cell outgrowth or SCOUT.

AIM 3: To exploit the molecular alterations discerned to make molecular probes that will detect those alterations that place women at risk for the disease, either in the fallopian tubes or lower genital tract fluids.

What was accomplished

AIM 1: 1) Major activity: isolate and grow fallopian tube stem cells in culture. 2) The specific objective was to identify stem cell characteristics that distinguished tumor associated (but normal appearing) stem cells from normal controls. 3) Significant results: a) We successfully cloned stem cells from normal fallopian tubes and showed that these cells were capable of both ciliated and squamous differentiation, in parallel with the histology of the fallopian tube (Figure 1). b) We generated a "stem cell" specific signature by comparing gene expression between undifferentiated stem cells and those grown on an air-liquid interface, which permitted ciliated differentiation. This was the first ever successful cloning, propagation and maturation of fallopian tube stem cells¹. What we have not accomplished is to show that stem cells from normal tubal epithelium in cancers can be distinguished from

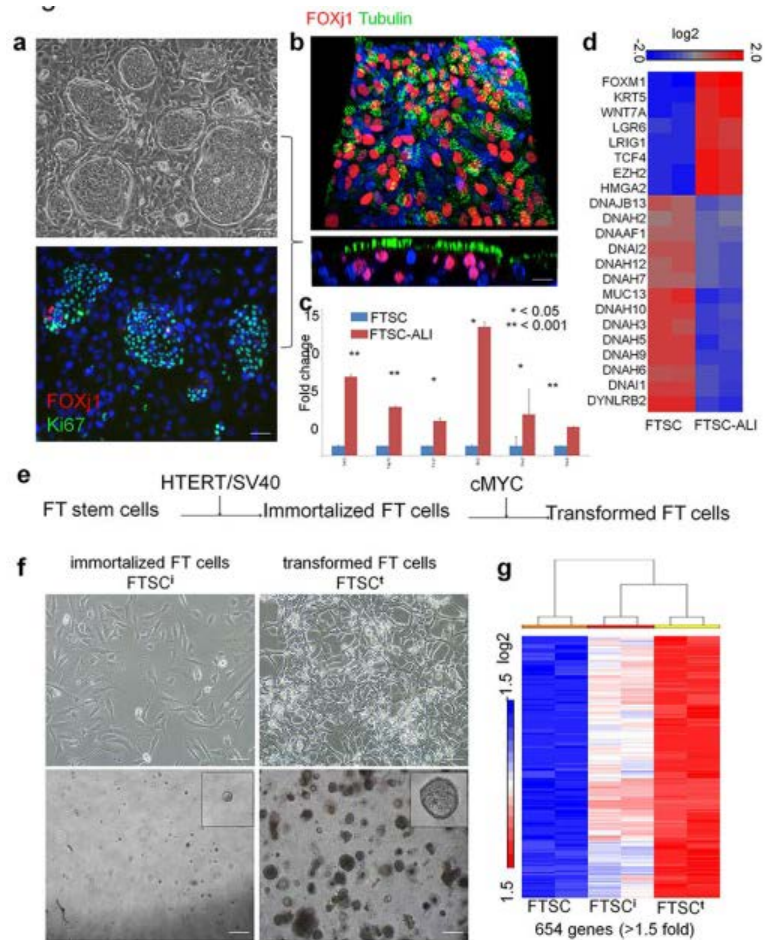


Figure 1. Upper panels show differences in expression between putative stem cells and cells undergoing in vitro ciliated differentiation. Lower panels depict progressive changes in gene expression with immortal and transformed cells

epithelium from normal controls. b) An additional achievement, however, was to identify potential stem cell markers that were novel and might be used to unearth potential stem cells in the general pool of non-ciliated tubal epithelium². Moreover, we showed remarkable parallels between stem cells and putative stem cell outgrowths as well as HGSC precursors. c) Another added achievement was to demonstrate a parallel between immortalized and transformed stem cells and precursor and malignant HGSCs in the fallopian tube (Figures 2&3). D) Still another added achievement was a successful experiment focusing on propagating potential cancer stem cells. In this study we identified subpopulations that were Taxane resistant and were able to identify the same cells in Taxane naive cell cultures (Ning et al, submitted). This suggests that there is a small population of chemo-resistant cells that is inborn and not created by chemotherapy per se.

AIM 2 1) Major activity: Translating the in vitro findings to histopathology. 2) Objective : To link the disturbances observed in isolated stem cells to stem cell outgrowths (SCOUTs) and serous cancer precursors (STICs). This was shown in the papers by Ning et al and Yamamoto et al^{1,2}. Additional achievements: During these studies we took the opportunity to address an issue fundamental to the aims, which is the origin of HGSC. Because a high grade precancerous process (or STIC) can only be uncovered in subset of cancers, we felt it important to address this issue, the goal being to better understand the potential origins of these neoplasms. This was done in a series of studies. First, we showed a potential dualistic model for HGSC with a lower association with STIC seen for tumors with certain morphologic features³ (Howitt 2015). This suggests that there could be more than one pathway to HGSC including one where STIC is not the primary predecessor. Second, we showed that certain histologic patterns were associated with specific gene mutations (Ritterhouse 2016) again suggesting more than one pathogenetic route to HGSC. Third, we recently sequenced cases with bilateral STICs and have shown them to contain identical p53 mutations. This raises the critical question that not all STICs develop de novo but may signify mucosal metastases from either the opposite tube or another site (Meserve et al in preparation). Fourth, we have concluded the preliminary phase of an ambitious project that has exhaustively analyzed fallopian tubes of women with HGSC but no STIC. In these tubes we have seen non-cancerous epithelium with p53 mutations. On comparing the p53 mutations status between these non-cancerous epithelia and the associated (and physically removed) HGSCs we have discovered identical mutations. This suggests the possibility that pelvic HGSCs could be derived from minor atypias within genetically altered stem cell proliferations (Figure 2). This has potentially profound implications in that it suggests that the serous carcinogenic sequence can initiate in the tube but continue beyond the confines of the oviduct at an unknown pelvic location (Soong et al manuscript in preparation).

AIM3: 1) Major activity: To develop a means to detect the presence of biomarkers unique to serous cancer or serous cancer risk in the uterus or lower genital tract. Objective: To employ deep sequencing to identify p53 mutations in the lower genital tract tissues or fluids that would indicate the presence of an upper genital tract neoplasm. Significant results: We decided to use a novel approach to this problem by first identifying cases of HGSC and then searching the archive for prior formalin-fixed, paraffin embedded endometrial specimens from diagnostic procedures. DNA from serial sections of this material was extracted and then analyzed on a platform targeting p53 mutations. These mutations were compared to those found in the tumors at a later date. We identified 5 samples in which information was available. In two (40%) we detected p53 mutation in prior endometrial samples that matched those found in the subsequent tumor. The intervals from detecting the mutations to the diagnosis of the tumors were 2 weeks and 2 months.

What opportunities for training and professional development has the project provided?

This grant has provided opportunities to several young investigators both at BWH and at collaborating institutions, including the following:

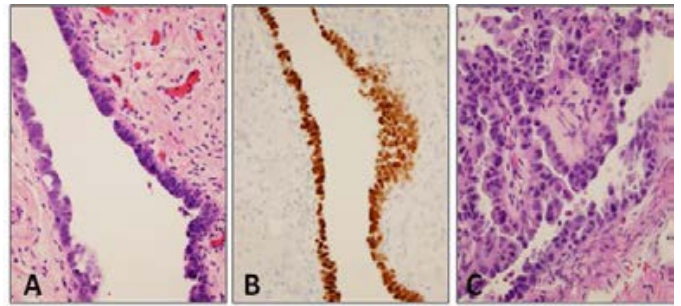


Figure 2. A mild atypia is the only abnormality in the tubal mucosa (A) of a patient with widespread metastatic HGSC (C) and stains strongly for p53 (B). Both lesions shared an identical p53 mutation following sequence analysis, supporting the concept of "precursor escape" (Soong R, Howitt BE, Crum CP unpublished).

- 1) Collaboration in year 1 with the Xian laboratory at the Jackson laboratory (Farmington CT). This collaboration centered on sharing of samples with the Xian laboratory, primarily examining fallopian tubes for stem cells and characterizing putative stem cell in the fallopian tube. Young investigators Yamamoto and Ning were first authors on publications coming from this collaboration.
- 2) Career development for Dr. Xian (collaborator in the first year). Dr. Xian has recently obtained a Teal award stemming in part from opportunities created during this collaboration.
- 3) Career development for Brooke Howitt at BWH. Dr. Howitt is a young faculty member at BWH who was involved in several projects including genomic analysis of ovarian cancers, and was the lead author on a study proposing a dualistic model of high grade serous carcinogenesis. She is currently applying for funding to expand her protected time for ovarian cancer research.
- 4) Career development for visiting scholar Jan Brouwer. Mr. Brouwer is an MD PhD candidate from the Netherlands who worked for 6 months on a project dissecting the immunophenotype of putative stem cells in the fallopian tube.
- 5) Career development for Kyle Strickland, Thing Rinda Soong, and Lauren Ritterhouse. These trainees have been involved in projects supported by this grant. Dr. Strickland will be taking a position at Duke in gynecologic pathology and cancer research, Dr. Ritterhouse will be joining the Pathology Department at the University of Chicago Medical Center in Molecular Diagnostics and Dr. Soong is scheduled to spend the next year in clinical and research in breast and gynecologic neoplasia.
- 6) Support of colleagues. In addition to the above, we have supported colleagues at the Dana Farber Cancer Institute, including Dr. Alan D'Andrea's laboratory.

How were the results disseminated to the communities of interest?

Results were published in the pathology and gynecology journals and presented at yearly meetings (see Products below)

How do you plan during the next reporting period to accomplish these goals?

This is the second year of the grant. However, we received a no-cost extension and will file a final report at the end of March 2017.

4. IMPACT

What was the impact on the development of the principal discipline of the project?

The purpose of this project was broaden our understanding of the cells involved in the pathogenesis of high grade serous cancer. It is currently assumed by many that the fallopian tube is the only source of these tumors - prompting efforts to prevent cancer by salpingectomy alone - yet we cannot prove this based on the pathologic evidence. The stem cell work has elucidated immuno-phenotypes that bear further study as cancer stem cells that can be searched for in extra tubal site such as the ovary. Moreover, the studies of fallopian tubes from women with cancer raise the intriguing question that earlier precursors might escape to produce tumors elsewhere. The identification of mutations in lower genital tract samples has raised the hope that a molecular Pap smear will be possible. However, our identification of p53 mutations in normal tubal mucosa raises concerns about the specificity of such a test, which is becoming more obvious.

What was the impact on other disciplines?

These studies are highly relevant to the epidemiology of ovarian cancer and expectations from surgical approaches.

What was the impact on technology transfer?

The discovery of genes deregulated in putative stem cells (Yamamoto et al) has been made public.

What was the impact on society beyond science and technology?

The fallopian tube and its role in ovarian cancer has had broad impact on women in general including those with or without genetic predisposition. The publications from this group have always addressed the strengths and limitations of the fallopian tube hypothesis with an eye on patient care.

5. CHANGES/PROBLEMS:

We encountered no major challenges in completing this work.

6. PRODUCTS

Publications

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Presentations, conference publications

Past presentations

Jelena Mirkovic, Amy DiVasta, Stacey Missmer, Brooke Howitt, Christopher Crum, Marc Laufer, Sara Vargas. The Histologic Spectrum of Adolescent Endometriosis. USCAP meeting, Boston, March 2015

Andre Pinto, Brooke Howitt, Christopher Crum. The Variable Spectrum of Tubal Intraepithelial Neoplasia in Women With High Grade Serous Carcinoma. USCAP meeting, Boston, March 2015

Lauren Ritterhouse, Christopher Crum, Lynette Sholl, Neal Lindeman, Brooke Howitt. Morphologic and Molecular Evaluation of Extra-Uterine Mullerian Carcinoma. USCAP meeting, Boston, March 2015

Presentations at the USCAP 2016 meeting

Emily E Meserve, Jelena Mirkovic, James R Conner, Eric Yang, Brooke E Howitt, Christopher P Crum. Detection of serous tubal intraepithelial carcinoma (STIC) in incidentally removed fallopian tubes from low-risk women.

Thing Rinda Soong, Christopher P Crum, Brooke E Howitt. Serial Sectioning of Distal Fallopian Tubes and its Role in the Discovery of Occult Serous Tubal Intraepithelial Carcinoma in Women with High Grade Ovarian Serous Carcinoma.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name:	<i>Wa Xian, PhD</i>
Project Role:	<i>Co-investigator, Assistant Professor, Center for Stem Cell & Regenerative Medicine CPRIT Scholar in Cancer Research, University of Texas Health Sciences Center,</i>
Researcher Identifier	<i>NA</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Xian collaborated on the fallopian stem cell culture and stem cell studies.</i>
Funding Support:	<i>Currently recipient of a Teal Award</i>

8. SPECIAL REPORTING REQUIREMENTS

Not applicable

9. APPENDICES

Selected manuscripts

Howitt et al 2015

Ning et al 2014

Yamamoto et al 2016

Sherman et al 2016

Ritterhouse et al 2016

Evidence for a Dualistic Model of High-grade Serous Carcinoma

BRCA Mutation Status, Histology, and Tubal Intraepithelial Carcinoma

Brooke E. Howitt, MD,* Suchanan Hanamornroongruang, MD,† Douglas I. Lin, MD, PhD,*
James E. Conner, MD, PhD,* Stephanie Schulte, MD, PhD,* Neil Horowitz, MD,‡§
Christopher P. Crum, MD,* and Emily E. Meserve, MD, MPH*

Abstract: Most early adnexal carcinomas detected in asymptomatic women with germline *BRCA* mutations (*BRCA*⁺) present as serous tubal intraepithelial carcinomas (STIC). However, STICs are found in only ~40% of symptomatic high-grade serous carcinomas (HGSCs) and less frequently in pseudoendometrioid variants of HGSC. Consecutive cases of untreated HGSC from *BRCA*⁺ and *BRCA*[−] women with detailed fallopian tube examination (SEE-FIM protocol) were compared. STIC status (+/−) was determined, and tumors were classified morphologically as SET (“SET”, >50% solid, pseudoendometrioid, or transitional) or classic predominate (“Classic”). SET tumors trended toward a higher frequency in *BRCA*⁺ versus *BRCA*[−] women (50% vs. 28%, *P* = 0.11), had a significantly younger mean age than those with classic HGSC in *BRCA*[−] women (mean 56.2 vs. 64.8 y, *P* = 0.04), and displayed a better clinical outcome in both groups combined (*P* = 0.024). STIC was significantly more frequent in tumors from the *BRCA*[−] cohort (66% vs. 31%, *P* = 0.017) and specifically the *BRCA*[−] tumors with classic morphology (83%) versus those with SET morphology (22%, *P* = 0.003). Overall, several covariables—histology, *BRCA* status, age, coexisting STIC, and response to therapy—define 2 categories of HGSC with differences in precursor (STIC) frequency, morphology, and outcome. We introduce a dualistic HGSC model that could shed light on the differences in frequency of STIC between symptomatic and asymptomatic women with HGSC. This model emphasizes the

need for further study of HGSC precursors to determine their relevance to the prevention of this lethal malignancy.

Key Words: fallopian tube, neoplasia, serous carcinoma, *BRCA*, endometrioid, tubal intraepithelial carcinoma, risk-reduction salpingo-oophorectomy

(*Am J Surg Pathol* 2015;39:287–293)

In the last decade, the origin of ovarian cancer has become the subject of intense study, and the distal fallopian tube has emerged as a potential origin for a significant proportion of high-grade serous carcinomas (HGSCs).^{1,2} Evidence in support of the distal fallopian tube as a site of origin has been (1) the discovery of tubal epithelial atypia in women with *BRCA1* or *BRCA2* mutations, (2) detection of high-grade serous tubal intraepithelial neoplasia (STIC) in risk-reducing salpingo-oophorectomies (RRSOs), (3) the finding of STIC in fallopian tubes of women with advanced carcinoma, and (4) identification of a credible precursor spectrum spanning both normal and neoplastic tubal mucosa.^{1,3–5} The latter has been characterized by evidence of DNA damage, *TP53* mutation, and progressive molecular perturbations that have been reproduced in both cell culture and animal models.^{6–8}

The percentage of HGSCs whose origins can be traced to the distal fallopian tube has increased, in part attributable to the use of sampling protocols (SEE-FIM) that more thoroughly examine the distal fallopian tube and fimbria. Identification of STIC supports, if not confirms, a tubal origin in 18% to 60% of cases of advanced or symptomatic HGSCs.² Still, in a significant percentage of cases, a tubal carcinogenic sequence has not been confirmed by detection of an intramucosal carcinoma. In contrast, when HGSCs are discovered early or in asymptomatic women (RRSO), approximately 80% coexist with STIC.^{9–11}

In recent publications, the spectrum of HGSC has been expanded to include endometrioid or endometrioid-like (pseudoendometrioid) tumors, largely because of the observation of an identical immunophenotype using p53, PTEN, and Pax2 as well as similar rates of *TP53* mutation.¹² Of interest, Roh et al¹³ found that the

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C.P.C. and E.E.M. contributed equally.

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endometrioid subset had a lower frequency of associated STIC. This finding was of borderline statistical significance but raised the possibility that certain tumor morphologies might be less likely to arise from STIC. Recently, Soslow et al¹⁴ reported that particular patterns of HGSC, namely solid, pseudoendometrioid, and transitional (SET; Fig. 1), were seen more commonly in association with *BRCA1/2* mutations. This, combined with the observations of Roh and colleagues, suggested that endometrioid or SET histology might not only be more commonly found in women with *BRCA* mutations but paradoxically less likely to be associated with a STIC as currently described. The purpose of this study was to examine this paradox from the perspective of symptomatic malignancies in women with (*BRCA*⁺) and without (*BRCA*⁻) germline mutations in the *BRCA1* or *BRCA2* genes.

MATERIALS AND METHODS

Patient Samples and Case Selection

This study was approved by the institutional review boards at Brigham and Women's Hospital (BWH) and Dana Farber Cancer Institute (DFCI). All cases of HGSC resected at BWH from 2005 to 2013 were identified from archival records. Careful examination of the tubes and ovaries, including the SEE-FIM protocol, was performed in all cases as previously described.³ This cohort was cross-indexed with genetic testing records in the Center for Cancer Genetics and Prevention at DFCI to identify 2 cohorts of women with HGSC: those who were confirmed to have *BRCA1/2* germline mutations and those documented to be negative for these mutations. Patients who received neoadjuvant chemotherapy were excluded from this study. Between 2005 and 2013, 387

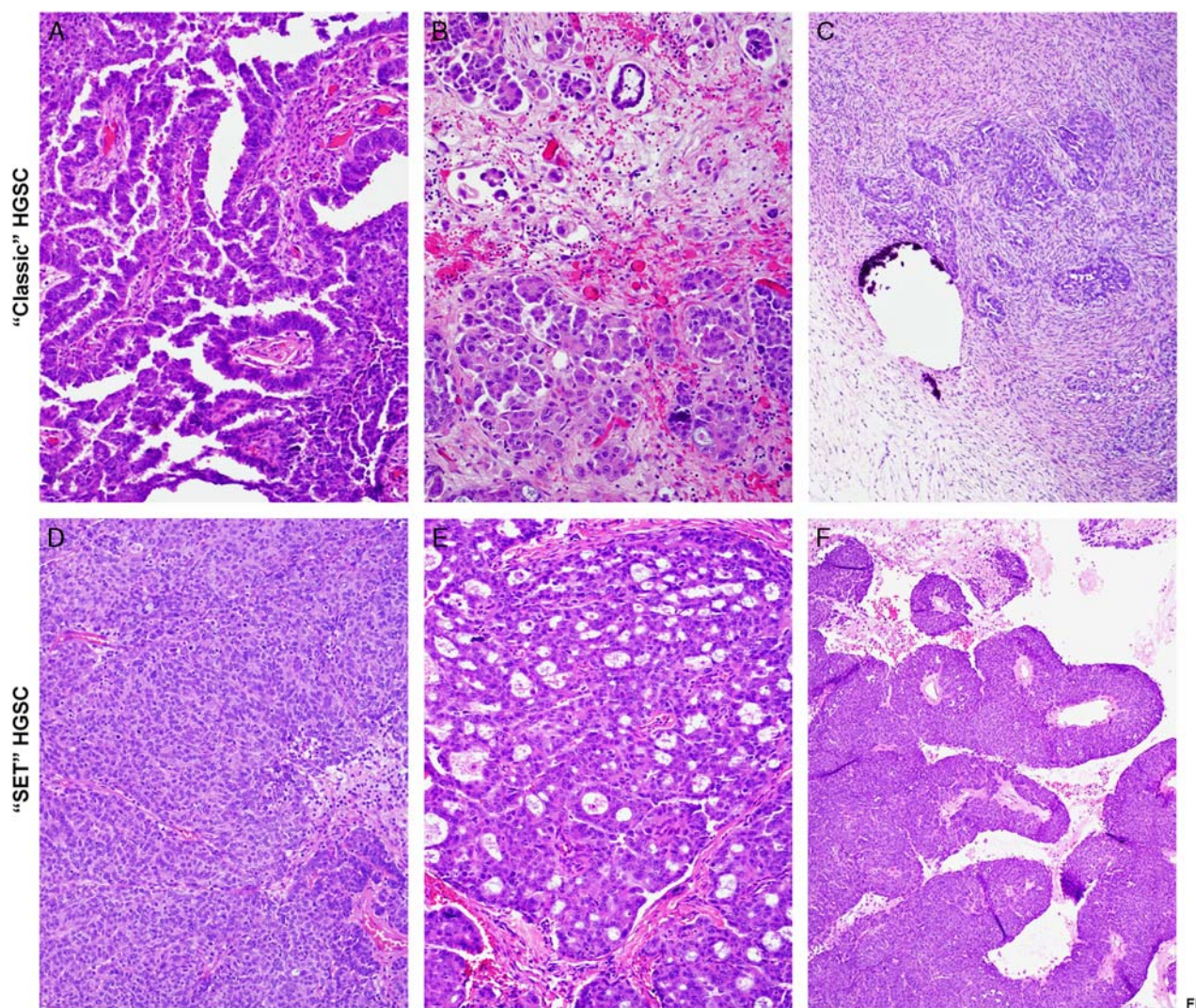


FIGURE 1. Histologic features of HGSC. Classic patterns include papillary (A), micropapillary (B), and infiltrative (C). SET patterns include solid (D), endometrioid-like (E), and transitional (F).

patients underwent surgery at BWH for a diagnosis of HGSC. Within this group, 116 underwent germline testing for *BRCA1* or *BRCA2* mutations. A germline mutation in either *BRCA1* or *BRCA2* (*BRCA*⁺) was detected by direct sequence analysis in 47 patients. No *BRCA1* or *BRCA2* germline mutation was detected in 69 patients (*BRCA*[−]). Forty-one patients had undergone neoadjuvant chemotherapy treatment (10 *BRCA*⁺, 31 *BRCA*[−]) and were excluded from the study. Slides were unavailable for histologic review in 17 cases (11 *BRCA*⁺, 6 *BRCA*[−]). Clinical outcome data including the time to last follow-up, and clinical status at last follow-up were extracted from the electronic medical record.

Histologic Review and Classification

Invasive Tumors

Tumors were reviewed without knowledge of their *BRCA* mutation status and classified as previously described into the following groups by 2 coauthors (B.E.H., C.P.C.):

- **Classic Predominate HGSC Histology ("Classic"):** >50% of the tumor demonstrates papillary, micropapillary, or infiltrative architecture, and often desmoplastic stroma.
- **SET Predominate HGSC Histology ("SET"):** >50% of the tumor displays 1 or more variant features, including solid growth, gland formation, and papillary transitional patterns.

The percentage of tumor containing SET versus classic histology was estimated in each case (in increments of 10%).

STIC Diagnosis

STIC was identified and confirmed as previously described.^{4,5}

Statistical Analysis

Age and predominant morphologic pattern were compared between *BRCA*⁺ and *BRCA*[−] women by the Student *t* test and χ^2 test. Age was compared between tumors with SET and classic predominant morphology by the Student *t* test. Subgroup analyses of frequency of STIC were performed by the Fisher exact test. Analysis of survival using Kaplan-Meier curves and log rank tests was performed.

RESULTS

Study Population

The study group comprised 26 *BRCA*⁺ cases including 21 advanced (stage 3-4) and 5 early (stage 1-2) carcinomas and 32 advanced *BRCA*[−] cases, all of which were evaluated by the SEE-FIM protocol. The 5 early carcinomas were all identified in RRSO specimens. Table 1 summarizes the 2 groups.

In 16 (8 *BRCA*⁺ and 8 *BRCA*[−]) of the 58 cases, a grossly normal-appearing portion of distal fallopian tube was sampled (one half of 1 fimbriated end) for research immediately after excision and before processing. In six of

TABLE 1. Summary of *BRCA*⁺ and *BRCA*[−] Tumor Histology, STIC Status, and Age

	Age (y)	No. STIC ⁺ Cases (%)
<i>BRCA</i> ⁺ (all; n = 26)	52.9*	8 (31)
SET (n = 13)	49.7	3 (23)
Classic (n = 13)	55.4	5 (38)
<i>BRCA</i> [−] (all; n = 32)	62.5*	21 (66)
SET (n = 9)	56.2**	2 (22)***
Classic (n = 23)	64.8**	19 (83)***

**P* = 0.0007.

***P* = 0.04.

****P* = 0.003.

these, a STIC was ultimately identified in permanent sections from the main specimen. Ten samples taken for special studies were from specimens in which no STIC was identified in the permanent sections. Of these, 8 were examined by frozen section of the banked tissue, none of which revealed a STIC. No pathologic information was available on the remaining two samples taken for special studies.

BRCA⁺ Women With HGSC Are Significantly Younger Than *BRCA*[−] Women

The study group included 17 women with *BRCA1* and 9 with *BRCA2* mutations. The mean age of *BRCA*⁺ women was 52.9 years (range 42 to 76 years) and was significantly younger than that of the *BRCA*[−] population (62.5 y; range 38 to 80 years, *P* = 0.0007).

