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14. ABSTRACT The goal of this project is to systematically characterize immune cell enrichment in the microenvironment of ovarian cancers and identify immune cell subsets that are prognostically relevant for patient care and/or can be used to develop more effective therapies targeting the tumor microenvironment. We compared the transcriptomes of primary tumors and omental metastases to elucidate if metastases have unique molecular characteristics that could be used as therapeutic targets or predictors of treatment success. We showed that biomarkers of clinical outcomes are different in primary tumors and metastases. Since biopsies of omental metastases are more accessible than primary tumors and increasingly used to determine the appropriate course of treatment for ovarian cancer patients, we generated an omental metastasis sample-based 8-gene signature as an independent risk factor for overall survival in ovarian cancer patients. We also identified different subsets of T cells in the epithelial and stromal tumor components in patient matched primary, metastatic, and recurrent ovarian cancer; conducted computational analyses of the imaging data; and identified correlations between patient survival and the presence of different T cell subsets in the epithelial and/or stromal tumor components.					
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1. INTRODUCTION:

It is becoming increasingly clear that the tumor microenvironment plays an important role in tumor evolution, including the evolution of immune tolerance. In particular, cancer-associated fibroblasts (CAFs) are emerging as critical regulators of immune cell recruitment and function. CAFs physically hinder trafficking of functional immune cells to the tumor bed. CAFs are also a rich source of biologically active molecules that modify immune cell behavior and attract or repel certain immune cell types. While tumor progression is typically accompanied by the increased presence of CAFs, the configuration of pro-tumorigenic and anti-tumorigenic immune cell types during tumor progression is currently unknown. Our integrative approach is providing a comprehensive view of immune cell enrichment in the tumor microenvironment during tumor progression. Our major finding is that the presence of CAFs negates the beneficial effects of immune infiltrates on patient survival. In addition to identifying combinations of cell subsets that are prognostically significant for patient treatment with existing immunotherapies, our study could prove that immune cell infiltration can be effectively manipulated by targeting CAFs, which may provide a novel approach to immunotherapy. Our spatiotemporal quantitative map of individual immune cell subsets in matched primary, metastatic, and recurrent ovarian tumors could serve as a foundation for therapeutic targeting of the CAF/immune cell interface to develop more effective and less toxic therapies that selectively deplete the tumor-promoting stromal cell subsets in different stages of tumor progression.

KEYWORDS:

Ovarian cancer, tumor microenvironment, tumor progression, cancer-associated fibroblasts, immune infiltrates

ACCOMPLISHMENTS:

- What were the major goals of the project?

Specific Aim 1 (specified in proposal)	Timeline	Percent Completed
Major Task 1 Identify biomarkers and therapeutic targets in the tumor stroma by correlating stromal factors with clinical outcomes.	Months	Cedars-Sinai Medical Center
Subtask 1 Normalize transcriptome data from 5 public Affymetrix-based ovarian cancer datasets and combine into one dataset with transcriptome and clinical information for 1,228 primary ovarian cancer samples.	1-2	100%
Subtask 2 Computationally (implementing the BASE algorithm) deduce hematopoietic cell type enrichment in human ovarian cancer samples using the ImmGen dataset (131 mouse hematopoietic	3-5	100%

cell types). Validate the enrichment of 22 major hematopoietic cell types using the LM22 dataset (leukocyte 547-gene signature that distinguishes 22 major human hematopoietic cell types).		
Subtask 3 Use statistical methods to associate enrichments in individual hematopoietic cell types (or specific groups of cell types) with distinct molecular and clinical phenotypes, such as the molecular subtype, chemosensitivity, metastasis, and survival.	6-7	90%
Subtask 4 Write and publish the computational part of the manuscript.	8-15	80%
Milestone Achieved Identified and validated association between immune cell enrichment and molecular and clinical parameters (molecular subtype, stromal content, overall survival, disease-free survival, and cisplatin sensitivity) in 1,228 primary ovarian cancer samples.	15	
Specific Aim 2 (specified in proposal)	Timeline	Site 1
Major Task 2 Validate the identified stromal factors and generate a spatiotemporal map of stromal activity associated with tumor progression and chemoresistance.	Months	Cedars-Sinai Medical Center
Subtask 1 Amend approved IRB 44852 protocol for local approval and send related material for DoD's approval.	Upon award notice	100%
Subtask 2 Design an immune cell panel for multi-color flow cytometry/immunohistochemistry (IHC)/immunofluorescence (IF); purchase reagents; plan experiments.	1-30	100%
Subtask 3 Prospectively collect surgically isolated matched primary and metastatic ovarian cancer samples from 40 patients. Process tissues in 4 ways: 1) disaggregate into viable single-cell suspensions for flow cytometry; 2) fix in formalin/embed in paraffin (FFPE) for IHC; 3) snap freeze for IF; 4) grow fresh omental metastasis tissue (5-10 patients) in culture for isolation of carcinoma-associated fibroblasts (CAFs).	1-30	30%
Subtask 4 Isolate and culture CAFs from metastasis samples from 5-10 patients.	1-12	10%
Subtask 5 Conduct multi-color flow cytometry and multi-color IHC or IF on prospectively collected samples from 40 patients or on an existing tissue microarray (TMA) of patient-matched primary, metastatic, and recurrent cancer samples from 42 patients.	1-30	80%

Subtask 6 Acquire images from TMAs and process for image analysis.	3-30	100%
Subtask 7 If ambiguous results are obtained with TMA, validate IHC and IF data on full tumor sections.	1-36	5%
Subtask 8 Analyze data from multi-color flow cytometry and multi-color IHC or IF and correlate with stromal content and clinical parameters, including debulking status, cisplatin sensitivity, disease-free survival, and overall survival.	3-36	40%
Milestone Achieved Obtained a detailed spatiotemporal map of immune cell subsets in primary and metastatic tumor samples and correlated data with stromal content and clinical parameters, including debulking status, cisplatin sensitivity, disease-free survival, and overall survival. Identified actionable immune biomarkers and therapeutic targets.	36	
Specific Aim 3 (specified in proposal)	Timeline	Site 1
Major Task 3 Demonstrate that targeted ablation of stromal cell subsets impedes tumor progression.	Months	Cedars-Sinai Medical Center
Subtask 1 Amend approved IACUC 5318 protocol for local approval and send related material for DoD's approval.	Upon award notice	Dr. Sandra Orsulic
Subtask 2 Purchase FVB mice, drugs and reagents; design a panel of mouse immunomarkers for multi-color flow cytometry and IHC/IF; select targeted stromal agents for treatment; plan experiments.	1-2	100%
Subtask 3 Implant FVB mice with mouse ovarian cancer cells. Treat mice with selected targeted stromal agents.	3-25	100%
Subtask 4 Collect and process tissues from experimental and control mice, including ascites, blood, tumor, spleen and lymph nodes, for multi-color flow cytometry and IHC or IF.	5-30	100%
Subtask 5 Conduct multi-color flow cytometry and multi-color IHC or IF on collected tissues from experimental and control mice, including ascites, blood, tumor, spleen and lymph nodes.	5-30	50%
Subtask 6 Analyze data using statistical methods; replicate experiments if necessary; prepare manuscripts.	5-36	20%

Milestone Achieved Tested the effect of stroma-targeted therapy on immune response. Identified potential immunomodulators for cancer treatment.	36	
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▪ **What was accomplished under these goals?**

1) major activities

Our activities focused on finalizing all three Specific Aims, analyzing the results, and submitting datasets to public repositories, publishing the results, and preparing additional manuscripts for publication.

2) specific objectives

Aim 1. Identify biomarkers and therapeutic targets in the tumor stroma by correlating stromal factors with clinical outcomes. Objectives: Publish completed projects, finalize outstanding projects and draft manuscripts for publication.

Aim 2. Generate a spatiotemporal map of stromal activity associated with tumor progression and chemoresistance. Finalize projects and draft manuscripts for publication.

Aim 3. Demonstrate that targeting CAFs improves the immune response to tumors. Objectives: Publish completed projects, finalize outstanding projects and draft manuscripts for publication.

3) significant results or key outcomes, including major findings, developments, or conclusions

Aim 1. Identify biomarkers and therapeutic targets in the tumor stroma by correlating stromal factors with clinical outcomes

Aim 1, major achievement #1. Our expression profile analysis of CAFs in surgical samples from ovarian cancer patients revealed a gene signature very similar to the stromal gene signature previously identified in patients with Diffuse Large B-cell Lymphoma (DLBCL) (Gentles et al., NEJM, 2015). Paradoxically, this signature is associated with good survival in DLBCL and several other lymphomas and poor survival in ovarian cancer and most other solid malignancies. We published a manuscript showing that the DLBCL stromal gene signature is enriched in lymphoid fibroblasts in normal lymph nodes and in CAFs in ovarian cancer. Based on these findings, we proposed several possible mechanisms by which CAFs may contribute to opposite survival outcomes in B-cell lymphomas and carcinomas.

Haro M, Orsulic S. A Paradoxical Correlation of Cancer-Associated Fibroblasts With Survival Outcomes in B-Cell Lymphomas and Carcinomas. Frontiers in Cell and Developmental Biology 2018; Aug 28;6:98. doi: 10.3389/fcell.2018.00098. eCollection 2018.

Aim 1, major achievement #2. Our computational analyses of the four major molecular subtypes of primary HGSC (Immunoreactive, Proliferative, Differentiated, and Mesenchymal) revealed that the Mesenchymal subtype (Mes-HGSC) most likely comprise clusters of cancer and associated stromal cells that detached from tumors in the upper abdomen/omentum and disseminated in the peritoneal cavity, including to the ovary. Using comparative analyses of multiple transcriptomic data sets, we provided the following evidence that the phenotype of Mes-HGSC matches the phenotype of tumors in the upper abdomen/omentum: 1) irrespective of the primary ovarian HGSC molecular subtype, matched upper abdominal/omental metastases were typically of the Mes subtype, 2) the Mes subtype was present at the ovarian site only in patients with concurrent upper abdominal/omental metastases and not in those with HGSC confined to the ovary, and 3) ovarian Mes-HGSC had an expression profile characteristic of stromal cells in the upper abdominal/omental metastases. We suggest that ovarian Mes-HGSC signifies advanced intraperitoneal tumor dissemination to the ovary rather than a subtype of primary ovarian HGSC. This is consistent with the presence of upper abdominal/omental disease, suboptimal debulking, and worst survival previously reported in patients with ovarian Mes-HGSC compared to other molecular subtypes. These data were presented in the following publication:

Hu Y, Taylor-Harding B, Haro M, Raz Y, Recouvreux M, Taylan E, Walts AE, Karlan BY, Orsulic S. Are epithelial ovarian cancers of the mesenchymal subtype actually intraperitoneal metastases to the ovary? Frontiers in Cell and Developmental Biology 2020

We also generated 2 new transcriptome datasets with a large number of chemotherapy-naïve samples from patients with HGSOC and deposited raw and normalized data into the public GEO archive under the following accession numbers: GSE135712 and GSE133296.

Aim 1, major achievement #3. The computational analyses in this project resulted in several CAF- and immunocyte-specific genes that are associated with survival. While we were focusing on ongoing functional studies of individual genes, we teamed up with the OTTA-SPOT (Ovarian Tumor Tissue Analysis consortium - Stratified Prognosis of Ovarian Tumours) to analyze our candidate survival genes along with their ~500 candidate genes in chemotherapy-naïve ovarian cancer samples from 1,455 patients. One of our genes (FBN1) was identified in the top 5 genes strongly associated with survival in this study. It is expected that this 5-gene expression signature will improve risk stratification in clinical trials by identifying patients who are least likely to achieve 5-year survival. The identified novel genes associated with the outcome may also yield opportunities for the development of targeted therapeutic approaches. These data were published in the following manuscript:

Millstein J, Budden T, Goode EL, Anglesio MS, Talhouk A, Intermaggio MP, Leong HS, Chen S, Elatre W, Gilks B, Nazeran T, Volchek M, Bentley RC, Wang C, Chiu DS, Kommoss S, Leung SCY, Senz J, Lum A, Chow V, Sudderuddin H, Mackenzie R, George J; AOCs Group, Fereday S, Hendley J, Traficante N, Steed H, Koziak JM, Köbel M, McNeish IA, Goranova T, Ennis D, Macintyre G, Silva D, Ramón Y Cajal T, García-Donas J, Polo SH, Rodriguez GC, Cushing-Haugen KL, Harris HR, Greene CS, Zelaya RA, Behrens S, Fortner RT, Sinn P, Herpel E, Lester J, Lubiński J, Oszurek O, Tołoczko A, Cybulski C, Menkiszak J, Pearce CL, Pike MC, Tseng C, Alsop J, Rhenius V, Song H, Jimenez-Linan M, Piskorz A, Gentry-Maharaj A, Karpinskyj C,

Widschwendter M, Singh N, Kennedy CJ, Sharma R, Harnett PR, Gao B, Johnatty SE, Sayer R, Boros J, Winham SJ, Keeney GL, Kaufmann SH, Larson MC, Luk H, Hernandez BY, Thompson PJ, Wilkens LR, Carney ME, Trabert B, Lissowska J, Brinton L, Sherman ME, Bodelon C, Hinsley S, Lewsley LA, Glasspool R, Banerjee SN, Stronach EA, Haluska P, Ray-Coquard I, Mahner S, Winterhoff B, Slamon D, Levine DA, Kelemen LE, Benitez J, Chang-Claude J, Gronwald J, Wu AH, Menon U, Goodman MT, Schildkraut JM, Wentzensen N, Brown R, Berchuck A, Chenevix-Trench G, A deFazio, Gayther SA, García MJ, Henderson M, Rossing MA, Beeghly-Fadiel A, Fasching PA, **Orsulic S**, Karlan BY, Konecny GE, Huntsman DG, Bowtell DD, Brenton JD, Doherty JA, Pharoah PDP, Ramus SJ. Prognostic gene expression signature for high-grade serous ovarian cancer. *Annals of Oncology* 2020; May 27. pii: S0923-7534(20)39841-0. doi: 10.1016/j.annonc.2020.05.019.

Aim 1, major achievement #4. The hypothesis for Aim 1 was that individual immune cell types associated with specific tumor states and clinical outcomes in ovarian cancer can be identified by superimposing transcriptomes of tumor samples with transcriptomes of isolated hematopoietic cell types. We proved that the presence of CAFs is correlated with a skewed proportion of immune cell subsets and showed that the mesenchymal molecular subtype of ovarian cancer is enriched for tolerogenic immune cell subsets (*the data were described in our previous progress reports*). We are currently validating these data in our samples from HGSC patients.

Aim 1, major achievement #5. The Cancer Genome Atlas Project (TCGA) and other comprehensive molecular analyses of clinically-annotated ovarian cancer samples have greatly enriched our molecular understanding of the most common type of ovarian cancer, high grade serous ovarian carcinoma (HGSOC). This wealth of molecular data is currently used by investigators worldwide to identify novel therapeutic targets and delineate biomarkers of clinical outcomes in HGSOC. However, the TCGA molecular data have been derived from primary ovarian cancer samples located in the ovary. Such data may not be fully representative of HGSOC, which is characterized by widespread peritoneal metastases at the time of diagnosis and upon relapse. To effectively treat metastatic disease, it is important to understand the distinct molecular characteristics of metastases. We suspect that transcriptome of a metastasis to the omentum (the most common metastatic site in HGSOC patients) is probably more representative of disease progression and has a closer association with survival than the transcriptome of the primary tumor. Another reason to pursue potential biomarkers of clinical outcomes in omental metastases is that biopsies of omental metastases are more accessible than primary tumors and increasingly used to determine the appropriate course of treatment for ovarian cancer patients (neoadjuvant chemotherapy with secondary cytoreduction vs. primary cytoreduction followed by chemotherapy).

We generated an RNA-sequencing transcriptome dataset comprising chemotherapy-naïve samples of omental metastases from 130 patients with HGSOC and deposited raw and normalized data into the public GEO archive under the accession number GSE138866.

To elucidate if metastases have unique molecular characteristics that could be used as therapeutic targets or predictors of treatment success, we determined by RNA sequencing expression patterns in formalin-fixed paraffin-embedded (FFPE) samples of chemotherapy-naïve omental

metastases from 127 patients diagnosed with HGSOC. We identified two main groups of samples associated with survival. One group, consisting primarily of mesenchymal and proliferative tumors, was associated with poor survival while the other group, which was associated with a strong DNA repair signature, was associated with good survival. We further studied CAFs in the group associated with poor survival and identified INHBA (Activin) as a functional gene in these CAFs. This is important because Activin has a natural inhibitor,

Follistatin, that could potentially be harnessed to develop new therapy for metastatic HGSOC. We are currently preparing a manuscript describing these data. Major findings resulting from this project are described in the figures below.

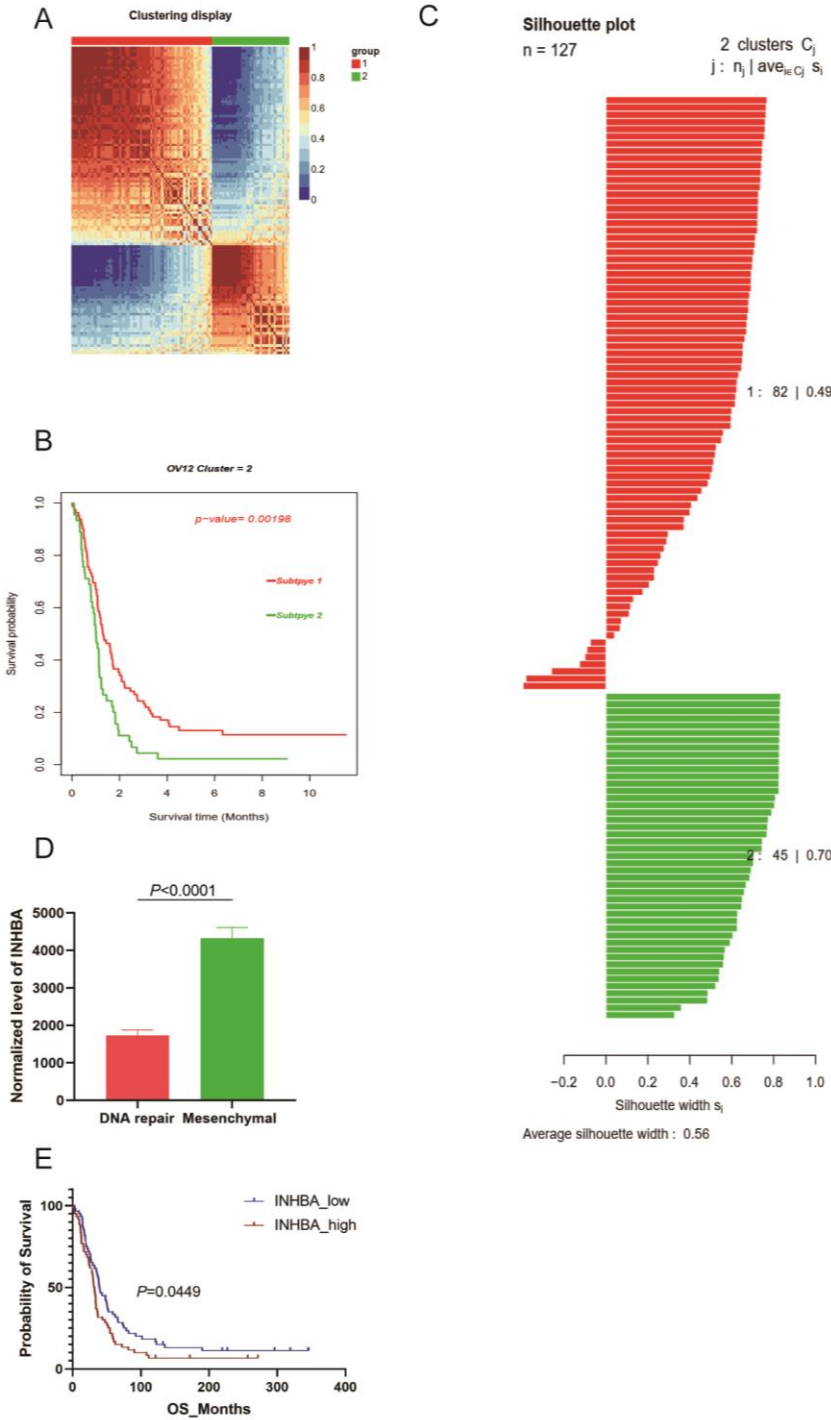


Figure 1. Ovarian cancer (OvCa) omental metastases (OMs) can be classified into two molecular subtypes associated with patient survival.

(A) Heatmap of sample similarity matrix for the identified molecular subtypes (DNA repair and Mesenchymal) based on RNA sequencing expression data and survival data in 127 OvCa OMs. (B) Kaplan-Meier survival curves for the two molecular subtypes of OvCa OMs. (C) Silhouette width plots for the two molecular subtypes of OvCa OMs. Each horizontal line represents a sample in the Silhouette plot. The length of the line is the silhouette width. 120 samples with positive silhouette values were selected for further analyses. (D) INHBA expression levels in 120 OvCa OMs classified into DNA repair and Mesenchymal subtypes. (E) Kaplan-Meier survival curves for the 120 OvCa OMs divided into two groups based on INHBA expression level.

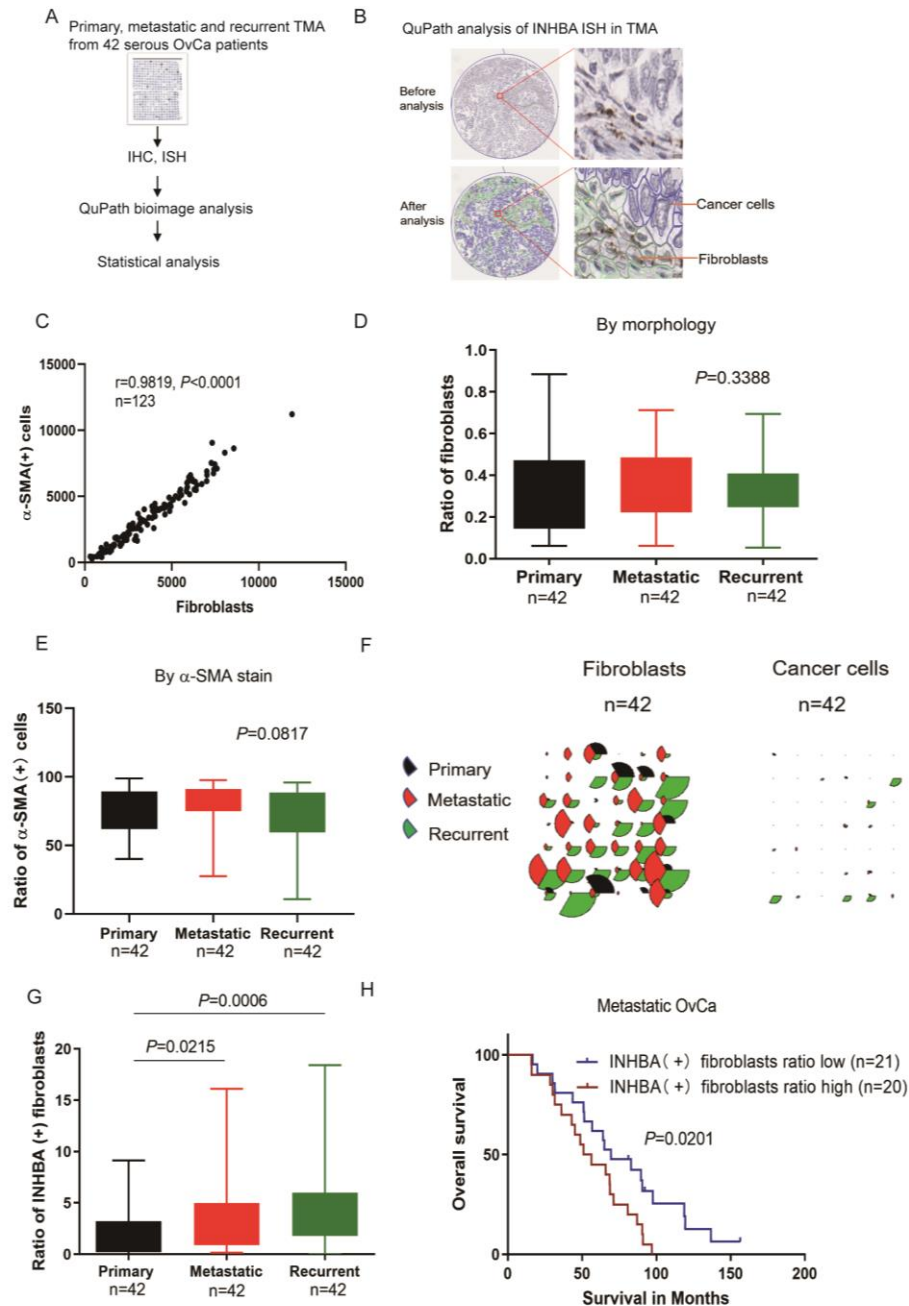


Figure 2. Cancer associated fibroblasts (CAF) and immunocytes correlate with OvCa progression and overall survival.

(A) A schematic flowchart of the process of image analysis in the OvCa TMA comprising patient-matched primary, metastatic, and recurrent OvCa samples from 42 patients with HGSOC (aka OvCa Progression TMA). INHBA was detected by ISH while CD4, CD8, and α -SMA were detected by IHC. The slides were scanned and digital images were analyzed with the QuPath software, followed by statistical analysis.

(B) A representative TMA core before and after QuPath annotation. Cells in the TMA cores were annotated as tumor cells, fibroblasts, immunocytes, and other based on combinations of cell feature measurements integrated in the QuPath platform. Each cell is color-coded. DAB staining intensity classification was applied to identify positive/negative stained cells.

(C) Correlation between the number of fibroblasts (defined by QuPath morphology-based classification on H&E-

stained sections) and α -SMA(+) cells in OvCa TMA as revealed by QuPath ($P<0.0001$, Pearson correlation, $n=123$; OVTP58, OVTP62 and OVTP114 samples were excluded because of insufficient cancer tissue for analysis).

(D) Statistical analysis of the ratio of fibroblasts in the OvCa Progression TMA (ANOVA test, $P=0.3388$).

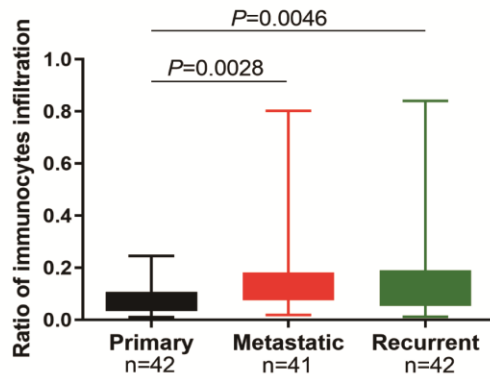
(E) Statistical analysis of the ratio of α -SMA(+) fibroblasts in the OvCa Progression TMA (ANOVA test, $P=0.0817$).

(F) Fan graph showing INHBA(+) cell proportion in fibroblasts compared with cancer cells in the OvCa Progression TMA.

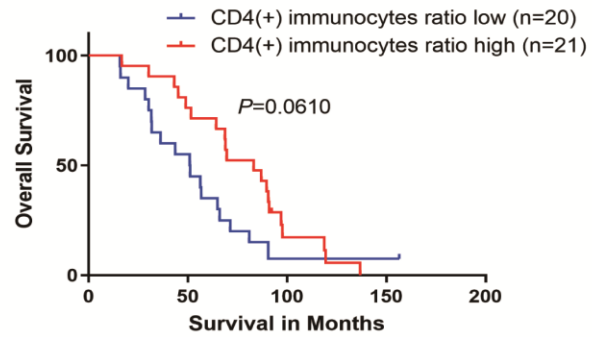
(G) Statistical analysis of the proportion of INHBA(+) fibroblasts in the OvCa Progression TMA (paired t test, primary vs metastatic: $P<0.05$, primary vs recurrent: $P<0.001$).

(H) Kaplan-Meier survival plot comparing overall survival of patients with a low INHBA(+) fibroblast ratio ($n=21$) and a high INHBA(+) fibroblast ratio ($n=20$; one sample with few cancer cells was excluded) in the OvCa Progression TMA (log-rank test, $P<0.05$).

I



J



H

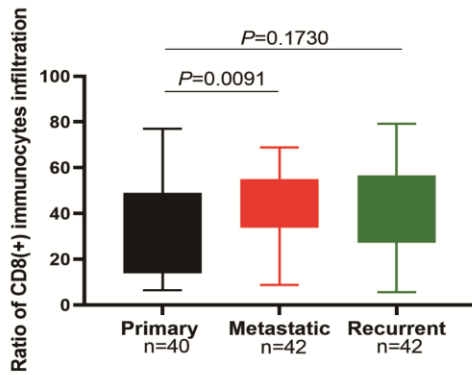


Figure 2 (continued)

(I) Statistical analysis of the immunocyte ratio (amount of immunocyte/(amount of fibroblasts + immunocytes + tumor cells)) in the OvCa Progression TMA. The y-axis indicates the ratio of immunocyte infiltration, which was significantly higher in the metastatic and recurrent tumor samples than in the primary samples (paired *t* test, primary vs metastatic: $P=0.0028$, primary vs recurrent: $P=0.0046$).

(J) Kaplan-Meier survival plot comparing overall survival of patients with a low CD4(+) immunocyte ratio and a high CD4(+) immunocyte ratio (log-rank test, $P=0.0610$).

(H) Statistical analysis of CD8(+) immunocyte ratio (amount of CD8(+) immunocytes/amount of immunocytes in the OvCa Progression TMA). The y-axis indicates the ratio of CD8(+) immunocyte infiltration (paired *t* test, primary vs metastatic: $P=0.0091$, primary vs recurrent: $P=0.1730$).

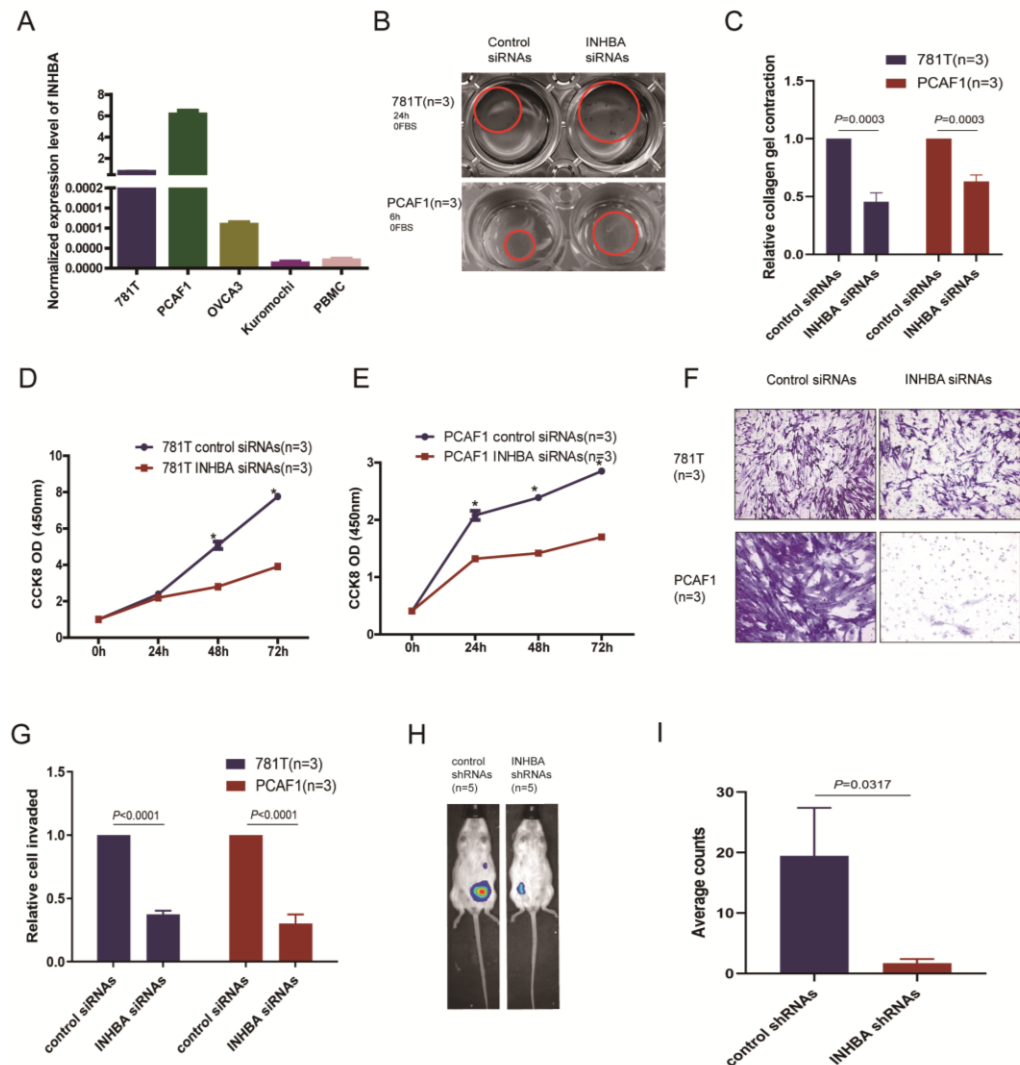


Figure 3. INHBA regulates CAF function and impacts OvCa progression.

(A) INHBA levels measured by RT-qPCR in an immortalized CAF cell line (781T), primary CAF (PCAF1), human OvCa cell lines (OVCA3 and Kuromochi), and PBMCs from a healthy donor.

(B) The collagen gel contraction assay with control ovarian CAFs and ovarian CAFs in which expression of INHBA was suppressed by siRNAs.

(C) Quantitation of gel contraction shown in (B) based on 3 independent experiments.

(D, E) Proliferation of 781T cells and PCAF1 cells treated with control siRNA or INHBA siRNAs. Cell growth rates were determined by the CCK-8 viability assay. The x-axis shows the time after transfection. The y-axis shows the readout of the CCK-8 assay (absorbance at 450 nm).

(F) Representative images of the Transwell invasion assay with 781T cells and PCAF1 cells. The crystal violet-stained images show the abundance of cells that penetrated the porous membrane.

(G) Quantitation of cell invasion shown in (F) based on 3 independent experiments.

(H) Representative intravital bioluminescence images of tumors in an immunocompetent mouse ovarian cancer model. The mouse OvCa cell line BR-luc was injected intraperitoneally into syngeneic FVB mice to establish a xenograft model. Five mice per group were treated with control siRNAs or INHBA siRNAs 3 times a week for 4 weeks. Tumors in both groups were measured by intravital bioluminescence imaging 30 days after the first injection of siRNAs.

(I) Quantitation of intravital bioluminescence signal intensity shown in (H).

All quantitation data are presented as the mean $\pm$ SEM in 3 or more independent experiments.

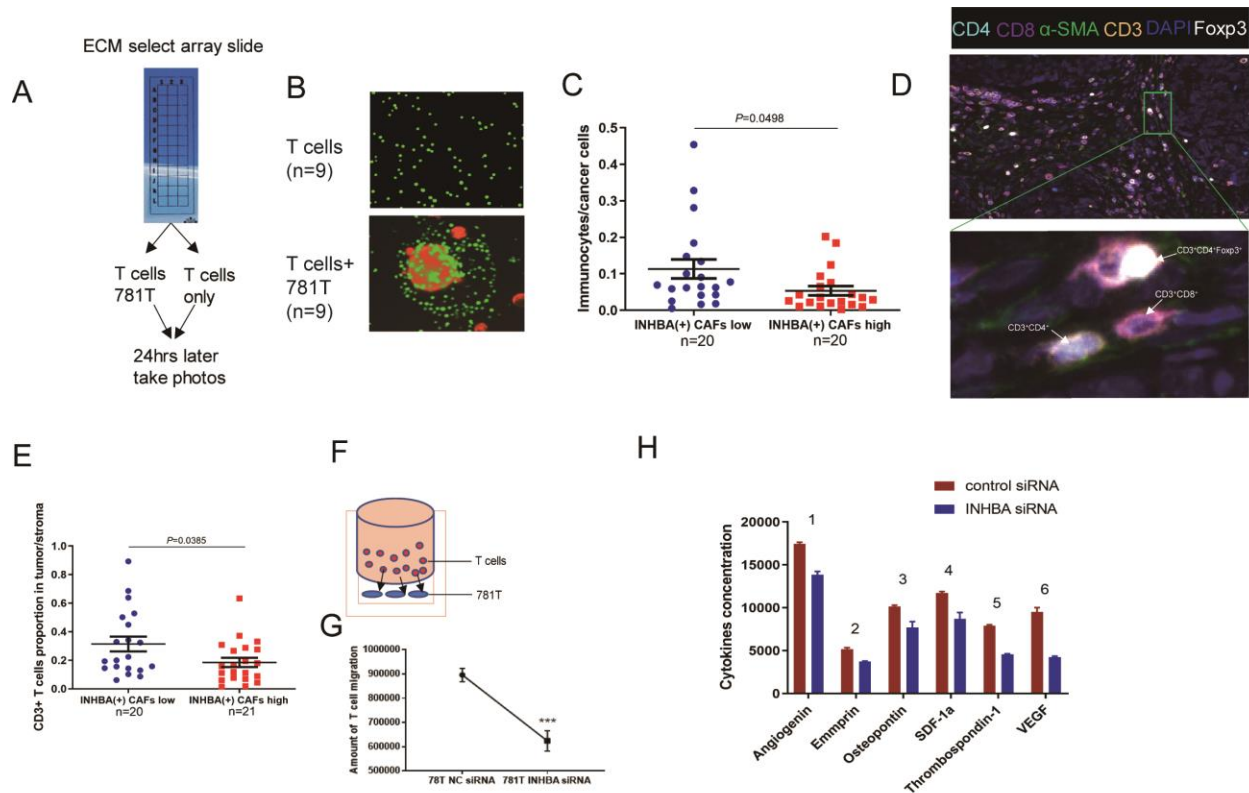


Figure 4. INHBA (+) CAFs create an immunosuppressive tumor microenvironment (TME) in OvCa metastases.

(A) Schematic flowchart of cell adhesion assay using the ECM select array. T cells alone or mixed with 781T CAFs were plated onto the ECM select array slide. After 24 hours in culture, images were taken to measure the cell attachment to various combinations of recombinant ECM proteins. Each combination of ECM proteins was printed as 9 replicate spots (400 μ m in diameter) onto a microscope slide.

(B) Representative images of a printed ECM spot with T cells alone (upper panel) and T cells co-cultured with 781T CAFs (lower panel). T cells were stained with CellTracker™ Green CMFDA Dye and 781T CAFs were stained with CellTracker™ Orange CMTMR Dye.

(C) Statistical analysis of the immunocytes infiltration ratio (amount of immunocytes/amount of tumor cells) in tumors with a low proportion of INHBA(+) fibroblasts and tumors with a high proportion of INHBA(+) fibroblasts. The immunocytes infiltration ratio in the two groups was compared using the *t* test ($P<0.05$).

(D) Representative image of multiplex immunofluorescence (IF) staining of OvCa TMA from 42-paired primary, metastatic and recurrent samples.

(E) Statistical analysis of the CD3(+) T cells proportion ratio associated with cancer cells and stroma (CD3(+) T cells/cancer cells: CD3(+) T cells/stroma) in tumors with high and low proportions of INHBA(+) fibroblasts. The proportion of CD3(+) T cells in the cancer cell area vs stromal area in the two groups was compared using the *t* test ($P<0.05$).

(F) A Transwell migration assay of T cells toward 781T CAFs treated with control siRNA or INHBA siRNAs. Human pan T cells were plated in the upper Transwell chamber while, 781T CAFs treated with control siRNA or INHBA siRNAs were plated in the bottom chamber for 24h-36h.

(G) Quantitation of T cells in (F) that migrated to the bottom chamber.

(H) Quantitation of differentially expressed proteins between supernatants of 781T CAFs treated with control siRNA and supernatants of 781T CAFs treated with INHBA siRNAs. Protein levels were determined using the Proteome Profiler Human XL Cytokine Array.

All quantitation data are presented as the mean $\pm$ SEM in 3 or more independent experiments.

