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TITLE: Improving Immune Response in Ovarian Cancer by Modulating the Wnt Pathway

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14. ABSTRACT

Even though 80% of ovarian cancer patients will achieve a complete remission with a combination of surgery and chemotherapy, almost all will recur due to the development of chemoresistance. The WNT/beta-catenin pathway is involved in ovarian cancer growth and suppressing the ability of the immune system to fight off the cancer. Many ovarian cancers have a deficiency in the ability to repair its own DNA, called DNA repair deficiency. Recent clinical efforts have focused on using immune-directed therapies for the treatment of cancer, and specifically ovarian cancer. Although only a small subset of ovarian cancer patients respond to immunotherapy. Our goal to gain a better understanding of how DNA repair deficiency and upregulation of the WNT/beta-catenin pathway effect immune response and patient outcomes in ovarian cancer. Our preliminary data show that using a WNT inhibitor for the treatment of ovarian cancer in a mouse model improves the immune system's ability to fight off the cancer. Our central hypothesis is that inhibition of the WNT/beta-catenin signaling pathway will promote antitumor immune response and repress tumor growth, thereby improving clinical response. WNT/beta-catenin genes regulate cell proliferation, thereby mediating cancer initiation and progression and we have successfully targeted this pathway in cells isolated from patients with ovarian cancer and shown that a WNT inhibitor downregulates the WNT pathway and, in a subset of patient samples, caused cell kill. This project will examine the inhibition of the WNT/beta-catenin pathway using a mouse model of spontaneously developing ovarian cancer. In addition, we will also implant ovarian cancer cells with and without DNA repair deficiency into mice that have an intact immune system. The specific aims of this project are: 1) To determine the relationship between WNT/beta-catenin signaling, the DNA repair pathway, T cell responses and clinical outcomes in ovarian cancer. 2) To determine how inactivation of the WNT pathway by the tumor and dendritic immune cells impacts T cell responses and tumor growth using mouse models. 3) To determine whether mutations that affect DNA repair impact T cell responses following treatment with WNT inhibitors. The proposed research is significant because it will investigate the role of WNT-mediated T cell exclusion from the tumor and elucidate the therapeutic potential of novel WNT inhibitors against ovarian cancer cells with and without a mutation that causes deficiency in the DNA damage repair mechanism.

15. SUBJECT TERMS

None listed

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1. **INTRODUCTION:** Ovarian cancer is the fifth leading cause of death in women in the USA and the most lethal gynecologic malignancy. Given the poor overall survival, high recurrence rates, and rapid development of resistance to chemotherapy in HGSOc, we urgently require new methods to treat this disease. In addition to classic prognostic factors like stage and debulking status, mutations in *BRCA1* and *BRCA2*, components of the homologous recombination (HR) DNA repair pathway, are linked to long-term prognosis in HGSOc. *BRCA1/2* mutations are found in ~20% of HGSOc tumors and are associated with improved prognosis. Interestingly, most BRCA-deficient tumors have activated tumor-infiltrating lymphocytes (TILs), which are also linked to improved outcomes. In contrast, immunosuppressive T regulatory cells (Tregs) and tumor-associated macrophages (TAMs), correlate with a worse prognosis. Despite the link between TILs and positive clinical outcomes, the use of immune checkpoint inhibitors (ICIs) in HGSOc has been disappointing in part due to the “cold” immune landscape surrounding these tumors. A “cold” immune landscape is characterized by the lack of TILs, which sensitize tumors to ICI. Thus, in order to develop better treatments for HGSOc, we need to understand the mechanisms that regulate anti-tumor immune responses, including the role of BRCA1/2 and the HR DNA repair pathway. WNT/ β -catenin signaling is also linked to the progression of HGSOc by directly triggering tumor growth (1, 23), and promoting resistance to platinum agents. Interestingly, melanoma-intrinsic activation of the WNT pathway leads to the exclusion of TILs and immune escape by repressing local chemokine expression. In addition, WNT/ β -catenin signaling in DCs triggers the expression of immunosuppressive molecules like IL- 10, TGF β and RALDH, which in turn promote the differentiation of Tregs. Given that infiltrating Tregs and TAMs correlate with a worse prognosis in HGSOc, and that these cells are associated with WNT/ β -catenin signaling in other tumors, it is likely that WNT signaling in ovarian cancer also leads to immune suppression. In fact, expression data from 8890 tumor samples (including HGSOc) in The Cancer Genome Atlas (TCGA), show that activating mutations in the WNT/ β -catenin pathway, such as *CTNNB1*, encoding β -catenin, and inactivating mutations in negative regulators, such as Axin1, Axin2, APC1, and APC2, are inversely related to inflammatory T cell gene expression signatures. Thus, the purpose of our research is to investigate the inhibition of WNT/ β -catenin signaling and its effects on tumor growth and anti-tumor immune responses in ovarian cancer patients with an intact or altered HR DNA repair pathway.
2. **KEYWORDS:** Ovarian cancer, murine ovarian cancer, WNT, β -catenin, WNT inhibition, tumor microenvironment, ID8 cells, ID8p53^{-/-} cells, PORCN, WNT-974, CGX-132, DKK1, DKN-01
3. **ACCOMPLISHMENTS:**

- What were the major goals of the project?

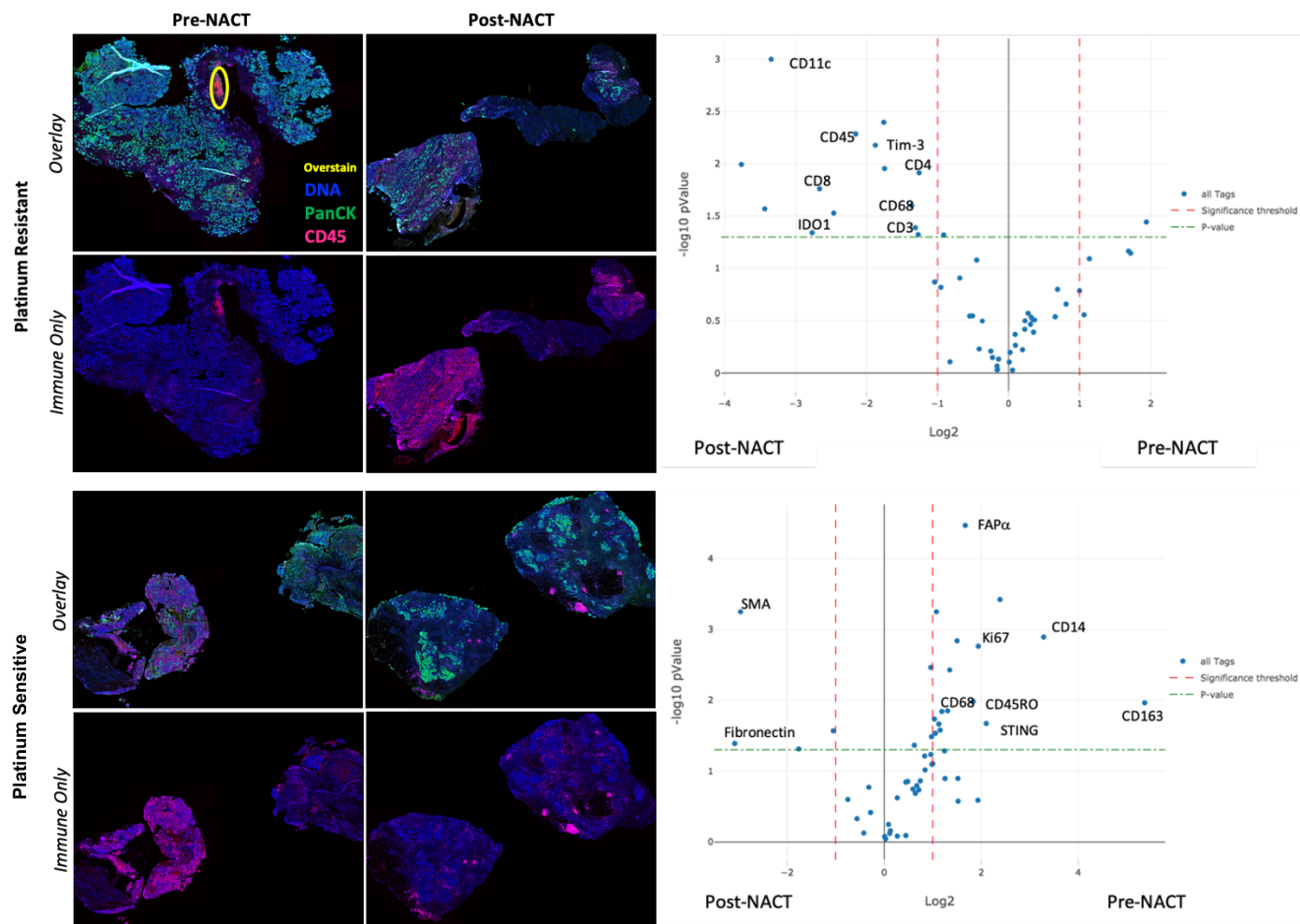
	Timeline (Months)	% of Completion
Specific Aim 1		
Major Task 1: Obtain RNA sequencing results from 917 patients from GOG 218	2	50
Major Task 2: Categorize the patients into T cell inflammation subtype, WNT pathway score, HRD status, correlate with survival stratified by treatment	5	75
Milestone(s) Proposed: (1) RNA sequencing data downloaded for 917 patients with HGSOc (2) The relationship between WNT signaling, DNA repair pathway, T cell inflammation subtype and clinical outcomes in HGSC		
Specific Aim 2		
Major Task 1: Determine whether inhibition or activation of the WNT pathway impacts T cell response	9	75

Major Task 2: Create a CRISPR/Cas9-mediated β -catenin knockout model and access T cell response	12	75
Milestone(s) Proposed: (1) Identify the T cell response and tumor response in mice treated with a T cell inhibitor and T cell activator (2) Validate the role of β -catenin in T cell and dendritic cell response		
Specific Aim 3		
Major Task 1: Evaluate the number and types of T cells that are present after treating BRCA2 mutated and wild-type cells	32	75
Milestone(s) Proposed: Determine the difference between immune response to WNT inhibition and activation in cells with and without HR DNA repair deficiency		

- What was accomplished under these goals?

Specific Aim 1							
Major Task 1: Obtain RNA sequencing results from 917 patients from GOG 218							
Subtask 1: Identify location of data							
Subtask 2: Download data in a format that can be transferred to Hudson Alpha Institute for analysis							
Goals not met: We are still attempting to secure data from GOG 218 trial.							
Major Task 2: Categorize the patient data into T cell inflammation subtype, WNT pathway score, HRD status, correlate with survival stratified by treatment							
Subtask 1: Create categories based on the ovarian TCGA analysis of “hot” and “cold” tumors							
Since we have yet to obtain data from the GOG 218 trial, we decided to move forward with an analysis of matched tumor samples from individual patients with high-grade serous ovarian cancer (HGSOC) pre- and post-neoadjuvant chemotherapy (pre- and post-NACT, respectively) (18-Study). We were able to obtain unstained slides from formalin-fixed paraffin-embedded (FFPE) blocks from each timepoint from 18 patients and sent them to NanoString for GeoMx Digital Spatial Profiling (DSP) Analysis using their Human Immuno-Oncology Protein Panel. This panel allowed for high-plex and high-throughput spatial profiling that enabled us to rapidly and quantitatively assess over 70 proteins, highlighted in table below, of interest simultaneously.							
Immune Cell Profiling Panel		IO Drug Target Panel	Immune Activation Status Panel	Immune Cell Typing Panel	Pan-Tumor Panel	Cell Death Panel	PI3K/AKT Signaling Panel
CD3	CTLA-4	4-1BB CD137	CD25	CD14	Bcl-2	BAD	p-AKT (S473)
CD4	Fibronectin	ARG1	CD27	CD34	EpCAM	BCL6	p-GSK (S9)
CD8	GZMB	B7-H3	CD40	CD45RO	ER alpha	BCLXL	p-GSK3ab (S21/S9)
CD11c	HLA-DR	GITR	CD44	CD66b	HER2	BIM	INPP4B
CD20	Ki-67	IDO1	CD80	CD163	MART1	CD95/Fas	MET
CD45	Pan-cytokeratin	LAG3	CD127	FAPalpha	NY-ESO-1	Cleaved caspase 9	Pan-AKT
CD56	PD-1	OX40L	ICOS	FOXP3	PR	GZMA	PLCG1
CD68	PD-L1	STING	PD-L2		OTEN	NF1	p-PRAS40 (T246)
	SMA	Tim-3			S100B	p53	p-Tuberin (T1462)
		VISTA				PARP	
DSP allows for 6 regions of interest (ROIs) to be further segmented into tumor (PanCK+CD45-) and immune (PanCK-CD45+) areas resulting in 6 tumor and 6 immune areas with 70 protein readouts per area. The innovation behind DSP is the ability to quantitate tumor vs. immune markers of interest, capture heterogeneity across patient tissue, and visualize the spatial distribution of tumor and immune cells. The immunosuppressive proteins of interest included (CTLA-4, PD-1, PD-L1, ARG1, Lag3, Tim-3, CD25, CD163 and FOXP3). Furthermore, we were assisted by NanoString's TAP Team lead by trained scientists and research staff to perform and aid in DSP data analysis. Due to the expensive nature of this analysis we were only able to send in 2 patients matched pre- and post-NACT tissue; one patients was deemed platinum sensitive (PS) and 1 patient platinum							

resistant (PR). **Conclusion:** PS patient had low WNT activity with high expression of interferon (STING), immune memory (CD45RO,) and proliferation (Ki67) markers in pre-NACT. The PR patient had high WNT activity in pre-NACT tissue, and elevated immune exhaustion (Tim-3 and IDO1) in their post-NACT tissues. The PS patient did not have an increase in these markers in response to NACT. However, the PS patient did have increased.



Subtask 2: Analyze the previously collected data (BROCA-HR assay) as it relates to the “hot” and “cold” signature

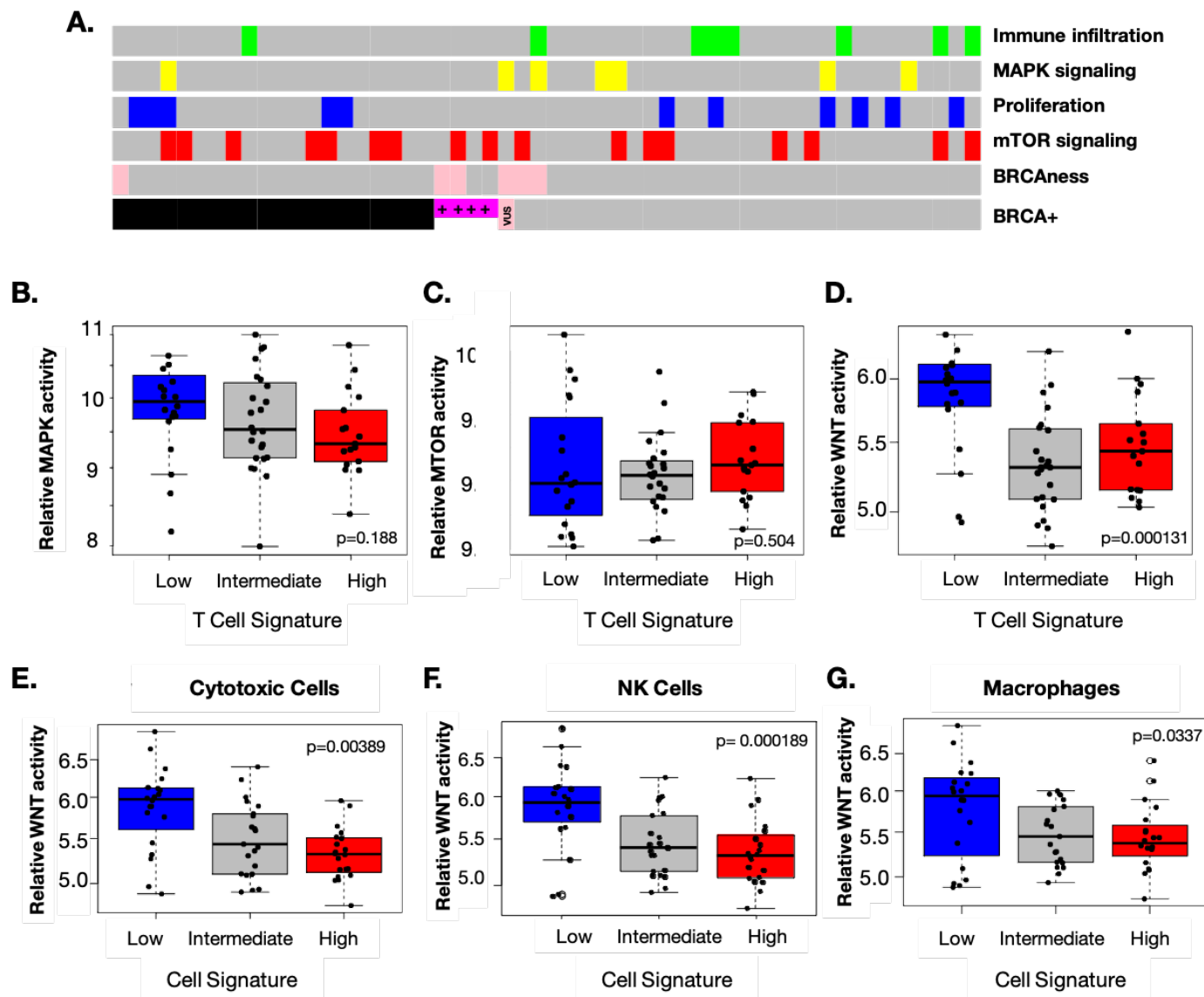
Subtask 3: Analyze the RNA sequencing data pertinent to HRD status

The HGSOC patient samples we were able to analyze from the pre- and post-NACT timepoint were also screened for HR status. Unfortunately, many of the patients had unknown HR status, potentially due to the lack of HR testing at the time of tissue collection. However, of the patients who had known status (n=11), only 25% of patients whose WNT signaling increased in response to NACT were BRCA(+) = HRd, and 34% of patients with high WNT signaling post-NACT were BRCA+.

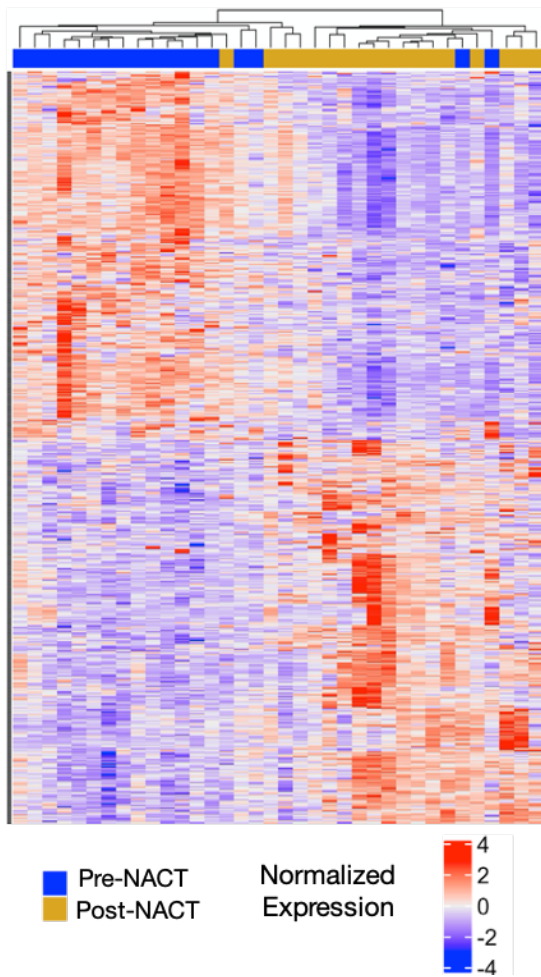
Subtask 4: Categorize patient data based on WNT pathway gene expression

Transcriptional signatures were used to identify tumor tissues with evidence of alterations in (A) immune infiltration, MAPK signaling, increased proliferation, mTOR signaling, BRCAness, and/or BRCA+. Activity of (B) MAPK, (C) mTOR, and (D) WNT pathways was compared to T cell Infiltration Signature. A more detailed analysis of specific immune cell types estimated from transcriptional profiles using CIBERSORT was performed to calculate association of WNT signaling activity is negatively associated with infiltration of (E) cytotoxic cells, (F) natural killer (NK) cells, and (G) macrophages. **Conclusion:** WNT signaling was the only pathway that inversely correlated with

T cell infiltration signature, and it also inversely correlated with cytotoxic, NK, and macrophage infiltration.

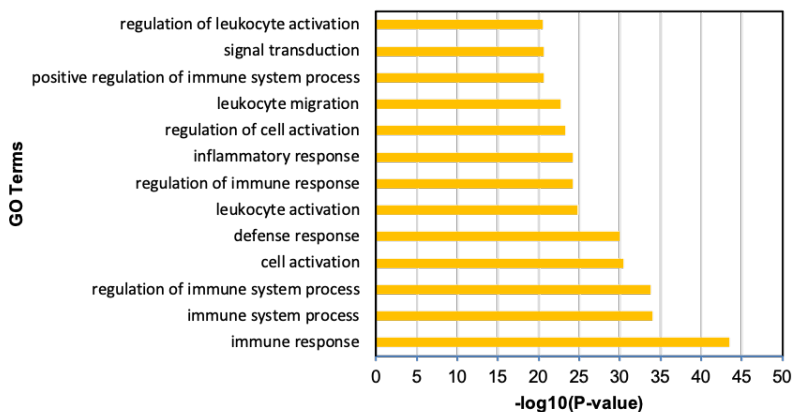


For 18 patients from which we collected treatment naïve tissue (pre-NACT) and transcriptomic data, we also analyzed tissue collected at the time of resection after 3-6 cycles of NACT (post-NACT). Heatmap of transcriptomic profiles from top differentially expressed genes (DEGs) (FDR<0.05) associated with NACT. **Conclusion: NACT causes significant alterations in gene expression profiles in HGSOC.**

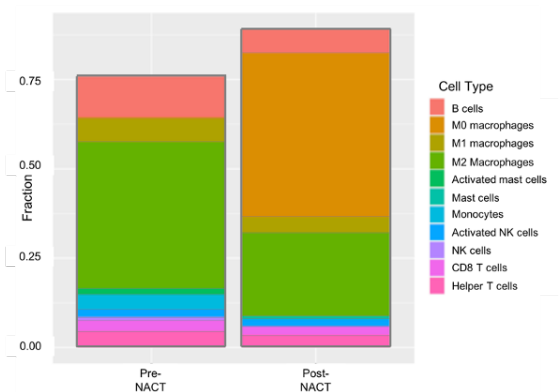


DEGs from pre- and post-NACT tumor samples were used for (A) pathway analysis. (B) Immune cell types/fractions were estimated from bulk RNA-seq data. **Conclusions: NACT enriched genes associated with immune response and activation pathways. M0 (inactive) macrophage population was the only immune cell population that increased in response to NACT in our 18 matched pre- and post-NACT patient samples.**

A.



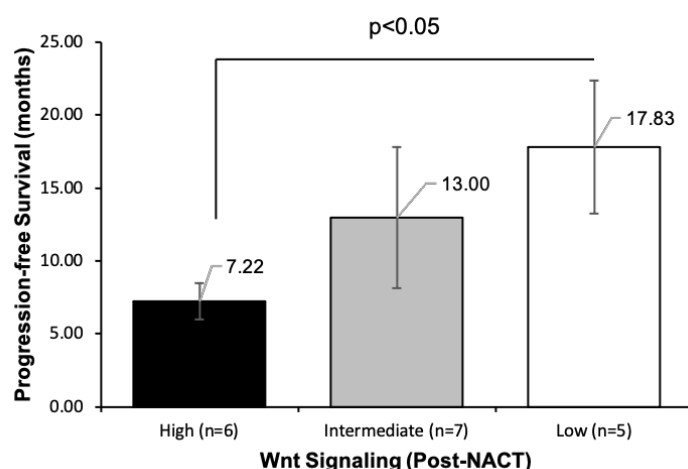
B.



Subtask 5: Combine the data collected in subtask 1-4 and correlate to patient outcomes and treatment

In our samples from 18-Study, we calculate WNT activity score (high, intermediate, or low) from gene expression profiles. We calculated a score that represents WNT and immune signatures from gene expression data. First, we used the variant StabilizingTransformation function in the DESeq2 package to normalize all data. Next, we calculated a score for WNT signaling by determining the median normalized gene expression levels of all genes associated with WNT signaling function.

Conclusion: HGSOc patients who had increased WNT signaling in response to NACT had decreased PFS compared to patients with decreased WNT signaling.



Goals not met: Once GOG 218 data is received, we can perform proposed tasks with that data.

Specific Aim 2

Major Task 1: Determine whether inhibition or activation of the WNT pathway impacts T cell response

Subtask 1: Breed enough female *TgMISIR-Tag-Low* mice (n=40)

Subtask 2: Inject mice with MOVCAR-luc cells

Subtask 3: Treat mice with vehicle control, WNT974, WNT7A (n=10 in each group) with one untreated group. (DKN-01 used as surrogate for WNT7a)

Subtask 4: Sac 5 mice at day 14 and the remainder on day 28 and quantify tumor-specific CD8+ T cells, test whether the T cells are activated, and send whole tumor for RNA seq

Subtask 5: Dissociate fresh tumor tissue and sort-purify cells into CD3/4+ or CD3/8+ T cells

Subtask 6: Extract RNA from sorted cells and use TCR repertoire sequencing

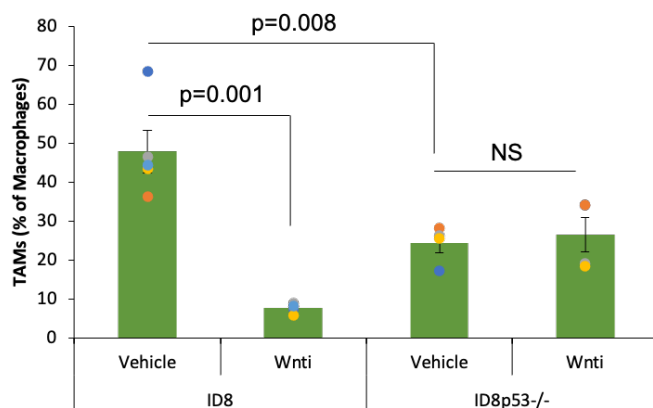
Goals not met: We have not been able to complete TCR repertoire sequencing.

Major Task 2: Create a CRISPR/Cas9-mediated β -catenin knockout model and assess T cell response

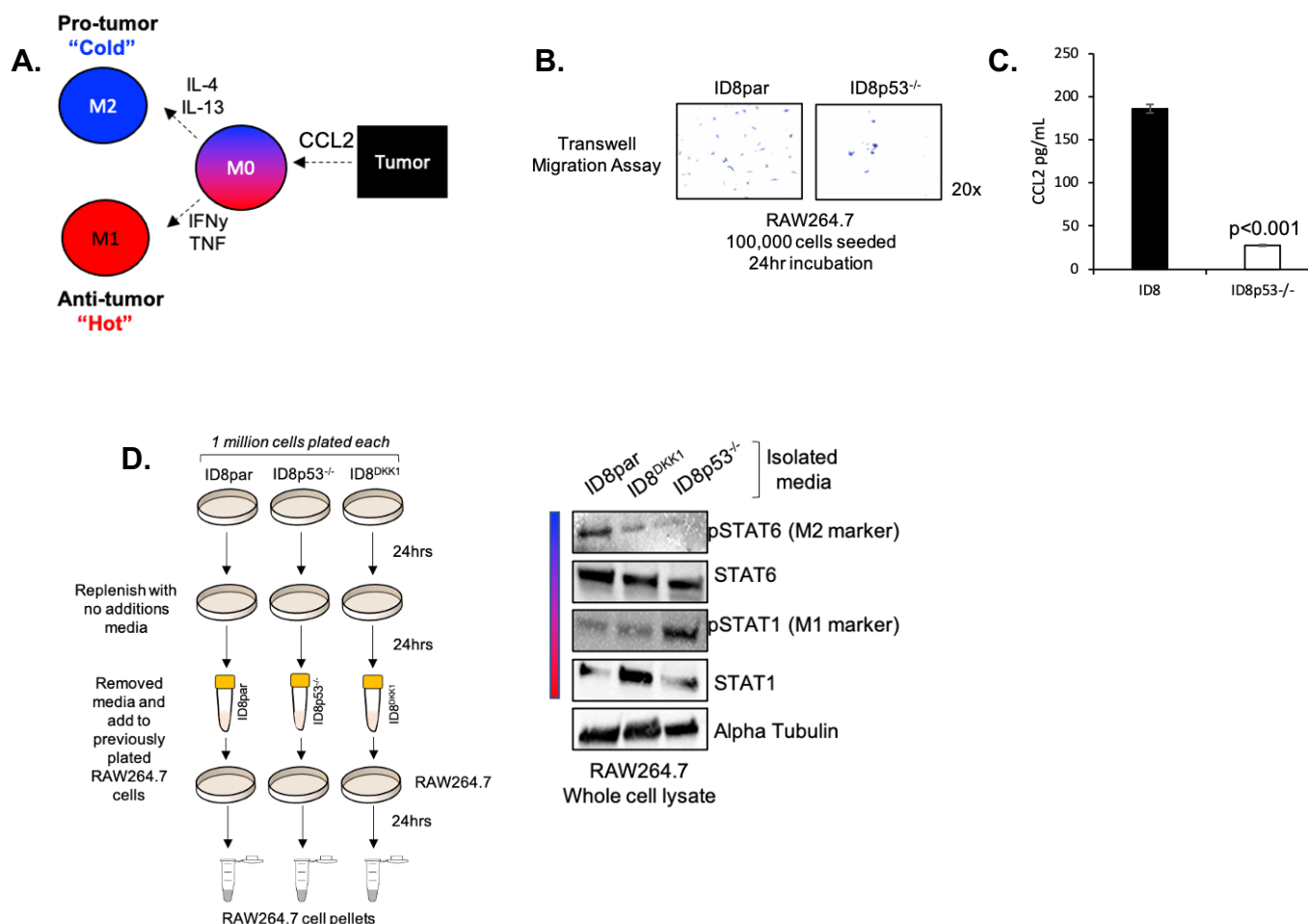
Subtask 1: Create MOVCAR knockout cell line

Subtask 2: Compare T cell infiltration, proportion of TAMs, proportion of Tregs, presence of CD103+ dendritic cells in the β -catenin knockout model compared to wild-type. (ID8p53^{-/-} model was used as a surrogate for β -catenin knock-out due to its decreased β -catenin expression compared to ID8 model)

Flow cytometry (FACS) analysis of total tumor-associated macrophages (CD45+F4/80+CD206+) presence in the omenta of female, C57BL/6 mice injected with either ID8 or ID8p53^{-/-} cells treated with vehicle or WNT inhibitor (WNTi). **Conclusion: ID8 model had elevated TAM infiltration in omenta compared to ID8p53^{-/-} model. In addition, WNT inhibition only resulted in decreased TAM infiltration in ID8 model compared to ID8p53^{-/-} model.**



Macrophage (A) recruitment and polarization in the tumor microenvironment (TME). We performed (B) transwell migration assays to measure macrophage migration in response to isolated ID8 model media, as well as (C) ELISA assays to determine the level of CCL2, macrophage chemokine, in the isolated media from ID8 models. In addition, we performed (D) western blot analyses on macrophages that had been exposed to isolated media from ID8 models to determine protein expression of pSTAT6 (M2 macrophage) and pSTAT1 (M1 marker). **Conclusion: ID8 cells with high WNT activity had enhanced CCL2 secretion and enhanced macrophage migration compared to ID8p53^{-/-} cells with low WNT activity. In addition, isolated media from ID8 cells increased macrophage polarization towards an M2-like state compared to isolated media from ID8p53^{-/-} cells shifting macrophages more towards an M1-like state.**



Goals not met: We recently got our *TgMISIIR-Tag-Low* colony back and will continue analyses proposed with this model. However, we have found that our ID8p53^{-/-} mice have low beta-catenin compared to ID8 parental cells and will use this model to substitute for beta-catenin knockout model.