BRCA⁺ Tumors Trend Toward a Higher Frequency of SET Morphology

Figure 2 and Table 1 summarize the comparison of *BRCA*⁺ and *BRCA*[−] patients by predominant morphologic pattern. *BRCA*⁺ tumors were more likely to have SET morphology (50%) when compared with *BRCA*[−] tumors, of which 28% were SET predominate (*P* = 0.11).

In *BRCA*[−] Tumors, SET Morphology Is Seen in Younger Patients Compared With Those With Classic Morphology

Within the *BRCA*[−] cohort, tumors with SET morphology had a younger mean age compared with those with classic morphology (56.2 vs. 64.8 years, *P* = 0.04). The *BRCA*⁺ patients with SET morphology tended to be younger (mean 49.7 vs. 55.4 years), although this difference was not statistically significant (*P* = 0.13).

SET Morphology in *BRCA*[−] Tumors Is Less Likely to be Associated With STIC

Figure 2 and Table 1 summarize and graphically illustrate the frequency and morphology of STIC in *BRCA*⁺ and *BRCA*[−] tumors. Eight of 26 (31%) *BRCA*⁺ and 21 of 32 (66%) *BRCA*[−] tumors contained STIC (*P* = 0.017). In the *BRCA*⁺ group, 38% and 23% of classic and SET tumors, respectively, were associated with STIC (*P* = 0.3). In contrast, 83% and 22% of classic and SET *BRCA*[−] tumors were associated with STIC, respectively (*P* = 0.003). When both *BRCA*⁺ and *BRCA*[−] groups were combined, the

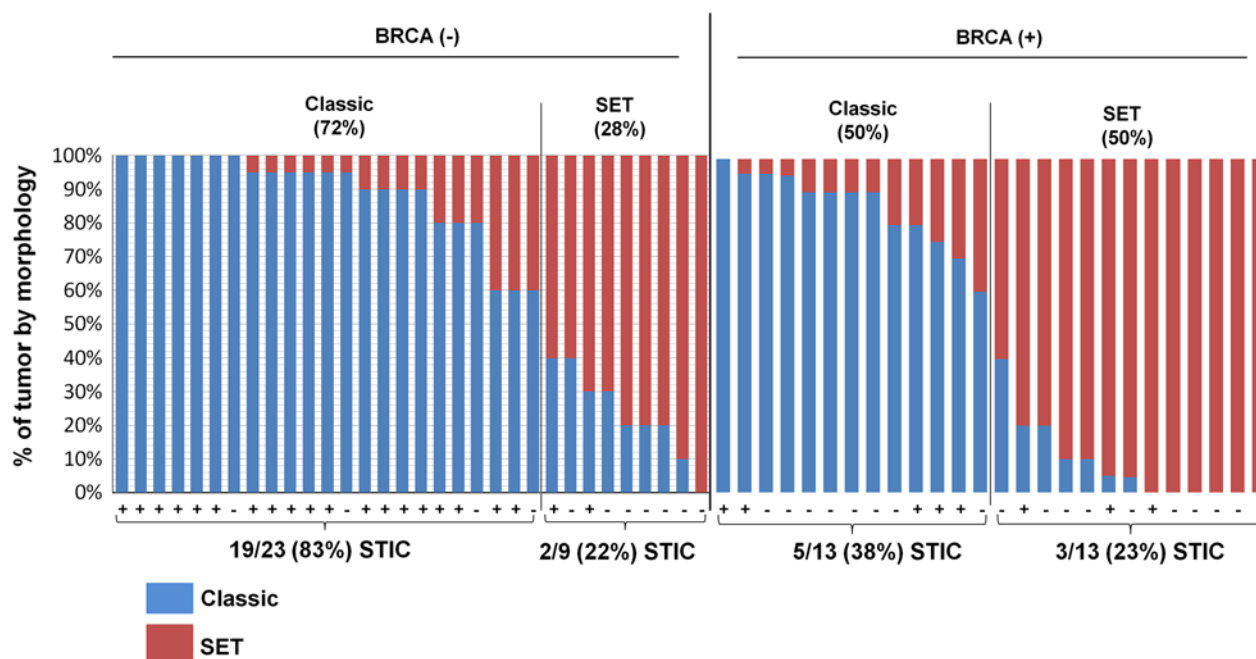


FIGURE 2. Comparison of $BRCA^+$ and $BRCA^-$ patients by predominant ($>50\%$) morphologic pattern and STIC status. Each column represents a single patient's tumor, with percent tumor demonstrating SET morphology quantified in red and classic morphology in blue.

frequency of STIC in classic and SET tumors was 67% and 23%, respectively ($P = 0.003$).

Although most STICs associated with SET tumors were morphologically indistinguishable from those with classic tumors, one $BRCA^+$ case revealed a STIC with a more solid morphology similar to that seen in the associated SET tumor (Fig. 3). The significance of this is unclear at this point.

Clinical Outcome of Tumors With SET Versus Classic Morphology

Table 2 summarizes the clinical outcome of advanced cases with SET versus classic morphology. In general, $BRCA^+$ patients were less likely to be dead of disease (DOD) at last clinical follow-up; however, 1 $BRCA^+$ patient with SET morphology who died also had widely metastatic breast carcinoma and thus was excluded from this analysis. Five $BRCA^+$ HGSCs were early stage and detected in RRSO and were also excluded from the clinical outcome analysis. Of note, 4 of 5 (80%) early HGSCs demonstrated SET predominant morphology, whereas 1 of 5 (20%) demonstrated a predominantly classic pattern. All 5 of these $BRCA^+$ patients were alive without evidence of disease (ANED) at last follow-up (mean 72.5 months; range 48.3 to 106.5 months). Of the $BRCA^+$ patients with advanced disease (mean follow-up time 48.5 mo; range 6.4 to 107.2 mo), 5 of 9 (56%) patients with SET tumors were ANED, in contrast to 3 of 12 (25%) patients with classic morphology who were ANED. Within the $BRCA^-$ cohort (mean follow-up time 30.4 months; range 8.5 to 55.5 months), a favorable effect on clinical outcome was seen in patients with SET predominant tumors when compared with those with classic

morphology (1/12 [8%] DOD vs. 8/20 [40%] DOD). After combining both $BRCA^+$ and $BRCA^-$ women, SET tumors were significantly more likely to trend toward a less severe outcome ($P = 0.024$, χ^2 test for trend).

DISCUSSION

Beginning in 2000, a wealth of information has progressively linked the distal fallopian tube to the origin of HGSC by the discovery of HGSC precursors in the tubal mucosa. This has produced a paradigm shift in the field of ovarian cancer and prompted recent claims that this disease can be partially prevented by opportunistic salpingectomy. The strongest endorsement of a tubal origin has been studies of early carcinomas which are discovered in approximately 5% to 10% of asymptomatic $BRCA^+$ women; 80% are associated with a tubal intra-epithelial carcinoma (STIC). The distribution of disease in this early "snapshot" of HGSC was heavily weighted toward the fimbria and prompted speculation that the tube was responsible for virtually all HGSCs. However, 2 subsequent observations suggested that the pathogenesis (and possibly origin) of HGSC is more complex. First, a multitude of studies have detected STICs in only 40% (on average) of advanced HGSC. Second, a study showed that "endometrioid" variants of HGSC had a lower frequency of STIC (8%) than HGSC with classic morphology, albeit not significant ($P = 0.09$).¹³ Another study showed that tumors with SET morphology (solid, pseudoendometrioid, transitional) were more likely to be associated with $BRCA^+$ status.¹⁴ Taken together, these studies raised the possibilities that the pathway to HGSC

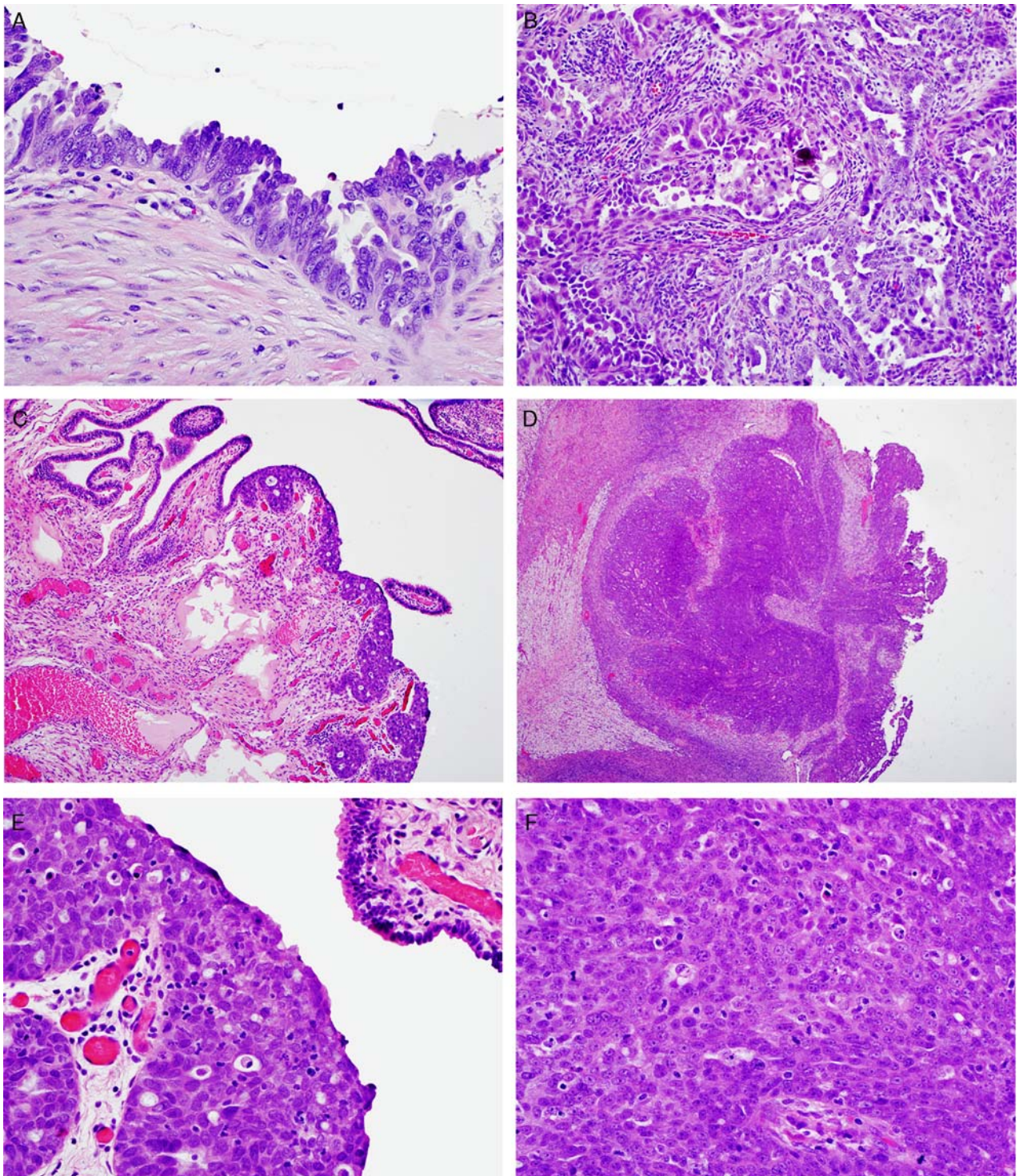


FIGURE 3. Histology of intraepithelial carcinoma (A, C, E) and associated invasive tumor (B, D, F) in a *BRCA*⁻ (A and B) case and *BRCA*⁺ (C–F) case. At higher magnification, the similarity in morphology of 1 *BRCA*⁺ STIC (E) and associated tumor with SET morphology (F) is evident.

might be more diverse than expected, both in precursor and cell type.

This study establishes further that HGSC, in both *BRCA*⁺ and *BRCA*⁻ women, is not a homogenous disease.

In our study of *BRCA*⁺ and *BRCA*⁻ groups, we found that several variables, including histology, coexisting STIC, age, and clinical outcome segregated 2 general tumor groups. Further studies will be needed to flesh out the particulars,

TABLE 2. Clinical Outcome in Advanced HGSC by *BRCA* Status and Tumor Morphology

	<i>BRCA</i> ⁺		<i>BRCA</i> [−]	
	SET	Classic	SET	Classic
ANED	5	3	6	4
AWD	1	7	5	8
DOD	2	2	1	8
Total	9	12	12	20

SET tumors displayed a statistically significant trend toward a more favorable outcome ($P = 0.024$, χ^2 test for trend).

AWD indicates alive with evidence of disease.

but the following observations from this study—witnessed in both *BRCA*⁺ and/or *BRCA*[−] tumors—suggest greater than 1 form of HGSC: (1) Multiple histologic patterns are observed in HGSC, and the SET pattern seems more common in *BRCA*⁺ women albeit the significance of this association varies depending on the criteria for defining SET morphology and the study population.¹⁴ (2) A significant inverse correlation exists between SET histology and STIC in *BRCA*[−] tumors. (3) SET histology in *BRCA*[−] tumors is associated with a significantly younger mean age. (4) A generally more favorable outcome exists for women with SET histology. Further study is warranted to determine whether there is a consistent association of SET histology with chemo-responsiveness and whether the SET group of HGSCs explains why *BRCA*⁺ tumors are generally more chemo-responsive.

The negative correlation between SET histology and coexisting STIC observed in both *BRCA*⁺ and *BRCA*[−] tumors in this study suggests that the differences in morphology may reflect different pathways of tumor evolution.^{13,14} First, we found that SET tumors were associated with a significantly younger mean age than classic HGSCs. Second, a recent report looking at fallopian tubes from low-

risk women who underwent surgery for benign conditions suggests that isolated STICs in asymptomatic women are more common than previously appreciated.¹⁵ Moreover, in *BRCA*⁺ women STICs in isolation confer a low risk for HGSC, and when HGSC does develop, there is a lag period of 2 to 5 years after discovery of the STIC.^{9,10} The implications are that progression from STIC to HGSC does not always occur, and when it does it may take years. The classic tumors—which have high association with STIC—fit nicely into the model proposed by Brown and Palmer¹⁶ that allows an interval of a few years between initiation of carcinogenesis and symptomatic disease, during which a defined precursor (STIC) develops and evolves. With the observed younger mean age of discovery and lower frequency of STIC, SET tumors do not fit as cleanly into this model.

Figure 4 accounts for the possibility of a second pathway to HGSC and depicts a dualistic model of tumor development in HGSC that incorporates the 2 variables of a defined precursor (STIC) and tumor histology (SET vs. classic). At one pole of this model is classic STIC, which develops and may spread, but demonstrates lag phases both from precursor to STIC and from STIC to symptomatic metastatic tumor, leading to an older average age of clinical presentation. At the other pole are SET tumors that could arise from STIC, from some other tubal precursor, or from elsewhere and become clinically apparent at a younger mean age. This admixture of 2 different tumor biologies would explain one prior observation, which is the rather narrow age gap between *BRCA*⁺ women with STIC versus women with more advanced disease.^{9,10}

This study separated classic and SET tumors on the basis of the assignment of the predominant histologic pattern, and it remains to be determined how this translates biologically and whether the associations seen will be reproduced and consistently reveal 2 separable entities. However, it should be emphasized that type II “ovarian” cancers (HGSCs) are increasingly being subdivided, and the notion that these tumors invariably evolve rapidly may be an oversimplification. In the breast, multiple pathogenetic tumor types have emerged, with *BRCA1* disease most frequently associated with a basal (including triple negative) breast cancer phenotype and an obscure or particularly high-grade precursor that evolves rapidly.^{17,18} Another tumor phenotype, luminal A, is associated with defined precursors, develops more slowly, and as expected is more likely to be detected on screening studies.^{19–21} Additional studies are introducing dualistic classifications of HGSC in *BRCA*⁺ and *BRCA*[−] women that correlate molecular profiles with outcome.^{22,23} In parallel with arguments for multiple tumor origins, others have divided tumors prognostically into ovarian surface or tubal origin on the basis of expression signatures.^{24,25} Such studies beg the correlation of molecular pathways, tumor morphology, and outcome with closer examination of the fallopian tube and its environs to identify novel precursors to explain different biological behaviors within the spectrum of HGSC. The answers bear not only on our understanding of type II HGSCs but also on expectations both from serologic screening and prophylactic salpingectomy.

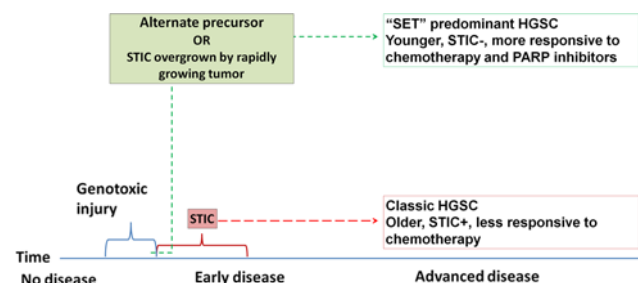


FIGURE 4. A summative model of HGSC pathogenesis with respect to clinical features (age, outcome), precursor lesion, and tumor evolution. In this model, tumor morphology in HGSC could signal a greater likelihood of a certain tumor cell origin and/or biological behavior. The classic pathway involves a STIC and possibly a protracted period from precursor to HGSC. The SET pathway evolves differently as implied by lower frequency of STIC and a younger mean age at discovery. The 2 pathways also seem to differ in terms of responsiveness to chemotherapy and PARP inhibitors.

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The PAX2-null immunophenotype defines multiple lineages with common expression signatures in benign and neoplastic oviductal epithelium

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Abstract

The oviducts contain high grade serous cancer (HGSC) precursors (serous tubal intraepithelial neoplasia or STINs), which are γ -H2AX^P- and *TP53* mutation-positive. Although they express wild type p53, secretory cell outgrowths (SCOUTs) are associated with older age and serous cancer; moreover both STINs and SCOUTs share a loss of PAX2 expression (PAX2ⁿ). We evaluated PAX2 expression in proliferating adult and embryonic oviductal cells, normal mucosa, SCOUTs, Walthard cell nests (WCNs), STINs and HGSCs, and the expression of genes chosen empirically or from SCOUT expression arrays. Clones generated *in vitro* from embryonic gynecologic tract and adult fallopian tube were Krt7^P/PAX2ⁿ/EZH2^P and underwent ciliated (PAX2ⁿ/EZH2ⁿ/FOXJ1^P) and basal (Krt7ⁿ/EZH2ⁿ/Krt5^P) differentiation. Similarly non-ciliated cells in normal mucosa were PAX2^P but became PAX2ⁿ in multilayered epithelium undergoing ciliated or basal (Walthard cell nests or WCN) cell differentiation. PAX2ⁿ SCOUTs fell into two groups; Type I were secretory or secretory/ciliated with a “tubal” phenotype and were ALDH1ⁿ and β -catenin^{mem} (membraneous only). Type II displayed a columnar to pseudostratified (endometrioid) phenotype, with an EZH2^P, ALDH1^P, β -catenin^{nc} (nuclear and cytoplasmic), stathmin^P, LEF1^P, RCN1^P and RUNX2^P expression signature. STINs and HGSCs shared the Type I immunophenotype of PAX2ⁿ, ALDH1ⁿ, β -catenin^{mem}, but highly expressed EZH2^P, LEF1^P, RCN1^P, and stathmin^P. This study, for the first time, links PAX2ⁿ with proliferating fetal and adult oviductal cells undergoing basal and ciliated differentiation and shows that this expression state is maintained in SCOUTs, STINs and HGSCs. All three entities can demonstrate a consistent perturbation of genes involved in potential tumor suppressor gene silencing (EZH2),

[^]Shared equal responsibility as first and senior authors.

The authors declare that they have no conflicts of interest.

Author contributions

Contributions of the coauthors to Design (1), Data collection (2), Data analysis (3), Data interpretation (4), Literature search (5), Figures (6) and Manuscript writing (7) were as follows: Ning (1,2,3,4,6,7); Bijron (1,2,3,4,6,7); Yamamoto (1,2,3,6); Wang (2,3); Howitt (1,2,4,5); Herfs (1,2,3,4); Yang (1,2,3,5); Hong (2,3,4); Cornille (2,3,4); Wu(2,3,4); Hanamor (2,3,4); McKeon (1,4,7); Crum (1,4,7); Xian (1,3,4,7).

transcriptional regulation (LEF1), regulation of differentiation (RUNX2), calcium binding (RCN1) and oncogenesis (stathmin). This shared expression signature between benign and neoplastic entities links normal progenitor cell expansion to abnormal and neoplastic outgrowth in the oviduct and exposes a common pathway that could be a target for early prevention.

Keywords

fallopian tube; serous carcinoma; stem cell; PAX2; ALDH1

Introduction

Recent discoveries have strengthened the relationship between the distal fallopian tube and epithelial malignancies traditionally attributed to the ovary, specifically high-grade serous carcinomas (HGSC), the most lethal of ovarian cancers.^{1, 2, 3} With these discoveries has emerged a collective effort to resolve the sequence of histologic and molecular events giving rise to these tumors in the fallopian tube. The serous carcinogenic sequence involves not only frank malignancies with metastatic spread, but serous cancer precursors, including latent precursors—the p53 signature—and serous tubal intraepithelial neoplasms (STINs). The latter include intramucosal carcinomas (STICs) and lesser but immunophenotypically similar atypias that are considered premalignant intraepithelial lesions (STILs).^{4, 5} Virtually all serous cancer precursors contain mutations in *TP53*, evidence of a DNA damage response (γ -H2AX^p) and predominate in the distal fallopian tube.⁴ Contiguous benign (p53 signatures) and malignant (STICs) epithelia have been documented with shared mutations in specific codons of *TP53*.^{4, 6} In addition, further studies have unearthed other benign epithelial alterations, termed secretory cell outgrowths—SCOUTs—that do not contain *TP53* mutations or evidence of a DNA damage response, yet share with precursors and carcinomas loss of PAX2 expression.^{7, 8, 9} SCOUTs do not appear directly linked to HGSC, but have been documented at higher frequency in the normal tubes of postmenopausal women and those with HGSC.^{8, 9} Based on these properties we have designated SCOUTs as “surrogate precursors” and hypothesize that both SCOUTs and serous cancer precursors share properties or similar mechanisms in their pathogenesis albeit with different potential outcomes.

The shared loss of PAX2 expression in both SCOUTs and many “true” serous cancer precursors suggests that inactivation of this gene, while integral to neoplasia, has a wider range of associations and may signify a generic pathway common to epithelial cell expansion. The goals of this study were to first determine the breadth of the PAX2ⁿ immunophenotype in the fallopian tube by examining “normal” cell growth and differentiation *in vitro* and *in vivo*. Secondly, we wanted to characterize more fully the alterations in expression that typified SCOUTs by array analysis and employ a biomarker profile to determine whether the SCOUT signature was recapitulated in STINs and HGSCs.

Methods

Case material

This study was approved by the Brigham and Women's human investigation committee and involved the use of discarded fresh and archived tissues. Case material for antibody staining consisted of the following epithelia/lesions: 1) normal salpingeal epithelium (n = 15), 2) SCOUTs (n = 44) and other outgrowths such as transitional-like metaplasia (Walthard cell nests, n = 5), 3) serous tubal intraepithelial neoplasms (STINs) (n = 18) and 4) metastatic or invasive serous carcinomas (n = 39). In addition, cultured clonogenic cells from normal fallopian tubes were examined for selected marker expression. Cases for immunohistochemistry were selected by one of us (CPC) using previously described criteria (Figure 1).¹⁰

Cell culture

Fimbrial tissue was obtained from discarded surgical specimens of women undergoing benign procedures. Discarded fetal oviductal tissues were obtained by parental consent under an approved IRB protocol. Disaggregated cells were cultivated onto a feeder layer of lethally irradiated 3T3-J2 cells in stem cell culturing media (Jackson Laboratory, scm003). Clonal analysis and in vitro 3D differentiation were based on previously described methods for lung epithelial stem cells.¹¹

Microarray and bioinformatics

In order to identify genes expressed in PAX2ⁿ epithelium, expression arrays were generated from formalin-fixed, laser-capture-micro-dissected (LCM) PAX2ⁿ SCOUTs and benign control oviductal epithelium. RNAs obtained from the LCM procedure were amplified using the Ovation FFPE WTA System, WT-Ovation Exon Module and Encore Biotin Module (NuGEN Technologies) and hybridized onto GeneChip® Human Exon 1.0 ST Arrays. GeneChip operating software was used to process all the Cel files and calculate probe intensity values. To validate sample quality, hybridization ratios were calculated using Affymetrix Expression Console software. The intensity values were log₂-transformed and imported into the Partek Genomics Suite. Exons were summarized to genes and a 1-way ANOVA was performed to identify differentially expressed genes. *P* values and fold-change were calculated for each analysis. Heatmaps were generated using Pearson's correlation and Ward's method with selected genes based on *p* value. Pathway analyses were performed using Gene Set Enrichment Analysis (GSEA) software. Candidate biomarkers were culled from these arrays and are summarized in Table 1.

Immunohistochemistry

Immunostaining was performed with attention to the biomarkers in Supplementary Table 1, in which product information and dilutions are included. When normal-appearing epithelia were scanned for putative PAX2ⁿ secretory cells, sections were immunostained with two antibodies concurrently; PAX2, which stains non-ciliated cells, and FOXJ1, a ciliated cell marker. Antibodies to leukocyte common antigen (LCM) for CD3, as well as FASCIN, were also used to track intraepithelial lymphocytes and dendritic cells, which are normally

PAX2ⁿ. Detection was completed with the Vectastain ABC kit (Cat. No. PK-6102; Vector Laboratories, Inc) with a liquid DAB-plus substrate kit (Cat. No. 00-2020). Slides were counterstained with Hematoxylin Stain 3 (Cat. No. CS402-1D). Antibody information is summarized in Supplementary Table 1. Reaction to antibody staining is indicated by superscripted “p” or “n” for positive or negative (PAX2, ALDH1, FOXJ1 etc), “m” or “wt” for mutated or wild type (p53) and “nc” or “mem” for nuclear and cytoplasmic vs. membrane localization (β-catenin). Immunohistochemistry, immunofluorescence staining and image acquisition were performed as previously described.^{9,11} Proliferating clones were identified and immunostained for PAX2, PAX8, FOXJ1, Krt7, Krt5, p63, EZH2 and Ki67. Evidence of ciliated cell differentiation was identified by immunostaining for FOXJ1 and acetylated alpha-tubulin. Basal cells were identified by Krt5 or p63 immunostaining.