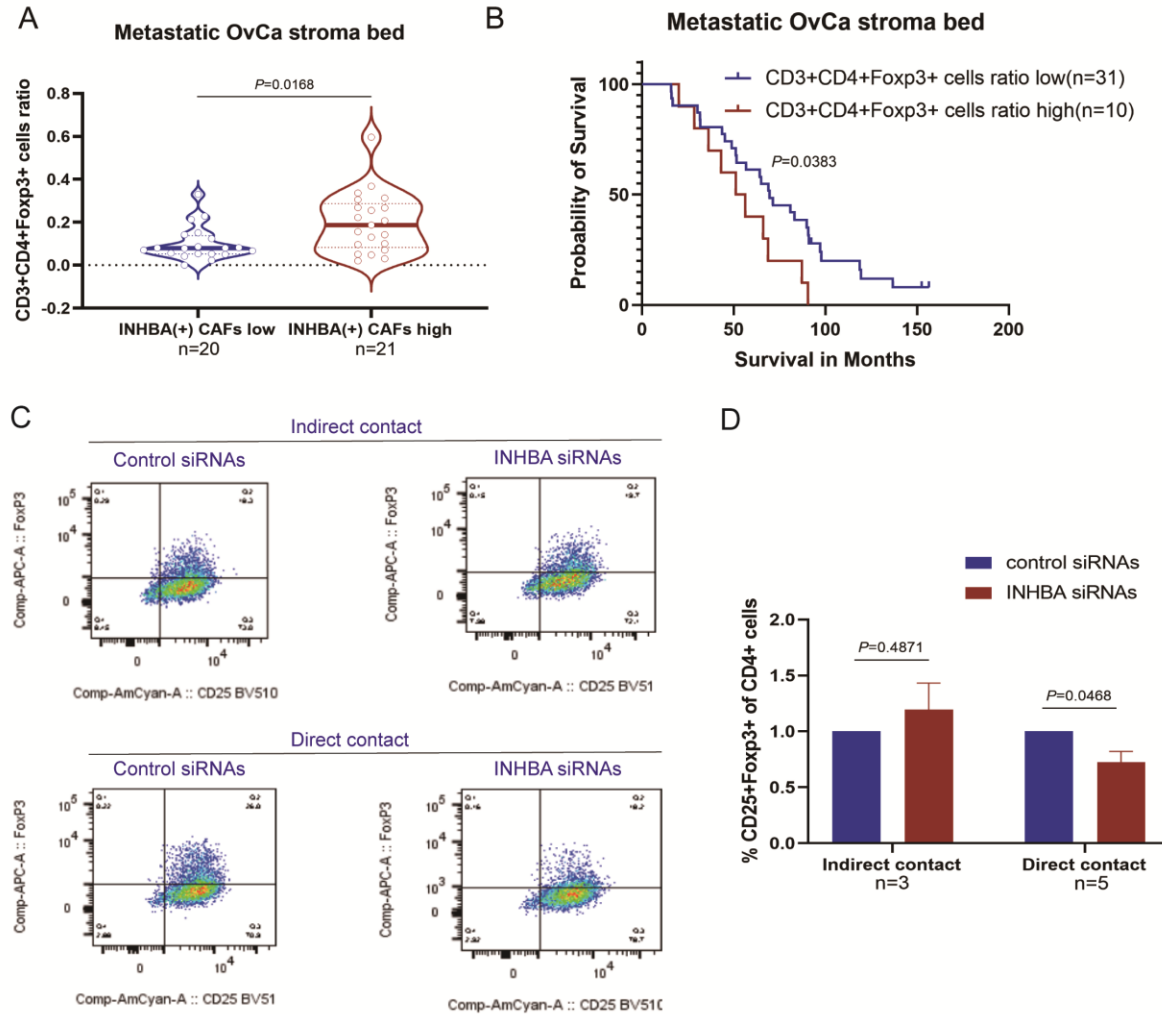


Figure 5. INHBA creates an immunosuppressive tumor microenvironment through upregulation of PD-L1.

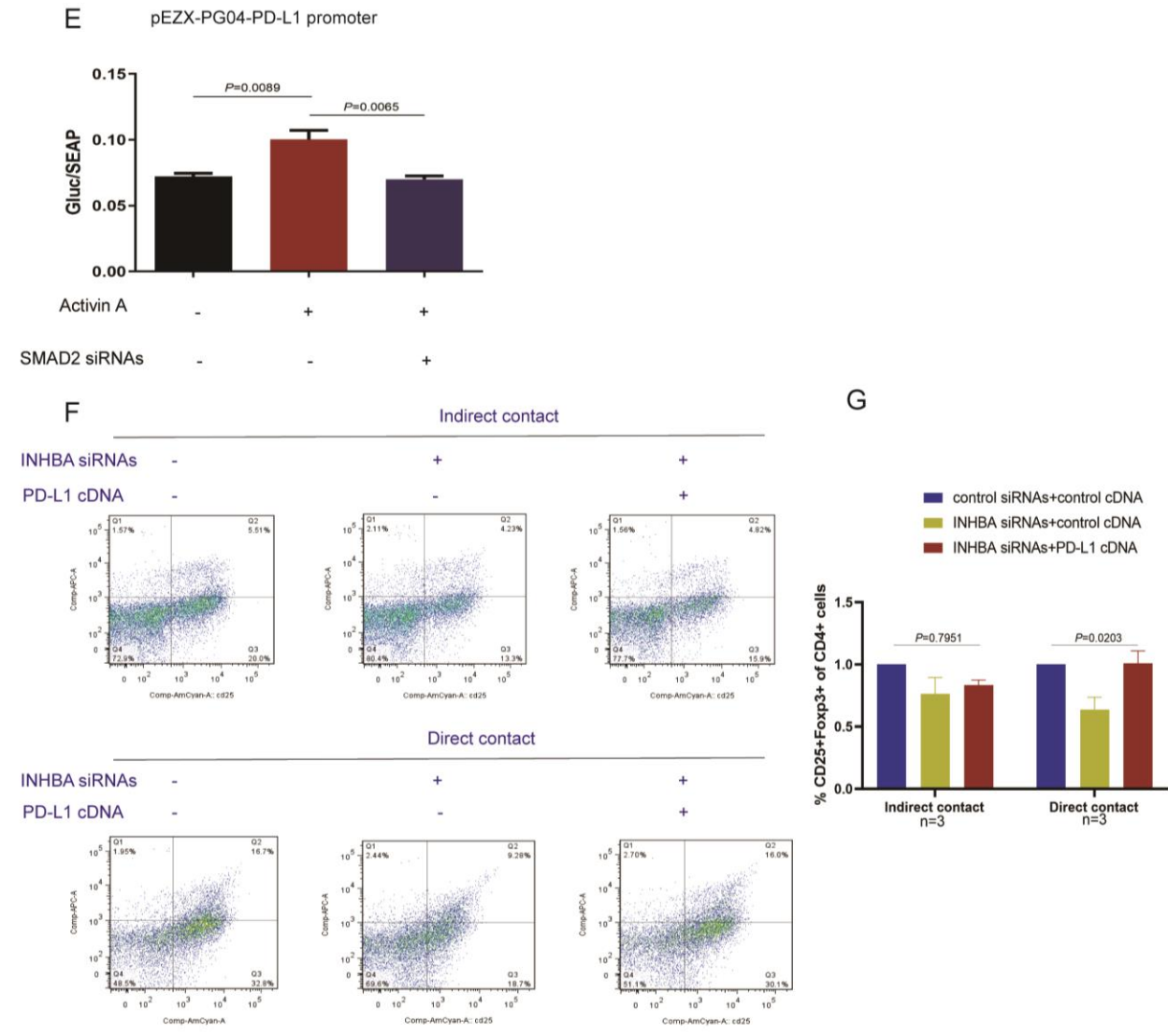
(A) Statistical analysis of the T_{reg} infiltration ratio (amount of CD3(+) CD4(+) Foxp3(+) cells / amount of CD3(+) CD4(+) cells) in tumors with a low proportion of INHBA(+) fibroblast and tumors with a high proportion of INHBA(+) fibroblast. The T_{reg} infiltration ratio in the two groups was compared using the t test ($P=0.0168$, Mann Whitney U test).

(B) Kaplan-Meier survival curves between patients with low and high T_{reg} infiltration ratios in samples of OvCa metastases (log-rank test, $P=0.0383$).

(C) Representative flow cytometry graphs showing the frequency of CD3(+) CD4(+) Foxp3(+) T_{reg} when CD3/CD28 activated T cells are co-cultured under direct contact (5 independent experiments) or indirect contact (3 independent experiments) with 781T CAFs treated with control siRNA or INHBA siRNAs. Flow cytometric assay showed with direct contact (5 independent experiments), less Tregs were observed in 781T

(D) Quantitation of T_{reg} from (C) in co-cultures of activated T cells and CAFs in direct contact (5 independent experiments) and indirect contact (3 independent experiments).

Figure 5. continued



(E) Activin A upregulates transactivity of PD-L1 through pSMAD2. The promoter sequence of PD-L1 mRNA was inserted upstream of a luciferase gene. The reporter vector was transfected to 781T cells, which were treated by a combination of Activin A and pSMAD2 siRNAs or control siRNA. The luciferase signal (Gluc/SEAP) of the reporter gene was significantly increased when cells were treated with Activin A, and the effect of Activin A on luciferase signal decreased when the cells were also treated with SMAD2 siRNAs (student t-test, *P* values are indicated).

(F) Representative flow cytometry images. Silencing of INHBA in 781T cells suppressed CD3(+) CD4(+) Foxp3(+) T_{reg} expansion in a co-culture system, which can be rescued by promoting PD-L1 expression. 781T cells were transfected with INHBA siRNAs in the presence or absence of cDNA for PD-L1, and the frequency of T_{reg} cells was determined by flow cytometry. Fewer T_{reg} cells were observed in indirect (but not direct) co-culture with 781T cells with INHBA siRNAs compared to 781T cells with INHBA control siRNA and this effect was compromised by PD-L1 overexpression.

(G) Quantitation of the data in (F) presented as the mean $\pm$ SEM in 3 independent experiments.

Aim 2. Generate a spatiotemporal map of stromal activity associated with tumor progression and chemoresistance

Multiple studies have reported an uneven distribution of different immune cell types and/or different maturation stages of the same cell type in different parts of the tumor. These observations formed the basis for developing “immunoscores” as predictors of survival, metastasis, and therapeutic response. A recent anti-PD-1 clinical trial in melanoma has revealed that, in addition to immunomarkers, a mesenchymal gene signature strongly correlates with therapy resistance. Our group has shown that this gene signature is primarily expressed in cancer-associated fibroblasts (CAFs), suggesting that CAFs may contribute to therapy resistance through immunosuppression. However, mechanisms by which CAFs control immune responses in cancer are unclear. Our goal was to test the hypotheses that the accumulation of CAFs during tumor progression skews the immune infiltrate toward the immuno-suppressive profile and/or prevents access of immune cells to the tumor bed. The spatiotemporal view of stromal dynamics during ovarian cancer progression will assist in decisionmaking about the most appropriate therapy regimen for different stages of cancer progression.

Aim 2, major achievement #1. We generated data on the identity of cell types, extracellular matrix, and soluble factors involved in ovarian cancer progression and metastasis. Ovarian cancer cells have a predilection to metastasize to the omentum although it is still unknown which factors associated with the omentum make this site attractive to ovarian cancer cell seeding. With the goal of elucidating expression patterns in the microenvironment of omental metastases, we profiled expression of immune- and cancer progression-related genes in pre-treatment omental metastasis samples from **152 patients** diagnosed with HGSOC. This is the largest study of omental metastasis transcriptome in HGSOC to date. Among other analyses, we quantitated fibroblasts, immune cells, and cancer cells and identified genes correlated with these cell types. *These data have been described in our previous progress reports. We are currently drafting a manuscript describing the results of these analyses.*

Aim 2, major achievement #2. We obtained data on the spatial distribution of fibroblasts, immune cells, and cancer cells during ovarian cancer progression using a TMA comprising patient-matched samples of primary, metastatic, and recurrent HGSC from 42 patients. *The data were described in our last progress report and we are currently drafting a manuscript describing the results of these analyses.*

Aim 3. Demonstrate that targeting CAFs improves the immune response to tumors

Aim 3, major achievement #1. We published a research manuscript that establishes the basis of our experiments in targeting CAFs for improved immune response in our immunocompetent mouse model of ovarian cancer.

Jia D, Nagaoka Y, Katsumata M, Orsulic S. Inflammation is a key contributor to ovarian cancer cell seeding. Scientific Reports 2018; Aug 17;8(1):12394. doi: 10.1038/s41598-018-30261-8.

Aim 3, major achievement #2. In collaboration with my former postdoctoral fellow, we published a manuscript that elucidates the underlying mechanism by which the CAF-secreted

COL11A1 contributes to cisplatin resistance and demonstrated that targeting this pathway is effective in inducing apoptotic cell death in cisplatin-resistant ovarian cancer.

Nallanthighal S, Rada M, Heiserman JP, Cha J, Sage J, Zhou B, Yang W, Hu Y, Korgaonkar C, Hanos CT, Ashkavand Z, Norman K, **Orsulic S**, Cheon DJ. Inhibition of collagen XI alpha 1-induced fatty acid oxidation triggers apoptotic cell death in cisplatin-resistant ovarian cancer. *Cell Death and Disease* 2020; Apr 20;11(4):258. doi: 10.1038/s41419-020-2442-z.

Aim 3, major achievement #3. Using human ovarian cancer cell lines and our immunocompetent mouse model for ovarian cancer, we published a manuscript demonstrating that dual inhibition of GSK3B and HDACs is effective in reducing growth of cisplatin-resistant cells and reducing ovarian cancer progression in our syngeneic mouse model.

Taylan E, Zayou F, Murali R, Karlan BY, Pandol SJ, Edderkaoui M, **Orsulic S**. Dual targeting of GSK3B and HDACs reduces tumor growth and improves survival in an ovarian cancer mouse model. *Gynecologic Oncology* 2020; Jul 19:S0090-8258(20)32380-5. doi: 10.1016/j.ygyno.2020.07.005. Online ahead of print. PMID: 32698955

Aim 3. Major achievement #4. Using a peritoneal injury/fibrosis syngeneic mouse model of ovarian cancer, we demonstrated that neutrophils are critical for initial ovarian cancer cell attachment to the peritoneal surface and that ovarian cancer progression is reduced upon neutrophil depletion. These data are summarized below:

To identify cell types and molecules involved in attraction of cancer cells to the injured mesothelium, we compared RNA sequencing data from the peritoneal walls of 5 PBS and 5 NaOCl mice on days 1, 3, and 7 after NaOCl injection. Analysis of the top 24 upregulated genes in the NaOCl mice revealed a strong gene signature of neutrophils (Lcn2, Tnfrsf23, Tnfaip2, Skap2, Gadd45a, and Flot1) on day 1 (data not shown) and signatures of neutrophils, monocytes, and macrophages on day 3 (**Fig. 6**).

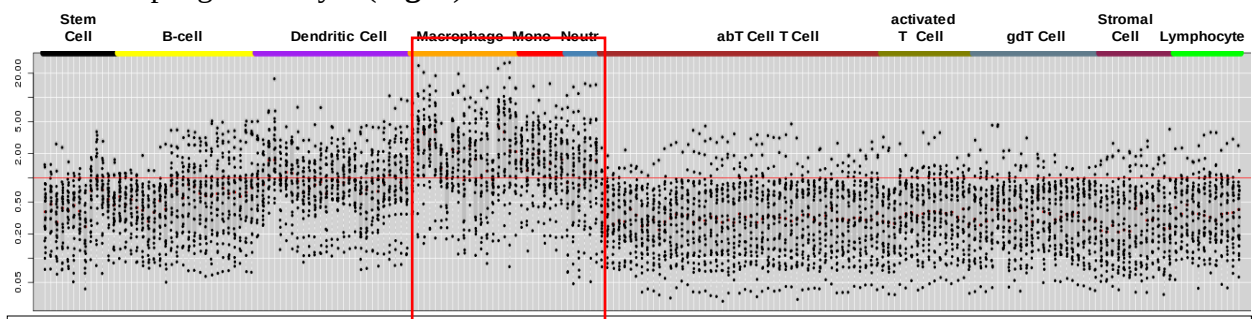


Fig. 6. Differential transcriptomes in peritoneal wall samples from PBS and NaOCl mice on day 3 after treatment. Expression levels of the top 24 genes upregulated in NaOCl mice in different immune and stromal cell subtypes in the ImmGen dataset reveal that these genes are primarily enriched in the transcriptomes of macrophages, monocytes, and neutrophils.

Neutrophils are typically the first immune cells to be recruited to the site of injury. Importantly, neutrophils were recently identified as the key facilitators of pre-metastatic niche formation in ovarian cancer. We confirmed the presence of neutrophils (and/or gMDSCs, which are

phenotypically indistinguishable from neutrophils) by flow cytometry of peritoneal lavages and dissociated peritoneal walls at 4h and 24h after treatment with PBS or NaOCl. Increased frequencies of live neutrophils/gMDSCs in peritoneal lavages and walls were observed at both time points (**Fig. 7A-B**). Immunohistochemical staining of peritoneal wall sections with the neutrophil marker - neutrophil gelatinase-associated lipocalin (NGAL, gene name LCN2) - confirmed that neutrophils infiltrated blood vessels and sub-mesothelial connective tissue as early as 4h after peritoneal injury (**Fig. 7C**) and remained in these tissues 24h and 72h after treatment (data not shown).

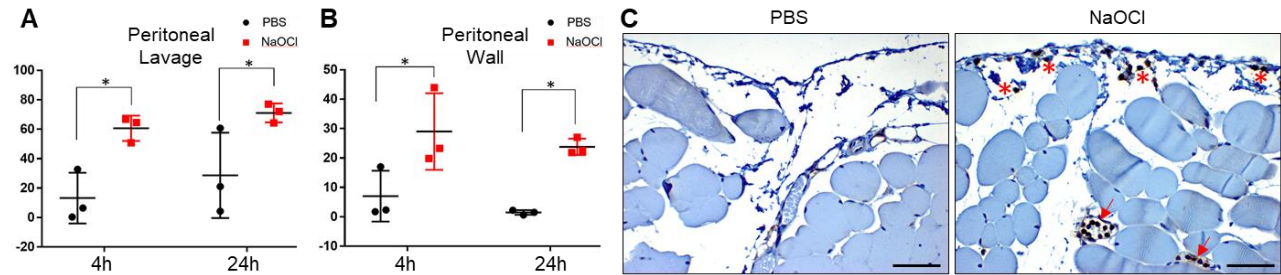


Fig. 7. Early influx of neutrophils in the peritoneum of NaOCl mice. (A-B) Peritoneal lavage (A) and dissociated body wall (B) neutrophils or granulocytic MDSC (frequency of live cells) 4 hours and 24 hours after intraperitoneal injection of 1 ml PBS or 1 ml 0.05% NaOCl diluted in PBS (n=3 mice/group). *Immune cell marker panel: CD3, CD4, CD8, CD11b, CD11c, CD19, CD45, F4/80, Ly6G, live/dead cells.* * $p < 0.05$. (C) Representative images of peritoneal walls (n=5 mice/group) fixed 4 hours after intraperitoneal injection of PBS or NaOCl. Neutrophil gelatinase-associated lipocalin (NGAL) staining shows the influx of neutrophils in the blood vessels (arrows) and submesothelial connective tissue (asterisks) in the peritoneal walls of NaOCl mice. Scale bar: 50 μ m.

To test the possible role of neutrophils in early stages of ovarian cancer cell attachment to the mesothelium and intraperitoneal dissemination, neutrophils were depleted using anti-Ly6G antibody, which is specifically expressed on mouse neutrophils. Six-week-old female FVB mice (n=10 mice/group) were depleted for neutrophils by intraperitoneal injection of 200 μ g anti-Ly6G antibody or control antibody every 3 days starting 6 days before intraperitoneal injection of 0.5 ml of 0.05% NaOCl diluted in PBS. 10^6 BR-luc cells were intraperitoneally injected one day after treatment with NaOCl. The mice were imaged weekly by intravital luciferase imaging (**Fig. 8** and data not shown).

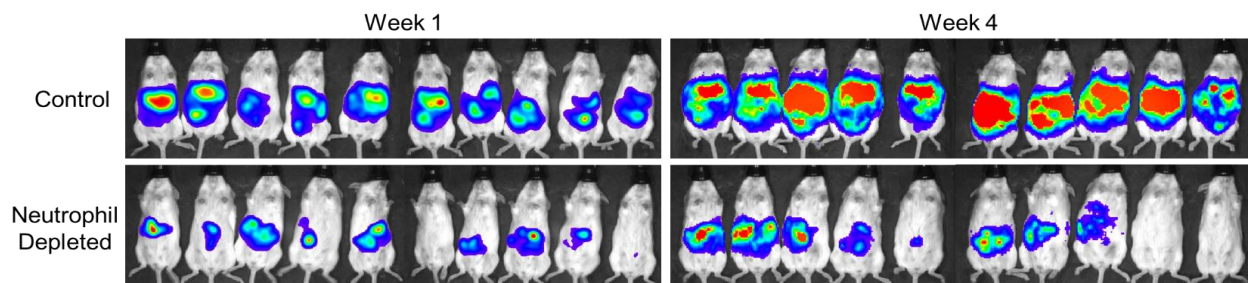


Fig. 9. Neutrophil depletion inhibits metastatic dissemination of cancer cells in NaOCl mice. (A) Representative *in vivo* luciferase imaging of FVB mice 1 week and 4 weeks after intraperitoneal injection of 10^6 BR-luc cells one day after treatment with 0.5 ml of 0.05% NaOCl diluted in PBS. Neutrophil depletion was performed by intraperitoneal injection of 200 μ g of anti-Ly6G antibody (n=10 mice/group) every 3 days starting 6 days before NaOCl injection.

4) *other achievements*

Nothing to report.

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

Nothing to report.

b. How were the results disseminated to communities of interest?

Nothing to report.

c. What do you plan to do during the next reporting period to accomplish the goals?

N/A

3. IMPACT:

a. What was the impact on the development of the principal discipline(s) of the project?

Nothing to report.

b. What was the impact on other disciplines?

Nothing to report.

c. What was the impact on technology transfer?

Nothing to report.

d. What was the impact on society beyond science and technology?

Nothing to report.

4. CHANGES/PROBLEMS:

a. **Changes in approach and reasons for change.** Yes

b. **Actual or anticipated problems or delays and actions or plans to resolve them.**

Due to our laboratory's move from Cedar-Sinai Medical Center to UCLA at the end of January, 2020 and subsequent research ramp-down at UCLA, we could not continue with the proposed bench research and have thus focused on computational analyses and preparation of manuscripts.

c. **Changes that had a significant impact on expenditures.** No

d. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.** No

e. **Significant changes in use or care of human subjects.** Yes

We suspended all collection of human tissues in January of 2020 because of our laboratory's move to UCLA.

f. **Significant changes in use or care of vertebrate animals.** Yes

We suspended all animal work in January of 2020 because of our laboratory's move to UCLA.

g. **Significant changes in use of biohazards and/or select agents.** No

5. PRODUCTS:

Nothing to report.

a. **Publications, conference papers, and presentations**

i. **Journal publications.**

Jia D, Nagaoka Y, Katsumata M, **Orsulic S**. Inflammation is a key contributor to ovarian cancer cell seeding. Scientific Reports 2018; Aug 17;8(1):12394. doi: 10.1038/s41598-018-30261-8.

Haro M, **Orsulic S**. A Paradoxical Correlation of Cancer-Associated Fibroblasts With Survival Outcomes in B-Cell Lymphomas and Carcinomas. Frontiers in Cell and Developmental Biology 2018; Aug 28;6:98. doi: 10.3389/fcell.2018.00098. eCollection 2018.

Taylan E, Zayou F, Murali R, Karlan BY, Pandol SJ, Edderkaoui M, **Orsulic S**. Dual targeting of GSK3B and HDACs reduces tumor growth and improves survival in an ovarian cancer mouse model. Gynecologic Oncology 2020; Jul 19:S0090-8258(20)32380-5. doi: 10.1016/j.ygyno.2020.07.005. Online ahead of print. PMID: 32698955

Nallanthighal S, Rada M, Heiserman JP, Cha J, Sage J, Zhou B, Yang W, Hu Y, Korgaonkar C, Hanos CT, Ashkavand Z, Norman K, **Orsulic S**, Cheon DJ. Inhibition of collagen XI alpha 1-induced fatty acid oxidation triggers apoptotic cell death in cisplatin-resistant ovarian cancer. Cell Death and Disease 2020; Apr 20;11(4):258. doi: 10.1038/s41419-020-2442-z.

Hu Y, Taylor-Harding B, Haro M, Raz Y, Recouvreux M, Taylan E, Walts AE, Karlan BY, **Orsulic S**. Are epithelial ovarian cancers of the mesenchymal subtype actually intraperitoneal metastases to the ovary? *Frontiers in Cell and Developmental Biology* 2020

Millstein J, Budden T, Goode EL, Anglesio MS, Talhouk A, Intermaggio MP, Leong HS, Chen S, Elatre W, Gilks B, Nazeran T, Volchek M, Bentley RC, Wang C, Chiu DS, Kommoss S, Leung SCY, Senz J, Lum A, Chow V, Sudderuddin H, Mackenzie R, George J; AOCs Group, Fereday S, Hendley J, Traficante N, Steed H, Koziak JM, Köbel M, McNeish IA, Goranova T, Ennis D, Macintyre G, Silva D, Ramón Y Cajal T, García-Donas J, Polo SH, Rodriguez GC, Cushing-Haugen KL, Harris HR, Greene CS, Zelaya RA, Behrens S, Fortner RT, Sinn P, Herpel E, Lester J, Lubiński J, Oszurek O, Tołoczko A, Cybulski C, Menkiszak J, Pearce CL, Pike MC, Tseng C, Alsop J, Rhenius V, Song H, Jimenez-Linan M, Piskorz A, Gentry-Maharaj A, Karpinskyj C, Widschwendter M, Singh N, Kennedy CJ, Sharma R, Harnett PR, Gao B, Johnatty SE, Sayer R, Boros J, Winham SJ, Keeney GL, Kaufmann SH, Larson MC, Luk H, Hernandez BY, Thompson PJ, Wilkens LR, Carney ME, Trabert B, Lissowska J, Brinton L, Sherman ME, Bodelon C, Hinsley S, Lewsley LA, Glasspool R, Banerjee SN, Stronach EA, Haluska P, Ray-Coquard I, Mahner S, Winterhoff B, Slamon D, Levine DA, Kelemen LE, Benitez J, Chang-Claude J, Gronwald J, Wu AH, Menon U, Goodman MT, Schildkraut JM, Wentzensen N, Brown R, Berchuck A, Chenevix-Trench G, A deFazio, Gayther SA, García MJ, Henderson M, Rossing MA, Beeghly-Fadiel A, Fasching PA, **Orsulic S**, Karlan BY, Konecny GE, Huntsman DG, Bowtell DD, Brenton JD, Doherty JA, Pharoah PDP, Ramus SJ. Prognostic gene expression signature for high-grade serous ovarian cancer. *Annals of Oncology* 2020; May 27. pii: S0923-7534(20)39841-0. doi: 10.1016/j.annonc.2020.05.019. [Epub ahead of print]

ii. **Books or other non-periodical, one-time publications.** N/A

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

Oral presentations (funding acknowledged):

Tumor Microenvironment. Cancer Biology Seminar, Mayo Clinic, Jacksonville, FL. February 23, 2018.

Cancer-Associated Fibroblasts Offset the Benefits of Immune Cell Infiltration. Biomedical and Translational Science Seminar Series, Cedars-Sinai Medical Center. Los Angeles, CA. May 2, 2018.

Tumor microenvironment: a gold mine of biomarkers and therapeutic targets. Department of Obstetrics and Gynecology Grand Rounds, David Geffen School of Medicine, UCLA. Los Angeles, CA. March 20, 2019.

b. **Website(s) or other Internet site(s).**

The following three datasets have been deposited into the GEO public repository:

GSE135712

NanoString expression profiling in metastatic ovarian cancer. A total of 152 specimens from patients with HGSOC were profiled for gene expression and classified by clinicopathological characteristics including age, race, BMI, PFS, OS, debulking and BRCA mutation status.

GSE133296

Matched primary ovarian cancer, omental metastases, and non-omental metastases from 10 patients with high-grade serous ovarian carcinoma. Matched high-grade serous ovarian carcinoma samples collected from the ovary (ov), omental metastasis (om-met), and non-omental intraperitoneal metastasis (met) from 10 patients at the time of primary debulking surgery were analyzed for RNA expression by RNA sequencing.

GSE138866

RNA sequencing expression data from 130 patients with HGSOC metastases to the omentum. NanoString expression profiling in metastatic ovarian cancer. The expression dataset is linked to multiple clinicopathological characteristics including age, race, BMI, PFS, OS, debulking and BRCA mutation status.

c. **Technologies or techniques.** N/A

d. **Inventions, patent applications, and/or licenses.** N/A

e. **Other Products.** N/A

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. What individuals have worked on the project?

Name:	Sandra Orsulic
Project Role:	PI
Nearest person month worked:	2.00
Contribution to Project:	Dr. Orsulic oversaw projects for all three specific aims, including experimental design, execution, and data analysis and interpretation. She submitted manuscripts and prepared presentations as well as the progress report.

Name:	Enes Taylan
Project Role:	Postdoctoral Fellow
Nearest person month worked:	10.00
Contribution to Project:	Dr. Taylan conducted all experiments in this proposal and assisted in data acquisition and analysis. He wrote one published paper and contributed to parts of a forthcoming manuscript describing the results of analyses conducted in Aim 1.

Name:	Barbie Taylor-Harding, PhD
Project Role:	Research Associate
Nearest person month worked:	3.00
Contribution to Project:	Dr. Taylor-Harding conducted all experiments in Aim 3 that involved testing different combinations of treatments in the immunocompetent mouse model of ovarian cancer and assisted in data acquisition and analysis.

- b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? No**
- c. What other organizations were involved as partners? None**
- 7. SPECIAL REPORTING REQUIREMENTS**
 - a. COLLABORATIVE AWARDS: N/A**
 - b. QUAD CHARTS: N/A**
- 8. APPENDICES: 6 publications**



A Paradoxical Correlation of Cancer-Associated Fibroblasts With Survival Outcomes in B-Cell Lymphomas and Carcinomas

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The tumor microenvironment is increasingly recognized as an active participant in tumor progression. A recent pan-cancer genomic profile analysis has revealed that gene signatures representing components of the tumor microenvironment are robust predictors of survival. A stromal gene signature representing fibroblasts and extracellular matrix components has been associated with good survival in diffuse large B-cell lymphoma (DLBCL). Paradoxically, a closely related gene signature has been shown to correlate with poor survival in carcinomas, including breast, ovarian, pancreatic, and colorectal cancer. To date, there has been no explanation for this paradoxical inverse correlation with survival outcomes in DLBCL and carcinomas. Using public gene data sets, we confirm that the DLBCL stromal gene signature is associated with good survival in DLBCL and several other B-cell lymphomas while it is associated with poor survival in ovarian cancer and several other solid tumors. We show that the DLBCL stromal gene signature is enriched in lymphoid fibroblasts in normal lymph nodes and in cancer-associated fibroblasts (CAFs) in ovarian cancer. Based on these findings, we propose several possible mechanisms by which CAFs may contribute to opposite survival outcomes in B-cell lymphomas and carcinomas.

Keywords: B cells, B-cell lymphoma, CAFs, cancer-associated fibroblasts, DLBCL, gene signature, ovarian cancer, tumor microenvironment

INTRODUCTION

During the past decade, gene expression profile analyses of frozen tumor pieces have been widely used to quantify various biological characteristics of malignant tumor cells and the microenvironment in which they reside. Individual biological characteristics and dominant molecular pathways in tumors are frequently associated with expression of a defined set of genes,

Abbreviations: CAF, cancer-associated fibroblast; CD, cluster of differentiation; CXCL, C-X-C motif chemokine ligand; DC, dendritic cells; DLBCL, diffuse large B-cell lymphoma; ECM, extracellular matrix; FDC, follicular dendritic cells; FRC, fibroblastic reticular cells; GC, germinal center; Ig, immunoglobulin; ImmGen, immunological genome project; IPA, ingenuity pathway analysis; MRC, marginal reticular cells; NK, natural killer; PDGFR α , platelet-derived growth factor receptor α ; PDPN, podoplanin; PRECOG, PREDiction of Clinical Outcomes from genomic profiles; TCGA, the Cancer genome atlas project; TGF β , transforming growth factor β ; TIL, tumor infiltrating lymphocyte; TLS, tertiary lymphoid structure.

known as a gene expression signature. Since phenotypic features represented by gene expression signatures are sometimes associated with clinical features, such as the length of survival of cancer patients or their response to therapy, gene expression signatures can be used as quantitative predictors of clinical outcomes. A recent pan-cancer PREdiction of Clinical Outcomes from Genomic Profiles (PRECOG) analysis revealed that genes in the tumor microenvironment are better predictors of survival than genes expressed in malignant tumor cells (Gentles et al., 2015). The two most prominent components in the microenvironment of solid tumors are fibroblasts and immune cells (Aran et al., 2017). Generally, in carcinomas, genes expressed in fibroblasts are associated with poor survival while genes expressed in immune cells, particularly leukocytes, are associated with good survival (Gentles et al., 2015). Tumor infiltrating lymphocytes (TILs) and tertiary lymphoid structures (TLS) are generally associated with improved clinical outcomes as evidenced by the improved overall survival and disease-free survival in various types of tumors (Fridman et al., 2012; Dieu-Nosjean et al., 2014; Barnes and Amir, 2017). However, depending on the type of tumor, tumor stage, and location of TILs within the tumor (tumor bed, invasive margin and stroma), different types of TILs have been associated with both positive and negative prognosis. For example, cytotoxic CD8⁺ T cells, memory T cells, and CD4⁺ T helper cells are generally associated with a better prognosis, whereas T regulatory cells, tumor associated macrophages, and myeloid-derived suppressor cells are associated with poor prognosis and can promote tumor progression (Fridman et al., 2012; Kitamura et al., 2015; Barnes and Amir, 2017). Furthermore, fibroblasts in the tumor microenvironment are phenotypically heterogeneous and may exhibit both a pro- and anti-tumorigenic phenotype (Augsten, 2014). Thus, the tumor microenvironment is a complex network of interaction between tumor cells and components of the stroma, including the extracellular matrix (ECM), and it is currently unclear which factors in the tumor microenvironment control the quantity and distribution of different immune cell subtypes. Specifically, it is unknown if fibroblasts and immune cells affect prognosis independently or through an interdependent interaction.

The functional interaction between fibroblasts and immune cells has been most thoroughly studied in normal lymph nodes and the spleen, where specialized fibroblasts produce ECM to form a network that allows for lymphocyte movement along the matrix in response to chemokine signaling. The presence of lymphoid fibroblasts is necessary for functional attraction, retention, compartmentalization, and survival of immune cells (Koning and Mebius, 2012). Lymphoid fibroblasts are crucial for lymphocyte homeostasis as well as controlling and expanding the lymphocyte pool (Mueller and Germain, 2009). Lymphoid fibroblasts are also key players in mediating functional immune cell interactions in the lymph nodes through direct contact or via secreted molecules (Chang and Turley, 2015). Follicular dendritic cells (FDC) attract B cells to the germinal center (GC) by secreting C-X-C motif chemokine

ligand 13 (CXCL13), while marginal reticular cells (MRC) use a network of follicular conduits to deliver antigens to cognate B cells (Chang and Turley, 2015). By secreting C-C motif chemokine ligands 19 and 21 (CCL19 and CCL21), fibroblastic reticular cells (FRC) recruit mature dendritic cells (DC) and naïve B and T cells to promote cell-cell interactions within the T cell zone (Mueller and Germain, 2009; Brown and Turley, 2015; Fletcher et al., 2015). Recent studies have shown that FRC are important for B-cell homeostasis (Cremasco et al., 2014). This function has been previously ascribed to FDC, however, cell-specific depletion experiments demonstrated that only FRC are crucial for B-cell survival. The mechanism by which FRC support B-cell survival is not entirely clear, but it is thought to involve crosstalk with B cells to control the boundaries of primary B-cell follicles (Cyster, 2010; Mionnet et al., 2013; Cremasco et al., 2014).

Similar to lymphoid fibroblasts in normal lymph nodes, cancer-associated fibroblasts (CAFs) are stromal cells that produce ECM, provide scaffolding, and exert regulatory functions through growth factors, cytokines, and chemokines that can promote tumor growth, angiogenesis, invasion, and metastasis (Kalluri and Zeisberg, 2006; Levental et al., 2009; Lu et al., 2012; Spano and Zollo, 2012; Harper and Sainson, 2014). Recent studies provide evidence that CAFs can also directly or indirectly contribute to immune cell fate and survival (Harper and Sainson, 2014; Costa et al., 2018; Mariathasan et al., 2018; Tauriello et al., 2018). It has recently been shown that a gene signature representing activated CAFs is present in most epithelial tumors (Jia et al., 2016) despite the diversity of resident fibroblasts in different organs and the presence of multiple fibroblast populations within a single tumor type (Costa et al., 2018). Activated CAFs in breast cancer, and possibly in other carcinomas, are associated with immunosuppressive populations of T lymphocytes (Costa et al., 2018). It is unclear if activated CAFs in carcinomas are also associated with immunosuppressive populations of B cells due to poorly defined markers for such cells (Sarvaria et al., 2017). Moreover, studies investigating the associations of B cell subsets with tumor progression using defined B-cell markers have produced conflicting results even within the same tumor type (Guy et al., 2016). An insufficient understanding of the roles of B cells in carcinomas has hindered the development of rational clinical trials targeting B-cells in carcinomas. The remarkable success of B-cell depletion with the cluster of differentiation 20 (CD20) monoclonal antibody, rituximab, in lymphomas and rheumatoid arthritis has sparked interest in rituximab and other B-cell targeted antibodies as possible therapies in carcinomas (Gundersen and Coussens, 2013). Although many carcinomas have significant B cell infiltration (Germain et al., 2014), clinical trials have shown limited benefits of B-cell depletion in carcinomas (Barbera-Guillem et al., 2000; Aklilu et al., 2004), possibly because B cells can have pro-tumorigenic or anti-tumorigenic properties depending on their maturation stage and other conditions that have not yet been defined (Sarvaria et al., 2017).

TABLE 1 | DLBCL “stromal-1” signature genes are inversely correlated with survival outcomes in B-cell lymphomas and other malignancies.