Furthermore, we need to further profile macrophage subtypes in response to Wnt signaling alteration in our models.

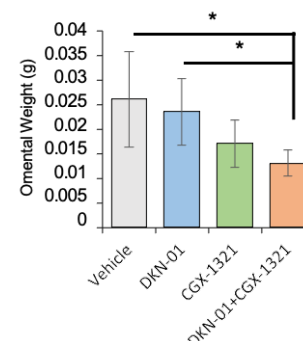
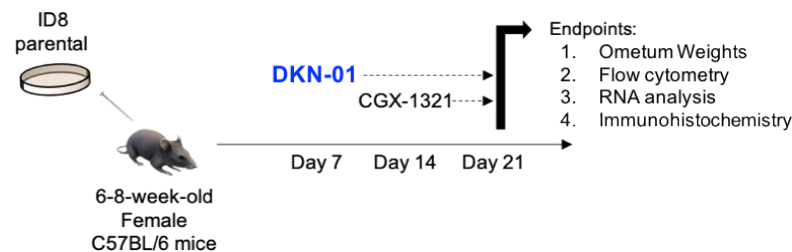
Specific Aim 3

Major Task 1: Evaluate the number and types of T cells that are present after treating BRCA2 mutated and wild-type cells

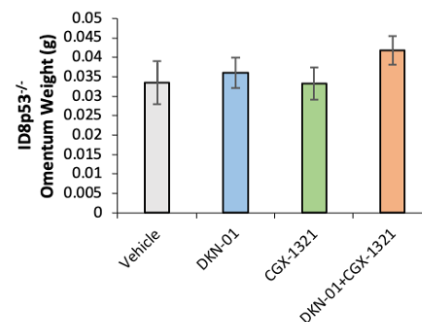
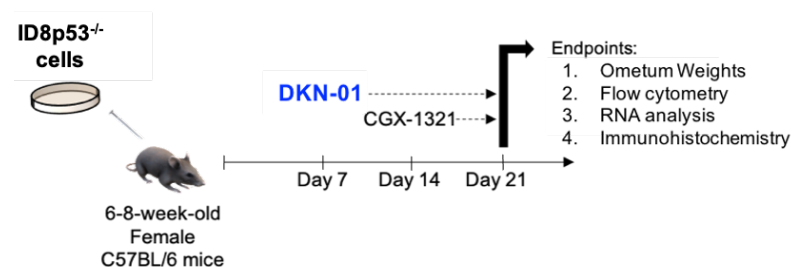
Subtask 1: Implant C57BL/6 mice with ID8 cells that have been modified using CRISPR/Cas9 to lack TRP53 or both TRP53 and BRCA2.

Subtask 2: Treat mice with vehicle control, WNT-974*, WNT7A** (n=10 in each group) with one untreated group (*CGX-1321 was used as a surrogate for WNT-97; **DKN-01 was used as a surrogate for WNT7A)

Schema of ID8 model sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week), n=8 per treatment arm and day 21 tumor burdens measured by omental weight (g). **Conclusion: sequential therapy with DKN-01 and CGX-1321 in ID8 model resulted in decreased tumor burden in combo treatment group compared to DKN-01 and vehicle monotherapy groups.**

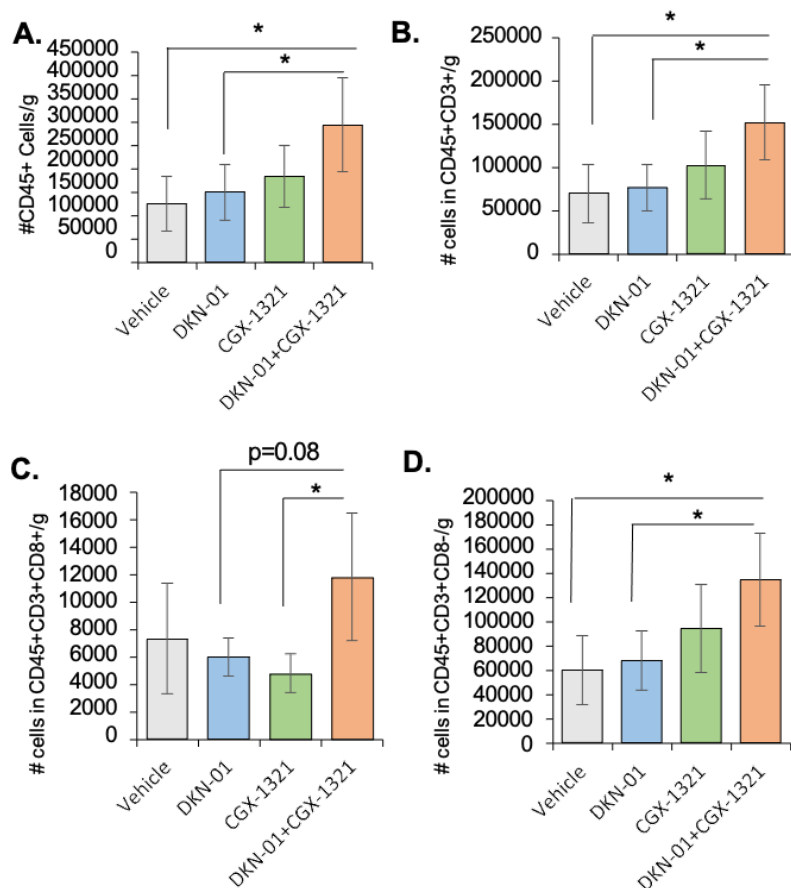


Schema of ID8p53^{-/-} model sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week), n=8 per treatment arm and day 21 tumor burdens measured by omental weight (g). **Conclusion: sequential therapy with DKN-01 and CGX-1321 in ID8p53^{-/-} model did not result in decreased tumor burden in combo treatment group compared to DKN-01 and vehicle monotherapy groups.**

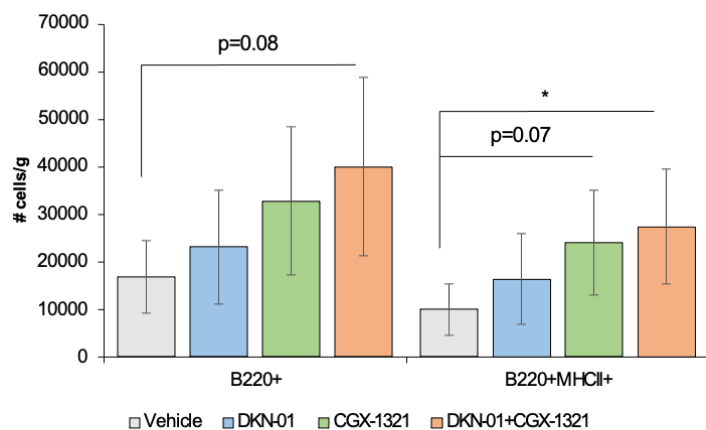


Subtask 3: Quantify and sort CD8⁺ T cells, CD4⁺ T cells, and dendritic cells

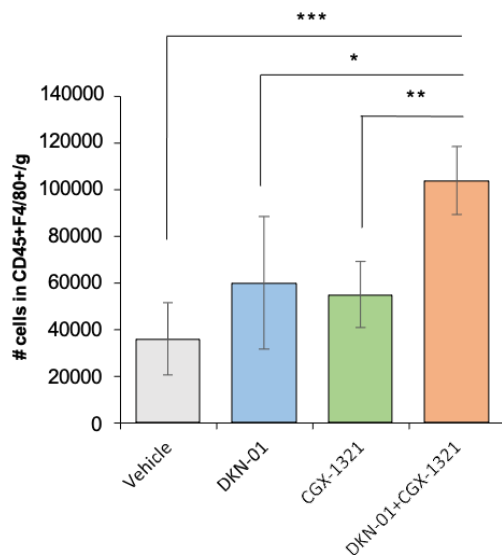
Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model (schema same as above), n=8 per treatment arm. FACS analysis of total (A) immune cells (CD45⁺), (B) T cells (CD45⁺CD3⁺), (C) CD8⁺ T cells (CD45⁺CD3⁺CD8⁺), and (D) presumptive CD4⁺ T cells (CD45⁺CD3⁺CD8⁻) presence in the omenta of mice. **Conclusion: sequential therapy of DKN-01 and CGX-1321 resulted in increased immune infiltration and T cell populations in combo treatment group versus vehicle and monotherapy groups.**



Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. FACS analysis of total B cell (CD45+B220+) and activated B cell (CD45+B220+MHCII+) presence in the omenta of mice. **Conclusion: sequential therapy of DKN-01 and CGX-1321 resulted in increased total B cell and activated B cells in combo treatment group compared to vehicle and DKN-01 monotherapy groups.**

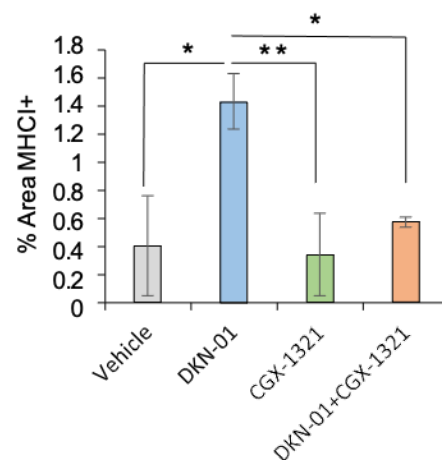
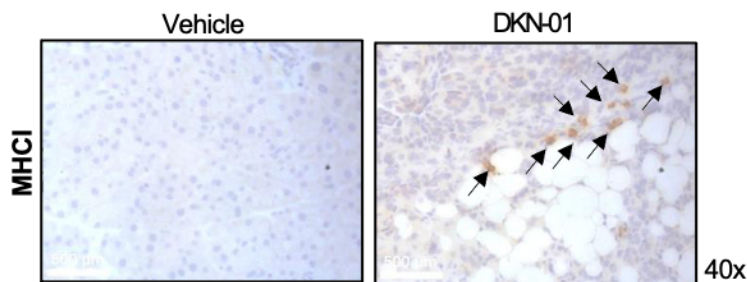


Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. FACS analysis of total macrophage (CD45+F4/80+) presence in the omenta of mice female, C57BL/6 mice 21-days post tumor injection. **Conclusion: sequential therapy of DKN-01 and CGX-1321 resulted in increased macrophages in omenta.**

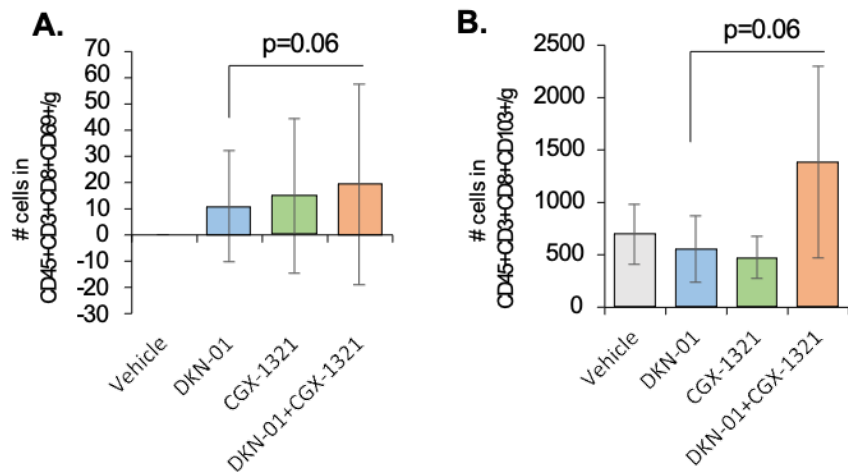


Subtask 4: Monitor T-cell function

Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. Immunohistochemistry (IHC) demonstrates of MHCII expression on ID8 cells with DKN-01 administration (*p<0.05). **Conclusion: DKN-01 treatment in ID8 model results in increased MHCII staining, suggesting this treatment can increase tumor-immune recognition.**

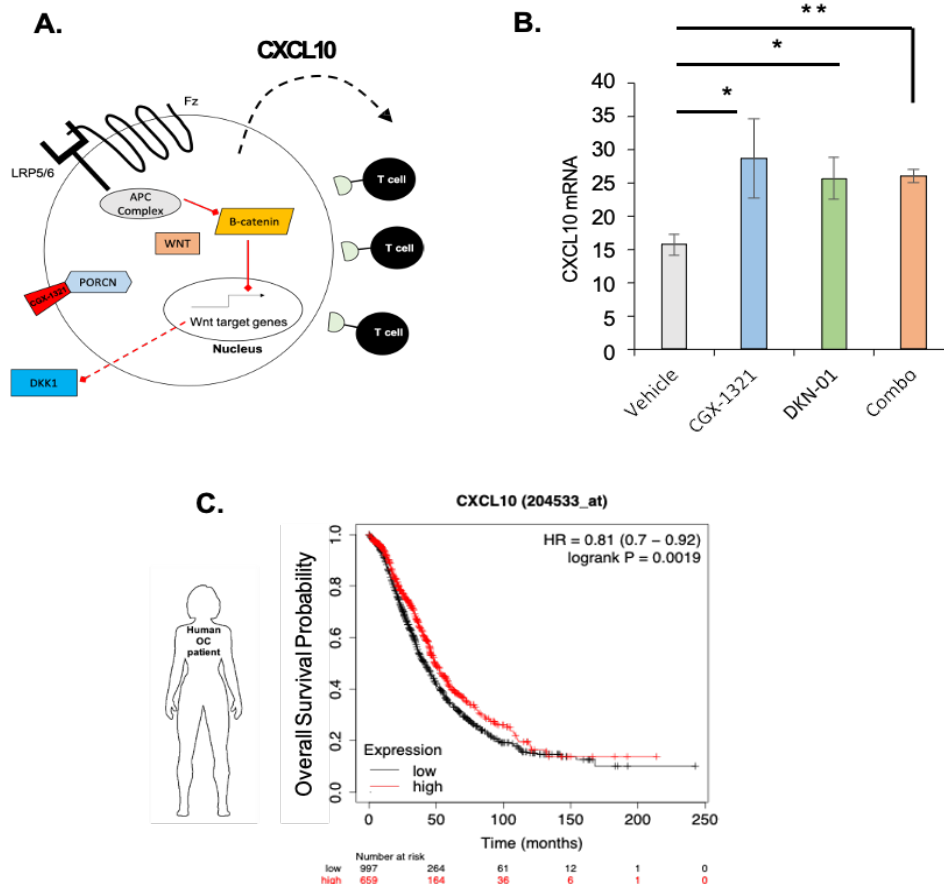


Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. FACS analysis of abundance of activated CD8+ T cells (A) CD8+CD69+ and (B) CD3+CD8+CD103+ in omenta of mice in response to treatments. **Conclusion: sequential therapy of DKN-01 and CGX-1321 resulted in increased CD8+ T cell activation compared to monotherapy groups.**



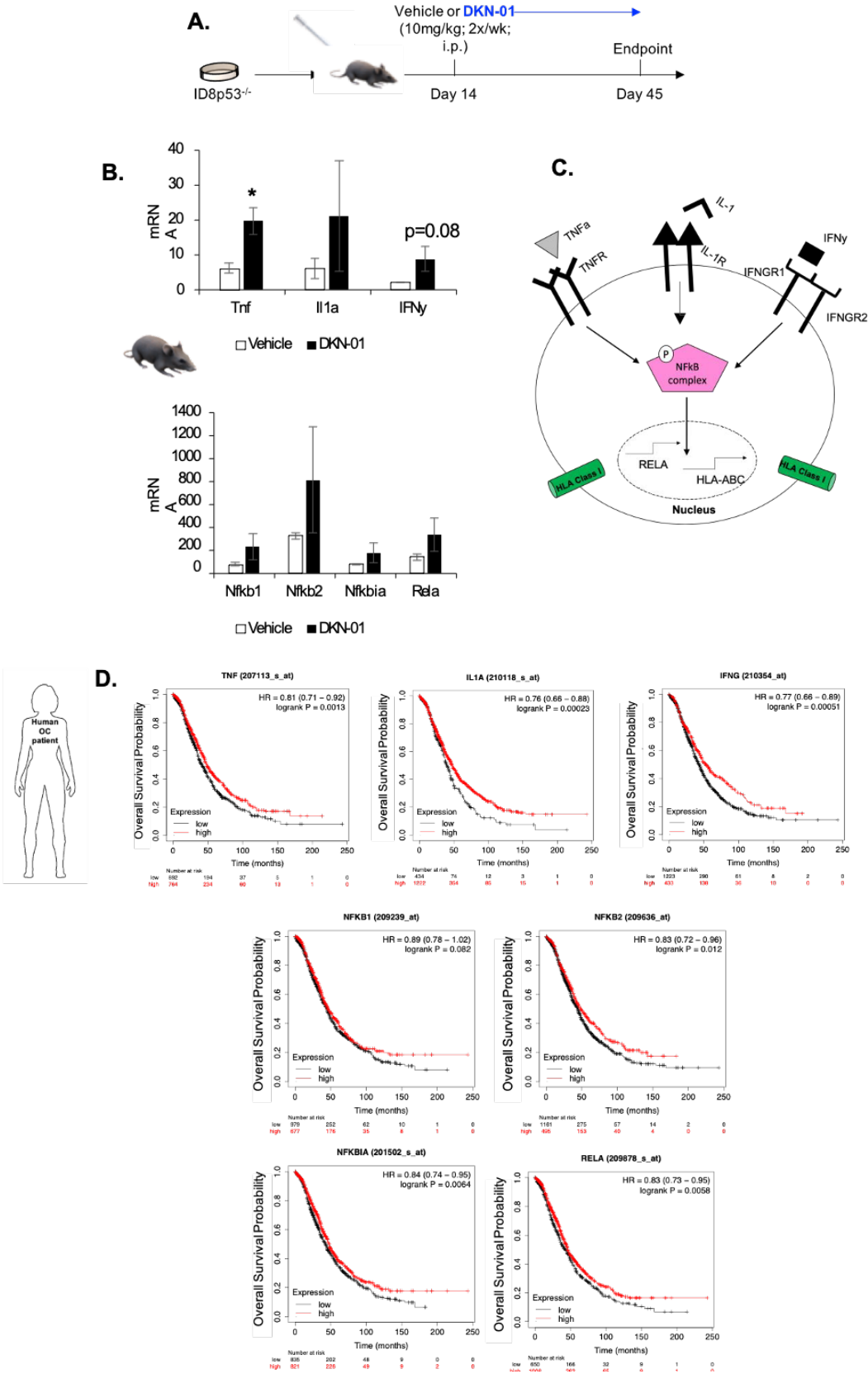
Subtask 5: Monitor gene expression by RNA seq (WNT, immune, HRD signatures)

Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. **(A)** Tumor expression of chemotactic factor CXCL10 stimulates T cell infiltration to TME. **(B)** RNAseq data from omenta. **(C)** Kaplan Meier (KM) curve of CXCL10 expression effects on ovarian cancer (OC) overall survival (OS). **Conclusion: sequential therapy increases CXCL10 (*p<0.05, **p=0.01) mRNA expression in tumors compared to vehicle. KM curve shows increased OS in OC patients with high expression of CXCL10.**



Schematic of **(A)** treatment timeline with either vehicle (n=2) or DKN-01 (n=3) monotherapy. **(B)** RNAseq data of TNF, IL-1a, IFN γ , NF κ B, and RELA mRNA expression in response to DKN-01 therapy. **(C)** TNF, IL-1, and IFN γ signaling activation of NF κ B increases HLA transcription. **(D)**

Kaplan-Meier curves of OS in OC patients with high expression of the indicated factors.
Conclusion: DKN-01 up-regulates signaling pathways related to HLA stabilization in a syngeneic ID8p53^{-/-} murine model. These signaling components correspond to increased OC patient OS.



Subtask 6: Extract RNA and use the iRep technique

Goals not met: We need to investigate our findings on the effects of WNT signaling alteration in the ID8 model with homozygous BRCA2 knock-out.

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

My bi-monthly, one-on-one lab meetings with Dr. Birrer, Dr. Odunsi, and myself discussing my career development and research progress continued in this year of funding. In addition, my weekly lab meetings with Dr. Sara Cooper from HudsonAlpha and Dr. Troy Randall from UAB continued to discuss on-going projects (*as previously outlined in my 2020 Annual Progress Report*). Furthermore, I started weekly meetings with Dr. Lyse Norian who is one of my collaborators and another expert in the field of immunology to help with manuscript preparations and grant submissions. My lab members and myself have each attended webinars and sought expertise on virtual platforms that can be used to investigate large datasets (e.g., cBioPortal). In addition, we have optimized two systems, bioreactor and 3-D magnetized spheroids, that we are currently using as models to study patient-derived tissues and their tumor-intrinsic and immune responses to targeted and immune checkpoint blockade (ICB) therapies.

I have continued serving on committees/positions at both the institutional and national levels. In addition, I am still fostering collaborations with investigators and physicians both at UAB and other institutions (*as previously outlined in my 2020 Annual Progress Report*).

Gynecologic oncology clinical fellow, Dr. Jhalak Dholakia was trained on this grant. She had the opportunity to perform basic science research, exploring the role of the WNT signaling pathway in progression of ovarian cancer and how it effects immune response in the tumor microenvironment. In addition, she wrote a review and research article pertaining to her projects. She was required to present her research findings at weekly lab meetings and once each semester to the Department of OB/GYN – Gynecologic Oncology Division. Furthermore, she presented her research project with an oral presentation at SGO. Furthermore, I obtained a postdoctoral research fellow, Dr. Carly Bess Scalise, who has a background in cell and molecular pharmacology and immunology and fellowship training in immunotherapy and clinical trials.

I have continued to mentor several other PhD, MD, and MD/PhD students directly and indirectly, each with presentations at local and/or national conferences.

- **How were the results disseminated to communities of interest?**

The results from these studies were presented at local and national conferences to audiences that are in similar areas of research and/or medicine.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Specific Aim 1

Major Task 1: Obtain RNA sequencing results from 917 patients from GOG 218

Subtask 1: Identify location of data

Subtask 2: Download data in a format that can be transferred to Hudson Alpha Institute for analysis

Proposed experiments: We are awaiting data from the GOG 218 trial, to perform the same analyses as in Major Task #2.

Major Task 2: Categorize the patient data into T cell inflammation subtype, WNT pathway score, HRD status, correlate with survival stratified by treatment

Subtask 1: Create categories based on the ovarian TCGA analysis of “hot” and “cold” tumors
Subtask 2: Analyze the previously collected data (BROCA-HR assay) as it relates to the “hot” and “cold” signature
Subtask 3: Analyze the RNA sequencing data pertinent to HRD status
Subtask 4: Categorize patient data based on WNT pathway gene expression
Proposed Experiments: Analyze the GOG 218 (once acquired) to determine “hot” and “cold” signatures, RNA sequencing data as it related to HRD status, and categorize data based on WNT pathway gene expression.

Specific Aim 2

Major Task 1: Determine whether inhibition or activation of the WNT pathway impacts T cell response
Subtask 1: Breed enough female TgMISIIR-Tag-Low mice (n=40)
Subtask 2: Inject mice with MOVCAR-luc cells
Subtask 3: Treat mice with vehicle control, WNT974, WNT7A (n=10 in each group) with one untreated group
Subtask 4: Sac 5 mice at day 14 and the remainder on day 28 and quantify tumor-specific CD8+ T cells, test whether the T cells are activated, and send whole tumor for RNA seq
Subtask 5: Dissociate fresh tumor tissue and sort-purify cells into CD3/4+ or CD3/8+ T cells
Subtask 6: Extract RNA from sorted cells and use TCR repertoire sequencing
Proposed Experiments: Moving forward, we need to complete TCR repertoire sequencing.
Major Task 2: Create a CRISPR/Cas9-mediated β-catenin knockout model and assess T cell response
Subtask 1: Create MOVCAR knockout cell line
Subtask 2: Compare T cell infiltration, proportion of TAMs, proportion of Tregs, presence of CD103+ dendritic cells in the β-catenin knockout model compared to wild-type
Proposed Experiments: Moving forward, we need to further profile macrophage subtypes in response to WNT signaling alteration in our models and elucidate the mechanisms behind WNT-mediate macrophage recruitment and polarization

Specific Aim 3

Major Task 1: Evaluate the number and types of T cells that are present after treating mutated and wild-type cells
Subtask 1: Implant C57BL/6 mice with ID8 cells that have been modified using CRISPR/Cas9 to lack TRP53 or both TRP53 and BRCA2
Subtask 2: Treat mice with vehicle control, WNT974, WNT7A (n=10 in each group*4 groups/ cell line) with one untreated group.
Subtask 3: Quantify and sort CD8+ T cells, CD4+ T cells, and dendritic cells
Subtask 4: Monitor T-cell function
Subtask 5: Monitor gene expression by RNA seq (WNT, immune, HRD signatures)
Subtask 6: Extract RNA and use the iRep technique
Proposed Experiments: Further characterize the immune responses by specifically looking at immune cell functional markers in more immune cell populations. In addition, we need to investigate our findings on the effects of Wnt signaling alteration in the ID8 model with homozygous BRCA2 knock-out.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

The data obtained from these studies lay the foundation for treatment strategies that will optimize ovarian cancer patient response to immunotherapy and to determine whether WNT signaling inhibition could be beneficial in other treatment settings such as in combination with neoadjuvant chemotherapy (NACT).

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

The data obtained from these studies lay the foundation for treatment strategies that could enhance cancer patient immune response, and ultimately lead to improved therapeutic success via increased sensitivity to immunotherapy and/or decreased chemoresistance.

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

Nothing to Report.

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

Nothing to Report.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

In **Specific Aim 1**, we have yet to secure data from GOG 218 trial hence we moved forward with analyzing data from our 18-Study with pre- (treatment naïve) and post-NACT matched patient samples.

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

COVID-19 outbreak significantly limited in-lab research during the months of July 2020-December 2020. This also impacted in-person training on lab techniques and training. Furthermore, the SGO and ASCO conferences were virtual this year, and this platform limited collaborative conversations with other researchers and clinicians in the field.

- **Changes that had a significant impact on expenditures**

COVID-19 outbreak during the months of July 2020-December 2020 delayed the hiring of a research technician due to a hiring freeze implemented by UAB. This, in addition to last years unused funds due to COVID-19, provided us with enough monetary support for an additional research technician that officially joined the lab in May 2021. With the impact that COVID-19 has had on our research and expenditures since February 2020, we would like to request a 1-year extension of this award in order to continue our research projects into 2022.

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

Nothing to Report.

- **Significant changes in use or care of human subjects.**

Nothing to Report.

- **Significant changes in use or care of vertebrate animals.**

Nothing to Report.

- **Significant changes in use of biohazards and/or select agents.**

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

- **Journal publications.**

1. Wall JA, Meza-Perez S, Scalise CB, Katre AA, Londono, AI, Turbitt WJ, Randall TD, Norian LA, Arend RC. "Manipulating the Wnt/ β -catenin signaling pathway to promote anti-tumor immune infiltration into the TME to sensitize ovarian cancer to ICB therapy." *Gynecol Oncol*. 2020 Nov 7. PMID: 33168307.
2. Arend RC, Fisher AJ, Chou J, Jacobs I, Monk BJ. "Ovarian cancer: new strategies and emerging targets for the treatment of patients with advanced disease." *Crit Rev Oncol Hematol*. 2021 Jan 11. PMID: 33427569
3. Dholakia JJ, Scalise CB, Arend RC. "Assessing Preclinical Research Models for Immunotherapy for Gynecologic Malignancies." *Cancers (Basel)*. 2021 Apr 2. PMID: 33918476
4. Martinez A, Buckley M, Scalise CB, Katre AA, Dholakia JJ, Crossman D, Birrer MJ, Berry J, Arend RC. "Understanding the effect of mechanical forces on ovarian cancer progression." *Gynecol Oncol*. 2021 Apr 19. PMID: 33888338
5. Dholakia J, Scalise CB, Katre AA, Goldsberry WN, Meza-Perez S, Randall TD, Norian LA, Arend RC. "Sequential modulation of the Wnt/ β -catenin signaling pathway enhances tumor-intrinsic MHC I expression and tumor clearance." *Gynecol Oncol* – under review.
6. Martinez, A., Buckley, M.S, Scalise, C.B., Wang, D., Katre, A., Birrer, M.J., Berry, J., and Arend, R.C. (2021). Utilization of a 3-D tissue engineered model to investigate the effects of perfusion on gynecologic cancer biology. *Journal of Tissue Engineering* – under review.

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

Nothing to Report.

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

- Oral Presentations

- Dholakia J, Wall JA, Scalise CB, Katre AA, Arend RC. Towards a 'hot' tumor phenotype: DKN-01 sensitizes the tumor micro-

environment via pro-immune cell cytokine release in vitro and ex vivo. *Featured Oral Presentation*, SGO Annual Meeting, Virtual, March 2021.

- Arend RC, Castro C, Matulonis UA, Hamilton EP, Gunderson C, Lybarger KS, Goodman HM, Duska L, Mahdi H, ElNaggar AC, Kagey M, Barroilhet LM, Bradley WH, Sachdev J, Sirard C, O'Malley DM, Birrer MJ. Patients with recurrent gynecologic cancers and Wnt activating mutations demonstrated greater clinical benefit when treated with DKN-01 therapy. *Featured Oral Presentation*, SGO Annual Meeting, Virtual, March 2021.
- Poster Presentations
 - Arend RC, Castro C, Matulonis UA, Hamilton EP, Gunderson C, Lybarger KS, Goodman HM, Duska L, Mahdi H, ElNaggar AC, Kagey M, Barroilhet LM, Bradley WH, Sachdev J, Sirard C, O'Malley DM, Birrer MJ. Patients with recurrent epithelial endometrial cancers (EEC) and Wnt signaling alterations demonstrated greater clinical benefit when treated with DKN-01 monotherapy. *Poster Presentation*, AACR Virtual Special Conference on Endometrial Cancer: New Biology Driving Research and Treatment, November 2020.
 - Dholakia, J. and Arend, R. Immunotherapeutic potential of non-immunotherapy drugs. University of Alabama at Birmingham O'Neal Cancer Center Young Investigator Symposium, November 2020.
- Abstracts
 - Dholakia J, Arend RC. Modification of intrinsic ovarian cancer tumor factors by Wnt-pathway agents influences macrophage recruitment and activity. *Abstract*, ASCO Annual Meeting, Virtual, June 2021.
 - Martinez A, Buckley M, Berry J, Birrer MJ, Arend RC. Using 3D perfusion bioreactor system to studying cell biology of Ovarian Cancer. *Abstract*, ASCO Annual Meeting, Virtual, June 2021.
- Lectures:
 - "Immunotherapeutic Potential of Non-Immunotherapy Drugs: Turing Cold tumors Hot" *Mayo GYN Seminar Series* – October 30, 2020, Virtual.
 - "Invited Distillant, Focused Plenary VI: Pre-Clinical Innovation - Exploring New Horizons" *SGO Annual Meeting* – March 21, 2021, Virtual.
 - "Other Potential Biomarkers in Ovarian Cancer" *University of Colorado Reproductive Sciences Seminar* – April 6, 2021, Virtual.
 - "Immunotherapeutic Potential of Non-Immunotherapy Drugs: Turing Cold tumors Hot" *University of Colorado OB-Gyn Grand Rounds* – April 7, 2021, Virtual.

- “Invited Discussant, Gynecologic Cancer Poster Discussion Session: Using Biomarkers to Improve Outcomes in Ovarian Cancer” *ASCO Annual Meeting* – June 4, 2021, Virtual.