Results

Histologic sub-classification of SCOUTs and STINs

Lesions under study are illustrated in Figure 1. Based on previous studies, SCOUTs were subdivided into two general histologic categories.^{8, 12} The first, designated as Type 1 SCOUTs, consisted of a typical mono or biphasic tubal epithelial composition with either single layers of tubal non-ciliated cells or (more commonly) a combination of non-ciliated and ciliated cells. The second, arbitrarily labeled Type 2 SCOUTs, consisted of proliferations with mildly pseudostratified and closely arranged elongated fusiform nuclei, similar to endometrial epithelium, and also termed “endometrioid” SCOUTs. Cells with ciliated differentiation (FOXJ1^p) were present, but were typically less than 30% of the cells and scattered throughout the epithelium. Walthard cell nests (WCNs), consisting of basal cell outgrowth with a squamo-transitional phenotype were also studied because they signify another form of outgrowth derived from columnar epithelial cells, albeit metaplastic. STINs were sub-classified as previously described and contained strong p53 immuno-staining and evidence of DNA damage by H2AX staining.⁵ Those with mild or moderate atypia and preserved epithelial polarity were classified as low grade and are identical to lesions classified as “STILs”, “TILTs” and atypical hyperplasia in other reports.^{13, 14, 15} Those with conspicuous loss of epithelial polarity were classified as high grade, synonymous with serous tubal intraepithelial carcinoma (STIC). The latter have a 0–11% outcome risk of HGSC, based on recent studies.^{16, 17, 18} The HGSC outcome risk of lower grade STINs is unknown but presumed to be less than that of high grade STINs.

In vitro and *in vivo* expression of PAX2 in the fallopian tube mucosa

Cultured epithelial cells from the gynecologic tract, both in adults and at 20 weeks gestation, were plated and colonies of clonogenic cells were characterized. The dominant immunophenotype associated with highly-proliferative clonogenic cell outgrowth was Krt7^p/PAX8^p/EZH2^p/PAX2ⁿ/Krt5ⁿ/p63ⁿ (Figure 2A, Figure 3A and Supplementary Figure 2A). FOXJ1 expression indicating ciliated cell differentiation was also seen occasionally in the non-proliferative cells that were not stained positively with Ki67 (Figure 2A). To examine the differentiation ability of these cloned cells at the single-cell level, we established single-cell pedigree lines by subsequent rounds of plating and clone selection (Figure 2B). Pedigree lines of these cloned oviductal progenitor cells were differentiated in

either air-liquid interface (ALI) cell culture system or 3D Matrigel cultures for 10–20 days. In 3D Matrigel cultures, PAX8^P oviductal progenitor cells differentiated into columnar epithelium comprised of acetylated tubulin^P/FOXJ1^P/PAX8ⁿ ciliated cells and PAX8^P non-ciliated cells, which resembles the human oviduct histology (Figure 2C). In the ALI culture system, a series of images of acetylated tubulin expression were taken at different time points during the differentiation and showed that the oviductal progenitor cells started to differentiate into ciliated cells at day 3 and became maturely differentiated at day 10 (Supplementary Figure 2B). At day 10 in the ALI culture system, the cloned oviductal progenitor cells formed a simple epithelium with ciliated cells marked by FOXJ1 and acetylated tubulin and non-ciliated cells marked by PAX2 (Supplementary Figure 2C). It is noteworthy that while the proliferating population is PAX2ⁿ (Figure 2A), PAX2 expression was reclaimed in some non-ciliated (secretory) cells. This further indicates that the progeny of a single oviductal progenitor cell can give rise to all epithelial lineages typically found in the oviduct, including not only mature ciliated cells but non-ciliated (secretory) cells.

Immunostaining of both fetal and adult fallopian tubes was performed to ascertain the distribution of PAX2-expressing cells and address the possibility that the PAX2ⁿ immunophenotype was programmed earlier in development. Histologic sections of fetal (at 21 weeks) and adult fallopian tubes were examined. Fetal tubes contained an abundance of PAX2^P cells, with occasional interspersed ciliated cells (Supplemental Figure 1A). Expression of PAX8 was similar in distribution (Supplemental Figure 1B). Similarly, in normal adult tubes, PAX2 staining was extensive in cells that were not undergoing ciliated (tubulin^P) differentiation (Supplemental Figure 1C). A summary of immunophenotypes for progenitor and adult cells is displayed in Supplementary Table 2.

In the adult tubes, sections were also stained with FOXJ1, and/or LCA to account for other PAX2ⁿ cells that were either undergoing ciliated differentiation or were non-epithelial. Mono-layered or mildly pseudostratified normal fallopian tube mucosa typically contained cells either expressing PAX2 or FOXJ1 (Figure 2D1). In occasional foci of prominent multilayered epithelium with some cells staining positive with FOXJ1, loss of PAX2 nuclear staining could be seen (Figure 2D2) giving the impression that loss of PAX2 expression in non-ciliated cells was coordinated with cell growth in multilayered epithelium. Albeit less so, FOXJ1 staining was also seen in STINs, supporting ciliated differentiation in PAX2ⁿ neoplastic growth (Figure 2D3).

Metaplastic (Walthard cell nests) differentiation of PAX2ⁿ columnar cells *in vitro* and *in vivo*

Walthard cell nests are foci of transitional-like metaplasia in the fimbria or adjacent peritoneal surface and are emblematic of basal cell outgrowth that can develop near the junctions of disparate epithelial types.¹⁹ Other sites include the gastro-esophageal and cervical squamo-columnar junctions. Both have been designated as sites harboring residual embryonic cells and studies of the latter have suggested that basal or reserve cells emerge from the overlying columnar cells and then undergo squamous metaplasia.^{20, 21} This process has been termed “top down” differentiation, *i.e.* the progeny (basal cells) emerge from beneath the progenitor population. However, no study has ever displayed this sequence

in vitro. Fetal tubal cells propagated *in vitro* were strongly positive for both Krt7 and PAX8 was seen, in keeping with Mullerian epithelium (Figure 2A and Figure 3A). Moreover, these progenitor cells did not express Krt5 or p63 (Figure 3A). Interestingly, when pedigree lines of these cloned oviductal progenitor cells were differentiated in 3D Matrigel cultures for 10–20 days, in addition to the typical ciliated cell differentiation (Figure 2C), subjacent p63/Krt5^p basal cells emerged (Figure 3B1) and expanded (Figure 3B3) in a pattern similar to that seen in p63/Krt5^p cells in Walthard cell nests (WCNs) in the adult tube (Figures 3B2 and 3B4). *In vitro*, the Krt5 and p63 immunopositive cells were superimposed, although the Krt5 staining index was higher (Supplementary Figure 2E). Analysis of WCNs in tissue sections (Figure 3C) revealed a strikingly similar pattern of growth and differentiation, arising from either beneath Krt7^p epithelial cells or in continuity with columnar epithelium typical of Type I SCOUTs. The result was a PAX2ⁿ/ALDH1ⁿ transitional-like outgrowth that was strongly Krt5^p but stathminⁿ (not shown). Taken in the context of the *in vitro* findings, this observation further linked the PAX2ⁿ immunophenotype to cell outgrowth and a Krt7^p progenitor cell to the development of not only terminal (FOXJ1⁺) but also metaplastic (Krt5⁺) differentiation in the fallopian tube.

Altered gene expression in PAX2ⁿ proliferations (SCOUTs, STINs and HGSC)

Supplementary Table 3 is a list of genes selected for analysis and found to be differentially expressed in SCOUTs relative to normal appearing epithelium. Arrays generated from RNA extracted from formalin-fixed laser-capture micro-dissected SCOUTs yielded differentially expressed genes, illustrated in the representative heat map (Figure 4A and B, Supplemental Figures 4&5). When stained with selected markers, Type 1 SCOUTs varied from strictly secretory to mixed secretory and ciliated and were ALDH1ⁿ, β-catenin^{mem} and stained weakly or negative for LEF1, RCN1, RUNX2 and EZH2 (Figures 4C, Figure 5, and Supplemental Figure 3). Type 2 SCOUTs stained variably for ciliated cell differentiation and were β-catenin^{nc} and ALDH1, LEF1, RCN1, EZH2, RUNX2 (not shown) and stathmin positive (Figure 4C and Figure 5 and Supplemental Figure 3). Basal cell differentiation, signifying WCN development, was associated with PAX2ⁿ columnar epithelium, suggesting this pathway of differentiation might initiate within Type I PAX2ⁿ SCOUTs.

Figure 5 and Supplemental Figure 6 summarize the staining patterns observed in the different lesions. STINs and HGSCs shared expression of several markers with SCOUTs. Expression patterns for ALDH and β-catenin were identical to Type 1 SCOUTs (ALDHⁿ and β-catenin^{mem}). In addition, like Type 2 SCOUTs, there was increased staining for EZH2, Stathmin, LEF1, RCN, Krt5, RUNX2 (not shown). Not surprisingly, no marker in this group separated STINs or HGSCs from SCOUTs. This is in contrast to other published markers such as Ki67, Cyclin E, p16 and others, which are significantly more commonly expressed in STINs and HGSCs relative to benign fallopian tube mucosa.^{4, 5, 15, 22}

Discussion

Analysis of arrays generated from high-grade serous cancer has confirmed a transcriptome that parallels oviductal epithelium.²³ Given that these tumors are strongly positive for biomarkers (such as PAX8) typically assigned to non-ciliated (so-called secretory) cells, the

assumption has been that the secretory cell is the cell of origin.¹ Levanon et al. showed that PAX8-expressing (secretory) cells of the tube were uniquely susceptible to DNA damage imposed by irradiation, a finding that parallels similar observations in latent precursors (p53 signatures) and STINs that contain p53 mutations.^{5, 24} However, with the discovery of PAX2ⁿ SCOUTs and a similar PAX2ⁿ expression pattern in many STINs, it became clear that there may be a relationship between the two entities, despite the fact that SCOUTs are more ubiquitous in the fallopian tube and do not arise in the setting of a DNA damage response and loss of p53 function. Although altered PAX2 expression has been focused on in neoplasia, we hypothesized that the PAX2ⁿ immunophenotype typified a “generic” series of molecular events that were the underpinning of stem cell expansion common to many proliferations.

We addressed PAX2 expression or loss in the fallopian tube from three perspectives. The first was by analyzing expression and differentiation in proliferating normal adult and fetal cells propagated *in vitro*. The second was by comparing the *in vitro* findings to expression in tissue sections from fetal and adult tubes. The third was to look for shared expression across PAX2ⁿ cells in cell proliferation and expansion (SCOUTs, STINs and HGSCs). We discovered that the PAX2ⁿ immunophenotype was particularly linked to *in vitro* and *in vivo* cell growth, not infrequently with an increase in EZH2 expression. Moreover, in highly clonogenic Krt7^P/FOXJ1ⁿ oviductal progenitor cells grown *in vitro*, we demonstrated for the first time that PAX2ⁿ expanding populations were capable of both ciliated (FOXJ1) and basal cell (Krt5) differentiation. This sequence of cell growth and differentiation was recapitulated in SCOUTs, STINs and HGSCs, with progressively reduced ciliated differentiation in the Type 2 SCOUTs, STINs and HGSCs. We thus concluded that all of these entities were related to a similar progenitor cell.

The next goal was to determine if the cells involved in benign and neoplastic outgrowth shared common expression patterns and we chose to use the least proliferative lesions (SCOUTs) as the reference. One advantage of this approach is to identify events that occur prior to the more dramatic molecular changes that characterize malignancy that may have profound influences on expression. The study delineated two general groups of SCOUTs; the first (Type 1) closely resembled normal tubal epithelium, histologically and in their expression profile (Figure 1D). The second (Type 2) was composed of proliferations with less pronounced ciliated differentiation, many noticeably “endometrial” like (Figure 1E). Accordingly, there was minimal difference in expression between Type 1 SCOUTs and control epithelium, although they were consistently ALDH1ⁿ. In contrast, type 2 SCOUTs demonstrated nuclear and cytoplasmic β -catenin staining plus increased BCL2 (see ref 7), ALDH1, and Krt5 staining. This diversity in phenotype underscores the complexity of cell growth and differentiation that can occur in the fallopian tubes with age. Type I SCOUTs appear to signify very minor genomic changes as evidenced by the similarities in transcription to normal controls. Thus, the alterations in transcription are limited to absence of ALDH1 expression. In contrast, Type 2 SCOUTs, which exhibit a more divergent histology, have a common biomarker signature—stathmin, EZH2, LEF1, RCN1 and RUNX2—that is more similar to premalignant (STIN) and malignant (HGSCs) entities in the tube (Figure 5).

A fundamental question stemming from the above observation is the relevance of the gene signature found in SCOUTs, STINs and HGSCs to both stem cell biology and neoplasia. ALDH1 has been identified as a marker of epithelial stem cells. Its expression can be both increased or absent, the latter more typical of STINs and HGSCs.^{25, 26} EZH2 is a polycomb suppressor that is implicated in stem cell maintenance and regulation of differentiation. It is noteworthy that EZH2 expression typically increased in areas of cell expansion, in keeping with the coordinated suppression of PAX2 expression.²⁷ EZH2 is also a potential suppressor of tumor suppressor genes.²⁸ LEF1 is likewise expressed during lineage differentiation.²⁹ The function of RCN1 is less clear but this gene product is a calcium binder that is weakly expressed in renal tubular cells and up-regulated in renal cell carcinomas.³⁰ RUNX2 is a gene involved in morphogenesis and osteoblastic differentiation.³¹ Functions attributed to stathmin are multiple. It is a marker of P13 kinase activation that has been linked to serous neoplasia in some studies, tumor progression and metastases in others, and regulates p53 stability in still others.^{32, 33, 34} Its range of expression, including normal epithelium, SCOUTs and STINs, is similar to that of these other markers, several of which (ALDH1, PAX2, EZH2) have also been linked to not only stem cells but outcome or resistance to chemotherapy.^{35, 36, 37} The significance of the unique β -catenin staining in Type 2 SCOUTs, with a shift in distribution from the membrane to cytoplasm and nucleus is unclear, but it is emblematic of Wnt pathway activation, and mutations in β -catenin are commonly found in endometrial and colon carcinomas.³⁸

Walthard cell nests are a common benign condition seen in the distal fallopian tube mucosa or the adjacent peritoneal reflection.¹⁹ They bear a close resemblance to the cervical squamo-columnar junction where columnar cells are undermined by p63-positive basal cells. These cells could be envisioned to either originate from the columnar epithelium or give rise to the overlying Krt7-positive epithelial cells. This study has made two novel observations. First, based on the matrigel cell culture data, the basal cells emerge from the Krt7-positive columnar cells. Second, this process is marked by not only loss of PAX2 and but also ALDH1 expression, similar to that seen in Type 1 SCOUTs. The initiating cell, the Krt7^P non-ciliated epithelial cell, is remarkably similar to the cells seen in the SC junction of the cervix from which squamous metaplasia is derived and this process is similar to so-called “top-down” differentiation reported in the squamocolumnar junction.²¹ The fact that WCNs are not considered direct precursors to malignancy is not surprising, in as much as they are terminally differentiated relative to their progenitors. This is similar to the cervix, where the progenitor cells in the SC junction are considered more vulnerable to neoplastic transformation than their metaplastic progeny.²¹ What is interesting is the fact that WCNs underscore the existence of multi-potential cells in the distal fallopian tube.¹² Given that 40–60 percent of HGSCs do not have a documented source (or STIN) in the fallopian tube mucosa, coupled with the fact that a subset of HGSCs are strongly Krt5 positive, the possibility that cells involved in alternate differentiation pathways might contribute to a subset of these malignancies deserves further study (Hanamornroongruang S, Howitt BE, Crum CP, unpublished).^{5, 25}

Epithelia in virtually every organ (breast being a prime example) display a wide range of clonal expansions, some of which may be direct precursors to malignancy and others of which serve as risk factors for a malignant outcome. The model depicted in Figure 6 reflects

a similar but novel scenario in the oviduct, with multiple categories of putative monoclonal cell outgrowth and striking similarities in expression across multiple genes between surrogate precursors and lesions that are considered premalignant or pre-metastatic. These findings emphasize the complexity of molecular and phenotypic perturbations that can take place in the fallopian tubes during and following menopause. This complexity invites caution when considering the role (or diagnostic value) of newly discovered biomarkers as specific indicators of neoplasia. More importantly, it reveals a consistent disturbance in progenitor cell biology in keeping with a common pathway that is triggered by more than one initiating event. Thus, it introduces two approaches to cancer prevention, one directed at the initiating event and the other at the early perturbations in the pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

STIN	serous tubal intraepithelial neoplasia
SCOUT	secretory cell outgrowth
ALI	air-liquid interface culture
WCN	Walthard cell nest

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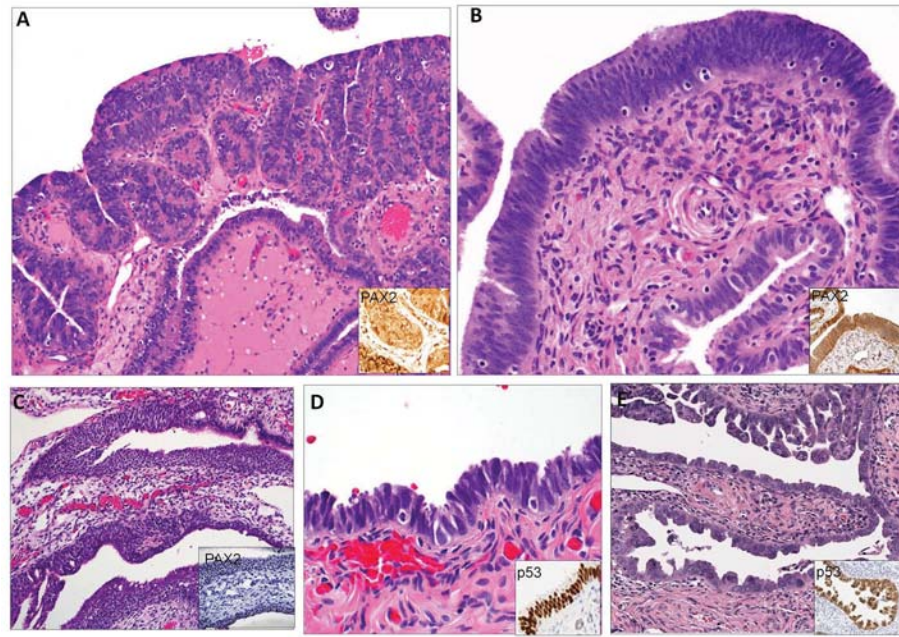


Figure 1.

Entities associated with PAX2^{hi} immunophenotype included (A) Type 1 secretory cell outgrowths (SCOUT), (B) Type 2 SCOUTs, (C) Walthard cell nests and (D) low- and (E) high-(serous tubal intraepithelial carcinoma) grade tubal intraepithelial neoplasia.

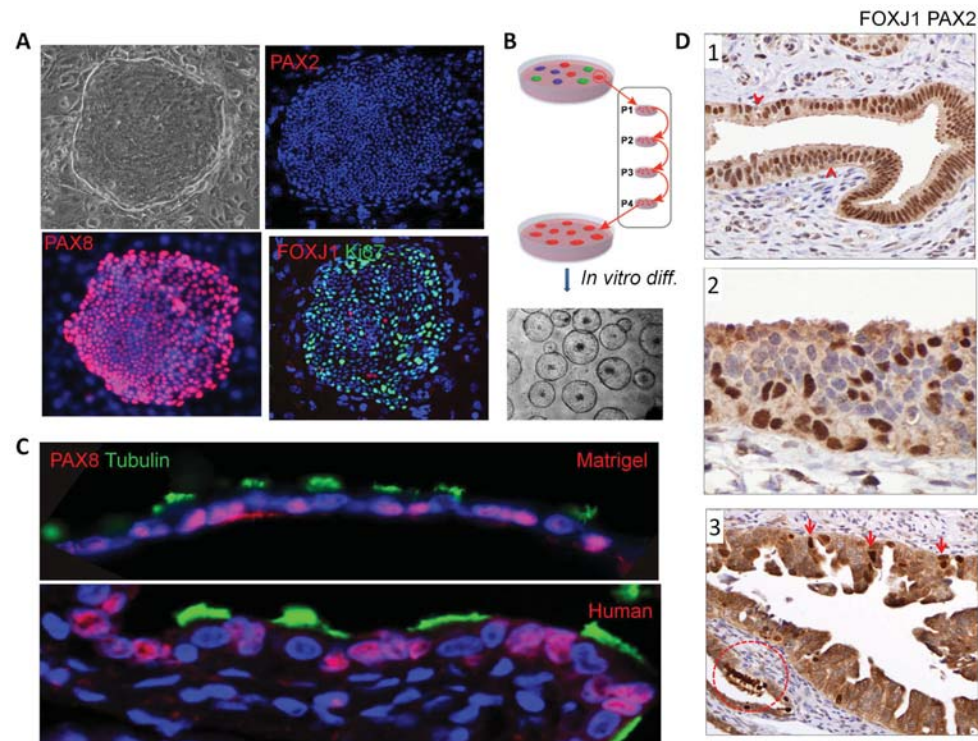


Figure 2.

In vitro propagation and differentiation of oviductal progenitor cells. (A) The cells cloned from fetal or adult oviduct are PAX2ⁿ, PAX8^p and occasionally express differentiation marker (FOXJ1) in non-proliferative cells (Ki67ⁿ). (B) Schematic diagram of pedigree cell line establishment. (C) *Upper panel*, representative image of fetal (20-week) oviductal progenitor cells differentiated in 3D Matrigel culture system. *Lower panel*, immunofluorescence image of human adult oviduct epithelium. Acetyl- α -tubulin (green) indicates ciliated cell differentiation. PAX8 (red) indicates non-ciliated cells. DAPI stains nuclei (blue). (D1) Combined staining of histologic sections of normal tube with both PAX2 and FOXJ1 reveals widespread nuclear staining, except occasional lymphocytes (arrows). (D2) Occasional foci of multilayered epithelium undergoing ciliated cell differentiation (positive nuclei) consist of some cells negative for PAX2. (D3) Tubal intraepithelial carcinoma with focal FOXJ1 staining (arrowheads) indicating ciliated cell differentiation. Circled focus of normal ciliated cells is an internal positive control.

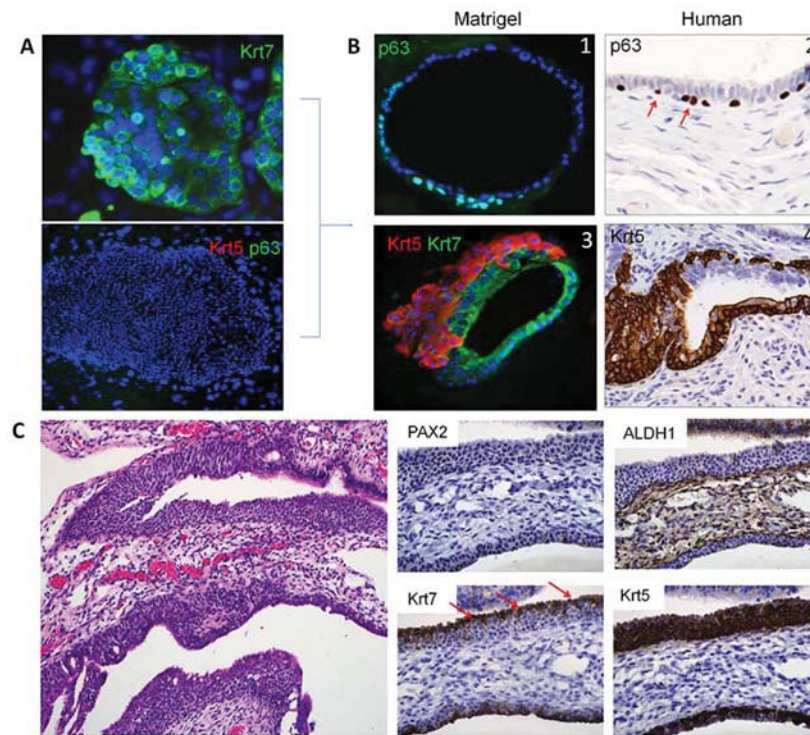


Figure 3.

In vitro and *in vivo* basal cell differentiation in the oviduct. (A) Colonies of Krt7^P/Krt5^N/p63^N cells from a 20 week old fetal oviduct. (B1, B3) single (p63, green) and multilayered (Krt5, red) basal cell outgrowth seen in matrigel cultures. (B2, B4) similar basal cell growth highlighted by p63 and Krt5 in the adult fimbria. (C) Walthard cell nest in the adult tube is typically PAX2 and ALDH1 negative. Residual Krt7-positive cells (arrows) are displaced from beneath by an expanding Krt5 population.

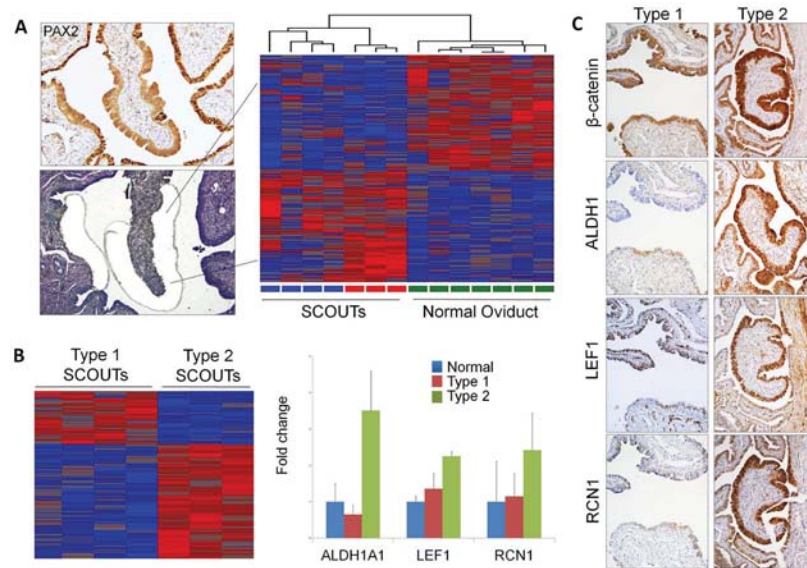


Figure 4.