Gene	B-cell lymphoma						Solid tumor					
	BL	CLL	DLBCL	FL	MCL	MM	Bladder	Astro cytoma	Glioma	Colon	Head and neck	Ovarian
ACTN1	−0.928	−3.216	−6.211	−1.901	−0.94	0.658	3.312	3.22	4.557	2.36	1.988	1.552
ADAM12	0.746	−0.084	−7.809	−1.749	−0.866	−0.395	0.537	1.653	4.405	1.675	2.051	2.99
BGN	0.842	1.309	−4.115	−1.775	0	−2.627	1.438	2.341	3.643	2.33	3.559	3.09
CEBPA	−1.516	−3.127	−5.644	−1.639	0	−0.977	1.001	−0.041	2.652	−2.664	−1.578	−1.442
COL13A1	−0.313	−1.513	−2.402	0.332	0	−0.001	2.23	2.006	1.613	2.164	1.74	0.893
COL16A1	−0.481	0.252	−3.89	−0.6	0.333	−0.477	2.214	2.49	5.005	−0.546	1.263	4.542
COL1A1	0.349	−1.476	−4.621	−1.581	0	−1.951	3.592	3.326	3.77	1.544	3.354	3.929
COL1A2	−0.097	−0.879	−6.264	−1.605	0	−0.573	2.745	4.432	4.391	2.42	2.634	3.771
COL5A1	0.715	−0.675	−3.366	0.127	0	−0.467	1.957	3.528	4.438	2.328	3.686	3.65
COL5A2	0.969	1.124	−3.962	−1.597	0	−0.777	3.47	3.588	7.322	2.437	3.26	5.256
COL6A2	0.677	−1.368	−3.719	−0.749	−1.415	0.14	2.369	4.591	5.693	1.301	3.12	2.11
COL6A3	1.194	−0.129	−4.502	−1.442	1.37	2.684	1.282	3.005	3.071	2.403	3.141	3.178
COL8A2	−0.212	−0.894	−3.046	0.069	0	−0.905	−0.085	2.942	3.077	−0.007	1.779	2.908
CSF2RA	−1.84	0	−2.861	0	0	−2.39	−0.046	0.193	0	0	0	−1.959
CTGF	−0.5	0.796	−5.525	−0.73	−1.387	−0.775	1.651	1.676	−1.132	2.024	2.381	2.974
CYR61	1.159	0.092	−1.865	0.074	1.837	−0.123	3.342	1.159	3.807	1.678	1.757	3.607
DCN	0.819	0.185	−3.731	−0.026	0	−0.794	0.472	1.113	2.414	1.303	0.917	4.604
EFEMP2	1.823	1.113	−2.797	0.307	0	−5.014	2.112	4.044	7.62	1.684	3.53	2.576
EMP2	−0.057	0.044	−4.122	0.147	0	−0.579	−1.125	4.55	2.985	−0.368	0.452	−1.446
FAP	−1.551	0.374	−7.496	−0.76	−1.266	−0.536	3.522	2.321	3.736	2.366	2.874	4.814
FBN1	1.125	1.079	−4.907	−1.854	0	−0.044	2.151	1.518	2.239	2.311	1.906	4.676
FN1	−1.025	−0.496	−5.638	−1.852	−1.352	2.973	3.251	2.852	5.499	2.628	2.46	4.439
GPNMB	−1.638	−0.153	−6.899	0.513	0	1.112	1.281	3.946	5.214	1.74	−2.745	1.476
HSPG2	−0.267	2.244	−2.792	−1.63	0	0.845	−0.02	4.261	2.989	1.313	2.108	2.396
IL1R1	−1.566	−2.791	−4.858	−0.432	0.804	−1.789	−0.186	1.194	1.217	1.275	0.897	−0.137
ITGAV	0.897	−2.698	−6.933	0.614	−2.033	−0.212	0.402	0.945	0.226	2.253	1.503	1.792
ITGB2	−1.522	−2.053	−5.68	0.558	0.343	−1.803	0.886	0.4	4.299	−0.086	−2.064	−2.339
KITLG	0.896	−0.172	−1.923	1.04	−1.197	0.454	1.113	−0.331	1.091	1.164	−0.721	−0.504
LAMA4	0.445	2.207	−3.683	0.453	0	−3.155	2.474	0.028	3.397	2.415	2.021	2.168
LAMB2	−0.635	0.504	−1.974	−1.052	0	−0.728	0.926	1.686	5.906	0.913	1.836	2.326
LAMB3	1.291	−1.315	−2.703	0.256	0	0.265	−0.927	1.977	3.542	1.516	2.039	−1.966
LOXL1	−1.453	−1.007	−4.202	−1.287	0	−1.92	0.711	3.9	6.299	1.697	0.751	3.664
LTBP2	0.219	−1.562	−7.565	−0.187	0	−1.848	2.849	1.197	3.314	0.542	2.718	1.541
LUM	−0.357	−1.043	−5.663	−0.089	0	−1.859	1.442	3.796	3.723	1.447	1.428	4.841
MFAP2	0.862	0.01	−2.835	0.608	0	−0.68	3.151	3.543	3.011	0.874	1.666	5.462
MMP14	−1.105	2.746	−3.319	0.69	0.681	−1.647	2.046	1.787	4.691	1.786	1.168	2.297
MMP2	−1.227	−0.269	−5.709	−1.128	0.014	−0.545	0.66	1.792	3.631	1.567	3.12	3.084
MMP9	−0.819	−1.238	−7.734	−0.401	−0.12	−0.892	1.8	2.739	5.06	−0.723	0.039	−3.208
PDGFC	0.62	−3.08	−4.268	0.632	0	−0.486	2.788	−3.419	3.639	1.987	2.096	−0.167
PLAU	−1.723	−1.701	−7.712	0.205	0.528	−0.749	2.515	2.302	4.592	0.627	1.521	2.334
POSTN	1.565	0.675	−5.031	−1.266	−0.77	−1.157	3.246	2.76	5.46	2.632	2.092	4.696
SDC2	−0.209	−1.963	−3.763	−0.47	−0.383	−0.664	−1.091	1.405	5.736	2.239	1.659	1.424
SERPINH1	−1.173	2.067	−2.912	−1.224	0	1.565	1.422	3.846	5.397	3.044	2.065	2.07
SPARC	0.487	−3.125	−7.236	−1.599	1.012	−2.767	2.24	−1.998	−0.074	2.412	2.933	4.188
TGFB11	−0.842	−1.479	−2.367	0.662	0	−1.787	1.518	2.783	4.58	1.523	3.557	4.265
THBS1	1.462	−3.212	−2.038	−1.38	0.238	−1.674	1.673	2.947	3.122	0.799	2.328	3.565
TIMP2	−0.677	−2.448	−1.399	1.006	0.343	0.83	2.608	1.584	1.251	2.73	2.271	2.495
VCAN	1.459	−3.803	−3.177	−0.588	0	−2.078	3.133	−3.546	−3.171	2.264	2.238	4.277

Analysis of the DLBCL “stromal-1” geneset in the PREdiction of Clinical Outcomes from Genomic Profiles (PRECOG) public dataset (<https://precog.stanford.edu>). Each gene is assigned z scores associated with survival in different cancer types. Scores less than or equal to zero (red) are associated with good survival while positive scores (blue) are associated with poor survival. BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma.

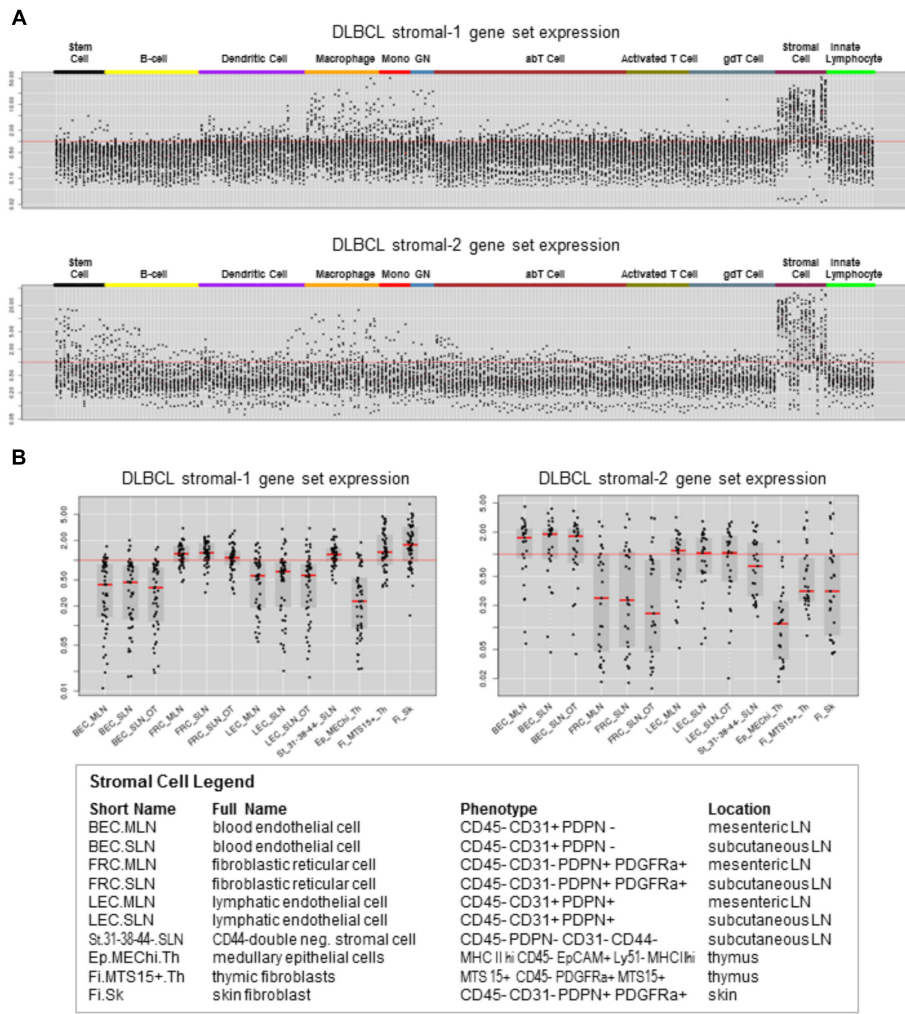


FIGURE 1 | DLBCL stromal-1 and stromal-2 signature genes are enriched in different stromal cell types. Expression of the DLBCL stromal-1 and stromal-2 signature genes in the Immunological Genome Project (ImmGen) data set. **(A)** Gene expression values normalized across 249 mouse immunological cell types. **(B)** Detailed view of gene expression values normalized to the stromal cell types shown in the legend. The graphs were generated using data from ImmGen (<http://www.immgen.org>).

THE DLBCL STROMAL-1 GENE SIGNATURE IS INVERSELY CORRELATED WITH SURVIVAL OUTCOMES IN B-CELL LYMPHOMAS AND OTHER SOLID TUMORS

Using expression profile analysis of DLBCL biopsy samples from treatment-naïve newly diagnosed patients, Lenz et al. identified two stromal gene signatures, stromal-1 and stromal-2, of which the stromal-1 gene signature was found to be associated with good survival in DLBCL patients (Lenz et al., 2008). However, gene signatures similar to the DLBCL stromal-1 gene signatures have been associated with poor survival in carcinomas, including ovarian cancer (Cheon et al., 2014), breast cancer (Farmer et al., 2009), colorectal cancer (Calon et al., 2015; Isella et al., 2015), and pancreatic cancer (Moffitt et al., 2015).

To systematically explore the association of the DLBCL stromal-1 gene signature with survival in cancer patients, we used PRECOG, a pan-cancer database of expression signatures in which each tumor type is represented by multiple independent expression profile data sets and associated survival data. This extensive database is ideal for multi-data set validation of prognostic signatures that have been identified in individual data sets. Using the DLBCL stromal-1 gene signature represented by 50 genes (Lenz et al., 2008), we confirmed that the signature is associated with poor survival in carcinomas and brain tumors and good survival in DLBCL and several other B-cell lymphomas (Table 1). This pattern of inverse association with survival between B-cell lymphomas and carcinomas/brain tumors was specific to the DLBCL stromal-1 gene signature, and was not associated with the DLBCL stromal-2 gene signature represented by 34 genes (Lenz et al., 2008) (data not shown).

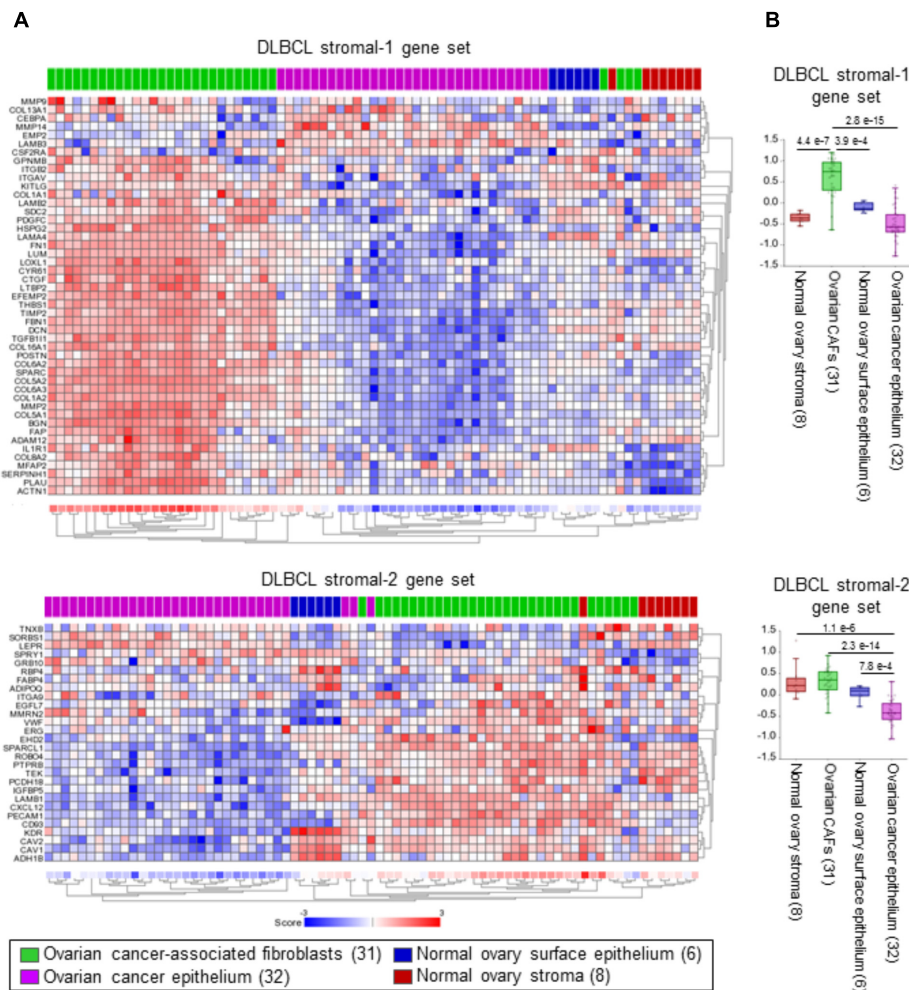


FIGURE 2 | DLBCL stromal-1 signature genes are enriched in cancer-associated fibroblasts (CAFs). **(A)** Non-centered gene set clustering analysis of the stromal and epithelial cell types in ovarian cancer and the normal ovary in the GSE40595 dataset using the DLBCL stromal-1 and stromal-2 gene sets. The number of samples in each group is indicated in parentheses. The gene set clustering analysis and image acquisition was performed using the R2 Genomics Analysis and Visualization Platform (<https://hgserver1.amc.nl>). **(B)** The same data are shown as box dot plots with *P*-values for differential expression of the DLBCL stromal-1 and stromal-2 gene signatures in different cell types.

IN NORMAL LYMPH NODES, DLBCL STROMAL-1 AND STROMAL-2 GENE SIGNATURES ARE ENRICHED IN STROMAL FIBROBLASTS AND ENDOTHELIAL CELLS, RESPECTIVELY

To identify immune cell types that express the DLBCL stromal-1 and stromal-2 signature genes, we looked for enrichment of these genes in the transcriptomes of 249 normal immunological cell types that had been isolated from mice and characterized by the Immunological Genome Project (ImmGen) (Heng and Painter, 2008; Shay and Kang, 2013). This analysis identified stromal cells as the most likely source of both gene signatures, although some of the genes were also expressed in macrophages, monocytes, granulocytes, and stem cells (Figure 1A). Closer examination of the stromal cell subtypes revealed that the DLBCL

stromal-1 and stromal-2 signature genes were preferentially expressed in different types of stromal cells. DLBCL stromal-1 signature genes were particularly enriched in cells characterized by expression of podoplanin (PDPN) and platelet-derived growth factor receptor α (PDGFR α), including FRC from mesenteric and subcutaneous lymph nodes and the so-called double-negative stromal cells, while stromal-2 signature genes were enriched in blood and lymphatic endothelial cells (Figure 1B).

THE DLBCL STROMAL-1 GENE SIGNATURE IS ENRICHED IN OVARIAN CAFs

To identify cells that express the DLBCL stromal-1 and stromal-2 signature genes in an epithelial tumor, we selected

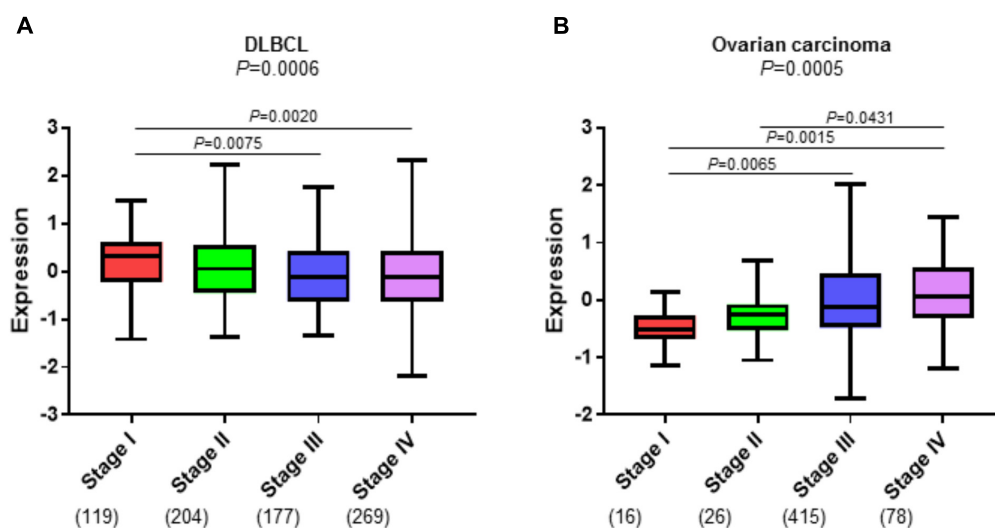


FIGURE 3 | CAFs have an inverse association with tumor stage in DLBCL and ovarian carcinoma. Enrichment of the DLBCL stromal-1 gene signature in progression stages I-IV in **(A)** three DLBCL microarray datasets (GSE10846, GSE87371, and GSE4475) that were combined into one dataset, and **(B)** The Cancer Genome Atlas (TCGA) ovarian carcinoma dataset (<https://cancergenome.nih.gov>). The gene signature enrichment analysis was performed using the R2 Genomics Analysis and Visualization Platform (<https://hgserver1.amc.nl>). The y axis shows relative enrichment of the DLBCL stromal-1 gene signature. The x axis shows tumor stage. The number of samples for each tumor stage is indicated in parentheses.

ovarian cancer because of the existing microarray data set (GSE40595) in which a large number of ovarian cancers have been laser capture microdissected into epithelial and stromal components (Yeung et al., 2013). For comparison with normal tissue, a small number of samples in this data set were microdissected from the normal ovary epithelium and stroma (Yeung et al., 2013). Our gene signature enrichment analysis revealed strong enrichment of the DLBCL stromal-1 gene signature in CAFs in comparison to cancer cells, normal ovary fibroblasts, and normal ovary epithelial cells (Figure 2). The DLBCL stromal-2 gene signature was enriched in CAFs but also in the normal ovary stroma (Figure 2).

POSSIBLE MECHANISMS BY WHICH CAFs CONTRIBUTE TO INVERSE SURVIVAL OUTCOMES IN B-CELL LYMPHOMAS AND CARCINOMAS

It is unusual for a gene signature to be associated with inverse survival outcomes in B-cell lymphomas and carcinomas. This is unlikely to be a technical error related to microarray technology as several individual genes from the DLBCL stromal-1 signature have been validated as predictors of good survival in DLBCL by independent technologies, such as immunohistochemistry and qPCR in formalin-fixed paraffin-embedded tissues (Lossos et al., 2004; Meyer et al., 2011; Tekin et al., 2016). Similarly, various technologies have been used to validate many of the signature genes as predictors of poor survival in carcinomas (Farmer et al., 2009; Cheon et al., 2014; Calon et al., 2015; Isella et al., 2015;

TABLE 2 | Upstream regulators of genes in the DLBCL stromal gene signature-1 and stromal gene signature-2.

Upstream regulator	Molecule type	p-value of overlap
DLBCL stromal-1 gene signature		
TGFB1	Growth factor	4.78E-31
COLQ	Other	2.70E-20
Bleomycin	Chemical drug	1.97E-18
SPDEF	Transcription regulator	2.73E-18
Tgf beta	Group	3.95E-18
TGFB3	Growth factor	8.04E-18
TNF	Cytokine	1.53E-17
DLBCL stromal-2 gene signature		
KLF2	Transcription regulator	1.89E-09
Rosiglitazone	Chemical drug	5.82E-09
VEGFA	Growth factor	5.90E-09
PPARG	Ligand-dependent nuclear receptor	1.36E-08
10E,12Z-octadecadienoic acid	Chemical – endogenous Mammalian	4.98E-08
WNT3A	Cytokine	6.02E-08
MGEA5	Enzyme	1.08E-07

The identification of upstream regulators was done using Ingenuity Pathway Analysis (www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/).

Moffitt et al., 2015; Jia et al., 2016). While the mechanism by which the DLBCL stromal-1 signature genes could contribute to good survival in DLBCL is still unclear, multiple mechanisms by which CAFs contribute to poor outcomes in carcinomas

have been proposed, including the promotion of tumor growth, angiogenesis, invasion and metastasis, the provision of protective niches for cancer stem cells, and the obstruction of access of chemotherapies and immunotherapies (Jain, 2013; Kalluri, 2016). Here, we will specifically focus on the possible direct or indirect roles of CAFs that could contribute to inverse survival outcomes in DLBCL and carcinomas.

Cancer-associated fibroblasts share structural and molecular features with the reticular fiber networks of secondary lymphoid organs, which are known to guide and compartmentalize specific immune cell types and play key roles in mediating functional immune cell interactions (Acton et al., 2012; Astarita et al., 2012; Cremasco et al., 2014; Chang and Turley, 2015; Fletcher et al., 2015; Turley et al., 2015). However, in addition to being sites in which immune responses are initiated, secondary lymphoid organs are also sites that foster immune privilege that prevents autoimmunity by inducing tolerance and deleting autoreactive T cells, suppressing effector T cell proliferation, and supporting regulatory T cells (Fletcher et al., 2011, 2014, 2015; Brown and Turley, 2015). Currently, lymph node fibroblasts are being explored for their therapeutic potential to circumvent unwanted inflammation in autoimmune diseases, sepsis, and graft rejection after organ transplantation (Fletcher et al., 2011, 2014, 2015). Based on the molecular similarity between CAFs and lymph node fibroblasts, we propose that CAFs primarily play an immunosuppressive role in tumors using similar molecular mechanisms to those used by lymph node fibroblasts in regulating immune cell tolerance and homeostasis. In support of this hypothesis, CAF-derived factors have been shown to contribute to immune editing *in vivo* to avoid tumor detection and rejection by the host immune system (Stover et al., 2007; Kraman et al., 2010). Specific to B cells, several *in vitro* models have shown the ability of different types of fibroblasts to modulate B cell differentiation, activation, and function. Adipose tissue-derived fibroblasts have been shown to suppress plasmablast formation and induce formation of regulatory B cells (Franquesa et al., 2015) while rheumatoid synovial fibroblasts have been shown to induce immunoglobulin (Ig) class-switch recombination and IgG/IgA production in IgD⁺ B cells (Bombardieri et al., 2011). We envision that the immunoregulatory functions of CAFs may lead to improved survival in DLBCL and other B-cell lymphomas where malignant cells themselves are subject to functional alteration. In contrast, immunosuppression by CAFs in carcinomas may lead to an ineffective immune defense against malignant cells, which is associated with poor survival.

Cancer-associated fibroblasts are also capable of modifying the immune landscape by selective attraction, recruitment, retention, activation, and suppression of different immune cell types (Karin, 2010; Raz and Erez, 2013; Harper and Sainson, 2014). Recent studies provide evidence that CAFs can directly contribute to immune cell fate and survival (Harper and Sainson, 2014). In mouse models, CAFs have been shown to attract macrophages, neutrophils, and subsets

of T cells that promote tumor progression (Silzle et al., 2003; Grum-Schwensen et al., 2010; Elkabets et al., 2011). One possible underlying mechanism for the association of the DLBCL stromal-1 gene signature with good survival in patients with DLBCL is that fibroblasts and the associated ECM attract and trap malignant B cells thereby impeding their spread to new anatomical locations. We show a small but consistent inverse association of the DLBCL stromal-1 gene signature expression with DLBCL tumor stage (a measure of lymph node groups and extranodal sites to which malignant cells have metastasized) (**Figure 3A**). The decrease in stromal gene signature expression in the later stages of DLBCL may indicate that the stroma plays a role in localizing the lymphoma cells to the lymph nodes during the earlier stages of the disease. In contrast, DLBCL stromal-1 gene signature expression is typically increased with increased tumor stage in epithelial carcinomas, such as ovarian cancer (**Figure 3B**). The increase in CAFs in the later stages of carcinomas may prevent immune cells from reaching the tumor parenchyma by trapping the immune cells in the stroma thereby preventing an anti-tumor response. A recent study of immune cell infiltration in metastatic urothelial carcinomas showed that patients whose tumors were classified as immune-excluded (immune cells localized in the CAF-rich stroma) had increased disease progression and decreased response to immunotherapy (Mariathasan et al., 2018). Therefore, we hypothesize that CAFs aid in retaining DLBCL in the lymph node, which is associated with better prognosis, whereas in carcinomas CAFs trap immune cells, which is associated with decreased anti-tumor immune activity and a worse prognosis.

One of the key modulators of the cancer microenvironment is the multifunctional cytokine, transforming growth factor β (TGF β). TGF β induces CAF activation and fibroblast-to-myofibroblast transition with consequent linearization of collagen fibers and stiffening of the ECM. In turn, activated CAFs induce TGF β signaling to perpetually maintain the activated state (Calon et al., 2014; Beach et al., 2016; Erdogan and Webb, 2017). Consistent with the DLBCL stromal-1 signature representing CAFs, our Ingenuity Pathway Analysis (IPA) of the DLBCL gene signatures implicates TGF β signaling as the main upstream regulator of the DLBCL stromal-1 gene signature (**Table 2**). In carcinomas, TGF β has been shown to promote tumor progression by inhibiting immunosurveillance through multiple mechanisms (Flavell et al., 2010; Sheng et al., 2015), including the recruitment of macrophages (Byrne et al., 2008) and limited efficacy of immunotherapy by excluding CD8⁺ T cells from the tumor parenchyma (Mariathasan et al., 2018; Tauriello et al., 2018). It is likely that TGF β also plays an immunosuppressive role in lymphomas. However, TGF β is also a potent negative regulator of B-cell survival, proliferation, activation, and differentiation (Sanjabi et al., 2017). Stroma-derived TGF β has been shown to induce senescence and apoptosis in mouse models of B-cell lymphoma (Reimann et al., 2010; Stelling et al., 2018). Thus, the DLBCL stromal-1 gene signature may be primarily associated with tumor-promoting immunosuppression in carcinomas, while the same immunosuppression may lead to the eradication of

B cells, which represent the malignant component of B-cell lymphoma.

CONCLUSION

Past clinical trials have taught us that successful targeted therapies in one disease do not always yield the desired results in another disease despite the presence of the same target. One example is the poor response of B-cell-infiltrated carcinomas to rituximab, which has shown remarkable success in lymphomas and rheumatoid arthritis. The opposite survival outcomes associated with the presence of stromal cells in B-cell lymphomas and carcinomas should serve as a warning that targeting the tumor microenvironment may produce opposite effects in B-cell lymphomas and carcinomas.

DATABASE LINKS

GEO Data Sets (<https://www.ncbi.nlm.nih.gov/gds>)
Immunological Genome Project (<https://www.immgen.org>)
PRECOC – PRediction of Clinical Outcomes from Genomic Profiles (<https://precoc.stanford.edu>)
R2: Genomics Analysis

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AUTHOR CONTRIBUTIONS

SO analyzed the public data sets. SO and MH wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Inflammation is a key contributor to ovarian cancer cell seeding

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The incidence of ovarian cancer dramatically increases in early menopause but the factors contributing to cancer onset are unclear. Most ovarian cancers originate in the fallopian tube with subsequent implantation of malignant cells into the ovary. However, the events and conditions that lead to cancer cell implantation are unknown. To quantify which conditions are conducive to the seeding of cancer cells in an immunocompetent mouse model, we surgically implanted mouse ovarian cancer cells into the oviducts of syngeneic mice and simulated conditions associated with ovulatory wound repair, incessant ovulation, ovarian surface scarring, and aging. We found that the dominant site of cancer cell seeding was not the ovary but the nearby surgical wound site, which was associated with a strong and persistent inflammatory reaction. Conditions in the ovary associated with inflammation, such as acute ovulatory wound repair, active healing of the scarred ovarian surface, and mouse aging, contributed to increased seeding of the cancer cells to the surgical wound site and tissues surrounding the ovary. Changes in the ovary not accompanied by inflammation, such as completed ovulatory cycles and fully-healed scars on the ovarian surface, did not contribute to increased cancer cell seeding. We conclude that inflammation is the most likely mechanism by which ovulation and postmenopausal events contribute to the increased risk of ovarian cancer.

Despite modern day cytoreductive surgical techniques and combination chemotherapies for high-grade ovarian cancer, five-year survival rates remain below 40%¹. However, when found early, the survival rate dramatically rises to 90%^{1,2}. Thus, the ability to detect ovarian cancer in its earliest stages is critical to a cure. It is increasingly accepted that high-grade ovarian cancers actually originate in the fallopian tube with malignant cells shedding to the adjacent ovary^{3–7}. Since the bulk of the tumor typically forms in the ovary, rather than the fallopian tube, ovaries must play a significant role in the early stages of cancer development. Discovering which cellular and molecular processes promote and inhibit the seeding of malignant cells to the ovary could facilitate the development of markers for early detection as well as the identification of rate-limiting events in the early stages of ovarian cancer development. If contextual molecular cues provided by the ovary are required for the clinical development of ovarian cancer, such molecules could serve as novel therapeutic targets to prevent cancer progression in the early stages, when cures are more viable.

Epithelial ovarian cancer is predominantly a disease of postmenopausal women⁸. Many theories of postmenopausal onset of ovarian cancer have been proposed, including incessant ovulation and inflammation, hormonal changes, reduced immunity, increased cell senescence, and uncontrolled production of reactive oxygen species^{9–13}. Epidemiologic data consistently show that the risk of ovarian cancer increases with the number of ovulatory cycles^{14–16}, indicating that ovulation plays a significant role in ovarian cancer etiology. However, the peak incidence of menopause occurs at age 51, while the peak incidence of invasive epithelial ovarian cancer occurs at age 63¹. Thus, most women develop ovarian cancer years after their last ovulatory cycle. Currently, it is unknown which conditions in the ovary promote tumor growth but the fact that more than 80% of ovarian cancer cases occur after menopause suggests that the events associated with menopause and aging are major contributing factors⁸.

During the postmenopausal years, ovarian follicles are largely depleted and much of the remaining ovary is reduced to a collagenous scar tissue¹⁷. If the microenvironment of postmenopausal ovaries is conducive to the implantation of cancer cells, simulating postmenopausal conditions should result in more cancer cell deposits in the ovary. A better understanding of ovarian cancer pathogenesis, specifically the role of the early postmenopausal

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ovarian microenvironment in supporting the seeding and survival of malignant cells in the ovary, is necessary to develop strategies for ovarian cancer prevention and detection. Experiments in mice provide a convenient system in which both the effect and the outcome of specific conditions can be examined and quantified. Previously, we used a mouse model to study events associated with ovulation and ovulatory wound repair, including epithelial cell entrapment and the formation of epithelial inclusion cysts¹⁸. Here, we extended those studies by simulating various postmenopausal conditions in mice and quantifying cancer cell deposits for each condition. The goal of the study was to determine whether conditions associated with ovulation and aging increase the spread of cancer cells from the oviduct to the ovary. To account for a possible role of the immune system in ovarian cancer cell seeding, we used an immunocompetent FVB mouse model with syngeneic ovarian cancer cell aggregates implanted into the fallopian tube. Our data show that premenopausal and postmenopausal conditions contribute to increased cancer cell seeding only in the presence of an inflammatory reaction.

Materials and Methods

Cancer cell line. The FVB-syngeneic mouse ovarian cancer cell line, BR, was engineered with combinations of genetic alterations (p53^{-/-}, Brca1^{-/-}, myc, and Akt) as described¹⁹. We have shown that this ovarian cancer model recapitulates human serous histology, pattern of metastatic spread, and response to standard and targeted therapies^{19–23}. The BR cells were subsequently transduced with luciferase lentiviral plasmid pLenti-CMVpuro-LUC (Addgene, w168-1) to generate BR-luc cells.

Preparation of cell aggregates. BR-luc cells were seeded at a density of 1×10^6 cells per well in Costar ultra-low attachment 6-well plates (Corning). The cells were incubated with 3 ml DMEM media in 5% CO₂ at 37 °C. After 2 days, culture media were collected in 15 ml conical tubes and cells were precipitated at 1000 rpm for 0.5 minutes. After two rounds of washing with phosphate buffered saline (PBS), large cell aggregates were separated into small aggregates by multiple pipetting through a 1 ml pipette tip.

Injection of cell aggregates into oviducts. All procedures in mice were performed in accordance with the approved Cedars-Sinai IACUC protocol (IACUC5318). The procedures were performed in an AAALAC-accredited facility at Cedars-Sinai Medical Center. The surgical procedures were performed according to the method described for embryo transfer into the oviduct (Manipulating the Mouse Embryo: A Laboratory Manual, 3rd Edition, ISBN-978-087969591-0). Under the dissecting microscope, a small incision between the infundibulum and the ampulla of the oviduct (equivalent to human fallopian tube) was created using Vannas scissors (Supplementary Video 1). The transfer pipette loaded with cell aggregates in PBS was inserted into the incision with the tip pointing toward the ovary and approximately 200 cell aggregates in 2 µl volume were injected into each oviduct (Supplementary Video 1).

Simulation of ovulatory and menopausal conditions. Mice were superovulated by intraperitoneal injection of pregnant mare serum (PMS) and human chorionic gonadotropin (hCG) as previously described¹⁸. In the control mice, PBS was injected instead of PMS and hCG. To generate scar tissue, bursa (a thin membrane covering the ovary in mice) was removed (Supplementary Video 2) and the ovarian surface was burned with a hand-held battery-powered cauterizer (Gemini Cautery System) (Supplementary Video 3).

Quantification of cancer cell deposits. Mice were euthanized by CO₂ asphyxiation followed by cervical dislocation prior to harvesting the ovaries and surrounding tissues. To quantify macroscopic tumors, dimensions (length, width, height) were measured by calipers. Tumor volume (mm³) was calculated using the equation $V = (L \times W \times H)/2$, where V is tumor volume, L is tumor length, W is tumor width, and H is tumor height. For the flat, superficial tumors that typically formed on the surgical wounds/scars, tumor area (mm²) was measured using the equation $A = L \times W$, where A is tumor area size, L is tumor length, and W is tumor width. To quantify microscopic cancer cell deposits, the ovaries, oviducts, and surrounding fat tissues were fixed in formalin and embedded in paraffin. One 4 µm-thick section per sample was stained with hematoxylin and eosin (H&E) and evaluated under the light microscope for visible cancer cell deposits.

Statistical analyses. The statistical analyses were performed using GraphPad Prism (version 6.0; GraphPad Software). Intergroup differences were assessed by the Student's *t*-test.

Data availability. No datasets were generated or analyzed during the current study.

Results

Our ability to screen for early stage ovarian cancer is hampered by deficiencies in the understanding of the molecular and morphological steps involved in ovarian carcinogenesis. It is currently unknown why cancer cells in the fallopian tube have the propensity to migrate to the ovary where they tend to form a large tumor mass. To determine which ovarian conditions are most conducive to implantation of detached tubal cells, we simulated in mice conditions associated with ovulatory wound healing, incessant ovulation, atrophy/scarring, and aging.

Inflammatory events associated with ovulatory wound repair contribute to increased cancer cell seeding to tissues surrounding the ovary but are not directly associated with the implantation of cancer cells to the ovary. To simulate cancer cell seeding and entrapment during ovulatory wound healing, superovulation was induced in 4 week-old female FVB mice by intraperitoneal injection of PMS and hCG hormones (superovulated group, N = 6) or PBS (control group, N = 6). This combination of hormones induces ovulation of a large number of follicles to form 10–30 acute ovulatory wounds within one ovulatory cycle¹⁸. Two days after hCG (or control PBS) injection, when ovulatory wound repair is at its peak¹⁸, cancer cell

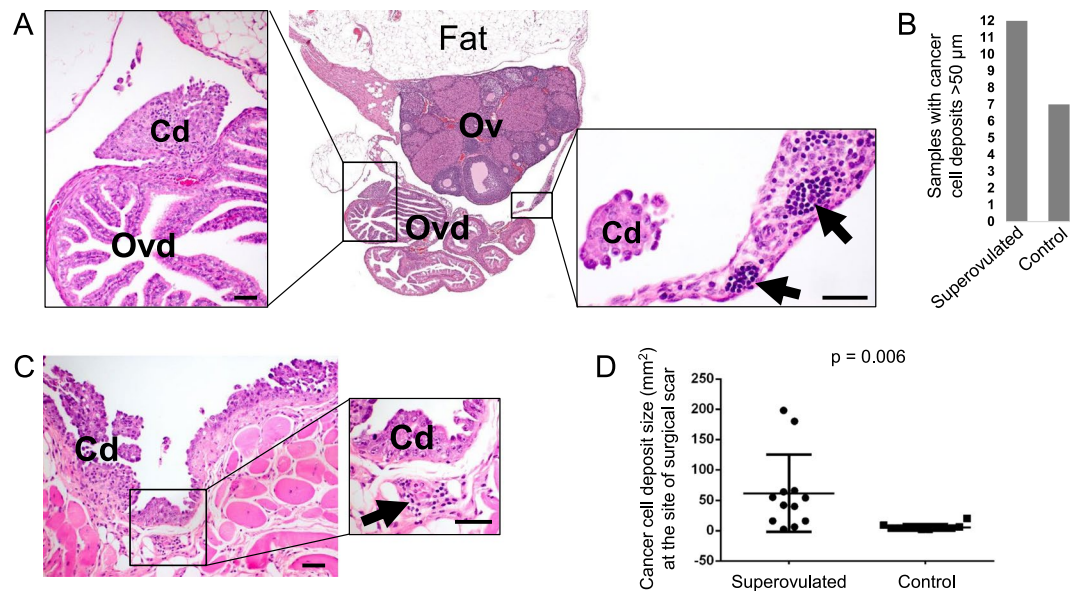


Figure 1. Assessing the effect of ovulatory wound repair on cancer cell seeding from the oviduct to the ovary and adjacent tissues. **(A)** Representative H&E-stained section of cancer cell deposits on the oviduct and ovary. Arrows indicate immune cell infiltrates. Size bars: 50 μm. Cd, cancer cell deposit; Ov, ovary; Ovd, oviduct. **(B)** Graph indicates the number of superovulated and control ovary/oviduct samples containing cancer cell deposits larger than 50 μm in diameter (out of 12 ovaries in each group). **(C)** H&E-stained section representing ‘carpeting’ of cancer cells along the surgical wound/scar site in the peritoneal wall. Arrow indicates an immune cell infiltrate. Size bars: 50 μm. Cd, cancer cell deposit. **(D)** Comparison of cancer cell deposit size at the surgical wound/scar site in superovulated and control mice.

aggregates were bilaterally implanted into the mouse oviduct. Three weeks later, intraperitoneal tumor dissemination was evaluated by recording the presence of ascites and measurable tumor deposits inside of the peritoneal cavity. Macroscopically visible swelling was observed in 4/12 ovaries from the superovulated mice and in 0/12 ovaries from the control mice. Microscopic cancer cell deposits in the oviducts, ovaries, and surrounding fat were quantified by pathologic examination of H&E-stained sections under the 4x objective, and the presence of cancer cells was further verified under higher magnification (Fig. 1A). The deposits in tissues surrounding the ovary were frequently associated with immune cell infiltrates (Fig. 1A). Cancer cell deposits larger than 50 μm were present in tissues surrounding the ovary (oviduct, bursa, and space between the fat and ovarian surface) in 12/12 samples from the superovulated mice and in 7/12 samples from the control mice (Fig. 1B). However, neither group of mice exhibited cell deposits directly on the ovarian surface or as intraovarian inclusions. These results suggest that ovulatory wound healing is not directly associated with the implantation of cancer cells to the ovary. In both groups of mice, the largest cancer cell deposits presented as carpeting of the abdominal wall at the sites of surgical wounds/scars (Fig. 1C). The surgical wound/scar cancer cell deposits were frequently associated with immune cell infiltrates (Fig. 1C) and were significantly larger in superovulated mice than in control mice (Fig. 1D). Taken together, our results indicate that events associated with ovulatory wound healing contributed to increased seeding of cancer cells to the surgical site and tissues surrounding the ovary. The lack of cancer cell deposits attached to the ovarian surface indicates that re-epithelialization of the ovarian surface does not significantly contribute to cancer cell seeding. It is more likely that ovulatory events contributed to increased inflammatory infiltrates, which attracted cancer cells and/or supported their survival and expansion.

Ovarian atrophy resulting from previous incessant ovulation is not associated with increased cancer cell seeding. To simulate cancer cell seeding in ovaries that endured repeated damage and repair due to multiple cycles of ovulation, six week-old female FVB mice were subjected to nine weeks of weekly intraperitoneal PMS and hCG hormone injections (repeatedly superovulated group, N = 7) or PBS injections (control group, N = 7). To mimic conditions in postmenopausal women whose ovaries have not been actively cycling for years, we waited 12 weeks after the last superovulation to implant BR-luc cell aggregates into the oviducts of the repeatedly superovulated and control mice. Eight weeks after cancer cell implantation, the tumor burden was evaluated in both groups. The majority of tumor deposits were found at the surgical wound/scar tissue, which was frequently fused with the adjacent fat and infiltrated with immune cells (data not shown). There were no macroscopically or microscopically visible cancer cell deposits on the ovaries and oviducts in either group of mice (data not shown). Thus, in the absence of acute inflammation, ovaries that have undergone repetitive superovulations do not appear to attract cancer cells any more than age-matched ovaries with a normal number of ovulatory cycles. One caveat to this experiment is that we did not achieve complete depletion of the oocytes pool despite nine cycles of superovulation, possibly because mice become unresponsive to hormone induction after reaching reproductive maturity²⁴.