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

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

- ***Biospecimen collections:***

During the course of study, we have continued to bank multiple ovarian cancer tissues and matching blood from patients at UAB. These specimens will be used in patient-derived xenografts (PDX) and 3-dimensional (3-D) organoid models to further study the effects of WNT pathway alteration on immune and chemotherapy response.

- ***Clinical interventions:***

The results from this study provide evidence for the use of WNT and or DKK1 inhibitors as a means to enhance ovarian cancer's anti-tumor immune response. Importantly, these interventions could sensitize ovarian cancer to immune checkpoint blockade (ICB) therapy.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

		Status
Name:	Dr. Rebecca Arend	Unchanged from 2020 Annual Progression Report (APR)
	Dr. Michael Birrer	Unchanged from 2020 APR
	Dr. Kunle Odunsi	Unchanged from 2020 APR
	Dr. Troy Randall	Unchanged from 2020 APR
	Dr. Sara Cooper	Unchanged from 2020 APR
	Ashwini Katre	Unchanged from 2020 APR

Name:	Dr. Jhalak Dholakia
Project Role:	Clinical Gynecologic Oncology Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Dr. Dholakia has performed non-animal and animal experiments described in the study.
Funding Support:	N/A

Name:	Dr. Lyse Norian
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Norian has provided guidance in designing experiments, professional guidance and analyzing data.
Funding Support:	N/A

Name:	Dr. Carly Bess Scalise
Project Role:	Postdoctoral Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Dr. Scalise has aided in experimental design and data analysis.
Funding Support:	N/A

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

Nothing to Report.

- What other organizations were involved as partners?

8. Organization Name	LEAP Therapeutics
Location of Organization	Massachusetts
Partner's contribution to the project	
Financial support	N/A
In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);	Supplied DKN-01 monoclonal antibody for studies.
Facilities (e.g., project staff use the partner's facilities for project activities);	N/A
Collaboration (e.g., partner's staff work with project staff on the project);	N/A
Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site);	N/A
Other	N/A

Organization Name	Curegenix
Location of Organization	San Francisco Bay Area
Partner's contribution to the project	
Financial support	N/A
In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);	Supplied CGX-1321 compound for studies
Facilities (e.g., project staff use the partner's facilities for project activities);	N/A
Collaboration (e.g., partner's staff work with project staff on the project);	N/A
Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site);	N/A
Other	N/A

		Status
Organization	HudsonAlpha Institution for Biotechnology	Unchanged from 2020 APR

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**

Nothing to Report.

- **QUAD CHARTS:**

Nothing to Report.

9. APPENDICES:



Award Log Number: Improving Immune Response in Ovarian Cancer by Modulating the Wnt Pathway

PI: Rebecca C. Arend, University of Alabama at Birmingham, AL

Budget: \$1,074,771

Topic Area: Ovarian cancer WNT signaling

Mechanism: DOD Early Career Investigator Award

Research Area(s): Ovarian Cancer WNT/ β -catenin signaling

Award Status: 1 July 2020-30 June 2021

Study Goals: Study if the inhibition of WNT/ β -catenin signaling will repress tumor growth and promote anti-tumor immune responses, thereby improving clinical outcomes

Specific Aims:

- 1: To determine the relationship between WNT/ β -catenin signaling, the DNA repair pathway, T cell responses and clinical outcomes in ovarian cancer.
- 2: To determine how tumor-intrinsic and DC-intrinsic inactivation of the WNT pathway impacts T cell responses and tumor growth using immunocompetent mouse models of ovarian cancer.
- 3: To determine whether mutations that affect the HR DNA repair pathway impact T cell responses following treatment with WNT inhibitors using immunocompetent mouse models of ovarian cancer.

Key Accomplishments and Outcomes:

Publications:

1. Martinez A, Buckley M, Scalise CB, Katre AA, Dholakia JJ, Crossman D, Birrer MJ, Berry J, Arend RC. "Understanding the effect of mechanical forces on ovarian cancer progression." *Gynecol Oncol*. 2021 Apr 19. PMID: 33888338
2. Arend RC, Fisher AJ, Chou J, Jacobs I, Monk BJ. "Ovarian cancer: new strategies and emerging targets for the treatment of patients with advanced disease." *Crit Rev Oncol Hematol*. 2021 Jan 11. PMID: 33427569
3. Dholakia JJ, Scalise CB, Arend RC. "Assessing Preclinical Research Models for Immunotherapy for Gynecologic Malignancies." *Cancers (Basel)*. 2021 Apr 2. PMID: 33918476
4. Wall JA, Meza-Perez S, Scalise CB, Katre AA, Londono AI, Turbitt WJ, Randall TD, Norian LA, Arend RC. "Manipulating the Wnt/ β -catenin signaling pathway to promote anti-tumor immune infiltration into the TME to sensitize ovarian cancer to ICB therapy." *Gynecol Oncol*. 2020 Nov 7. PMID: 33168307.
5. Goel N, Foxall ME, Scalise CB, Wall JA, Arend RC. Strategies in Overcoming Homologous Recombination Proficiency and PARP Inhibitor Resistance. *Mol Cancer Ther*. 2021 Sep;20(9):1542-1549. doi: 10.1158/1535-7163.MCT-20-0992. Epub 2021 Jun 25. PMID: 34172532.

Patents: None to date

Funding Obtained: Dr. Arend received award of \$57,455 through Research Agreement between - Arend and Champions Oncology, Protocol, #000531086 for ovarian cancer research

Dr. Arend Received award of \$7,500 through ECMT2 Consortium Agreement UAB- PI Rebecca Arend and Duke University, protocol # 248035 for Ovarian cancer research

REVIEW



Ovarian cancer: new strategies and emerging targets for the treatment of patients with advanced disease

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ABSTRACT

Recently approved therapies have contributed to a significant progress in the management of ovarian cancer; yet, more options are needed to further improve outcomes in patients with advanced disease. Here we review the rationale and ongoing clinical trials of novel combination strategies involving chemotherapy, poly ADP ribose polymerase, programmed death 1 (PD-1)/PD-ligand 1 immune checkpoint and/or vascular endothelial growth factor receptor inhibitors. Further, we discuss novel agents aimed at targets associated with ovarian cancer growth or progression that are emerging as potential new treatment approaches. Among them, agents targeted to folate receptor α , tissue factor, and protein kinase-mediated pathways (WEE1 kinase, phosphatidylinositol-3 kinase α , cell cycle checkpoint kinase 1/2, ATR kinase) are currently in clinical development as mono- or combination therapies. If successful, findings from these extensive development efforts may further transform treatment of patients with advanced ovarian cancer.

ARTICLE HISTORY

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KEYWORDS

Ovarian cancer;
angiogenesis; PARP
inhibitors; PD-1/PD-L1
immune checkpoint
inhibitors; targeted agents

I. Introduction

Although incidence rates of ovarian cancer have been decreasing by about 1–2% each year, ~21,750 new cases are expected in the United States in 2020, with a diagnosis of epithelial ovarian cancer in ~90% of these patients.^{1,2} Primary peritoneal and fallopian tube cancers may be referred to as part of this group of malignancies. The adoption of more effective treatment options with novel chemotherapeutic regimens and targeted agents has significantly contributed to the improvements in patient outcomes observed in the past decade (Figure 1).^{1–4}

Nonetheless, a substantial proportion of patients with ovarian cancer are primarily resistant to treatment with available agents or develop secondary resistance over time, with disease progression and a poorer prognosis. In 2020, ~13,940 deaths are estimated to occur in the United States due to ovarian cancer. The current, overall 5-year survival rate is 48% and only 29% in the most advanced disease stages.² This limited survival underscores the need of identifying additional safe and effective treatments to improve outcomes in patients with ovarian cancer, particularly in advanced stage disease. The development of novel agents or regimens with improved tolerability may also contribute to provide treatment options associated with a better health-related quality of life for this patient population.

In this review, we discuss new strategies that are emerging for the treatment of patients with advanced ovarian cancer across a broad range of mechanisms of action, outlining the rationale underlying the selection of new targets in ovarian cancer and the novel combination approaches currently being evaluated in clinical trials.

II. Angiogenesis, genomic instability, and the immune microenvironment in ovarian cancer

Angiogenesis and vascular endothelial growth factor receptor (VEGFR) inhibition

Multiple lines of evidence have demonstrated that angiogenesis may play a key role in the survival and progression of various tumor types, including advanced ovarian cancers.⁵ Once released within the tumor microenvironment, proangiogenic growth factors such as VEGF can induce activation and proliferation of vascular endothelial cells, promote tumor-associated angiogenesis and contribute to the survival of endothelial cells in the newly formed vessels. In addition, proangiogenic factors modify the vascular tone and increase vascular permeability thereby contributing to further support the growth and survival of tumor cells.⁵

In ovarian cancer, overexpression of VEGF leads to an increase in tumor microvessel density, which has been found to be associated with disease progression and worse prognosis.⁵ Consistently, VEGF inhibition by bevacizumab in combination with chemotherapy, followed by maintenance therapy, has proven effective in delaying disease progression in ovarian cancer when administered after initial surgical resection or in recurrent platinum-sensitive disease, and as monotherapy or combined with chemotherapy in patients with platinum-resistant disease.^{6–8}

However, various mechanisms may allow escape of tumor cells from therapeutic control, including selection of tumor clones with increased expression of compensatory signaling pathways or clones with an increased capacity to grow and invade normal tissues in conditions of limited angiogenic support.^{9,10} Multiple

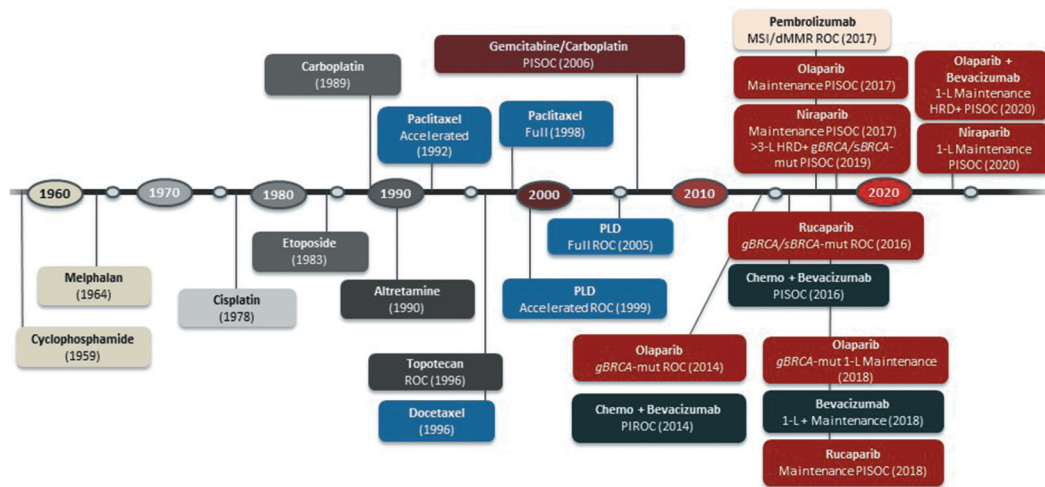


Figure 1. FDA-approved drugs in advanced ovarian cancer. dMMR: deficient mismatch repair, FDA: US Food and Drug Administration, HRD: homologous recombination deficiency, L: line of treatment, MSI: microsatellite instability high, mut: mutated, PISOC: platinum-sensitive ovarian cancer, PIROC: platinum-resistant ovarian cancer, PLD: pegylated liposomal doxorubicin.

approaches are thus being investigated to further improve clinical outcomes, through the development and characterization of novel antiangiogenic agents, and their use in combination regimens with agents targeting other key pathways in tumor cells (i.e. DNA repair) or immune checkpoints (i.e. programmed death receptor 1 [PD1]/PD-ligand 1 [PD-L1]).¹¹ Both VEGF receptor (VEGFR) 1 and 2 are expressed by microvascular endothelial cells in malignant ovarian tumors and borderline lesions suggesting their potential usefulness as targets for new therapeutic approaches.^{5,12}

Nintedanib is a triple angiokinase inhibitor of the VEGFR, platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR).¹³ Combined treatment with this small-molecule inhibitor and chemotherapy (carboplatin/paclitaxel) in the AGO-OVAR12 phase III trial showed activity in patients with newly diagnosed, advanced ovarian cancer, with an improvement in median progression-free survival (mPFS) versus chemotherapy. However, treatment was associated with limited tolerability and a higher incidence of gastrointestinal adverse events (AEs) compared with the control group.¹³

Targeted inhibition of VEGFR2 with the small-molecule, tyrosine kinase inhibitor apatinib has demonstrated activity in advanced gastric cancer and other malignancies. Single-agent administration in an initial phase I study induced responses, although of limited duration, in patients with recurrent, platinum-resistant ovarian cancer.¹⁴ Treatment with apatinib was most frequently associated with hand-foot syndrome, hypertension, nausea, and vomiting. Further evaluation is ongoing in combination with the DNA topoisomerase II inhibitor etoposide or the anti-PD-1 antibody camrelizumab in patients with advanced, platinum-resistant disease.¹⁴

In addition to VEGFR targeting, selective inhibition of angiopoietin binding to the Tie2 tyrosine kinase receptor may result in impairment of tumor-associated angiogenesis.¹⁵ Investigation of combination treatment with the angiopoietin inhibitor trebananib (AMG386) plus paclitaxel showed a significant prolongation in mPFS versus paclitaxel alone in patients with recurrent disease (platinum-free interval <12 months), in the randomized phase III TRINOVA-1 trial. Edema (of any grade) was reported

in more than half of patients receiving combination treatment, but most of the AEs usually related to VEGF-targeted therapy (i.e. hypertension, proteinuria, thrombotic events, wound-healing complications, gastrointestinal perforation) were infrequently observed.¹⁵ At final analysis of this study, OS was significantly prolonged only in the patient subset with baseline ascites.¹⁶ Combination treatment with trebananib plus pegylated liposomal doxorubicin in patients with recurrent, partially platinum-sensitive or resistant ovarian cancer (platinum-free interval ≤12 months, ENGOT-ov6/TRINOVA-2 phase III trial) demonstrated an improvement in objective response rate (ORR), but not in mPFS, versus chemotherapy alone.¹⁷ Recent findings from the randomized ENGOT-ov2/TRINOVA-3/GOG-3001 phase III trial of trebananib plus carboplatin/paclitaxel as first-line treatment for advanced disease followed by maintenance with trebananib or placebo, showed no significant improvement in mPFS, the primary endpoint, compared with chemotherapy.¹⁸ A phase Ib study is currently evaluating trebananib in combination with the PD-1 immune checkpoint inhibitor pembrolizumab in patients with advanced, platinum-resistant ovarian cancer.

Genomic instability and poly ADP ribose polymerase (PARP) inhibition

As in other tumor types, genomic instability may arise in ovarian cancer from the genetic abnormalities associated with malignant transformation, which often affects genes encoding homologous recombination (HR) DNA repair factors such as BRCA1 and BRCA2.^{19–21} The PARP1 and PARP2 nuclear enzymes mediate repair of single-stranded (ss) DNA breaks, induced by ultraviolet light, radiation, chemotherapy, and other DNA-damaging agents, by base-excision repair.^{22,23} In patients with germline or somatic defects in genes involved in HR repair (i.e. *BRCA1*, *BRCA2*), who cannot repair (ds) DNA breaks, inhibition of PARP activity blocks DNA repair and induces cell death.^{22,23} Inhibition of PARP becomes synthetically lethal in the context of an inactivating *BRCA* mutation,

because HR defects make *BRCA*-mutated tumors ‘addicted to’ (dependent on) other DNA repair pathways.²⁴ Although PARP inhibitors were first shown to be effective in *BRCA*-mutated tumors, responses and clinical benefit have been observed in the presence of wild-type *BRCA1* or 2 genes, suggesting that other factors and genetic mutations, involved in HR DNA repair, may confer sensitivity to PARP inhibitors.^{19,25,26} Beyond *BRCA*, genes encoding proteins involved in HR repair that may be mutated and associated with HR deficiency (HRD) in ovarian cancer include the Fanconi anemia genes *RAD51C*, *RAD51D*, *RAD50*, *BRIPI1*, *BARD1*, *MRE11A*, and *PALB2* as well as the DNA mismatch repair genes *MLH1*, *MSH2*, *MSH6*, and *PMS2*.²⁷ In patients with ovarian cancer, germline mutations in HR genes have been identified more frequently than somatic tumor mutations (<10% of cases).²⁸

Findings from multiple trials have demonstrated that PARP inhibition is an effective therapeutic strategy in ovarian cancer (Table 1). Treatment with single-agent rucaparib or olaparib is standard of care for patients with recurrent, platinum-sensitive or platinum-resistant *BRCA*-mutated disease, after 2 or 3 prior lines of chemotherapy, respectively.^{4,25,26,29} In addition, the QUADRA study has shown efficacy of niraparib monotherapy in patients with HRD, including *BRCA*-mutated and non-mutated, recurrent ovarian cancer after four or more lines of chemotherapy (ORR, 28%).³⁰ These results supported the approval of niraparib by the Food and Drug Administration (FDA) for heavily pretreated patients with HRD+ disease.

Based on the results of the SOLO2, study 19, NOVA, and ARIEL 3 studies, olaparib, niraparib, and rucaparib are indicated for the maintenance treatment of patients with recurrent ovarian cancer who are in complete or partial response following prior platinum-based therapy, independent of their biomarker status.^{4,25,26,29} Results from the randomized phase III SOLO1 trial have demonstrated that maintenance treatment with a PARP inhibitor is effective in newly diagnosed patients following first-line platinum-based chemotherapy.³¹ Based on the extent of benefit observed, olaparib was approved by the FDA for clinical use in this indication for patients with *BRCA*-mutated tumors. Furthermore, the phase III studies ENGOT-

OV26/PRIMA, VELIA, and PAOLA-1 have shown that both niraparib and veliparib, and the combination of olaparib plus bevacizumab are effective as first-line maintenance therapy in patients with advanced ovarian cancer, following response to prior platinum-based chemotherapy. Detailed study results are presented in Table 2 for the intent-to-treat populations and the subgroups analyzed (i.e. HRD+, *BRCA*-mutation+ patients). Although all three trials were positive in the intention-to-treat population, which included ‘all-comers’ despite specific genetic abnormalities, the patients that derived most benefit were HRD+, either due to a *BRCA* mutation or other HR defect.^{32–35}

Nonetheless, ovarian tumors may display primary or secondary resistance to treatment with PARP inhibitors, prompting an extensive evaluation of biomarkers that may help to determine the underlying resistance mechanisms and contribute to the identification and selection of therapies/combination regimens, timing, and sequencing of treatments suitable for each patient.^{36–41} Secondary mutations have been detected in patients with acquired resistance to PARP inhibitor therapy, including somatic mutations that restore *BRCA1/2* gene functions, through elimination of the open reading frame shift or by reverse mutation within the coding region.^{37,41–43} Analysis of baseline and on treatment samples showed that the presence of heterogeneous *BRCA2* reversion mutations was associated with resistance to PARP inhibition in prostate and ovarian cancers.^{42,43} In one of these studies, *BRCA* reversion mutations were detected in pretreatment samples in 18% of platinum-refractory v 2% of platinum-sensitive, high-grade ovarian carcinomas ($p = .049$). Patients without *BRCA* reversion mutations at baseline had significantly longer PFS following treatment with rucaparib compared with patients who had *BRCA* reversion mutations (median PFS, 9.0 v 1.8 months; HR, 0.12; $p < .0001$).⁴³ Additionally, heterogeneous mutations were identified in some patients after PARP inhibitor therapy.⁴³

Primary and acquired resistance to PARP inhibition in patients with high-grade ovarian cancers were also found associated with secondary, somatic mutations, including a truncation mutation,

Table 1. Pivotal trials and FDA approvals of PARP and VEGF inhibitors in advanced ovarian cancer.

Drug	Maintenance	Later-Line Treatment	FDA Approval
Olaparib ^a	SOLO-2 (<i>BRCA</i> -mutated) Study 19 (Aug 17, 2017) SOLO-1 (<i>BRCA</i> -mutated) (Dec 19, 2018) PAOLA-1 (HRD-positive) (May 8, 2020)	Study 42 (<i>BRCA</i> -mutated) (Dec 19, 2014)	> 3 rd line, germline <i>BRCA</i> , treatment (Dec 19, 2014) > 2 nd line, no biomarker, maintenance after response to platinum (Aug 17, 2017) Front-line, germline and somatic <i>BRCA</i> , maintenance after response to platinum (Dec 19, 2018) Front-line olaparib plus bevacizumab, HRD-positive, maintenance after response to platinum (May 8, 2020)
Niraparib ^b	NOVA (Mar 27, 2017) ENGOT-OV26/PRIMA (April 29, 2020)	QUADRA (Oct 23, 2019)	2 nd line, no biomarker, maintenance after response to platinum (March 27, 2017) > 3 rd line, HRD+, germline and somatic <i>BRCA</i> , platinum-sensitive, treatment (Oct 23, 2019) Front-line maintenance after response to platinum (April 29, 2020)
Rucaparib ^c	ARIEL3 (April 6, 2018)	Study 10 (<i>BRCA</i> -mutated) ARIEL2 (<i>BRCA</i> -mutated) (Dec 19, 2016)	> 2 nd line, germline and somatic <i>BRCA</i> , treatment (Dec 19, 2016) > 2 nd line, no biomarker, maintenance after response to platinum (Apr 6, 2018)
Bevacizumab ^d	GOG218 (June 13, 2018) OCEANS – GOG213 (Dec 6, 2016)	AURELIA (Nov 14, 2014)	1 st and later-line treatment plus maintenance (Nov 14, 2014; Dec 6, 2016; June 13, 2018)

^aOlaparib Prescribing Information. AstraZeneca Pharmaceuticals LP, May 2020; ^bNiraparib Prescribing Information. Tesaro Inc., April 2020; ^cRucaparib Prescribing Information. Clovis Oncology Inc., May 2020; ^dBevacizumab Prescribing Information. Genentech/Roche, May 2020.

FDA: US Food and Drug Administration, HRD: homologous recombination deficiency, PARP: poly ADP ribose polymerase, VEGF: vascular endothelial growth factor.

Table 2. Pivotal trial results for front-line maintenance therapy in patients with newly diagnosed, advanced ovarian cancer after response to platinum-based therapy.

Drug/Regimen	Phase III Trial	Patient Population	N	Outcomes	Reference
Niraparib vs placebo	ENGOT-OV26 /PRIMA	ITT	733	mPFS, 13.8 v 8.2 mo, HR 0.62, $p < .001$;	Gonzalez-Martin et al., 2019
		HRD+ (including <i>BRCA</i> -mt+)	373	mOS at 24 mo, 84% v 77%, HR 0.70 mPFS, 21.9 v 10.4 mo, HR 0.43, $p < .001$	
Veliparib vs placebo	VELIA	ITT	1140	mPFS, 23.5 v 17.3 mo, HR 0.68, $p < .001$	Coleman et al., 2019
		HRD+ (including <i>BRCA</i> -mt+)	421	mPFS, 31.9 v 20.5 mo, HR 0.57, $p < .001$	
Olaparib plus bev vs placebo plus bev	PAOLA-1	<i>BRCA</i> -mt+	200	mPFS, 34.7 v 22.0 mo, HR 0.44, $p < .001$	Ray-Coquard et al., 2019
		ITT	806	mPFS, 22.1 v 16.6 mo, HR 0.59, $p < .001$	
		HRD+ (including <i>BRCA</i> -mt+)	387	mPFS, 37.2 v 17.7 mo, HR 0.33	
		HRD+ (not including <i>BRCA</i> -mt+)	150	mPFS, 28.1 v 16.6 mo, HR 0.43	
		<i>BRCA</i> -mt+	237	mPFS, 37.2 v 21.7 mo, HR 0.31	
		HRD-negative	277	mPFS, 16.6 v 16.2 mo, HR 1.00	

Bev: bevacizumab, HRD: homologous recombination deficiency, HR: hazard ratio, ITT: intent to treat, mo: month, mOS: median overall survival, mPFS: median progression-free survival.

in the genes *RAD51C* and *RAD51D*, which encode proteins involved in (ds) DNA break repair by HR.⁴⁴ Complementation assays confirmed the association between the presence of these mutations and resistance to PARP inhibition therapy.⁴⁴ Combination with other targeted inhibitors as well as earlier administration of a PARP inhibitor in the course of the disease may help to decrease or delay the development of resistance in ovarian cancer, and thus improve treatment outcomes for patients with advanced disease.^{25,44}

The immune microenvironment, activity of PD-1/PD-L1-targeted immune checkpoint inhibitors and other immunotherapeutic approaches

The tumor immune microenvironment in ovarian cancers is quite complex with infiltration by helper or effector T cells and antigen-presenting cells (i.e. dendritic cells), production of interferon (INF) γ , interleukin (IL) 2 and IL-16 (a chemoattractant), which may result in antitumor immune responses. Conversely, the activity of VEGF and other ‘pro-tumor’ immune factors such as transforming growth factor (TGF) β , PD-L1, tumor necrosis (TNF) α , IL-6, and IL-10 often contribute to tumor survival and escape from immune control.^{45–48}

Overexpression of VEGF may induce downregulation of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion-molecule-1 (VCAM-1) and thus reduce the ability of lymphocytes to adhere to the tumor vascular endothelial cells and migrate into the tumor tissues. TGF- β can suppress the activation and proliferation of tumor-infiltrating lymphocytes.⁴⁵

Engagement of the PD-1 receptor on the immune cells by PD-L1 expressed by tumor cells may lead to a decrease in T cell activation, inhibition of T cell proliferation, and suppression of antitumor CD8 + T cell responses. In ovarian cancer, PD-L1 is detectable in about a third of advanced tumors, but most of the tumor-infiltrating lymphocytes express PD-1.^{46,47} High-grade tumors have been reported to express PD-L1 to a greater extent than low-grade ovarian tumors (i.e. 42% v 8%). A higher expression of PD-L1 may be associated with a worse prognosis compared with patients with lower PD-L1 levels, although further investigations are warranted to define the prognostic value of PD-L1 expression in ovarian cancer.^{46,47} Nonetheless,

infiltrating CD3+, CD8+, and CD4 + T cells detected in the tumor tissues of approximately half of the ovarian cancers investigated (‘hot tumors’) were associated with better outcomes in patients with advanced ovarian cancer, with prolonged PFS and OS after treatment compared with patients with no infiltrates, suggesting that therapeutic approaches aimed at restoring active antitumor responses may provide benefit in this setting.^{45–47} However, the complexity of the immune microenvironment associated with ovarian cancer, with the occurrence of immunosuppressive mediators and immunoregulatory cells (i.e. T regulatory cells [Treg] and myeloid-derived suppressor cells),^{45–47,49} the potential for T cell exhaustion^{49,50} and the effects of chemotherapy or surgery on antitumor immune responses, are all factors that need to be considered in the selection/sequencing of novel immunotherapeutic approaches and the design of combination regimens. Assessment of the pharmacodynamic effects of novel immunomodulatory approaches, currently ongoing in clinical trials, may also contribute to a better understanding of their impact on antitumor immune responses in patients with advanced ovarian cancer.

At initial clinical evaluation of PD-1/PD-L1 immune checkpoint inhibition therapies, both anti-PD-1 (i.e. nivolumab, pembrolizumab, PF-06801591) and anti-PD-L1 antibodies (i.e. atezolizumab, avelumab), have shown some single-agent, immunomodulatory and anti-tumor activity in patients with advanced ovarian cancer (7%–22%), albeit to a lower extent compared with other anti-PD-1/PD-L1 responsive tumor types (i.e. melanoma, non-small-cell lung cancer) (Table 3).^{51–59} For immune checkpoint inhibition to work in any cancer, T cells must be present in the tumor microenvironment. A substantial proportion of patients with ovarian cancer are resistant to treatment with anti-PD-1/PD-L1 antibodies due to limited infiltration by antitumor immune cells, the activity of immunosuppressive cells and cytokines in the tumor microenvironment, and/or a low expression of PD-1/PD-L1, suggesting that multi-targeted combination approaches including PARP inhibitors, chemotherapeutic agents, angiogenesis inhibitors, radiation, other immunomodulatory agents, vaccines, dendritic cell therapy, adoptive T cell therapy, or other targeted therapy may be more effective to achieve disease control.^{51–60}

Table 3. Single-agent treatment with PD-1/PD-L1 immune checkpoint inhibitors in patients with advanced ovarian cancer.

Agent	Study Phase	Patient Population	N	Outcomes	Reference
Anti-PD-1 antibodies					
Nivolumab	II	Platinum-resistant OvCa	20	ORR, 15%; durable CR in 2 patients; DCR, 45%; mPFS, 3.5 months; mOS, 20 months	Hamanishi et al., 2015
Nivolumab vs gemcitabine or pegylated liposomal doxorubicin	III (NINJA) randomized	Advanced or recurrent platinum-resistant OvCa	316	ORR, 8% v 13%; Duration of response: 18.7 v 7.4 months; mPFS, 2.1 v 3.8 months (HR, 1.46; $p = .002$); mOS: 10.1 v 12.1 months (HR, 1.03; $p = .808$).	Omatsu et al., 2020
Pembrolizumab	Ib	Advanced OvCa (73.1% of patients with ≥ 3 prior lines of therapy.	26, PD-L1+	ORR, 11.5%; mPFS, 1.9 months; mOS, 13.8 months	Varga et al., 2019
Pembrolizumab	II	Recurrent OvCa (1–3 prior lines of therapy, PFI/TFI 3–12 months)	285 (cohort A)	ORR, 7.4%; mDOR 8.2 months; DCR, 37.2%; mPFS, 2.1 months; mOS not reached	Matulonis et al., 2019
Pembrolizumab	II	Recurrent OvCa (4–6 prior lines of therapy, PFI/TFI ≥ 3 months)	91 (cohort B)	ORR, 9.9%; mDOR not reached; DCR, 37.4%; mPFS, 2.1 months; mOS 17.6 months	Matulonis et al., 2019
PF-06801591	I	Advanced OvCa	15	ORR, 20%; mPFS, 5.3 months; mOS, not reached	Johnson et al., 2019
Anti-PD-L1 antibodies					
Atezolizumab	Ia	Recurrent OvCa	12	ORR, 22%; durable PR in 2 patients; mPFS, 2.9 months; mOS, 11.3 months	Infante et al., 2016; Liu et al., 2019
Avelumab	Ib	Recurrent or refractory OvCa (median 3 prior lines of therapy)	125	ORR, 9.6%; mDOR, 10.4 months; DCR, 42%; mPFS, 2.6 months; mOS, 11.2 months	Disis et al., 2019

CR: complete response, DCR: disease control rate, mDOR: median duration of response, mOS: median overall survival, mPFS: median progression-free survival, OvCa: ovarian cancer, PD-1: programmed death receptor 1, PD-L1: PD ligand 1, PFI: platinum-free interval, PR: partial response, TFI: treatment-free interval.