(A) Laser-captured microdissected SCOUTs (left) and a Heatmap comparison of SCOUTs and normal oviduct (right). (B) Arrays generated from PAX2ⁿ SCOUTs revealed genes differentially expressed across Type 1 and Type 2 SCOUTs, including ALDH1, LEF1 and RCN1 (right). (C) Coordinated expression of the above genes distinguish Type 1 SCOUTs, which show membranous β -catenin localization and absent ALDH1 staining plus negative or weak staining for LEF1 and RCN1 staining (right) from Type 2 SCOUTs, with nuclear and cytoplasmic β -catenin, strong ALDH1, LEF1 and RCN1 staining.

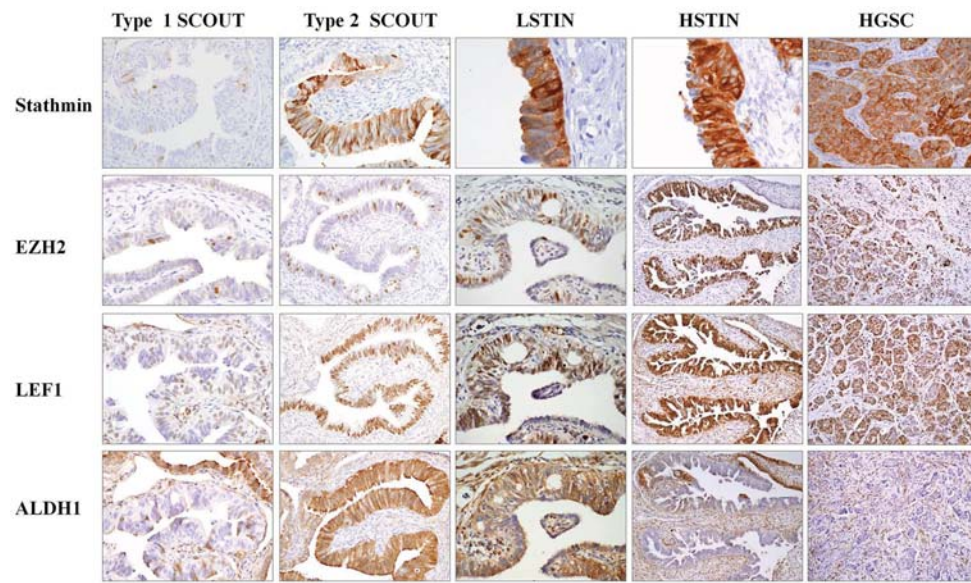


Figure 5.

Shared expression of SCOUT markers with low (LSTIN) and high (HSTIN or STIC) grade serous tubal intraepithelial neoplasia and high-grade serous carcinoma (HGSC). Neoplasms (STINs, HGSCs) share with Type 1 SCOUTS loss of PAX2, ALDH1 and with Type 2 SCOUTs, increased LEF1, EZH2 and Stathmin and other markers (see text).

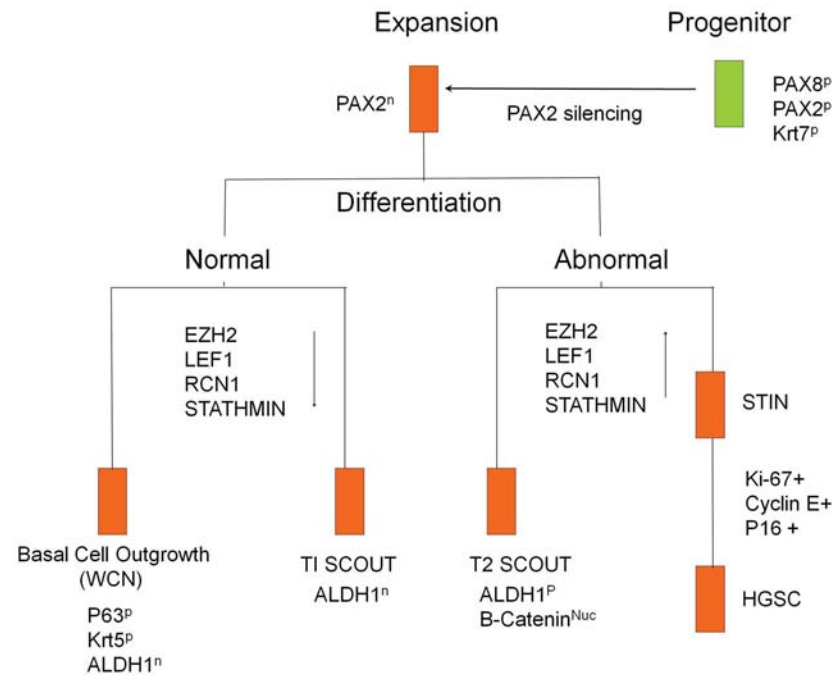


Figure 6.

A progenitor cell model for the fallopian tube in which Krt7 identifies the progenitor cell and PAX2ⁿ defines progenitor cell expansion. Expanding PAX2ⁿ cells can differentiate into basal or ciliated cells in WCNs or Type 1 SCOUTs, both of which approximate normal differentiation pathways, with loss of ALDH1 and normal or minimally increased expression of LEF1, RCN1, Stathmin and EZH2. In contrast, Type 2 SCOUTs and STINs (right) share a different expression signature characterized by multiple genes, including EZH2, LEF1, RCN1, and stathmin and others involved in a divergent pathway of progenitor cell growth.



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***In vitro* and *in vivo* correlates of physiological and neoplastic human Fallopian tube stem cells**

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Author contribution statement

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Figure S1. Pathway enrichment analyses.

Figure S2. GSEA and heatmaps of differentially expressed genes.

Figure S3. PTTG1 and CCNE1 expression and enriched pathways in STIC and invasive cancer versus normal epithelium.

Table S1. List of antibodies.

Table S2. Differentially expressed genes in FTSC and ALI structures.

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Table S9. List of genes overexpressed in normal Fallopian tube epithelium.

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Abstract

High-grade serous cancer (HGSC) progresses to advanced stages without symptoms and the 5-year survival rate is a dismal 30%. Recent studies of ovaries and Fallopian tubes in patients with *BRCA1* or *BRCA2* mutations have documented a pre-metastatic intramucosal neoplasm that is found almost exclusively in the Fallopian tube, termed ‘serous tubal intraepithelial carcinoma’ or STIC. Moreover, other proliferations, termed p53 signatures, secretory cell outgrowths (SCOUTs), and lower-grade serous tubal intraepithelial neoplasms (STINs) fall short of STIC but share similar alterations in expression, in keeping with an underpinning of genomic disturbances involved in, or occurring in parallel with, serous carcinogenesis. To gain insight into the cellular origins of this unique tubal pathway to high-grade serous cancer, we cloned and both immortalized and transformed Fallopian tube stem cells (FTSCs). We demonstrated that pedigrees of FTSCs were capable of multipotent differentiation and that the tumours derived from transformed FTSCs shared the histological and molecular features of HGSC. We also demonstrated that altered expression of some biomarkers seen in transformed FTSCs and HGSCs (stathmin, EZH2, CXCR4, CXCL12, and FOXM1) could be seen as well in immortalized cells and their *in vivo* counterparts SCOUTs and STINs. Thus, a whole-genome transcriptome analysis comparing FTSCs, immortalized FTSCs, and transformed FTSCs showed a clear molecular progression sequence that is recapitulated by the spectrum of accumulated perturbations characterizing the range of proliferations seen *in vivo*. Biomarkers unique to STIC relative to normal tubal epithelium provide a basis for novel detection approaches to early HGSC, but must be viewed critically given their potential expression in lesser proliferations. Perturbations shared by both immortalized and transformed FTSCs may provide unique early targets for prevention strategies. Central to these efforts has been the ability to clone and perpetuate multipotent FTSCs.

Keywords

Fallopian tubes; ovary; cell culture; neoplasia

Introduction

Epithelial ovarian cancer (EOC) is the fifth most common cause of death from cancer in women, and the most common type – high-grade serous carcinoma or HGSC – is the most lethal. One in 200 women will develop ovarian cancer between their 50th and 70th birthdays. Worldwide, there are 225 000 new cases of ovarian cancer diagnosed annually and an estimated 140 163 disease-related deaths [1]. Up to 80% of women present with stages III/IV disease, and the 5-year survival rate is a dismal 30%. Mortality for this disease has not markedly changed since the 1930s [2], because ovarian cancer cannot be detected at low stage by current screening programmes. Resolving this dilemma will require effective

tools and methods to interrupt the carcinogenic sequence at a point that permits either cure or prevention of tumour-specific mortality.

There is cogent clinical and molecular evidence to suggest that many, if not all, cases of HGSC arise from the Fallopian tube. From the clinical end, it has been known that reducing the risk of *BRCA1* and *BRCA2* patients required removal of the Fallopian tube in addition to the ovary [3]. Molecular analyses have shown that HGSC has gene expression profiles more akin to those of Fallopian tube epithelium than to ovarian surface epithelium [4]. Finally, and most significantly, the pathological examination of risk reduction salpingo-oophorectomies for germ-line *BRCA1* and *BRCA2* mutations has uncovered pre-metastatic stages of HGSC (serous tubal intraepithelial carcinoma or STIC) as well as premalignant tubal intraepithelial neoplasia (or serous tubal intraepithelial lesions) [5,6]. In the Fallopian tube model, STIC is considered the earliest morphological manifestation of serous carcinoma. STICs are composed of ‘secretory cells’, the non-ciliated population of the endosalpinx. These cells, when neoplastic, exhibit features including variable stratification, increased proliferation, and loss of nuclear polarity [7].

Most STICs are marked by mutant p53, as are their metastatic form, high-grade serous cancers. Further analyses of *BRCA1/BRCA2* mutation-associated Fallopian tubes have revealed the presence as well of a ‘latent precancer’ – the ‘p53 signature’, which has mutant p53 overexpression but retains cell polarity and lacks excessive cell proliferation. Interestingly, p53 signatures have been found adjacent to STICs and in several revealing examples have been shown to share the same *p53* mutation as HGSC, suggesting a lineage relationship [8]. These compelling results demonstrate that the Fallopian tube is a site of origin of HGSC, the development of which follows the classic multi-step carcinogenesis model. Importantly, latent precancers are common in the tubes of women who are not at genetic risk, and between 40% and 60% of the serous cancers in *BRCA* mutation-negative women also co-exist with STIC [7,8] with a genetic link between the two [9,10]. Thus, STIC represents the earliest phase of most pelvic serous cancers and targeted treatment or prevention of STIC is a valid goal in cancer prevention. In parallel with the serous carcinogenic sequence is one characterized by putative stem cell outgrowths, termed SCOUTs. These proliferations lack *p53* mutations but share many attributes with intraepithelial neoplasms, one being altered expression levels of genes including *ALDH1*, *PAX2*, *EZH2*, *LEF1*, and others. The impression from these collective entities is that the tube is prone to both self-limited and potentially malignant intraepithelial proliferations.

HGSCs presumably arise from non-ciliated cells of the Fallopian tube (secretory cells) but the precise relationship between these cells and stem cells in the tube is not understood, in part because of a lack of detailed *in vitro* studies of putative stem cells. Herein, we report a Fallopian tube stem cell model based on a cell culture paradigm of both limited (immortalization) and aggressive (transformation) cell outgrowth. This model is superimposed on a similar *in vivo* paradigm of proliferative lesions seen in the Fallopian tube. The goal of this exercise was to discern not only molecular perturbations marking the transition from STIC to metastatic disease but also those that highlight the loss of growth control in the early phases of neoplasia.

Materials and methods

Case material

This study was approved by the Brigham and Women's Human Investigation Committee and involved the use of discarded fresh and archived tissues. Case material for gene expression analysis and histology consisted of the following epithelia/lesions: (1) normal oviduct and HGSC paired samples ($n = 10$) and (2) normal oviduct, STIC, and invasive HGSC lesions from each patient section ($n = 6$). Cases for immunohistochemistry were selected by one of us (CPC) using criteria that have been previously described [10].

Stem cell culture and differentiation

Fimbriae of Fallopian tube tissue were obtained from discarded surgical specimens of women undergoing benign procedures. Discarded fetal Fallopian tube tissues were obtained under an approved IRB protocol. Tissues were digested in 2 mg/ml collagenase A (Roche, Indianapolis, IN, USA) at 37 °C for 1.5 h. Disaggregated cells were cultured on a feeder layer of lethally irradiated 3 T3-J2 cells in stem cell culturing media (SCM-6 F8) [11]. Clonal analysis and *in vitro* ALI differentiation were based on previously described methods for lung epithelial stem cells [12].

Xenografts of transformed FTSCs

Fallopian tube stem cells were infected with retroviruses expressing c-Myc, hTERT, and SV40 large T antigen (SV40 large T and hTERT for immortalization; and SV40 large T, hTERT, and c-Myc for transformation). The PMN-MYC-IRES-GFP retroviral vector expressing full-length human c-MYC was a gift from Yu Qiang [13]. PBABE-puro SV40 LT was a gift from Thomas Roberts (Addgene plasmid #13970; Addgene, Cambridge, MA, USA) [14] and PBABE-puro-hTERT was a gift from Bob Weinberg (Addgene plasmid #1771) [15]. Recombinant retroviral particles were prepared by transient transfection of GP2-293 T cells (ATCC) along with packaging plasmids (pCMV-VSVG). The medium containing recombinant retrovirus was harvested 48 h after transfection. Cellular debris was removed by centrifugation and filtration through a 0.45 µm filter (Millipore, Billerica, MA, USA). For infection, in brief, 200 000 stem cells were plated onto a lawn of feeder cells in 3 cm culture dishes and transduced 3 days later. After 48 h, cells were split 1:5 onto new lawns and grown and passaged for 4 weeks before plating onto culture plates without feeder cells for an additional 4 weeks. Individual colonies were selected and tested for growth in soft agar, and positive colonies selected for expansion and transplantation. Two thousand transformed cells (expressing SV40 large T antigen, hTERT, and c-myc) were injected subcutaneously into 6-week-old female NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice following the protocols approved by BRC IACUC #110643 at the Agency for Science Technology and Research (A*STAR) Singapore. Visible tumours appeared typically at 2 weeks and were harvested following euthanasia and analysed by histology and expression microarray.

Histology and immunofluorescence

Histology, immunohistochemistry (IHC), and immunofluorescence (IF) were performed following standard laboratory protocols. All the primary antibodies used in this study and staining conditions are summarized in Supplementary Table 1. For IHC, detection was completed with the Vectastain ABC kit (Catalog No PK-6102; Vector Laboratories, Inc, Burlingame, CA, USA) with a liquid DAB-plus substrate kit (Catalog No 00-2020). Slides were counterstained with Hematoxylin Stain 3 (Catalog No CS402-1D). Stained slides were stored at 4 °C in the dark and all images for section slides were captured by using an Inverted Eclipse Ti-Series microscope (Nikon, Japan) with Lumencor SOLA light engine and Andor Technology Clara Interline CCD camera and NIS-Elements Advanced Research v.4.13 software (Nikon, Japan) or an LSM 780 confocal microscope (Carl Zeiss, Germany) with LSM software. Bright field cell culture images were obtained on an Eclipse TS100 microscope (Nikon, Japan) with a Digital Sight DSFi1 camera (Nikon, Japan) and NIS-Elements F3.0 software (Nikon, Japan).

Laser captured microdissection

Fresh surgical specimens from ten independent women for normal oviduct and HGSC and six independent women for normal, STIC, and invasive STIC were embedded in OCT, sectioned on a cryostat, and stained with haematoxylin to morphologically identify each region. Twelve serial frozen sections of each tissue sample were microdissected using a PALM microbeam instrument (Carl Zeiss, Germany) and each selected cell population from different slides of the same patient was pooled. Total RNAs were extracted using the Pico Pure RNA extraction kit (Life Technologies, Grand Island, NY, USA).

Microarray

For normal Fallopian tube epithelium and paired HGSC samples from ten patients and FTSC (stem cells, immortalized, transformed, and xenograft) samples, total RNA processing and hybridization were performed on Affymetrix human U133 plus 2.0 Array chips (Affymetrix, CA, USA). For normal Fallopian tube epithelium, STIC, and invasive STIC from six patients, total RNAs were amplified using the WT Pico RNA Amplification System V2 and Encore Biotin Module (NuGEN Technologies, San Carlos, CA, USA). Amplified DNA samples were prepared according to the manufacturer's instructions and hybridized onto a GeneChip Human Exon 1.0 ST Array (Affymetrix). GeneChip operating software was used to process all the Cel files and calculate probe intensity values. To validate sample quality, a quality check was conducted using Affymetrix Expression Console software. The intensity values were log₂-transformed and imported into the Partek Genomics Suite 6.6 (Partek Inc, Chesterfield, MO, USA). For the GeneChip Human Exon 1.0 ST Array, exons were summarized to genes and a one-way ANOVA was performed to identify differentially expressed genes. *p* values and fold-change numbers were calculated for each analysis.

Bioinformatics for gene expression

Unsupervised clustering and heatmap generation were performed with sorted datasets by Euclidean distance on average linkage clustering with selected probe sets by Partek Genomics Suite 6.6. Gene set enrichment analysis (GSEA) [16] was performed to compare

(1) STIC, invasive cancer, and normal; and (2) FTSCs and immortalized and transformed FTSCs (FTSCⁱ and FTSC^t). DAVID bioinformatics resources (<http://david.abcc.ncifcrf.gov/>) were used to find enriched pathways [17]. In Figure 1g, 1.5-fold progressively up-regulated genes are selected from FTSCs to FTSTⁱ to FTSC^t in order to create a heatmap. In Figures 2d, 4a and 4c, 2-fold or more and $p < 0.05$ differentially expressed genes are chosen as significantly changed genes for further data analysis. Datasets generated for this study have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under GSE69428 for normal oviduct and HGSC from ten patients; GSE69453 for FTSC-ALI and transformed FTSCs (stem cell, immortalized, transformed, and xenografts); and GSE69429 for normal Fallopian tubal epithelium, STIC, and invasive STIC from six patients.

Results

Cloning, immortalizing, and transforming Fallopian tube stem cells

If the Fallopian tube is the origin of serous cancer, one possible mechanism for the evolution of cancer is a dysregulation of indigenous stem cells. We therefore set out to clone the stem cells of the Fallopian tube using methods to clone columnar epithelial stem cells such as human intestinal stem cells [11]. Using this method, we were able to generate clones of Fallopian tube stem cells which contained many small, undifferentiated, and highly proliferative (Ki67+) cells that can propagate through multiple passages (Figure 1a). These stem cell clones show strong and consistent staining with markers of Fallopian tube epithelial cells (PAX8) (Figure 2a). In our recent work on cloning adult stem cells from human airway and human intestine [11,12], we established a pedigree analysis method to examine the multipotential differentiation ability from a single stem cell. The high clonogenic capacity of Fallopian tube stem cells (consistently 10–15% throughout passages; data not shown) allowed us to use the same approach to rapidly generate single cell ‘pedigree’ lines of expansion and characterization of their lineage fates upon induced differentiation in air–liquid interface (ALI) cultures. Following FTSC differentiation, we found through immune staining with specific antibodies and RT-PCR with specific primers that one single Fallopian tube stem cell (Foxj1–, acetylated tubulin–, TAp73–, Sall2–, BCL2–, PAX2–) can give rise to both ciliated cells (Foxj1+, acetylated tubulin+, TAp73+, Sall2+) and secretory cells (Foxj1–, acetylated tubulin–, BCL2+, PAX2+) [18] (Figures 1b and 1c). Moreover, the same FTSC pedigree line can be induced to differentiate into squamous metaplasia (p63+/Krt5+) in 3D Matrigel assay [19]. We next compared the FTSCs and their differentiated structure in ALI by gene expression. FTSCs showed high expression of several known adult stem cell markers such as Lrig1 [20] and Lgr6 [21] and regulators of self-renewal such as EZH2 [22], FOXM1 [23], and TCF4 [24]. Interestingly, we did not find high expression of Lgr5 [25] in FTSCs. While the differentiated cells lost expression of stem cell markers, they showed increased expression of genes associated with ciliated cell and secretory cell differentiation such as genes in the dynein family [26] (DNAH3, DNAH2, DNAI2, DNAH12, DNAH7, DNAH10, DNAH5, DNAH9, DNAH6, DNAI1, and *DYNLRB2*) and *MUC13* [27] (Figure 1d and Supplementary Table 2).

To examine whether the Fallopian tube stem cells are the cell of origin of high-grade serous cancer, we introduced SV40/hTERT or SV40/hTERT/c-MYC into these cells by retroviral infection to induce immortalization or transformation of these cells (Figure 1e). We showed that while both immortalized and transformed FTSCs gained the new property of growing without the support of an irradiated 3 T3-J2 fibroblast feeder, the transformed FTSCs lost contact inhibition and showed fibroblast-like morphology (Figure 1f, upper). Moreover, both immortalized and transformed FTSCs formed sphere structures in growth factor-reduced Matrigel in 5 days, but transformed FTSCs generated the irregular structures around ten times larger in comparison with small and round spheres derived from immortalized FTSCs (Figure 1f, lower). A heatmap of differentially expressed genes in whole-genome transcriptome analysis of FTSCs and immortalized and transformed FTSCs showed distinct expression profile differences between normal FTSCs and transformed FTSCs (Figure 1d and Supplementary Table 2). Interestingly, immortalized FTSCs expressed at the moderate level many genes that are highly expressed in transformed FTSCs (Figure 1g and Supplementary Table 3). Gene ontology analysis was performed to identify the gene pathways significantly enriched in transformed cells including DNA replication and DNA repair (Supplementary Figure 1a). In addition, a highly amplified gene in ovarian cancer, *c-MYC*, was used as the transforming agent [28]. Consistently, we observed that downstream genes of *c-MYC* are highly enriched in transformed FTSCs but not in immortalized FTSCs (Supplementary Figure 1b).

Transformed Fallopian tube stem cells gave rise to high-grade serous cancer

To examine whether the transformed Fallopian tube stem cells belong in the serous carcinogenic pathway, we injected 2000 transformed Fallopian tube stem cells subcutaneously into immunodeficient (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice [29] and observed the formation of palpable tumour in 2 weeks. The xenografted tumours demonstrate all the pathological and immunological hallmarks of human high-grade serous cancer, such as gain of p53, WT1, EZH2, and MUC4 expression (Figure 2a) [19,30-32]. To determine whether FTSC^t xenograft tumours resemble human high-grade serous cancer at the level of gene expression, we first compared the RNA from ten microdissected histologically normal Fallopian tube epithelium and paired high-grade serous cancer tumour samples on expression microarray chips. These data revealed a significant two-or-more-fold alteration ($p < 0.5$) of the levels of 2395 genes, which is presented in a heatmap (Figure 2b and Supplementary Table 4). Of the 2395 genes, 1017 are up-regulated and 1378 are down-regulated in tumour samples. Further analysis showed that FTSC^t xenograft tumours, just like HGSC, also expressed these HGSC-related genes in a similar manner.

To uncover the genes or pathways that could be targeted to lead to tumour cell death for therapeutic purposes, we uncovered a number of druggable targets in HGSC such as enhancer of zeste homolog 2 (EZH2). EZH2 is a histone methyl transferase (HMT) and a member of the polycomb group of genes (PcG) regulating (suppressing) transcription through nucleosome modification, chromatin remodeling, and interaction with other transcription factors. Several studies have demonstrated that EZH2 is involved in oncogenesis, and high EZH2 transcript and protein levels have consistently been associated with aggressive tumour behavior, chemo-resistant tumour stem-like side populations, and

overall poor clinical outcome in ovarian, breast, prostate, and bladder cancer patients [33]. We showed that the protein expression of EZH2 is up-regulated in precancerous lesions of HSGC (p53 signature) [34], STIC, invasive serous cancer, and FTSC^t xenograft tumours (Figure 2c). Consistent with the increased expression of EZH2 in HGSC and xenograft tumours, the expression of downstream targets of EZH2 was significantly down-regulated compared with normal Fallopian tube epithelium (Figure 2d).

Resolving molecular alterations in STIC and its progression to invasive serous cancer

Although advanced serous cancer has been proposed to result from STIC progression, there has not been any transcriptome analysis to demonstrate the molecular progression from STIC to advanced serous cancer. To better understand the potential links between these entities via markers of progression, we used the LCM approach to isolate normal Fallopian tube epithelium, STIC, and advanced serous cancer from the same patient (Figure 3a). A heatmap of differentially expressed genes in these datasets showed a distinct expression profile between normal Fallopian tubal epithelium and advanced serous cancer among six patients. In contrast, STIC showed a significant overlap with both normal epithelium and advanced cancer (Figure 3b). Microarray and gene set enrichment analysis (GSEA) were performed to identify the genes and pathways significantly enriched in both STIC and advanced cancer (Supplementary Figures 2c and 3b and Supplementary Table 9) or uniquely changed in advanced serous cancer (Figure 3c, Supplementary Figures 2a and 2b, and Supplementary Tables 5 and 8). The pathways involved in cell proliferation, genomic instability, and survival are aberrantly expressed at the early stage of serous carcinogenesis and are followed by deregulation of the pathways involved in cell migration and cell adhesion.

Furthermore, we particularly focused on the secreted proteins that are highly expressed in STIC or invasive serous cancer with the goal to use them as biomarkers for early detection of HGSC. We identified eight genes significantly up-regulated in invasive cancer and three of them are already up-regulated in the localized tubal tumour (STIC) (Figure 3d). Among them, *SPPI* (osteopontin), *SPARC* (osteonectin), and *VCAN* (versican) have been reported to be overexpressed in various human cancers. In particular, osteopontin levels in plasma were significantly higher in patients with epithelial ovarian cancer compared with those of healthy controls and patients with other gynaecological cancers [35].