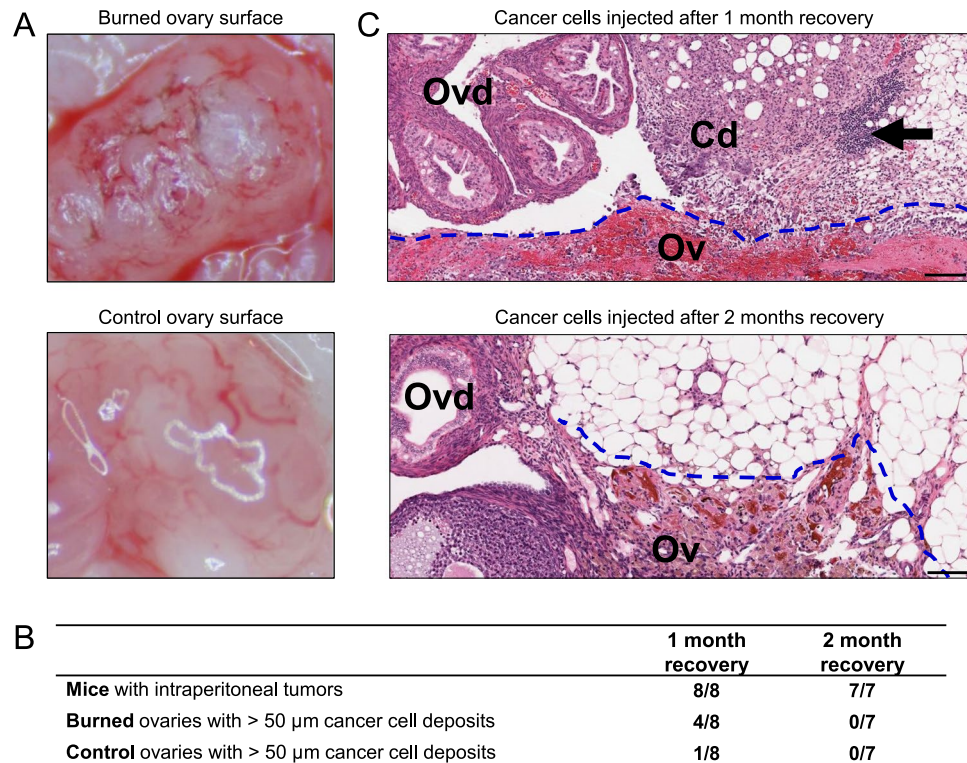


Figure 2. Assessing the effect of burn-induced ovary scarring on the seeding of intraperitoneally injected cancer cells. **(A)** Representative images of ovarian surface immediately after manipulation. Control ovaries were surgically released from the bursa while burned ovaries were first surgically released from the bursa, then superficially burned with a cauterizer. **(B)** Comparison of seeding efficiency of intraperitoneally injected cancer cells one or two months after surgical ovary manipulation. **(C)** Representative H&E-stained sections of cancer cell deposits on the ovaries and oviducts of mice that were injected one or two months after surgical ovary manipulation and euthanized four weeks later. Arrow indicates an immune cell infiltrate. Size bars: 100 μ m. Cd, cancer cell deposit; Ov, ovary; Ovd, oviduct.

Burn-induced scarring of the ovarian surface is associated with increased cancer cell seeding to the ovaries and surrounding tissues only in the presence of active scar wound healing. To simulate events associated with postmenopausal ovary atrophy and connective tissue scarring, burn-induced scars were generated on the ovarian surface of the six week-old female FVB mice. In each mouse, one ovary was surgically released from the ovarian bursa (Supplementary Video 2) and superficially burned with a cauterizer (Fig. 2A and Supplementary Video 3). The contralateral ovary was surgically released from the bursa but not burned (control ovary) (Fig. 2A). Mice were intraperitoneally injected with a single-cell suspension of BR-luc cells ($\sim 1 \times 10^6$ cells) after one month recovery (N = 8) or two months recovery (N = 7). Four weeks after intraperitoneal cell injection, mice were euthanized for tumor burden quantification. Regardless of whether mice were intraperitoneally injected with cancer cells one month or two months after surgery, BR-luc cells formed multiple small tumor nodules on the mesothelial surfaces of the omentum, pancreas, diaphragm, spleen and abdominal lining; however, there were no visible tumor cell deposits on the surface of the burned or control ovaries. Therefore, we assessed microscopic cancer cell deposits in H&E-stained sections of ovaries/oviducts and adjacent fat. For the one month recovery group (N = 8), cancer cell deposits larger than 50 μ m were present in the tissues surrounding the ovary (fat, oviduct, and bursa) in 4/8 burned ovaries and in 1/8 control ovaries (Fig. 2B). All ovaries that contained tumor deposits also had abundant immune cell infiltrates (Fig. 2C). For the two months recovery group (N = 7), none of the ovary sections contained cancer cell deposits (Fig. 2B). Although burn-induced scars on the ovarian surface were detectable two months later, the scars were no longer associated with abundant immune cell infiltrates (Fig. 2C). These results suggest that burn-induced scars attract cancer cells but only in the presence of inflammation.

Events associated with aging contribute to increased cancer cell seeding to the ovaries and surrounding tissues. BR-luc cancer cell aggregates were bilaterally implanted into the oviducts of eight week-old (N = 10) and greater than one year-old (age range 14–19 months; N = 10) female FVB mice. Mice were euthanized for analysis four weeks after cancer cell implantation. Both groups of mice developed multiple intraperitoneal metastases with the largest tumor masses present on the omentum and abdominal wall. Omental and abdominal wall masses were more frequent in aged mice (Fig. 3A). Three of the aged mice also exhibited unilateral or bilateral uterine horn hyperplasia (data not shown). H&E-stained sections showed that the ovaries from young mice contained multiple follicles in different phases of maturation (data not shown), while the ovaries from old mice were devoid of follicles (Fig. 3B). Microscopic examination of the ovaries and adjacent tissues (oviduct,

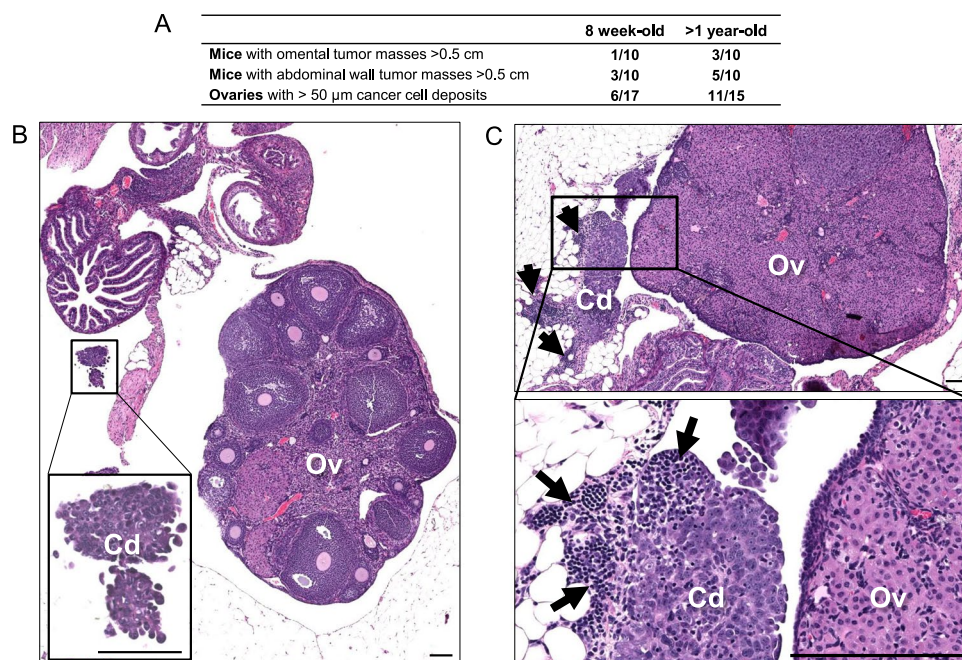


Figure 3. Assessing the effect of aging on cancer cell seeding from the oviduct to the ovary and adjacent tissues. **(A)** Comparison of seeding efficiency of cancer cells implanted into the oviducts of young (8 weeks) and aged (>1 year) mice. In H&E-stained sections, several ovaries were excluded from the analysis because they were either missing from the slide section or the tissue was insufficient for evaluation. **(B,C)** Representative H&E-stained section of cancer cell deposits on ovaries and oviducts four weeks after surgical implantation of cancer cells into the oviducts of **(B)** 8-week-old mice and **(C)** >1-year-old mice. Arrow indicates an immune cell infiltrate. Size bars: 100 μ m. Cd, cancer cell deposit; Ov, ovary.

bursa, and adjacent fat) revealed that tumor cell deposits were more frequent in aged mice (Fig. 3A), which also contained more abundant immune cell infiltrates (Fig. 3B). These results suggest that ovaries from aged mice are more conducive to cancer cell seeding than ovaries from young mice.

Discussion

A poor understanding of the initiating events in ovarian cancer has significantly hampered our efforts towards early ovarian cancer detection and prevention. Most early stage cancers in the tubal fimbria are associated with a dominant mass in the ovary, indicating that the ovarian microenvironment is essential for tumor growth. However, conditions that promote cancer cell seeding and growth in the ovary are still unknown. Recently, Yang-Hartwich and colleagues used a mouse xenograft model to test the role of ovulatory wound repair in the migration of cancer cells from the injection site in the uterus toward the ovary²⁵. Consistent with epidemiologic data that increased ovulation is strongly associated with ovarian cancer^{15,16}, they showed that superovulation in mice enhances the migration and adhesion of malignant cells to the ovary and that this attraction is mediated through the release of cytokines/chemokines from the surface wound created by oocyte release²⁵. Using a syngeneic immunocompetent mouse model with cancer cells surgically implanted into the oviduct, we confirmed that superovulation contributes to ovarian cancer cell seeding. Tumor cell deposits were accompanied by immune infiltrates, indicating that ovulation-induced inflammation may play an important role in cancer cell seeding. It is possible that the inflammatory reaction is the only factor that contributes to increased cancer cell seeding because the largest cancer cell deposits typically formed in the abdominal wall along surgical wounds, which were associated with extensive immune infiltrates. It appears that the wounded surface of the superovulated ovary did not play a direct role in cancer cell attraction as there were no cancer cells attached to the ovarian surface epithelium or inside the ovarian stroma. The importance of the inflammatory reaction, rather than the damaged ovarian surface in cancer cell seeding, was illustrated by the next two sets of experiments in which we repeatedly wounded the ovarian surface by multiple rounds of superovulation or burned the ovarian surface to induce scarring. The wounded/scarred ovarian surface proved to be attractive to cancer cells only if the wounds were ‘fresh’. If ovarian wounds/scars were allowed to recover for two months, cancer cells were no longer attracted to the ovarian surface but were still attracted to other sites in the peritoneal cavity where inflammation persisted. It is well established that aging is characterized by subclinical, chronic inflammation²⁶. Consistent with multiple studies showing that the overall proinflammatory status in older mice is associated with increased tumor burden²⁷, our results show that oviductal implantation of cancer cells in aged mice resulted in increased tumor burden throughout the peritoneal cavity.

Our finding that surgical wounds in mice attract cancer cells is consistent with an observation in clinical practice that wound trauma in patients is associated with cancer recurrence^{28,29}. It has been shown that an early peak of breast cancer recurrence is due to surgery-driven intervention³⁰. The exact reasons for surgery-related cancer

attraction are not fully understood but possible factors include surgery-related acute wound healing process, inflammation, and activation of dormant cancer cells by surgery-driven growth factors^{31–33}. If inflammation is a key factor in cancer cell seeding, what are the contributions of other factors strongly associated with increased cancer incidence, such as ovulation, oocyte depletion and atrophy, and aging? Our data in a mouse model are consistent with the concept that most of the factors implicated in ovarian cancer incidence converge on inflammation as a common denominator. One successful path to ovarian cancer prevention has been controlling factors that induce inflammation, such as the use of oral contraceptives to suppress ovulation³⁴. Epidemiologic data show that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) can be beneficial in the prevention of multiple cancers, including ovarian^{35,36}. Although factors associated with the increased risk of ovarian cancer, such as aging and menopause cannot be prevented, the risk can be reduced by suppressing inflammation. The results of our study in a mouse model confirm previous results that inflammation is a key factor in promoting ovarian cancer cell seeding. An understanding of the mechanisms by which inflammation plays a role in the early stages ovarian cancer will be necessary for effective ovarian cancer prevention.

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Author Contributions

D.J. performed all experiments described in this study except mouse surgery, which was performed by Y.N. and M.K. D.J. also participated in data collection, analysis, and interpretation. S.O. conceived and designed the study, participated in data analysis and interpretation, and wrote the manuscript. All authors contributed to manuscript revisions and approved the submitted version.

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Are Epithelial Ovarian Cancers of the Mesenchymal Subtype Actually Intraperitoneal Metastases to the Ovary?

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Primary ovarian high-grade serous carcinoma (HGSC) has been classified into 4 molecular subtypes: Immunoreactive, Proliferative, Differentiated, and Mesenchymal (Mes), of which the Mes subtype (Mes-HGSC) is associated with the worst clinical outcomes. We propose that Mes-HGSC comprise clusters of cancer and associated stromal cells that detached from tumors in the upper abdomen/omentum and disseminated in the peritoneal cavity, including to the ovary. Using comparative analyses of multiple transcriptomic data sets, we provide the following evidence that the phenotype of Mes-HGSC matches the phenotype of tumors in the upper abdomen/omentum: (1) irrespective of the primary ovarian HGSC molecular subtype, matched upper abdominal/omental metastases were typically of the Mes subtype, (2) the Mes subtype was present at the ovarian site only in patients with concurrent upper abdominal/omental metastases and not in those with HGSC confined to the ovary, and (3) ovarian Mes-HGSC had an expression profile characteristic of stromal cells in the upper abdominal/omental metastases. We suggest that ovarian Mes-HGSC signifies advanced intraperitoneal tumor dissemination to the ovary rather than a subtype of primary ovarian HGSC. This is consistent with the presence of upper abdominal/omental disease, suboptimal debulking, and worst survival previously reported in patients with ovarian Mes-HGSC compared to other molecular subtypes.

Keywords: ovarian cancer, mesenchymal, molecular subtype, metastases, desmoplasia

INTRODUCTION

High-grade serous carcinoma is the most common and most deadly type of ovarian cancer (Matulonis et al., 2016). The majority of ovarian cancer patients with HGSC are diagnosed with tumors involving one or both ovaries and various additional intraperitoneal sites including the upper abdomen/omentum (FIGO stage III) (Matulonis et al., 2016). HGSC can arise from the

fallopian tube, the ovarian surface serous epithelium, or extraovarian peritoneal tissues as primary peritoneal carcinoma (PPC) (**Figure 1A**). Currently, it is thought that ovarian cancer cells shed from the primary tumor into the peritoneal fluid and disseminate in the peritoneal cavity, typically from the ovary to the upper abdomen/omentum (**Figure 1A**). However, the model of primarily unidirectional HGSC metastasis from the pelvis to the upper abdomen/omentum seems simplistic within a cavity that lacks internal physical barriers to cancer dissemination. We propose that in stage III HGSC, metastases and PPC in the upper abdomen/omentum shed cancer cell-stroma aggregates into the peritoneum, resulting in intraperitoneal dissemination that includes secondary metastases to the primary tumor in the pelvis (**Figure 1B**). Patterns of cancer dissemination within the peritoneal cavity have been difficult to discern using genomic data because genomic instability is an early event in HGSC and copy number profiles and mutational patterns are typically shared across different anatomic sites (Brodsky et al., 2014; Lee et al., 2015; Schwarz et al., 2015; Eckert et al., 2016; McPherson et al., 2016). However, individual clones have been tracked using whole-genome and single-nucleus sequencing of patient-matched tumor deposits at different anatomic locations. These studies identified evidence of metastases to the ovary or the fallopian tube in 4 out of 15 patients, thereby demonstrating that re-seeding of the primary tumor site by clones from peritoneal metastases is not a rare event (Eckert et al., 2016; McPherson et al., 2016).

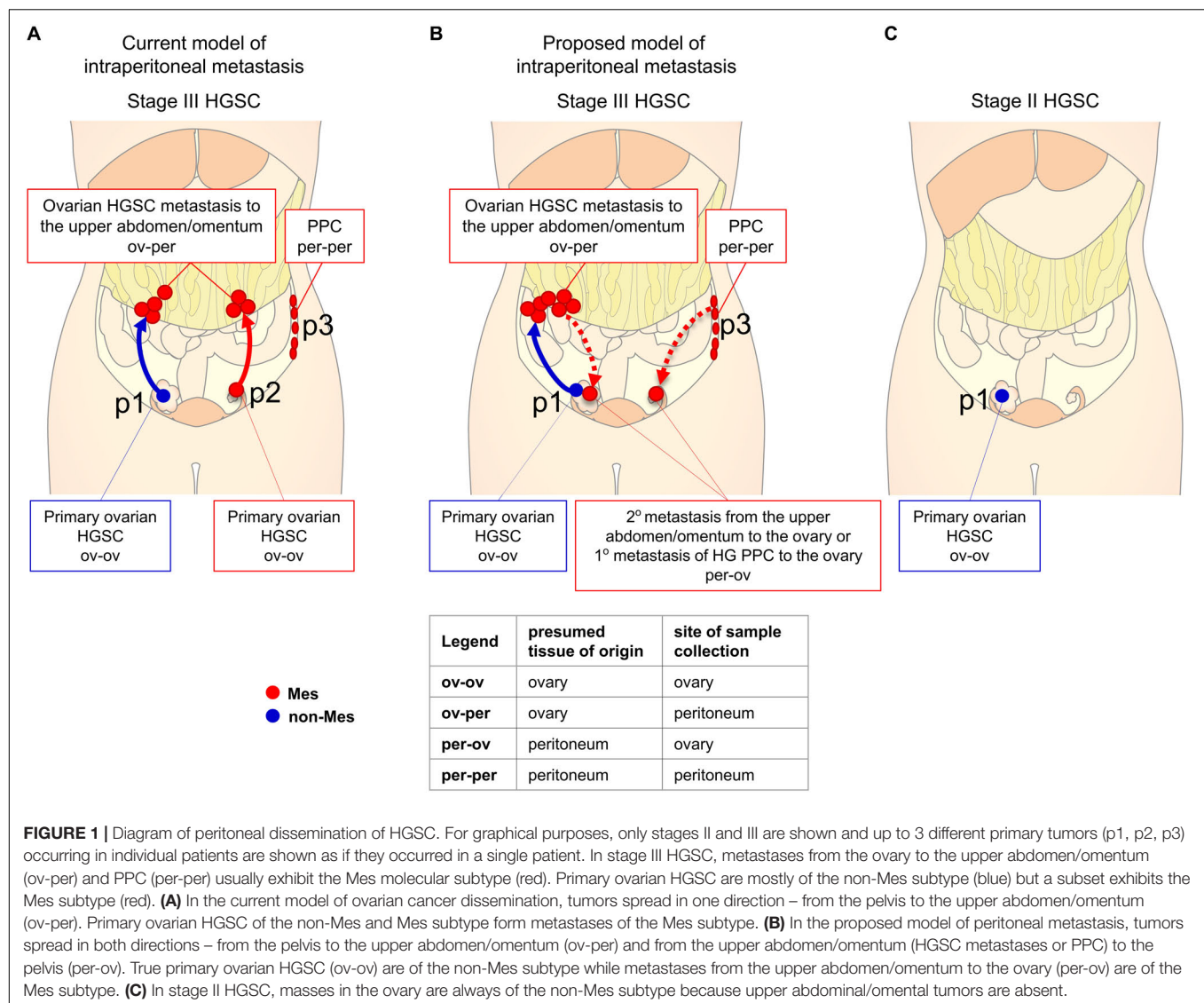
Transcriptomic analyses have clustered primary ovarian HGSC into 4 main molecular subtypes: Immunoreactive, Mesenchymal (Mes), Proliferative and Differentiated (The Cancer Genome Atlas Research Network, 2011; Verhaak et al., 2013). The feature that distinguishes primary ovarian HGSC of the Mes molecular subtype (Mes-HGSC) from the other 3 subtypes (non-Mes subtypes) is the elevated expression of myofibroblast/extracellular matrix (ECM) remodeling genes (Zhang et al., 2015, 2018; Jia et al., 2016). In addition to this distinct transcriptome, primary ovarian Mes-HGSC is more frequently associated with the presence of upper abdominal/omental metastases (Vargas et al., 2015; Torres et al., 2017), suboptimal surgical debulking (presence of residual macroscopic disease after cytoreductive surgery) (Liu et al., 2015; Torres et al., 2017; Wang et al., 2017), severe postoperative complications (Torres et al., 2017, 2018a), and reduced overall survival (Liu et al., 2015; Vargas et al., 2015; Torres et al., 2017, 2018a; Wang et al., 2017; Shilpi et al., 2019) in comparison to the primary ovarian non-Mes subtypes. The current theory suggests that cancer cells in primary ovarian Mes-HGSC recruit myofibroblasts or convert the local ovarian stroma into myofibroblasts, which equip cancer cells with greater metastatic ability (Torres et al., 2017, 2018b; Zhang et al., 2018). However, this theory does not explain why metastases are predominantly of the Mes phenotype even when the primary tumor is Immunoreactive, Proliferative, or Differentiated subtype (Tan et al., 2018). PPC is also typically of the Mes phenotype (Gao et al., 2016), suggesting that this phenotype is an inherent feature of peritoneal lesions.

In this study, we used transcriptomic analyses of tumor samples with annotated presumed sites of tumor origin and sites of sample collection (**Supplementary Table S1**) to show that the Mes gene signature is expressed in the stromal component of tumors in the upper abdomen/omentum but not in most primary ovarian HGSC. However, if a tumor in the ovary expresses the Mes gene signature, we propose that this tumor contains the microenvironment from tumors in the upper abdomen/omentum. This can occur by two mechanisms (**Figure 1B**). The first mechanism involves a primary tumor in the ovary/pelvis that metastasizes to the omentum/upper abdomen, then the metastases, which have now acquired the Mes phenotype, continue to seed the rest of the peritoneal cavity, including the ovary where the tumor initially originated. The second mechanism involves PPC from the upper abdomen/omentum that metastasizes throughout the peritoneal cavity, including to the ovary. If our hypothesis is correct, we predict that stage II primary ovarian HGSC (tumors confined to the pelvis) cannot exhibit the Mes subtype because of the absence of cancer/stroma cell aggregates from the upper abdomen/omentum as a source of metastases to the ovary (**Figure 1C**).

RESULTS

The Mes Subtype Reflects Tumor Location

We examined whether the Mes phenotype varies across patient-matched samples of primary, metastatic, and recurrent HGSC. The Mes phenotype was determined by threshold expression of 15 mesenchymal genes (section “Materials and Methods”; **Supplementary Table S2**). Notably, these 15 genes were expressed at significantly higher levels in the tumor stroma in comparison to epithelial cancer cells in both the primary HGSC data set of laser-capture-microdissected stromal and epithelial cells (GSE40595 data set) (Yeung et al., 2013; **Supplementary Figure S1A**) and flow cytometry-isolated individual cells in colorectal cancer (GSE39395 data set) (Calon et al., 2012; **Supplementary Figure S1B**). The 15 genes were among the top 100 genes used by Verhaak et al. (2013) to define the Mes subtype by expression profile analysis (Mes 100-gene set) (**Supplementary Table S2**). In the HGSC TCGA data set, this Mes 15-gene signature was associated with poor overall and progression-free survival (**Supplementary Figure S1C**) and was equivalent to the Mes 100-gene signature in classifying samples of the Mes subtype (**Supplementary Figure S2**). All 15 genes were expressed at higher levels in the metastatic and recurrent tumors compared to the matched primary tumors (**Figure 2A**). While only 20% of the primary ovarian HGSC were classified as Mes, 79% of the concurrent and 58% of the recurrent HGSC metastases were classified as Mes (**Figure 2B**). The higher percentage of the Mes subtype in the concurrent metastases than in the recurrent metastases may be attributed to different sites of sample collection. The omentum was the most common collection site for concurrent metastases but not for recurrent metastases because most patients had a partial omentectomy during primary



debulking surgery (**Figure 2B**). Notably, 15 of 17 metastatic and recurrent samples collected from the omentum were classified as Mes and none of the 4 metastatic and recurrent samples collected from the lymph nodes was classified as Mes (**Figure 2B**). In 2 patients, omental metastases were classified as non-Mes; their expression levels of the 15 Mes genes were high (**Supplementary Table S3**) but did not reach the threshold we set for Mes subtype classification (see section “Materials and Methods”).

Since most metastases located in the upper abdomen/omentum were classified as Mes (**Figure 2B**), we hypothesized that primary ovarian HGSC of the Mes subtype are actually metastases from the upper abdomen/omentum to the ovary. If this is correct, HGSC collected from patients with cancer confined to the pelvis (stage I-II) should not exhibit the Mes phenotype because of the absence of upper abdominal/omental disease as a source of metastatic tumor clusters able to seed the ovary (**Figure 1C**). We tested this hypothesis in 2 public data sets in which samples had been

divided into 4 molecular subtypes. In this study, we used the original molecular classifications for the TCGA and GSE9891 data sets since this classification is most commonly used in the literature (Tothill et al., 2008; The Cancer Genome Atlas Research Network, 2011). Of note, subsequent studies may have used different classification algorithms, which resulted in different molecular subtype assignments to the same samples. In a recent study, 22% of the samples had been reclassified to a different molecular subtype, which resulted in better correlation with survival outcomes (Shilpi et al., 2019).

In the ovarian TCGA data set, samples had been divided into 4 molecular subtypes: Immunoreactive, Mesenchymal (Mes), Proliferative, and Differentiated (The Cancer Genome Atlas Research Network, 2011). According to the strict TCGA guidelines for sample collection, all of the ovarian TCGA tumor samples presumably originated in the ovary and were collected from the ovary (ov-ov) (**Figure 2C**). Of 23 stage II HGSC samples, only 1 was classified as Mes (**Figure 2C**). Notably,

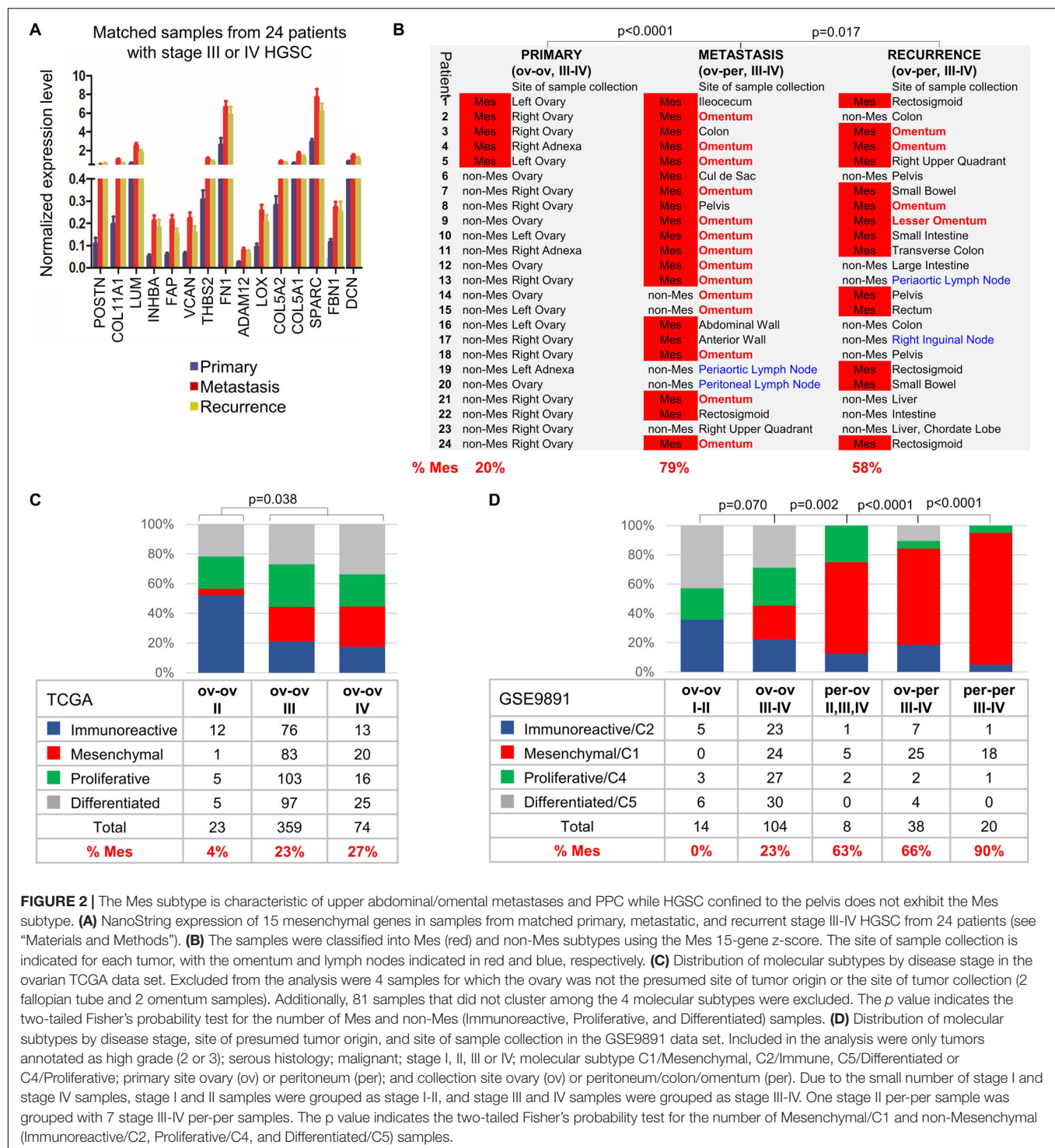


FIGURE 2 | The Mes subtype is characteristic of upper abdominal/omental metastases and PPC while HGSC confined to the pelvis does not exhibit the Mes subtype. **(A)** NanoString expression of 15 mesenchymal genes in samples from matched primary, metastatic, and recurrent stage III-IV HGSC from 24 patients (see “Materials and Methods”). **(B)** The samples were classified into Mes (red) and non-Mes subtypes using the Mes 15-gene z-score. The site of sample collection is indicated for each tumor, with the omentum and lymph nodes indicated in red and blue, respectively. **(C)** Distribution of molecular subtypes by disease stage in the ovarian TCGA data set. Excluded from the analysis were 4 samples for which the ovary was not the presumed site of tumor origin or the site of tumor collection (2 fallopian tube and 2 omentum samples). Additionally, 81 samples that did not cluster among the 4 molecular subtypes were excluded. The *p* value indicates the two-tailed Fisher’s probability test for the number of Mes and non-Mes (Immunoreactive, Proliferative, and Differentiated) samples. **(D)** Distribution of molecular subtypes by disease stage, site of presumed tumor origin, and site of sample collection in the GSE9891 data set. Included in the analysis were only tumors annotated as high grade (2 or 3); serous histology; malignant; stage I, II, III or IV; molecular subtype C1/Mesenchymal, C2/Immune, C5/Differentiated or C4/Proliferative; primary site ovary (ov) or peritoneum (per); and collection site ovary (ov) or peritoneum/colon/omentum (per). Due to the small number of stage I and stage IV samples, stage I and II samples were grouped as stage I-II, and stage III and IV samples were grouped as stage III-IV. One stage II per-per sample was grouped with 7 stage III-IV per-per samples. The *p* value indicates the two-tailed Fisher’s probability test for the number of Mesenchymal/C1 and non-Mesenchymal (Immunoreactive/C2, Proliferative/C4, and Differentiated/C5) samples.

that sample (TCGA-61-2133) had features of an aggressive malignancy despite its stage II designation: it was annotated as stage IIc, grade 3 with extensive lymphovascular permeation, positive pelvic lymph nodes and the shortest overall survival among patients with stage IIc HGSC who died from the disease (676 days vs 1,380 days mean survival). In contrast to stage II HGSC, out of 359 stage III HGSC and 74 stage IV

HGSC, Mes tumors contributed to 23 and 27% of samples, respectively (Figure 2C).

In the GSE9891 data set, HGSC samples had been clustered into C1/Mesenchymal, C2/Immune, C4/Proliferative, and C5/Differentiated molecular subtypes (Tothill et al., 2008) and annotated by their presumed tissue of origin and the site of specimen collection as ov-ov, per-ov, ov-per, and per-per

(Figure 2D). Due to a small number of stage I and stage IV samples, we grouped stage I and II samples as stage I-II, and stage III and IV samples as stage III-IV. None of the 14 ov-ov stage I-II HGSC was classified as Mes/C1 (Figure 2D). Of the 104 ov-ov stage III-IV HGSC, 23% were classified as C1/Mes. Of the 38 ov-per stage III-IV HGSC, 66% were classified as C1/Mes (Figure 2D). Of the 8 per-ov HGSC (including 1 stage II and 7 stage III-IV), 63% were classified as C1/Mes (Figure 2D). According to our hypothesis, all metastases that originated in the upper abdomen/omentum as PPC and then spread to the ovary (per-ov) should be of the C1/Mes subtype. However, it is important to note that these 8 per-ov tumors were reported by pathologists as PPC based only on the impression of disease distribution gathered from the surgeon's description of the intraoperative findings in the operative report and the tissue samples that surgeons had elected to excise. Interestingly, and as noted by pathologists in some of these surgical pathology reports, pathologists were not always certain or in agreement about the origin of the tumor (primary ovarian vs. PPC). Of the 20 per-per stage III-IV HGSCs, 90% were classified as C1/Mes. It is unknown if some of these PPC samples were located in the pelvis, in which case we predict the non-Mes phenotype. As an independent method of classification, we used the Mes 15-gene signature (Supplementary Table S2) to classify samples in the TCGA (Figure 2C) and GSE9891 (Figure 2D) data sets into Mes and non-Mes subtypes. This classification resulted in similar proportions of Mes samples in each respective group (Supplementary Tables S4, S5 and Figure S3) as the original classification of the molecular subtypes in Figures 2C,D.

Together, we conclude that ov-ov HGSC stage I-II (confined to the pelvis) are almost never of the Mes subtype while ~20–30% of ov-ov HGSC stage III-IV (presence of concurrent upper abdominal/omental metastases) are of the Mes subtype. HGSC samples collected from the peritoneal cavity (ov-per and per-per) as well as samples presumed to be peritoneal metastases to the ovary (per-ov) are typically of the Mes subtype.

The Mes Molecular Subtype Is Defined by the Metastatic Microenvironment, Not the Epithelial Cancer Cells

To determine which cell type expresses the Mes 15-gene signature, we used digital image analysis for the annotation of fibroblasts, epithelial cancer cells, and immune cells in H&E-stained full sections of omental metastases collected during primary debulking surgery from 152 HGSC patients (GSE135712) (Figure 3A). The Mes 15-gene z-score was determined for each patient (Supplementary Figure S4A) and correlated with the content of each of the 3 annotated cell types. The two prevalent cell types in omental metastases were epithelial cancer cells and fibroblasts, while the content of immune cells was variable across 152 samples (data not shown). The Mes 15-gene z-score correlated with the fibroblast content ($r = 0.660$; $p = 2.4 \times 10^{-20}$), inversely correlated with the epithelial cancer cell content ($r = -0.619$; $p = 1.8 \times 10^{-17}$) and showed no significant correlation with the immune cell content ($r = -0.035$; $p = 0.67$) (Figure 3B), suggesting that among these 3 cell types in omental

metastases, fibroblasts are the most likely source of the Mes 15-gene signature.

To study the correlation of the Mes 15-gene z-score with fibroblast content in primary and metastatic tumors, we used concurrent primary ovarian HGSC, omental metastases, and non-omental intraperitoneal metastases collected at the time of primary debulking surgery from 10 HGSC patients (GSE133296). The average fibroblast content did not differ significantly between primary ovarian HGSC, omental metastases, and non-omental metastases (Figure 3C). The Mes 15-gene z-score (Supplementary Figure S4B) was significantly correlated with the fibroblast content in omental ($r = 0.703$; $p = 0.02$) and non-omental intraperitoneal ($r = 0.893$; $p = 5.0 \times 10^{-4}$) metastases but not in primary tumors ($r = 0.170$; $p = 0.64$) (Figure 3D), suggesting that primary tumor fibroblasts were not expressing high levels of the 15 Mes genes. This result is consistent with our prior *in situ* hybridization findings that only a small number of patients (~20%) expressed COL11A1 in cancer-associated fibroblasts (CAFs) in primary ovarian HGSC, while the majority of patient-matched metastases expressed COL11A1 in CAFs (Cheon et al., 2014).

The Mes 15-gene z-score was inversely correlated with the content of epithelial cancer cells and was not significantly different between primary ovarian HGSC, omental metastases, and non-omental metastases (data not shown). The Mes 15-gene z-score was not enriched in metastases if metastatic epithelial cancer cells were stripped of their microenvironment. EpCAM-positive epithelial cancer cells isolated from matched primary ovarian HGSC, ascites, and metastasis from 5 patients (3 with replicate samples) (GSE73168) (Gao et al., 2019) exhibited equivalent relative values of the Mes 15-gene z-score (Supplementary Figures S4C,D). Together, these results show that the Mes phenotype is determined by the metastatic microenvironment rather than by the intrinsic molecular subtype of epithelial cancer cells.

Primary Ovarian HGSC of the Mes Subtype Are Enriched for a Gene Signature Characteristic of Stromal Cells in Metastases Located in the Upper Abdomen/Omentum

We were interested to know if the stroma in primary ovarian HGSC differs from the stroma in HGSC metastases located in various tissue sites in the peritoneal cavity. To completely exclude the epithelial cancer cell transcriptome from the analysis, we used published stromal gene signatures derived from proteome data of laser-capture-microdissected stromal cells from primary ovarian HGSC and matched omental metastases from 11 HGSC patients (Eckert et al., 2019). We first validated the 2 stromal gene signatures (Eckert et al., 2019) in our own data set (GSE133296) of matched primary ovarian HGSC, omental metastases, and non-omental metastases from 10 HGSC patients. The primary ovarian HGSC stromal gene signature was overexpressed in a subset of primary ovarian HGSC while the omental metastasis stromal gene signature

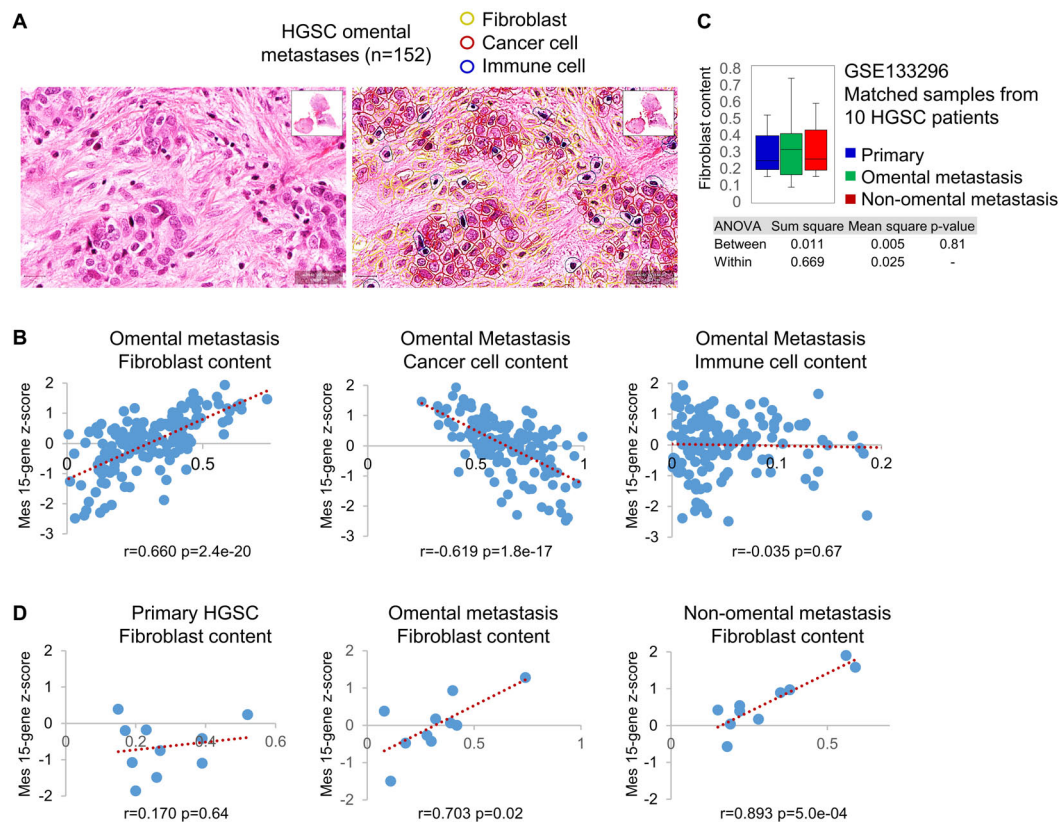


FIGURE 3 | The Mes gene signature expression correlates with fibroblast content in HGSC metastases but not primary ovarian HGSC. **(A)** A representative example of cell type (fibroblast, epithelial cancer cell, immune cell) annotation by QuPath analysis of H&E-stained full sections of omental metastases isolated from 152 HGSC patients at the time of primary debulking surgery (GSE135712). **(B)** Correlation of the Mes 15-gene z-score (Y axis) with the content of fibroblasts, cancer cells, and immune cells in omental metastases isolated from 152 HGSC patients (GSE135712). The content of each cell type was determined as the percent of one cell type in the 3 annotated cell types (fibroblasts, cancer cells, immune cells) in each sample (X axis). **(C)** Fibroblast content in matched primary tumors, omental metastases, and non-omental peritoneal metastases isolated at the time of primary debulking surgery from 10 HGSC patients (GSE133296). Fibroblast content was determined as the percent of fibroblasts in the 3 annotated cell types in each sample. **(D)** Correlation of the Mes 15-gene z-score (Y axis) with the content of fibroblasts (X axis) individually in matched primary tumors, omental metastases, and non-omental intraperitoneal metastases from 10 HGSC patients (GSE133296).

was overexpressed in a subset of omental and non-omental metastases (**Figure 4A**). Thus, application of the published proteome-derived stromal gene signatures to our data set shows that the 2 stromal gene signatures are differentially enriched in primary and metastatic tumors in most patients (**Figure 4A**). To assign a quantitative value to the difference in enrichment of the 2 stromal gene signatures, we used an unweighted ratio of the stromal gene signature z-scores (positive value for the omental metastasis gene signature and negative value for the primary ovarian HGSC gene signature). The average unweighted ratio of the z-scores from Omental metastasis/Primary HGSC stromal gene signatures was significantly lower in primary ovarian HGSC compared to patient-matched omental or non-omental metastases in the GSE133296 data set (**Figure 4B**).