Inhibition of DNA damage repair by a PARP inhibitor may induce an increase in the overall tumor mutational burden (TMB) and release of immunogenic, tumor-associated neoantigens. In addition, it promotes type-I interferon signaling, which in turn induces recruitment of T cells. The cell death induced by exposure to chemotherapeutic agents also generates new, tumor-related, immunogenic determinants that can facilitate the antitumor immune responses restored by PD-1/PD-L1 inhibition.⁶⁰

VEGFR inhibition may result in tumor vessel normalization and facilitate migration of immune cells into the tumor microenvironment.^{11,45} Consistently, VEGF inhibition by bevacizumab in combination with atezolizumab was shown to attenuate progression of platinum-resistant ovarian cancer through synergic anti-tumor activity, based on *in vitro* and *in vivo* analyses.⁶¹ Clinical activity has also been observed with nivolumab plus bevacizumab combination treatment in a phase II trial conducted in patients with recurrent ovarian cancer.⁶² Furthermore, preclinical investigations have shown that treatment with niraparib increased the activity of IFN- γ and IFN- γ signaling pathways as well as intra-tumor infiltration by CD8+ and CD4+ T cells. Combined administration of niraparib and an anti-PD-1 antibody in breast and ovarian cancer mouse models demonstrated synergic antitumor activity against both mutated *BRCA*+ and wild-type *BRCA* tumors, indicating a potential applicability of this combination.⁶³ In addition, a study demonstrated that PARP inhibition by olaparib or talazoparib can induce upregulation of PD-L1 expression in breast cancer cell lines *in vitro* and in xenograft tumor models *in vivo*.⁶⁴ Block of PD-L1 by a targeted antibody was found to restore sensitivity of PARP inhibitor-treated cancer cells to T-cell-mediated cytotoxicity. Combined administration of a PARP inhibitor and an anti-PD-L1 antibody produced greater antitumor activity *in vivo* versus either agent alone in this animal model, suggesting potential benefit from combined treatment with these agents in humans.⁶⁴

In addition to PD-1/PD-L1 immune checkpoint inhibitors, other immunotherapeutic approaches such as dendritic cell vaccines, tumor-infiltrating lymphocytes (TILs), and chimeric antigen receptor (CAR)-T cells are being pursued for the treatment of advanced ovarian cancer.^{60,65–69} Investigational, autologous ovarian cancer vaccines generated by pulsing dendritic cells with oxidized, whole-tumor-cell lysates obtained from individual patients have been evaluated in a pilot study, following intranodal injection in immune-naïve patients with recurrent, platinum-pretreated advanced ovarian cancer.⁶⁵ A subgroup of patients also received IV bevacizumab to enhance tumor infiltration by immune cells and low-dose cyclophosphamide to decrease infiltration by Treg cells. Vaccination was associated with a tolerable safety profile, high-affinity T-cell responses to autologous tumor antigens, and prolonged survival. Anti-tumor antigen responses were higher in vaccinated patients who had received concomitant treatment with cyclophosphamide. The OS at 2 years was greater in vaccinated patients (78%) who had received bevacizumab plus cyclophosphamide compared with an historical, institutional group of matched patients treated only with bevacizumab plus cyclophosphamide (44%). Although different factors (i.e. tumor sample availability, low lysate immunogenicity, complex production process) may limit the clinical applicability of autologous,

dendritic cell vaccines for each patient, these findings indicate the feasibility of inducing beneficial, tumor-specific immune responses in advanced ovarian cancer.⁶⁵

In a pilot study, adoptive cell therapy with TILs and progressively decreasing IV doses of IL-2 in patients with metastatic, platinum-resistant ovarian cancer, was associated with a manageable toxicity and early signals of clinical activity, although all treated patients ultimately progressed, mostly due to the development of new lesions. Infused TILs were found to express biomarkers potentially associated with T-cell exhaustion (i.e. LAG3 and PD-1), while tumor tissues demonstrated expression of major histocompatibility complex class II antigens and PD-L1, suggesting activation of inhibitory immune checkpoint pathways.⁶⁶

Results from an adoptive immunotherapy study of gene-modified CAR T cells redirected to the tumor-associated antigen folate receptor alpha (FRA) plus high-dose IL-2 showed large numbers of transferred T cells in the peripheral blood of treated patients within the first 2 days after infusion, followed, however, by a decline to very low levels within 1 month and mostly no localization in tumors.⁶⁷ A lack of responsiveness to growth factors *in vivo*, T-cell exhaustion after prolonged *in vitro* culture, and/or the IV route of administration with intra-organ sequestration may have contributed to the limited *in vivo* survival and tumor homing of the transferred, gene-modified T cells observed in this study. Optimization of *in vitro* culture conditions and costimulatory signals as well as selection of an IP delivery route may contribute to improved survival and *in vivo* antitumor activity of CAR T cells against ovarian cancers.⁶⁷ In other studies, CAR T cells targeted to the tumor-associated antigens mesothelin or mucin 16 (MUC16), known to be overexpressed on ovarian cancer cells, demonstrated cytotoxic activity *in vitro* and antitumor activity *in vivo*, resulting in growth inhibition of ovarian tumors in preclinical animal models.^{68,69} Combinations with immunomodulatory signals, such as costimulation through CD28 or PD-1/PD-L1 immune checkpoint inhibition, or with growth factors (i.e. IL-12), can contribute to enhance the *in vivo* efficacy of CAR T-cell-based approaches.^{68,69}

Combination studies of chemotherapy, PARP inhibitors, anti-PD-1/PD-L1 antibodies, and/or VEGFR inhibitors in patients with advanced ovarian cancer

Based on the findings outlined above, multiple combination strategies including PARP inhibition and/or an anti-PD-1/PD-L1 antibody and/or a VEGFR inhibitor are being evaluated in patients with a) newly diagnosed, b) relapsed, platinum-sensitive or c) platinum-resistant disease (Table 4).

Chemotherapy-based combination regimens ($\pm$ PARP inhibitor $\pm$ anti-PD-1/PD-L1 antibody $\pm$ VEGFR inhibitor)

PD-1/PD-L1 targeted immune checkpoint inhibitors are being administered in combination with standard of care platinum/taxane-based chemotherapy in various phase III trials for the treatment of patients with stage III–IV, newly diagnosed ovarian cancer with or without bevacizumab, followed by maintenance therapy with a PARP inhibitor with or without an anti-

PD-1/PD-L1 antibody and/or bevacizumab. Details of these trials are presented in Table 4.

JAVELIN ovarian PARP 100, which was a phase III study in newly diagnosed ovarian cancer patients, evaluating avelumab in combination with platinum-based chemotherapy followed by maintenance with avelumab and talazoparib versus chemotherapy plus talazoparib maintenance or chemotherapy plus bevacizumab followed by bevacizumab maintenance, was terminated early as the extent of benefit observed with avelumab did not support continuation of this study in an unselected patient population.

Results from the randomized, phase III trial IMagyn050/GOG 3015/ENGOT-ov39 conducted in newly diagnosed patients with stage III–IV ovarian cancer have shown that addition of a PD-L1 inhibitor, atezolizumab, to standard chemotherapy (carboplatin/paclitaxel) plus bevacizumab followed by maintenance with bevacizumab did not significantly improve mPFS (primary endpoint) in the intent-to-treat population (19.5 v 18.4 months; hazard ratio, 0.92) or in PD-L1-positive patients with $\geq 1\%$ expression (20.8 v 18.5 months; hazard ratio, 0.80). However, an exploratory analysis suggested a trend toward improved mPFS with addition of atezolizumab in the subgroup of patients with PD-L1 expression $\geq 5\%$.⁷⁰

Table 4 summarizes phase III trials being conducted in patients with recurrent, platinum-sensitive disease, which are evaluating chemotherapy in combination with a PD-1/PD-L1 inhibitor with or without bevacizumab and maintenance treatment with an anti-PD-1/PD-L1 antibody plus/minus a PARP inhibitor and/or bevacizumab.

PARP inhibitor + VEGFR inhibitor combinations

VEGFR inhibition can produce impairment of homologous recombination DNA repair with downregulation of BRCA1/2 and RAD51 in addition to inhibition of tumor-associated angiogenesis, potentially sensitizing target cells to the antitumor activity of PARP inhibitors.⁷¹ Results from randomized trials have demonstrated this combination as superior to PARP inhibition alone.^{72,73}

Cediranib is a small-molecule, VEGFR 1–3 tyrosine kinase inhibitor, reported to induce sensitivity to PARP inhibitors (i.e. olaparib) by inducing tumor hypoxia and inhibiting PDGFR, which result in decreased expression of BRCA1/2 and RAD51 and reduced activity of HR DNA repair in target cells.⁷¹ In a randomized phase II study, combination treatment with cediranib and olaparib demonstrated a significant improvement in mPFS and overall survival versus olaparib alone in patients with germline BRCA wild-type/unknown, relapsed, platinum-sensitive ovarian cancer.⁷² A significant improvement in mPFS with VEGF plus PARP inhibition has also been reported with bevacizumab plus niraparib in patients with recurrent, platinum-sensitive disease, in the randomized phase II trial NSGO-AVANOVA2/ENGOT-ov24.⁷³

Thus, phase III trials have been initiated to evaluate combinations of VEGFR and PARP inhibition for potential additive/synergistic effects in relapsed platinum-sensitive or platinum-resistant/refractory, advanced ovarian cancer (Table 4). Combination of cediranib with olaparib in the randomized, phase III NRG-GY004 trial has recently demonstrated an mPFS (primary endpoint) comparable rather than significantly improved versus standard platinum-based chemotherapy with

Table 4. Investigational combination regimens in patients with advanced ovarian cancer.

Clinical Trial	CT	P	I	A	Treatment	Patient Population ^a	Phase	Primary EP
Chemotherapy-based combinations (CT ± PARPi ± anti-PD-1/PD-L1 antibody ± VEGFRI)								
FIRST/ENGOT-ov44 (NCT03602859)	m				Platinum-based CT + TSR-042 (dostarlimab) followed by niraparib + TSR-042 vs CT followed by niraparib maintenance vs CT	1 st line, stage III–IV OvCa	Randomized, double-blind, phase III	PFS
KEYLYNK-001/ENGOT-ov43 (NCT03740165)	m				CT (carboplatin/paclitaxel) + pembrolizumab followed by olaparib maintenance or CT (carboplatin/paclitaxel) + pembrolizumab vs CT (carboplatin/paclitaxel) (study arms may include bev)	1 st line, BRCA non-mutated OvCa	Randomized, double-blind, phase III	PFS, OS
IMagyn050/GOG 3015/ENGOT-ov39 (NCT03038100)					CT (carboplatin/paclitaxel) + atezolizumab + bev followed by maintenance atezolizumab + bev vs CT (carboplatin/paclitaxel) + bev followed by bev	Stage III–IV OvCa after tumor-reductive surgery	Randomized, double-blind, phase III	PFS, OS
DUO-O/ENGOT-ov46 (NCT03737643)	m			m	CT + durvalumab followed by maintenance durvalumab + olaparib + optional bev	Newly diagnosed OvCa, BRCA-mutated	Randomized, open-label, phase III	PFS
DUO-O/ENGOT-ov46 (NCT03737643)	m			m	CT + durvalumab + bev followed by maintenance durvalumab + olaparib + bev vs CT + durvalumab + bev followed by maintenance durvalumab + bev vs CT + bev followed by maintenance bev	Newly diagnosed OvCa, non BRCA-mutated	Randomized, double-blind, phase III	PFS
ATHENA/ENGOT-ov45 (NCT03522246)	m			m	Platinum-based CT followed by maintenance rucaparib + nivolumab vs rucaparib vs nivolumab vs placebo	Responders to front-line chemotherapy	Randomized, double-blind, phase III	PFS
ICON 9 (NCT03278717)	m			m	Platinum-based CT followed by maintenance olaparib + cediranib vs platinum-based CT followed by maintenance olaparib	Relapsed, platinum-sensitive OvCa (responders ≥4 cycles of CT)	Randomized, open-label, phase III	PFS, OS
ANITA/ENGOT-ov41/GEICO 69-O (NCT03598270)	m			m	Platinum-based CT (investigator's choice) + atezolizumab followed by maintenance niraparib + atezolizumab vs CT (investigator's choice) followed by maintenance niraparib	Recurrent OvCa, TFIp >6 mos	Randomized, double-blind, phase III	PFS
ATALANTE (NCT02891824)					Platinum-based CT + atezolizumab + bev ± maintenance atezolizumab + bev vs platinum-based CT + bev followed by maintenance bev	Recurrent OvCa, TFIp >6 mos	Randomized, double-blind, phase III	PFS
NRG-GY009 (NCT02839707)					Pegylated liposomal doxorubicin (PLD) + atezolizumab or PLD + atezolizumab + bevacizumab vs PLD + bevacizumab	Platinum-resistant OvCa	Randomized, open-label, phase II–III	PFS, OS
PARPi + VEGFRI combinations								
NSGO-AVANOVA2/ENGOT-ov24 (NCT02354131)					Niraparib + bev vs niraparib	Relapsed, platinum-sensitive OvCa	Randomized, open-label, phase II	PFS
NRG-GY004 (NCT02446600)					Olaparib + cediranib or olaparib vs standard platinum-based CT	Relapsed, platinum-sensitive OvCa	Randomized, open-label, phase III	PFS
NRG-GY005/COCOS (NCT02502266)					Cediranib + olaparib vs cediranib vs SOC CT (physician's choice)	Recurrent, platinum-resistant/refractory OvCa (high-grade for BRCA1/2 non-mutation carriers)	Randomized, open-label, phase III	PFS, OS
OCTOVA (NCT03117933)					Olaparib + cediranib vs olaparib vs paclitaxel	Platinum-resistant OvCa	Randomized, open-label, phase II	PFS
PARPi + anti-PD-1/PD-L1 antibody combinations								
TOPACIO/KEYNOTE-162 (NCT02657889)					Niraparib + pembrolizumab	Recurrent OvCa	Open-label, phase I–II	ORR

(Continued)

Table 4. (Continued).

Clinical Trial	CT	P	I	A	Treatment	Patient Population ^a	Phase	Primary EP
Javelin PARP Medley (NCT03330405)					Talazoparib + avelumab	Recurrent, platinum-sensitive including BRCA-mutated OvCa	Open-label, phase Ib-II	ORR
NCT02660034					BGB-290 (pamiparib) + BGB-A317 (tislelizumab)	Recurrent, platinum-sensitive (TFIP >6 mos), high-grade OvCa	Open-label, phase I	ORR
ARIES (NCT03824704)					Rucaparib + nivolumab	Relapsed, platinum-sensitive OvCa	Open-label, phase II	ORR
NCT02484404					Olaparib + durvalumab	Recurrent, platinum-resistant OvCa	Open-label, phase II	ORR
PARPi + anti-PD-1/PD-L1 antibody + VEGFRi combinations								
NCT02484404					Durvalumab + olaparib + cediranib, durvalumab + olaparib, and durvalumab + cediranib	Recurrent, platinum-sensitive or resistant OvCa	Non-randomized, open-label, phase II	ORR
MEDIOLA (NCT02734004)					Durvalumab + olaparib + bevacizumab and durvalumab + olaparib	Relapsed, platinum-sensitive OvCa	Non-randomized, open-label, phase II	ORR
NCT02873962					Nivolumab + rucaparib + bevacizumab, nivolumab + bevacizumab	Relapsed, platinum-sensitive or resistant OvCa	Non-randomized, open-label, phase II	ORR
NSGO/AVANOVA-Triplet (NCT03806049)					TSR-042 + niraparib + bevacizumab, niraparib + bevacizumab, and carboplatin + paclitaxel	Recurrent, platinum-sensitive OvCa	Randomized, open-label, phase III	PFS
OPAL (NCT03574779)					TSR-042 + niraparib + bevacizumab	Recurrent, platinum-resistant OvCa	Open-label, phase II	ORR
anti-PD-1/PD-L1 antibody + VEGFRi combination								
NCT04068974					Camrelizumab + apatinib	Recurrent, platinum-resistant OvCa	Open-label, phase II	ORR

^aClinical trials may include patients with other tumor types in addition to patients with advanced ovarian cancer. A: angiogenesis inhibition, bev: bevacizumab, CT: chemotherapy, m: maintenance, EP: endpoint, I: PD-1/PD-L1 immune checkpoint inhibition, ORR: objective response rate, OS: overall survival, OvCa: ovarian cancer, P: PARP inhibition, PARPi: PARP inhibitor, PD-1: programmed death 1 receptor, PD-L1: PD-ligand 1, PFS: progression-free survival, SOC: standard of care, TFIP: platinum treatment-free interval, VEGFRi: vascular endothelial growth factor receptor inhibitor.

carboplatin/paclitaxel, carboplatin/gemcitabine, or carboplatin/pegylated liposomal doxorubicin (10.4 v 10.3 months, hazard ratio 0.86), in the intent-to-treat population of patients with relapsed, platinum-sensitive OvCa.⁷⁴ However, in a planned subset analysis of patients with germline *BRCA* mutations, the hazard ratio for improvement in mPFS versus chemotherapy was 0.55 for cediranib plus olaparib and 0.63 for olaparib alone.⁷⁴ Further phase II or III clinical trials are in progress evaluating cediranib in various combinations with PARP inhibitors, chemotherapy, or anti-PD-1/PD-L1 antibodies.

PARP inhibitor + anti-PD-1/PD-L1 antibody ± VEGFR inhibitor combinations

As previously discussed, preclinical studies have demonstrated synergy between PARP inhibitors and immune checkpoint inhibitors in mediating antitumor activity.^{63,64} Thus, a number of clinical trials are currently evaluating combination treatment with these two classes of agents in patients with recurrent ovarian cancer, including niraparib plus pembrolizumab, talazoparib plus avelumab, BGB-290 (pamiparib) plus BGB-A317 (tislelizumab), rucaparib plus nivolumab, and olaparib plus durvalumab. In addition, multiple phase II or III studies are assessing triple combinations of a PARP inhibitor with an anti-PD-1/PD-L1 antibody and a VEGFR inhibitor in platinum-sensitive or resistant disease (Table 4).

Results from the phase I–II study (TOPACIO/Keynote-162) of niraparib in combination with pembrolizumab showed an ORR of 18% in patients with recurrent, advanced ovarian cancer, with no new safety signals from the combination treatment. The majority of patients had platinum-resistant/refractory disease.⁷⁵ This compares with historical ORRs of ≤10% with PD-1 checkpoint inhibitor treatment (irrespective of PD-1 levels) or PARPi monotherapy in similar patient populations without *BRCA*-mutations.⁷⁵

Initial findings from the phase II study (NCT02484404) of olaparib and durvalumab in patients with mostly platinum-

resistant, recurrent disease, showed an overall ORR of ~15%, with responses in both *BRCA*-mutated and *BRCA*-wild type patients. Grade 3–4 anemia and lymphopenia were reported in 26% and 14% of patients, respectively. Dose reductions of olaparib were required in <1% of patients.⁷⁶ Preliminary results from the same phase II study of a triple combination of olaparib, durvalumab, and cediranib in a small number of patients with recurrent ovarian cancer have shown tolerability, antitumor activity with partial responses, and correlation of clinical benefit with PD-L1 expression levels in tumors.⁷⁷ Furthermore, a triple combination of olaparib, durvalumab, and bevacizumab investigated in the phase II, non-randomized MEDIOLA trial, in patients with relapsed, platinum-sensitive ovarian cancer (non-germline *BRCA*-mutated) demonstrated a high 24-week disease control rate (77.4%) and confirmed ORR (77.4%), with a mPFS of 14.7 months. In the olaparib plus durvalumab arm, the ORR was 31.3% and the mPFS was 5.5 months.^{78,79} Anemia, hypertension, fatigue, increased lipase levels, and neutropenia were the grade ≥3 AEs most frequently observed with the triple combination; 16% of patients discontinued one or more study drugs in this combination regimen.⁷⁹

III. Targeting tumor-associated antigens and signaling pathways in ovarian cancer

A number of novel agents to tumor targets associated with ovarian cancer growth and progression are currently in clinical development, to identify new options for single-agent or combination treatment in patients with advanced ovarian cancer (Table 5). Details of the clinical studies in progress with these agents are presented in Table 6.

Folate receptor alpha (FRα)

FRα is a transmembrane glycoprotein mediating transport of folate into cells, which is overexpressed in the majority of

Table 5. Targeted agents in development for the treatment of patients with advanced ovarian cancer.

Agent	Target/MOA	Structure	Company	Phase
Folate receptor α (FRα) targeting				
Mirvetuximab soravtansine (IMGN853)	FRα/microtubule inhibitor	ADC	ImmunoGen	I–III
(M9346A antibody + maytansinoid DM4)				
MORAb-202	FRα/microtubule inhibitor	ADC	Eisai	I–II
(farletuzumab + eribulin)				
Tissue factor targeting				
Tisotumab vedotin (HuMax-TF-ADC)	Tissue factor targeting/microtubule inhibitor	ADC	Genmab/Seattle Genetics	II
(antibody + MMAE)				
PTK7 targeting				
PF-06647020	PTK7 targeting/microtubule inhibitor	ADC	Pfizer/AbbVie	I
(antibody + auristatin 0101)				
Protein kinase inhibition				
Adavosertib (AZD1775)	WEE1 TK inhibitor	SMI	AstraZeneca	II
Alpelisib (BYL719)	PI3K-α inhibitor	SMI	Novartis	Ib
Ralimetinib (LY2228820 dimesylate)	p38 MAPK1 inhibitor	SMI	Lilly	Ib/II
Prexasertib (LY2606368)	CHK 1/2 inhibitor	SMI	Lilly	II
AZD6738	ATR PK inhibitor	SMI	AstraZeneca	II
Berzosertib (M6620, VX-970/VE-822)	ATR PK inhibitor	SMI	Merck Serono	II

ADC: antibody-drug conjugate, ATR: ataxia telangiectasia mutated and Rad3-related kinase, CHK: cell cycle checkpoint, FGF: fibroblast growth factor, HDAC: histone deacetylase, Hsp: heat shock protein, MAPK: mitogen-activated protein kinase, MMAE: monomethyl auristatin E, MOA: mechanism of action, PDGF: platelet-derived growth factor, PI3K: phosphatidylinositol 3-kinase, PK: protein kinase, SMI: small-molecule inhibitor, TK: tyrosine kinase, VEGF: vascular endothelial growth factor, VEGFR2: VEGF receptor 2.

Table 6. Clinical trials evaluating emerging agents for the treatment of patients with advanced ovarian cancer.

Clinical Trial	Treatment	Patient Population ^a	Phase	Primary EP
Targeting folate receptor α (FRα)				
FORWARD I (NCT02631876)	Mirvetuximab soravtansine vs CT of choice (paclitaxel, pegylated liposomal doxorubicin or topotecan)	Platinum-resistant, FR α -positive OvCa	Randomized, open-label phase III	PFS
MIRASOL (NCT04209855)	Mirvetuximab soravtansine vs CT of choice (paclitaxel, pegylated liposomal doxorubicin, or topotecan)	Platinum-resistant OvCa with high FR α expression	Randomized, open-label phase III	PFS
SORAYA (NCT04296890)	Mirvetuximab soravtansine	Platinum-resistant OvCa with high FR α expression	Single-arm, open-label phase III	ORR
KEYNOTE PN409/FORWARD II (NCT02606305)	Mirvetuximab soravtansine + carboplatin or bev or pegylated liposomal doxorubicin or pembrolizumab or carboplatin/bev	FR α -positive OvCa	Phase Ib-II	ORR
NCT03552471	Mirvetuximab soravtansine + rucaparib	FR α -positive, recurrent <i>BRCA</i> -mutated or platinum-resistant OvCa	Phase I	RP2D
NCT04300556	Morab-202	FR α -positive, platinum-resistant OvCa and other solid tumors	Phase I–II	Safety, RP2D, ORR
Tissue factor (TF) targeting				
NCT02552121	Tisotumab vedotin	Relapsed/metastatic tumors known to express TF	Phase I–II	Safety, RP2D
InnovaTV 208 (NCT03657043)	Tisotumab vedotin (standard or dose-dense regimen)	Platinum-resistant OvCa	Randomized, open-label phase II	ORR
Protein kinase inhibition				
NCT03579316	Adavosertib single agent or + olaparib	Recurrent OvCa with progression after prior PARPi therapy	Randomized, open-label phase II	ORR
NCI MATCH screening trial/subprotocol Z11 <i>BRCA</i> -mutated tumors (NCT02465060)	Single-agent adavosertib	Advanced, <i>BRCA</i> -mutated, refractory OvCa	Phase II	ORR
NCT01623349	Alpelisib (BYL 719) or buparlisib (BKM120) + olaparib	Recurrent high-grade OvCa after prior platinum-based therapy	Phase Ib	MTD, RP2D
NCT01663857	Ralimetinib + CT (gemcitabine and carboplatin) vs CT	Recurrent OvCa, TFlp >6 months	Randomized, double-blind, phase Ib-II	PFS
NCT02203513	Prexasertib (LY2606368)	Recurrent <i>BRCA</i> -mutated and <i>BRCA</i> -non mutated OvCa	Phase II	ORR
NCT03414047	Prexasertib (LY2606368)	Recurrent, <i>BRCA</i> -mutated and <i>BRCA</i> -non mutated, platinum resistant/refractory OvCa	Phase II	ORR
CAPRI (NCT03462342)	AZD6738 + olaparib	Recurrent platinum-sensitive and platinum-resistant OvCa	Phase II	Safety
NCT02595892	Berzosertib (M6620) + gemcitabine vs gemcitabine	Recurrent, platinum-resistant OvCa	Randomized, open-label phase II	PFS
NCT02627443	Berzosertib (M6620) + carboplatin and gemcitabine	Recurrent, platinum-sensitive OvCa	Phase I	MTD, safety

^aClinical trials may include patients with other tumor types in addition to advanced ovarian cancer. AE: adverse event, Bev: bevacizumab, CT: chemotherapy, DLT: dose-limiting toxicity, EP: endpoint, MTD: maximum tolerated dose, ORR: objective response rate, OvCa: ovarian cancer, PARPi: PARP inhibitor, PFS: progression-free survival, q2 weeks: every 2 weeks, q3 weeks: every 3 weeks, RP2D: recommended phase II dose, SOC: standard of care, TFlp: platinum treatment-free interval.

epithelial ovarian cancers but absent in normal epithelial cells of the ovary.⁸⁰ Such selective expression in tumor cells and the ability to internalize following ligand binding make FR α a suitable target for antibody-drug conjugates (ADCs) designed to deliver cytotoxic payloads to tumor cells.⁸⁰

Mirvetuximab soravtansine is an FR α -targeted ADC composed of the anti-FR α M9346A antibody and the microtubule-disrupting agent maytansinoid DM4. The presence of a cleavable linker also allows release of active DM4 molecules and killing of proximal tumor cells.^{80,81} Combination treatment with mirvetuximab soravtansine plus carboplatin followed by maintenance with mirvetuximab soravtansine had an acceptable tolerability profile in a phase I study of patients with recurrent, platinum-sensitive ovarian cancer, with an ORR of 71% and median PFS of 15 months. The most frequent

AEs were nausea, diarrhea, thrombocytopenia, blurred vision, and fatigue (mostly $\leq$ grade 2).⁸⁰

The randomized phase III FORWARD I trial evaluated safety and efficacy of mirvetuximab soravtansine versus chemotherapy of choice in patients with FR α -positive, platinum-resistant ovarian cancer, who had received up to three lines of prior treatment.⁸¹ Although the response rate was higher in the experimental arm, the study did not meet the primary endpoint of a significant improvement in mPFS. Nonetheless, patients with high levels of FR α expression treated with mirvetuximab soravtansine had longer mPFS (4.8 v 3.3 months) and a higher ORR (24% v 10%), with less grade ≥ 3 AEs and treatment discontinuations due to AEs compared with chemotherapy, thus suggesting a potentially favorable risk/benefit profile in this patient population.⁸² The most frequent AEs reported with

mirvetuximab soravtansine in this study were nausea (54%), diarrhea (44%), and blurred vision (43%).⁸² Two phase III trials, MIRASOL and SORAYA, are further evaluating mirvetuximab soravtansine in patients with platinum-resistant ovarian cancer and high FR α expression. In MIRASOL, safety and efficacy of mirvetuximab soravtansine are assessed versus chemotherapy of choice with paclitaxel, pegylated liposomal doxorubicin, or topotecan (primary endpoint, mPFS) (Table 6).

Furthermore, mirvetuximab soravtansine is being evaluated in combination with chemotherapy, bevacizumab, the anti-PD-1 antibody pembrolizumab, or the PARP inhibitor rucaparib in phase I–II studies, to identify potential synergies with these inhibitors (Table 6).

Results from cohorts of patients with platinum-resistant, ovarian cancer treated with mirvetuximab soravtansine and bevacizumab in the FORWARD II study have shown an ORR of 39% in all patients (mPFS, 6.9 months) and 56% in bevacizumab-naïve patients with medium/high FR α expression ($\geq 50\%$ positive cells) (mPFS, 9.9 months). AEs were generally mild or moderate in severity; 9% of patients developed grade 1–2 pneumonitis.⁸³

In addition, a triple combination of mirvetuximab soravtansine with carboplatin and bevacizumab (followed by mirvetuximab soravtansine/bevacizumab maintenance), in a phase Ib/II study of patients with recurrent, platinum-sensitive ovarian cancer and medium/high FR α expression, was associated with a manageable safety profile, confirmed responses in 81% of patients (median duration, 10.7 months) and a mPFS of 12.0 months. The most frequently reported treatment-related AEs were diarrhea, nausea, fatigue, and blurred vision, consistent with the known safety profile of mirvetuximab soravtansine. Grade 2 peripheral neuropathy was observed in 22% of patients.⁸⁴

The ADC MORAb-202 consists of the humanized anti-FR α antibody farletuzumab conjugated to the microtubule-targeted agent eribulin (maleimido-PEG2-valine-citrulline-*p*-aminobenzylcarbamyl-eribulin) through reduced inter-chain disulfide bonds. Preclinical studies demonstrated durable antitumor responses in human cell lines and patient-derived xenograft tumor models, supporting evaluation of this novel ADC for the treatment of patients with FR α -positive tumors.⁸⁵ Administration of MORAb-202 was associated with complete and partial responses in patients with advanced ovarian cancer enrolled in a first-in-human, phase I study. Leukopenia and neutropenia, observed in approximately half of the patients, were the most frequent treatment-related AEs.⁸⁶ Further evaluation of MORAb-202 is ongoing in patients with FR α -positive, platinum-resistant ovarian cancer and other selected tumor types in advanced stage (endometrial, non-small-cell lung, and triple-negative breast cancer) (Table 6).