Uncovering early molecular changes associated with STIC

The data presented here support the existing hypothesis that the Fallopian tube is the site of origin of high-grade serous cancer and that STIC is the non-invasive, pre-metastatic form of high-grade serous cancer. A heatmap including 62 genes (>2-fold, $p < 0.05$) was generated to show genes up-regulated in common between STIC and matched invasive cancer (Figure 4a and Supplementary Table 6). Among these 62 genes, pituitary tumour-transforming gene (*PTTG1*) and cyclin E1 (*CCNE1*) (Supplementary Figure 3a) are particularly interesting because they have been implicated in early oncogenesis through their driving role in cellular transformation [36,37]. To validate the expression of some of these genes, we next performed the immuno-histochemistry using antibodies for PTTG1 and CCNE1 on patient-matched sections of normal Fallopian tube epithelium, STIC, and invasive cancer. While

these two markers are barely detectable in normal Fallopian tube epithelium, they are highly expressed in STIC and invasive cancer (Figure 4b). We next hypothesized that among the aberrantly expressed genes in STIC, there is a group expressed during cellular immortalization, which is the first step towards malignancy [38]. To test this, we compared up-regulated genes in immortalized FTSCs and STIC and uncovered 123 genes (> 2 -fold, $p < 0.05$) that overlap in these two entities (Figure 4c and Supplementary Table 7). Amongst these, stathmin 1, a microtubule destabilizing protein [39]; Ect2, a Rho guanine nucleotide exchange factor [40]; and forkhead box M1 (FOXM1), a transcription factor regulating cell cycle [41], have been suggested to play critical roles in HGSC initiation (Figure 4d).

Discussion

The perception of ovarian epithelial carcinogenesis is changing rapidly since the proposal that many of these tumours appear to originate in the Fallopian tube [34]. In 2012, Gilbert *et al* further supported this hypothesis – among patients with ‘early’ HGSC, the cancer had originated from the Fallopian tube, peritoneum, or both in 78% [42]. Further evidence for the tubal origin of high-grade serous carcinomas comes from a recent report noting that non-uterine high-grade serous carcinomas incidentally discovered in the general patient population arise in the Fallopian tube in most cases [43].

In this study, we cloned stem cells from human Fallopian tube and demonstrated that transformed Fallopian tube stem cells (FTSCs) can develop to aggressive HGSC in mouse xenograft models in a short time. The xenografted tumour shared all the hallmark features with HGSC, further supporting the Fallopian tube as the site of origin of serous cancer. In our working model (Figure 5), we hypothesize that the immortalized FTSCs correlate with STIN/SCOUTs and the transformed FTSCs correlate with STIC. Gene expression array and genomic analysis of cloned cells from STIN/SCOUTs or STIC will help to further examine this hypothesis and provide valuable information of the multi-step carcinogenesis of HGSC *in vivo*.

Attempts to culture oviductal epithelial cells have been made previously, including efforts to model HGSC [14,44-47]. In addition, a study using a mouse model specifically targeting BRCA, p53, and pTEN in Fallopian tube further supports it as the site of origin for high-grade serous cancer [48]. However, none of these studies addressed the existence of FTSCs and their role in serous carcinogenesis. Herein, we report that stem cells of Fallopian tube can be maintained in culture in their elemental state and, using the pedigree approach, are capable of multipotent differentiation from one single stem cell in the Fallopian tube. Importantly, this platform of culturing FTSCs faithfully and robustly *in vitro* provided us a unique opportunity to functionally study putative oncogenes or tumour suppressors discovered in recent cancer genome analyses through genetic editing of patient-derived FTSCs. Moreover, in this study, we found that several putative oncogenes were significantly overexpressed at the step of FTSC immortalization prior to the occurrence of transformation. Among them, stathmin 1 has been proposed as a marker expressed in early pelvic serous carcinomas [39]; CXCR4 and its ligand CXCL12 have been suggested as the key determinants of tumour initiation and metastasis of ovarian cancer [49]; and forkhead box M1 (FOXM1) has been reported as a key regulator of tumourigenesis by increasing

proliferative activity and leading to uncontrolled cell division [41]. EZH2, a negative regulator of transcription, was also up-regulated two-fold with immortalization. Interestingly, our previous study showed that the PAX2-null progenitor cell growth (secretory cell outgrowth, SCOUTs) in the Fallopian tube also strongly expressed both EZH2 and stathmin 1 [19]. This correlation led us to propose the link between FTSC immortalization and the development of early proliferations in the tube, either SCOUTs or lower-grade STINs. Based on these correlations, it appears that these gene perturbations associated with immortalization might occur prior to or even in the absence of the serous cancer, in which case their value as predictors of malignancy could be limited by their lack of specificity as actionable values. However, they could conceivably be targeted as co-determinants of neoplastic progression, with the goal of depriving the serous carcinogenic sequence of a participating pathway.

The stage at which an ovarian cancer is detected is the single most important factor influencing outcome, and interrupting ovarian cancer when it is curable will require addressing early disease in the distal Fallopian tube. Early molecular signatures that are specific for these neoplasms are of paramount importance given their potential value in detecting neoplasia via analysis of fluids in the lower genital tract [50]. Given the fact that many STICs do not have a HGSC outcome, we believe that there is a window of opportunity where patients with potentially lethal precursors can be identified through screening and spared death from this malignancy. It is hoped that a molecular analysis of early lesions might provide an array of targets that are either secreted by these cells or presented on the cell surface for screening and therapeutic value, respectively. Monoclonal antibodies to secreted proteins have the potential to form the basis of population-wide screening methods from blood or cervical fluid for those at risk who might benefit from salpingectomy. Monoclonal antibodies to cell surface markers in these lesions might assist in alternative detection via imaging technologies as the technology evolves. An important question that must be addressed is whether the parallels between immortalized cells *in vitro* and proliferations *in vivo* signify a background of accrued biological events that precede – and are needed for – progression to malignancy. Thus, the challenge will be to tease out those molecular events that are biologically significant and, when intervened, will prevent subsequent malignancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

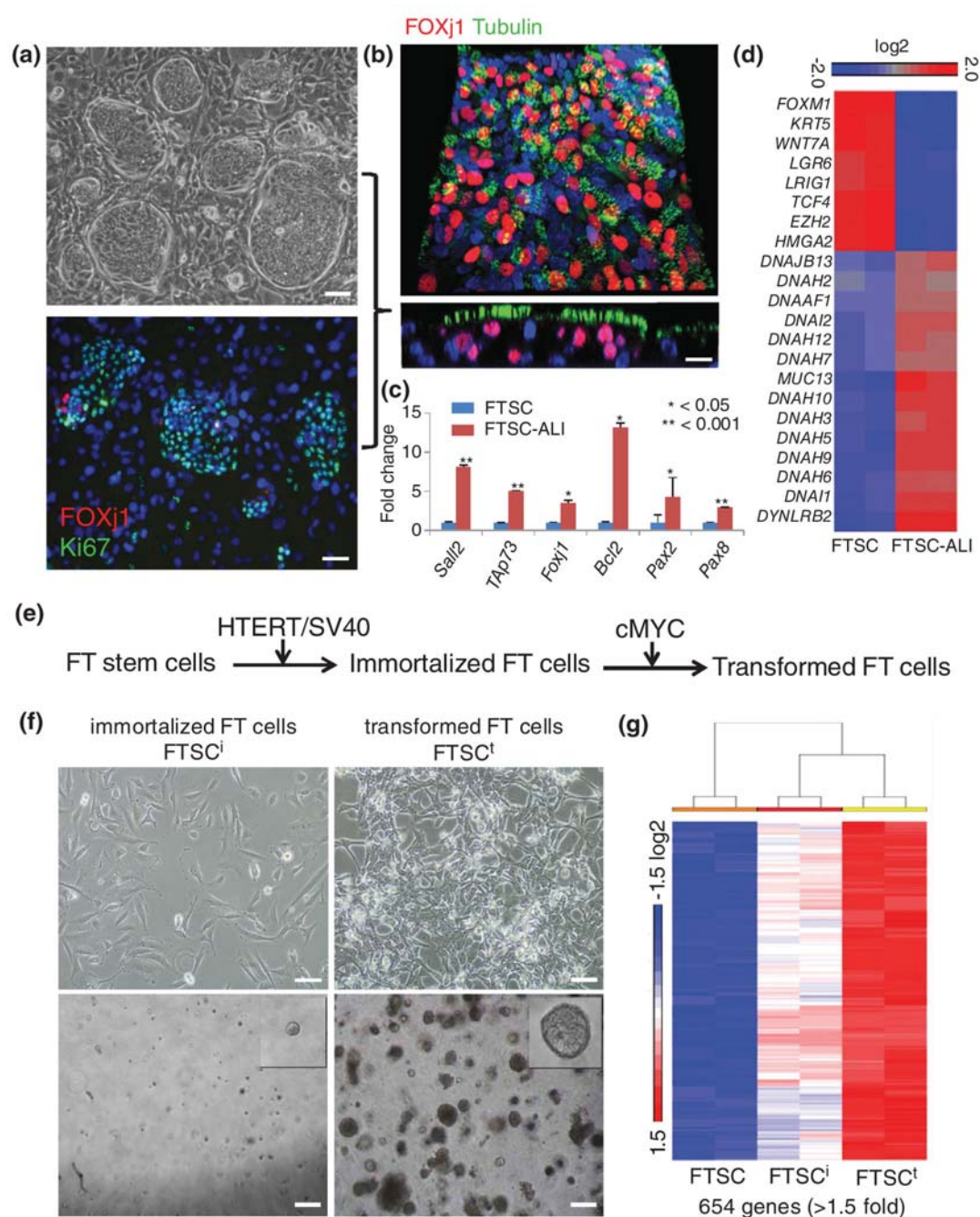
ALI	air–liquid interface culture
FTSCs	Fallopian tube stem cells
HGSC	high-grade serous cancer
STIC	serous tubal intraepithelial carcinoma

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**Figure 1.**

Cloning, immortalization, and transformation of the Fallopian tube stem cells. (a) Cloned FTSCs with proliferation marker Ki67 (green) and ciliated marker FOXJ1 (red). Scale bar = 50 μ m. (b) ALI differentiation culture of FTSCs stained with FOXJ1 (red) and acetylated tubulin (green). Scale bar = 25 μ m. (c) RT-PCR of selected markers ($n = 2$; error bars, SD). (d) Heatmap of selected genes from whole-genome transcriptome analysis. (e) Schematic of FTSC immortalization and transformation *in vitro*. (f) Morphology of immortalized (FTSCⁱ) and transformed FT stem cells (FTSC^t) on plastic culture dishes and in 3D Matrigel assay.

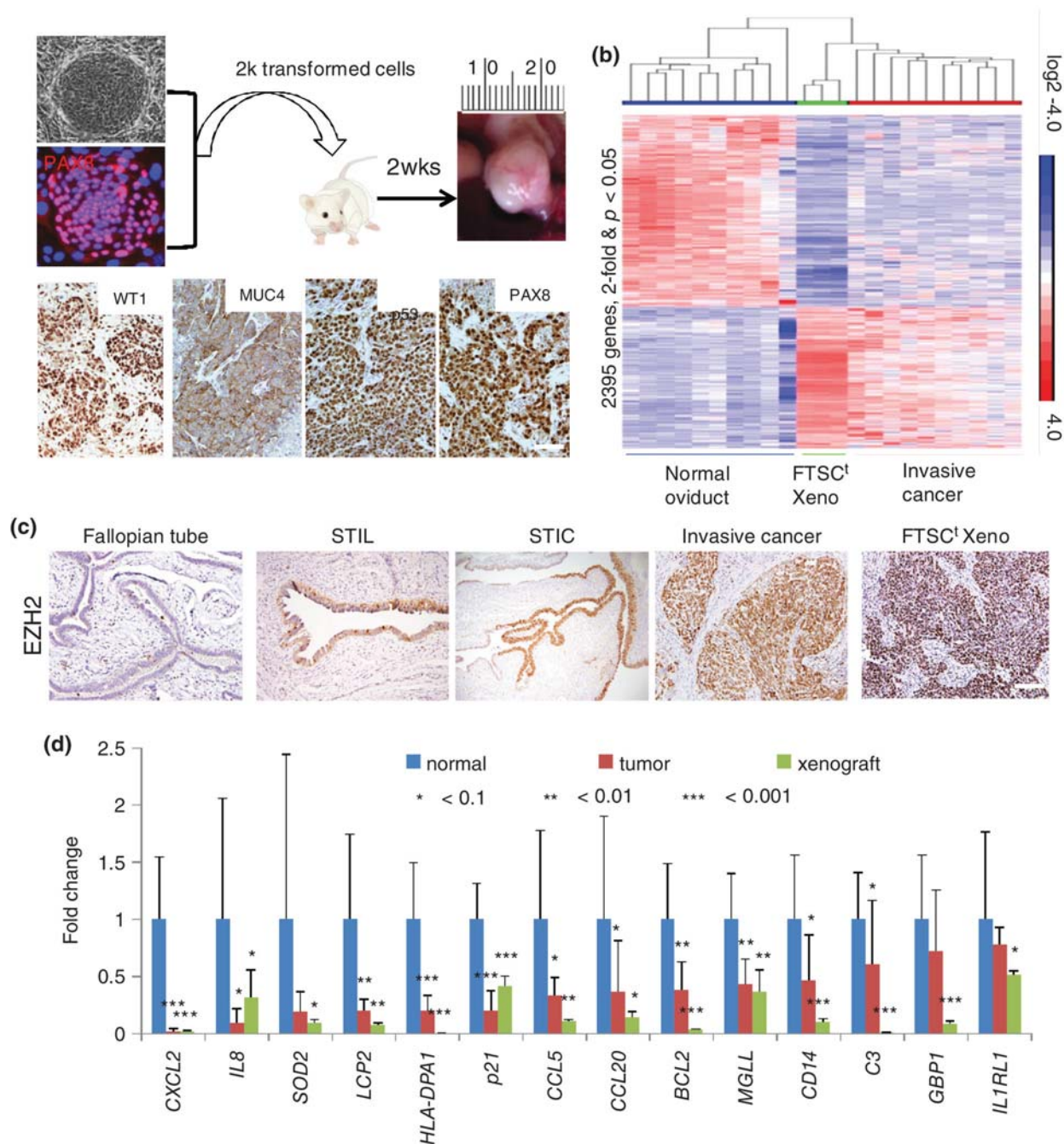
Scale bar = 25 μm . (g) Progressive change of gene expression among FTSCs, FTSCⁱ, and FTSC^t ($n = 2$ each). Genes with increased expression (> 1.5 -fold and $p < 0.05$, 654 genes) following transformation were selected for heatmap production.

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**Figure 2.**

FTSC^t xenograft tumour resembles human high-grade serous cancer. (a) Upper panel: 2000 FTSC^t cells (PAX8, red) were injected into NSG mice and palpable tumour was observed at 2 weeks. Lower panel: xenograft tumour expressed HGSC hallmarks MUC4, p53, and PAX8. Scale bar = 50 μ m. (b) Heatmap showing that FTSC^t xenograft tumours and invasive SC share similar gene expression profiles (FTSC^t tumour: $n = 3$; invasive SC: $n = 10$; and paired normal oviduct: $n = 10$; 2395 genes selected, > 2-fold and $p < 0.05$). (c) EZH2 protein

in multiple stages of HGSC development. Scale bar = 1 mm. (d) EZH2 target genes in FTSC^t xenograft tumours and invasive SC ($n > 3$; error bars, SD).

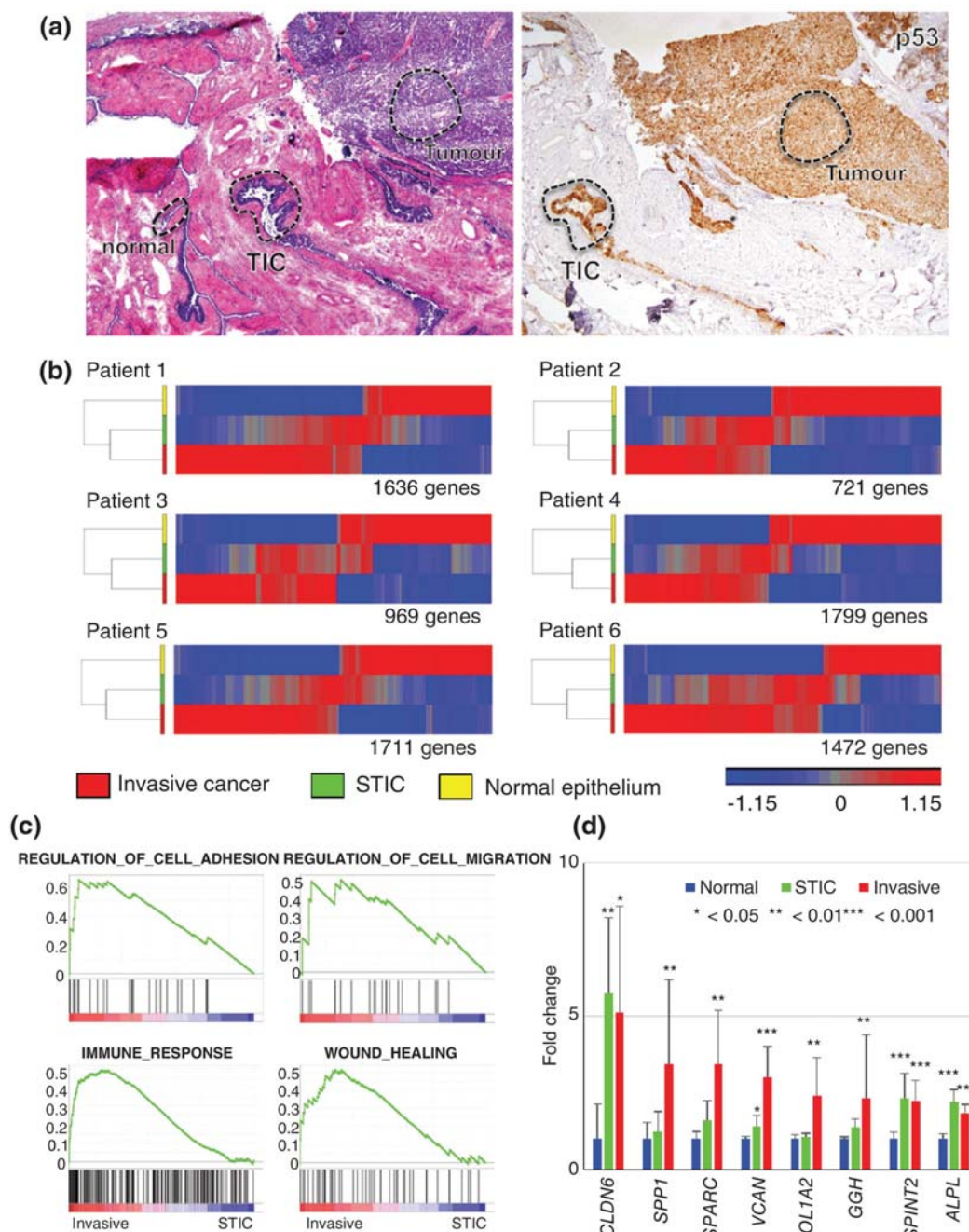


Figure 3.

Molecular correlates of progression from STIC to invasive cancer. (a) Left: histology of the sections used for laser captured microdissection (LCM) of normal Fallopian tube epithelium, STIC, and invasive cancer. Right: p53 antibody staining showing high levels in STIC and invasive cancer. Scale bar = 1 mm. (b) Heatmaps showing progressive gene expression from STIC to invasive cancer in six individual patients (genes differentially expressed in invasive cancer compared with normal FT epithelium were selected, > 2-fold and $p < 0.05$). (c) Gene set enrichment analysis (GSEA) of invasive cancer versus STIC highlighting angiogenesis

and regulation of cell adhesion in invasive cancer. (d) Plots of selected genes highly expressed in STIC and invasive cancer (normal Fallopian tube: $n = 6$; STIC: $n = 6$; invasive SC: $n = 6$; error bars: SD).

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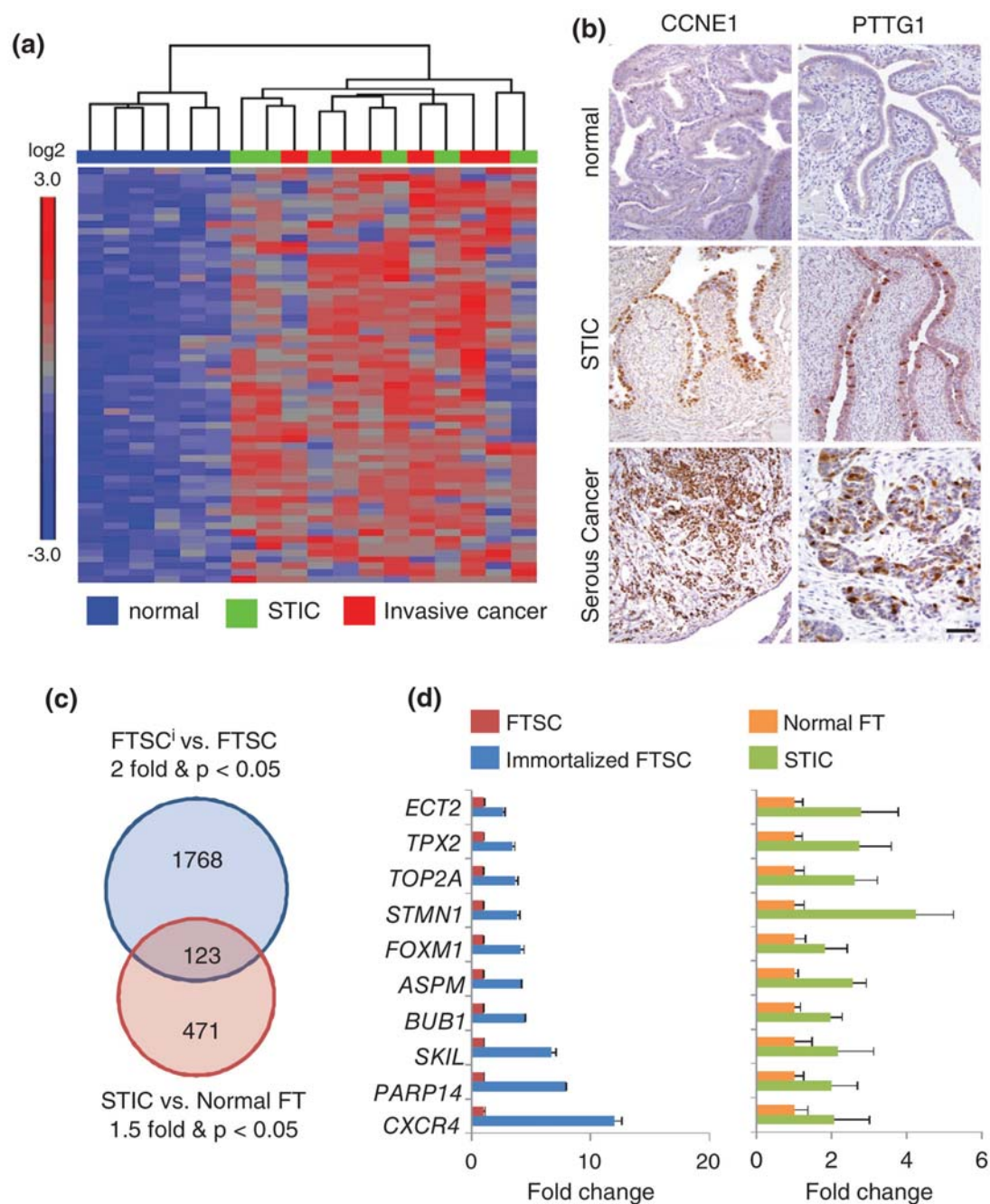


Figure 4.

Early molecular changes associated with FTSC immortalization and STIC. (a) Heatmap showing 62 genes (> 2-fold, $p < 0.05$) commonly overexpressed between STIC and matched invasive serous cancer. (b) Representative images of CCNE1 and PTTG1 immunostaining on normal FT epithelium, STIC, and invasive serous cancer. Scale bar = 1 mm. (c) Venn diagram of genes overexpressed in STIC (> 1.5-fold, $p < 0.05$) and immortalized FTSCs (> 2-fold, $p < 0.05$). (d) Selected overlapping genes and fold change. $n = 2$; error bars: SD.

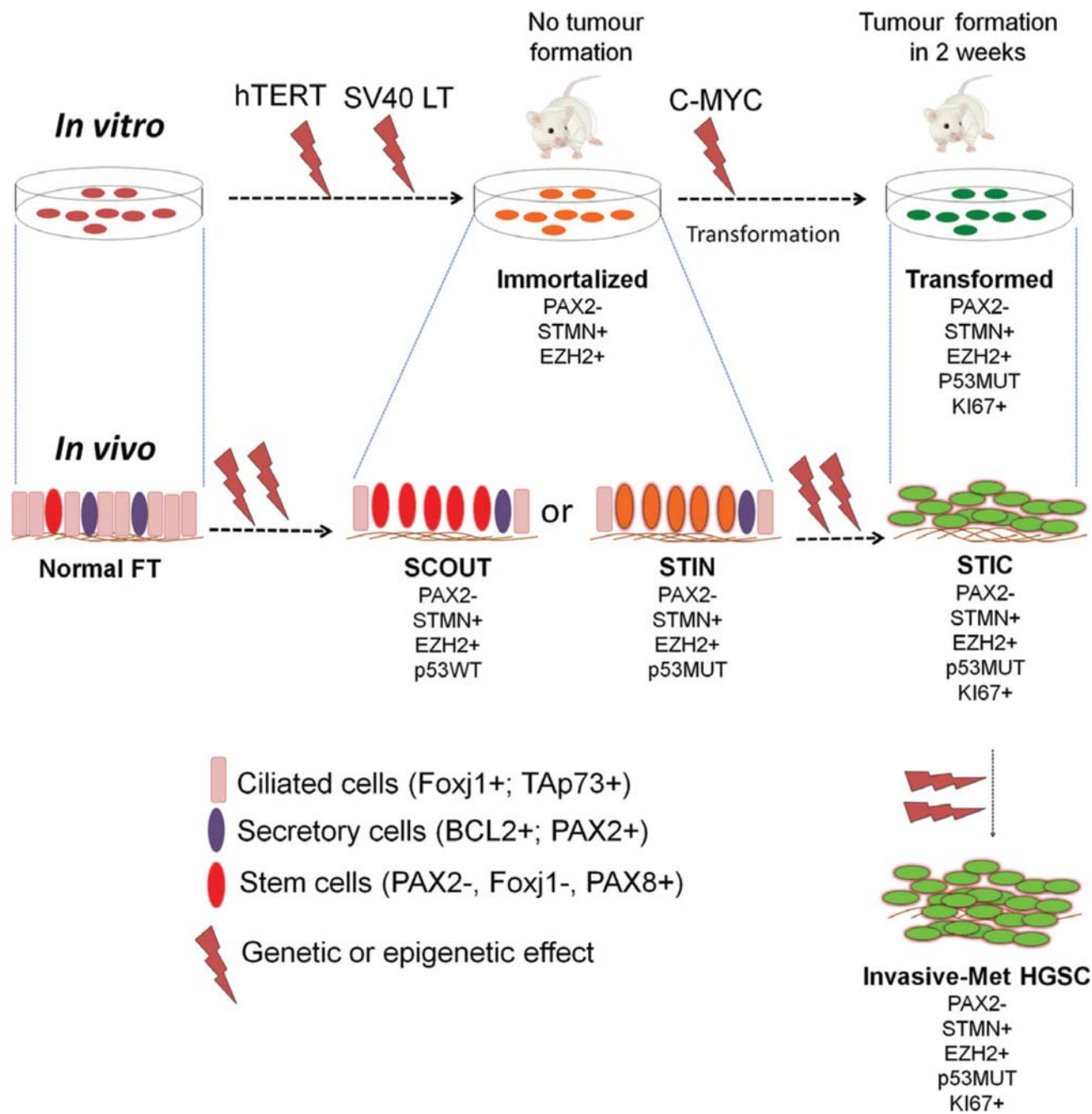


Figure 5.
In vitro and *in vivo* correlations proposing a model of multi-step development of HGSC originating from Fallopian tube stem cells.