Using 2 large transcriptomic data sets in which subsets of samples have been annotated by the site of sample collection (GSE9891 and GSE2109), we showed that the average unweighted ratio of the z-scores from Omental metastasis/Primary HGSC stromal gene signatures was lower in

tumors located retroperitoneally or in the pelvis (ovary, uterus, and fallopian tube) than in tumors located outside of the pelvis (omentum, colon/intestine, abdominal wall, peritoneum, and diaphragm) (**Figures 4C,D**). Together, these data suggest that intraperitoneal tumors located in the pelvis are enriched for a stromal gene signature of primary ovarian HGSC while tumors outside of the pelvis are enriched for a stromal gene signature of omental metastases.

To determine whether the Omental metastasis and Primary HGSC stromal gene signatures are associated with molecular subtypes in primary ovarian HGSC, we used the ovarian TCGA data set (The Cancer Genome Atlas Research Network, 2011). Overlay of the Omental metastasis and Primary HGSC stromal gene signatures with the ovarian TCGA data set showed strong enrichment of the Omental metastasis gene signature in the Mes subtype while the Primary HGSC stromal gene signature was not significantly enriched in any specific molecular subtype (**Figure 5A**). The average unweighted ratio of the z-scores from Omental metastasis/Primary HGSC stromal gene signatures was significantly enriched in the Mes

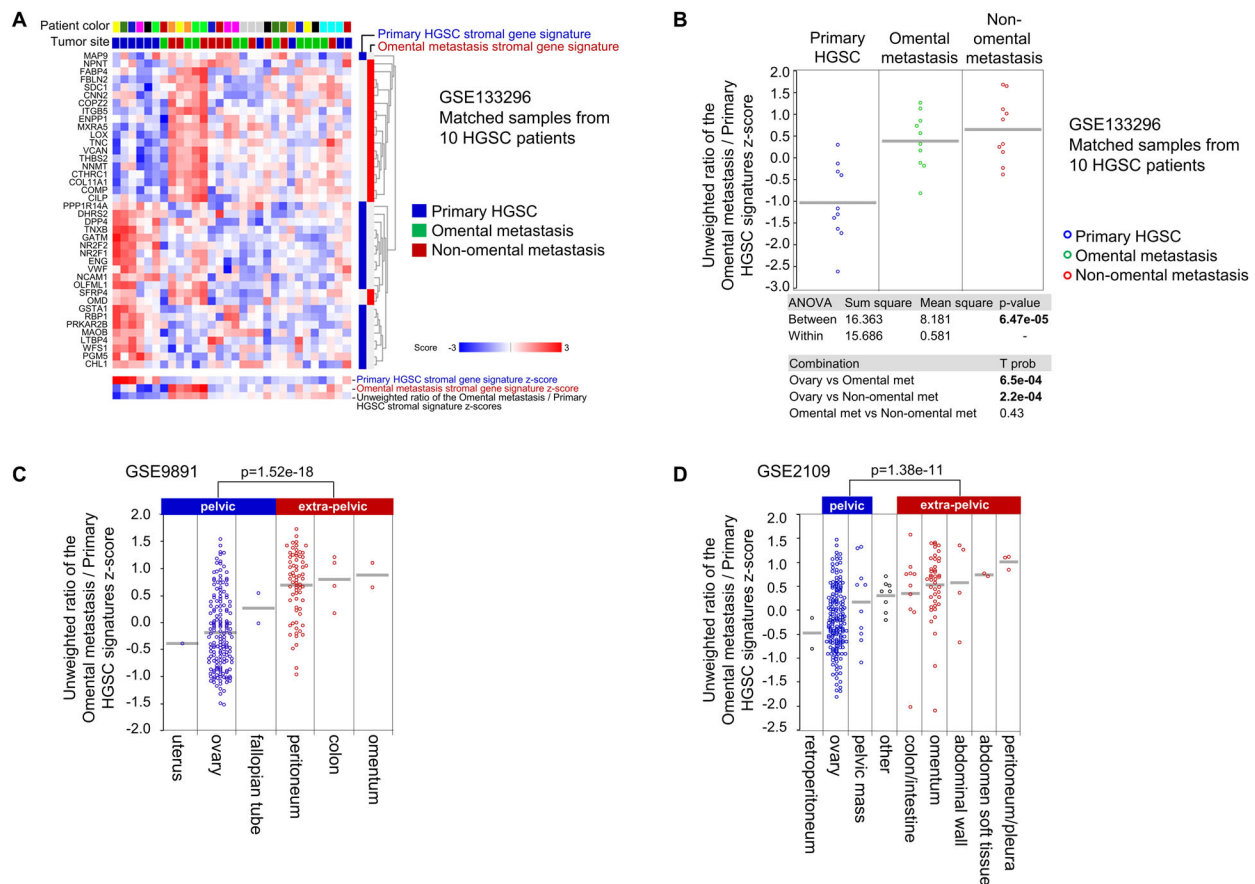


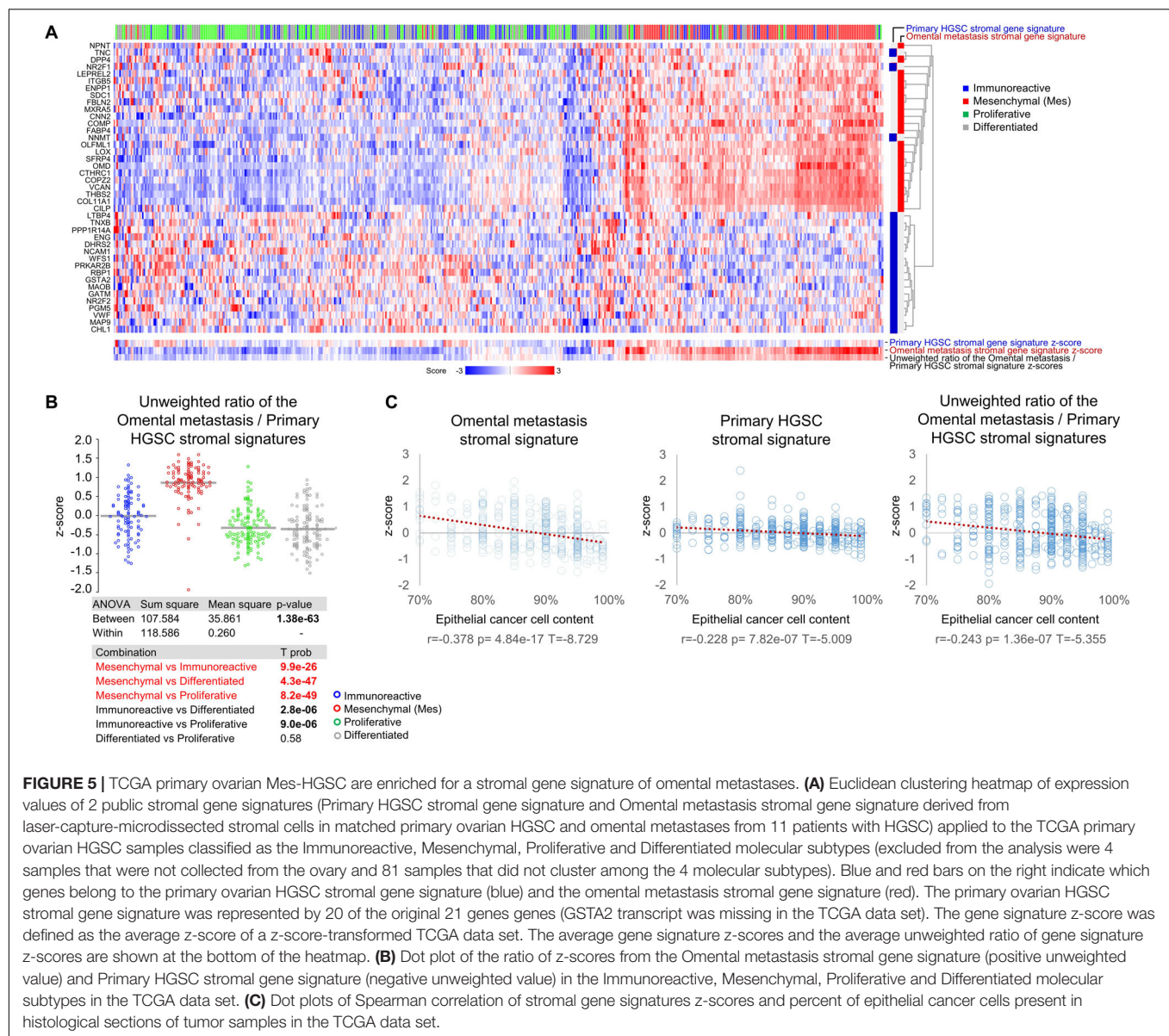
FIGURE 4 | The stroma in HGSC metastases has different molecular features than the stroma in primary ovarian HGSC. **(A)** Euclidean clustering heatmap of expression values of 2 public stromal gene signatures (Primary HGSC stromal gene signature and Omental metastasis stromal gene signature derived from laser-capture-microdissected stromal cells in matched primary ovarian HGSC and mental metastases from 11 patients with HGSC) applied to the GSE133296 transcriptome data set of matched primary ovarian HGSC, omental metastases, and non-omental metastases from 10 HGSC patients. Blue and red bars on the right indicate which genes belong to the primary ovarian HGSC stromal gene signature (blue) and the omental metastasis stromal gene signature (red). Transcripts for GSTA2 (from the original primary ovarian HGSC stromal gene signature) and LPREL2 (from the original omental metastasis stromal gene signature) were missing in the GSE133296 data set. The gene signature z-score was defined as the average z-score of a z-score-transformed GSE133296 data set. The average gene signature z-scores and the average unweighted ratio of the gene signature z-scores are shown at the bottom of the heatmap. **(B–D)** Dot plots of the ratio of z-scores from the Omental metastasis stromal gene signature (positive unweighted value) and Primary HGSC stromal gene signature (negative unweighted value) in panel **(B)** primary ovarian HGSC, omental metastases, and non-omental metastases in the GSE133296 data set; **(C)** different sites of sample collection in the GSE9891 data set (excluded from the analysis were tumors of low malignant potential, non-serous tumors, one bone metastasis, and tumors lacking annotation of the collection site); and **(D)** different sites of sample collection in the GSE2109 data set (included in the analysis were ovarian tumors of all types and histologies that have been annotated by the site of sample collection; some sites have been grouped in this graph; for original annotation, please see **Supplementary Table S6**). The GSTA2 transcript was missing in the GSE9891 and GSE2109 data sets. The bars indicate average ratio of z-scores in each group.

subtype in comparison to the Immunoreactive, Differentiated, and Proliferative molecular subtypes (**Figure 5B**). Since the Immunoreactive and Mesenchymal primary ovarian HGSC subtypes have been shown to contain more stroma (less epithelial cancer cells) than the Differentiated and Proliferative subtypes (Aran et al., 2015; Zhang et al., 2015, 2018; Chen et al., 2018), we considered the possibility that the stromal gene signatures are overexpressed in samples with high stromal content and underexpressed in samples with high epithelial cancer cell content. However, we show little correlation between the stromal gene signatures and epithelial cancer cell content in the TCGA data set (**Figure 5C**), suggesting that the strong enrichment of the average unweighted ratio of the z-scores from Omental

metastasis/Primary HGSC stromal gene signatures in Mes-HGSC (**Figure 5B**) reflects a molecularly different type of stroma rather than an increased presence of the stroma in the Mes subtype. Together, these results indicate that the Mes-HGSC subtype is enriched for cells that exhibit the phenotype of stromal cells in omental metastases rather than stromal cells in primary ovarian HGSC.

DISCUSSION

Molecular profiling studies have identified 4 distinct molecular subtypes of primary ovarian HGSC of which the Mes subtype



has the lowest rate of optimal surgical debulking and the worst overall survival (Tothill et al., 2008; Tan et al., 2013; Verhaak et al., 2013; Konecny et al., 2014; Zhang et al., 2015, 2016; Torres et al., 2017; Wang et al., 2017), and is almost always associated with coexisting upper abdominal/omental metastases (Torres et al., 2017; Wang et al., 2017). It has been shown that cancer-associated stroma and ECM largely contribute to the Mes gene signature (Zhang et al., 2015, 2018; Jia et al., 2016). Additionally, it has been shown that the transcriptome of primary ovarian Mes-HGSC is strongly correlated with the stromal gene signature of omental metastases (Eckert et al., 2019). Considering the phenotypic similarity between primary ovarian Mes-HGSC and peritoneal metastases and the frequent coexistence of primary ovarian Mes-HGSC with upper abdominal/omental metastases, we propose that primary ovarian Mes-HGSC might actually be cancer-stroma aggregates that detached from tumors located in the

upper abdomen/omentum. Indeed, whole-genome and single-nucleus sequencing analyses have demonstrated that metastases are not always unidirectional and that the re-seeding of peritoneal metastasis to the fallopian tube or ovary can occur (Eckert et al., 2016; McPherson et al., 2016). Unfortunately, the gene expression based molecular subtypes of such samples cannot be determined as these studies isolated high purity epithelial tumor cells rather than stromal cells, which frequently contribute to gene expression signatures that define molecular subtypes.

The signature genes in the Mes molecular subtype are frequently referred to in literature as the signature genes of epithelial-mesenchymal transition (EMT) because the same genes can be upregulated in epithelial cells in cell culture upon induction of EMT, i.e., by knockout of E-cadherin (Onder et al., 2008), overexpression of Twist (Yang et al., 2004), and exposure of epithelial cancer cells to TGF β or conditioned media

from cancer-associated fibroblasts (Beach et al., 2016). We have shown that laser-capture-microdissected epithelial cells from primary HGSC express negligible levels of the 15 Mes genes in comparison to laser-capture-microdissected stromal cells. Thus, even if epithelial cancer cells undergo partial EMT, the levels of mesenchymal genes expressed in cancer cells would be dwarfed by the levels of mesenchymal genes expressed in fibroblasts in mixed tumor samples. It has been suggested that one possible source of CAFs are epithelial cancer cells that undergo full EMT and thus are indistinguishable from fibroblasts (Petersen et al., 2003). However, studies in different solid tumors, including breast and ovarian carcinomas, have shown that CAFs typically do not contain genetic mutations present in epithelial cancer cells, suggesting that CAFs do not originate from cancer cells (Qiu et al., 2008; Hosein et al., 2010). While it is possible that a minor fraction of CAFs in the mesenchymal subtype of ovarian cancer arises through EMT, this fraction of CAFs is probably below the detection levels of RNA profiling technologies employed in the data sets (**Supplementary Table S1**) used in our analyses.

Studies in cell co-cultures and in mouse models demonstrated the existence of heterotypic aggregates of cancer cells and stroma, in which stromal cells support epithelial cancer cell survival and guide peritoneal invasion, and can accompany epithelial cancer cells to a new metastatic site and actively reconstitute the tumor stroma in newly formed metastases (Duda et al., 2010; Gao et al., 2019). However, it has been shown that HGSC metastases rarely contain CAFs from primary ovarian HGSC (Gao et al., 2019), suggesting that stromal cells in primary ovarian tumors are not overly efficient in accompanying cancer cells to a new metastatic site and/or are not proficient in re-building the stroma at a new site. It is likely that implantation of cancer cell-stroma aggregates at a new metastatic site requires significant remodeling of the local stroma or recruitment of new stroma. Indeed, stroma in the upper abdominal/omental metastases is frequently enriched for markers of myofibroblasts and ECM remodeling, such as POSTN, COL11A1, LOX, VCAN, TNC, and THBS2 (Gao et al., 2016; Jia et al., 2016; Eckert et al., 2019). It is possible that the upper abdominal/omental metastasis stroma is more efficient than primary ovarian cancer stroma in accompanying metastatic cancer cells and reconstituting the stroma at secondary metastatic sites. For example, omental adipocytes have been shown to promote ovarian cancer metastasis and provide energy for rapid tumor growth (Nieman et al., 2011, 2013). Presently, it would be difficult to design an experimental system for lineage tracing of CAFs to prove our hypothesis in a mouse model. The existing genetically engineered mouse models that develop upper abdominal metastases from the fallopian tube/ovarian lesions are triple or quadruple transgenics; it is not feasible to combine them with additional lines of transgenic mice for lineage tracing. Additionally, since autochthonous ovarian cancer models have been generated relatively recently, it is unknown if the patterns of ovarian cancer dissemination in the mouse peritoneal cavity recapitulate the patterns of intraperitoneal dissemination in women with ovarian cancer.

Our result that the majority of patient-matched metastatic or recurrent HGSC samples were classified as Mes-HGSC

irrespective of the primary cancer subtype is consistent with the results of a recent study in a different cohort of patients (Tan et al., 2018) as well as a study showing that the majority of PPC are classified as the Mes subtype (Gao et al., 2016). According to our hypothesis that the Mes gene signature is a signature of stromal cells in the upper abdominal/omental HGSC, all HGSC in the upper abdomen/omentum should be classified as Mes. Yet in our study, only 90% of PPC and 66% of peritoneal metastases were classified as Mes. It is also expected that all PPC metastases to the ovary are Mes but only 63% were classified as Mes in our study. Multiple technical reasons could explain why some metastases to the ovary did not classify as Mes including imperfections in algorithms that had been used for the Mes subtype classification in the original publications, unknown precise site of sample collection in the peritoneal cavity (pelvis vs upper abdomen/omentum), and/or inclusion of samples that had been annotated as PPC based on the tumor distribution but are actually primary ovarian or fallopian tube HGSC. A biological explanation for the existence of non-Mes metastases in the upper abdomen/omentum could be that metastases are initially associated with accompanying stroma from the primary ovarian HGSC until cancer cells can recruit and/or remodel the stroma at the metastatic site.

Similar to HGSC, colorectal cancer (CRC) has been classified into 4 molecular subtypes (CMS1-4) (Chen et al., 2018). The (Mes) molecular subtype (CMS4) is associated with poor survival (Calon et al., 2015; Guinney et al., 2015). It has been shown that the CMS4 subtype is largely defined by genes expressed in CAFs (Guinney et al., 2015; Dunne et al., 2016; Ubink et al., 2018), challenging the assumption that poor prognosis tumors acquire stem-like characteristics by undergoing widespread EMT. The rationale for classifying tumors by molecular subtypes has been challenged by a study demonstrating that CRC from an individual patient can be simultaneously classified into multiple molecular subtypes based purely on the degree of stromal infiltration in the tumoral region (Dunne et al., 2016). The observed intratumoral heterogeneity was confirmed in the study by Ubink et al. where the CMS4 subtype was defined by qRT-PCR expression of 4 mesenchymal markers (PDGFRA, PDGFRB, PDGFC, and KIT) (Ubink et al., 2017). The issue of intratumoral heterogeneity was somewhat clarified by another study by Ubink et al. in which primary CRC were compared with concurrent peritoneal metastases. In that study, 60% of primary CRC (a higher proportion than in the original CMS classification study (Guinney et al., 2015) and 75% of peritoneal metastases were classified as CMS4. The observed high proportion of the CMS4 subtype in both primary and metastatic CRC is consistent with our data showing that the Mes subtype is determined by the tumor location, which is primarily in the upper abdomen/omentum for both primary and metastatic CRC. Due to differences in the genes used to classify the Mes subtype, we could not directly compare HGSC and CRC classification, however, it is worth noting that expression levels of the Mes 15-gene z-score was higher in primary CRC than in primary HGSC in the TCGA data set (data not shown). Similar to our results in HGSC, CMS4 status of matched primary and metastatic CRC did not correlate with the percentage of stromal cells in the sample,

suggesting that the CMS4 phenotype was determined by the functional differences in the type of stroma (Ubink et al., 2017).

Major limitations of our study are the correlative nature of the data and a small number of samples in some of the data sets used for the analyses. Thus, we cannot completely exclude the possibility that cancer cells in primary ovarian Mes-HGSC are capable of converting the resident ovarian stromal cells into myofibroblasts or recruiting myofibroblast-like stroma to the ovary. However, if this were true, it would be expected that some of the stage I-II primary ovarian HGSC were of the Mes subtype. Of the 37 stage I-II primary ovarian HGSC samples that satisfied our inclusion criteria in the TCGA and GSE9891 data sets, only 1 was classified as Mes and that tumor exhibited features of a highly aggressive malignancy (stage IIc with lympho-vascular invasion and early death from the disease), suggesting the potential presence of malignant ascites containing microscopic cancer cell-stroma aggregates from the upper abdomen/omentum.

Although the main purpose of this study was to present a new perspective in the understanding of intraperitoneal HGSC dissemination, our results have clinical relevance. We suggest that the Mes gene signature in primary ovarian HGSC signifies advanced/high-stage intraperitoneal metastatic dissemination that includes metastasis to the ovary by cancer cell-stroma aggregates from the upper abdomen/omentum. From this perspective, stage III Mes-HGSC could be considered “more advanced” than stage III non-Mes-HGSC. Additionally, our results may be relevant to the future clinical use of molecular subtype biomarkers to triage patients to primary cytoreductive surgery or neoadjuvant chemotherapy. Genes associated with the Mes subtype have been associated with suboptimal debulking and increased postoperative morbidity and mortality (Riester et al., 2014; Tucker et al., 2014; Torres et al., 2017; Wang et al., 2017), suggesting that the Mes subtype could be helpful as a biomarker to triage patients toward neoadjuvant chemotherapy (Orsulic and Karlan, 2019). Some medical centers are already using preoperative biopsy to assess resectability and triage patients for neoadjuvant chemotherapy (Spencer et al., 2010; Nick et al., 2015). Results of the current analysis show that the site of tumor biopsy is important in determining the Mes subtype. Although large omental metastases are most easily accessed (Spencer et al., 2010), they are not reliable for patient stratification by tumor molecular subtype classification because they usually exhibit the Mes subtype. If classification by molecular subtype is to be used to inform clinical management, our findings underscore that biopsies submitted for molecular analysis should be obtained from the ovarian mass, even though it may be more difficult to obtain than an omental biopsy (Nagamine et al., 2017; Rutten et al., 2017).

MATERIALS AND METHODS

Patient Samples and Gene Expression Analyses

Formalin-fixed paraffin-embedded (FFPE) blocks were retrieved from the pathology archives at Cedars-Sinai Medical Center under an approved IRB protocol. FFPE blocks were sectioned

onto uncharged glass slides. One 4 μ m H&E-stained section was used by a pathologist to circle the tumor areas and delineate them from the adjacent normal tissue. Depending on the tumor size, 1–3 unstained 10 μ m sections were macrodissected (removal of non-tumor areas based on the H&E template) with a clean razor blade. Total RNA was isolated using the miRNeasy FFPE kit according to the manufacturer's instructions (Qiagen). For the GSE135712 data set, samples of omental metastases collected from 152 HGSC patients at the time of primary debulking surgery were analyzed for RNA expression of 1,067 genes by NanoString nCounter technology (NanoString Technologies). Data were normalized using nSolver software (NanoString Technologies). In a separate NanoString data set, matched primary, metastatic, and recurrent HGSC samples from 29 patients were analyzed for RNA expression of 15 genes by NanoString nCounter. Five patients were excluded from the analysis due to missing tissue or missing mRNA data for one of the matched tumors. In 4 patients where more than one matched metastatic or recurrent tumor sample was available, one sample was randomly selected for the study. For the GSE133296 data set, matched HGSC samples collected from the ovary, omental metastasis, and non-omental intraperitoneal metastasis from 10 patients at the time of primary debulking surgery were analyzed for RNA expression by RNA sequencing using the SMARTer Stranded Total RNA-Seq Kit v2 on the Illumina HiSeqX platform (MedGenome). Unwanted sequences (non-polyA tailed RNAs from the sample, mitochondrial genome sequences, ribosomal RNAs, transfer RNAs, adapter sequences and others) were removed using Bowtie2 (version 2.2.4). The paired-end reads were aligned to the reference human genome downloaded from the UCSC database (GRCh37/hg19). STAR (2.4.1) aligner was used for read alignment. Reads mapping to ribosomal and mitochondrial genomes were removed before alignment was performed. The raw read counts were estimated using HTSeq-0.6.1. Read count data were normalized using DESeq2.

Expression Data Sets

For the ovarian TCGA data set, level 3 data (gene merged) on the AgilentG4502A_07_3 platform was used for analyses. The GSE2109, GSE9891, GSE39395, GSE40595, and GSE73168 data sets were obtained from the Gene Expression Omnibus (GEO) repository. Raw and normalized data for GSE135712 and GSE133296 were deposited into the GEO archive. Data sets used in this study and their associated publications are listed in **Supplementary Table S1**.

Gene Signatures Distinguishing Mes-HGSC From Non-Mes-HGSC

For the Mes 15-gene signature, matched primary, metastatic, and recurrent FFPE tumor samples from 24 patients with HGSC were profiled with NanoString nCounter for expression of 15 genes (**Supplementary Table S2**) that we previously found to be associated with poor survival in HGSC (Cheon et al., 2014) and/or belonged to the pan-cancer gene signature of activated CAFs (Jia et al., 2016). A threshold for each of the 15 genes was

determined by its median expression level in primary tumors in the data set of 24 patients with paired primary, metastatic and recurrent tumors. For TCGA and GSE9891 data sets, the threshold for each of the 15 genes was determined by median expression levels in all tumors in each corresponding data set. A score of 1 was given if the expression exceeded the threshold, otherwise a score of 0 was given (**Supplementary Tables S3–S5**). Once 15 individual scores corresponding to 15 mesenchymal genes were obtained, they were used to create a Mes score. The Mes score was normalized to a range between 0 and 1, in which 1 indicated Mes-HGSC while all other values indicated non-Mes-HGSC. To test the concordance of the Mes 15-gene signature and the original classification of the Mes molecular subtype in the TCGA and GSE9891 data sets, we calculated Cohen's kappa coefficient between the two classifications. Cohen's kappa coefficient for Mes and non-Mes classifications in the TCGA data set was 0.733. Application of this 15-gene score algorithm to the TCGA data set correctly classified 95 of 104 (91%) samples annotated as the Mes subtype and 314 of 352 (89%) samples annotated as the non-Mes subtype (Immunoreactive, Proliferative or Differentiated) (**Supplementary Table S4**). Cohen's kappa coefficient for Mes and non-Mes classifications in the GSE9891 data set was 0.792. Application of this 15-gene score algorithm to the GSE9891 data set correctly classified 71 of 72 (99%) samples annotated as the Mes/C1 subtype and 94 of 112 (84%) samples annotated as the non-Mes subtype (**Supplementary Table S5**). For the 100-gene set mesenchymal HGSC gene signature, we used the top 100 genes that distinguished the Mes subtype from other subtypes, according to the study by Verhaak et al. (2013; **Supplementary Table S2**). The 21-gene stromal signature of primary ovarian HGSC and the 21-gene stromal signature of omental metastasis have been described (Eckert et al., 2019).

Data Analyses

The R2: Genomic Analysis and Visualization Platform¹ was used for analyses of RNA expression levels and correlation between gene signatures and sample groups in different data sets. The gene signature z-score was defined as the average z-score of a z-score-transformed data set. For digital image data analyses, H&E stained slides were scanned at 20× magnification using Aperio AT Turbo. The image analysis was performed using the QuPath software. The image analysis workflow consisted of cell/nucleus detection, annotation of regions containing 3 different cell types (fibroblast, epithelial cancer cell, immune cell), creating the cell detection classifier, and applying the classifier to all cells in the circled regions of the slide.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human tissues were reviewed and approved by Cedars-Sinai Medical Center IRB Committee.

AUTHOR CONTRIBUTIONS

SO conceived the hypothesis, analyzed data sets and wrote the manuscript. JL reviewed and categorized clinical information. AW reviewed the pathology and selected tissue blocks for mRNA isolation. BT-H isolated RNA from FFPE samples and conducted NanoString nCounter analyses. YH, YR, MH, MR, and ET contributed to data analyses. JM analyzed the NanoString nCounter data for matched primary, metastatic, and recurrent tumor samples. AW and BK provided critical input and contributed to the writing of the manuscript. All authors participated in manuscript revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.00647/full#supplementary-material>

¹<http://hgserver1.amc.nl/cgi-bin/r2/main.cgi>

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Conflict of Interest: SO and BK have a patent “Molecular Signatures of Ovarian Cancer (US010253368 and EU2908913)”.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation with several of the authors YH, BT-H, YR, MH, MR, ET, JM, BY, and SO at time of review.

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ORIGINAL ARTICLE

Prognostic gene expression signature for high-grade serous ovarian cancer

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Background: Median overall survival (OS) for women with high-grade serous ovarian cancer (HGSOC) is ~4 years, yet survival varies widely between patients. There are no well-established, gene expression signatures associated with prognosis. The aim of this study was to develop a robust prognostic signature for OS in patients with HGSOC.

Patients and methods: Expression of 513 genes, selected from a meta-analysis of 1455 tumours and other candidates, was measured using NanoString technology from formalin-fixed paraffin-embedded tumour tissue collected from 3769 women with HGSOC from multiple studies. Elastic net regularization for survival analysis was applied to develop a prognostic model for 5-year OS, trained on 2702 tumours from 15 studies and evaluated on an independent set of 1067 tumours from six studies.

Results: Expression levels of 276 genes were associated with OS (false discovery rate < 0.05) in covariate-adjusted single-gene analyses. The top five genes were *TAP1*, *ZFHX4*, *CXCL9*, *FBN1* and *PTGER3* ($P < 0.001$). The best performing prognostic signature included 101 genes enriched in pathways with treatment implications. Each gain of one standard deviation in the gene expression score conferred a greater than twofold increase in risk of death [hazard ratio (HR) 2.35, 95% confidence interval (CI) 2.02–2.71; $P < 0.001$]. Median survival [HR (95% CI)] by gene expression score quintile was 9.5 (8.3 to –), 5.4 (4.6–7.0), 3.8 (3.3–4.6), 3.2 (2.9–3.7) and 2.3 (2.1–2.6) years.

Conclusion: The OTTA-SPOT (Ovarian Tumor Tissue Analysis consortium - Stratified Prognosis of Ovarian Tumours) gene expression signature may improve risk stratification in clinical trials by identifying patients who are least likely to achieve 5-year survival. The identified novel genes associated with the outcome may also yield opportunities for the development of targeted therapeutic approaches.

Key words: formalin-fixed paraffin-embedded, gene expression, high-grade serous ovarian cancer, overall survival, prognosis

INTRODUCTION

Epithelial ovarian cancer (EOC) causes ~125 000 deaths globally every year, and long-term survival rates have changed little in the past three decades.¹ Approximately 70% of women with EOC are diagnosed with advanced stage disease (stages III/IV), and fewer than 50% will survive more than 5 years.² There are five major EOC histotypes: high-grade serous, low-grade serous, endometrioid, clear cell and mucinous.³ High-grade serous ovarian cancer (HGSOC) comprises about two-thirds of cases, is responsible for most deaths and is characterized by profound genomic and clinical heterogeneity.

The most informative prognostic factors for HGSOC are International Federation of Gynecology and Obstetrics (FIGO) stage, residual disease following debulking surgery,⁴ *BRCA1* or *BRCA2* germline mutation^{5,6} and tumour-infiltrating lymphocyte scores.^{7,8} Patients with HGSOC who carry a loss-of-function germline mutation in *BRCA1* or *BRCA2* have an increased sensitivity to platinum-based chemotherapy and PARP inhibitor treatment^{9,10} and a medium-term survival advantage.⁵ However, the frequent development of drug-resistant disease⁶ limits the effectiveness of current therapies.

Gene expression data have been used to define four tumour molecular subtypes of HGSOC (C1/mesenchymal, C2/immune, C4/differentiated and C5/proliferative).^{11,12}

Using transcriptome-wide data from fresh frozen tissues, The Cancer Genome Atlas (TCGA) project used 215 tumours to identify an overall survival (OS) expression signature of 193 genes that has been validated on three other HGSOC gene expression datasets.¹²

Despite these findings, gene expression biomarkers have not been implemented clinically owing to several important shortcomings. The majority of the individual markers comprising the 193 gene signature were not statistically significant across all studies, suggesting that the signature may not be robust. The sample sizes in other discovery efforts have been too small for robust statistical inference.¹² In addition, previous studies used fresh frozen samples, resulting in logistic and cost barriers to examining large clinically relevant datasets, and translation to the clinical setting.

The aim of this study was to identify a robust and clinic-ready prognostic HGSOC profile that can be applied to formalin-fixed paraffin-embedded (FFPE) tumour tissue.

PATIENTS AND METHODS

Twenty studies provided pretreatment FFPE tumour samples from 4071 women diagnosed with HGSOC (supplementary Table S1, available at *Annals of Oncology* online). All HGSOC cases with available tissue were included. During this period, patients with HGSOC were treated with chemotherapy (carboplatin and paclitaxel) after primary debulking surgery. Study protocols were approved by the respective Institutional Review Board/Ethics Approval Committee for each site (supplementary Table S1, available at *Annals of Oncology* online).

A schematic of the overall study design is shown in Figure 1. There were four main components: gene selection, gene expression assay, development of prognostic gene signature in a training set and validation of prognostic signature in an independent validation set.

Gene selection

Candidate prognostic genes were identified by carrying out an individual participant meta-analysis of six transcriptome-wide microarray studies,^{11–16} which included tumour samples from 1455 participants. Association of gene expression with OS was evaluated by Cox proportional hazards regression adjusted for molecular subtype (supplementary Table S2, available at *Annals of Oncology* online). In total, 200 genes from the meta-analysis, most achieving a permutation-based false discovery rate (FDR)¹⁷ of <0.05, and an additional 313 candidate genes based on the literature and unpublished data were selected (supplementary Tables S3 and S4, and supplementary Figure S1, available at *Annals of Oncology* online; for more details see supplementary Material, available at *Annals of Oncology* online). Five genes, *RPL19*, *ACTB*, *PGK1*, *SDHA* and *POLR1B*, were included as house-keeping genes for normalization.

Gene expression assay in tumour samples from study participants

FFPE tumour samples were processed with the NanoString nCounter technology at three different locations: Vancouver, Los Angeles and Melbourne. A control set of 48 FFPE tumour samples was run at each location and the average intraclass correlation coefficient was 0.987. Approximately 2% of the samples were run in duplicate and the average Spearman's correlation coefficient r_s was 0.995. Single-patient classification methods were used with reference samples to control for batch effects.¹⁸ The data in this publication have been deposited in NCBI's Gene Expression Omnibus¹⁹; GEO Series accession number GSE132342 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132342>). A total of 3329 samples passed quality control of which 3769 had survival data and assessable gene expression for 513 genes. Data can be found in NCBI GEO: Accession numbers GSE132342 and GPL26748.

Overall survival analysis of individual genes

Samples that contributed to the meta-analysis dataset ($n = 211$) were removed from subsequent selected analyses to enforce independence of study samples between the gene selection and final survival analysis. Time-to-event analyses were carried out for OS with right censoring at 10 years and left truncation of prevalent cases. Associations between log-transformed normalized gene expression and survival time were tested using likelihood ratio tests with Cox proportional hazards models adjusted for age, race and stage, and stratified by study. Patients with missing race or stage information were assigned to 'unknown' categories. Age was modelled using a B-spline with a knot at the median age, which yielded a better fit than using knots at quartiles or categorical variables. Stage was dichotomized into early (FIGO stage I/II) and advanced (FIGO stage III/IV). Genes were scaled to have a standard deviation of one, so hazard ratios (HRs) correspond to a change of one standard deviation. A Benjamini–Hochberg FDR of <0.05 was used to identify notable associations. Because the expression of genes can be correlated, an analysis of correlated genes was performed using data from TCGA. Advanced stage ovarian cancer usually has disease spread throughout the abdomen, and therefore sensitivity analyses were performed to assess effects of the anatomical location of tumour samples included in the study by removing observations corresponding to samples known to be extraovarian ($n = 437$).

Prognostic signature development and validation

Studies were initially randomized to training set ($N = 14$) and validation set ($N = 6$). The TRI study was randomized to the validation set, but, because 107 samples were part of the meta-analysis data used for gene selection, the study was split, so those 107 samples were included in the model training dataset. Thus 2702 samples from 15 studies were used for model training and 1067 samples from 6 studies were used for validation (supplementary Table S1, available at *Annals of Oncology* online). In the training set, four

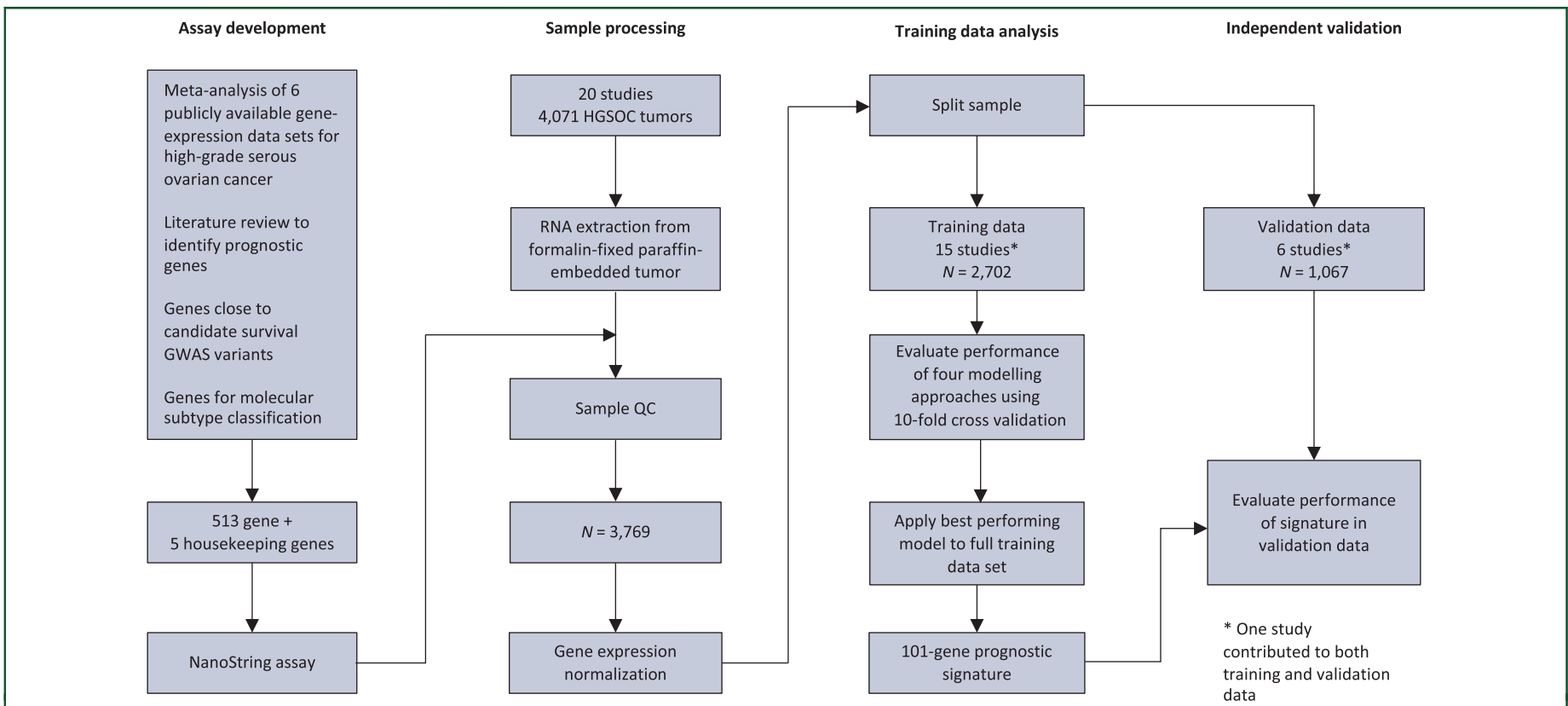


Figure 1. Schematic of study design.