Tissue factor (TF)

TF, usually involved as a cofactor in the coagulation process, can be abnormally expressed on the surface of cancer cells in various tumor types and thus provide a potential new target for anticancer therapy.^{87,88} Tisotumab vedotin is a TF-targeted ADC consisting of a human IgG1 antibody conjugated to monomethyl auristatin E (MMAE) through a protease-cleavable valine citrulline linker.⁸⁷ Durable tumor responses were achieved with tisotumab vedotin in TF-positive xenograft solid tumor models, including patient-derived xenografts.⁸⁷

Preliminary findings from a phase I–II study of tisotumab vedotin in patients with advanced solid malignancies, unselected for predefined TF expression levels, showed an ORR of $\sim 16\%$. The most common AEs were epistaxis, fatigue, nausea, alopecia, and conjunctivitis; grade ≥ 3 AEs (fatigue, anemia, abdominal pain, and hypokalemia) were reported in $\leq 10\%$ of patients.⁸⁷ A randomized, open-label phase II study (innovaTV 208) is evaluating a standard and a dose-dense regimen of tisotumab vedotin in patients with advanced, platinum-resistant ovarian cancer.⁸⁹

Protein tyrosine kinase 7 (PTK7)

PTK7 is a catalytically inactive receptor tyrosine kinase, involved in the Wnt signaling pathway, which was shown to be enriched in tumor-initiating cells (TICs) in patient tumor xenografts.⁹⁰ Thus, PTK7 represents a tumor target that can lead to elimination of cancer cells responsible for tumor recurrence and dissemination. The anti-PTK7 ADC PF-06647020 consists of a humanized, monoclonal antibody joined to the auristatin microtubule inhibitor Aur0101 by a cleavable valine-citrulline-based linker. Following internalization and cleavage, auristatin-0101 inhibits tubulin polymerization leading to apoptotic cell death in target cells.⁹⁰ In preclinical, patient-derived tumor xenograft models, PF-06647020 induced durable responses and showed greater efficacy compared with chemotherapy.⁹⁰

Results from a phase I study of PF-06647020 demonstrated a manageable safety profile, with a disease control rate of 73% (ORR, 27%) in patients with advanced ovarian cancer.⁹¹ The majority of the treatment-related AEs were grade 1–2, including most frequently nausea, alopecia, fatigue, headache, neutropenia, and vomiting.⁹¹ Biomarker analysis showed that clinical responses to PF-06647020 correlated with higher baseline PTK7 tumor expression levels.⁹²

Protein kinase-mediated pathways

The WEE1 kinase is a key intracellular checkpoint at the G₂-M transition that mediates arrest of the cell cycle to allow for premitotic DNA repair in the G₂ phase. Differently from normal cells, DNA repair in cancer cells occurs more frequently in G₂ than in G₁ arrest, thus conferring tumor selectivity to WEE1 targeting. Inhibition of WEE1 in the presence of DNA damaging agents can result in mitotic catastrophe due to initiation of mitosis with unrepaired lethal DNA damage.⁹³ Consistently, preclinical studies have demonstrated increased cell death, reduced tumor burden, and prolonged survival in experimental animal models following WEE1 inhibition.⁹³

Adavosertib (AZD1775) is a WEE1 tyrosine kinase inhibitor which has demonstrated antitumor activity in a small number of treatment-refractory patients with *BRCA*-mutated tumors including ovarian cancer.⁹⁴ Supraventricular tachyarrhythmia and myelosuppression were reported as dose-limiting toxicities, while myelosuppression and diarrhea were common treatment-related AEs.⁹⁴ Evaluation of adavosertib plus carboplatin in patients with *TP53*-mutated, platinum-resistant/refractory ovarian cancer showed an ORR of 43% and a median PFS of 5.3 months, with responses lasting more than 31 months in 2 of

24 patients. The most frequent, grade 3–4, treatment-related AEs were thrombocytopenia and neutropenia.⁹⁵ Further trials are in progress investigating combination treatment with adavosertib and olaparib in a phase II study of patients with ovarian cancer progressing after prior PARP inhibitor therapy, and adavosertib monotherapy in the phase II NCI MATCH screening trial/subprotocol Z11 in patients with *BRCA*-mutated tumors.⁹⁶

Alpelisib (BYL719) is a small-molecule, selective inhibitor of the phosphatidylinositol 3-kinase (PI3K) α subunit, approved by the FDA for the treatment of hormone receptor positive, HER2-negative, PIK3CA mutation-positive breast cancer.⁹⁷ Preclinical studies have shown that PI3K inhibitors may impair homologous recombination repair and sensitize ovarian cancer cells to PARP inhibitors.⁹⁸ Results from a phase Ib combination study of alpelisib with olaparib, in patients with recurrent ovarian cancer after prior platinum-based therapy, indicated a partial response rate of 36% and stable disease in 50% of patients. Treatment-related grade 3–4 AEs included hyperglycemia, nausea, and increased alanine aminotransferase levels. Hyperglycemia and neutropenic fever occurred as dose-limiting toxicities.⁹⁸

Ralimetinib mesylate (LY2228820 dimesylate) is a small-molecule inhibitor of p38 α and β mitogen-activated protein kinase 1 (MAPK1), a kinase that may facilitate cell survival and resistance to standard treatment by modulation of cytokine production in the tumor microenvironment. Pharmacodynamic studies showed inhibition of p38 MAPK-induced phosphorylation of MAP kinase-activated protein kinase 2 (MAPKAP-K2) by ralimetinib in patient peripheral blood mononuclear cells.⁹⁹ Preliminary evaluation of ralimetinib in a randomized phase Ib-II study in combination with gemcitabine and carboplatin chemotherapy demonstrated a significant, although limited prolongation in median PFS (10.3 v 7.9 months), but no significant difference in ORR and median OS, in patients with recurrent, platinum-sensitive ovarian cancer. Neutropenia, thrombocytopenia, and anemia were the most frequent grade 3–4 AEs in both treatment arms; grade 3–4 elevations in alanine aminotransferase levels were observed more frequently in patients receiving the triple combination.¹⁰⁰

A further, novel kinase inhibitor that has shown activity against ovarian cancer in preclinical and early clinical studies is prexasertib (LY2606368), a selective, ATP-competitive inhibitor of the cell cycle checkpoint kinase 1 and 2 (CHK 1/2), which are expressed at higher levels in cancer cells compared with normal tissues.^{101,102} Inhibition of CHK 1/2 activity leads to replication catastrophe, thereby inducing cell death and sensitizing cancer cells to the antitumor activity of PARP inhibitors. Prexasertib in combination with olaparib has shown antitumor activity in patient-derived, ovarian cancer xenograft models with acquired resistance to olaparib and in olaparib-sensitive models with an increase in the extent and durability of the antitumor responses observed.¹⁰¹ Initial findings from a phase II study of prexasertib in patients with recurrent, mostly platinum-resistant/refractory, *BRCA*-non mutated, high-grade ovarian cancer demonstrated a 33% response rate in evaluable patients.¹⁰² Neutropenia, leukopenia, and thrombocytopenia were the most frequent grade 3–4 AEs associated with treatment ($\geq 25\%$ of patients). However, grade 4 neutropenia observed in $\sim 79\%$ of cases after first dose administration appeared transient and improved without growth factor therapy.¹⁰² Further

evaluation of prexasertib is in progress in patients with advanced, *BRCA*-mutated ovarian cancer.¹⁰³

Acting in concert with CHKs (i.e. CHK1), the ataxia telangiectasia mutated and Rad3-related (ATR) serine threonine protein kinase contributes to genomic stability by regulating initiation of DNA replication and DNA repair.¹⁰⁴ The ATR kinase inhibitor AZD6738 is an ATP-competitive, small-molecule inhibitor of ATR which inhibits phosphorylation of CHK1 Ser345, leading to an impairment in cell cycle progression and cell proliferation.¹⁰⁵ Synergistic activity was observed against tumor cells with AZD6738 and DNA-damaging agents (i.e. cisplatin, carboplatin, gemcitabine), ionizing radiation, or PARP inhibitors.¹⁰⁶ A phase II study is assessing AZD6738 in combination with olaparib in patients with recurrent platinum-sensitive or platinum-resistant ovarian cancer.

Consistent with its mechanism of action, the selective ATR kinase inhibitor berzosertib (M6620/VX-970/VE-822) has shown enhanced induction of double-strand DNA breaks and antitumor activity in combination with the topoisomerase I inhibitor topotecan in patients with platinum-refractory, solid tumors.^{107,108} The most frequent treatment-related AEs were hematologic, with grade 3–4 anemia, leukopenia, and neutropenia observed in 19% and thrombocytopenia in 10% of treated patients.¹⁰⁸ Preliminary results from a randomized phase II study in platinum-resistant ovarian cancer have recently demonstrated a prolongation in mPFS with berzosertib in combination with gemcitabine versus gemcitabine alone in the overall intent-to-treat population (22.9 v 14.7 weeks, $p = .047$), which was mainly due to the benefit observed in a subgroup analysis of the patients stratified for platinum-free interval ≤ 3 months.¹⁰⁹ Berzosertib is currently being evaluated in a further, phase I study in triple combination with carboplatin and gemcitabine in recurrent, platinum-sensitive disease (Table 6).

Conclusions

The identification of new, effective combination regimens including chemotherapeutic agents, PARP inhibitors, angiogenesis inhibitors and/or other novel, targeted agents may provide therapeutic options that could prove beneficial for a substantial proportion of patients, particularly in the early lines of treatment for advanced disease. Accordingly, based on the findings reported from multiple phase III trials and recent FDA approvals, PARP inhibitors may become a new standard of care for maintenance treatment of newly diagnosed patients with advanced ovarian cancer. Validation of key biomarkers able to predict response to agents in each drug class may further result in improved drug selection at treatment initiation, maintenance phase, or switch to a new therapeutic option in case of tumor resistance and disease progression.

Chemotherapy-free regimens of potentially comparable or greater efficacy, based on various combinations of PARP inhibitors, PD-1/PD-L1 antibodies, and/or VEGFR inhibitors are being actively pursued to reduce the burden of toxicity associated with treatment. The eagerly awaited results from the ongoing phase II–III trials outlined in this review will provide evidence on the feasibility of these new therapeutic approaches and their potential to change the current standards of care for

advanced disease. Investigational agents with novel mechanisms of action (i.e. inhibition of key factors in the cell cycle, DNA repair and protein kinase pathways), designed to overcome the limitations imposed by primary and secondary tumor resistance to available therapies, also appear to be opening new, promising avenues for selective targeting of ovarian tumors in concert with other DNA-damaging or targeted agents and PD-1/PD-L1 immune-checkpoint inhibitors.

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Strategies in Overcoming Homologous Recombination (HR) Proficiency and Poly (ADP-Ribose) Polymerase Inhibitor (PARPi) Resistance

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ABSTRACT

Ovarian cancer is the second most common gynecologic malignancy in the United States (US) and the most common cause of gynecologic cancer-related death. The majority of ovarian cancers ultimately recur despite excellent response rates to upfront platinum and taxane-based chemotherapy. Maintenance therapy after frontline treatment has emerged in recent years as an effective tool for extending the platinum-free interval of these patients. Maintenance therapy with poly (ADP-ribose) polymerase inhibitors (PARPi) in particular has become part of standard of care in the upfront setting and in patients with platinum-sensitive disease. HR deficient (HRD) tumors have a nonfunctioning homologous recombination repair (HRR) pathway and respond well to PARPi, which takes advantage of synthetic lethality by concomitantly impairing DNA repair mechanisms. Conversely, patients with a functioning HRR pathway, i.e. HR proficient (HRP) tumors, can still elicit benefit from PARPi, but the efficacy is not as remarkable as what is seen in HRD tumors. PARPi are ineffective in some patients due to HR proficiency, which is either inherent to the tumor or potentially acquired as a method of therapeutic resistance. This review seeks to outline current strategies employed by clinicians and scientists to overcome PARPi resistance – either acquired or inherent to the tumor.

I. Introduction

Gynecologic malignancies are among the leading cause of cancer-related death in women in the United States (US) with ovarian cancer being the deadliest. The American Cancer Society (ACS) predicts that over 21,000 women will be diagnosed with ovarian cancer in 2020 in the United States (US), and approximately 14,000 women will die from their disease [1]. Historically, patients with HGSOV have been treated with a combination of surgical cytoreduction with platinum- and/or taxane-based chemotherapy. Despite promising initial responses to therapy that include complete responses (CRs), approximately 80% of women experience disease progression or recurrence [2]. For this reason, further research into superior treatment options for these patients is of necessary.

Poly (ADP-Ribose) Polymerase (PARP) proteins are involved in the repair of both single- and double-stranded DNA breaks (SSB and DSB) through several repair pathways, including mismatch repair (MMR), nonhomologous end joining (NHEJ), and nucleotide excision repair (NER) [3]. Notably, PARP proteins are integral to base excision repair in which a single misplaced base is exchanged with the correct base (**Figure 1**). In cells with a non-functional homologous recombination (HR) repair pathway, PARP-mediated base excision is utilized for cell survival. Inhibiting these pathways via PARP inhibition (PARPi) causes an accumulation of SSBs that eventually stall replication forks and prohibit replication, causing an accumulation of DSBs. The failed repair of DSBs due to HRD can result in insurmountable DNA damage that ultimately leads to cancer cell death. [4]. HRD can arise from mutations in the tumor suppressor genes BRCA1/2, and other genes involved in the HRR pathway, including ATM, CHEK2, BRIP1, RAD51C, and PALB2.

Once it was discovered that cells with mutations in *BRCA1/2* had increased sensitivity to PARPi, [5], the concept of synthetic lethality quickly became the focus of many preclinical and clinical

studies. Synthetic lethality occurs when cells that are genetically predisposed to the inactivation of one pathway are targeted by the intentional inactivation of a second pathway, whereas the inactivation of either pathway alone would not be enough to kill the cell [6]. The SOLO1 trial (NCT01844986) led to the Food and Drug Administration (FDA) approval of a PARPi, olaparib, for upfront maintenance therapy in *BRCA*-mutated patients and demonstrated an unprecedented improvement in progression free survival (PFS) of 36 months compared to placebo [7]. Similarly, the SOLO2 trial evaluated the use of maintenance olaparib in recurrent, platinum sensitive, *BRCA1/2*-mutated ovarian cancers and demonstrated an improvement of PFS in the olaparib group. The recently published SOLO2 data showed a benefit of 13 months in OS, further proving the efficacy of maintenance PARPi in this patient population [8]. Additionally, niraparib maintenance therapy has now been approved in the frontline setting regardless of *BRCA* or HR status based on the PRIMA trial (NCT02655016), although the magnitude of benefit is not as great in the HR proficient (HRP) population [9]. Three PARPi's (niraparib, olaparib, and rucaparib) are currently approved for use in the maintenance setting of treatment for platinum sensitive HGSOc.

II. Mechanisms of PARPi Resistance

Despite the revolutionary impact of PARPi on the HRD population, PARPi resistance (PIR) has been observed in many patients [10]. Among those mechanisms of resistance studied includes the upregulation of drug efflux pumps such as p-glycoprotein. This upregulation was seen after treatment of *BRCA-1* deficient breast cancer cells in mice that developed PARPi resistance [11]. This mechanism of resistance through drug efflux is also a commonly described mechanism of resistance to platinum-based chemotherapy through a variety of transporters including CTR1, CTR2, ATP7A, and ATP7B [12].

The vast majority of PIR mechanisms revolve around the restoration of HRR in cells that are deficient. Previous studies have shown that the restoration of wild-type *BRCA1/2* (*wtBRCA*) phenotype in patients whose tumors harbor *BRCA1/2* mutations – either through secondary mutations or epigenetic modifications – can cause significant PIR [13, 14]. One study showed that nearly 50% of platinum-resistant ovarian carcinomas had a secondary mutation that restored the *wt-BRCA* gene and thus HR function [13]. Other HR gene mutations have been shown to be reversed by secondary mutations, including *PALB2*, *RAD51C*, and *RAD51D*. In fact, this mechanism of resistance via restoration of HR has been shown to play a role in the resistance of ovarian cancer cells to platinum-based chemotherapy as well [15]. Particular sensitivity of ovarian cancer cells with HRD to platinum-based chemotherapy has been described, as it allows for the accumulation of DNA DSBs that are rendered irreparable by the lack of HR functionality. Subsequently, the theoretical resistance to platinum-based therapy as a result of regaining HR function has been proven true as seen by *BRCA* reversion mutations in patients with platinum-resistant ovarian cancer [16]. This overlap in resistance allows for the further evasion of HGSOC from advanced therapy.

An additional mechanism of PIR that has been described is the rewiring of HRR and the DNA damage response by replication fork protection. HR proteins such as *BRCA1/2* function to prevent replication fork stress and therefore protect genomic stability [17]. Resistant cells have been shown to bypass the loss of fork protection through mechanisms like the downregulation of *MRE11* or loss of *CDH4* [18]. Since restoring replication fork protection is independent of DNA repair, combination therapy targeting this mechanism could result in synergistic lethality with PARPi.

Downregulation of *PARP1* proteins is another classically described mechanism of PIR. During PARPi treatment, the cytotoxicity that occurs as a result of *PARP1*-trapped DNA complexes can

lead to a depletion of PARP protein expression rather than cell death [19]. Moreover, a clinical case of PIR has been seen as a result of a *PARP1* mutation [20]. The loss of PARP1 protein in cancer cells was only found in patients with either some or all of the functioning HR pathway. Thus, it can be conferred that patients with complete loss of HR do not show this mechanism of PARPi resistance.

According to the Cancer Genome Atlas (TCGA), approximately half of ovarian cancers are considered to be HRP [21]. Additionally, PIR occurs in over 50% of patients with ovarian cancer, which poses a question of how clinicians can overcome both acquired and *de novo* HRP. These tumors have an overall worse response to platinum-based chemotherapy and dismal survival outcomes, as shown by a worse OS in *wtBRCA* cases compared to *BRCA*-mutated cases (median OS ~40 months vs ~60 months, respectively) [22]. Because of the unmet need to improve outcomes in HRP patients, many studies are actively trying to better understand ways to “switch” an HRP tumor to have an HRD phenotype. This review focuses on research regarding the utilization of various targeted agents (**Tables 1 and 2**) to sensitize HRP ovarian cancers to PARPi or re-sensitize HRD patients that have become resistant to PARPi.

III. ATR inhibitors

Historically, ataxia telangiectasia Rad3-related (ATR) is known to be a major regulator of a cell cycle checkpoint signaling pathway controlled by checkpoint kinase 1 (Chk1). This pathway functions during the G2/M phase of the cell cycle to recognize DNA irregularities and induce cell cycle arrest for DNA repair [23]. Beyond its role in the Chk1 pathway of DNA repair, ATR has also been implicated as a regulator of several proteins in the HRR pathway, including activation of BRCA1, PALB2, and RAD51, which suggests the potential for mechanistic synergism between ATR inhibitors (ATRi) and PARPi [24]. In fact, the use of ATRi has been shown to sensitize both HRP and HRD ovarian cancer cells to the use of PARPi in preclinical models [25].

Several clinical trials have explored this combination treatment, including an ongoing phase I trial investigating the combination therapy of cisplatin, veliparib, and VX-970 (ATRi) in the treatment of refractory solid tumors (NCT02723864).

ATRi have also been found to impair replication fork protection, a known mechanism of PIR. They target this mechanism by inhibiting Rad51-loading onto stalled replication forks in HRD cells, subsequently subjecting the cell to synthetic lethality induced by PARPi [26]. The ATRi VE-821 has been shown to enhance the degradation of stalled replication forks using *ex vivo* DNA fiber analyses. Unfortunately, this effect was not seen in cells with wtBRCA proteins, showing that the use of ATRi may not extend to innately HRP tumors [27].

Previous studies have demonstrated a relationship between PARPi response and the expression of SLFN11, a gene involved in cell-lethal replication inhibition [28]. By prolonging cell cycle arrest and inducing replication fork damage, high *SLFN11* expression can cause hypersensitivity to PARPi [29]. PIR was further demonstrated after the SLFN11 gene was inactivated in preclinical models [28]. These cells were no longer able to go through PARP trapping at prolonged cell arrest points and thereafter increased the reliance on ATR checkpoints to promote cell survival [28]. Using this data as a foundation, ATRi could be of particular use for PARPi sensitization in cancer cells with PIR.

IV. PI3K inhibitors

The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway has been heavily investigated given its multiple roles in cancer progression. Aberrations in this pathway can contribute to both the initial development and the survival of ovarian cancer cells, providing an opportunity for targeted therapies. Despite this, there has been minimal clinical benefit seen when treating EOC patients with single-agent PI3K inhibitors (PI3Ki) [30]. In contrast, there is an

abundance of preclinical data supporting the synergism between PARPi and PI3Ki as PI3Ki can increase the antimetabolic effects of PARPi [31]. One study showed that PI3Ki caused the cessation of DNA synthesis as well as cytoskeletal functions, processes that are further exacerbated by PARPi. Utilizing mouse models of mammary epithelial cells, treatment with PARPi + PI3Ki resulted in a decrease in S-phase cell cycle progression due to impairment of nucleotide synthesis [32].

When considering the combinational use of PARPi and PI3Ki in the treatment of ovarian cancer, the crosstalk of the PI3K and HRR pathways must be taken into account. Beyond its mechanisms of cell cycle regulation and metabolic influence, PI3K helps control the repair of DSBs by acting as a sensor of genomic instability and detecting DSBs [33]. One preclinical study showed that PI3Ki resulted in the downregulation of *BRCA1/2* and induced a HRD phenotype in the cell [34]. Additionally, a phase Ib study published in 2019 showed that combining the PI3Ki alpelisib with olaparib in recurrent HGSOC was safe; and potential efficacy was seen with an overall response rate of 33% [35]. Currently, there is a phase I clinical trial (NCT01623349) examining the oral PI3Ki's, BKM120 and BYL719, in conjunction with olaparib in patients with HGSOC and triple-negative breast cancer (TNBC). Preliminary data from this trial has shown a 29% response rate in patients with wt*BRCA1/2* tumors regardless of platinum sensitivity, recommending phase II studies to further examine this relationship [31]. The current data fully supports continued investigation of the clinical synergism between PI3Ki and PARPi.

V. Glutaminase inhibitors

The metabolic alterations in tumor cells have been thoroughly investigated, with particular focus on the adjustments that tumor cells make to enable their survival in hypoxic conditions. It has been demonstrated that cancer cells have a decreased ability for glucose to enter the tricarboxylic acid (TCA) cycle, which results in reliance on other carbon sources for the cell,

including glutamine [36]. This shift towards using glutamine is an integral part of the development and progression of invasive and advanced ovarian cancer.

Previous data has shown that inhibition of glutaminase can cause an arrest in the growth of HGSOC [37]. When exploring this mechanism as a potential cancer therapeutic target, one must consider the role of phosphate-activated mitochondrial glutaminase (GLS1), a key enzyme involved in glutamine metabolism that allows the conversion of glutamine to glutamate [38]. A study investigating the effects of the GLS1 inhibitor compound 968 found that glutaminase inhibition caused cessation of ovarian cancer cell proliferation and reduced the glutamine metabolism for the sustainment of cancer cells [39]. This inhibitor also increased ROS formation, cell apoptosis, and induced cell cycle arrest leading to tumor cell destruction. The use of glutaminase inhibitors to sensitize ovarian cancer to PARPi is still being explored. Glutamine is a necessary nitrogen source for nucleotide synthesis and subsequent DNA synthesis. Thus, by inhibiting the metabolism of glutamine, the cell would experience a depletion of completed nucleotides and would be unable to repair DNA effectively [40]. This buildup of DNA replication stress can thereafter be exploited by PARPi in cancer cells. Okazaki *et al.* found synergism between glutaminase inhibitors and PARPi in the treatment of renal cell carcinoma due to von Hippel-Lindau (VHL) disease [41]. Currently, a phase I/II clinical trial is investigating the results of treating metastatic solid tumors with a combination of the glutaminase inhibitor, CB-839, and PARPi, talazoparib (NCT03875313). Additionally, an upcoming investigator-initiated clinical trial at our institution will explore the efficacy of combining CB-839 with niraparib in the treatment of platinum resistant, wtBRCA1/2 ovarian cancer (NCT03944902). This could provide insight as to possible mechanism of overcoming HRP, although more research exploring the potential to reverse this phenotype with glutaminase inhibitors is warranted.

VI. HDAC Inhibitors

HDAC Class 1 expression is prevalent in ovarian cancers and has been examined as a potential cause of resistance to platinum-based chemotherapy [42]. HDAC expression in HGSOC is associated with a poor prognosis and provides an opportunity for targeted pharmacotherapy. HDAC inhibitors (HDACi) have been shown to downregulate the transcription of wild-type HRR genes such as *RAD51*, leading to a further increase of irreparable DNA damage and providing an opportunity for combination treatment with PARPi [43].

The HDACi romidepsin has been shown to enhance the antitumor effects of cisplatin by further inflicting DNA damage [44]. In a study using the HDACi sodium butyrate, tumor radiosensitivity was enhanced due to the DNA damage caused by downregulation of HRR genes [43]. This theory was further explored by Konstantinopoulos *et al.*, who showed through preclinical microarray assays that vorinostat, also known as suberanilohydroxamic acid (SAHA), induced the downregulation of critical HRR genes *RAD51* and *BRCA* [45]. Currently, there is a phase I clinical trial underway looking at the combinational treatment of olaparib with vorinostat in the treatment of metastatic breast cancer (NCT03742245). Future work could explore this combination therapy in patients with *wtBRCA* or *BRCA*-mutated ovarian cancer resistant to PARPi therapy.

VII. Immune Checkpoint Inhibitors

The response of HGSOC to immune checkpoint modulators has been variable, usually seen only in patients with high microsatellite instability (MSI-H) [46]. For this reason, other agents such as PARPi have been explored to be used in conjunction with immunotherapy in the treatment of HGSOC. It has previously been demonstrated that HRD tumors are more sensitive to immune checkpoint blockade (ICB) therapies than HRP tumors [47]. In a preclinical study investigating the efficacy of treating ovarian cancer cells with a PARPi prior to the use of ICB

therapy, combining niraparib with full dose anti-PD-1 resulted in significant tumor growth inhibition compared to either drug used alone [48]. Although preclinical studies strongly suggest that the use of PARPi and ICB therapy is of clinical benefit in patients with HRD, the utility was not demonstrated in patients with HRP [47].

A single-arm phase I/II clinical trial (NCT02657889) investigating the clinical response of patients with HGSOC to both PARPi and ICB therapy using pembrolizumab demonstrated promising antitumor activity. In fact, even patients with a functioning HR pathway had higher antitumor activity than those treated with monotherapy niraparib or pembrolizumab [49]. These findings suggest the potential for clinical combination of ICB therapies and PARPi in the setting of HRP.

VIII. VEGFR and EGFR inhibitors

The vascular endothelial growth factor (VEGF) protein family consists of growth factors that promote increased vascularity and angiogenesis in response to hypoxic conditions. VEGF is induced by hypoxia inducible factors (HIFs) that are upregulated to allow tumors to thrive in a hypoxic environment, which plays a part in several diseases when over-expressed [50]. Various agents targeting VEGF have been clinically utilized in several malignancies with the intention of destroying the tumor vascular supply [51].

A phase II clinical trial investigated the combination of olaparib and cediranib, a potent inhibitor of VEGF receptors (VEGFR), in the treatment of platinum resistant ovarian cancers (NCT01116648). The combination treatment resulted in an increase in PFS compared treatment with olaparib alone [52]. Specifically, the added activity of cediranib increased tumor regression even in patients with *wtBRCA1/2*, suggesting that synergism induced by cediranib extends to HRP tumors [52]. Despite this, GY004 (NCT02446600), a recent phase III open-label clinical

trial that was designed to expand on NCT01116648, showed no significant improvement in the PFS of patients treated with the combination treatment compared to olaparib alone (regardless of HR status). This mechanism could be explained by data showing that VEGFR inhibition in ovarian cancer cells is associated with decreased expression of *wtBRCA1/2* [52].

Furthermore, the epidermal growth factor receptor (EGFR/ERBB/HER) has been shown to be associated with accelerated tumor growth when overexpressed. The use of the EGFR inhibitor neratinib has been shown in preclinical studies to act in synergy with niraparib to accentuate ovarian cell death via exploitation of synthetic lethality to heighten levels of DNA damage [53]. This is being further explored in an ongoing Phase I/Ib clinical trial utilizing the combination therapy in the treatment of platinum-resistant ovarian cancer (NCT04502602). Overall, the use of EGFR inhibitor therapy in combination with PARPi should be explored further to understand its use in the treatment of HRP ovarian cancers and whether it can overcome that barrier of treatment.

IX. WEE1 inhibitors

Wee-1-like kinase (WEE1) is highly upregulated in certain cancers, including HGSOC, as well as glioblastoma, osteosarcoma, melanoma, and breast and vulvar carcinoma [54]. By targeting the G2-M checkpoint via WEE1 inhibition (WEE1i), tumor cells with dysfunctional G1-S checkpoints are selectively targeted and ultimately undergo cell death due to irreparable DNA damage [55].

In recent years, WEE1 has been linked to the HRR pathway via its interaction with DNA repair mechanisms, making it an interesting target to study in combination with PARPi. A study using the WEE1i AZD1775 in combination with gemcitabine-radiation in pancreatic cancer cells

showed that upon treatment with AZD1775 and chemoradiation, only HRP cells were sensitive to chemoradiation [56].

One proposed mechanism for WEE1's role in HRR is through nucleotide exhaustion and PARP trapping. A previous study investigating the combination AZD1775 with olaparib found that only *KRAS*-mutant non-small cell lung cancer (NSCLC) were more sensitive to radiotherapy than cells treated with either agent alone [57]. Additionally, through experiments using nucleotide depletion, it was observed that PARP1 trapping must be present for WEE1i to sensitize tumors to radiotherapy. Although PARPi alone can radio-sensitize a tumor regardless of PARP1 trapping activity, the current theory for the synergy of WEE1i with PARPi is that the two agents impair DNA replication at multiple time points [57]. More experiments must be done to further characterize how WEE1i impacts the HRR pathway.

X. BET inhibitors

Similar to WEE1i, BET inhibitors (BETi) have been increasingly linked to the HRR pathway by synergizing with PARPi via induction of DNA damage [58]. The BET family of proteins includes BRD2, BRD3, BRD4, and BRDT, each with a conserved N-terminal bromodomain. One study has shown that *BRD4* is a necessary factor for survival of ovarian cancer cell lines such as OVCAR8 [59]. Additionally, it was observed that HGSOC tumors with *BRD4* amplifications may derive the most clinical benefit from BETi [60]. This finding is particularly interesting as the *BRD4* overexpressing subtype often do not harbor *BRCA1/2* mutations, rendering these cancers limited in their treatment options.

BRD4 expression has also been recognized as a component of PIR. In a study investigating aldehyde dehydrogenase (ALDH) expression in ovarian cancer cell lines, cells with acquired resistance to olaparib demonstrated increased activity in ALDH via elevated expression of

ALDH1A1 [61]. This increase in *ALDH1A1* expression stems from *BRD4* over-expression, which can be induced by exposure to olaparib. BETi has been shown to suppress the expression of both *WEE1* and *TOPBP1*, which sensitized wt*BRCA1/2* cells to a PARPi. Additionally, BETi treatment was able to re-sensitize m*BRCA2* cells with acquired olaparib resistance to PARPi [58]. Inhibition of *BRD4* has also been shown to result in inhibition of *ALDH1A1* expression, which assisted in overcoming PIR in HGSOC cells [61].

The *BRD4* inhibitor INCB054329 caused decreased activity in both *BRCA1* and *RAD51* as well as HR reporter activity in an HRP ovarian cancer cell line, supporting its ability to induce an HRD phenotype [62]. Additionally, cell lines treated with the combination of INCB054329 and olaparib showed increased tumor cytotoxicity compared to cells treated with either agent alone [62]. A similar study demonstrated that the three BETi: JQ1, I-BET762, and OTX015 were able to sensitize HRP cells to PARPi [63]. Cumulatively, these findings suggest that the combination of BET and PARP inhibition warrants further investigation in HRP tumors.

XI. BCL2 Inhibitors

B-cell lymphoma 2 (*BCL2*) is a group of proteins that are key cell death regulators, making it a potential key player to target cancer cells [64]. The expression of *BCL2* genes have been shown to be associated with increased chemotherapy resistance, specifically taxane and platinum-based therapies [65].

Synergy has been observed between rucaparib and the *BCL2* inhibitor navitoclax – the combination therapy induced more apoptosis than navitoclax monotherapy [64]. Preferential apoptotic activity was seen in cells with mutations in HRR genes, indicating that cells with a dysfunctional HRR system may rely more heavily on the anti-apoptotic *BCL* group of proteins. Furthermore, Stover et.al. demonstrated the effectiveness of *BCL2* inhibition in sensitizing

HGSOC cells to PARPi [65]. Lastly, the use of combination therapy with navitoclax and talazoparib in the treatment of three *wt-BRCA* HGSOC cell lines showed a greater cytotoxic effect than seen with monotherapy, suggesting a potential clinical benefit in HRP tumors [66].

Currently, an ongoing phase II clinical trial (NCT02591095) is seeking to determine survival of patients with platinum-resistant/refractory ovarian cancer while treated with single agent use of the BCL2 inhibitor ABT263. Further clinical trials are warranted investigating the clinical use of combinational therapy with BCL2 inhibitors and PARPi in the treatment of ovarian cancer.

XII. CDK1 inhibitors

Cyclin dependent kinase 1 (CDK1) is a key cell cycle regulator whose function is essential for cell proliferation [67]. It forms a complex with cyclins to regulate G1/S phase gene transcription, ultimately promoting cell cycle progression through the phosphorylation of cell cycle regulators. It has previously been demonstrated that CDK1 modulates the BRCA1 protein, and a preclinical study demonstrated the cessation of S phase checkpoint activation due to short hairpin RNA (shRNA)-mediated CDK1 depletion in TNBC [68]. This inhibition causes a decrease in the phosphorylation of cell cycle regulator, BRCA1, resulting in the inhibition of BRCA1-mediated foci formation at sites of DNA damage [68]. By disrupting BRCA1 function, it was theorized that CDK1 could inhibit not only cell cycle checkpoints, but other critical DNA damage repair pathways as well.