Rationale for Developing a Specimen Bank to Study the Pathogenesis of High-Grade Serous Carcinoma: A Review of the Evidence

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Abstract

Women with clinically detected high-grade serous carcinomas (HGSC) generally present with advanced-stage disease, which portends a poor prognosis, despite extensive surgery and intensive chemotherapy. Historically, HGSCs were presumed to arise from the ovarian surface epithelium (OSE), but the inability to identify early-stage HGSCs and their putative precursors in the ovary dimmed prospects for advancing our knowledge of the pathogenesis of these tumors and translating these findings into effective prevention strategies. Over the last decade, increased *BRCA1/2* mutation testing coupled with performance of risk-reducing surgeries has enabled studies that have provided strong evidence

that many, but probably not all, HGSCs among *BRCA1/2* mutation carriers appear to arise from the fallopian tubes, rather than from the ovaries. This shift in our understanding of the pathogenesis of HGSCs provides an important opportunity to achieve practice changing advances; however, the scarcity of clinically annotated tissues containing early lesions, particularly among women at average risk, poses challenges to progress. Accordingly, we review studies that have kindled our evolving understanding of the pathogenesis of HGSC and present the rationale for developing an epidemiologically annotated national specimen resource to support this research. *Cancer Prev Res*; 9(9): 713–20. ©2016 AACR.

Overview of the Problem

Ovarian carcinoma accounts for more than 22,000 incident cases and 14,000 deaths annually in the United States (1). The

most common histopathologic subtype of ovarian carcinoma is high-grade serous carcinoma (HGSC), which characteristically presents with symptomatic, late-stage, high-volume disease. Even with aggressive treatment, the prognosis of advanced-stage HGSC is poor, with 5-year survival rates estimated at less than 50% (2).

Among women with deleterious *BRCA1/2* mutations, risk-reducing salpingo-oophorectomy (RRSO) is effective in reducing ovarian cancer incidence and mortality (3). Unexpectedly, early pathology studies of RRSO specimens led to the identification of putative clinically occult HGSC precursors in the fimbria of the fallopian tubes, rather than in the ovarian surface epithelium (OSE), as anticipated (4). Subsequently, many studies have described putative HGSC precursors in tubes of *BRCA1/2* mutation carriers (reviewed in ref. 5); however, descriptions of these lesions among noncarriers, especially in the absence of concurrent HGSC, remain rare (6, 7), and developing the specimen resource required to investigate such lesions is challenging. Herein, we review recent advances in the understanding of the pathogenesis of HGSC and provide evidence that the development of a tissue bank may facilitate translation of recent findings into improved prevention strategies.

Screening and Prevention Approaches for HGSC

To date, approaches for ovarian/tubal cancer screening and prevention in the general population (8–10) have been disappointing. Screening using CA-125 blood testing at a fixed threshold in combination with pelvic ultrasound did not reduce ovarian cancer mortality in the Prostate, Lung, Colorectal and Ovarian

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Cancer Screening Trial (11) or earlier studies (summarized in ref. 12). In the United Kingdom Collaborative Trial of Ovarian Cancer Screening), serial CA-125 serum levels analyzed with the risk of ovarian cancer algorithm in combination with transvaginal ultrasound also did not demonstrate a statistically significant mortality reduction (13), despite a favorably stage shift (14). Although long-term use of oral contraceptives reduces risk of developing ovarian cancer by up to 50% (15), uptake for this indication has been limited by concerns related to increased risks of thrombotic complications, stroke, and breast cancer (16).

Despite the aforementioned challenges, the discovery that many HGSCs found among asymptomatic *BRCA1/2* mutation carriers seem to arise from the fallopian tubes offers hope of achieving a breakthrough in the early detection and prevention of this disease. However, the percentage of HGSCs that originate in the fallopian tube among *BRCA1/2* mutation carriers and non-carriers is unclear. Further, lack of sufficiently annotated benign gynecologic tissues, putative HGSC precursors and early-stage HGSCs from non-carriers poses an obstacle to pursuit of this work.

Evolving Views on the Molecular Histology and Pathology of the Fallopian Tube

Prior to implementation of RRSO as a prevention strategy among *BRCA1/2* mutation carriers, pathologists rarely encountered specimens containing low-volume HGSC, and when such tumors were identified, attention was routinely focused on the ovaries (17). HGSC was presumed to develop from OSE because tumor was frequently present on the ovarian surface, OSE was presumed to represent the source of a unique progenitor of HGSC, and the risk of HGSC increases with a woman's number of lifetime ovulations. In this model, each ovulation would subject the OSE to injury and repair that could lead to accumulation of deleterious mutations (18). Among cases of HGSC, ovarian and peritoneal involvement is often extensive, whereas tubal involvement is comparatively subtle, easily overlooked, and was seldom sought historically. Thus, the failure to identify dysplastic changes in OSE in older studies was generally ascribed to destructive overgrowth of invasive carcinoma (19).

Recognition that *BRCA1/2* mutations confer lifetime risks of HGSC of 18% to 40% (20) led to increased use of RRSO, enabling Piek and colleagues (21), Crum and colleagues and others (22–25) to identify serous tubal intraepithelial carcinoma (STIC) in the fallopian tube epithelium (predominantly the fimbria) in the context of preserved microanatomy. When STIC and HGSC were present concurrently, the relatedness of the lesions was often suggested by the following: similar morphology with marked cytologic atypia; identical *TP53* mutations in paired lesions (26, 27), comparable immunohistochemical staining for p53, Ki-67, apoptotic markers, and DNA damage response proteins (28–31), and topographic continuity (32). Further, STICs demonstrated shorter telomeres than adjacent normal appearing tubal epithelial cells, suggesting their status as a possible precursor of HGSC (33). In one study, 61% of *TP53* mutations were missense and demonstrated strong p53 protein staining by immunohistochemistry; the remaining cases showed frameshift, splice junction, or nonsense mutations, which were p53 null by immunohistochemistry (26). Thus, most STICs overexpress p53 protein, but a minority is null, and may be identified with other immu-

nohistochemical stains, such as stathmin 1, p16INK4A, and laminin C1 (34–36). Other studies have also reported STICs that were negative by p53 immunostaining (6, 7).

STIC (alone or with concurrent carcinoma) has been identified in 2% to 8% of RRSO specimens, reflecting differences among populations, intensity of sampling for microscopic pathology, and diagnostic criteria (5, 7, 19, 37, 38). In the general population, STIC has been found concurrently with HGSC in approximately 20% to 70% of cases when the tube is extensively scrutinized (39–41), but the presence of cancer limits inferences regarding whether STIC is a cancer precursor. Further, the frequency of detecting STIC may vary with the histopathologic pattern of the associated HGSC and the patient's *BRCA1/2* mutation status, but studies have not identified an alternate origin of HGSC when STIC is not found (42, 43). Thus, at this point, many, but probably not all, HGSCs among *BRCA1/2* carriers appear to arise from STICs, although little is known about the frequency of STICs in the general population (44–46).

STICs have been found in approximately 0.5% of RRSO specimens removed from women at elevated risk of developing HGSC related to a positive family history who tested negative for *BRCA1/2* mutations (5), and anecdotally in tubes removed for benign indications among women in the general population (7, 19, 47). Sensitive protocols for pathology processing to optimize histologic detection of tubal precursors of HGSC have been developed (48, 49), and as pathologists apply these methods more routinely, detection will certainly increase, providing more opportunities for research. Utility of these tissues is enhanced by targeted next-generation sequencing methods that may enable molecular characterization of these lesions in fixed tissues, despite their minimal size (50). These studies may also provide molecular evidence suggesting that some "STIC" lesions represent secondary deposits from endometrial carcinomas (50) and that the clonal relationships of multiple foci of STIC and carcinoma within a single woman are complex (51, 52).

In addition, the development of genetically engineered mouse models that recapitulate the origin of HGSC from the fallopian tube, provide opportunities to perform mechanistic studies that will complement clinical research (53–56). Studies aimed at understanding how ovulation might damage fallopian tube epithelium may suggest new prevention strategies (57, 58).

Approaches to HGSC Research in the General Population

Translating advances in our understanding of the early pathogenesis of HGSC among *BRCA1/2* mutation carriers to the general population is limited by several factors, including: (i) rarity of detecting STIC among women who are *not* *BRCA1/2* mutation carriers and who do *not* have advanced-stage HGSC; (ii) the microscopic size of almost all STIC lesions; (iii) incomplete standardization of the extent of pathology processing of gynecologic tissue specimens (especially when performed for benign indications; refs. 59, 60); and (iv) limited epidemiologic and clinical annotation of samples. Given that STIC requires salpingectomy for diagnosis, the natural history of these lesions will likely remain unknown. Consequently, comparative molecular analysis of STIC, early-stage HGSC, and benign tissues may

represent the best available approach to study the biology of these lesions.

Detection and Characterization of HGSC and Putative Precursors

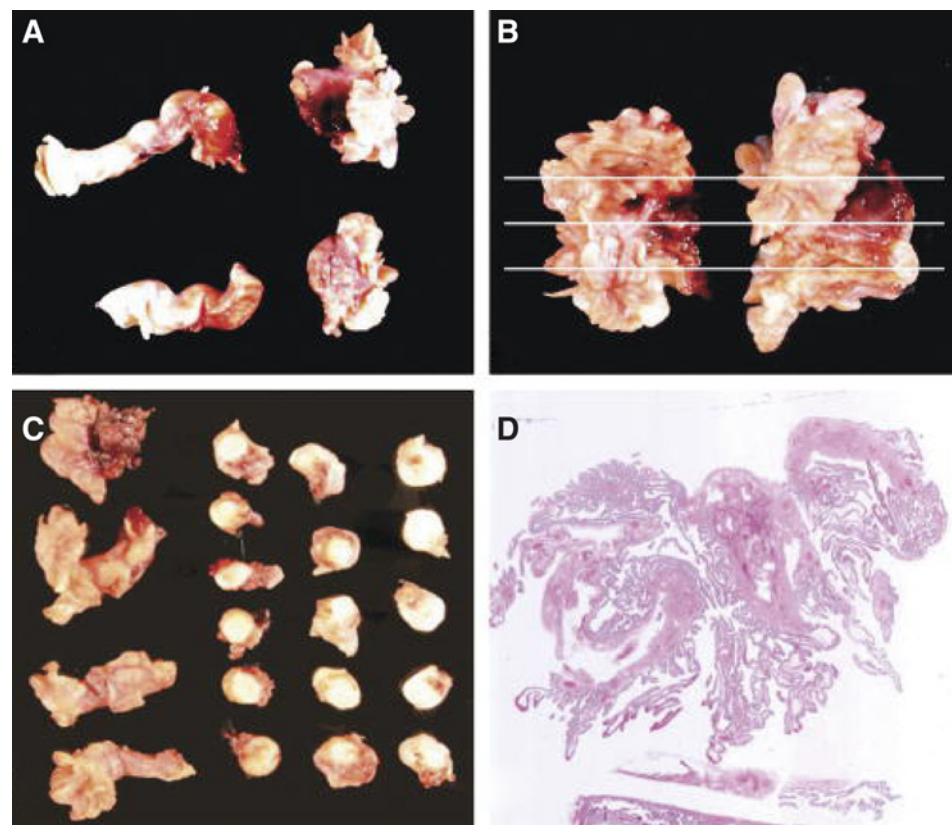
The Sectioning and Extensively Examining the Fimbria pathology protocol ("SEE-Fim") was developed to enable detailed comprehensive microscopic study of the fallopian tube in RRSO specimens (Fig. 1; ref. 49). Dissemination of data regarding detection of STIC at RRSO, and guidelines that emphasize microscopic examination of the tube when cancer is present, have undoubtedly led to increased use of SEE-Fim (61). However, pathology processing of surgical specimens removed from women with wild-type *BRCA1/2* for benign indications is likely more variable, particularly if the tubes and the ovaries appear unremarkable on microscopic examination of the "representative sections" initially submitted for histologic processing.

Among 523 sequential surgical pathology specimens removed for benign indications that were processed according to a modified SEE-Fim approach for research, 4 STICs and 11 additional examples of epithelial atypia were identified (47). A recent study found STICs in 3 (0.17%) of 1,747 specimens from women 50 years of age and older who neither harbored a concurrent pelvic or uterine HGSC, nor were known *BRCA1/2* mutation carriers (E. Meserve and C. Crum, unpublished). Experience suggests that if these specimens had been processed routinely, many STIC lesions may have been missed. In contrast, among 966 high-risk women with or without deleterious *BRCA1/2* mutations who elected immediate risk-reducing surgery in Gynecologic Oncology

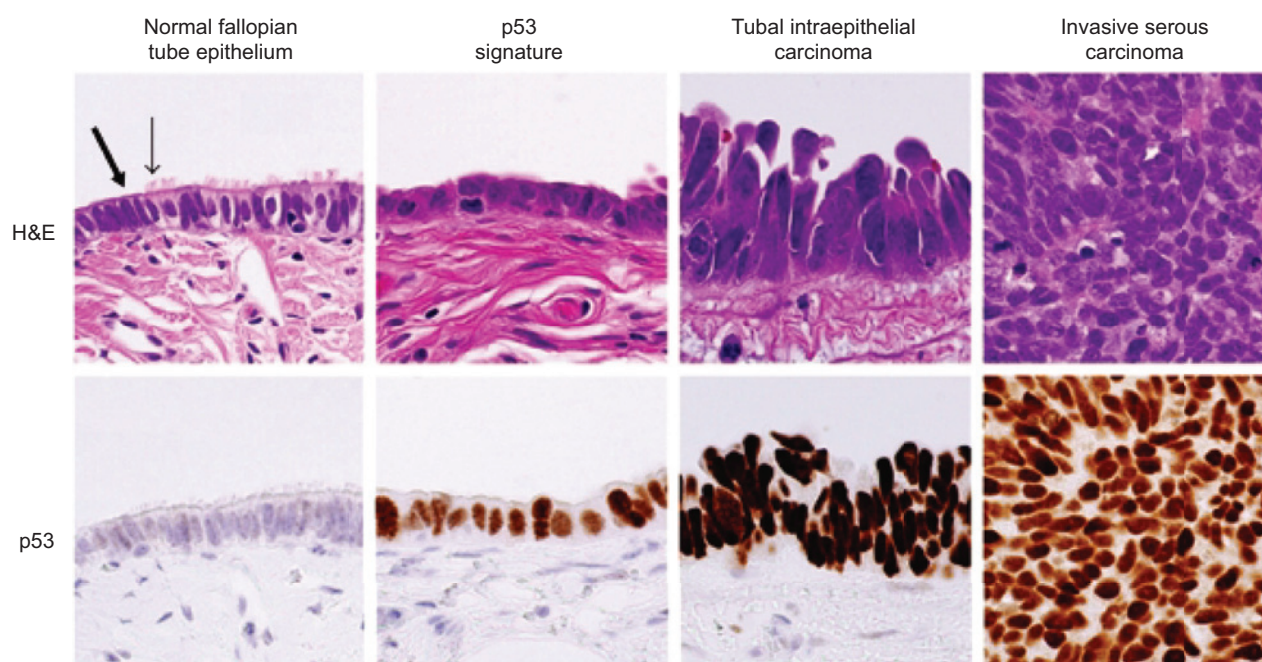
Group Protocol-0199, STICs were identified in four and invasive fallopian tube cancers in five women (5). Among women who are not *BRCA1/2* carriers, STIC is infrequent; however, the *absolute number* of STICs in this group may be substantial given that these women account for 85% to 90% of HGSCs in the population. Further, germline mutations in genes other than *BRCA1/2* may increase risk of HGSC and these women may also harbor STIC or other cancer precursors (62).

The "molecular histology" of the fallopian tube, broadly conceptualized as the morphology, molecular biology, and function of benign tubal tissues in relation to risk exposures has not been extensively studied; however, similarities have been found between the transcriptome of benign tubal epithelium of *BRCA1/2* carriers and HGSC (63, 64), prompting a hypothesis that mutation carriers may respond abnormally to post-ovulatory inflammation (65). In addition, stretches of p53 immunopositive cells have been identified in approximately 24% of carriers of *BRCA1/2* mutations and 33% of women undergoing benign surgery (ref. 27; Fig. 2). These "p53 signatures," which may appear cytologically normal or show only mild cytologic atypia, are not highly proliferative, but frequently demonstrate *TP53* mutations and stain positively for γ H2Ax, a histone that is phosphorylated by ATM kinase at sites of double-strand DNA breaks. Compared with STIC and HGSC, p53 signatures are much more common, especially with intensive scrutiny (59), suggesting that many would not progress to neoplasia if left intact, although a minority of such lesions may represent early steps in carcinogenesis. Areas of secretory cell outgrowths (SCOUTs) composed of stretches of non-ciliated cells expressing wild-type p53 have also been recognized in otherwise histopathologically unremarkable fallopian

Figure 1. Macroscopic appearance of fallopian tube demonstrating SEE-Fim protocol (A–C). Approach to longitudinal sectioning of fimbria (B) and preparing cross-sections of tubes (C). Hematoxylin and eosin-stained section of fimbria (D). This figure was published in *Diagnostic Gynecology and Obstetrics Pathology*, Christopher Crum, Marissa Nucci and Kenneth Lee, Chapter 21, *The Fallopian Tube and Broad Ligaments*, p. 701, copyright Elsevier.



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**Figure 2.**

Sections of fallopian tube epithelium stained with hematoxylin and eosin top and immunohistochemistry for p53 bottom, showing normal, p53 signature, STIC, and invasive serous carcinoma (left to right). Adapted from: Ovarian cancer pathogenesis: A model in evolution. Karst AM, Drapkin R. *J Oncol* 2010.

tube epithelium, but whether this is a variant of normal or a subtle alteration associated with greater cancer risk is also uncertain (66).

Fallopian Tube Pathology in Clinical Practice and Translational Research

The interobserver reproducibility of the diagnosis of STIC based on morphology is suboptimal. Although use of immunohistochemical stains may improve agreement (28, 31, 67–69), expert consensus is the only available measure of diagnostic accuracy. Establishing reproducible and accurate diagnoses of STIC is a prerequisite for developing clinical studies to improve management. Accurate diagnosis of STIC will likely pose an increasing clinical problem, as *BRCA1/2* mutation testing, performance of RRSO, and meticulous examination of surgically removed fallopian tube increases. Moreover, only 6% to 10% of STICs encountered in RRSOs of women with *BRCA1/2* mutations have an outcome of metastatic HGSC, raising important questions about the risk of progression of this putative early form of HGSC (70, 71).

"Opportunistic salpingectomy" has been proposed as a public health strategy to lower incidence rates of ovarian/tubal cancer (72–76). Salpingectomy with deferred oophorectomy offers the potential to prevent HGSC while limiting harms associated with premature estrogen deprivation. Opportunities to perform incidental salpingectomy occur in conjunction with (i) sterilization (in place of tubal ligation); (ii) hysterectomy for benign diseases and (iii) non-gynecologic abdominal or pelvic surgery. Opportunistic salpingectomy offers considerable theoretical appeal; however, prospective proof-of-safety and effectiveness will require decades of surveillance. Population-based registry analyses from Scandinavia have demonstrated that women that have

undergone salpingectomy, particularly if bilateral, have a substantially reduced incidence of "ovarian cancer," supporting the hypothesis that a sizeable percentage of HGSC arises from the fallopian tube (77, 78).

Anecdotal observations suggest that cells from STIC lesions may exfoliate from the fallopian tube mucosa and implant on the ovary or peritoneum without invading through the basement membrane of the tube (79). Staging procedures may demonstrate invasive HGSC in cases initially diagnosed as STIC (80). Interest in the topic of prophylactic salpingectomy with deferred oophorectomy will likely magnify unaddressed concerns regarding whether detection of STIC or STIC-like lesions necessitates immediate oophorectomy, and possibly, formal cancer staging. In fact, clinical observations (81) and studies of animal models (53) suggest that ovarian involvement may potentiate the malignant behavior of early HGSC. Further, the value of offering *BRCA1/2* genetic testing to women with incidental STIC is unknown. It is also unclear whether high-risk women who undergo salpingectomy will return for delayed oophorectomy, and if so, when that should be performed to maximize cancer risk reduction, while minimizing negative effects of estrogen deprivation, including osteoporosis and cardiovascular disease.

National Gynecological Specimen Bank: Considerations

The overarching goal of creating a national gynecological specimen bank would be to provide epidemiologically annotated samples to the research community to pursue high-quality research related to the pathogenesis of early-stage HGSC. Although investigators have collected RRSO samples, and a campaign promoting "opportunistic salpingectomy" with benign

hysterectomy as a means of lowering the incidence of HGSC has been promulgated in British Columbia (73), these resources have limitations, including (i) rare numbers of STIC lesions and early cancers, (ii) exhaustion of small lesions by histopathology processing and molecular testing, (iii) variable pathology processing, (iv) incomplete epidemiological and clinical annotation, and (v) lack of associated germline DNA. The goal of the proposed bank is to augment available resources and to complement registry efforts, such as the recently established Pelvic-Ovarian Cancer Interception (POINT) Project (Pointproject.org/POINT/).

Historically, pathologists have examined grossly unremarkable fallopian tubes sparingly, mainly for documentation purposes; however, clinical practices are likely changing. Thus, by leveraging the shift toward routinely examining tubes more thoroughly, it may be practical to efficiently identify the rare cases of STIC among non-carriers of *BRCA1/2* mutations, without vastly modifying routine pathology protocols for research. Specifically, electronic searches of surgical pathology reports may be sufficient to identify a useful number of women with STIC, even if such cases are rare. Further, more extensive sampling of the ovary and endometrium may reveal unsuspected non-tubal HGSC precursors, such as endometrial intraepithelial carcinoma, the probable precursor of uterine serous carcinoma (82).

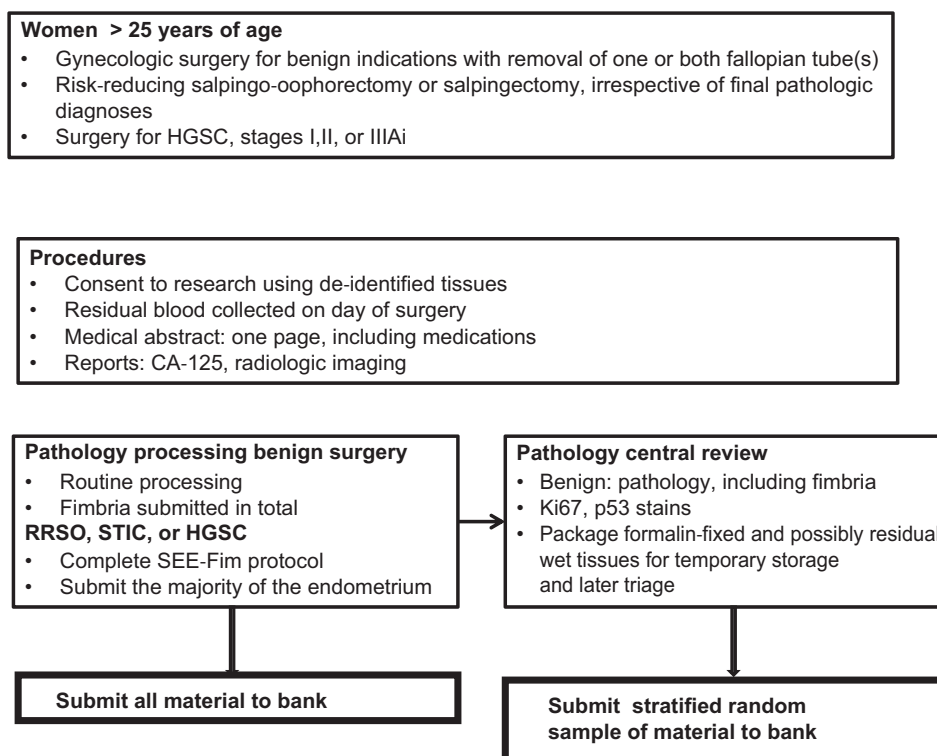
BRCA1/2 carriers are diagnosed with HGSC at earlier ages, respond better to treatment, and in a recent meta-analysis, had improved survival compared with non-carriers at a median of 6.3 years (83). Further, studies suggest HGSC comprises multiple histopathologic patterns, which may be differentially associated with loss of *BRCA1/2* function, STIC, age at onset or prognosis (42, 43). Similarly, HGSC may include multiple molecular subtypes with different clinical behaviors (84). Accordingly, the hypothesis that most HGSCs among non-carriers develop from

STICs represents an untested hypothesis, which could be evaluated using tissue bank resources. Defining whether tubal lesions are associated with HGSC among women who are not carriers of *BRCA1/2* mutations would be useful, either confirming a common approach to HGSC prevention, irrespective of mutation status, or redirecting attention to other approaches.