*The TRI study was split across the training and validation sets due to 107 samples overlapping with the meta-analysis. GWAS, genome-wide association studies; HGSOC, high-grade serous ovarian cancer.

modelling approaches (stepwise regression, elastic net regularized regression, boosting and random survival forests) were applied to construct competing gene expression–based biomarkers. Each was evaluated in the training data using 10-fold cross-validation for its prognostic value for OS at 2 and 5 years of follow-up using an area under the curve (AUC) measure derived from receiver operator characteristic analysis (see [supplementary Material](#), available at *Annals of Oncology* online, for additional details). The best performing method, elastic net regularized regression, was applied to the full training set to determine the final gene signature and scoring method, which was then evaluated using the independent testing set. All models were constrained to include age and stage, where age was modelled as categorical based on quartiles of the training dataset with groups aged <53, 53–59, 60–66, and ≥67. Stage was modelled as described earlier for the OS individual gene analysis.

RESULTS

Association of expression of individual genes with OS in HGSOc

In a gene-by-gene analysis of the full dataset adjusted for age, race and stage, and stratified by study, 276 of the 513 selected genes were associated with OS (FDR < 0.05). Of these, 138 were selected from the meta-analysis of six published microarray studies ([supplementary Table S2](#), available at *Annals of Oncology* online)^{11–16} and 144 from candidate gene approaches ([supplementary Tables S5 and S6](#), available at *Annals of Oncology* online). HRs for one standard deviation change in gene expression ranged from 0.84 to 1.19, with multiple genes exhibiting associations at very stringent significance levels (e.g. 19 genes with $P < 1 \times 10^{-8}$; [supplementary Tables S5 and S6](#), available at *Annals of Oncology* online). The five most significant genes were *TAP1*, *ZFHX4*, *CXCL9*, *FBN1* and *PTGER3* ([Table 1](#)). We did not find extensive evidence of high co-expression between these five genes and genes measured in TCGA project ([supplementary Table S7](#), available at *Annals of Oncology* online). In sensitivity analyses we found that excluding samples from omentum and other extra-ovarian sites did not substantially affect the results ([supplementary Tables S8 and S9](#), available at *Annals of Oncology* online).

Development of a novel prognostic gene signature

The four predictive modelling approaches that were evaluated in the training data using 10-fold cross-validation yielded median AUCs that ranged from 0.69 to 0.73 for 2-year OS and 0.69 to 0.74 for 5-year survival ([supplementary Figure S2](#), available at *Annals of Oncology* online) with better prediction of 5-year OS than of 2-year OS. The elastic net approach yielded the highest median AUC for both 2- and 5-year OS and was selected for final development of the signature. Using the model on the full training dataset resulted in a prognostic signature of 101 genes in addition to age and stage ([supplementary Table S10](#), available at *Annals of Oncology* online). Of these, 66 genes were associated with OS (FDR < 0.05) in the single gene models. There was no obvious subset of signature genes that performed as well or nearly as well as the full 101 gene signature ([supplementary Figure S3](#), available at *Annals of Oncology* online).

Performance of the signature including age and stage was AUC 0.69 [95% confidence interval (CI) 0.65–0.73] and AUC 0.75 (95% CI 0.72–0.78) for 2- and 5-year OS, respectively ([Figures 2 and 3](#); [supplementary Figure S4](#), available at *Annals of Oncology* online). This was substantially better than age and stage alone with AUC 0.61 (95% CI 0.57–0.65) and AUC 0.62 (95% CI 0.59–0.67) for 2- and 5-year OS, respectively, particularly for the 5-year OS outcome with non-overlapping 95% CI. One standard deviation change in the gene expression score was associated with an HR of 2.35 [(95% CI 2.02–2.71); $P = 5.1 \times 10^{-31}$], and median survival [HR (CI)] varied substantially across quintiles of the gene expression score [9.5 (8.3 to –), 5.4 (4.6–7.0), 3.8 (3.3–4.6), 3.2 (2.9–3.7) and 2.3 (2.1–2.6) years, respectively, from smallest to largest quintile (Q1–Q5); [Table 2](#)].

For a subset of cases, there was clinical and experimental data for known prognostic factors. All samples had molecular subtype classification (Talhouk et al.²⁰); residual disease was known for 1771 cases, primary chemotherapy treatment for 687, germline *BRCA* mutation status for 904 and nuclear CD8 tumour-infiltrating lymphocyte counts⁸ for 1111 ([supplementary Table S11](#), available at *Annals of Oncology* online). When examined by quintile of gene expression score there were differences, as expected, for each of the known prognostic factors, including age and stage that were included in the model ([Table 3](#)). However, in sensitivity analyses, applying the signature to specific

Table 1. Hazard ratios and 95% CIs for top five prognostic genes in covariate-adjusted single-gene analyses

Gene	HR (95% CI)	P	Selection	Correlated gene ^a	r_s
<i>TAP1</i>	0.84 (0.80–0.87)	8.3×10^{-18}	Meta	PSMB9	0.89
<i>ZFHX4</i>	1.19 (1.14–1.25)	1.4×10^{-15}	Meta	LOC100192378	0.74
<i>CXCL9</i>	0.85 (0.82–0.88)	1.8×10^{-15}	Meta and candidate	CXCR6	0.89
<i>FBN1</i>	1.18 (1.13–1.24)	4.2×10^{-14}	Candidate	SPARC ^b	0.91
<i>PTGER3</i>	1.18 (1.13–1.24)	1.2×10^{-13}	Meta	COL8A1	0.67

CI, confidence interval; HR, hazard ratio.

^a Most correlated gene according to Spearman's rank correlation coefficient, r_s , computed in The Cancer Genome Atlas (TCGA) Ovarian Serous Cystadenocarcinoma RNA-seq dataset.

^b SPARC was included in this project and was less significant.

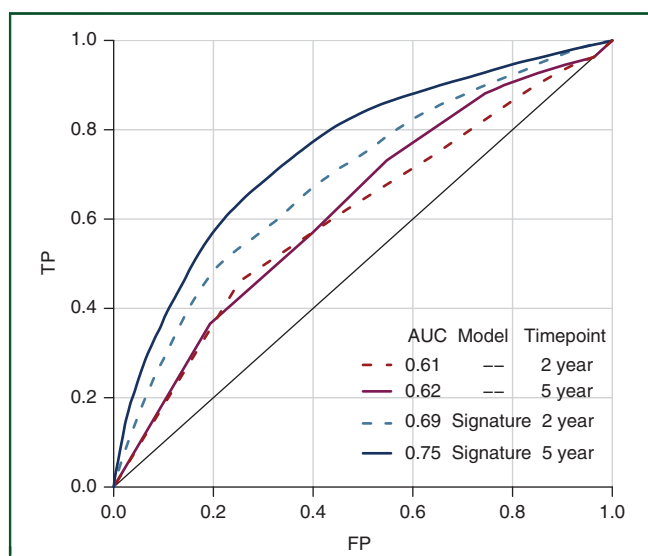


Figure 2. Receiver operator characteristic (ROC) curves for prognostic performance of the gene expression signature in independent high-grade serous ovarian cancer patients (testing data).

There was no overlap between studies or patient data used to develop models (training data) and construct ROC curves and calculate area under the curve (AUC) values shown here (testing data). All models included age and stage as described in Methods section. TP denotes the true positive rate (sensitivity) and FP denotes the false positive rate ($1 - \text{specificity}$).

patient groups, a robustness of stratification was demonstrated, suggesting that the prognostic power of the signature is not explained by the individual factors, residual disease, treatment, *BRCA* status or CD8 score (Figure 3 and supplementary Figures S5–S7, available at *Annals of Oncology* online). The signature score showed modest differences by molecular subtype (supplementary Figure S8, available at *Annals of Oncology* online), and adjusting for molecular subtype in the Cox analysis resulted in only minor changes to the HR estimates for signature quintiles (Table 2). The signature was shown to be prognostic within a homogenous group of 316 stage IIIC cases with no residual disease, within early stage cases (FIGO IA and IB) and within patients whose samples were collected from the omentum (supplementary Figures S9 and S10, available at *Annals of Oncology* online). Analysis of the signature score for paired ovarian and omental tissue from 42 of the cases showed a highly significant Pearson's correlation coefficient, $r = 0.79$ ($P = 5.4 \times 10^{-10}$; supplementary Figure S11, available at *Annals of Oncology* online).

A gene set enrichment analysis was performed for the 101 genes in the signature, as well as for genes correlated with signature genes achieving $r^2 > 0.75$ (supplementary Table S12, available at *Annals of Oncology* online). For the correlated gene analysis, the three most significant pathways involved the immune system, including the adaptive immune system and cytokine signalling. A further 10 immune pathways were significantly enriched and included interferon signalling, innate immune system and TCR signalling and antigen presentation pathways. Restricting to the signature genes only, there was also enrichment in the immune system, but the top two pathways were PI-3K (phosphoinositide 3-kinase) cascade and GPCR (G

protein-coupled receptor) ligand binding. Four other pathways were related to the cell cycle and mitosis, with the remaining enriched for fibroblast growth factor receptor (FGFR) and epidermal growth factor receptor (ERBB) signalling, and one pathway related to homologous combination repair.

DISCUSSION

In a large-scale study of patients with HGSOC, we identified a 101-gene expression signature able to predict clinically relevant differences in OS. Using methods that are both economical and applicable to standard clinical sampling techniques, we showed that the signature performs substantially better than age and stage alone for prognosis of both 2- and 5-year OS. The number of patients and samples included in this study is an order of magnitude greater than previous comparable studies of gene expression and OS in patients with HGSOC.^{12,21,22} Thus, we have been able to more precisely quantify the prognostic value of gene expression.

We report definitive associations between OS and expression of 276 genes. Of the five most significant genes (*TAP1*, *ZFHX4*, *CXCL9*, *FBN1* and *PTGER3*), four have been previously reported to be associated with survival in HGSOC. The top prognostic gene, *TAP1*, is involved in the antigen-presenting pathway. Expression was reduced in metastatic HGSOC, positively associated with OS,²³ as observed here, and linked to tumour regression in response to treatment.²⁴ Further, hypomethylation of *TAP1* was associated with improved time to disease recurrence.²⁵ *CXCL9* is a chemokine that mediates the recruitment of T cells to solid tumours.²⁶ High expression of intratumoural *CXCL9* was associated with higher OS²⁷ and higher lymphocytic infiltration, which is also a robust prognostic factor in HGSOC^{8,11,28} and a feature of the immunoreactive HGSOC molecular subtype.¹¹ *CXCL9* has also been proposed as a therapeutic target due to evidence that it inhibits angiogenesis and promotes antitumour adaptive immunity.^{29–31} Strikingly, the signature was able to further refine prognostic groups within patients with high tumour-infiltrating lymphocyte counts suggesting that *CXCL9* and *TAP1* expression may be strong indicators of immune competency in HGSOC.

FBN1 is an extracellular matrix protein previously found to be a biomarker associated with early recurrence in patients with ovarian cancer who are initially sensitive to chemotherapy³² and strongly correlated with desmoplasia in HGSOC. The prostaglandin E₂ receptor *PTGER3* is expressed in ovarian tumour cells and is associated with relapse-free survival.³³ By contrast, *ZFHX4* does not have previous associations with HGSOC.

Associations between the expression of specific genes in tumour tissues and OS in patients with HGSOC may suggest new drug targets and lead to insights into biological variation in treatment response. For example, cases in the Q5 quintile with the poorest outcome had increased expression of *IGF2*, *FGFR1* and *MYC*, a possible argument for the use of

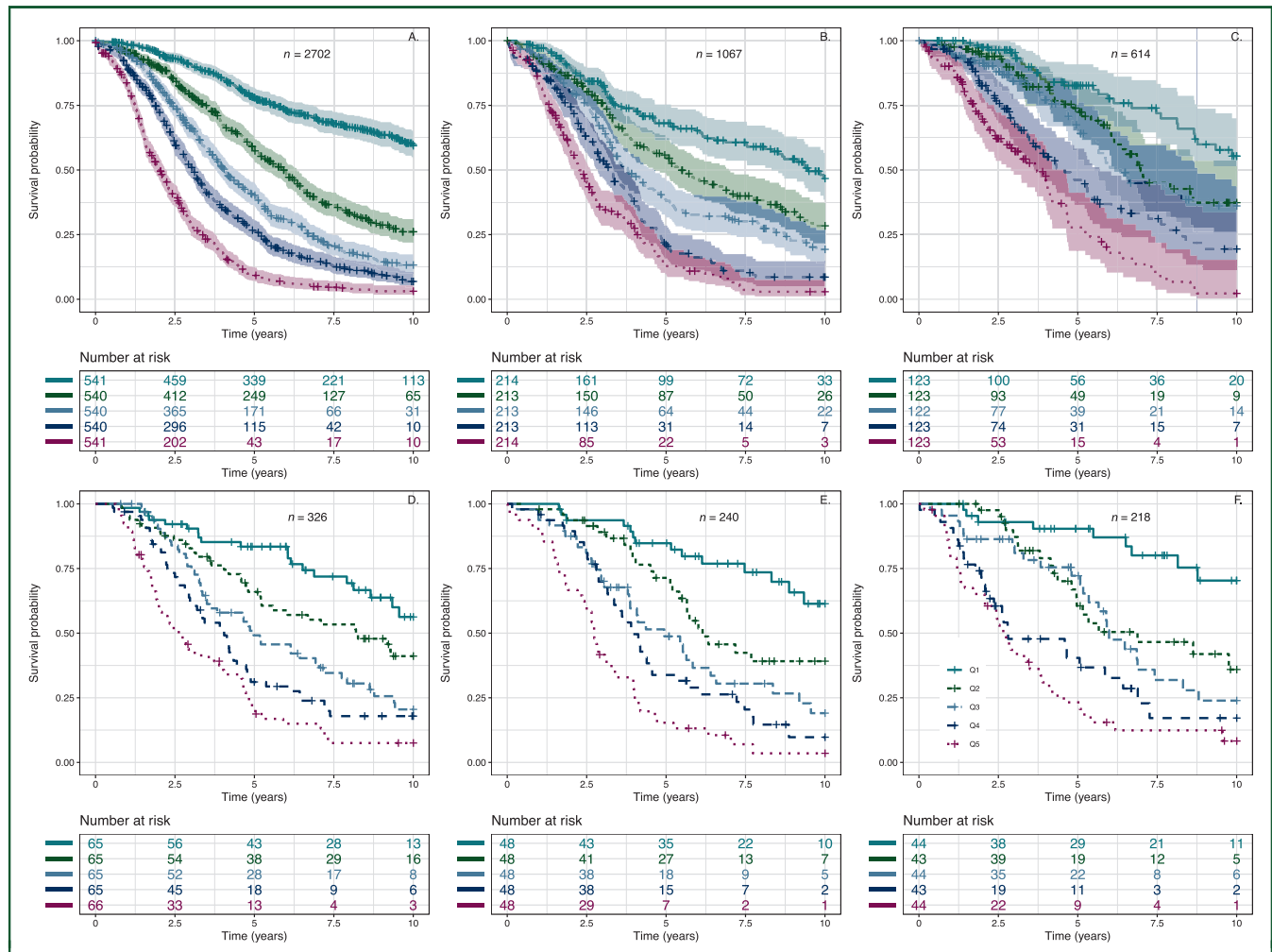


Figure 3. Kaplan-Meier curves of overall survival for patients (A) in the training and (B) testing sets. Patients were assigned to quintiles (Q1–Q5) of the signature score including age and stage. Shaded areas indicate 95% confidence regions, only included for plots representing larger sample sizes. Because of limited sample size, the following plots represent all such patients in the entire dataset, training or testing: (C) no macroscopic residual disease after debulking surgery, (D) primary chemotherapy treatment ≥ 4 cycles of intravenous (IV) carboplatin area under the curve (AUC) 5 or 6 and paclitaxel 135 or 175 mg/m² every 3 weeks (actual dose known or presumed), (E) *BRCA1* or *BRCA2* germline mutation and (F) CD8 > 19.

IGFR1, *FGFR*, bromodomain (*MYC*) or a combination of PARP and CDK4/6 inhibitors (*MYC*).³⁴ More immediately, the signature may help clinicians identify patients most in need of intervention, such as patients that could potentially benefit from neoadjuvant chemotherapy (NACT). Alternatively, in clinical trials it could be used to stratify randomization by patients' risk, thereby reducing heterogeneity within subgroups and increasing heterogeneity between

subgroups. The signature will be incorporated into future prospective clinical trials to determine if it can predict response to specific treatments.

Measurement of the signature required standard FFPE tissue used in routine histopathology. In addition, data preprocessing and normalization were conducted on an individual level, thus translatable to a general patient population. That is, 5-year OS prognosis of future patients

Table 2. Hazard ratios and 95% CIs for quintiles of the gene expression signature score in validation data

Quintile	N	Deaths	Median survival ^a	HR (95% CI)	Adjusted for age and stage	Adjusted for molecular subtype age and stage
					HR (95% CI)	HR (95% CI)
Q1	214	81	9.47 (8.32 to —)	0.44 (0.33–0.58)	0.34 (0.22–0.55)	0.37 (0.23–0.59)
Q2	213	117	5.38 (4.63–6.97)	0.73 (0.57–0.93)	0.71 (0.55–0.91)	0.74 (0.58–0.96)
Q3	213	145	3.80 (3.34–4.60)			
Q4	213	158	3.23 (2.85–3.68)	1.56 (1.25–1.96)	1.56 (1.24–1.97)	1.56 (1.24–1.96)
Q5	214	179	2.27 (2.09–2.62)	2.23 (1.78–2.78)	2.11 (1.67–2.67)	2.07 (1.63–2.61)

CI, confidence interval; HR, hazard ratio.

^a Median survival (95% CI) in years for patients in the validation set.

Table 3. Clinical data for the 3769 patients that passed quality control and the percentage of patients in each quintile of the gene expression score

	Total	Q1	Q2	Q3	Q4	Q5	P value
N	3769	754	754	753	754	754	
Median survival (years)	4.1	9.5	5.4	3.8	3.2	2.3	
% 5-year survival	41	75	57	39	25	10	
Age median	63	58	57	61	64	70	
Age range	25–89	39–78	25–86	36–82	27–89	39–86	
Age quartile Q1	894	30.8	31.3	20.0	13.4	4.5	$<1 \times 10^{-50}$
Age quartile Q2	838	21.5	20.0	22.9	21.2	14.3	
Age quartile Q3	961	16.0	20.2	21.4	23.6	18.7	
Age quartile Q4	1076	13.5	10.4	16.4	21.3	38.5	
FIGO stage I/II	607	97.4	2.6	0.0	0.0	0.0	$<1 \times 10^{-50}$
FIGO stage III/IV	3067	3.8	23.0	24.1	24.4	24.6	
Primary chemo ^a 1	136	16.2	22.1	23.5	19.1	19.1	0.163
Primary chemo ^a 2	190	16.3	20.0	21.6	22.1	20.0	
Primary chemo ^a 3	361	11.1	16.9	22.4	20.5	29.1	
Residual disease: No	614	32.4	22.1	17.8	15.5	12.2	$<1 \times 10^{-50}$
Residual disease: Yes	1157	6.0	19.2	24.1	24.5	26.2	
Germline BRCA1 mutation	130	23.8	31.5	26.2	11.5	6.9	2.22×10^{-7}
Germline BRCA2 mutation	71	28.2	26.8	18.3	18.3	8.5	
Germline no BRCA1/2 mutation	663	19.6	16.7	18.7	20.7	24.3	
CD8 TIL score 0	192	19.8	14.6	12.5	21.4	31.8	2.46×10^{-14}
CD8 TIL score 1–2	186	18.3	14.0	18.8	21.5	27.4	
CD8 TIL score 3–19	515	19.8	24.1	20.8	17.9	17.5	
CD8 TIL score >19	218	34.4	31.2	16.5	11.5	6.4	
Molecular subtype C1.MES	1105	5.4	10.4	20.7	27.4	36.0	$<1 \times 10^{-50}$
Molecular subtype C2.IMM	907	23.2	28.8	21.2	16.2	10.7	
Molecular subtype C4.DIF	1144	32.6	25.5	17.9	12.8	11.2	
Molecular subtype C5.PRO	613	18.1	14.0	20.7	25.8	21.4	
FIGO stage IA and IB	111	96.4	3.6	0.0	0.0	0.0	$<1 \times 10^{-50}$
FIGO stage IIIC	1979	3.1	23.7	24.6	24.1	24.6	$<1 \times 10^{-50}$
FIGO stage IIIC residual disease: No	316	6.3	31.0	24.4	20.9	17.4	6.24×10^{-45}
FIGO stage IIIC residual disease: Yes	846	2.6	21.5	25.3	24.6	26.0	

Q1 is the quintile with the best survival and Q5 the worst survival. Samples with missing data are reported in [supplementary Table S11](#), available at *Annals of Oncology* online. P values for BRCA1/2 mutation status were calculated for BRCA1 or BRCA2 mutation versus no mutation.

FIGO, International Federation of Gynecology and Obstetrics; TIL, tumour-infiltrating lymphocyte.

^a Treatment: 1 = known to have received first-line chemotherapy treatment of ≥ 4 cycles of IV carboplatin AUC 5 or 6 and paclitaxel 135 or 175 mg/m² every 3 weeks. 2 = known to have received first-line chemotherapy treatment of ≥ 4 cycles of IV carboplatin and paclitaxel three times weekly but at doses presumed to be carboplatin AUC 5 or 6 and paclitaxel 135 or 175 mg/m². 3 = all remaining cases with chemo regimens that do not fit criteria 1 or 2 and include unknown or no chemotherapy.

can be evaluated against the patient population reported here by (i) following the same steps described here for generating the normalized gene expression data, (ii) computing an individual signature score and (iii) assigning an HR based on the score or comparing it with the reported quintiles ([supplementary Material](#), available at *Annals of Oncology* online). NanoString gene expression is highly reproducible as seen by our quality control metrics ([supplementary Material](#), available at *Annals of Oncology* online) and the FDA approval of the Prosigna test for breast cancer.

The question of heterogeneity by ancestry or ethnicity was beyond the scope of this study but should be pursued in future research. Another important question is whether molecular subtype can improve biomarker performance. A substantial proportion of signature genes were identified by the subtype-adjusted meta-analysis, suggesting that the strong performance of the signature is not solely attributable to differences among molecular subtypes. In addition, all of the individual genes used in the molecular subtype classification were included in development of the signature.

Although the cases received chemotherapy, the FFPE samples used in this study were chemo-naïve, as few

patients had NACT during the calendar period in which these samples were collected. Because the signature appears to be prognostic in omentum samples, future studies may assess the value in NACT patients, using pretreatment omental biopsies or post-treatment tumour samples. Future work will also address if the signature can predict platinum-refractory patients.

We have developed a robust prognostic signature for HGSOC that can be used to stratify patients and identify those in need of alternative treatments. Gene set enrichment analysis applied to the signature indicates an important role for the immune system in OS and supports further investigation of immune therapy in ovarian cancer. More generally, the identification here of high-confidence prognostic genes may lead to new hypotheses for targeted treatments.

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DISCLOSURE

BYK served on Invitae Corporation's Advisory Board from 2017 to 2018. IAM has acted on the Advisory Boards for AstraZeneca, Clovis Oncology, Tesaro, Carrick Therapeutics and Takeda. His institution receives funding from AstraZeneca. RG is on the Advisory Boards for AstraZeneca, Tesaro, Clovis and Immunogen and does consultancy work for SOTIO. She has received support to attend conferences from AstraZeneca, Roche and Tesaro. Her institution has received research funding from Boehringer Ingelheim and Lilly/Ignyta and she is the national co-ordinating investigator for the UK for trials sponsored by AstraZeneca and Tesaro and site principal investigator for trials sponsored by AstraZeneca, Tesaro, Immunogen, Pfizer, Lilly and Clovis.

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ARTICLE

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Inhibition of collagen XI alpha 1-induced fatty acid oxidation triggers apoptotic cell death in cisplatin-resistant ovarian cancer

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Abstract

Collagen type XI alpha 1 (COL11A1) is a novel biomarker associated with cisplatin resistance in ovarian cancer. However, the mechanisms underlying how COL11A1 confers cisplatin resistance in ovarian cancer are poorly understood. We identified that fatty acid β -oxidation (FAO) is upregulated by COL11A1 in ovarian cancer cells and that COL11A1-driven cisplatin resistance can be abrogated by inhibition of FAO. Furthermore, our results demonstrate that COL11A1 also enhances the expression of proteins involved in fatty acid synthesis. Interestingly, COL11A1-induced upregulation of fatty acid synthesis and FAO is modulated by the same signaling molecules. We identified that binding of COL11A1 to its receptors, $\alpha 1\beta 1$ integrin and discoidin domain receptor 2 (DDR2), activates Src-Akt-AMPK signaling to increase the expression of both fatty acid synthesis and oxidation enzymes, although DDR2 seems to be the predominant receptor. Inhibition of fatty acid synthesis downregulates FAO despite the presence of COL11A1, suggesting that fatty acid synthesis might be a driver of FAO in ovarian cancer cells. Taken together, our results suggest that COL11A1 upregulates fatty acid metabolism in ovarian cancer cells in a DDR2-Src-Akt-AMPK dependent manner. Therefore, we propose that blocking FAO might serve as a promising therapeutic target to treat ovarian cancer, particularly cisplatin-resistant recurrent ovarian cancers which typically express high levels of COL11A1.

Introduction

The Warburg effect describes a phenomenon where cancer cells rely on glycolysis for their energy needs even in the presence of oxygen¹. However, increasing evidence suggests that other metabolic pathways also play important roles in supplying energy and biomass to cancer cells^{2–4}. For example, rapidly proliferating cancer cells rely on large amounts of fatty acids to support various biological processes including membrane formation and signaling. Thus, aberrant fatty acid metabolism has been

implicated in driving malignancy of several cancers, such as breast, prostate, leukemia, and ovarian cancer^{5–9}. Overexpression of fatty acid synthase (FASN), a key fatty acid synthesis enzyme, has also been reported in several cancer types and associated with poor prognosis and resistance to chemotherapy^{8–16}. In addition to synthesis, mitochondrial fatty acid oxidation (FAO) seems to be important for maintaining cancer cell survival. FAO breaks down fatty acids to produce excess ATP and NADPH to support cell survival. FAO is initiated by the conversion of the long-chain fatty acids into fatty acyl-CoAs by the action of ACSL1 enzyme followed by transport into the inner mitochondrial membrane through the activity of carnitine palmitoyl transferases, CPT1 and CPT2. In the mitochondria, FAO is characterized by a series of breakdown reactions catalyzed by

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four major enzymes encoded by ACADM, ECHS1, HADH/HADHA/HADHB, and ACAA2, which results in the generation of acetyl-CoA, NADH, and FADH₂. Recent studies have shown that blocking of FAO inhibits tumor cell proliferation and induces apoptosis in leukemia, myeloma, glioma, glioblastoma, prostate, breast, and ovarian cancer^{6,17–22}. Overall, fatty acid metabolism is particularly important for ovarian cancer cells as they frequently disseminate to fat-rich omentum and uptake fatty acids for their growth and survival^{23,24}. However, the molecular mechanisms by which ovarian cancer cells switch their metabolic phenotype to promote fatty acid metabolism and chemotherapy resistance are largely unknown.

Collagens are the major structural component of the tumor microenvironment and have emerged as an important contributor to cancer cell chemoresistance. Collagen type XI alpha 1 (COL11A1), a minor fibrillar collagen crucial for skeletal development and collagen fiber assembly, is a novel biomarker associated with poor survival and chemoresistance in several cancer types including ovarian cancer^{25–29}. COL11A1 expression is increased during ovarian cancer progression with the highest expression in cisplatin-resistant recurrent tumors²⁷. COL11A1 is expressed and secreted by a subset of cancer-associated fibroblasts (CAFs) adjacent to tumor cells and a small number of cancer cells including A2780cis cisplatin-resistant ovarian cancer cell line^{25–27,30}. We have previously shown that COL11A1 confers cisplatin resistance by engaging $\alpha 1\beta 1$ integrin and Discoidin domain receptor 2 (DDR2) on ovarian cancer cells to activate c-Src-Akt-NF κ B signaling to induce inhibitor of apoptosis proteins (IAPs)³¹. Here, we report another mechanism by which COL11A1 confers cisplatin resistance by regulating ovarian cancer cell metabolism. We show that COL11A1 upregulates both fatty acid synthesis and oxidation predominantly through DDR2-Src-Akt-AMPK dependent signaling to inhibit cisplatin-induced apoptosis in ovarian cancer cells. Our results provide novel therapeutic strategies to treat cisplatin-resistant recurrent ovarian cancers which typically express high levels of COL11A1.

Materials and methods

Cell lines

ES2 and OVCAR3 ovarian cancer cell lines and A204 cell line were purchased from ATCC. A2780 and A2780cis ovarian cancer cell lines were purchased from SIGMA. Lenti-X 293T cells were purchased from Clontech. Human CAFs were a generous gift from Dr. Nikki Cheng (The University of Kansas, Lawrence, USA). A204, CAFs, ES2, and Lenti-X 293T cell lines were cultured in DMEM (Gibco Life Technologies) supplemented with 10% FBS (Sigma-Aldrich) and 1 $\times$ penicillin/streptomycin

(Gibco Life Technologies). OVCAR3, A2780, and A2780cis cell lines were cultured in RPMI (Gibco Life Technologies) supplemented with 10% FBS, 1 $\times$ penicillin/streptomycin. All cells were cultured at 37 °C in 5% CO₂. All cell lines were authenticated and confirmed to be mycoplasma free.

Cell culture

Cancer cells were serum starved overnight prior to the experiments. Tissue culture plates were coated with 5 μ g/ml of type I collagen (COL1; Gibco Life Technologies) or COL11A1 extracted from A204-scrambled cells or A204 shCOL11A1 cells³¹ (Fig. S1B) for 2 h at 37 °C. The A204 cell line is a rhabdomyosarcoma cell line that has been previously shown to express and secrete high levels of COL11A1 and very low levels of COL5A2³². Cancer cells were trypsinized, resuspended in 1% FBS containing medium and cultured in collagen-coated plates for 72 h before the analysis. For co-culture, CAFs were seeded in 6-well inserts (Falcon #353090) and ovarian cancer cells were plated in the companion 6-well plates (Falcon #353502) in media supplemented with 1% FBS for 72 h. CAFs have been shown to express high levels of COL11A1 and co-culture with these CAFs increased cisplatin resistance in ovarian cancer cell lines^{25,27,31}. For drug treatments, cancer cells were serum starved and plated on COL11A1 extract for 48 h in media containing 1% FBS and treated with drugs (dasatinib, LY294002, MK2206 and dorsomorphin) for another 48 h. For cisplatin treatment, cancer cells were serum starved and cultured on uncoated plates, COL11A1-positive extract or COL11A1-negative extract in media containing 1% FBS for 24 h and treated with cisplatin for another 72 h.

Cell viability assays

Cell viability was measured by CellTiter-Glo luminescent cell viability assay (Promega # G7572) using a Glo-max Explorer Microplate Reader (Promega), according to the manufacturer's protocols. For the propidium iodide (PI) staining, cancer cells were collected, washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 1% FBS, 50 μ g/ml PI (Sigma-Aldrich #P4170) and 10 μ g/ml RNase A (Omega bio-tek #AC118). Flow cytometry analysis was performed using Becton Dickinson DXP flow cytometer and the data were analyzed by FlowJo software version 7.5 (Tree Star Inc., Ashland, OR, USA).

Lentiviral shRNA knockdown

Stable gene knockdown was performed using short hairpin RNAs (shRNAs) as described previously³¹. pCMV- Δ R 8.2 (Addgene #8455), pCMV-VSVG (Addgene #8454), and a lentiviral construct containing shRNA (Supplementary Table 1) were co-transfected into Lenti-X

293T cells using Lipofectamine 2000 (Life technologies) according to manufacturer's protocol. Lentivirus-containing media was harvested and filtered through a 0.45 µm polyvinylidene fluoride (PVDF) low protein-binding membrane filter (Celltreat) and mixed with polybrene (8 µg/ml). Cancer cells were incubated in the lentivirus-containing medium for 72 h at 37 °C in 5% CO₂ and then replaced with complete growth media. Transduced cells were selected by puromycin (5 µg/ml) treatment for 72 h and gene knockdown in these cells was confirmed by Western blotting.

Quantitative proteomic analysis

The tandem mass tag (TMT) labeling and liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis were performed by the Cedars-Sinai Mass Spectrometry and Biomarker Discovery core as described³³. Briefly, proteins were extracted from cell pellets using lysis buffer containing 80 mM Tris–HCl, 4% sodium dodecyl sulfate, 100 mM DTT, pH 7.4, alkylated with iodoacetamide and digested with trypsin using the filter-aided sample preparation method³⁴. Samples were then labeled with the TMT6plex Reagents (Thermo Scientific), mixed, and fractionated using the poly styrenedivinylbenzene reverse phase sulfonate approach³⁵. Peptide fractions were separated using a 50 cm EASY-Spray analytical column on an EASY nLC 1000 ultra-performance LC system and analyzed by an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific). Mass spectra were acquired in a data-dependent manner, selecting up to 15 most abundant precursor ions for higher-energy collision dissociation. Database searching was performed using Proteome Discoverer (v1.4) using the SEQUEST algorithm. Searching parameters were as follows: trypsin (Full); up to two missed cleavage; precursor ion tolerance of 10 ppm; fragment ion tolerance of 0.02 Da; carbamidomethylation of cysteines and TMT6plex modification of lysines and peptide N-term as fixed modifications; acetylation of protein N-term, oxidation of methionines and deamidation of asparagines and glutamines as variable modifications. A stringent 1% false-discovery rate (FDR) was set to filter peptide and protein identifications. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE³⁶ partner repository with the dataset identifier PXD016244. Differentially expressed proteins (DEPs) were identified based on the criterion of log₂-transformed fold changes >0.5 in both replicates. Ingenuity Pathway Analysis (IPA) was applied to identify significantly changed biological pathways.

qRT-PCR and Immunoblotting

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen) and 1 µg of total RNA was reverse

transcribed into cDNA using the Quantitect Reverse Transcription Kit (Qiagen). Fifty nanograms of cDNA was combined with primers and iQSYBR-Green Supermix (BioRad). The quantitative real-time polymerase chain reaction (qRT-PCR) reaction was performed using a CFX96 RT-PCR detection system (BioRad) and the data were analyzed by the $2^{-\Delta\Delta C(T)}$ method. Primer sequences used for these experiments are listed in Supplementary Table 3.

For Western blotting, total protein was extracted from cells using RIPA cell lysis buffer (SIGMA) containing protease and phosphatase inhibitors (Roche). Total protein was quantified by a BCA assay kit (Pierce). Equal amounts of protein were loaded for each sample and resolved on a 4–20% Mini-PROTEAN[®] TGX[™] Protein Gel (BioRad). Proteins were transferred onto PVDF membranes and probed with antibodies listed in Supplementary Table 2.

Seahorse XF FAO and glycolysis assays

The Seahorse XF FAO assay and the XF glycolysis stress assay were performed using a Seahorse XFP analyzer (Agilent) according to the manufacturer's protocols. Briefly, 50,000 ovarian cancer cells were cultured in COL11A1-coated (for ES2) or uncoated (for A2780cis) XFP cell culture plates in serum-free media overnight. For the FAO assay, the cells were supplemented with palmitate followed by sequential injections of oligomycin (1 µM) at 20 min, phenylhydrazine (FCCP; 0.125 µM) at 30 min and rotenone/antimycin (0.5 µM) at 60 min after palmitate addition and the real-time oxygen consumption rate (OCR) was measured at different time points. To measure FAO after CD36 blockade, cells were incubated with IgA isotype control or 2 µg/ml CD36 blocking antibody³⁷ (# AB23680, Abcam) overnight followed by washing to remove excess antibody. Next, oligomycin (1 µM), FCCP (0.125 µM), and rotenone/antimycin (0.5 µM) were sequentially injected and OCR is measured over time. For the glycolysis stress assay, the cells were incubated in medium without glucose or pyruvate, followed by sequential injections of glucose (10 mM), oligomycin (1 µM), and 2-deoxy glucose (2-DG; 50 mM) and the extracellular acidification rate (ECAR) was measured at different time points.

Measurement of cellular NADH and ATP

The NAD/NADH-Glo[™] Assay and Cell Titer-Glo assay (Promega) were performed to measure the levels of cellular NADH and ATP respectively, according to the manufacturer's instructions. Briefly, 5000 ES2 cells were cultured in COL11A1-coated plates or PBS-coated plates for 48 h and NAD/NADH-Glo[™] or CellTiter-Glo detection reagent was added to each well in 1:1 ratio. Luminescence was recorded over time using a Glomax

Explorer Microplate Reader (Promega) and was normalized to total protein content.

Patient data analysis

Kaplan–Meier plots were generated by the KM plotter (www.kmplot.com) using the overall survival data of 1207 ovarian cancer patients expressing high or low mean expression of mitochondrial FAO genes (CPT1A, HADHA, and ACAA2). The protein expression of CPT1A in normal ovaries and ovarian cancer was extracted from the Human Protein Atlas. Tissue microarray (TMA) and corresponding clinical data were obtained from Dr. Sandra Orsulic under an approved IRB protocol (Cedars-Sinai Medical Center). The clinical characteristics of 21 patient tumors used in TMA sections are shown in Supplementary table 5. COL11A1 in situ hybridization was performed as described²⁵. We have previously shown high concordance between COL11A1 staining by in situ hybridization and immunohistochemistry and determined that in situ hybridization is more reliable in pre-cut slides²⁷. Immunostaining with a CPT1A antibody was performed by the histology core (Albany Medical College) and visualized using DAB (3,3'-Diaminobenzidine) as a chromogen. Nanozoomer (Hamamatsu) was used to acquire complete images of stained TMA and analyzed in a blinded fashion by at least three independent investigators. Kaplan–Meier curves of ovarian cancer patients with increased CPT1A expression during recurrence were plotted using GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, CA, USA).