This theory was later investigated in a study which found that decreased CDK1-mediated BRCA1 phosphorylation was able to inhibit the HRR pathway [69]. Furthermore, CDK1 inhibition (CDK1i) resulted in an 80% decrease in RAD51 foci formation in an *in vitro* lung adenocarcinoma model. RAD51 function plays a critical role in the HRR pathway, and its decreased expression is associated with *BRCA1*-deficient cells [69]. This data demonstrated

that CDK1i could inflict similar damage to both *wtBRCA1/2* and *mBRCA1/2* cells, and therefore leaving the cells susceptible to PARPi. As such, combining a PARPi with a CDK1i, such as dinaciclib, could induce synthetic lethality within HRP tumors.

An ongoing phase I trial combining dinaciclib and veriparib in the treatment of advanced solid tumors (NCT01434316) is investigating both *wtBRCA* and *BRCA*-mutated tumors to determine their sensitivity to PARPi with and without a CDK1i. More preclinical and clinical data are needed to determine the effect on PARPi sensitization using dinaciclib in HGSOC patients with and without functioning HRR.

XIII. Hsp90 inhibitors

Mutated *BRCA* proteins can cause defective protein folding, leading to an unstable secondary structure that ultimately subjects the protein to degradation. Heat shock protein 90 (Hsp90) is a chaperone protein responsible for the stabilization of proteins against this very mechanism. The stabilization of *mBRCA* proteins via Hsp90 can result in enhanced RAD51 loading onto stalled replication forks that bypass the HRR pathway [70]. The interaction of Hsp90 with *BRCA* proteins as a mechanism of PIR has been a recent focus of investigation in cancer therapeutics.

Beyond the potential for Hsp90 inhibitors (Hsp90i) to reverse acquired PIR in HRD tumors, preclinical studies have shown that this could be applied even to HRP cells. In a study exposing HRP breast cancer cells to the HSP90i 17-AAG, treatment resulted in decreased activity of the HRR pathway, ATM serine/threonine kinase, and Fanconi Anemia DNA repair pathways [71]. Treatment with 17-AAG also down-regulated *wtBRCA1* and RAD51 levels, thus inducing an HRD state in initially HRP cancer cells. Most remarkably, the exposure of 17-AAG to HRP EOC cells sensitized the cells to olaparib and platinum-based chemotherapy, as measured by DNA damage using a γ H2AX assay measurement [71]. This sensitization was not seen in EOC cells

with HRD, suggesting a combination of PARPi and HSP90i could be explored clinically within
 HRP HGSOC patients.

CONCLUSION

Targeting the HRR pathway in ovarian cancer through PARPi has been a paradigm-shifting
 treatment strategy over the past decade. However, approximately half of patients have a limited
 response to PARPi due to HRP. Therefore, there has been a focus of research in the last
 decade as to whether innate or acquired HRP can be overcome in order to induce PARPi
 sensitivity. Various agents have shown promise in this area either by working synergistically
 with PARPi or by targeting the HRR pathway through an alternate strategy, both of which have
 potential clinical benefit in patients with otherwise limited options. The pharmacotherapies
 mentioned in this review are currently under preclinical or clinical investigation for use in
 combinational treatment with PARPi to improve response rates and survival outcomes. A variety
 of clinical trials across the country have the goal of maximizing clinical benefit from PARPi and
 helping to overcome or prevent therapeutic resistance. Genetic and molecular data are rapidly
 being integrated into cancer care. These analyses have immense potential to improve patient
 outcomes by providing more targeted treatments that can be used in combination with PARPi.
 As the scientific and clinical communities continue to better understand the underlying genetics
 of the disease and how to manipulate mechanisms that repair DNA damage, we are optimistic
 that these approaches will ultimately improve the survival of women with ovarian cancer.

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Table 1. Summary of pre-clinical studies utilizing PARPi combinational therapy

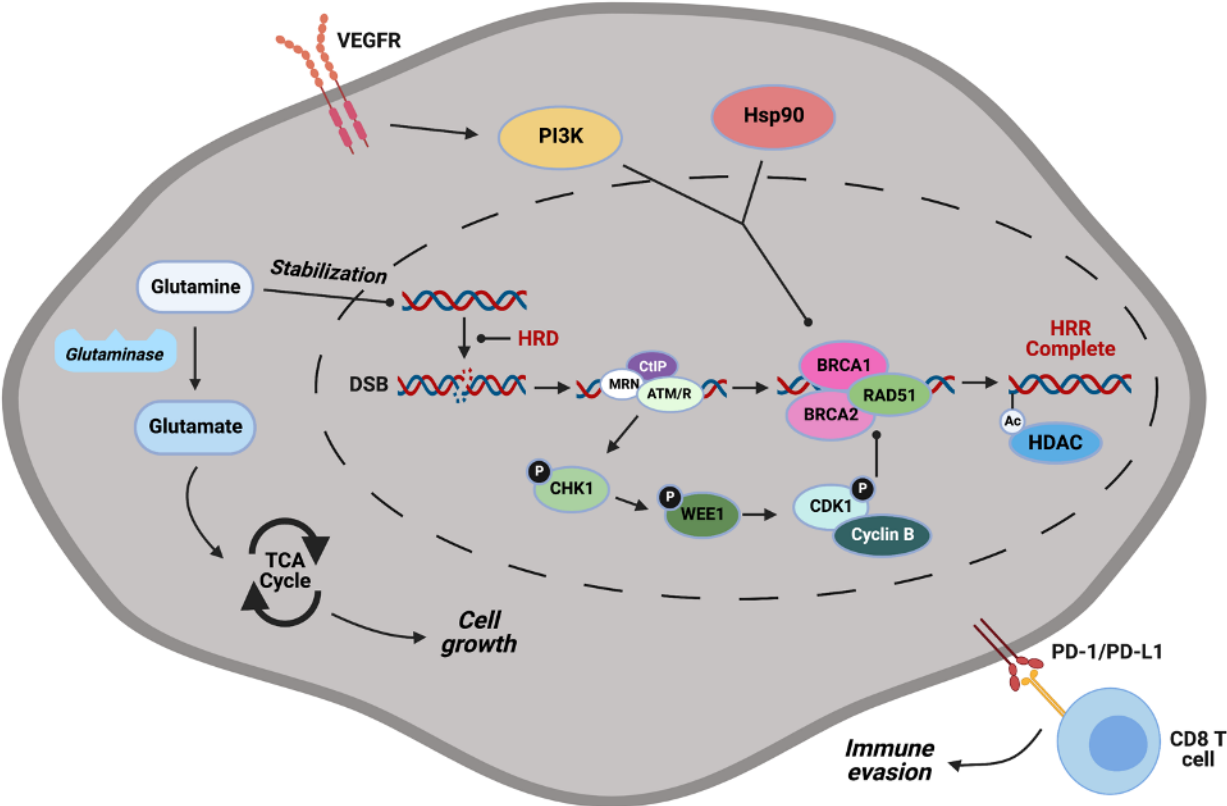
Drug Mechanism	First Author, Year	Drugs Used	Model	Summary of Results
ATR Inhibitors	Yazinski et.al., 2017	Olaparib + VE-821, AZ-20	<i>mBRCA1</i>	Disruption of resistance mechanisms and overcoming of resistance to PARPi
	Kim et. al., 2020	Olaparib + AZD6738	<i>mBRCA1</i> cells, <i>mBRCA2</i> PDX	Increased survival of PARPi-resistant cells
PI3K Inhibitors	Wang et.al., 2016	Olaparib + BKM120	<i>mBRCA2</i> OVCA433; <i>mBRCA1</i> OVCAR8; <i>wtBRCA1/2</i> OVCAR5	Attenuation of DNA repair impairment compared to PARPi treatment alone; decreased growth
	Juvekar et.al., 2012	Olaparib + BKM120	<i>mBRCA1</i> HCC1937	Delay in tumor growth
	Ibrahim et.al., 2012	Olaparib + BKM120	MDA-MB-468, MDA-MB-231, HCC70, HCC1143, BT20	Reduction in tumor growth and downregulation of <i>BRCA</i> expression
Glutaminase Inhibitors	Emberly et.al., 2018	Niraparib/Talazopraib + CB-839	TNBC, CRC, non-small cell lung carcinoma, ovarian and prostate cancer cells <i>in vivo</i> <i>VHL</i> -deficient/ <i>VHL</i> -replete	Enhanced anti-tumor activity
	Okazaki et.al., 2017	Olaparib + GLS1 inhibitors	UMRC2, UMRC3, RCC4, UOK102	Suppression of tumor cell growth
BET Inhibitors	Karakashev et.al., 2017	Olaparib + JQ1	<i>wtBRCA</i> OVCAR3	Synergistic increase in DNA damage and apoptosis
	Wilson et.al., 2018	Olaparib/Rucaparib + JQ1/INB054329/INCB057643	<i>wtBRCA1</i> OVCAR3, OVCAR4, SKOV3	Reduced HR activity and sensitized cell to PARPi, DNA damage, and cell death
	Yang et.al., 2017	Olaparib + JQ1/I-BET762/OTX015	<i>wtBRCA</i> OVCAR10	Impaired transcription of HR genes, sensitized tumors to PARPi
VEGFR Inhibitors	Kaplan et.al., 2019	Olaparib + Cediranib	IGROV1	Down-regulation of <i>BRCA</i> gene expression
WEE1 Inhibitors	Fang et.al., 2020	Talazoparib + Adavosertib	OVCAR8	Marked tumor regression with combination therapy
	Parsels et.al., 2018	Olaparib +AZD1775	Calu-6 and H23 NSCLC	Enhanced radiosensitization seen with combinational therapy
	Ha et.al., 2020	Olaparib +AZD1775	MDA-MB-157, MDA-MB-231, MDA-MB-468, HCC1143, BT-549, Hs 578 T	Induced apoptotic cell death with combinational therapy
CDK1 Inhibitors	Johnson et.al., 2011	Rucaparib + AG024322	MDA-MB-436	Sensitization of cells to PARPi <i>in vitro</i>
	Xia et.al., 2013	Olaparib + RO3306	MDA-MB-231, HCC1937, SK-BR-3, MCF-7	Decrease in cell growth with combinational therapy
Hsp90 Inhibitors	Choi et.al., 2014	Olaparib + 17-AAG	HR proficient: Hs578T, MCF-7, MDA-MB-157, T47D, MDA-MB-231 HR deficient: MBA-MD-436, HCC-1937, UACC3199	Sensitization of HR proficient cell lines to PARPi
	Gabbasov et.al., 2019	Talazoparib + Ganetespib	OVCAR3, OC-1, OC-16	Synergistic decrease in OC cell viability
	Jiang et.al., 2017	ABT-888 + Ganetespib	MCF7	Synergistic inhibition of tumor growth

4 Table 2: Summary of completed or currently active clinical trials utilizing combination PARPi therapy in ovarian cancer patients
5

Drug Mechanism	Study Phase	NCT ID #	Drugs Used	Study Status	Results Available
ATR inhibitors	Ib	NCT04267939	Niraparib + BAY 1895344	Recruiting	No
	I	NCT04149145	Niraparib + M4344	Not yet recruiting	No
	II	NCT04065269	Olaparib + AZD6738	Recruiting	No
	II	NCT03462342	Olaparib + AZD6738	Recruiting	No
	Ib	NCT02264678	Olaparib + Ceralasertib	Recruiting	No
PI3K Inhibitors	I	NCT01623349	Olaparib + BKM120 or BYL719	Active, not recruiting	Yes
	I	NCT04586335	Olaparib + CYH33	Recruiting	No
	I	NCT03586661	Niraparib + Copanlisib	Recruiting	No
Glutaminase Inhibitors	I	NCT03944902	Niraparib + CB-839	Not yet recruiting	No
VEGFR Inhibitors	II	NCT02340611	Olaparib + Cediranib	Completed	Yes
	I/II	NCT01116648	Olaparib + Cediranib	Active, not recruiting	Yes
	III	NCT02446600	Olaparib +/- Cediranib vs non-platinum chemo	Active, not recruiting	No
	III	NCT03278717	Olaparib +/- Cediranib	Recruiting	No
	II	NCT03326193	Niraparib + Bevacizumab	Active, not recruiting	No
	II	NCT02889900	Olaparib + Cediranib	Active, not recruiting	Yes
	II	NCT03314740	Olaparib + Cediranib vs Paclitaxel	Active, not recruiting	No
	I	NCT02855697	Olaparib +/- Cediranib	Active, not recruiting	No
	II	NCT03117933	Olaparib +/- Cediranib vs Paclitaxel	Active, not recruiting	No
	I	NCT02345265	Olaparib + Cediranib	Active, not recruiting	No
	I	NCT00989651	Carboplatin + Paclitaxel + Bevacizumab + Veliparib	Active, not recruiting	Yes
	II	NCT04566952	Olaparib + Anlotinib	Recruiting	No
WEE1 Inhibitors	II	NCT03579316	Adavosertib +/- Olaparib	Recruiting	No
CDK1 Inhibitors	I	NCT01434316	Veliparib + Dinaciclib	Recruiting	No
Hsp90 Inhibitors	II	NCT03783949	Niraparib + Ganetespib	Active, not recruiting	No
	I	NCT02898207	Olaparib + Onalespib	Active, not recruiting	No

6 **Figure 1:**

7 Overview of the cellular mechanisms that may be targeted to overcome Homologous Recombination Proficiency.



Molecular Cancer Therapeutics

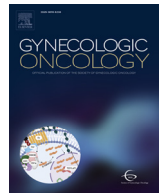
Strategies in Overcoming Homologous Recombination (HR) Proficiency and Poly (ADP-Ribose) Polymerase Inhibitor (PARPi) Resistance

Nidhi Goel, McKenzie E. Foxall, Carly Bess Scalise, et al.

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Understanding the effect of mechanical forces on ovarian cancer progression

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HIGHLIGHTS

- Oscillatory tension caused increased metastatic phenotypes to varying degrees in epithelial ovarian cancer cell lines.
- Several proteins and genes implied in cell growth, EMT, and metastasis were upregulated in cells under oscillatory tension.
- Mice were injected with cells placed under oscillating tension and developed tumors with increasingly aggressive phenotypes.

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ABSTRACT

Objective. Mechanical forces including tension, compression, and shear stress are increasingly implicated in tumor progression and metastasis. Understanding the mechanisms behind epithelial ovarian cancer (EOC) progression and metastasis is critical, and this study aimed to elucidate the effect of oscillatory and constant tension on EOC.

Methods. SKOV-3 and OVCAR-8 EOC cell lines were placed under oscillatory tension for 3 days and compared to cells placed under no tension. Cell proliferation, migration, and invasion were analyzed while RNAseq and Western Blots helped investigate the biological mechanisms underlying the increasingly aggressive state of the experimental cells. Finally, *in vivo* experiments using SCID mice assisted in confirming the *in vitro* results.

Results. Oscillatory tension (OT) and constant tension (CT) significantly increased SKOV-3 proliferation, while OT caused a significant increase in proliferative genes, migration, and invasion in this cell line. CT did not cause significant increases in these areas. Neither OT nor CT increased proliferation or invasion in OVCAR-8 cells, while both tension types significantly increased cellular migration. Two proteins involved in metastasis, E-cadherin and Snail, were both significantly affected by OT in both cell lines, with E-cadherin levels decreasing and Snail levels increasing. *In vivo*, tumor growth and weight for both cell types were significantly increased, and ascites development was significantly higher in the experimental OVCAR-8 group than in the control group.

Conclusions. This study found that mechanical forces are influential in EOC progression and metastasis. Further analysis of downstream mechanisms involved in EOC metastasis will be critical for improvements in EOC treatment.

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1. Introduction

Epithelial ovarian cancer (EOC), the leading cause of cancer-related mortality in women worldwide [1], will be diagnosed in over 20,000 women in the United States in 2020 with approximately 14,000 patients expected to die [2]. EOC accounts for the majority of ovarian

malignancies [3], which typically present in later stages [1]. Unfortunately, the majority of cases result in disease recurrence and/or chemoresistance [4]. Understanding how these tumors recur and metastasize is critical in developing successful treatment strategies.

Significant efforts have been made to understand the biochemical regulation and signaling pathways involved in EOC progression and metastasis [5–8]. However, the impact of mechanical forces on these parameters, known as mechanotransduction, in EOC progression has largely been ignored [9,10]. This process plays an essential role in normal tissue development and homeostasis as well as in cancer progression [10], but the exact mechanisms by which cancer cells experience

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mechanical forces and react during tumor progression, invasion, and dissemination to a metastatic site have yet to be elucidated. It is known that cancer cells experience intratumoral pressure due to uncontrolled cell growth and a stiffening extracellular matrix (ECM) that is subsequently remodeled during cancer progression [11–13]. There are two types of mechanical forces imparted on the tumor: solid forces, exerted by the solid parts of a tumor, or fluid forces, created from fluid that surrounds and penetrates the tumor (*i.e.*, ascitic fluid or blood) [14]. It is important to acknowledge that these mechanical forces are imparted on the tumor microenvironment (TME) of EOC primarily as shearing forces from ascites and interstitial fluid, tension on the tumor periphery from tumor expansion against the ECM and TME, and internal compression forces from tumor expansion and the hydrostatic pressure of ascites [15]. The importance of tension, compression, and fluid shear have been modeled in many studies on multiple different aspects of the tumor, including cancer-associated fibroblasts, angiogenesis and the resulting tumor vasculature, matrix remodeling, and cancer cells themselves [16–21]. However, there are very few mechanotransduction studies performed in EOC. This paper focuses on the response of EOC to constant and oscillatory tension.

The presence of ascites in EOC patients makes the TME of EOC particularly dynamic. Cells from the primary tumor detach and travel as spheroids through ascitic fluid into the peritoneal cavity and then implant in a secondary location *via* the transcoelomic metastasis pathway [22–24]. In order for the cells to detach from the tumor they undergo an epithelial to mesenchymal transition (EMT). In this process, the cancer cells lose their epithelial phenotype and gain mesenchymal characteristics to facilitate migration through the ECM [25]. EMT begins with the downregulation of several epithelial proteins (*i.e.*, E-cadherin, cytokeratins) with the simultaneous up-regulation of the transcription factors snail, slug, and twist as well as mesenchymal proteins including N-cadherin or vimentin [26–28].

Several efforts have been made to understand how shear [29–31] and compressive [32–34] forces affect EMT and tumor metastasis in EOC, while the effects of tension in EOC still remains poorly investigated. In addition, tensile forces have not been measured in EOC patients, and it remains unclear whether these forces follow an oscillatory pattern (periodically changing force) or constant pattern (force with unchanged

rate) (Fig. 1D). Prior studies in other cancer types suggest that patterns of both constant tension (CT) [35] and oscillatory tension (OT) [21,36–38] could have an impact on tumor progression. We aim to investigate the role of OT and CT imparted by the Flexcell FX 6000-T system in EOC to bring insight into EOC progression and metastasis.

2. Materials and methods

2.1. Flexcell FX-6000 T tension system setup

A Flexcell FX-6000 T Tension System was used to apply tensile forces to ovarian cancer cells. The Flexcell system consists of a baseplate (Fig. 1B) that holds four 6-well culture plates with a flexible bottom fabricated from a collagen coated silicone material (Fig. 1A). SKOV-3 or OVCAR-8 cells were cultured such that a ~ 80% confluent monolayer formed on the bottom of each well in the plates. Next, the well plates were placed on the baseplate which was placed in an incubator (37 °C, 5% CO₂) and connected to the Flexcell system. The baseplate is connected to the Flexlink control box, which connects to a vacuum pump, pressure reservoir, and air compressor. The Flexcell system creates a vacuum which pulls the sides of the silicon bottom of the well plate down (Fig. 1C) and either releases in an oscillatory manner or holds in a constant manner. Cells that were strained with sinusoidal OT were placed under 10% elongation and a frequency of 0.3 Hz while cells strained with CT were held at 10% elongation (Fig. 1D). Control plates (no tension) were placed in the same incubator.

2.2. Cell culture

The *luc*-SKOV-3 ovarian cancer cell line was provided by Dr. Michael Birrer (University of Arkansas), and the OVCAR-8 cell line was provided by Dr. Geeta Mehta (University of Michigan). The OVCAR-8 cell line was tagged with luciferase using retroviral infection with Firefly Luciferase Lentivirus Puromycin (Amsbio, MA). *Luc*-SKOV-3 and -OVAR-8 cells were cultured in RPMI 1640 1× medium supplemented with 10% FBS (Gibco; Waltham, MA), and 1% penicillin/streptomycin (ThermoFisher Scientific; Waltham, MA).

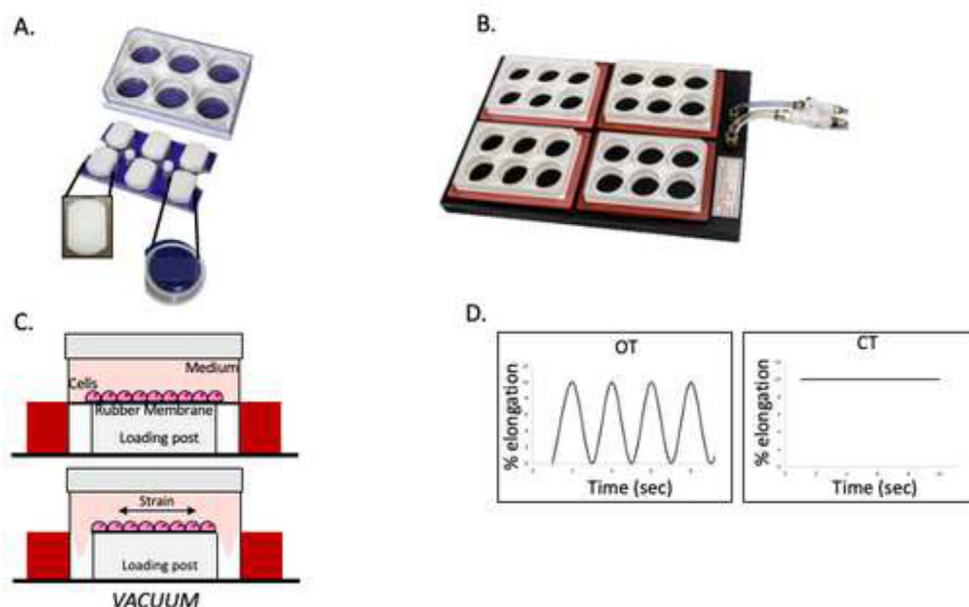


Fig. 1. Equipment for imparting uniaxial tension to a two-dimensional (2D) monolayer of SKOV-3 and OVCAR-8 cells. (A) Components of the Flexcell system include 6-well culture plates with a flexible bottom and a loading post for allowing uniaxial stretch. (B) Culture plates are placed on a baseplate allowing the vacuum system to apply tension. (C) Schematic of the manner in which the vacuum creates a tensile load. (D) Graphical representation of oscillatory tension (OT) and constant tension (CT) applied (% elongation vs. time).

2.3. Bioluminescent (BLI) cell growth assessment under mechanical tension

Luc-SKOV-3 and -OVCAR-8 cells were seeded at 2.5×10^5 cells/well in the flexible bottom 6-well culture plates and incubated (37 °C, 5% CO₂) for 24 h to reach ~80% confluency. After the initial 24 h, cell culture media was replenished, and 5 µg/mL d-luciferin (XenoLight D-Luciferin Potassium Salt, Perkin Elmer) was added for bioluminescent imaging (BLI) using the IVIS Lumina III system. After initial imaging, culture plates were placed either in the Flexcell FX-6000 T Tension System to undergo OT or CT or in the incubator under no tension (non-OT, non-CT) for 3 days, after which they were imaged again for cell growth comparison.

2.4. Transwell migration assay

A commercially available Boyden chamber (Corning®, NY, USA) was used to detect cell migration. The PET Membrane was 8.0 µm. After undergoing mechanical strain for 3 days, cancer cells were trypsinized from the Flexcell Uniflex® Culture Plates, centrifuged, and resuspended in serum free RPMI and counted. An equal number of cells (5×10^4 cells/well) were loaded onto the upper chamber. Medium enriched with 10% FBS was added in the lower chamber as a chemoattractant. After incubation (37 °C, 5% CO₂) for 24 h, non-migrated cells remained on the upper surface of the membrane and were removed; cells that migrated through the membrane were fixed with formalin and stained with Crystal violet (Fischer chemicals). Cells that had migrated were counted under a light microscope.

2.5. Transwell invasion assay

A commercially available BioCoat™ Matrigel® Invasion Chamber (Corning®, NY, USA) was used to measure cell invasion. The PET Membrane was 8.0 µm and coated with Matrigel®. After undergoing mechanical strain for 3 days, cancer cells were trypsinized from the Flexcell Uniflex® Culture Plates, centrifuged, and resuspended in serum free RPMI and counted. An equal number of cells (5×10^4 cells/well) were loaded onto the upper chamber of the invasion assay. Medium enriched with 10% FBS was added in the lower chamber as a chemoattractant. After incubation (37 °C, 5% CO₂) for 24 h, non-invaded cells remained on the upper surface of the membrane and were removed; cells that invaded through the membrane were fixed with formalin and stained with Crystal violet (Fischer chemicals). Cells that had invaded were counted under a light microscope.

2.6. Western blot

RIPA buffer containing PMSF and Protease Inhibitor (Sigma-Aldrich) were used to lyse the cell lines and total protein lysates were separated by Novex (ThermoFisher Scientific) 4–12% gel electrophoresis and transferred to membranes. Membranes were blocked with 5% non-fat dry milk in PBST (Sigma-Aldrich) for 60 min at room temperature and then incubated overnight (4 °C) with the primary antibody. The primary antibodies used were mouse anti-E-cadherin antibody (ab1416, AbCam) at 1:1000 dilution; goat anti-snail antibody (ab53519, AbCam) at 1:1000 dilution; anti-alpha Tubulin antibody - Loading Control (HRP) (AbCam), 1:2000.

After multiple washes, membranes were incubated for 1 h at room temperature with proper secondary HRP-conjugated antibodies (Bio-Rad Laboratories; Hercules, CA), except for primary antibodies already HRP conjugated. After multiple washes, images were developed using a mixed Peroxide and Luminol Enhancer Solution (Supersignal West Dura kit, ThermoFisher Scientific).

2.7. RNA sequencing

RNA was extracted from cultured cells using RNeasy Plus extraction kit (Qiagen). RNA integrity numbers were measured using BioAnalyzer (Agilent Technologies; Santa Clara, CA) and ranged from (9.5 to 10). Quantified RNA was processed by Illumina stranded mRNA Library Prep and Illumina NS500 Single-End 75 bp (SE75). Results were analyzed using Ingenuity software (Qiagen). Data assessment: STAR (version 2.7.3a) aligned the raw RNA-Seq fastq reads to the human (GRCh38 p13 Release 32) reference genome from Gencode using parameters `--outReadsUnmapped Fastx; --outSAMtype BAM SortedByCoordinate; --outSAMAttributes All; --outFilterIntronMotifs RemoveNoncanonicalUnannotated` [39]. Following alignment, HTSeq-count (version 0.11.2) counted the number of reads mapping to each gene using parameters `-m union; -r pos; -t exon; -i gene_id; -a 10; -s no; -f bam` [40]. Normalization and differential expression were then applied to the count files using DESeq2 using their default settings in the vignette (version 1.26.0) [40]. Systems Biology analysis. For generating networks, a data set containing gene identifiers and corresponding expression values was uploaded into Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). Each identifier was mapped to its corresponding object in Ingenuity's Knowledge Base. A log₂ fold change cutoff of ± 2 was set to identify molecules whose expression was significantly differentially regulated. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in Ingenuity's Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Molecules from the dataset that met the log₂ fold change cutoff of ± 2 and were associated with biological functions and/or diseases in Ingenuity's Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

2.8. Murine In Vivo experiment

All animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC), ethical code IACUC-20747. SCID mice (8–10 weeks old) from Charles River Laboratories (Wilmington, MA) were used according to IACUC protocols. Mice were injected with an intraperitoneal (IP) injection of 2.5×10^6 luc-SKOV-3 or -OVCAR-8 OT (experimental group) or non-OT (control group). Animals were injected weekly with 1 mL of d-luciferin (XenoLight D-Luciferin Potassium Salt, Perkin Elmer, 5 µg/mL) and imaged using the same IVIS Lumina III system described above. When animals started to show signs of sickness at 9 or 10 weeks for OVCAR-8 and SKOV-3 respectively, they were sacrificed according to the IACUC protocol. At sacrifice, open body final imaging was obtained as well as omentum and ascites imaging. The ascites and tumor were also collected.

2.9. Statistical analysis

Each *in vitro* experiment was performed at least in triplicate and results were expressed as mean $\pm$ standard error of the mean (SEM) from a representative experiment. All data were assessed for normality using Shapiro-Wilk test, and either parametric or nonparametric analyses were used to detect differences between treatment groups. $*P < 0.05$ was considered to be statistically significant. Two-way ANOVA was used for the animal experiment and the assessment of cell growth. Unpaired *t*-test was used for the remaining experiments. All statistical analyses were performed using GraphPad Prism 5.01 (GraphPad; La Jolla, CA).

3. Results

3.1. OT and CT significantly enhanced cell proliferation of SKOV-3 cells

The effect of OT and CT on EOC cellular proliferation was evaluated using bioluminescent imaging (BLI) of *luc*-SKOV-3 and *luc*-OVCAR-8 cells. After 3 days of OT, *luc*-SKOV-3 cell proliferation was significantly ($p = 0.001$) elevated compared to cells not exposed to oscillatory tension (non-OT) (Fig. 2A). In addition, when investigating differentially expressed genes (DEGs), there was a significant increase in *FOXS1* (positive log2 fold change, $p < 0.001$; FC = 2.48) and *ERVV-2* (positive log2 fold change, $p < 0.001$; FC = 2.44) expression in SKOV-3 cells exposed to OT versus non-OT (STable 1); both genes are associated with increased cell proliferation [41,42]. When analyzing the effect of CT on *luc*-SKOV-3 cellular proliferation, we found a significant ($p < 0.001$) increase in cells exposed to CT compared to non-CT (Fig. 2B). When evaluating the effect of OT and CT on *luc*-OVCAR-8 cell proliferation, neither OT nor CT had significant effects on cellular proliferation (Fig. 2C–D). Although the day 0 BLI images visually do not look the same (Fig. 2A, C), numerically, the total flux is the same as shown in the graphs (Fig. 2B, D). These results suggest that SKOV-3 cellular proliferation is subject to mechanical tension, while OVCAR-8 cell proliferation is not influenced by mechanical tension. While this imaging expounded on the impact of tension on cell proliferation, this is not always directly correlated with migration and invasion capabilities, so the effect of tensile forces on specific phenotypic needed to be clarified.