The proposed bank would collect pathology specimens from three contexts: (i) selected procedures performed for benign indications, such as hysterectomy or surgical sterilization; (ii) RRSO or risk-reducing salpingectomy; and (iii) HGSC, especially stages I, II, or IIIA (Fig. 3). An important aspect of the resource would be the collection of specimens from non-carriers that were removed for benign indications, but which revealed occult STIC or minimal HGSC on microscopic review. In addition, the bank would collect tissues from all RRSOs, HGSC cases, especially those defined as stage I or II or stage IIIA1i (disease volume ≤ 10 mm), and a judiciously selected sample of matching normal tissues from benign surgeries, including fallopian tubes. Each sample would be annotated with minimal medical history as required to estimate risk of developing HGSC within a reasonable logistical framework (85). Centers contributing specimens to the bank would agree to process pathology material according to a standard protocol (Fig. 1). Given that SEE-Fim processing is recommended for cases with STIC or HGSC (61) and that many pathologists are probably examining the tubal fimbria routinely, finding pathology laboratories that are currently processing samples that can identify HGSC precursors and early HGSC may be possible, without altering existing practices. This would enable a *post hoc* selection of a small percentage of specimens from a large pool by re-contacting patients after surgery for consent as needed and further collection of data and specimens. A survey of pathology

Figure 3.

Centers participating in the proposed bank would perform SEE-Fim on all fallopian tubes for microscopic examination. The bank would include the following specimens: RRSO, risk-reducing salpingectomy, any specimen with a diagnosis of STIC, or HGSC (multiple annotated samples of primary and metastatic deposits, SEE-Fim processing, and extensive endometrial sampling to assess the presence of early uterine serous carcinoma). Benign specimens would be selected randomly to create a set of tissues for comparison with those showing putative or diagnostic lesions. Clinical and epidemiologic annotation and source of germline DNA (e.g., unused blood drawn clinically) would be collected as permitted. Residual liquid-based cytology samples would also be banked.



laboratories to assess usual tissue sampling procedures for specimens by clinical indication as would be needed to develop a pilot project is ongoing.

The bank could be pilot tested in pathology laboratories that perform SEE-Fim on all tubes and meticulously sample ovaries and endometrium. Benign surgical pathology specimens removed from non-carriers could be handled using a two-stage approach. Specifically, the fimbria of fallopian tubes from procedures with a benign diagnosis would be processed in their entirety for clinical diagnosis and later centrally reviewed for research. On a rolling basis, a stratified random sample of benign specimens without STIC or HGSC would be chosen with oversampling of those at greatest risk (85). These samples could be used in comparative molecular analyses.

Goals of Research Using Banked Gynecologic Tissue Samples

Potentially, data from medical charts could be supplemented by questionnaires. Data and materials from the proposed bank could be used to address a wide range of potential questions related to the pathogenesis of HGSC, including (but not limited) to those defined below.

- Does the molecular histology of the fallopian tube, particularly the epithelium of the fimbria and/or its microenvironment, vary by critical factors including *BRCA1/2* mutation status, age, menopausal status, family history of breast or ovarian cancer, medications, parity or other factors?
 - Are factors associated with risk of developing HGSC associated with the "omic" profile of the benign appearing tubal epithelium?
 - How do molecular profiles of the fimbria and non-fimbria tubal epithelia compare, and what are the similarities and differences?
 - Does the frequency of detecting p53 protein over-expression by immunohistochemistry vary by risk of HGSC among carriers and among non-carriers?
 - Does the frequency, extent or molecular profile of microdissected "p53 signatures" vary by risk factors among non-carriers or carriers of deleterious *BRCA1/2* mutations? Are certain specific *p53* mutations in "p53 signatures" related to HGSC, while other mutations are not?
 - Are ovarian cancer risk factors associated with important characteristics of the microenvironment, including number and immunophenotype of mononuclear cells, microvessel density, collagen, or matrix factors or biophysical characteristics?
 - Are ovarian cancer risk factors associated with markers of cell stress, DNA damage, DNA repair, proliferation, apoptosis, inflammation, and telomere length in benign appearing tubal epithelium?

- How do molecular profiles of STIC, normal appearing epithelium adjacent to STIC and small foci of HGSC deposits compare within and between patients? What evidence is there for clonal relationships between classes of lesions and metastatic deposits and what specific molecular abnormalities are likely drivers of early events in the pathogenesis of these lesions?
- How do molecular profiles of benign appearing fallopian tube epithelium among women with small cancers that are not associated with STIC compare with those that are associated with STIC?
- How heterogeneous is the molecular profile of HGSC and does it vary by age and ovarian cancer risk factors? Do molecular signatures vary by proposed histological subtypes of HGSC?
 - Is there evidence of intratumoral molecular heterogeneity at the earliest stages of HGSC?
 - Given that ovarian involvement may be linked to accelerated dissemination of malignant cells, are there differences in gene expression between tubal and ovarian foci of HGSC?

Conclusions

The development of a national gynecologic tissue bank to study early-stage HGSC and its precursors holds promise for enabling researchers to identify improved methods for early cancer detection and prevention because an important challenge to conducting this research is the scarcity of carefully annotated tissue specimens representing different hypothesized stages in the development of HGSC. However, assembling this resource would require a complex multi-institutional effort, substantial investment, and equitable access based on objective merit of proposed studies. Accordingly, assessment of feasibility and pilot testing to define a cost-effective approach are important prerequisites for considering this project.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Prevention Research

Rationale for Developing a Specimen Bank to Study the Pathogenesis of High-Grade Serous Carcinoma: A Review of the Evidence

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Morphologic correlates of molecular alterations in extrauterine Müllerian carcinomas

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Extrauterine high-grade serous carcinomas can exhibit various histologic patterns including (1) classic architecture that is papillary, micropapillary and infiltrative and (2) solid, endometrioid, and transitional (ie, SET) patterns. Although the SET pattern has been associated with germline *BRCA* mutations, potential molecular underpinnings have not been fully investigated. DNA was isolated from 174 carcinomas of the fallopian tube, ovary, or peritoneum. Targeted next-generation sequencing was performed and single-nucleotide and copy number variants were correlated with morphologic subtype. Overall, 79% of tumors were classified as high-grade serous carcinoma ($n=138$), and the most common mutations in high-grade serous carcinomas were *TP53* (94%), *BRCA1* (25%), *BRCA2* (11%), and *ATM* (7%). Among chemotherapy-naïve high-grade serous carcinomas, 40 cases exhibited classic morphology and 40 cases had non-classic morphology (SET or ambiguous features). Mutations in homologous recombination pathways were seen across all tumor histotypes. High-grade serous carcinomas with homologous recombination mutations were six times more likely to be associated with non-classic histology ($P=0.002$) and were significantly more likely to be platinum sensitive and have improved progression-free survival (PFS) ($P=0.007$ and $P=0.004$, respectively). In a multivariate analysis adjusted for age, homologous recombination mutation status and increased copy number variants were independently associated with improved PFS ($P=0.008$ and $P=0.005$, respectively). These findings underscore the potential significance of variant morphologic patterns and comprehensive genomic analysis in high-grade serous carcinomas with potential implications for pathogenesis, as well as response to targeted therapies.

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Extrauterine Müllerian carcinomas (ovarian, fallopian tube, and peritoneal) are the eighth most common malignancy in women and the fifth most common cause of death from cancer among women in the United States.¹ The 5-year survival for high-

grade serous carcinoma, the most common and most lethal of all pelvic Müllerian carcinomas, is approximately 40%.² These tumors are difficult to detect in early stage and thus frequently present with metastatic disease. Although most high-grade serous carcinomas are associated with a poor prognosis, some patients with the disease have significantly better outcomes.^{3–5}

Germline mutations in *BRCA1* and *BRCA2* account for the majority of inherited cases of high-grade serous carcinomas, and the recognition of these germline mutations has led to the widespread use of prophylactic bilateral salpingo-oophorectomies to

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markedly reduce risk of developing high-grade serous carcinoma in this high-risk population.⁶ The *BRCA1/2* genes have an important role in homologous recombination, and other genes involved in homologous recombination, such as *BRIP1*, *RAD51C*, and *RAD51D*, have also been implicated as less common sources of genetic susceptibility to high-grade serous carcinoma.^{6–9} Homologous recombination-deficient high-grade serous carcinomas (including *BRCA1/2* mutations) depend on alternative, error-prone mechanisms for double-strand break repair, such as the Polθ/PARP1-mediated alternative end-joining pathway for DNA repair,^{10,11} and subsequently have been shown to have increased sensitivity to platinum chemotherapy and to poly ADP-ribose polymerase inhibitors (PARPi), and improved overall survival.^{3,4,12–19}

Studies from The Cancer Genome Atlas Research Network (TCGA) demonstrated that nearly one-third of ovarian high-grade serous carcinomas had *BRCA1/2* alterations, which included 20% with either germline or somatic mutations in *BRCA1/2* and an additional 11% with *BRCA1* epigenetic silencing via hypermethylation.²⁰ Interestingly, they found that improved survival was limited to high-grade serous carcinomas with mutations in *BRCA1/2*, and was not seen in high-grade serous carcinomas with epigenetically silenced *BRCA1*. In addition, it has recently been shown that platinum sensitivity and improved survival in high-grade serous carcinomas is not just limited to patients with *BRCA1/2* mutations but also extends to patients with either germline or somatic mutations in many of the other genes involved in the homologous recombination DNA repair pathway.²¹

Several studies have demonstrated specific morphologic features that are associated with *BRCA* mutations in high-grade serous carcinomas. The following features have been shown to be associated with *BRCA1* germline mutations: serous or undifferentiated histology, prominent tumor-infiltrating lymphocytes, marked nuclear atypia with bizarre nuclei, and a high mitotic index.^{22,23} Another study examined morphologic features in both *BRCA1*- and *BRCA2*-associated tumors that included both germline and somatic events and described a constellation of histologic features, termed ‘SET’, which included solid growth, pseudo-endometrioid architecture, and transitional cell carcinoma-like morphology.²³ This study also showed a higher mitotic index, an increase in tumor-infiltrating lymphocytes, and the presence of necrosis to be associated with *BRCA1* inactivated tumors. Variant high-grade serous carcinoma morphology is more commonly observed in women with *BRCA* germline mutations and is less likely to be associated with a serous tubal intraepithelial carcinoma, and furthermore, this morphologic phenotype may be associated with a better prognosis and a younger age of onset.^{23,24} However, the morphologic features of high-grade serous carcinomas harboring mutations in other DNA repair genes, including non-*BRCA*

homologous recombination genes, have yet to be described.

The purpose of this study was to characterize the molecular alterations present in ovarian, fallopian tube, and primary peritoneal carcinomas of all histotypes and to identify morphologic correlates with clinically significant and actionable molecular alterations in high-grade serous carcinomas.

Materials and methods

Case Selection

This study was approved by the Institutional Review Boards at Brigham and Women's Hospital and Dana Farber Cancer Institute, and included patients with ovarian, fallopian tube, or peritoneal carcinomas undergoing a targeted next-generation sequencing assay performed on tumor tissue at the Center for Advanced Molecular Diagnostics (Department of Pathology, Brigham and Women's Hospital).

Clinicopathologic Features

The following features were recorded in the cases examined: (1) age at diagnosis; (2) germline mutational status; (3) whether neoadjuvant chemotherapy had been administered before histologic assessment and tumor sequencing; (4) patients' treatment and status at last clinical follow-up based on the electronic medical record. Detailed histomorphologic review was performed on all available H&E slides for each case (mean number of slides per case 15, range 0–78); 54 cases had only a scanned digital slide available for review and 11 cases had no histology available. Tumors were classified as previously described^{23,24} into the following groups:

- Classic predominant high-grade serous carcinoma histology: >50% of the tumor demonstrates papillary, micropapillary, or infiltrative architecture, and often desmoplastic stroma (Figure 1a).
- Solid, endometrioid, or transitional patterns predominant high-grade serous carcinoma histology (‘SET’): >50% of the tumor displays one or more variant features, including solid growth, pseudo-gland formation, and transitional cell-like patterns (Figure 1b).
- High-grade serous carcinoma with ambiguous features: portions of the tumor contain areas diagnostic of high-grade serous carcinoma histology, other regions within the tumor exhibit indeterminate or ambiguous morphology suggestive of endometrioid or clear cell features, but falling short of a mixed type carcinoma diagnosis (Figure 1c).

The histologic subdivisions in high-grade serous carcinoma described above were applied to chemotherapy-naïve tumors on which we had available histologic slides or digital images ($n=80$) to avoid potential influence of chemotherapy on tumor

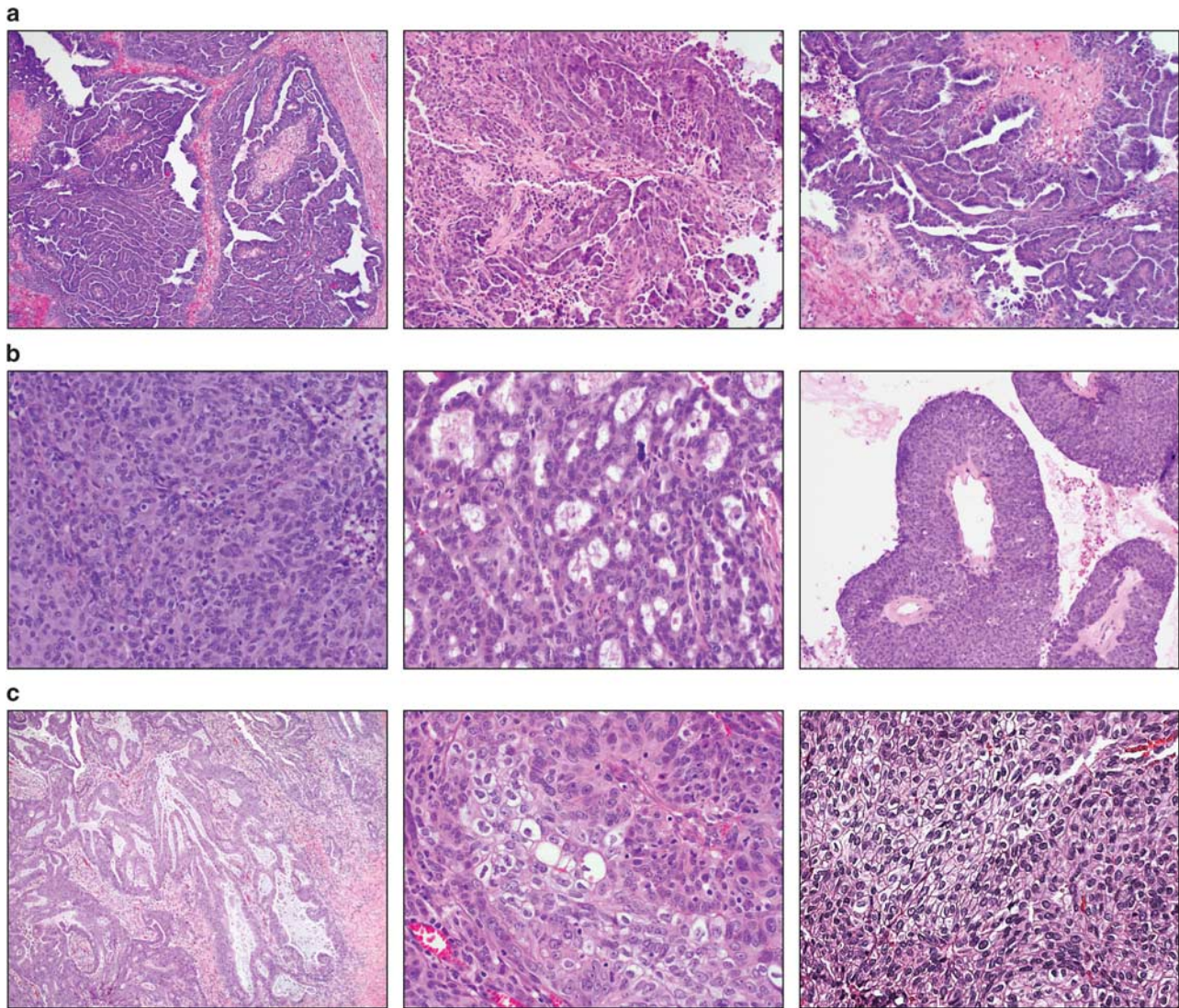


Figure 1 High-grade serous carcinoma histomorphology. (a) Classic morphology that includes papillary and micropapillary architecture. (b) 'SET' morphology that includes solid growth patterns (left), pseudo-endometrioid gland formation (middle), and transitional cell-like growth patterns (right). (c) Ambiguous morphology where portions of the tumor had diagnostic areas of either classic or SET histology as above, as well as regions within the tumor that exhibited endometrioid (left) or clear cell features (middle, right).

histology. The percentage of each tumor containing variant vs classic histology was estimated in each case (in increments of 10%). The presence of serous tubal intraepithelial carcinoma and the finding of endometriosis anywhere within the resection specimen was abstracted from the pathology report.

Targeted Tumor Genomic Sequencing

Formalin-fixed paraffin-embedded tumor samples were digested in proteinase K overnight and DNA was isolated according to the manufacturer's protocol (QIAamp DNA Mini Kit, QIAGEN, Gaithersburg, MD, USA). DNA concentration was assessed using PicoGreen ds DNA detection (Life Technologies, Carlsbad, CA, USA). All cases with at least 50 ng of

DNA were subjected to next-generation sequencing of a targeted panel that included the complete exons of 275 oncogenes and tumor-suppressor genes ($n=156$ cases) or a targeted panel that included the complete exons of 300 oncogenes and tumor-suppressor genes ($n=18$, a newer version of the panel). Ninety-one intronic regions across 30 genes were also included for the evaluation of structural rearrangements. The complete list of the genes interrogated is listed in Supplementary Table 1. Targeted sequences were captured using a solution-phase Agilent SureSelect hybrid capture kit (Agilent Technologies, Santa Clara, CA, USA), and massively parallel sequencing was performed on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA). Mutation calls were made using Mutect²⁵ and GATK software^{26–28} (Broad Institute, Cambridge,

MA, USA) and gene-level copy number alterations at the level of individual genes were assessed using VisCap Cancer (Dana Farber Cancer Institute, Boston, MA, USA). The sequence reads were aligned and processed through a bioinformatics pipeline to identify single-nucleotide variations and small insertions–deletions. Gene-level copy number variations were quantified as a ratio of fractional coverage of each exon in the tumor sample normalized against the fractional coverage of the corresponding exon in a panel of normal samples. Circular binary segmentation was then used to assemble exons into contiguous multi-exon regions. The copy number data for each segment were then displayed visually and interpreted manually by a laboratory scientist and molecular pathologist.

In addition, mutations in DNA repair genes (DNA repair genes highlighted in Supplementary Table 1) were manually reviewed and classified as either deleterious, variant of unknown significance, or single-nucleotide polymorphism. All nonsense, frame shift, out of frame insertion–deletion, splice site, and translation start site mutations were classified as deleterious. All missense mutations were classified based upon the annotation of the mutation in COSMIC (Catalogue of Somatic Mutations in Cancer; <http://cancer.sanger.ac.uk/cosmic>) and in the Exome Variant Server (<http://evs.gs.washington.edu/EVS/>). Mutations reported in COSMIC as confirmed somatic were included in our analysis. Missense mutations present at >0.1% minor allele frequency in the Exome Variant Server and without somatic confirmation in COSMIC were categorized as single-nucleotide polymorphisms. Finally, missense mutations that did not meet the requirements for a single-nucleotide polymorphism and were not present within the COSMIC database were classified as variants of unknown significance.

Germline Assessment

Germline status was determined by reviewing the electronic medical record of each patient in the study. A subset of the patients in this study ($n=115$) had germline genotyping performed as part of their standard clinical care, with 90 patients tested using Myriad myRisk™ (Myriad Genetics, Salt Lake City, UT, USA), 13 cases with OvaNext (Ambry Genetics, Aliso Viejo, CA, USA), and in 12 cases the testing platform was undocumented.

Statistical Analysis

The number of single-nucleotide and copy number variations between groups was analyzed using either an unpaired *t*-test or a Mann–Whitney test in instances of non-normal data distributions. Categorical data, including the frequency of mutations involving genes in the various DNA repair pathways, as well as the frequency of amplified or deleted

genes, was analyzed using Fisher's exact test. Survival curves were generated using the Kaplan–Meier method, and differences between survival curves were assessed for statistical significance with the log-rank test. Multivariate analysis was performed using Cox proportional hazards regression modeling. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and SPSS (SPSS Software v. 20.0, IBM, Armonk, NY, USA).

Results

Summary of Clinicopathologic Features of the Cohort

The study included 174 cases (104 cases from Brigham and Women's Hospital, and 70 cases from outside hospitals) with an average patient age of 58 years (range 25–84 years) and included the following histotypes: high-grade serous carcinoma ($n=138$), grade 1 endometrioid adenocarcinoma ($n=5$), grade 2 endometrioid adenocarcinoma ($n=6$), grade 3 endometrioid adenocarcinoma ($n=1$), clear cell carcinoma ($n=10$), low-grade serous carcinoma ($n=7$), mucinous adenocarcinoma ($n=4$), carcinosarcoma ($n=2$), and undifferentiated carcinoma ($n=1$) (Table 1). High-grade serous carcinoma was the only histotype for which a serous tubal intraepithelial carcinoma was reported (22% of cases). Endometriosis was present in over half of the endometrioid and clear cell carcinomas (60% and 56%, respectively) and was seen in association with high-grade serous carcinoma in 11% of cases ($P<0.0001$, high-grade serous carcinoma vs non- high-grade serous carcinoma).

Forty-nine of the high-grade serous carcinoma tumor samples were obtained status-post neoadjuvant chemotherapy and 89 of the samples were naive to chemotherapy. Eighty chemotherapy-naive high-grade serous carcinomas had histologic slides or digital images available for morphologic analysis; these were further subclassified into the following morphologic subtypes: classic (papillary, micropapillary, infiltrative growth) ($n=40$), SET (solid, endometrioid-like, or transitional-like)²³ ($n=12$), or 'ambiguous' (classic features as well as areas suggestive of either endometrioid or clear cell features) ($n=28$). There was no significant difference in age between the high-grade serous carcinoma morphologic subtypes, with the mean age of classic cases 60 years (range 31–77 years), SET cases 56 years (range 38–68 years), and ambiguous cases 57 years (range 45–70; $P=0.23$). For analysis purposes, SET and ambiguous high-grade serous carcinomas were grouped together as having 'non-classic' histology.

Overview of Targeted NGS Results

Across all 174 cases, 565 single-nucleotide variations (including variants of unknown significance) were

Table 1 Clinicopathologic features

Histotype	Mean, median age (range)	Germline testing	BRCA1/2 germline mutations (% of those tested)	Other non-BRCA germline mutations (% of those tested)	NACT	STIC	EMOSIS
High-grade serous carcinoma (n = 138)	60, 60 (31–85)	n = 91	35% (DEL) 1% (VUS)	1% (DEL) 9% (VUS)	36%	22%	11%
Classic (n = 40)	60, 61 (31–77)	n = 24	24% (DEL)	12% (VUS)	—	22%	5%
Non-classic (n = 40)	57, 58 (38–70)	n = 29	50% (DEL)	3% (VUS)	—	13%	8%
Endometrioid carcinoma (n = 12)	51, 50 (38–74)	n = 6	0%	0%	0%	0%	60%
Clear cell carcinoma (n = 10)	53, 51 (45–68)	n = 9	0%	11% (DEL) 11% (VUS)	0%	0%	56%
Low-grade serous carcinoma (n = 7)	63, 68 (48–79)	n = 4	0%	25% (VUS)	0%	0%	0%
Mucinous carcinoma (n = 4)	37, 34 (25–53)	n = 4	25% (DEL)	0%	0%	0%	25%
Carcinosarcoma (n = 2)	77 (74–79)	n = 1	0%	0%	0%	0%	100%
Undifferentiated carcinoma (n = 1)	57	n = 0	n/a	n/a	0%	0%	100%

Abbreviations: DEL, deleterious; EMOSIS, endometriosis; HGSC, high-grade serous carcinoma; NACT, neoadjuvant chemotherapy; STIC, serous tubal intraepithelial carcinoma; VUS, variant of unknown significance.

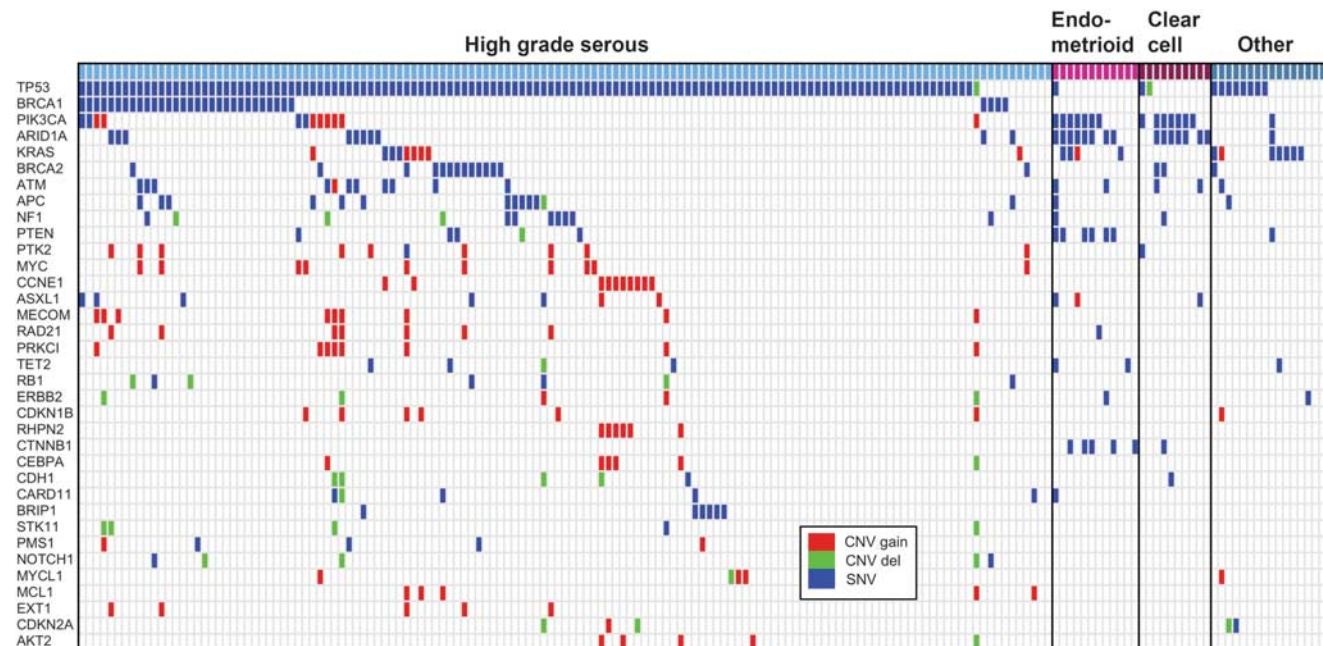


Figure 2 All genes with single-nucleotide variants, indels, or copy number variants identified in at least 3% of the cohort across all tumor histotypes arranged by rows from the most commonly altered gene (top) to the less frequently altered genes (bottom). Single-nucleotide variants and indels are indicated in blue, high copy number gains in red, and two-copy deletions in green.

identified. Figure 2 displays genes with either single-nucleotide or copy number variants identified in at least 3% of cases. A complete list of all single-nucleotide variants identified is available in Supplementary File 2. The most commonly mutated gene in the 12 endometrioid adenocarcinomas was *ARID1A*, comprising 13 mutations in 8 cases (67%), followed by *PIK3CA* mutations, present in 7 cases (58%) (Figure 2). *PTEN* and *CTNNB1* mutations were each seen in 50% of endometrioid adenocarcinomas, whereas only one (8%) had a *TP53* mutation (histologically FIGO grade 2). The most commonly mutated genes in clear cell carcinomas were *ARID1A*, which included 11 mutations present in 7 cases (70%) and *PIK3CA*, which included 8 mutations

present in 7 cases (70%); one clear cell carcinoma harbored a *TP53* mutation. *KRAS* mutations were frequently seen in low-grade serous carcinomas (57%).