Statistical analysis

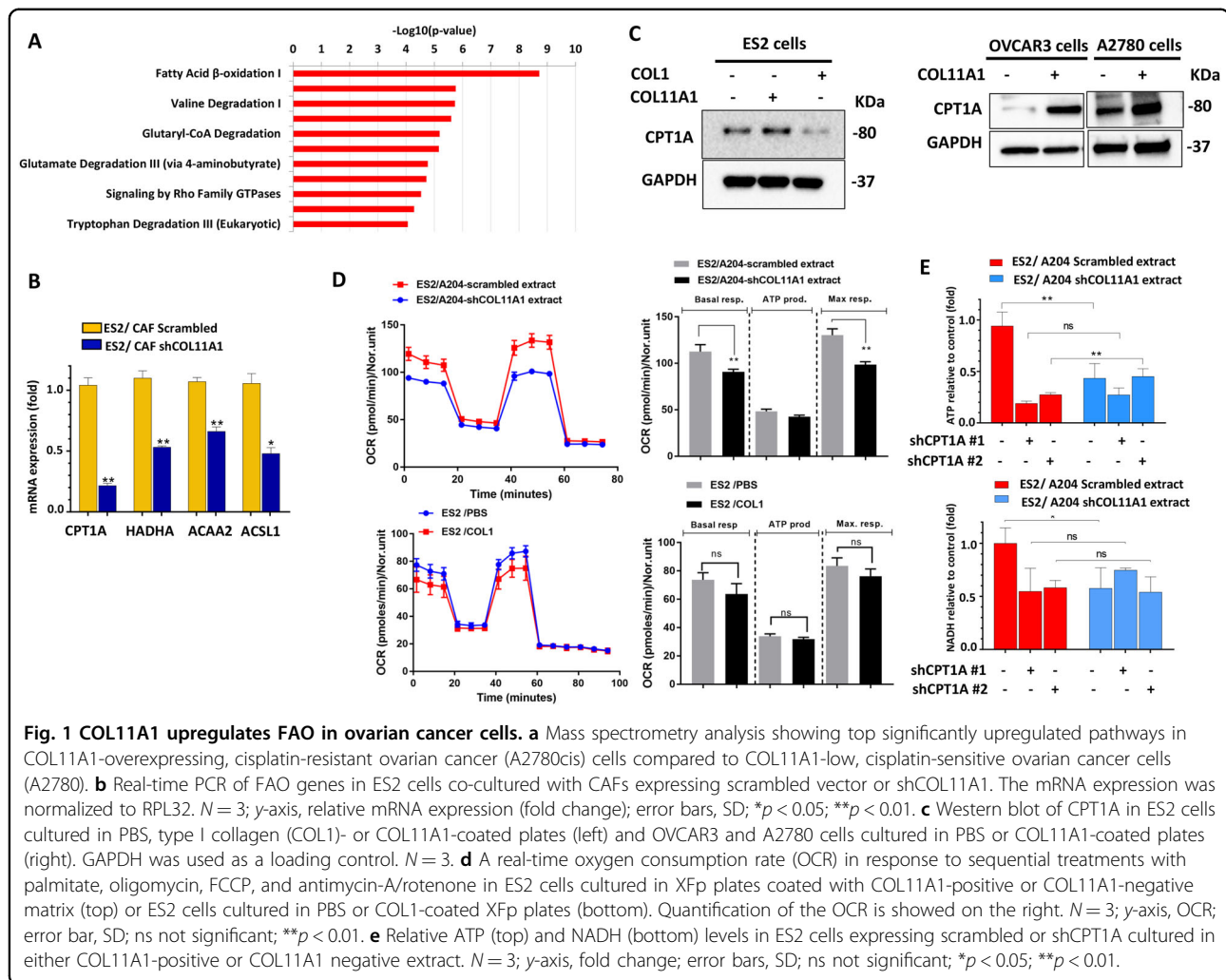
Statistical analyses were performed using GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA was applied to compare the mean difference of two or more groups. Mantel Cox (log rank) test was used to compare significance between survival curves. $P \leq 0.05$ was considered significant.

Results

COL11A1 induces FAO in ovarian cancer cells

To obtain mechanistic insight underlying COL11A1-mediated cisplatin resistance, we performed TMT-based quantitative proteomic analysis to compare the protein expression profiles of A2780 ovarian cancer cell line and A2780cis, a cisplatin-resistant variant of A2780 cell line which expresses high levels of COL11A1. Stringent analysis of this data with a FDR of less than 1% revealed 477 DEPs out of which 247 proteins were upregulated and 230 proteins were downregulated in A2780cis cells compared to A2780 cells (Supplementary Table 4). To identify major pathways that might contribute to COL11A1-mediated cisplatin resistance, we performed IPA on the proteins that were significantly upregulated in A2780cis cells.

Interestingly, proteins of the FAO pathway were predominantly overexpressed in A2780cis cells (Fig. 1a, S1A) compared to A2780 cells. To investigate whether COL11A1 directly upregulates the expression of FAO enzymes in ES2, OVCAR3, and A2780 ovarian cancer cells that do not express COL11A1 or express very low endogenous levels of COL11A1³¹, we (i) co-cultured ES2 ovarian cancer cell line with scrambled control CAFs or COL11A1-knockdown CAFs; (ii) cultured ES2, OVCAR3 and A2780 ovarian cancer cell lines in plates coated with COL11A1 extracted from A204 cell line (COL11A1 extract); or (iii) supplemented ES2 cells with recombinant COL11A1 protein. We measured the expression levels of COL11A1 and FAO enzymes in ovarian cancer cells by RT-PCR and Western blotting. Culturing COL11A1-low cells in the presence of COL11A1 extract or CAFs did not induce COL11A1 expression in the cancer cells (Fig. S1C). The mRNA expression of FAO enzymes was increased in ES2 cells co-cultured with scrambled CAFs (Fig. 1B) or supplemented with recombinant COL11A1 protein (Fig. S1D). In addition, the protein expression of carnitine palmitoyl transferase 1 (CPT1A), the rate-limiting enzyme of FAO, was upregulated in ES2 (Fig. 1C, left), OVCAR3, and A2780 cells (Fig. 1C, right) cultured in COL11A1-coated plates. In contrast, knockdown of COL11A1 reduced the mRNA expression of FAO enzymes CPT1A, ACSL1, ACAA2, and HADHA in A2780cis cells (Fig. S1E, F). To further validate whether COL11A1 increases the FAO rate in ovarian cancer cells, we supplemented a long-chain fatty acid substrate, palmitate, to ES2 cells cultured on COL11A1 extract, and measured OCR using the Seahorse XF analyzer. ES2 cells grown on COL11A1-positive extract (A204-scrambled extract) showed increased OCR after palmitate addition (indicative of increased FAO rate) compared to ES2 cells grown on COL11A1-negative extract (A204-shCOL11A1 extract) (Fig. 1D, top). In contrast, knockdown of COL11A1 resulted in decreased FAO rate in A2780cis cells (Fig. S2D). COL11A1 also increased the levels of ATP and NADH, byproducts of FAO, in ES2 cells cultured on COL11A1 extract (Fig. 1E), which was attenuated by CPT1A knockdown using shRNAs (Fig. 1E, S1F–G) or treatment with a pharmacological inhibitor of CPT1A, etomoxir (Fig. S2A, B). Similarly, etomoxir treatment significantly reduced COL11A1-induced NADH levels in OVCAR3 cells (Fig. S2C), suggesting that COL11A1 increases ATP and NADPH production through FAO in ovarian cancer cells. Interestingly, the upregulation of FAO enzyme expression or FAO rate was not observed in ES2 cells cultured in type I collagen (COL1)-coated plates (Fig. 1C, D, bottom), implying that FAO upregulation might be specific to COL11A1. Of note, the levels of glycolysis, measured by ECAR after glucose addition, were not altered in ES2 cells cultured on COL11A1 extract



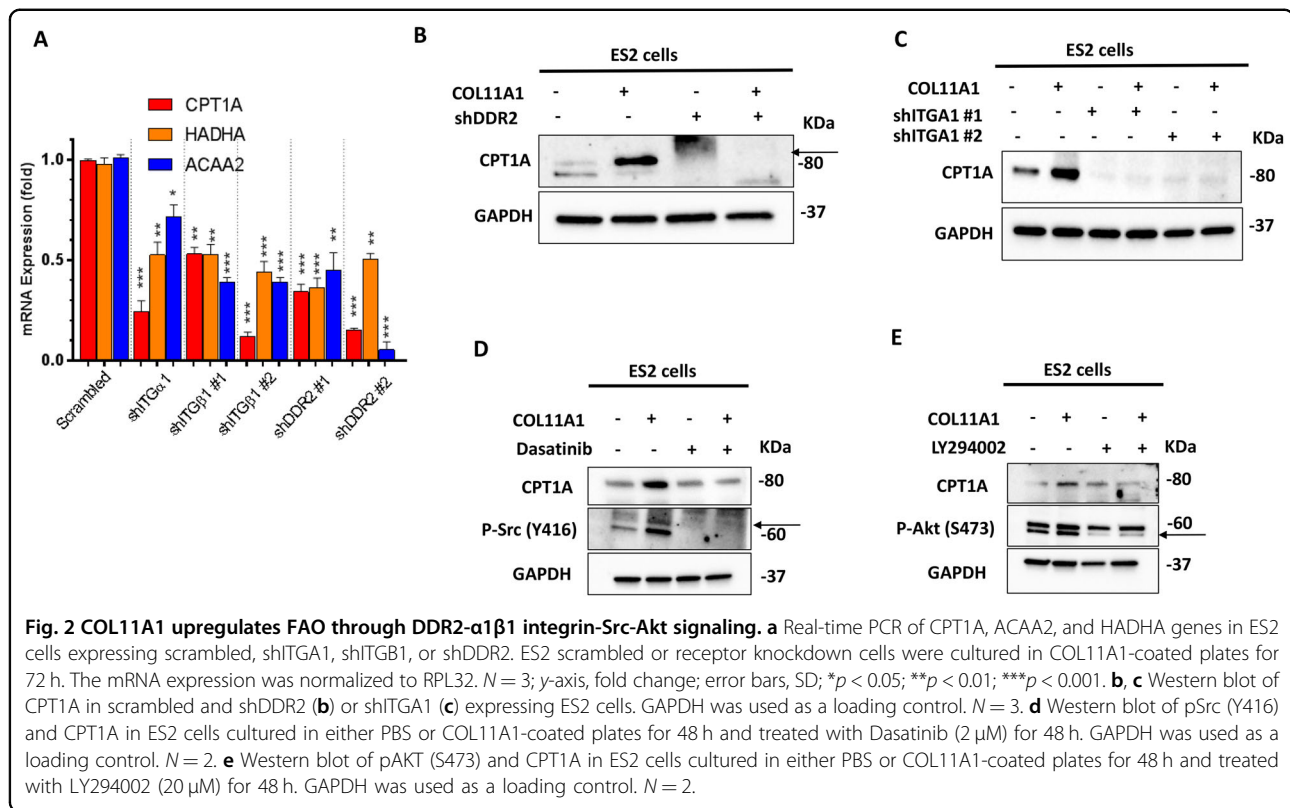
(Fig. S2E) or A2780cis cells with or without COL11A1 expression (Fig. S2F). Taken together, these results suggest that COL11A1, but not type I collagen, upregulates the FAO process in ovarian cancer cells.

COL11A1 upregulates FAO through the activation of DDR2/ α 1 β 1 integrin-Src-Akt-AMPK signaling in ovarian cancer

We previously published that COL11A1 engages DDR2 receptor tyrosine kinase and α 1 β 1 integrin on cell surface to transduce downstream signaling in ovarian cancer cells³¹. Therefore, we tested whether COL11A1 engages the same receptors to upregulate FAO in ovarian cancer cells. To address this question, we knocked down DDR2, ITGA1, and ITGB1 in ES2 cells using shRNAs (Fig. S3A–C), cultured them in COL11A1-coated plates and measured the expression levels of FAO enzymes. Knockdown of DDR2, ITGA1, or ITGB1 significantly decreased the expression levels of FAO enzymes in ES2 cells (Fig. 2A–C) and A2780 cells (Fig. S3D–G) despite

the presence of COL11A1. This result suggests that COL11A1 engages DDR2 and α 1 β 1 integrin to induce FAO in ovarian cancer cells.

Since we have shown that Src and Akt are activated by COL11A1 binding to DDR2 and α 1 β 1 integrin³¹, we next determined whether activation of Src and Akt is necessary for the COL11A1-induced FAO upregulation in ovarian cancer cells. As expected, in ES2 cells, COL11A1 markedly increased the phosphorylation of Src and Akt (indicative of Src and Akt activation) as early as 2.5 h (Fig. S4A, C) which was retained even after 96 h of culture on COL11A1 (Fig. 2D, E). When we blocked Src activation using a pharmacological inhibitor dasatinib, it significantly reduced the expression of CPT1A even in the presence of COL11A1 in ES2, OVCAR3, and A2780 cells (Fig. 2d, S4D, E). Similarly, when we blocked Akt activation using pharmacological inhibitors LY294002 or MK2206, it significantly reduced the expression of CPT1A even in the presence of COL11A1 in ES2 and A2780 cells (Fig. 2e, S4F) and OVCAR3 cells (Fig. S4G). Collectively,



these results suggest that COL11A1 activates Src and Akt to upregulate FAO in ovarian cancer cells.

5' AMP-activated protein kinase (AMPK) plays a central role in maintaining energy homeostasis by upregulating FAO³⁸. Therefore, we questioned whether AMPK is activated by COL11A1 to upregulate FAO in ovarian cancer cells. We demonstrated that phosphorylation of AMPK (indicative of AMPK activation) was increased by COL11A1 as early as 2.5 h and was retained until 96 h in ovarian cancer cells. (Fig. 3a–h, S3B). In addition, using a Human Protein Kinase Array, we demonstrated that AMPK was one of the phosphoproteins upregulated in response to culturing ovarian cancer cells on COL11A1. (Fig. S4H). Treating ES2 and OVCAR3 cells with an AMPK inhibitor dorsomorphin (DM) abrogated COL11A1-dependent increase in AMPK phosphorylation and CPT1A expression (Fig. 3a, b). Furthermore, knocking down AMPK expression in ES2 cells using shRNAs also resulted in loss of FAO enzyme expression (Fig. S4I) suggesting that AMPK acts as an upstream regulator of FAO.

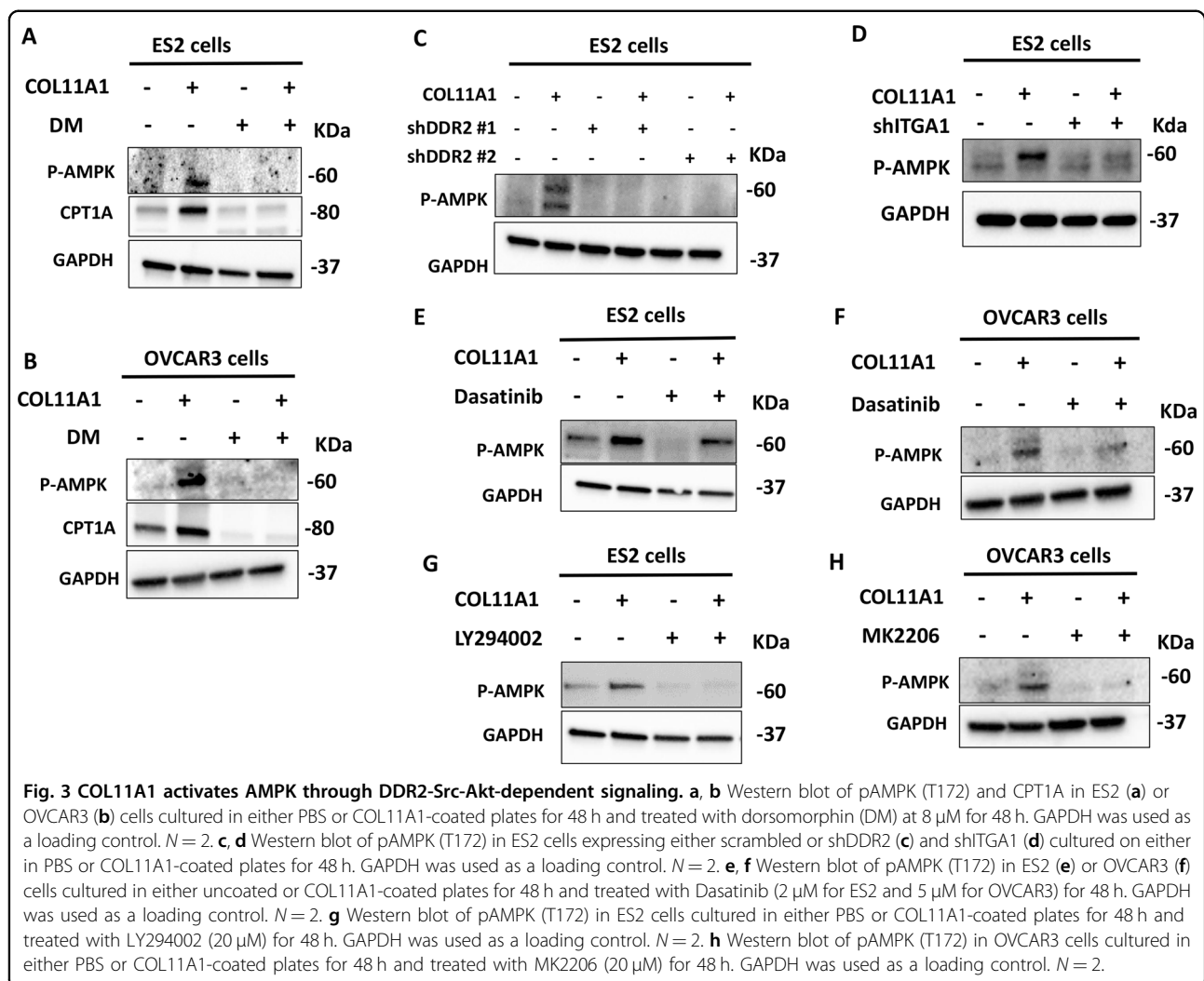
We next questioned whether DDR2- α 1 β 1 integrin-Src-Akt signaling is important for COL11A1-induced AMPK activation. Knockdown of DDR2 dramatically abrogated COL11A1-induced AMPK phosphorylation (Fig. 3c), while knockdown of ITGA1 yielded a moderate reduction in AMPK phosphorylation (Fig. 3d), suggesting that

DDR2 might be the predominant receptor that mediates COL11A1-induced AMPK activation. Treatment of ES2 and OVCAR3 cells with Src and Akt inhibitors also significantly attenuated AMPK phosphorylation despite the presence of COL11A1 (Fig. 3e–h). Taken together, these results suggest that AMPK acts as a downstream effector of COL11A1-DDR2-Src-Akt signaling to upregulate FAO in ovarian cancer cells.

COL11A1-mediated FAO upregulation is due to an increase in de novo fatty acid synthesis

Next, we investigated the source of fatty acids utilized for FAO. It has been shown that ovarian cancer cells uptake exogenous fatty acids from the omentum to acquire tumorigenic and metastatic phenotype^{24,27}. Therefore, we asked if COL11A1 also increases the uptake of extracellular fatty acids to promote FAO in ovarian cancer cells. We blocked CD36 (a long-chain fatty acid transporter) using blocking antibodies and measured the OCR after supplementation of exogenous palmitate (indicative of FAO rate). However, CD36 blocking antibodies only slightly decreased the FAO rate in ES2 and OVCAR3 cells cultured in COL11A1-coated plates (Fig. S5A, B), suggesting that exogenous fatty acids might not be a major source of fatty acids for FAO.

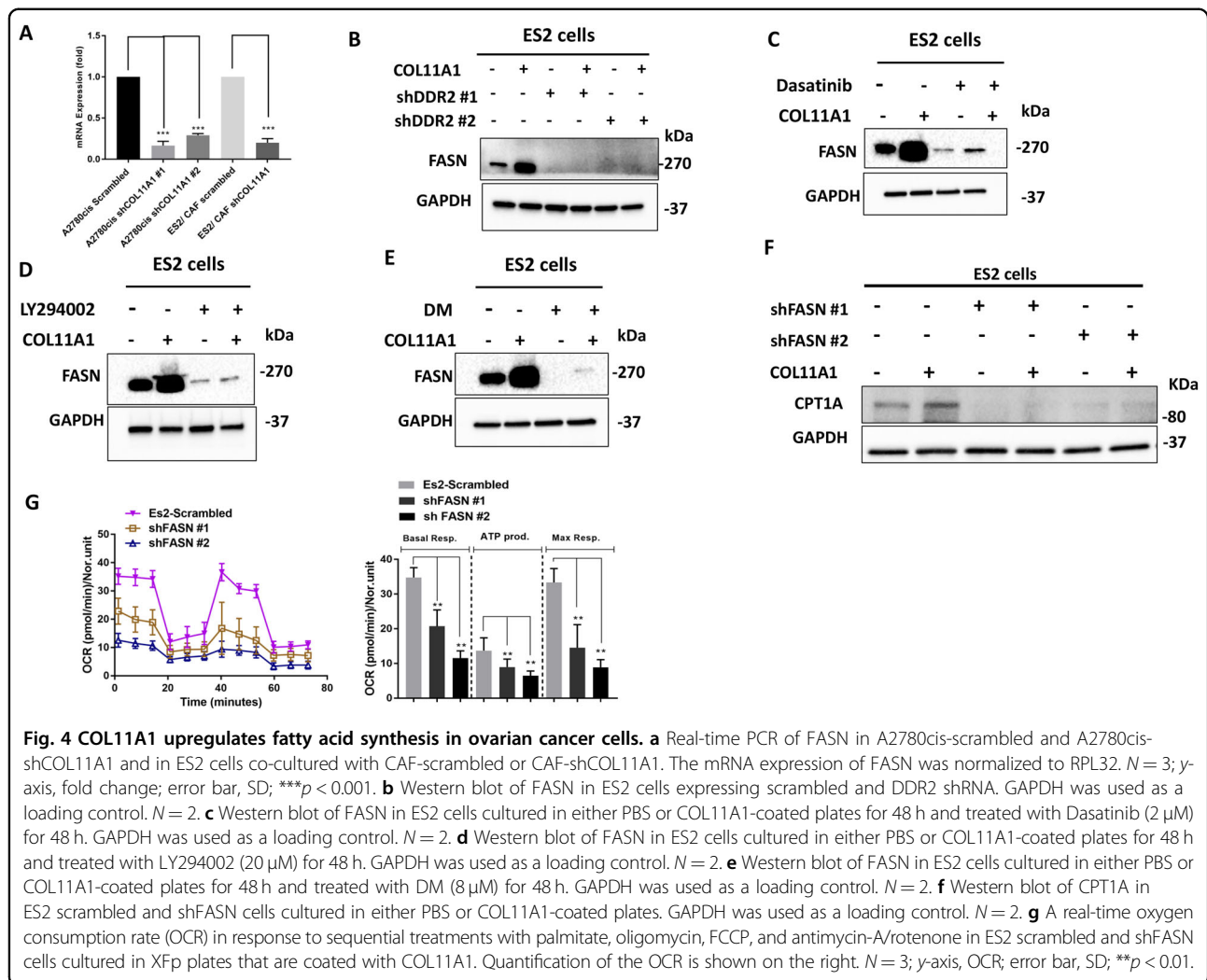
Cancer cells increase de novo fatty acid synthesis to provide fatty acid pools to generate essential structural



and signaling components and meet their high metabolic demands⁸. Therefore, we hypothesized that COL11A1 might upregulate fatty acid synthesis to supply cancer cells with fatty acids to promote FAO. Indeed, we observed an increased expression of FASN in ES2 cells co-cultured with COL11A1-expressing CAFs (Fig. 4A) or supplemented with recombinant COL11A1 (Fig. S1D) or cultured in COL11A1-coated plates (Fig. 4b–f), and in OVCAR3 and A2780 cells cultured in COL11A1-coated plates (Fig. S5C–F). Interestingly, FASN expression was not altered in ES2 cells cultured on type I collagen (data not shown). On the other hand, FASN expression was reduced by COL11A1 knockdown in A2780cis cells (Fig. 4A). Interestingly, DDR2 knockdown significantly abrogated COL11A1-induced FASN overexpression (Fig. 4B) while ITGA1 knockdown only slightly reduced FASN expression (data not shown), suggesting that DDR2 might be the predominant receptor that mediates COL11A1-induced FASN expression in ovarian cancer cells.

To determine whether the effectors of the COL11A1 signaling, Src, Akt, and AMPK, also play a role in upregulating FASN in ovarian cancer cells, we blocked these signaling molecules using pharmacological inhibitors and measured the expression of FASN. Inhibitors of Src (dasatinib), Akt (LY294002), and AMPK (DM) significantly reduced FASN protein expression even in the presence of COL11A1 in ES2 (Fig. 4c–e) and OVCAR3 cells (Fig. S5C–E). FASN mRNA expression was also reduced upon LY294002 treatment in A2780 cells (Fig. S5F).

To test whether fatty acid synthesis modulates FAO, we knocked down FASN in ES2 cells (Fig. S6A), cultured them in uncoated or COL11A1-coated plates and measured CPT1A expression and OCR. We observed a dramatic decrease in CPT1A expression and FAO rate in FASN-knockdown ES2 cells (Fig. 4F, G), suggesting that fatty acid synthesis might be an upstream regulator of FAO in ovarian cancer cells. Similarly, OVCAR3 cells

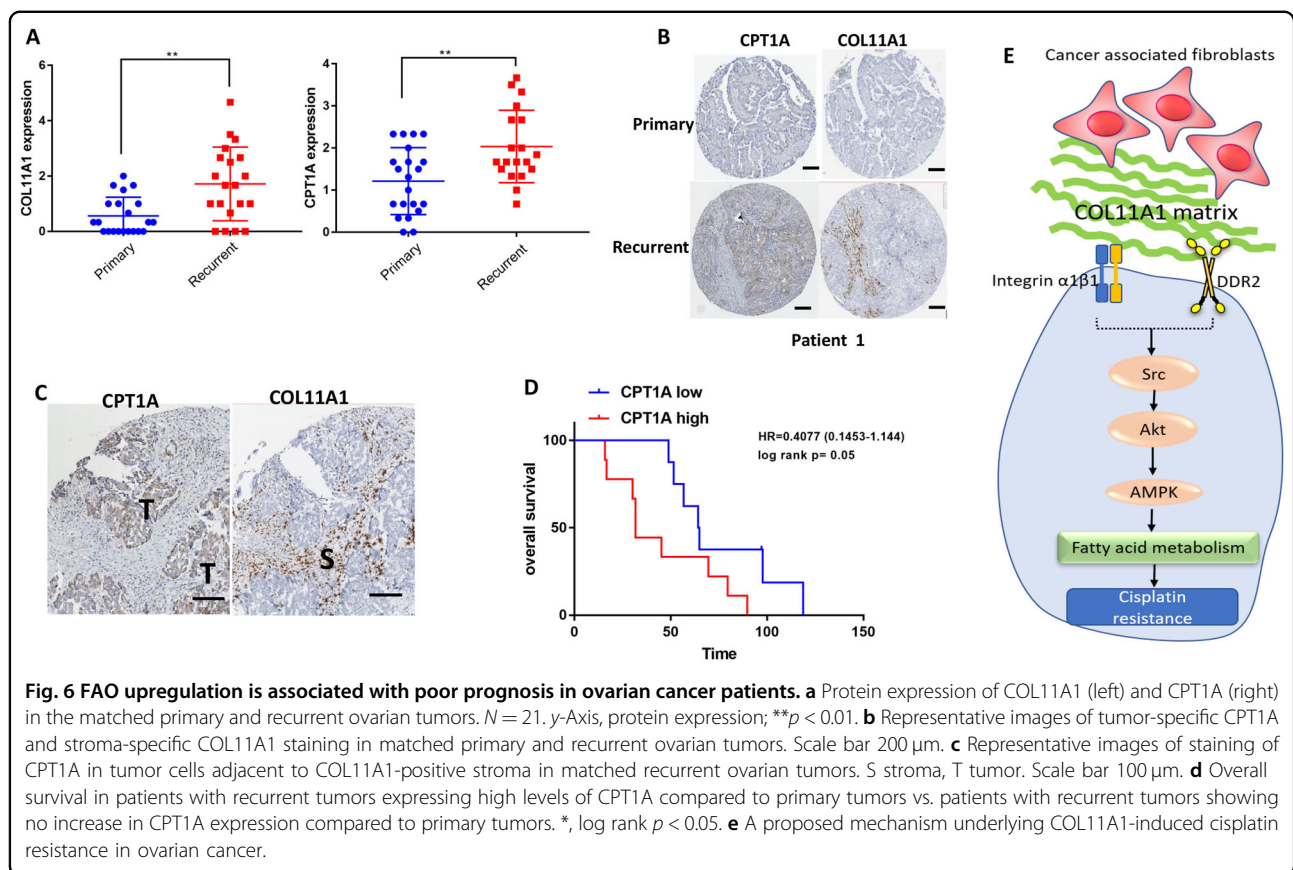


treated with C75, a competitive, irreversible FASN inhibitor, showed a marked reduction in CPT1A expression despite culturing on COL11A1 (Fig. S6B). Collectively, our results demonstrate that COL11A1 increases de novo fatty acid synthesis through DDR2-Src-Akt-AMPK signaling to upregulate FAO in ovarian cancer cells.

Inhibition of FAO restores sensitivity of ovarian cancer cells to cisplatin

Given our initial observation that FAO is upregulated in cisplatin-resistant variant of A2780 cell line (A2780cis), we sought to test whether FAO inhibition can attenuate COL11A1-induced cisplatin resistance in ovarian cancer cells. For this, we knocked down CPT1A in ES2 cells, cultured them on COL11A1 extract, and measured cell viability and apoptosis after cisplatin treatment. An appropriate dose of cisplatin was chosen after performing a dose response experiment in scrambled and shCPT1A

cells (Fig. S6C). ES2 cells cultured on COL11A1-positive extract exhibited a significant decrease in cell death upon cisplatin treatment, compared to ES2 cells cultured on COL11A1-negative extract (Fig. 5a; 13.2% vs. 21.6% cell death, $p < 0.01$). However, CPT1A-knockdown ES2 cells grown on COL11A1-positive extract exhibited similar percentage of cell death as CPT1A-knockdown ES2 cells on COL11A1-negative extract (Fig. 5a, b). Furthermore, CPT1A knockdown in ES2 cells significantly increased apoptosis (measured by cleaved caspase 3) upon cisplatin treatment despite the presence of COL11A1 (Fig. 5c; data not shown). Etomoxir treatment in ES2 cells also resulted in a significant decrease in mitochondrial membrane potential (measured by TMRE staining; Fig. S5F), an early event in apoptosis. Notably, FASN knockdown also resulted in decreased TMRE intensity despite the presence of COL11A1, further suggesting that fatty acid synthesis increases mitochondrial activity via FAO



the metabolic phenotype of ovarian cancer cells to confer chemotherapy resistance. In particular, we showed that COL11A1, a collagen subtype specifically overexpressed in cisplatin-resistant ovarian cancer^{25–27,30}, upregulates FAO to promote cisplatin resistance in ovarian cancer cells. Mechanistically, we demonstrate that COL11A1 predominantly binds to DDR2 and activates Src-Akt-AMPK signaling to induce FAO (Fig. 6e) and blocking FAO attenuates COL11A1-induced cisplatin resistance by increasing mitochondrial apoptosis pathway. Furthermore, we discovered that COL11A1 upregulates de novo fatty acid synthesis to derive fatty acids for oxidation in ovarian cancer cells. In patient tumors, FAO is increased concurrently with COL11A1 and is associated with poor survival in recurrent ovarian cancer.

The role of fatty acids as substrates that induce chemoresistance is less explored compared to glucose or glutamine⁴⁰. In this study, we showed that cisplatin-resistant ovarian cancer cells preferentially upregulate FAO and rely on fatty acids for their survival and the inhibition of FAO sensitizes them to cisplatin. Our work is further supported by the findings of others that chemoresistant ovarian cancers exhibit a high metabolically active phenotype with the ability to utilize fatty acids as fuel^{41,42}. FAO upregulation increases both mitochondrial

function and cellular pools of NADH and ATP (Fig. 1e, S6D). ATP and NADH are known to provide a survival advantage to cancer cells¹⁹ and thus might contribute to cisplatin resistance as well. In concordance with our study, FAO inhibition has been shown to cause chemosensitization by limiting ATP supply in prostate cancer⁴³ and leukemia cells⁴⁴.

The interplay between the ECM and cancer cells can regulate a plethora of cellular functions including cellular proliferation, differentiation, metabolism, and drug resistance^{45,46}. Several collagens have been associated with cancer progression and chemoresistance, including types I, III, V, VI, and XI^{27–29,33,47}, however, their role in regulating cancer cell metabolism has not been explored. Here, for the first time, we identified that type XI collagen in the tumor microenvironment can switch the metabolic preferences of ovarian cancer cells toward FAO. While type I collagen has been shown to upregulate glycolysis⁴⁸, our data show that type I collagen does not upregulate FAO in ovarian cancer cells. In contrast, COL11A1 does not change glycolysis, yet preferentially upregulates fatty acid metabolism to promote cisplatin resistance in ovarian cancer cells. It remains to be determined whether the differences in metabolic preferences induced by different collagen subtype arises from the binding to different cell

surface receptors and/or activation of different downstream signaling components. We identified that DDR2 might be a predominant receptor for COL11A1 to activate downstream signaling to upregulate fatty acid metabolism. Whether DDR2 also plays a role in type I collagen-mediated signaling and to what extent needs to be studied. To develop more effective therapy to block chemoresistance, it is important to understand which collagen subtypes are enriched in the tumor microenvironment at specific disease stages and how these collagen subtypes dictate specific bioenergetic phenotypes of cancer cells to confer chemoresistance.

Our study showed that a blockade of CD36, a fatty acid transporter known to drive FAO³⁷, did not affect FAO in the presence of COL11A1, suggesting that CD36-mediated fatty acid uptake only minimally supports FAO in ovarian cancer cells that reside in the COL11A1-rich microenvironment. Although this observation is somewhat in contrast with other studies showing a positive regulatory role of extracellular fatty acids on FAO^{23,24}, it is unclear at this point whether other fatty acid transporters might alter flux of fatty acids into the cells and promote FAO in the presence of COL11A1. Nevertheless, it is interesting that ovarian cancer cells can upregulate both synthesis and oxidation of fatty acids in the presence of COL11A1. Given that fatty acid synthesis and oxidation can negatively regulate each other, this poses an intriguing question as to how the ovarian cancer cells are able to overcome the regulatory effects of malonyl CoA, a substrate for fatty acid synthesis and an allosteric inhibitor of CPT1A. Although coupling of fatty acid synthesis with FAO seems to be bioenergetically unfavorable, this phenomenon has been documented in adipose tissue, muscle, and T cells^{49–51}. Furthermore, co-suppression of FAO and fatty acid synthesis showed synergistic effects in killing prostate cancer cells and myeloma cells and blocking metastasis of ovarian cancer cells^{23,52,53}, suggesting co-activation of these pathways. There are two possible explanations for co-activation of FAO and fatty acid synthesis. Firstly, ovarian cancer cells might oscillate between periods of FAO and fatty acid synthesis activation. Secondly, malonyl COA might be compartmentalized to prevent inhibition of CPT1A so that FAO is uninterrupted or rapidly utilized to meet cancer cells' high demand for energy and/or biomolecules.

In summary, we identified that COL11A1 increases FAO in ovarian cancer cells to confer cisplatin resistance and COL11A1 and FAO are concurrently upregulated in recurrent ovarian tumors. FAO inhibitors, such as etomoxir, perhexiline, and ranolazine, have been approved by FDA and/or EMA for heart diseases and are currently being evaluated in various stages of clinical trials as anticancer agents^{54–57}. Therefore, targeting FAO using these drugs might be a promising therapeutic strategy for

cisplatin-resistant recurrent ovarian cancer, which often overexpress COL11A1.

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Conflict of interest

The authors declare that they have no conflict of interest.

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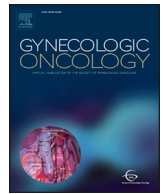
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Dual targeting of GSK3B and HDACs reduces tumor growth and improves survival in an ovarian cancer mouse model

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HIGHLIGHTS

- Dual inhibition of GSK3B and HDACs reduces cancer cell survival in both human and mouse ovarian cancer cell lines.
- GSK3B/HDACs dual inhibitor APCS-540 reduces ovarian cancer cell survival, migration and invasion.
- APCS-540 is cytotoxic to cisplatin-resistant human ovarian cancer cells and re-sensitizes cancer cells to cisplatin.
- APCS-540 reduces cancer stemness markers CD133, Oct4, Nanog and YAP in ovarian cancer cells.
- APCS-540 improves survival in a syngeneic ovarian cancer mouse model.

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ABSTRACT

Objective. To investigate the anti-tumor effect of a newly-developed dual inhibitor (APCS-540) of glycogen synthase kinase 3 beta (GSK3B) and histone deacetylases (HDACs) in ovarian cancer cells.

Methods. The effects of APCS-540 on cancer cell proliferation, migration, invasion and cancer stemness were investigated *in vitro* in human (KURAMOCHI, OVCA420, OVSAHO) and mouse (BR-Luc, ID8, MOSE-HRas-Myc) ovarian cancer cells. Cisplatin-sensitive (A2780) and cisplatin-resistant (A2780cis) cell lines were used to evaluate APCS-540's effect on chemoresistance. The immunocompetent syngeneic mouse model BR-Luc was used to test the effect of APCS-540 on ovarian cancer progression and survival.

Results. APCS-540 showed significant anti-tumor effects *in vitro* in both human and mouse ovarian cancer cells. Importantly, APCS-540 demonstrated marked cytotoxicity against cisplatin-resistant cancer cells and reversed cisplatin-resistance when used in combination with platinum. APCS-540 significantly decreased cancer cell invasion. A significant 66% increase in survival was observed in mice treated with APCS-540 compared to control mice.

Conclusion. Dual inhibition of GSK3B and HDACs via APCS-540 showed potent anti-tumor activity *in vitro* and *in vivo*, suggesting that APCS-540 may provide a novel treatment option for ovarian cancer, including the platinum-resistant disease.

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1. Introduction

Ovarian cancer is the deadliest gynecologic malignancy with approximately 22,000 new cases and more than 14,000 deaths each year in the United States [1]. With no effective means for early detection and no specific symptoms, the vast majority of women with ovarian cancer present with widely disseminated metastatic disease at diagnosis. Despite the work of committed physicians and scientists, the overall survival rate for the most common type of ovarian cancer, high-grade serous ovarian carcinoma, has not significantly improved in over

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40 years and remains at ~30%. Standard treatment for ovarian cancer includes cytoreductive surgery coupled with systemic chemotherapy to eliminate any remaining tumor cells and achieve clinical remission. Although remission can be achieved in about 80% of patients with high grade serous ovarian carcinoma, most of these patients experience recurrence due to the presence of chemotherapy-resistant clones of cancer cells. Recent clinical trials with various checkpoint inhibitors alone or in combination with standard therapy for ovarian cancer have been unsuccessful [2]. Thus, effective alternative therapies are urgently needed.

A promising approach to the development of more effective treatments for ovarian cancer is targeting multiple cellular pathways simultaneously to prevent the development of drug resistance in cancer cells, which is the major challenge for standard chemotherapy. Studies have shown that epigenetic dysregulation and alterations in metabolic pathways are crucial components in the development and progression of several solid tumors, including ovarian cancer [3,4]. Glycogen synthase kinase-3 beta (GSK3B) is a glycogen metabolism enzyme that upregulates NF- κ B activity, a key driver of proliferation and survival in ovarian cancer cells, and inhibition of GSK3B caused tumor shrinkage in mice [5–7]. Recent studies also showed that inhibition of histone deacetylases (HDACs) effectively suppressed ovarian cancer growth and metastasis by inhibiting expression of PAX8, a critical oncogene in ovarian cancer [8]. Therefore, we hypothesized that dual targeting of metabolic (GSK3B) and epigenetic (HDACs) pathways might significantly improve anti-tumor treatment and overcome chemoresistance in ovarian cancer.

We have previously reported the design, synthesis, and testing of a novel dual GSK3B and HDACs inhibitor Metavert for the treatment of pancreatic adenocarcinoma [9]. In this study, we investigated the anti-tumor effect of several dual inhibitors of HDACs and GSK3B on ovarian cancer cell proliferation, migration, and invasion *in vitro* in human and mouse ovarian cancer cell lines and identified the Metavert analog APCS-540 as the most effective compound against ovarian cancer compared to other Metavert analogs. We also tested the anti-tumor activity of APCS-540 in cisplatin-resistant human ovarian cancer cells (A2780cis) and examined its effectiveness in overcoming chemoresistance. Subsequently, we examined the effect of APCS-540 *in vivo* on disease progression in a syngeneic ovarian cancer mouse model.

2. Materials and methods

This study was reviewed and approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center, Los Angeles, California.

2.1. Cell lines

The syngeneic mouse ovarian cancer cell lines BR-Luc (*p53*–/–, *Brca1*–/–, *Myc*, *Myr-Akt*) and MOSE-HRas-Myc (*p53*–/–, *HRas*, *Myc*) were generated in the Orsulic Laboratory [10–12]. The ID8 cell line (*p53*–/–, *Brca2*–/–) was obtained from Dr. Ian McNeish (University of Glasgow, UK) [13]. The human ovarian cancer cell lines KURAMOCHI, OVCA420, and OVSAHO were kindly provided by Dr. Dennis Slamon (UCLA, USA). The Ishikawa (human endometrial adenocarcinoma) and SiHa (human cervical adenocarcinoma) cell lines were kindly provided by Dr. Kate Lawrenson (Cedars-Sinai Medical Center, USA). The ovarian cancer cell lines A2780 (cisplatin-sensitive) and A2780cis (cisplatin-resistant) were obtained from Fox Chase Cancer Center, PA, USA. None of the cell lines have been propagated in culture for more than 6 months. KURAMOCHI, OVCA420, OVSAHO, A2780 and A2780cis cells were cultured in RPMI 1640 media supplied with 10% fetal bovine serum (FBS). BR-Luc, ID8, Ishikawa, and SiHa cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplied with 10% FBS.