3.2. OT increased metastatic phenotypes of SKOV-3 and OVCAR-8 cells

The migratory and invasive tendencies of cells that were placed in the Flexcell system under OT for 3 days were compared to non-OT cells using transwell migration and invasion assays. After being placed in the transwell assays and allowing to incubate for 24 h (after the 3 days of oscillatory tension imparted by the Flexcell), both SKOV-3 and OVCAR-8 cells had significantly ($p < 0.0001$) increased migratory capacity compared to non-OT cells (Figs. 3A and 4A). However, OT significantly

($p < 0.0001$) increased SKOV-3 but not OVCAR-8 invasion (Figure Supplementary Figure 1A and C). In addition, we evaluated the effect of CT on cellular movement by using the same method described on cells that were placed under CT compared to non-CT cells. CT caused no significant effect on SKOV-3 cell migration or invasion (Fig. 3B and Figure Supplementary Figure 1B). There was a significant ($p < 0.0001$) increase in OVCAR-8 cell migration when exposed to CT compared to non-CT cells (Fig. 4B), but there was no significant effect of CT on invasion (Figure Supplementary Figure 1D). Since OT had a significant effect on cellular proliferation and had more of a significant effect on cellular migration and invasion, this method of tension was used in subsequent experiments. Western blot analyses were used to analyze the effects of OT on protein expression levels for E-cadherin and snail, two markers prevalent in EMT and metastasis. Patients with high SNAI2 expression have worse progression-free survival, while patients with high HCDH1 expression show better progression-free survival (Figure Supplementary Figure 3). After 3 days under OT, E-cadherin protein expression was lower in both SKOV-3 and OVCAR-8 cells ($p < 0.05$ for both) exposed to OT compared to non-OT (Figs. 3C and 4C). Conversely, snail protein expression was higher after 3 days of OT in both SKOV-3 and OVCAR-8 cells ($p < 0.05$ for both, Figs. 3D and 4D) compared to non-OT. Additionally, when investigating DEGs in response to tension, there was a significant increase in *KRT17* (positive log2 fold change, $p < 0.001$; FC = 2.21) and *KRT14* (positive log2 fold change, $p < 0.01$; FC = 1.16) in SKOV-3 and OVCAR-8 respectively, in response to OT compared to non-OT (Supplementary Table 1); both of these genes are associated with increased EMT phenotypes [43,44]. These findings suggest that both cell types are undergoing EMT when exposed to OT, thus enhancing their migratory capacity. Our DEG analysis also showed that there was a significant increase in *RBF3X3* (positive log2 fold change, $p < 0.05$; FC = 1.86) expression in SKOV-3 cells in response to tension, and an increase in *NDUFA4L2* (positive log2 fold change, $p < 0.05$; FC = 1.24) and *NRP2* (positive log2 fold change, $p < 0.05$; FC = 1.04) expression in OVCAR-8 cells in response to tension (Supplementary Table 1); these genes are associated with increased cell metastatic potential [45–47]. Overall, these results suggest that OT affects EMT and cell migration of both EOC cell lines, albeit through different signaling pathways and mechanisms.

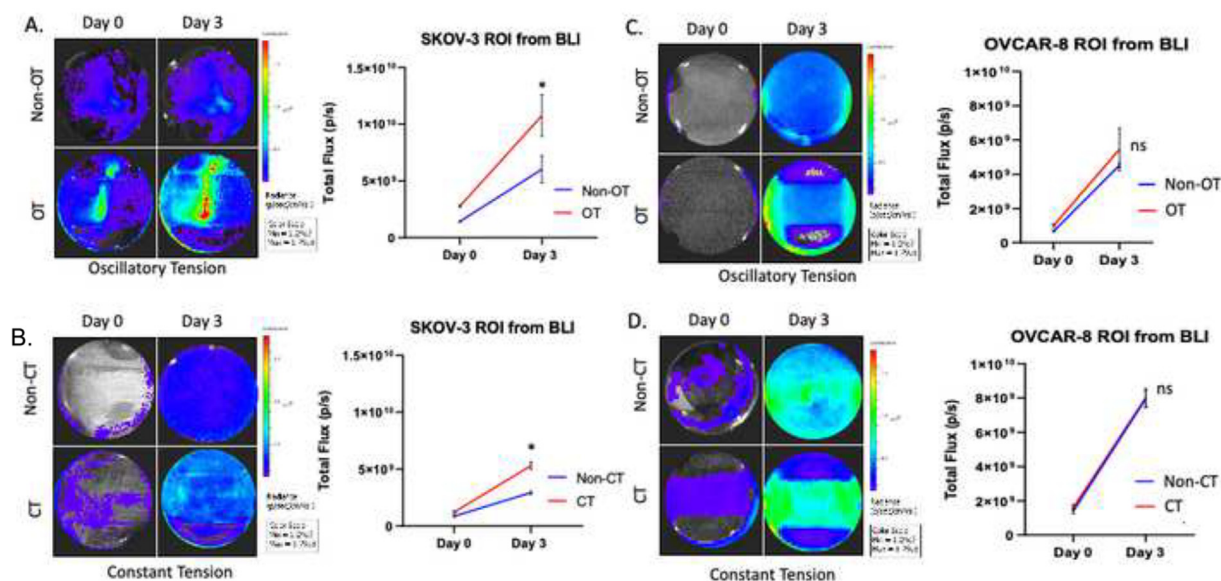


Fig. 2. Effects of OT and CT on cell proliferation of *luc*-SKOV-3 and *luc*-OVCAR-8 compared to no tension. (A) ROI (Region of Interest) signal from *luc*-SKOV-3 cells under oscillatory tension (OT) and BLI images showed a significant difference in cell growth compared to no tension (non-OT) controls after three days. (B) ROI signal from *luc*-SKOV-3 cells under constant tension (CT) and BLI images showed a significant difference in cell growth compared to no tension (non-CT) controls after three days. (C) ROI signal from *luc*-OVCAR-8 cells under OT and BLI images showed no significant difference in cell growth compared to non-OT controls after three days. (D) ROI signal from *luc*-OVCAR-8 cells under CT and BLI images showed no significant difference in cell growth compared to non-CT controls after three days. $N = 18$.

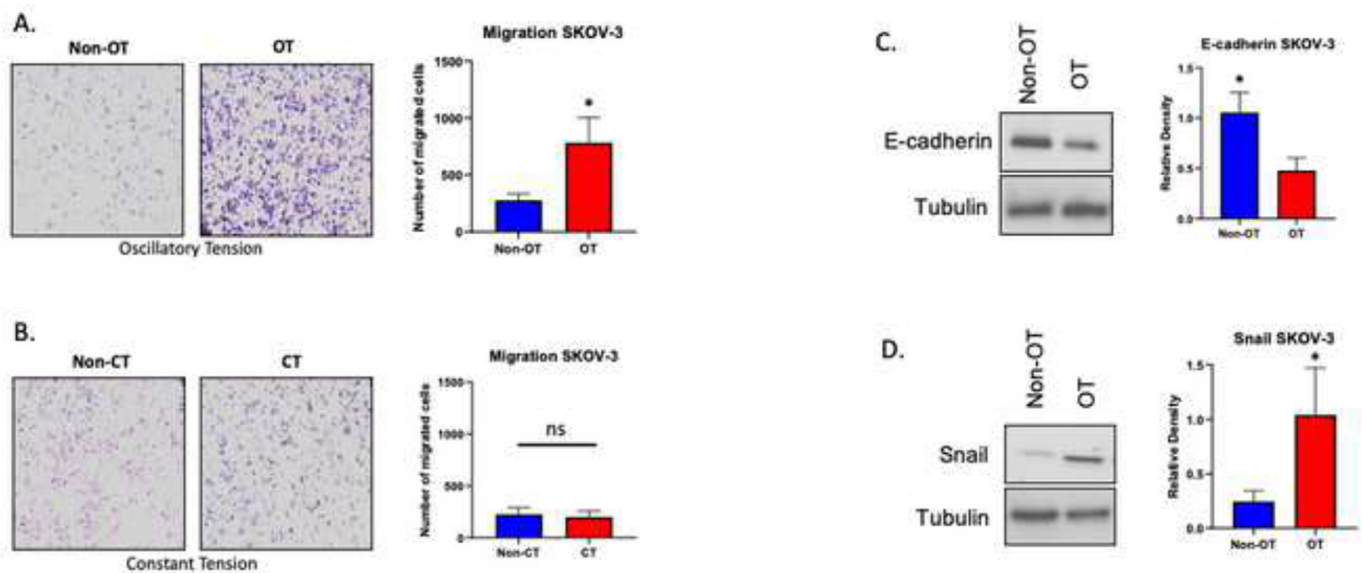


Fig. 3. OT increased migration and EMT protein expression in SKOV-3. (A) Quantification and representative picture of the transwell assay showing the number of migrated SKOV-3 cells after 3 days under OT and non-OT. There was a significant increase in migrated cells compared to non-OT controls. (B) Quantification and representative picture of the transwell assay showing the number of migrated SKOV-3 cells after 3 days under CT and non-CT. There was no significant difference in the number of migrated SKOV-3 cells compared to non-CT controls. In addition, SKOV-3 showed decreased expression of the epithelial marker (C) E-cadherin as well as increased expression of the mesenchymal marker (D) Snail of cells subjected to OT compared to non-OT controls. $N = 12$, * $P < 0.0001$, $N = 3$, * $P < 0.05$.

3.3. OT significantly increased *Luc-SKOV-3* and *-OVCAR-8* *In Vivo* tumor growth

We investigated the effects of OT on *in vivo* tumor growth by injecting SCID mice with *Luc-SKOV-3* or *-OVCAR-8* that were previously exposed to OT or non-OT (Figs. 5A and 6A). BLI was performed weekly to monitor tumor growth. At the end of week 9 (*Luc-OVCAR-8*) and 10 (*Luc-SKOV-3*), ROI signal from BLI images showed significantly increased late tumor growth in the experimental groups for both cell types compared to the controls ($p < 0.001$ in SKOV-3, $p < 0.05$ in OVCAR-8) (Figs. 5B and 6B). Tumors and ascites were collected from

mice and tumor weight was significantly higher in the experimental groups of mice compared to the control groups for both cell types ($p < 0.05$ in SKOV-3, $p < 0.01$ in OVCAR-8) (Figs. 5C and 6C). Additionally, mice injected with OVCAR-8 cells had more ascites than mice injected with SKOV-3 cells (Figs. 5D and 6D). Furthermore, mice injected with OVCAR-8 cells in the experimental group had significantly ($p < 0.05$) more ascites than mice in the control group (Fig. 6D). BLI imaging was used to access the metastatic potential of SKOV-3 cells or OVCAR-8 cells previously exposed to OT, and no significant differences were observed in the two groups of mice (Figs. 5E and 6E-G). Lastly, immunohistochemistry was performed on histologically fixed tumor samples

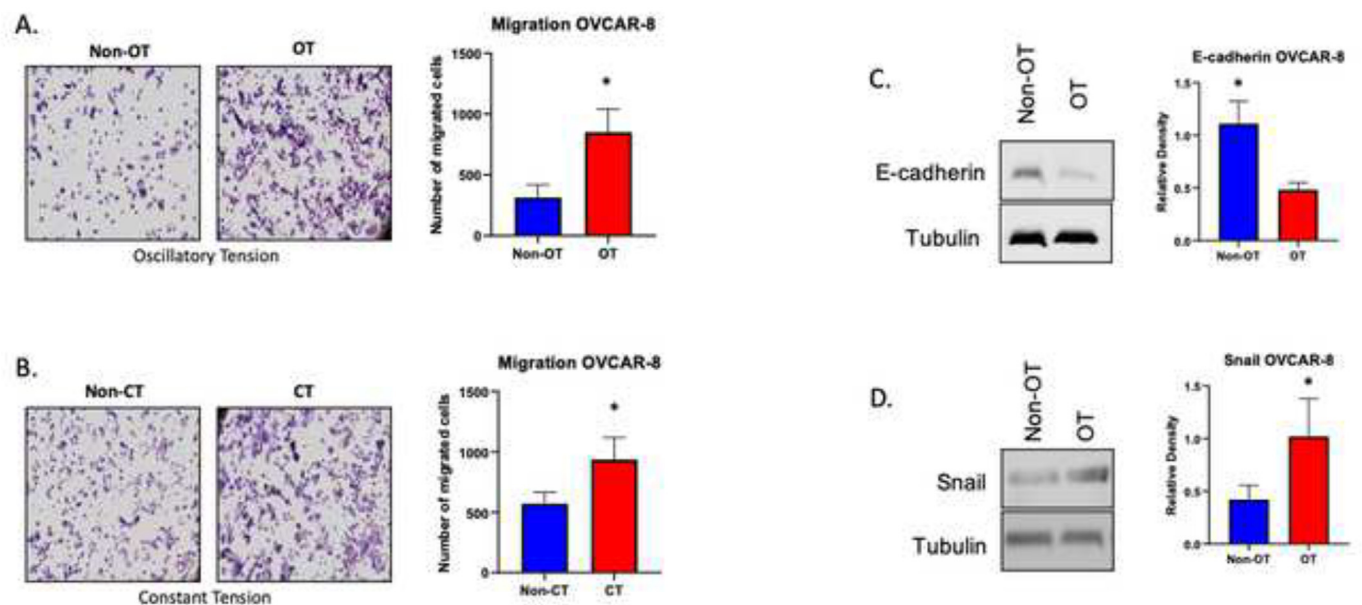


Fig. 4. OT increased migration and EMT protein expression in OVCAR-8. (A) Quantification and representative picture of the transwell assay showing the number of migrated OVCAR-8 cells after 3 days under OT and non-OT. There was a significant increase in migrated cells compared to non-OT controls. (B) Quantification and representative picture of the transwell assay showing the number of migrated OVCAR-8 cells after 3 days under CT and non-CT. There was a significant increase in the number of migrated OVCAR-8 cells compared to non-CT controls. In addition, OVCAR-8 showed decreased expression of the epithelial marker (C) E-cadherin as well as increased expression of the mesenchymal marker (D) Snail of cells subjected to OT compared to non-OT controls. $N = 12$, * $P < 0.0001$, $N = 3$, * $P < 0.05$.

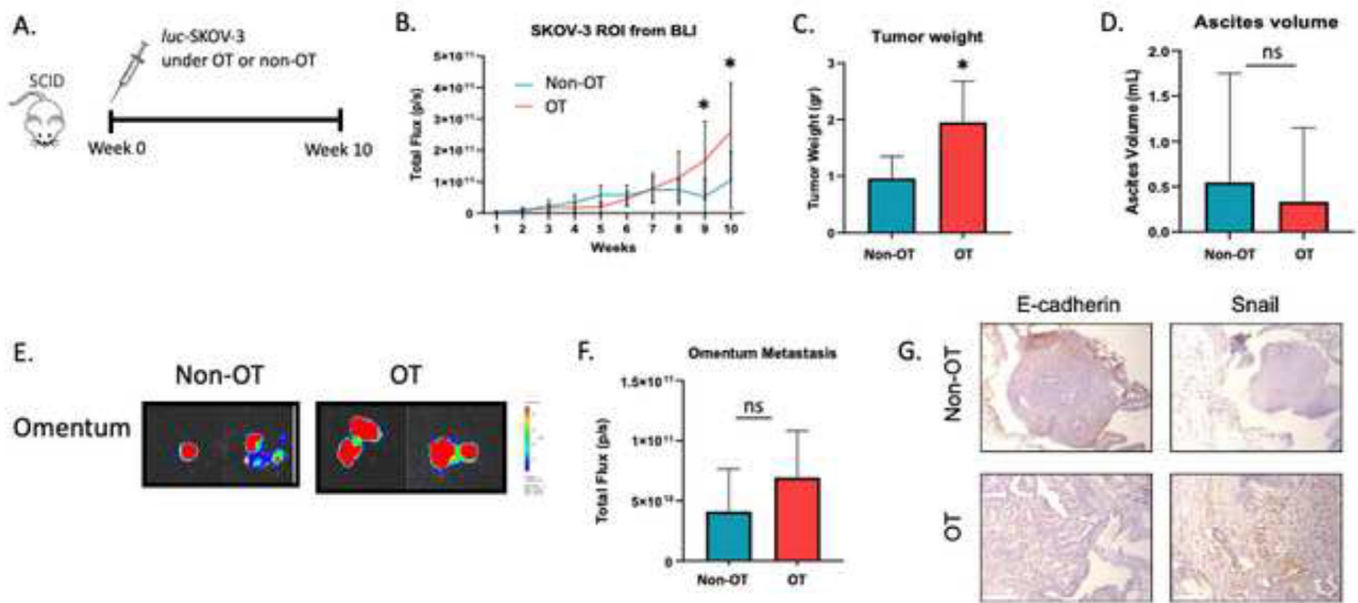


Fig. 5. OT significantly increased *luc*-SKOV-3 *in vivo* tumor growth. (A) Schematic of the experimental timeline. Mice were injected on week 0 with either OT cells (experimental group) or non-OT cells (control group) and were sacrificed in week 10. (B) ROI signal from BLI images showed a significant late tumor growth compared to non-OT controls. (C) Tumor weight (g) showed that the tumors developed by the animals injected with cells under OT were significantly larger compared to non-OT animals. (D) Ascites volume (mL) collected in the animals before they were sacrificed. The animals barely developed ascites and there was no difference in the OT animal group compared to the non-OT. (E) BLI images from metastatic omentum. Images are representative of two experiments (OT) and controls (non-OT). (F) ROI signal from BLI images taken of omentum metastasis organs showed no significant difference between the OT group and the non-OT group. (G) Photomicrographs of E-cadherin and snail immunohistochemical staining from mice tumors showed low E-cadherin presence and high presence of snail in the OT group. $N = 7$ per group, $^*P < 0.05$.

from experimental and control mice to determine the presence of E-cadherin and snail, which were shown to be significantly altered during the Western Blot analysis above. In both cell lines, E-cadherin presence was low and snail presence was high in the experimental groups compared to the controls. These data suggest that OT impacts tumor growth and EMT phenotypes in both cell types and ascites formation in the OVCAR-8 model.

4. Discussion

Preliminary studies show the importance of mechanical forces in cancer initiation and progression [21,36], specifically analyzing the role of mechanical forces resulting from ascites formation in ovarian cancer progression [22,23,29–31]. It has been revealed that shear forces from ascitic fluid help cancer cells acquire mesenchymal and stem cell-

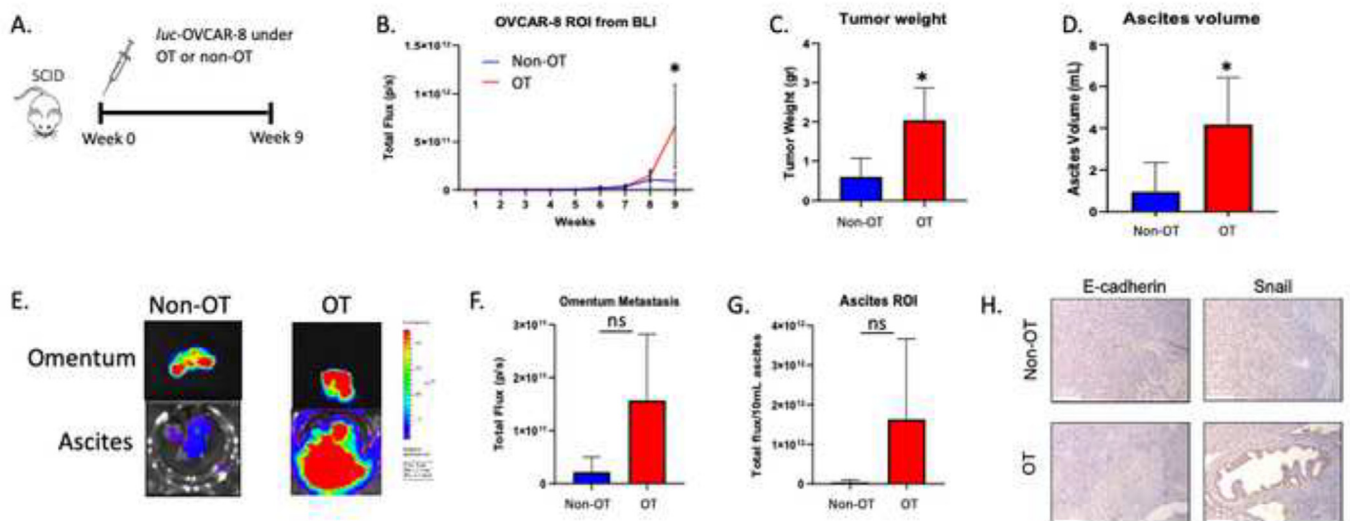


Fig. 6. OT significantly increased *luc*-OVCAR-8 *in vivo* tumor growth. (A) Schematic of the experimental timeline. Mice were injected on week 0 with either OT cells (experimental group) or non-OT cells (control group) and were sacrificed in week 9. (B) ROI signal from BLI images showed a significant late tumor growth compared to non-OT controls. (C) Tumor weight (g) showed that the tumors developed by the animals injected with cells under OT were significantly larger compared to non-OT animals. (D) Ascites volume (mL) collected in the animals before they were sacrificed. The ascites volume in the OT animal group was significantly higher compared to the non-OT group. (E) BLI images from metastatic omentum and ascites in a 24-well plate. Images are representative of one experiment (OT) and control (non-OT). ROI signal from BLI images taken of (F) omentum metastasis organs and (G) ascites showed no significant difference between the OT group and the non-OT group. (H) Photomicrographs of E-cadherin and snail immunohistochemical staining from mice tumors showed low E-cadherin presence and high presence of snail in the OT group. $N = 6$ per group, $^*P < 0.05$.

like phenotypes increasing their migratory/invasive potential and chemoresistance, thus allowing for dissemination throughout the peritoneal cavity [15,22–24,29–31,48]. Both CT and OT are present in normal physiology, however, recent studies have focused on these forces in cancer. There are also many downstream pathways involved in tumor progression that are affected by mechanotransductive transmission and sensing that result in a mechanoresponse [12,49,50]. Additionally, the ability for cells to sense their mechanical environment and develop a “mechanical memory” to the forces present has recently become an increasingly explored phenomenon [51–56].

In this study, we utilized two representative EOC cell lines, SKOV-3 and OVCAR-8. SKOV-3 cells represent a non-serous ovarian cancer whereas OVCAR-8 cells represent a serous ovarian cancer [57–60], and both have significant differences in mRNA profiles (Figure Supplementary Figure 4). A tensile force of 10% elongation was chosen based on previous studies [30] and 0.3 Hz was chosen for the frequency because of its consistency with the human respiration rate [61]. Although the forces used in this study were estimates based on previous studies in other cancers, they have not been quantified in ovarian cancer patients. Because of the unknown exact physiological forces, this is a parametric analysis to determine that the specific magnitudes, durations, and frequencies chosen are causing cellular changes. This study opens the door to a broader analysis where different magnitudes, durations, and frequencies can be compared to determine the mechanical threshold causing cancer progression, invasion, and metastasis. When analyzing the effects of OT and CT on SKOV-3 cells, we found that OT enhanced *in vitro* cell proliferation, EMT, and migration. In addition, SKOV-3 cells previously exposed to OT had increased *in vivo* tumor growth compared to non-OT cells. These findings could be explained by the up-regulation of *FOXS1*, *ERVV-2*, *KRT17*, and *RBFOX3* expression in response to OT. Studies have shown that *FOXS1* expression correlates with poor prognosis in gastric cancer and its over-expression accelerated tumor growth *in vivo* and increased cell migration and invasion through EMT promotion both *in vitro* and *in vivo* [42]. *ERVV-2* encodes a protein in the human endogenous retrovirus (HERV) group and blocking of HERV-K protein significantly reduced *in vitro* and *in vivo* growth rates of pancreatic cancer cell lines [41]. Additionally, several proteins of the HERV group are associated with cancer progression [62]. In gastric cancer, *KRT17* expression correlated with tumor size, lymph node metastasis, tumor node metastasis stage, and poor prognosis [63], and additional studies have shown that it facilitates oral tumor growth [64]. Lastly, *RBFOX3* promoted hepatocellular carcinoma growth and progression and predicted poor prognosis by activating the hTERT signaling [46].

When analyzing the effects of OT and CT on OVCAR-8 cells, neither affected *in vitro* cellular proliferation. However, OT triggered OVCAR-8 cellular EMT and migration. In addition, OVCAR-8 cells exposed to OT had increased *in vivo* tumor growth compared to non-OT cells. The difference in OT's effects on *in vitro* and *in vivo* cell proliferation could be due to components in the TME. Furthermore, these findings could be explained by the upregulation of *MYADM*, *NDUFA4L2*, *KRT14*, and *NRP2* expression in response to OT. It has been shown that *MYADM* knock-down exhibited reduced cell spreading and migration [65]. *NDUFA4L2* blockade in renal cell carcinoma showed decreased cell migratory capabilities and significantly decreased cell viability [47]. In addition, *KRT14* expression in ovarian cancer has been reported to contribute to a highly invasive phenotype, and the loss of *KRT14* completely abrogated this phenotype [43]. Finally, it has been reported that *NRP2* has a strong effect on EMT which results in enhanced migration, invasion, and metastasis [45,66].

Retention of mechanical memory has been shown extensively in mesenchymal stem cells, in which their fate is determined by the rigidity of the ECM that the cells have experienced in the past [53–56]. Additionally, epithelial cancer cells have been shown to remember past mechanical environments and act accordingly in new metastatic sites

[51,52]. These cells appear to retain biochemical characteristics, specifically YAP localization to the cell membrane and miRNA-21 expression, that were attained from the environment where they came from, which would explain the ability for the cells in this experiment which were placed under OT and placed in animal models to have long lasting morphological changes that resulted in more aggressive disease. It is unknown exactly how long this mechanical memory lasts and what specific genes and proteins are involved in this process in EOC, but our findings warrant further investigation in future studies.

Taken together, we have provided evidence that OT forces impact *in vitro* cell proliferation, EMT, and migration. In addition, cells pre-exposed to OT had increased *in vivo* tumor growth. These results are supported by the upregulation of genes involved in tumor progression. OVCAR-8 cells represent high-grade serous ovarian cancer [59,60], and SKOV-3 cells represent non-serous ovarian cancer based on genomic profiling and the histologic analysis of xenografts [57,58,67], and since these different histological subtypes of ovarian cancer present very differently in patients in regard to their mutational burden, response to chemotherapy, and survival rates and are clinically treated as distinct diseases entirely, this could explain the differences in results between the cell lines.

5. Conclusion

This study aimed to understand the role of tension in the progression and metastasis of epithelial ovarian cancer. This was done *in vitro* by placing two EOC cell lines, SKOV-3 and OVCAR-8, under oscillatory and constant tension and comparing their ability to proliferate, migrate, and begin an epithelial-to-mesenchymal transition to those cells under no tension. Through BLI imaging, transwell assay analysis, RNA sequencing, and Western Blot analysis, the proliferation of SKOV-3 cells and the migratory and EMT properties of both cell lines were enhanced under tensile forces. *In vivo* studies were then performed to further confirm these findings in animal models. It was determined that ascites growth in OVCAR-8 models and tumor growth of both SKOV-3 and OVCAR-8 models was escalated by placing the cells under OT before injection. These findings provide the opportunity for the discovery of biomarkers and development of novel cancer therapeutics through further investigations which would include altering the magnitude, duration, and frequency of forces imparted, adding additional stromal and ECM components for a more physiologically relevant model, and a deeper analysis of specific proteins and genes involved in mechanotransduction in EOC cells and the mechanical memory they achieve.

Author contributions

Methodology, A.M., M.B.; validation, A.M., M.B.; formal analysis, A.M., M.B., D.C.; investigation, A.M., M.B., A.A.K.; data curation, A.M., M.B., A.A.K., D.C.; writing, review and editing A.M., M.B., C.B.S., J.J.D., J.B., M.J.B., R.C.A.; supervision, C.B.S., J.B., M.J.B., R.C.A.; project administration, J.B., M.J.B., R.C.A. All authors have read and agree to the published version of the manuscript.

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Declaration of Competing Interest

R.C.A. has participated in Advisory Boards for Leap Therapeutics, AstraZeneca, GSK, Merck, VBL Therapeutics, and Caris Life Sciences. M.J.B. serves as an advisory board member for AstraZeneca, Clovis, and GSK. The remaining authors declare no conflict of interest.

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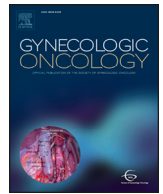
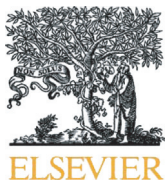
Appendix A. Supplementary data

The following are available online at www.mdpi.com/xxx/s1, Fig. S1: Oscillatory tension (OT) increased invasion in SKOV-3, Fig. S2: Comprehensive Western Blot with tubulin loading controls and molecular weight ladder, Table S1: Differentially Expressed Genes (DEGs) in the OT cells compared to non-OT cells revealed significant fold changes in genes implicated in tumor progression and metastasis. Supplementary data to this article can be found online at [doi: <https://doi.org/10.1016/j.ygyno.2021.04.003>].

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Manipulating the Wnt/ β -catenin signaling pathway to promote anti-tumor immune infiltration into the TME to sensitize ovarian cancer to ICB therapy

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HIGHLIGHTS

- DKN-01 suppresses MDSC gene signature.
- CGX-1321 increases CD8+ T cell infiltration into the omentum TME.
- Neither DKN-01 nor anti-PD-1 monotherapies enhanced the anti-tumor effects of CGX-1321.

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ABSTRACT

Objective. Immune checkpoint blockade (ICB) therapy shows limited efficacy in ovarian cancers due to the “cold” immune phenotype surrounding these tumors. Previous studies have shown that in ovarian cancer Wnt/ β -catenin pathway activation contributes to this immune phenotype. Here, we evaluated the anti-tumor and immune-enhancing properties of the Wnt inhibitor, CGX-1321, used alone or in combination with either DKN-01 or anti-PD-1 therapy, in pre-clinical ovarian cancer models.

Methods. The parental ID8 murine ovarian cancer model harboring a knock-out of *p53* (ID8p53^{-/-}) and MISIR-Tag spontaneous ovarian cancer models were used to test the effects of CGX-1321 alone or in combination therapies on tumor burden and immune cell landscape in the tumor microenvironment (TME). Flow cytometry and NanoString analyses were used to characterize the changes in tumor-intrinsic signaling and immune-related profiles in the TME of ovarian cancer in response to treatments.

Results. CGX-1321 significantly reduced tumor burden and constrained tumor progression in the ID8p53^{-/-} and MISIR-Tag models. Furthermore, CGX-1321 increased infiltrating CD8+ T cells in the TME. Combining CGX-1321 with either DKN-01 or anti-PD-1 therapy also decreased tumor burden and increased CD8+ T cell infiltration in the omentum TME but did not do so to a greater extent than CGX-1321 monotherapy.

Conclusions. CGX-1321 significantly reduced tumor burden and enhanced CD8+ T cell levels in ovarian cancer, nevertheless the addition of DKN-01 or anti-PD-1 therapies did not enhance these effects of CGX-1321. Further investigation is needed to determine if CGX-1321 + DKN-01 combination treatment sensitizes pre-clinical ovarian cancer to ICB therapy.

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1. Introduction

An emerging paradigm has classified tumor microenvironments (TMEs) into different immunological phenotypes including hot, altered-

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excluded, altered-immunosuppressed, and cold [1]. Cancers that are considered immunologically “cold” have a low immunoscore (low T and cytotoxic T (Tc) cell density in TME), low tumor mutational burden, poor antigen presentation, and/or a lack of T cell killing [1]. As the majority of ovarian cancers are considered “cold,” understanding how to reverse this phenotype is critical in order to develop new approaches to sensitize these tumors to ICB therapy in hopes to provide more durable therapeutic

responses. The Wnt/ β -catenin tumor-intrinsic signaling pathway has emerged as an immune evasion pathway, and data by our group and others show that Wnt/ β -catenin signaling and increased β -catenin levels correlates with a lack of T cell-mediated killing in ovarian cancer [2,3].

The Wnt/ β -catenin pathway is dysregulated in several cancers, including ovarian cancer [4,5]. Activated Wnt/ β -catenin signaling corresponds to non-T cell inflamed tumors [6] and chemo-resistance [7]. Animal studies from our group using a murine ovarian cancer model, highlighted a negative correlation between the degree of Wnt/ β -catenin signaling and T cell activity, and impairment of this signaling pathway increased T cell function, decreased tumor burden, and improved survival [3,8,9]. The Wnt/ β -catenin pathway is activated by Wnt ligands, which is secreted from cells after modification by the porcupine (PORCN) enzyme [10], and PORCN inhibitors (PORCNI's) can block the biogenesis of Wnt ligands thus suppressing Wnt/ β -catenin-dependent signaling (Fig. 1A).