There was no significant difference in the mean number of single-nucleotide variants present between neoadjuvant-treated ($n=49$) and chemotherapy-naïve ($n=89$) HGSC (5.3 vs 5.1, respectively; $P=0.75$). Among high-grade serous carcinomas with morphologic subtyping ($n=80$), there was no significant difference in the number of single-nucleotide variants in classic high-grade serous carcinomas ($n=40$) compared with non-classic high-grade serous carcinomas ($n=40$) (variant or ambiguous morphology), 4.9 vs 5.8, respectively ($P=0.11$). The most commonly mutated

genes in high-grade serous carcinomas were *TP53* (94%), *BRCA1* (25%), *BRCA2* (11%), and *ATM* (7%) (Figure 2).

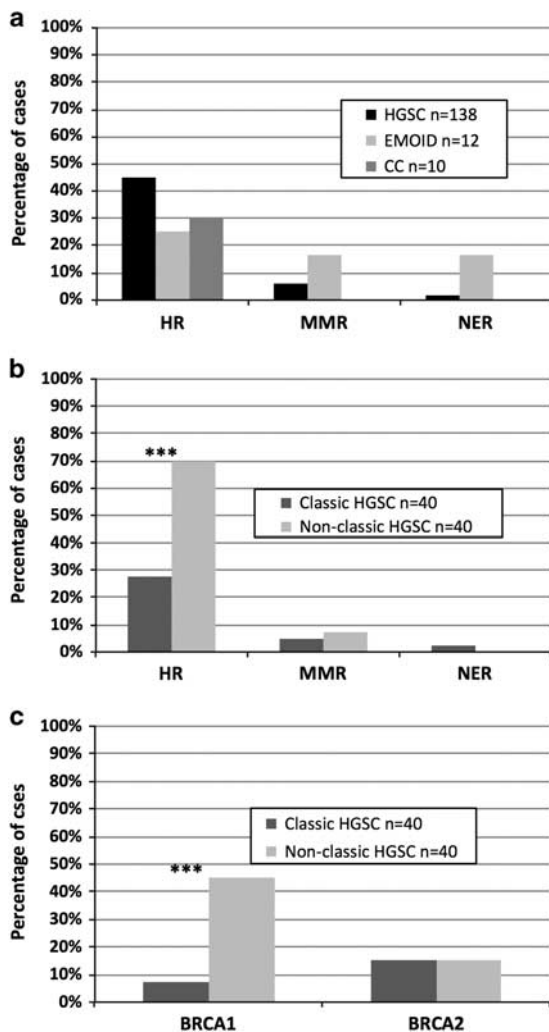


Figure 3 Frequency of DNA repair pathway mutations by morphology, including: (a) homologous recombination (HR), mismatch repair (MMR), and nucleotide excision repair (NER) by histotype. (b) Mutations in DNA repair pathways amongst high-grade serous carcinoma stratified by morphologic subtype. (c) *BRCA1/2* mutational status amongst high-grade serous carcinoma stratified by morphologic subtype. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Mutations Involving DNA Repair Pathway Genes

Mutations in DNA repair pathway genes were seen across all tumor histotypes (Figure 3a; Table 3). There was no significant difference in the frequency of homologous recombination mutations between the broad histotypes ($P = 0.29$) and mutations were seen in the following homologous recombination genes in high-grade serous carcinomas: *BRCA1* (34), *BRCA2* (15), *ATM* (9), *BRIP1* (5), *FANCC* (1), *FANCE* (1), and *FANCG* (1). An associated one copy gene loss, supporting biallelic inactivation, was identified in 42% of the homologous recombination mutations in high-grade serous carcinomas. Mutations involving mismatch repair (*MLH1*, *MSH6*, *PMS1*) and nucleotide excision repair (*ERCC2/3/4/5*, *XPA*) were also seen in a smaller fraction of cases across the histotypes (Figure 3; Table 2).

Within the high-grade serous carcinoma cases, homologous recombination gene mutations were significantly associated with non-classic histology (70% vs 28% with classic histology, OR = 6.2, 95% CI 2.3–16.2, $P = 0.0002$) and younger age (57.8 vs 62.1 years; $P = 0.005$; Figure 3b). Within the homologous recombination genes, *BRCA1* mutations were present in 45% of non-classic high-grade serous carcinomas compared with 8% of classic high-grade serous carcinomas (OR = 10.1, 95% CI 2.7–38.2; $P = 0.0002$; Figure 3c). In contrast, the same association was not seen with *BRCA2* mutations, which were present in 15% of both non-classic and classic high-grade serous carcinomas ($P = 1.00$). Ten percent of high-grade serous carcinomas contained non-*BRCA* homologous recombination mutations, with the most frequent being in *ATM* ($n = 6$) and *BRIP1* ($n = 5$) (Table 2). There was no significant difference in the frequency of mismatch repair or nucleotide excision repair mutations between classic and non-classic high-grade serous carcinomas (Figure 3b).

Correlation of Somatic and Germline Mutational Status

Germline *BRCA1* and *BRCA2* status was known in 115 cases (66%), which included 91 high-grade serous carcinomas (Table 3). Thirty-three (36%) high-grade serous carcinomas harbored known

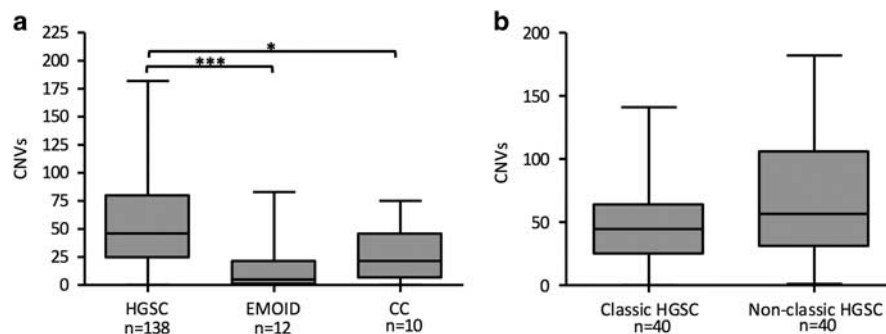
Table 2 Mutations in DNA repair pathway genes

Histotype	HR	BRCA1/2	Non-BRCA HR	MMR	NER
High-grade serous carcinoma ($n = 138$)	45%	25%	10%		
Classic ($n = 40$)	28%	8%	8%	5%	3%
Non-classic ($n = 40$)	70%	45%	10%	8%	0%
Endometrioid carcinoma ($n = 12$)	25%	0%	25%	17%	17%
Clear cell carcinoma ($n = 10$)	30%	20%	10%	0%	0%
Low-grade serous carcinoma ($n = 7$)	0%	0%	0%	0%	0%
Mucinous carcinoma ($n = 4$)	25%	25%	0%	0%	0%

Abbreviations: HGSC, high-grade serous carcinoma; HR, homologous recombination; MMR, mismatch repair; NER, nucleotide excision repair.

Table 3 Correlation between germline and somatic *BRCA1/2* mutations in HGSC

Histotype	<i>BRCA1</i>				<i>BRCA2</i>		
	Germline testing	Germline mutation	Present in tumor	Evidence of somatic inactivation	Germline mutation	Present in tumor	Evidence of somatic inactivation
High-grade serous carcinoma (n = 138)	n = 91	n = 27	n = 23	n = 10	n = 5	n = 5	n = 3
Classic (n = 40)	n = 24	n = 4	n = 2	n = 1	n = 2	n = 2	n = 1
Non-classic (n = 40)	n = 29	n = 13	n = 12	n = 4	n = 2	n = 2	n = 1

**Figure 4** Copy number variants. (a) Median, interquartile range (IQR) and range of overall number of CNVs by morphology. (b) Median, interquartile range (IQR) and range of CNVs in high-grade serous carcinoma by morphologic subtype. *** $P < 0.001$, * $P < 0.05$.

deleterious mutations and 9 (10%) had variants of unknown significance. Deleterious germline *BRCA1* mutations were seen in 27 high-grade serous carcinomas. In 23 of these instances, the same germline mutation was found in the tumor; there was evidence of biallelic inactivation by gene deletion in 10 cases and evidence of copy-neutral loss of heterozygosity in an additional 10 cases. Deleterious germline *BRCA2* mutations were seen in five high-grade serous carcinomas; in all cases the same mutation was found in the tumor and in three cases there was evidence of biallelic inactivation by gene deletion and in an additional case there was evidence of copy number neutral loss of heterozygosity.

Copy Number Variations

Across all 174 cases, 7512 gene-level copy number variations were identified. High-grade serous carcinomas had significantly increased numbers of copy number variants (median 43.5) compared with endometrioid adenocarcinomas (median 5.0; $P = 0.0003$) and clear cell carcinomas (median 21.5; $P = 0.045$; Figure 4a). In all, 206 (3%) of the copy number alterations were high copy number gains, of which the majority occurred in high-grade serous carcinomas (95%). Genes with two-copy (homozygous) deletions comprised 131 of the events (2% of total copy number variants) and occurred almost

exclusively in high-grade serous carcinomas (99%). The remaining copy number events represented low level gains or single copy losses.

Approximately 40% of high-grade serous carcinomas ($n = 54$) had high copy number gains of at least one gene, and overall high-grade serous carcinomas had a mean number of 1.4 genes with high copy number gains per case (range 0–10). There was no significant difference in the mean number of genes with high copy number gains in high-grade serous carcinoma with classic vs those with non-classic histology (1.2 and 0.8; $P = 0.24$). Genes with high copy number gains in high-grade serous carcinomas frequently occurred in tandem based on chromosomal location. The gene most commonly seen to have high level copy number gain in high-grade serous carcinomas was *CCNE1*, which was seen in 10 cases (7%) and co-amplified with *RHPN2* in 5 cases. Of the 10 cases with *CCNE1* amplifications, 4 had morphologic subtyping and all demonstrated classic high-grade serous carcinoma morphology. In addition, all 10 *CCNE1* amplified cases were wild-type for *BRCA1* and *BRCA2*. The second most commonly amplified gene with high copy number gains was *PTK2* (nine cases), of which six had co-amplification of *RAD21*. There were eight high-grade serous carcinomas that each had high level copy gains of *PIK3CA* and *PRKCI*, of which five occurred in tandem, and six cases with amplified *CDKN1B* and *KRAS*. There was a single case of clear cell carcinoma that had high copy number gains of *ZNF217*.

Approximately 19% of high-grade serous carcinomas ($n=26$) had at least one gene with two-copy deletion and overall high-grade serous carcinomas had a mean number of 0.9 two-copy deletions (range 0–35). A single case of clear cell carcinoma had one two-copy gene deletion (*TP53*). There was no significant difference in the mean number of genes with two-copy deletions within high-grade serous carcinoma subtypes, comparing classic vs those with non-classic histology (0.3 and 0.2; $P=0.598$). The most common genes with two-copy deletions in high-grade serous carcinoma were *STK11*, *GNA11*, and *CDH1*, each of which was present in four cases.

There was no significant difference in the number of copy number variants that were present in chemotherapy-naïve high-grade serous carcinomas and chemotherapy-treated high-grade serous carcinomas (58.1 vs 60.3, respectively; $P=0.79$). Although there was a trend for non-classic high-grade serous carcinomas to have a higher number of copy number variants (median 56.5, interquartile range (IQR) 30.3–106.0) than classic high-grade serous carcinomas (median 44.5, IQR 24.3–64.0), the difference was not significant ($P=0.11$; Figure 4b).

Platinum Sensitivity, Progression-Free and Overall Survival

Platinum sensitivity and progression-free survival data were available for 104 high-grade serous carcinomas. Among these, cases with mutations in homologous recombination genes ($n=48$) were significantly more likely to be platinum sensitive (79%) compared with high-grade serous carcinomas without mutations in homologous recombination pathways ($n=56$; 54%; OR = 3.3, $P=0.007$; Figure 5a). In addition, high-grade serous carcinomas with homologous recombination mutations had a significantly improved progression-free survival (11 months) compared with those without (7 months) (HR = 0.52, 95% CI 0.3–0.8; $P=0.004$; Figure 5b). There was no difference in progression-free survival between high-grade serous carcinomas with *BRCA1/2* mutations ($n=36$) and high-grade serous carcinomas with other non-*BRCA* homologous recombination mutations ($n=12$; $P=0.97$; Figure 5c). Progression-free survival data in patients with germline or confirmed somatic homologous recombination mutations were available in 25 and 7 cases, respectively. Although the numbers are small, progression-free survival in patients with high-grade serous carcinomas with germline homologous recombination mutations were not significantly different from those with confirmed somatic mutations ($P=0.6$). There was a trend for high-grade serous carcinomas with homologous recombination mutations to also have improved overall survival (94 months) compared with high-grade serous carcinomas without homologous recombination mutations (56 months; $P=0.15$).

Among all high-grade serous carcinomas, the presence of greater than or less than the median number of copy number variants (median = 44) was not significantly associated with platinum sensitivity ($P=0.22$; Figure 5d). However, when stratified by neoadjuvant status, neoadjuvant-treated high-grade serous carcinomas with ≤ 44 copy number variants had worse progression-free survival (6 months) compared with neoadjuvant-treated high-grade serous carcinomas with >44 copy number variants (8 months) (HR 3.5, 95% CI 1.5–8.2; $P=0.004$; Figure 5e). This same association between copy number variants and progression-free survival was not seen among high-grade serous carcinomas that were chemotherapy-naïve ($P=0.29$; Figure 5f). There was no significant difference in platinum sensitivity ($P=0.25$), progression-free survival ($P=0.18$), or overall survival ($P=0.56$) when high-grade serous carcinomas were stratified by morphologic subtype (classic vs non-classic).

Multivariate Cox regression model analysis was performed using age, neoadjuvant chemotherapy status, homologous recombination gene mutation status, and number of copy number variants (using the median CNV = 44 as a cutoff), and demonstrated that all of these variables, excluding age, were independent predictors of progression-free survival. In this multivariate model, homologous recombination deficiency was associated with improved progression-free survival (HR = 0.5, 95% CI 0.3–0.8, $P=0.008$), whereas low copy number variants (HR = 1.9, 95% CI 1.2–3.1, $P=0.005$) and neoadjuvant therapy (HR = 1.8, 95% CI 1.1–2.9, $P=0.02$) were associated with decreased progression-free survival.

Discussion

The goal of this study was to determine morphologic correlates of molecular alterations seen in 174 extrauterine Müllerian carcinomas and is currently the largest study in which detailed histomorphologic review was performed on all cases, including high-grade serous carcinomas. As has been previously demonstrated, we found that high-grade serous carcinomas were characterized by *TP53* mutations and increased number of copy number variants, an indicator for genomic instability.²⁰ In addition, we confirmed the prevalence of mutations in homologous recombination pathway genes across tumor histotypes, ranging from 25 to 45% of cases.²¹ However, it is possible that this number is an underestimate of the actual prevalence, owing to the fact that our targeted panel was not entirely inclusive of all genes involved in homologous recombination,²¹ as well as the fact that we were conservative by including only loss-of-function mutations or missense mutations that had been reported previously. In addition, although we did not evaluate epigenetic mechanisms of inactivating homologous recombination genes, such as promoter

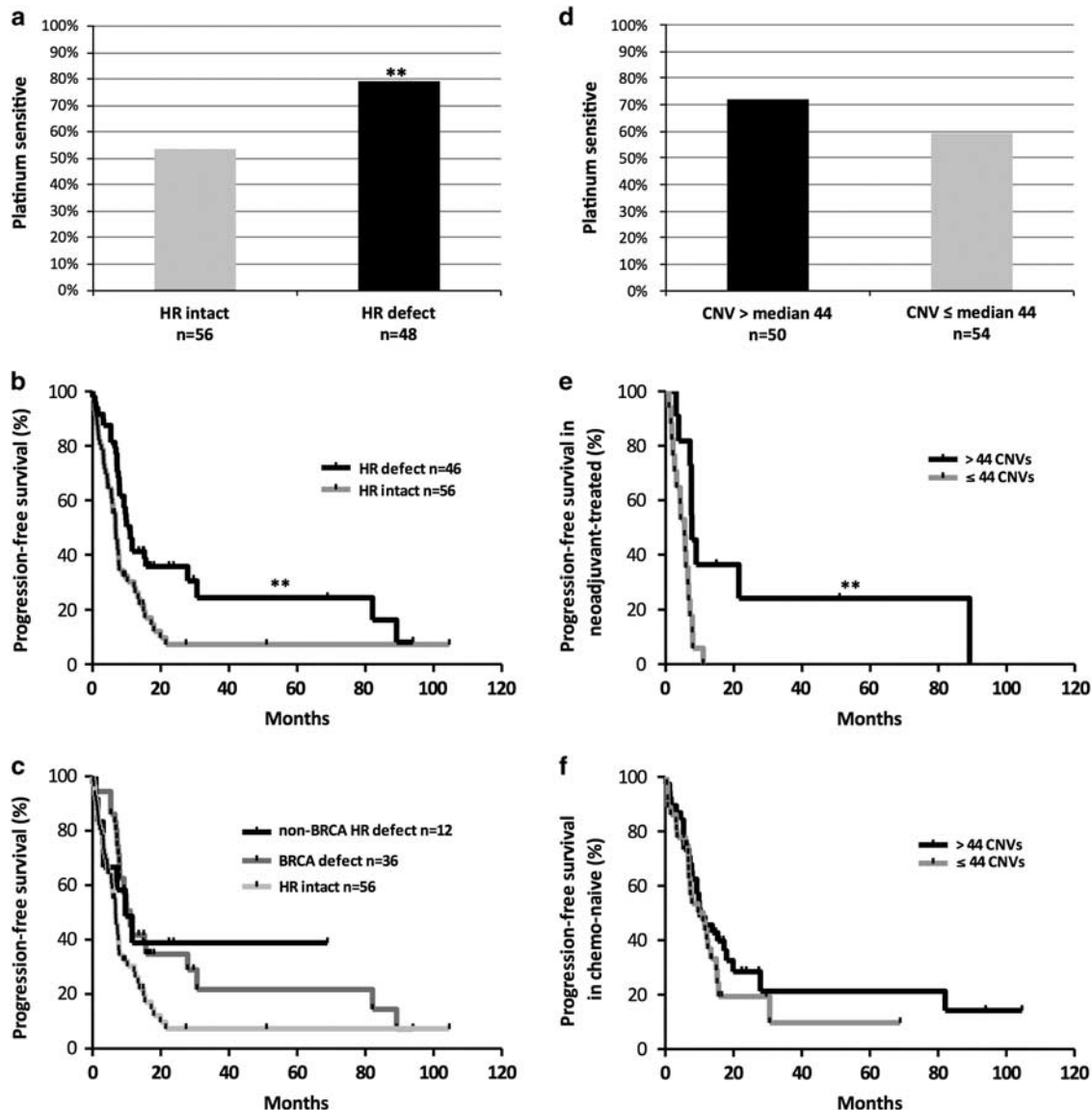


Figure 5 Platinum sensitivity and survival in high-grade serous carcinoma. (a) Frequency of platinum sensitivity stratified by homologous recombination gene mutational status. (b) Progression-free survival stratified by homologous recombination gene mutational status. (c) Progression-free survival stratified by BRCA mutation and by non-BRCA homologous recombination mutation status. (d) Frequency of platinum sensitivity stratified by number of copy number variations. (e) Progression-free survival in neoadjuvantly treated patients stratified by number of copy number variations (CNVs). (f) Progression-free survival in chemotherapy-naïve patients stratified by number of CNVs. ** $P < 0.01$.

methylation, it has been shown that patients with epigenetically silenced *BRCA1* have similar survival to patients with wild-type *BRCA1/2*.²⁰

Although the term high-grade serous carcinoma is currently applied to carcinomas with high nuclear grade and *TP53* mutations, this group of tumors displays a range of histologic patterns. In particular, classic features of high-grade serous carcinoma such as papillary or micropapillary architecture are often not present. Traditionally, high-grade Müllerian carcinomas have been subclassified based on the presence of serous and/or endometrioid features.²⁹ The concept of splitting vs lumping high-grade Müllerian carcinomas has been contentious in the

past, with the argument that the clinical outcome and treatment was the same regardless of the subclassification.³⁰ Since that time, Soslow *et al*²³ validated the significance of different histologic patterns by describing variant morphologies (SET) in tumors with *BRCA* germline mutations that are associated with a better prognosis and a better response to chemotherapy.^{3,4,12–19}

We previously reported on a separate cohort of high-grade serous carcinomas, showing an association between *BRCA*-positivity and variant histology (SET-like), younger age, a lower frequency of serous tubal intraepithelial carcinoma, and trends toward improved survival.²⁴ In this current study, we found

that mutations in homologous recombination genes were 6 times more likely to be associated with non-classic high-grade serous carcinoma histology (70%) than with classic high-grade serous carcinoma histology (28%) ($P < 0.001$), and *BRCA1* mutations were 10 times more likely to be associated with non-classic high-grade serous carcinoma histology ($P < 0.001$) than classic histology.

Mutations involving homologous recombination pathways have a significant influence on platinum sensitivity and survival. By multivariate analysis, we found that homologous recombination mutations were independently associated with improved progression-free survival (HR 0.5, $P = 0.008$), which correlated with a significantly increased rate of platinum sensitivity in these cases (OR = 3.3, $P = 0.007$). Interestingly, this improved progression-free survival was not limited to *BRCA1/2* mutations, but included defects in other homologous recombination genes. Homologous recombination-deficient tumors depend on other error-prone mechanisms for double-stranded break repair, such as the Polθ/PARP1-mediated alternative end-joining pathway.^{10,11} The reliance on alternative end-joining repair pathways subsequently renders homologous recombination-deficient tumors more sensitive to chemotherapy as well as to PARPi.^{3,4,12–19} Only one PARPi has been FDA approved for the treatment of ovarian cancer, olaparib, and this is only in the setting of *BRCA* mutation and at least three prior lines of chemotherapy. Our findings further support the work of Pennington *et al*,²¹ suggesting that a broader population of women with ovarian cancer may benefit from PARPi therapy.

It is important to emphasize that the histologic pattern and molecular correlates in high-grade serous carcinomas is not absolute, and may reflect variables yet to be uncovered or resolved. Although we saw improved platinum response and survival in high-grade serous carcinomas with homologous recombination mutations, the same associations were not seen when high-grade serous carcinomas were stratified by morphologic subtype, despite the significant association between homologous recombination mutations and non-classic histology. This could possibly be due to the fact that only a subset (58%) of the total high-grade serous carcinoma cases were available for morphologic subtyping, which limited the power of that analysis. The strategy for assigning tumor type, based on predominant growth pattern, is also subject to error. In addition, it has recently been shown that the morphologic pattern seen in metastatic lesions (infiltrative and micropapillary vs pushing border) of *BRCA*-associated high-grade serous carcinomas has a significant association with clinical outcome, with the more infiltrative metastatic lesions having a worse prognosis.³¹ The current study was limited by the number of slides available for morphologic review with the majority of slides consisting of sections from the primary ovarian/fallopian tube tumor;

therefore, it is possible that morphologic subtyping would have had a significant association with clinical outcome if we had been able to assess the morphology of metastatic lesions.

Ovarian high-grade serous carcinomas are known to have high levels of genomic instability, which is evidenced by frequent copy number alterations in this tumor type.²⁰ We demonstrated that among high-grade serous carcinomas that had received neo-adjuvant chemotherapy, the presence of increased copy number alterations was significantly associated with an improved progression-free survival ($P = 0.009$). This finding remained an independent predictor of progression-free survival in multivariate analyses ($P = 0.005$). Previous studies have focused on focal changes in copy number and their association with either platinum sensitivity or with survival;^{20,32,33} however, one study that looked at genome-wide copy number variants in 118 ovarian tumors noted that a greater number of significant copy number changes were detected in the therapy-responsive group compared with the therapy-resistant group.³³ This association has not been well characterized and could possibly serve as a predictive biomarker of response to chemotherapy.

In summary, this study has demonstrated that non-classic histology in high-grade serous carcinomas is strongly associated with mutations in homologous recombination genes. Defects in homologous recombination are not limited to this morphologic subtype, however, as high-grade serous carcinomas with classic morphology, as well as other histotypes also had homologous recombination mutations. Although further work will be needed to tease out the relevance of histologic phenotype, be it a meaningful differentiation pattern or marker for cell of origin, this study verifies that many women with high-grade serous carcinomas may benefit from targeted therapeutic approaches such as PARP inhibitors, and underscores the importance of non-classic histology in high-grade serous carcinomas and comprehensive genomic analysis to identify the candidates most likely to respond to targeted therapies.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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