2.2. Chemical compounds

Dual inhibitors of GSK3B and HDACs (APCS-540, APCS-643, APCS-644) were synthesized by Albany Molecular Research Institute (AMRI, Albany, NY, USA) and Metavert was synthesized by Royal Pharma (Mumbai, India). Metavert is a trademark owned by Avenzoar Pharmaceuticals, Encinitas, CA, USA and is currently in the preclinical stage for treating pancreatic adenocarcinoma. The Pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) was purchased from Cayman (Ann Arbor, MI, USA). The GSK3B inhibitor Tideglusib was purchased from Sigma-Aldrich (St. Louis, MO, USA). D-luciferin for *in vivo* bioluminescence imaging was purchased from Goldbio (St. Louis, MO, USA).

2.3. Cancer cell survival assay

Survival of *in vitro* cultured cancer cells was assessed by crystal violet staining. Crystal violet is a triarylmethane dye that can bind to ribose type molecules such as DNA in the nuclei. It offers an efficient method for *in vitro* cell viability or cytotoxicity assessment under various conditions and drug screening. The amount of staining in the assay is directly proportional to the cell biomass and can be measured at 560–570 nm absorbance. Briefly, 5×10^3 /well cancer cells were seeded into 24-well plates and cultured overnight. The cells were subsequently treated with different concentrations (0.6–4.8 μ M) of Metavert, APCS-540, APCS-643, APCS-644, SAHA, and Tideglusib and cultured for 72–96 h depending on the experimental setting. A cisplatin dose of 10 μ M was used in the experiments with A2780 and A2780cis cell lines. All cell culture experiments were performed in quadruplicate, and the survival of treated cancer cells was compared to untreated controls.

2.4. Transwell cancer cell migration and invasion assay

The effect of APCS-540 at different concentrations (0.6 μ M, 1.2 μ M, and 2.4 μ M) on migration and invasion ability of human and mouse ovarian cancer cell lines was evaluated using Boyden's chamber (Transwell Assay) with Matrigel-coated Transwell inserts with a pore size of 8 μ m. To create a chemotactic gradient, 10% FBS was added into the lower chamber media while cell media in the upper chamber were kept FBS-free. After 72 h of treatment, 50×10^3 cancer cells were reseeded on the Transwell insert overnight at 37 °C and 5% CO₂ to assess the effect of treatment on both migration and invasion. The Transwell inserts were later removed from the plate and residue culture media and cells that had not migrated from the upper side of the membranes were gently removed using a cotton-tipped applicator. Migrated cancer cells on the bottom side of the membranes were fixed with 10% formalin for 20 min, stained with crystal violet for 30–45 min, washed with PBS, and allowed to dry. Images of migrated cells were taken under an optical microscope and the cells were counted (average of 5 fields at 100 $\times$ magnification).

2.5. RNA isolation and real-time quantitative PCR

Total RNA was extracted using Trizol (ThermoFisher, Canoga Park, CA, USA), and reverse transcription reaction was carried out using High Capacity Reverse Transcription Kit (Thermo Fisher, Canoga Park, CA, USA). Real-time quantitative PCR (RT-qPCR) was used for quantifying mRNA levels using the iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) and BioRad cfx96 platform according to the manufacturer's protocol. Gene expression levels were normalized to that of GAPDH. Primers were purchased from Integrated DNA technologies (IDT), Coralville, IA, USA. The sequences of primers used for RT-PCR were as follow: h-Oct4-F; GAGAATTTGTTCTGCAGTGC, h-Oct4-R; GTTC CCAATTCCTCTTAGTG, h-CD133-F; GAGTCGGAACTGGCAGATAGCA, h-CD133-R; ACGCCTTGCTTGCTAGTGTG, h-Nanog-F; ACCTATGCC TGTGATTGTGG, h-Nanog-R; AAGAGTAGAGGCTGGGCTAGG, h-YAP-F; TAGCCCTGCGTAGCCAGTTA, h-YAP-R; TCATGCTTAGTCCACTGTCTGT, h-

GAPDH-F; CCAGGTGGTCTCCTCTGACTTCAACA, h-GAPDH-R; AGGGTC TCTCTCTCTCTTGTGCTC.

2.6. Western blot analysis

Cells were re-suspended in RIPA phosphorylation buffer (50 mM NaCl, 50 mM Tris/HCl pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 10 mM Na₂HPO₄ + NaH₂PO₄, 100 mM NaF, 2 mM Na₃VO₄, 80 μM glycerophosphate, 20% glycerol, 1 mM PMSF, 5 μg/mL each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin). Lysates were then centrifuged for 15 min at 16,000 × g at 4 °C. Proteins in the supernatant were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose or PVDF membranes. Non-specific binding was blocked for one hour with 5% bovine serum albumin or non-fat dry milk in Tris-buffered saline (4 mM Tris base, 100 mM NaCl, pH 7.5) containing 0.05% Tween 20. Membranes were incubated with primary antibody overnight at 4 °C, and then with peroxidase-conjugated secondary antibody for one hour. Blots were developed using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

2.7. Syngeneic mouse model of ovarian cancer and treatment protocol

For *in vivo* evaluation of the drug efficiency, 6-week-old female FVB mice (The Jackson Laboratory, Sacramento, CA, USA) were injected intraperitoneally (i.p.) with 1×10^6 BR-Luc cells to simulate metastatic ovarian cancer dissemination in the mouse. One week after i.p. injection of cancer cells, all mice were imaged by luciferin bioluminescence to confirm the intraabdominal development of tumors. The mice were subsequently assigned to two groups with 9 mice in each group: 1) control (vehicle), and 2) treatment (APCS-540, 10 mg/kg). Mice were injected i.p. 3 times per week by a researcher who was blinded to the study groups.

2.8. *In vivo* bioluminescence imaging

To confirm tumor growth and metastasis to the abdominal organs after i.p. injection of cancer cells, mice were imaged using the IVIS Spectrum CT *In Vivo* Imaging System (Perkin Elmer Inc., Waltham, MA, USA). One week after cancer cell injection, the mice were anesthetized with 2% isoflurane and i.p. injected with D-luciferin solution (10 μL per gram of body weight; concentration of 15 mg/mL diluted in PBS). After D-luciferin injection, we waited 10 min to allow for luciferase activity; then, the mice were placed in the imaging chamber of the IVIS system. Images were obtained every 30 s until the peak value of bioluminescence was achieved.

2.9. IVIS image analysis

The obtained images were analyzed for bioluminescence signal intensity per mouse via the advanced *in vivo* imaging software, Living Image (Perkin Elmer Inc., Waltham, MA, USA). A standardized rectangular region of interest (ROI) was selected in each image to include the abdominopelvic cavity where tumor growth and metastasis were anticipated. The average photon count per second in the selected ROI was calculated and recorded for every image. Statistical analysis was performed to compare photon intensity between the treatment and control groups using GraphPad Prism software (GraphPad Software, CA, USA).

2.10. Animal survival assay

All mice were monitored for signs of morbidity at least three times weekly until they became moribund due to advanced metastatic disease. The survival duration for each mouse was calculated as the number of days from the initiation of treatment to euthanasia.

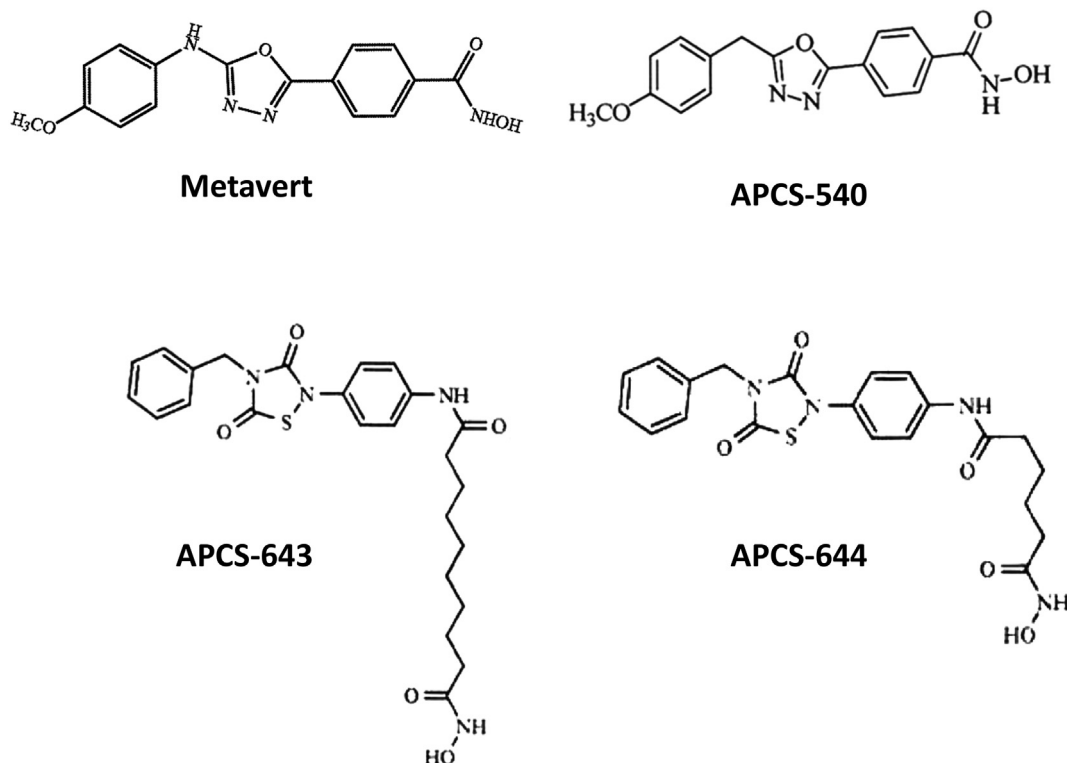


Fig. 1. Chemical structures of newly-developed dual GSK3B and HDACs inhibitors.

2.11. Statistical analysis

Collected data were analyzed using GraphPad Prism software. Comparisons were performed *via* Student *t*-test, Two-way ANOVA, or Fisher's exact test. For animal survival data analysis, the long-rank (Mantel-Cox) was utilized. A *p*-value <.05 was considered statistically significant.

3. Results

3.1. Dual inhibition of GSK3B and HDACs decreases human and mouse cancer cell survival

We first examined whether simultaneous inhibition of GSK3B and HDACs is more effective than inhibition of HDAC alone in reducing the survival of ovarian cancer cells *in vitro*. We designed and synthesized four dual inhibitors (Metavert, APCS-540, APCS-643, APCS-644), which were tested in comparison to the HDAC inhibitor SAHA in human (KURAMOCHI, OVSAHO, OVCA420) and mouse (MOSE-HRas-Myc) cell lines at concentrations of 1.2 μ M, 2.4 μ M, and 4.8 μ M vs. control. The chemical structures of Metavert and its analogs are shown in Fig. 1. All dual inhibitor analogs significantly reduced cancer cell survival in a dose-dependent manner in all human cancer cell lines with the most potent effect observed in OVCA420 cells (Fig. 2). At a concentration of 4.8 μ M, >95% of cancer cells were killed by APCS-540 and APCS-643 compared to control (p < .001 and p < .001, respectively). Even at 1.2 μ M concentration, APCS-540 reduced cancer cell survival significantly by 40% to 60% compared to controls in the KURAMOCHI, OVSAHO, and OVCA420 cell lines. SAHA showed no significant effect at 1.2 μ M concentration except in the OVCA420 cell line (p = .02 at 1.2 μ M vs. control). The anti-tumor effect was even more evident in

the cancer cells treated with APCS-540 and APCS-643. When we tested these agents in the MOSE-HRas-Myc mouse ovarian cancer cells, which have a highly aggressive proliferative behavior, only APCS-540 and APCS-643 (p = .03 at 2.4 μ M vs. control, and p = .02 at 4.8 μ M vs. control) demonstrated significant anti-tumor effects compared to Metavert, APCS-644, and SAHA (Fig. 2). Comparison of the effects of APCS-540 and Metavert on cancer cell survival showed that the effect of APCS-540 is significantly bigger than the effect of Metavert in most of the doses tested and in the 4 cell lines (Fig. 2).

After these initial experiments, which showed that analog APCS-540 possessed the highest anti-tumor activity, we investigated its effect on cancer cell survival in comparison to Tideglusib (GSK3B inhibitor) and SAHA (HDAC inhibitor) alone and in combination. APCS-540 reduced the viability of human and mouse ovarian cancer cells to a greater extent than Tideglusib and SAHA alone or in combination (Fig. 3). These results suggest that dual targeting of GSK3B and HDACs has better anti-tumor activity than the combination of agents singly targeting these oncogenic pathways.

Recent studies have shown that inhibition of GSK3B and HDACs induces anti-tumor effects in endometrial and cervical cancer [14–17]. To provide preliminary data on the anti-tumor potential of APCS-540 in these gynecologic malignancies, we treated human cervical (SiHa) and endometrial (Ishikawa) cancer cell lines with different concentrations of APCS-540. The results showed that 1.2 μ M concentration of APCS-540 significantly reduced survival in both cancer cell lines (p = .02 and p = .03, respectively). Combination treatment with SAHA and Tideglusib also effectively induced cell death in SiHa and Ishikawa cancer cell lines at 2.4 μ M concentration (Supplementary Fig. S1). These promising results suggest that dual inhibition of GSK3B and HDACs may be effective in a wider spectrum of gynecologic tumors.

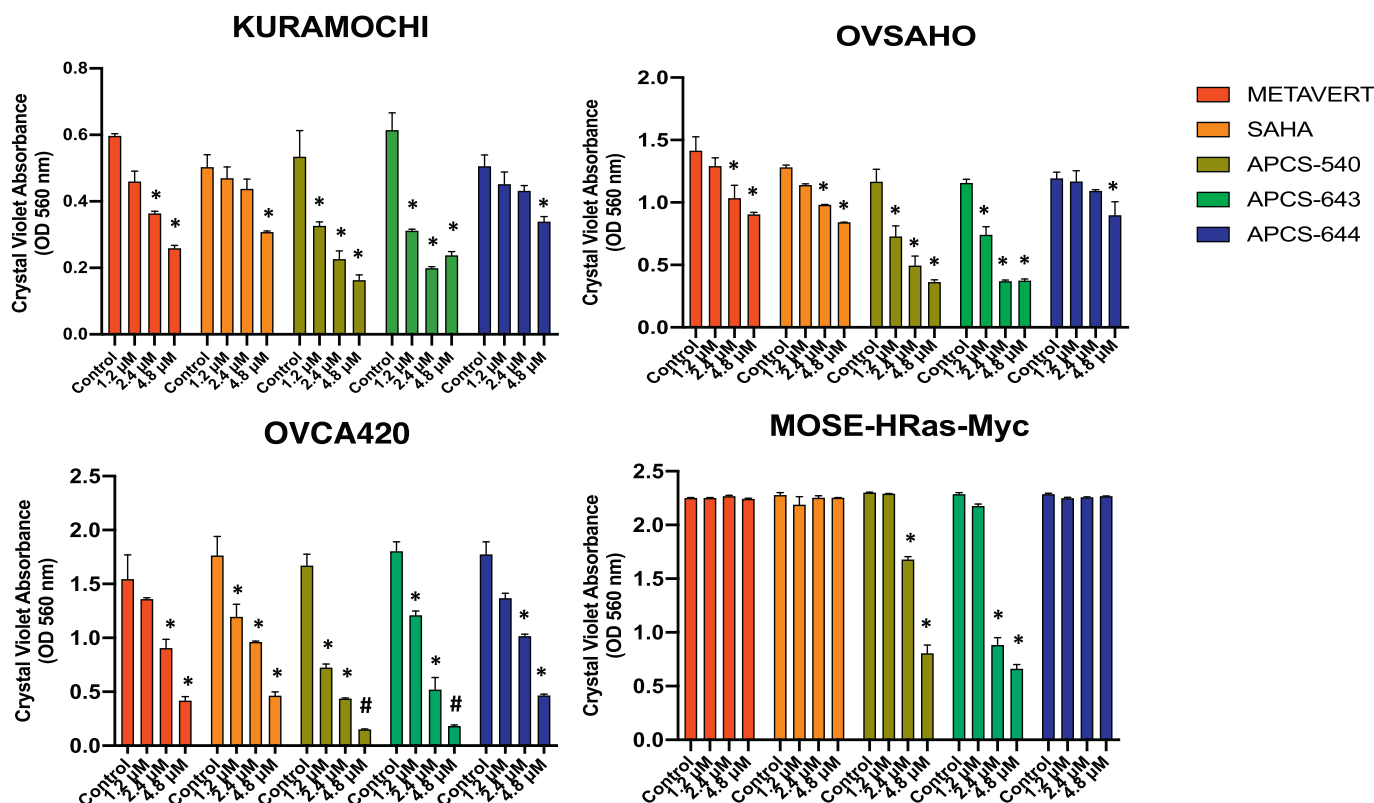


Fig. 2. The effects of dual GSK3B and HDACs inhibitor analogs on cancer cell survival compared to SAHA in three human (KURAMOCHI, OVCA420, OVSAHO) and one mouse (MOSE-HRas-Myc) ovarian cancer cell lines. * represents p < .05, and # represents p < .001 compared to control.

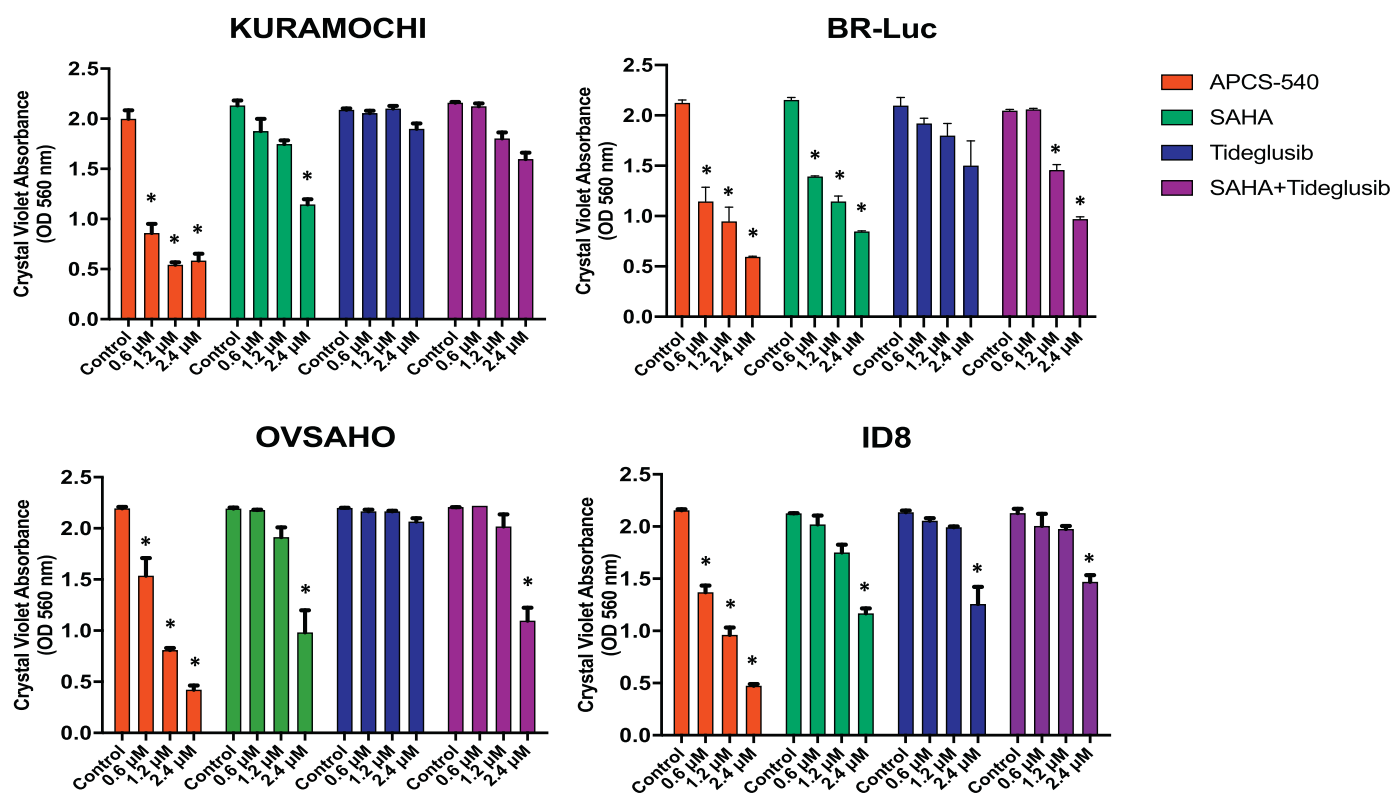


Fig. 3. The effects of GSK3B (Tideglusib) and HDAC (SAHA) inhibitors alone and in combination on cancer cell survival compared to APCS-540 in two human (KURAMOCHI, OVSAHO) and two mouse (BR-Luc, ID8) ovarian cancer cell lines. * represents $p < .05$.

3.2. Ovarian cancer cell migration and invasion are significantly reduced with APCS-540 treatment

After confirming the cytotoxic effect of APCS-540 in cancer cells, we examined its effect on cancer cell migration and invasion, which are the essential determinants of the metastatic capacity of cancer cells. Using a Matrigel-coated Transwell assay, we found that APCS-540 significantly reduced the ability of the highly invasive mouse ovarian cancer cell lines ID8 and BR-Luc to migrate and invade through Matrigel at 0.6 μ M and 1.2 μ M and almost completely inhibited migration and invasion at 2.4 μ M concentration (Fig. 4A, B). Similar results were observed in human ovarian cancer cell lines KURAMOCHI and OVSAHO (Fig. 4B). These results showed that APCS-540 not only reduced cancer cell viability but also inhibited the metastatic capacity, which is crucial for an effective treatment strategy.

3.3. APCS-540 reduces survival of cisplatin-resistant ovarian cancer cells and reverses chemo-resistance

Because chemoresistance is one of the major factors in tumor recurrence and worse prognosis, we aimed to evaluate the anti-tumor activity of APCS-540 in the cisplatin-sensitive human ovarian cancer cell line A2780 and its cisplatin-resistant derivative A2780cis alone or in combination with cisplatin (10 μ M). Western blot analysis of acetylated histone 3 lysine 9 (Ac-H3K9) and beta-catenin, which is the direct target of GSK3B, protein levels re-probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed that treatment with 0.6 μ M APCS-540 significantly inhibited HDAC and GSK3B pathways in both A2780 and A2780cis cancer cells compared to control (Supplementary Fig. S2A). Remarkably, APCS-540 reduced cancer cell viability in both A2780 and A2780cis cancer cells at a low concentration of 0.6 μ M ($p < .001$) (Fig. 5A). When combined with cisplatin, APCS-540 showed an even greater cytotoxic effect in both cell lines. These findings indicate that

APCS-540 can reverse chemoresistance and synergistically improve chemotherapy response in ovarian cancer cells.

3.4. APCS-540 reduces expression of stem cell markers in ovarian cancer cells

Additionally, we examined whether APCS-540 had any effect on cancer stemness, which is a critical factor mediating drug resistance. RT-qPCR analysis of stemness markers CD133, Oct4, and Nanog and pro-tumorigenic marker YAP in OVCA420 and BR-Luc cancer cells treated with 0.6 μ M APCS-540 showed a significant decrease in expression levels of these markers (Fig. 5B). Similarly, significant results were observed with Oct4 and Nanog in A2780 and A2780cis cancer cells (Supplementary Fig. S2B). These results, together with the reversal of chemoresistance, suggest APCS-540 is a promising anti-tumor agent for the treatment of ovarian cancer.

3.5. Treatment with APCS-540 increases survival in mice with ovarian cancer

To test whether APCS-540 can prevent ovarian cancer progression and metastasis, we used an immunocompetent syngeneic ovarian cancer mouse model, which successfully simulates human metastatic ovarian cancer [11]. BR-Luc mouse ovarian cancer cells were i.p. injected into twenty 6-week-old female FVB mice. One week after cancer cell injection, mice were imaged via IVIS bioluminescence imaging to confirm intraabdominal tumor growth. IVIS imaging showed 18 mice with growing tumors and 2 mice with no measurable luciferin activity. Hence, the 2 mice without tumors were excluded from the experiment. Mice with confirmed tumor growth were assigned randomly to treatment ($n = 9$) and control ($n = 9$) groups (Fig. 6A). Mice in the treatment group were i.p. injected three times a week with APCS-540 (10 mg/kg). The median survival in the control group was 21 days,

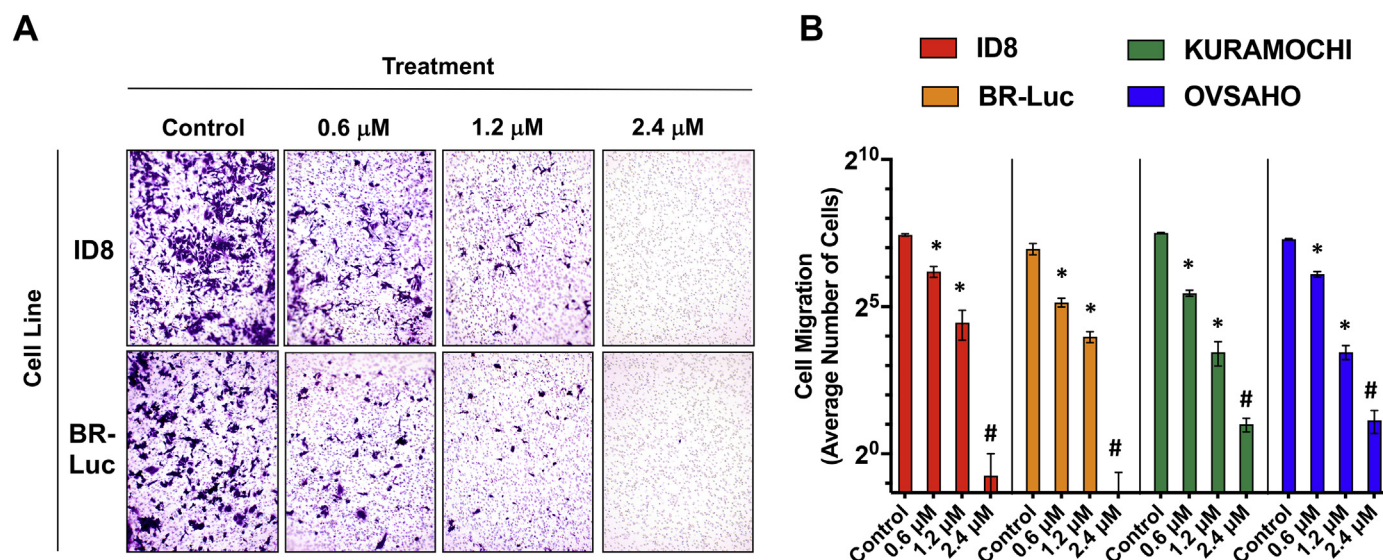


Fig. 4. Cancer cell migration and invasion assay results. A) Representative Transwell migration and invasion assay images (100 $\times$ magnification) from ID8 and BR-Luc cancer cells treated with different concentrations of APCS-540. Cancer cell migration and invasion abilities of both ID8 and BR-Luc cells were significantly reduced with 0.6 μ M of APCS-540, and completely inhibited at 2.4 μ M. B) Average number of migrated cancer cells treated with APCS-540 concentrations of 0.6 μ M, 1.2 μ M, and 2.4 μ M compared to control in mouse (ID8, BR-Luc) and human (KURAMOCHI, OVSAHO) ovarian cancer cells. Average number of cells is given at log2 scale. * represents $p < .05$, and # represents $p < .001$ compared to control.

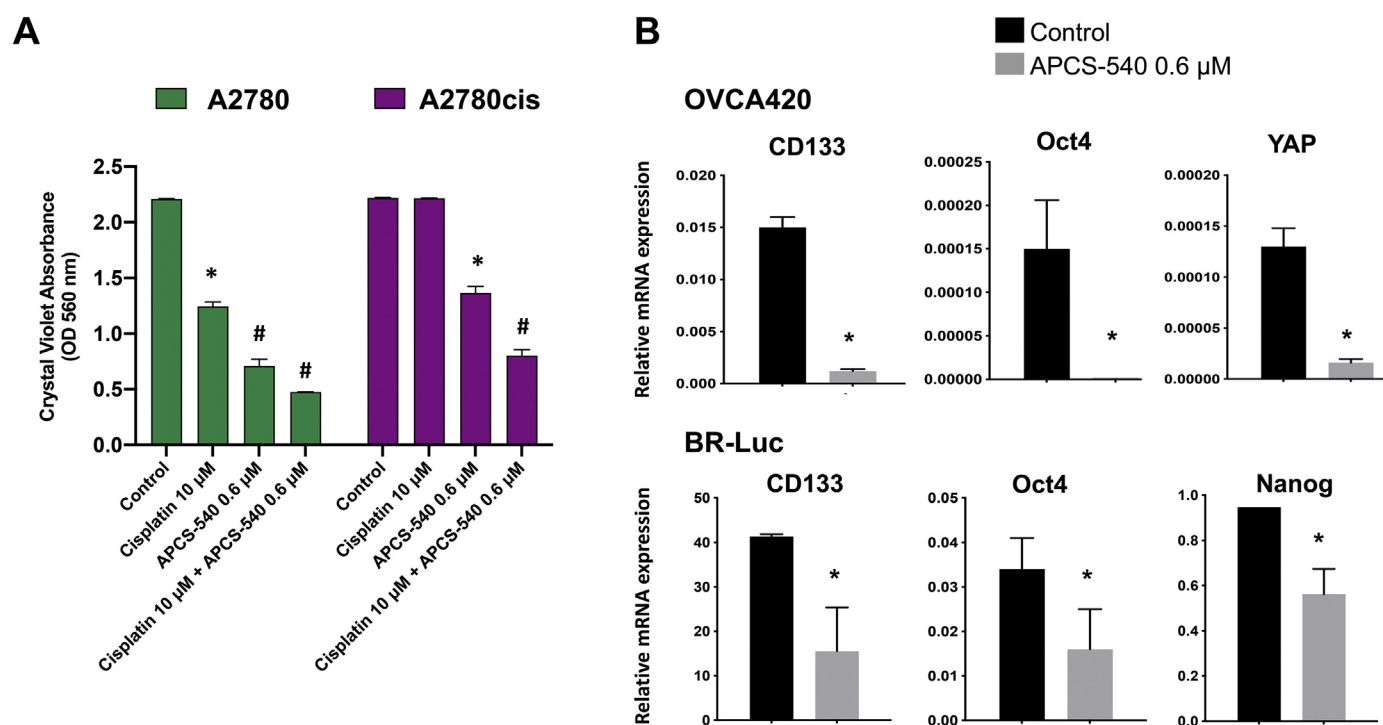


Fig. 5. A) The effect of APCS-540 on cisplatin-sensitive (A2780) and cisplatin-resistant (A2780cis) human ovarian cancer cells. B) APCS-540 (0.6 μ M) decreased relative mRNA expression levels of cancer stemness markers in human (OVCA420) and mouse (BR-Luc) ovarian cancer cell lines cultured for 48 h. Relative mRNA expression level was determined based on the housekeeping gene mRNA expression level. * represents $p < .05$, and # represents $p < .001$ compared to control.

whereas the median survival in the treatment group was 35 days (Fig. 6B). This result showed a 66% increase in survival when mice are treated with APCS-540 ($p = .03$).

Furthermore, during abdominal dissection of euthanized mice, we observed more disseminated disease in the control group with multiple tumor lesions in the peritoneum, omentum, intestines, and stomach. At the same time, less than half of the APCS-540 treated mice had tumors growing on the peritoneal surfaces of all of these organs, indicating a

significant decrease in tumor metastasis. This observation was in accordance with our *in vitro* results that showed reduced migration and invasion capability of BR-Luc cells treated with APCS-540.

4. Discussion

Despite remarkable advances in the treatment of several hematological cancers and solid tumors, there has been no significant

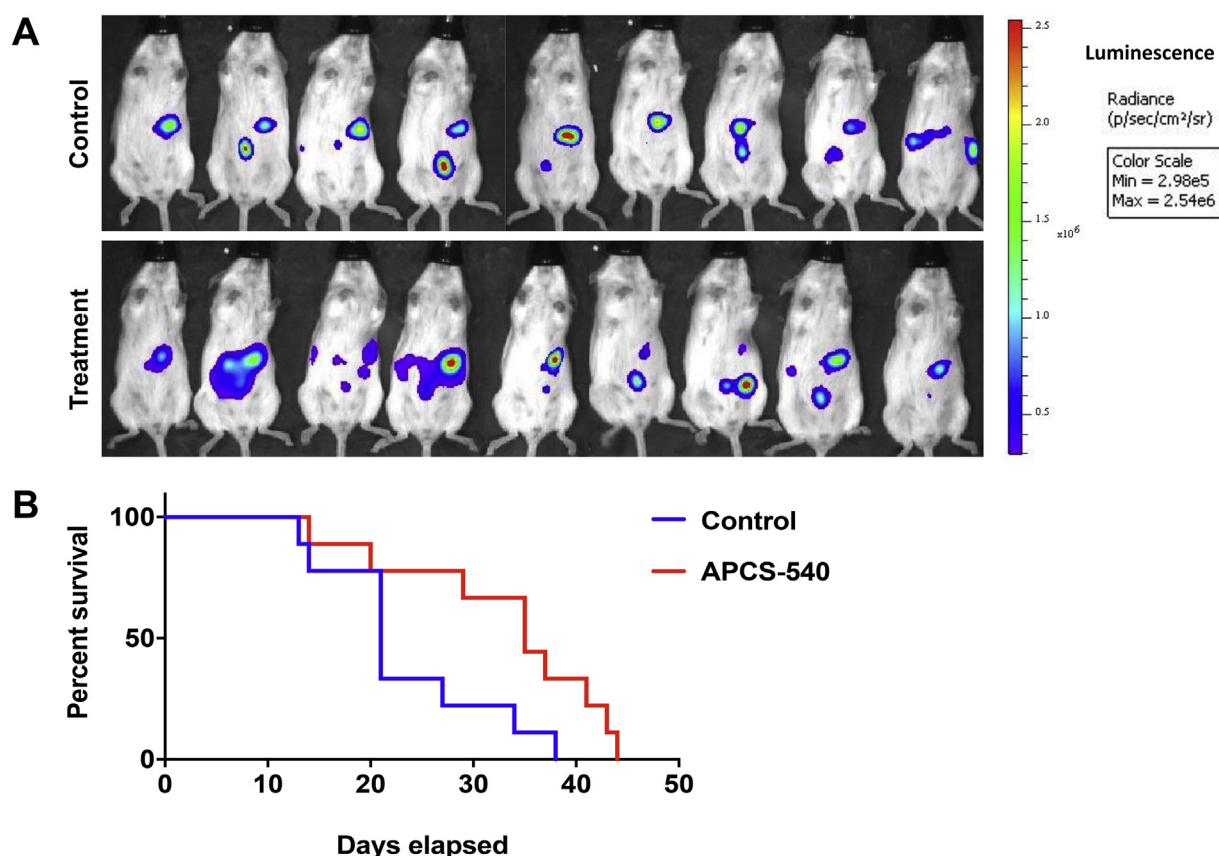


Fig. 6. A) Baseline (pre-treatment) IVIS imaging results of mice i.p. injected with BR-Luc cells and assigned to the control ($n = 9$) and treatment ($n = 9$) groups. Imaging confirmed that all mice in both groups harbor intra-abdominal growing tumors. No statistically significant difference was observed at baseline between two groups. B) Survival assay results for mice treated with APCS-540 vs. control. Treatment with APCS-540 significantly increased survival in tumor-bearing mice by 66%.

improvement in the treatment of ovarian cancer, which remains the deadliest gynecologic malignancy in the United States [1]. Standard of care treatment in ovarian cancer is debulking surgery combined with platinum/taxane-based chemotherapy. However, despite these aggressive treatments, tumor recurrence occurs in approximately 80% of patients. In the last decade, an increasing number of studies on cancer evolution have shown that evolving tumor clones that acquire chemoresistance are the primary cause of tumor recurrence [18,19]. Therefore, overcoming chemoresistance is a crucial challenge in developing an effective treatment for ovarian cancer.

Commonly used chemotherapeutic agents in ovarian cancer primarily target genomic integrity (platinum analogs) or cell division (taxanes). However, cancer cells can undergo extensive molecular changes at genetic, epigenetic, and metabolic activity levels as a response to different cellular stresses, such as chemotoxicity, and adapt to the environment by utilizing alternate cellular pathways, which results in increased cellular plasticity and chemoresistance [20]. A promising approach to overcome chemoresistance is simultaneous targeting of multiple key oncogenic pathways. Because the development of resistance to a single target is almost universal, combinations of drugs targeting different cellular pathways, such as epigenetic and metabolic pathways, may yield more effective anti-cancer therapies. A combination of multiple agents may provide an additive effect in killing cancer cells; however, it can also cause potential side effects due to drug toxicity and drug-drug interactions. Thus, a multitargeted single drug can provide superior pharmacokinetics and pharmacodynamics with lower side effects. Based on this knowledge, we focused on two oncogenic pathways, metabolic (GSK3B) and epigenetic (HDAC), and developed a novel drug that targets these two pathways simultaneously. To

our knowledge, this is the first report of using a dual GSK3B/HDAC inhibitor for the treatment of ovarian cancer.

GSK3B is a serine/threonine kinase and a glycogen metabolism enzyme involved in several NF- κ B signaling pathways. It has been proposed as a new therapeutic target for various cancers due to its essential roles in tumor proliferation and resistance to apoptosis [6]. Data from a recent study showed that inhibition of GSK3B using a small molecule (9ING41) significantly induced apoptosis in SKOV3 and OVCA432 ovarian cancer cell lines and reduced *in vivo* tumor growth in mice [7]. However, it should be noted that inhibiting GSK3B alone can also play a pro-cancer role, promoting epithelial to mesenchymal transition (EMT; a measure of metastatic potential) and cancer stemness (a measure of resistance to chemotherapy) [21,22]. Hence, single inhibition of GSK3B is an imperfect method that needs a complementary method to overcome these adverse effects.

Histone deacetylases (HDACs) are epigenetic modulators that play a crucial role in carcinogenesis by promoting cancer stemness and EMT. HDACs were shown to interact with EMT transcription factors leading to the promotion of EMT in cancer cells [23]. Successful results using HDAC inhibitors ultimately led to clinical trials and FDA-approved treatments for various cancers, such as advanced lymphoma and metastatic cancers [24–26]. In a recent study, HDAC inhibitors were reported to be effective in killing ovarian cancer cells and reducing tumor growth by suppressing oncogene PAX8 expression in a mouse model [8]. Considering this background, we hypothesized that simultaneously inhibiting GSK3B and HDACs would prevent cancer cell growth while also inhibiting EMT and its effect on metastasis and chemoresistance.

In this study, we investigated the effect of first-in-class dual inhibitor agents of GSK3B and HDACs for the treatment of ovarian cancer. Our results showed that dual inhibition of GSK3B and HDACs significantly

reduced cancer cell survival both in human and mouse ovarian cancer cell lines. Among the developed dual inhibitor agents, APCS-540 showed the best anti-tumor activity. *In vitro* experiments using both human and mouse ovarian cancer cell lines also demonstrated that APCS-540 significantly reduced cancer cell migration and invasion, demonstrating its effectiveness in inhibiting the metastasis capacity. More importantly, APCS-540 displayed significant cytotoxic activity in cisplatin-resistant ovarian cancer cells, and remarkably reversed chemoresistance when combined with cisplatin. In an immunocompetent syngeneic mouse model, APCS-540 significantly improved survival by 66%. Additionally, our preliminary *in vitro* results in cervical and endometrial adenocarcinoma cell lines showed that APCS-540 may be effective in a wider spectrum of gynecologic malignancies.

In conclusion, we showed significant effectiveness of simultaneous targeting of the cancer epigenome and metabolism in preclinical cancer models. Our results suggest APCS-540 as a promising therapeutic drug for ovarian cancer, including the platinum-resistant disease.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygyno.2020.07.005>.

Author contribution

E.T. conceptualized and designed the study. E.T. and F.Z. performed the experiments, collected the data, analyzed the results, and wrote the manuscript. R.M. contributed to the design of the structure of the CSAP chemicals. M.E., B.Y.K., S.J.P., and S.O. supervised the experiments, reviewed the results, and participated in manuscript revisions. All authors reviewed the finalized manuscript and approved the submission.

Declaration of Competing Interest

M.E., R.M., and S.J.P. are co-inventors in the patent related to APCS-540 and they disclose their relationship with Avenzoar Pharmaceuticals (U.S. patent No. 10,029,997 B2 and U.S. patent No. 10,266,505 B2). S.O. and B.Y.K. have patents on gene signatures in ovarian cancer that could potentially be used to triage patients for targeted therapies (US010253368 and EU2908913). The remaining authors have no potential conflict of interest to declare.

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