Dickkopf-1 (DKK1) is a Wnt-target gene that encodes the DKK1 protein that competes with Wnt ligand for LRP5/6 binding [11], thus creating a negative-feedback mechanism to suppress Wnt/ β -catenin-dependent signaling (Fig. 1B). Even though DKK1 serves as a negative regulator of the Wnt/ β -catenin pathway, increased levels of DKK1 corresponds to poor prognosis in various cancer types, including cervical [12] and ovarian cancer [13,14]. A prior study from our lab found that the over-expression of DKK1 in ovarian cancer promotes immune evasion by fostering myeloid-derived suppressor cells (MDSCs) within the TME of ovarian cancer [2]. These results suggest that blocking DKK1's function would hinder immune evasion in ovarian cancer, but this would also leave Wnt/ β -catenin signaling unregulated.

Both PORCN and DKK1 inhibitors have been investigated in clinical trials, (PORCN inhibitors: NCT02675946, NCT01351103; DKK1 inhibitor: NCT03395080, NCT02013154), but no studies have investigated dual inhibition of PORCN and DKK1. Therefore, we postulated that the combination of DKK1 inhibition and/or ICB therapy would increase the efficacy of PORCN inhibition to optimally decrease ovarian tumor progression.

2. Materials and methods

2.1. Cell lines and culture

The ID8 murine epithelial ovarian parental cancer cell line was provided by Dr. Yancey Gillespie (University of Alabama at Birmingham, Birmingham, Alabama). The ID8p53^{-/-} cell line is a derivative of the

ID8 parental cell line with CRISPR/Cas9 gene editing for a single gene mutation (Trp53^{-/-}) and was provided by Dr. Iain McNeish (Wolfson Wohl Cancer Research Centre, Institute of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom). Cells were maintained at 37 °C and 5% CO₂ and cultured in DMEM medium (Corning) supplemented with 4% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA), 1× insulin/selenium/transferrin (Invitrogen), penicillin/streptomycin, and 2 mM L-glutamine. Cells were maintained in culture fewer than 10 passages from the parent stock. Experiments were performed when cells were at 70–80% confluency.

2.2. Mouse studies

All animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC). C57BL/6 mice were obtained from NCI Charles River (Wilmington, MA, USA), and 7–8 week old C57BL/6 mice were challenged with 7 million ID8p53^{-/-} cells intraperitoneally (i.p.) on day 0. Treatment groups began with the same number of mice in each group. Treatments began either 10-, 14- or 28-days post-tumor challenge, and lasted until day 42 post-tumor challenge. Mice were treated with 1 mg/kg CGX-1321 (daily; oral gavage), 10 mg/kg DKN-01 (twice per week; i.p. injection), 250 µg/mouse anti-PD-1 antibody (every 3 days; i.p. injection), or vehicle/control. Doses were decided upon based on recommendations from the drug companies. The C57BL/6-MHSIR-Tag mice that spontaneously develop ovarian carcinoma due to expression of Simian Virus 40 T Antigen (SV40-TAG) under the control of the Mullerian Inhibitory Substance Type II Receptor promoter was provided by Dr. Troy Randall (University of Alabama at Birmingham). In this model, tumors spontaneously develop in the bilateral ovaries and metastasize to the omentum resulting in ascites [15]. Treatment with 1 mg/kg CGX-1321 (daily) was initiated when tumors had developed (approximately at 10 weeks of age) and continued for a total of eight weeks. Omentums and ascites were collected for endpoint analyses. Notably, mice intermittently died throughout the course of the experiments due to drug/experimental factors. This was variable between groups and thus impacted the group sizes reflected in the paper.

2.3. NanoString nCounter mRNA analysis

Omentum tumors were harvested and stored in RNeasy Lysis Buffer (Qiagen). RNA was isolated using Trizol Plus RNA Purification Kit

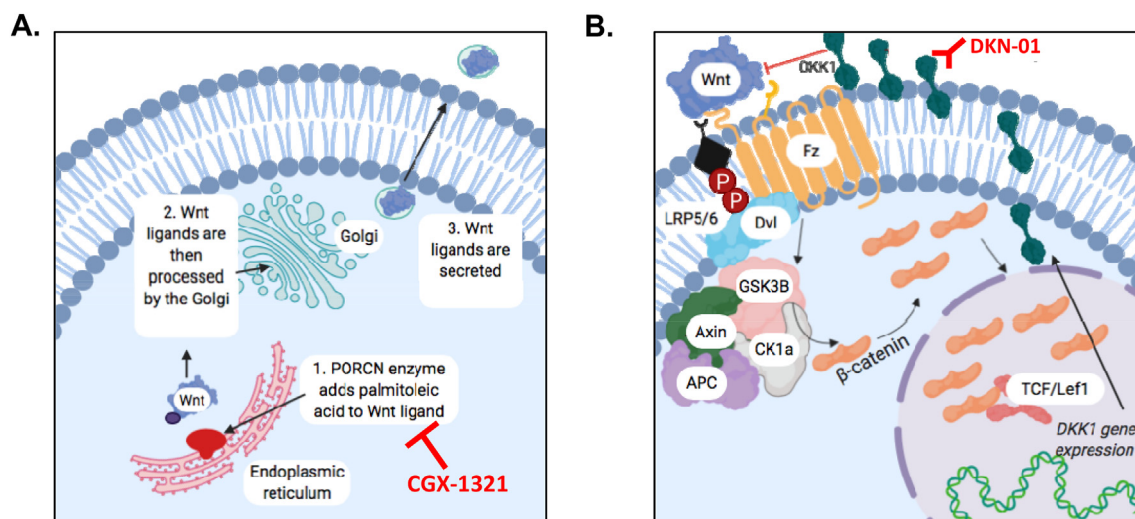


Fig. 1. Role of PORCN and DKK1 in Wnt/ β -catenin pathway signaling. (A) The porcupine (PORCN) enzyme is necessary for proper Wnt signaling to occur. PORCN is located within the endoplasmic reticulum and modifies Wnt ligands via the addition of adding palmitoleic acid. Ligands are then processed by the golgi body and ultimately secreted. Secretion of Wnt ligand can be blocked by PORCN inhibitors (e.g. CGX-1321). (B) Dickkopf-1 (DKK1) is a Wnt-target gene that encodes the DKK1 protein. As levels of DKK1 protein increase, Wnt signaling is eventually inhibited via a negative feedback mechanism. DKK1 can be inhibited by DKK1 antibody (e.g. DKN-01).

(Invitrogen) according to the manufacturer's instructions. Aqueous phase solutions were transferred to a spin cartridge found in the PureLink RNA Mini Kit and processed similarly. Samples of high purity ($A_{260}/A_{280} > 2$, $A_{260}/A_{230} > 1.4$) were processed on the NanoString nCounter Flex system (NanoString Technologies, Seattle, WA, USA) using the mouse PanCan Pathways and mouse PanCan Immune pre-made panels. Briefly, 100 ng of purified RNA was hybridized for at least 16 h with the respective Reporter Code set and Capture Probes for each panel separately. The samples were purified and immobilized on the NanoString Prep Station and counted on the NanoString Digital Analyzer. Analysis was performed using nSolver 3.0 software (NanoString Technologies). Cluster 3.0 and Java TreeView-1.1.6r4 were used to create heat maps. Expression levels were normalized to housekeeping genes that were not discarded by the gNorm program in the advanced analysis module.

2.4. Flow cytometry

Omentum tumors were harvested and placed in digestion buffer (DMEM 25 mM HEPES, 3.5% fatty acid free BSA plus 0.5 mg/ml collagenase + 70 µg/ml DNase I) (all from Corning) and fragmented with scissors. Tumor fragments were placed in an orbital shaker at 250–300 rpm for 30 min at 37 °C and subsequently filtered through 70 µm nylon mesh, washed with staining buffer (1× PBS, 2% BCS, and 2 mM EDTA) (all from Corning), and centrifuged at 800 xg for 10 min. The following antibodies were used for staining: CD3 (100214CD3), NK1.1 (108716) CD45.2 (109824), (all from Biolegend, San Diego, CA, USA), CD8a (551,162 A 1), CD5 (553023), CD19 (11-0191-85), zombie aqua (423101) (all from BD Biosciences, San Jose, CA, USA), CD4 (Q10092) (Invitrogen, Carlsbad, CA, USA) and live-dead discriminator 7-Amino-AMC (7AAD) (129935) (Calbiochem/Millipore Sigma, Burlington, MA, USA). Samples were run using a BD FACS Canto II (BD Biosciences), DIVA software 6.1.3. Data were analyzed with FlowJo version 10.9.6.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.0 (San Diego, CA, USA). NanoString data was analyzed separately. Determination of statistical significance between group means was done using multiple unpaired independent t-tests with an alpha of 0.05. For determination of significance between more than two group means, one-way or two-way ANOVA's were used correcting for multiple comparisons using Tukey's multiple comparisons test.

3. Results

3.1. CGX-1321 monotherapy decreases tumor burden in the ID8p53^{-/-} model, and ablated tumor progression in the MISIR-Tag model

Previously, our group investigated the effects of PORCN inhibitor, CGX-1321, on tumor growth in ID8 parental and ID8p53^{-/-} models [3]. To make our current study more translatable, we used the ID8p53^{-/-} model since the majority of ovarian cancers harbor p53 mutations [16], and also lengthened CGX-1321 treatment. Here, treatment began 14-days post-tumor challenge and lasted for a total of 31 days. In doing so, we found that CGX-1321 significantly decreased omental weights ($p = 0.008$) compared to vehicle/control-treated mice when treated for 31 days (Fig. 2A). There were no ascites present in mice receiving treatment with CGX-1321 compared to vehicle/control-treated mice (Fig. 2B). Additionally, we studied the effects of CGX-1321 treatment on tumor burden in the MISIR-Tag model. Treatment with CGX-1321 ablated the progression of tumors, as no increases were seen in omentum (Fig. 2C) or ovarian (Fig. 2D) weights compared to vehicle/control-treated mice.

3.2. The addition of DKN-01 does not enhance CGX-1321-mediated anti-tumor effects in the ID8p53^{-/-} model

Our previous study investigating the effects of DKK1 inhibitor, DKN-01, in the ID8 parental model suggested that DKN-01 does not directly impact tumor growth, but rather the immune components in the TME. Based on this, we believed that PORCN and DKK1 inhibition might have a synergistic anti-tumor effect through impacting both tumor intrinsic and immune components in the TME. In order to investigate the effects of CGX-1321 and DKN-01 treatments in the ID8p53^{-/-} model, we first performed western blot analysis on ID8p53^{-/-} whole cell lysates treated with control, CGX-1321, DKN-01, or CGX-1321 + DKN-01 to determine the effects of treatment on the phosphorylation of LRP6 (p-LRP6), a marker of Wnt signaling activation. We found that treatment with CGX-1321 (CGX) did not significantly alter p-LRP6 compared to control, but WNT3a (Wnt ligand) treatment increased p-LRP6 (activated Wnt signaling) compared to control (Fig. 3A). Similar to WNT3A, DKN-01 treatment increased p-LRP6 confirming that this antibody successfully blocked DKK1 negative regulation of Wnt signaling pathway (Fig. 3A). The combined treatment of CGX-1321 + DKN-01 decreased p-LRP6 as compared to DKN-01 single therapy (Fig. 3A). These results suggest that in combination, CGX-1321 can inhibit Wnt signaling by overriding effects of DKN-01, resulting in Wnt signaling inhibition.

Next, we sought to investigate the effects of DKN-01 alone and in combination with CGX-1321 on tumor growth in the ID8p53^{-/-} model. Following 31 days of treatment, CGX-1321 monotherapy significantly decreased omental weights ($p = 0.008$) compared to vehicle/control-treated mice (Fig. 3B). However, no significant difference in omental weights ($p = 0.40$) was observed in mice treated with DKN-01 compared to vehicle/control-treated mice (Fig. 3B). Combination treatment with CGX-1321 + DKN-01 significantly decreased omental weights ($p = 0.003$) compared to vehicle/control-treated mice, however no difference was observed between CGX-1321- and CGX-1321 + DKN-01-treated mice (Fig. 3B). Mice treated with CGX-1321 or CGX-1321 + DKN-01 did not develop any ascites, whereas four DKN-01-treated mice developed ascites although the volume was unmeasurable in two of these mice, and two vehicle/control-treated mice developed ascites (Fig. 3C).

We performed full RNA sequencing on 3 representative omental tumors from each treatment group, and heatmaps were generated to highlight differences in gene expression for a panel of known Wnt-related genes [17] in response to CGX-1321, DKN-01, and CGX-1321 + DKN-01 treatment. The CGX-1321 + DKN-01-treated tumors had overall decreased expression of Wnt-related genes compared to vehicle/control-treated samples, while also having a distinct profile from tumors treated with DKN-01 or CGX-1321 monotherapies (Supplemental Fig. 1A). These results suggest that the anti-tumor effects seen were CGX-1321-driven but did not address the effects that these therapies might have on immune landscape in the omentum TME.

3.3. CGX-1321 promote CD8+ T cell levels, while DKN-01 decreases MDSC-related gene signatures in ID8p53^{-/-} model

Our previous studies investigating PORCN's effects on the immune landscape of ovarian tumors found that treatment with PORCNI increased T cell gene signatures, infiltration, and function [3,9]. Even though DKN-01 did not affect tumor growth, our previous data suggested it may improve anti-tumor immune response by decreasing MDSCs and increasing natural killer (NK) cells and CD8+ T cells in the TME of ovarian cancer [2]. Therefore, we next utilized NanoString analysis to screen for patterns of gene expression changes in immune-related pathways following treatment with vehicle/control, CGX-1321, DKN-01, and CGX-1321 + DKN-01 (Fig. 4A). When utilizing the immune pathway score as a means for identifying groups of genes affected by treatment, DKN-01 monotherapy did not demonstrate notable

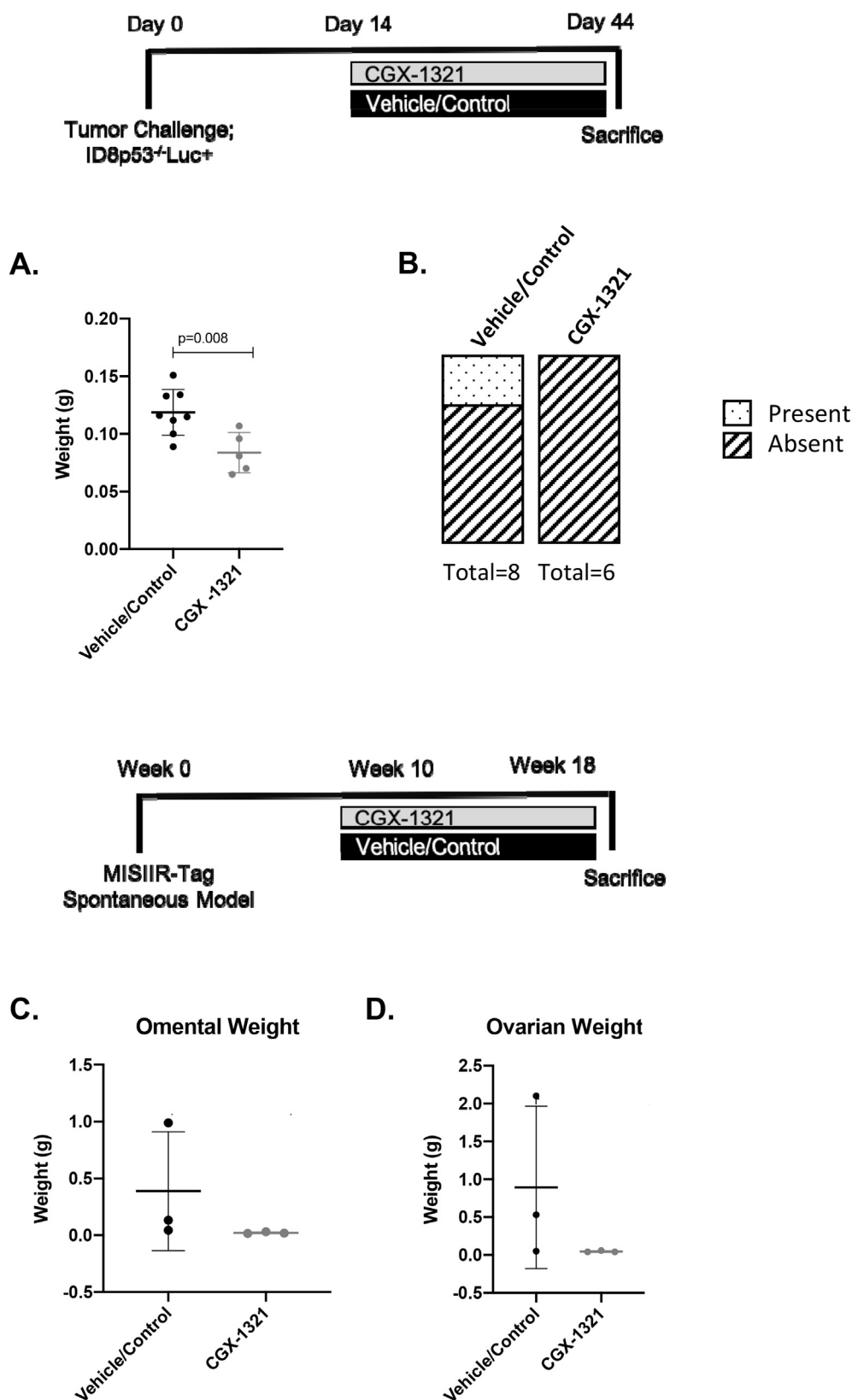


Fig. 2. CGX-1321 monotherapy decreases tumor burden in the ID8p53^{-/-} model, and ablated tumor progression in the MISIIR-Tag model. Female, 7–8 weeks old, C57BL/6 mice were challenged with 7 million ID8p53^{-/-} cells on day 0. Treatment consisted of 1 mg/kg CGX-1321 (oral gavage) or vehicle/control beginning 14 days post-tumor challenge. Mice were treated daily for 30 days until day 44. Mice were monitored throughout treatment and sacrificed on day 45, and (A) omentum weights (g) and (B) presence of ascites were measured. MISIIR-Tag spontaneous ovarian cancer model was also used, and treatments (as previously described) began when mouse omentums had tumors (~10 weeks of age) and treatment lasted for 8 weeks, and (C) omentum and (D) ovary weights were measured at the end of treatment.

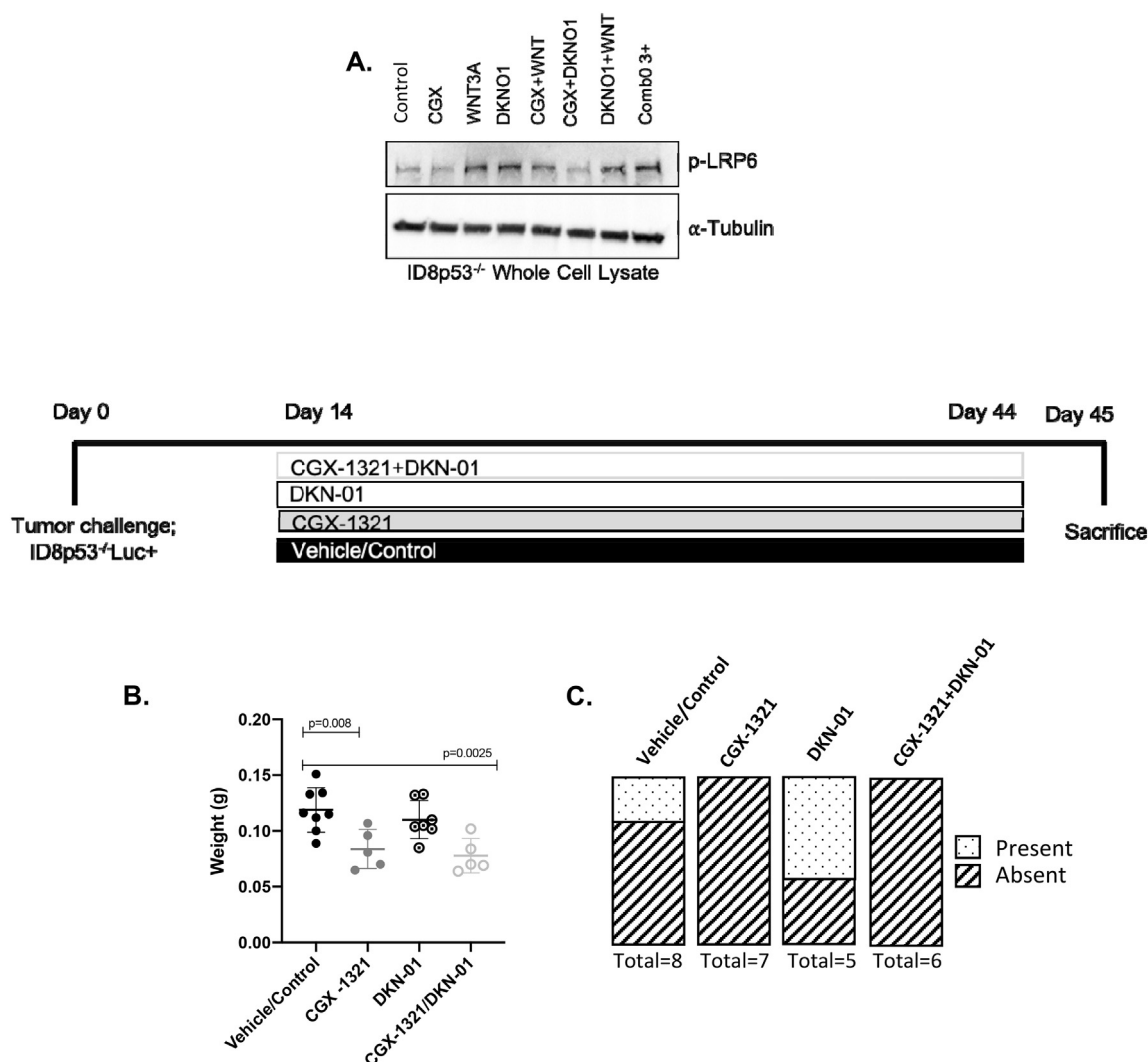


Fig. 3. The addition of DKN-01 does not enhance CGX-1321-mediated anti-tumor effects in the ID8p53^{-/-} model. (A) Western blot analysis on protein expression of phosphorylated LRP6 (p-LRP6) and α -Tubulin in ID8p53^{-/-} whole cell lysates. ID8p53^{-/-} model was initiated and treatment with 1 mg/kg CGX-1321 (oral gavage; daily), 10 mg/kg DKN-01 (intraperitoneal injection; 2 $\times$ /week), and vehicle/control started day-14 post-tumor challenge and continued through day 44, and (B) omental weights (g) and (C) presence of ascites were measured on day 45.

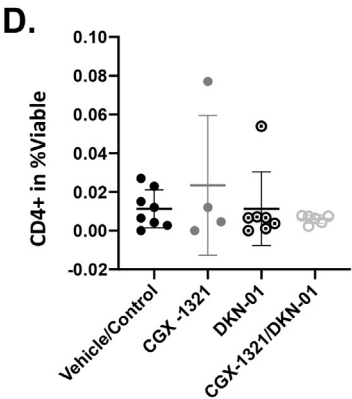
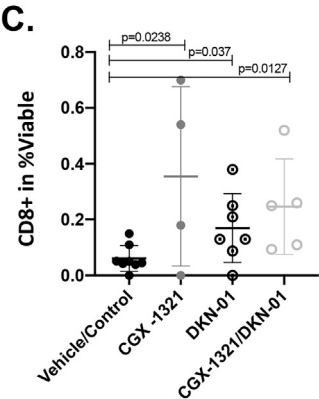
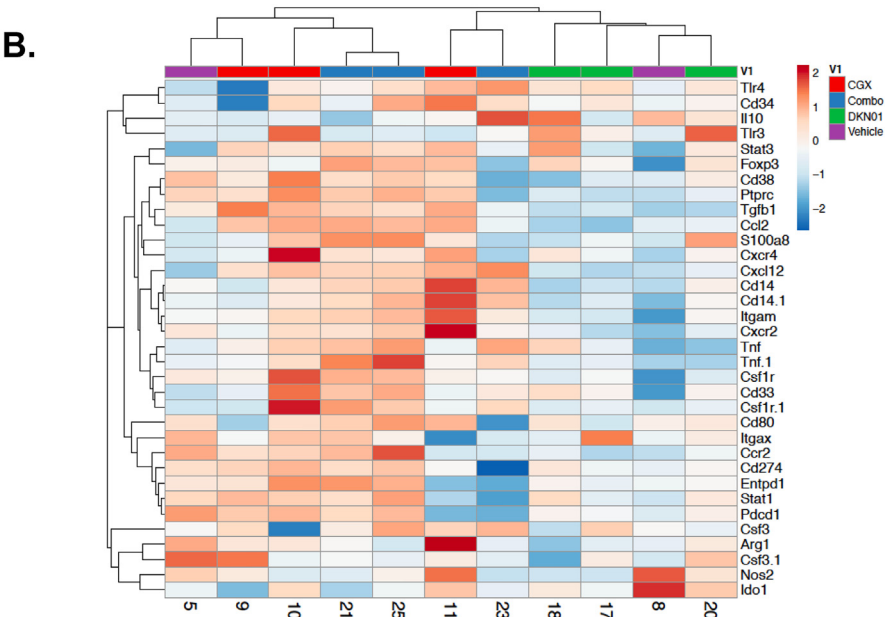
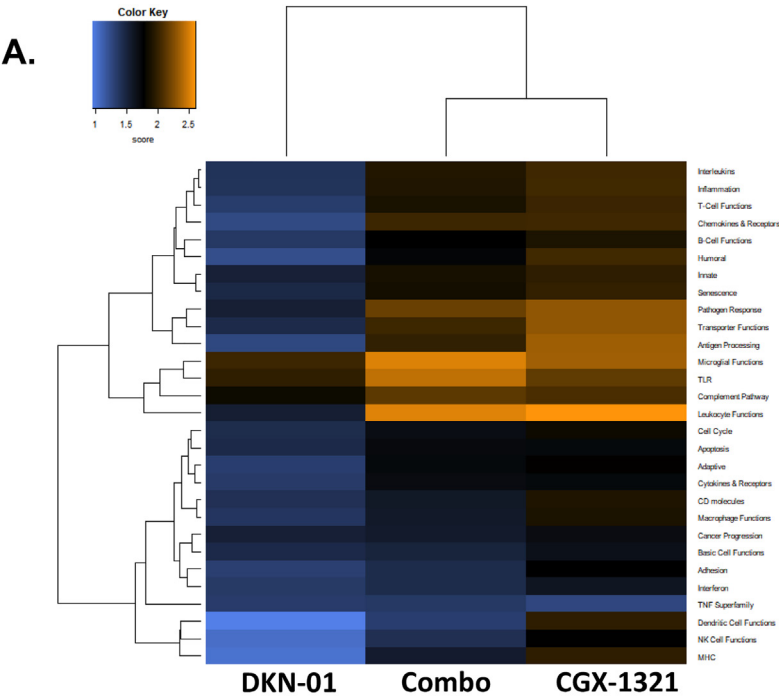
changes compared to vehicle/control-treated mice, although scores for dendritic cell (DC), NK, and major histocompatibility complex (MHC) functions were decreased in mice treated with DKN-01 compared to vehicle/control-treated mice (Fig. 4A). Both CGX-1321- and CGX-1321 + DKN-01-treated mice had increased scores for antigen processing and leukocyte function (Fig. 4A). These data suggest that the two therapies work differently in altering immune response, and together, elicit a diverse anti-tumor immune response.

Following NanoString analysis, we analyzed specific changes in MDSC-related gene expression following treatment with vehicle/control, CGX-1321, DKN-01, or CGX-1321 + DKN-01. Heatmaps were generated to highlight differences in gene expression for a panel of known MDSC-related genes [18], and consistent with previous findings, DKN-01 treatment decreased the expression of various MDSC-related genes (*Cd38*, *Ptprc*, *Tgfb1*, *Cxcr2*, *Tnf*, *Tnf.1*, *Ccr2*, *Entpd1*, *Nos2*, and *Arg1*) compared to vehicle/control-, CGX-1321-, or CGX-1321 + DKN-01-treated mice (Fig. 4B). Mice treated with CGX-1321 and CGX-1321 + DKN-01 had increased expression of the majority of these genes compared to vehicle/control-treated mice (Fig. 4B). These data suggest that DKN-01 efficiently suppresses pro-tumor-related immune gene signatures in the omentum TME.

Flow cytometry was utilized to characterize immune cell populations within the omental TME following treatment with vehicle/control, CGX-1321, DKN-01, and CGX-1321 + DKN-01. Compared to vehicle/control treatment, each treatment increased the percentage of CD8+ T cells (Fig. 4C), but not CD4+ helper T cells (Fig. 4D), in the omental TME. However, the combination of CGX-1321 + DKN-01 had a similar effect on CD8+ T cell infiltration as CGX-1321 monotherapy (Fig. 4C). These results suggest that CD8+ T cell infiltration in the omentum TME was driven primarily by CGX-1321 monotherapy. Altogether, these data suggest that CGX-1321 and DKN-01 therapies elicit different immune responses in the omentum TME that need to be further characterized.

3.4. The addition of anti-PD-1 therapy does not enhance CGX-1321-mediated anti-tumor effects in the ID8p53^{-/-} model

The programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1) axis is a common mechanism employed by cancer cells to evade the immune system; PD-1 on T cells binds PD-L1 on the tumor cell, and this interaction prevents anti-tumor T cell activity [19].



This axis is a common target in ICB therapy but has seen limited success in gynecologic malignancies due to the lack of cytotoxic CD8+ T cell infiltration into the TME. Since our current and previous [3,9] data suggest that PORCNI increases CD8+ T cell infiltration, we next asked whether this therapy would increase sensitivity to anti-PD-1 therapy in the ID8p53^{-/-} model.

Here, ID8p53^{-/-} tumors were initiated as previously described and 10-days post-tumor challenge treatment began. Following 33 days of treatment, there was a significant decrease in omental weights ($p = 0.0067$; $p = 0.008$) (Fig. 5A) and ascites volumes ($p < 0.0001$; $p = 0.0063$) (Fig. 5B) in CGX-1321- and CGX-1321 + anti-PD-1-treated mice compared to vehicle/control-treated mice. However, there was no difference observed in omental weights (Fig. 5A) or ascites volumes (Fig. 5B) between anti-PD-1-treated and vehicle/control-treated mice. In addition, there were no differences observed in omental weights (Fig. 5A) or ascites volumes (Fig. 5B) between CGX-1321- and CGX-1321 + anti-PD-1-treated mice. These results suggest that the anti-tumor effects seen were CGX-1321-driven, and CGX-1321 administration did not sensitize tumors to anti-PD-1 therapy.

To evaluate broad transcriptomic changes that may have arisen in response to CGX-1321 mono- or combinatorial therapies, we performed full RNA sequencing on 3 representative omentum tumors from each treatment group. Heatmaps were generated to highlight differences in gene expression for Wnt-signaling related genes [17] in response to vehicle/control, CGX-1321, anti-PD-1 therapy, and CGX-1321 + anti-PD-1 therapy. The general decrease in Wnt-related gene expressions by CGX-1321 was largely consistent with or without the addition of the anti-PD-1 therapy, which did not result in significant alternations of Wnt-related gene expressions (Supplemental Fig. 1B).

When investigating the effects of anti-PD-1 and CGX-1321 + anti-PD-1 on the immune landscape in the omental TME with flow cytometry, we found no change in total T cells (CD3+) in response to therapy (Fig. 5C). However, there was a significant increase in CD8+ T cells in omentum TME from mice treated with CGX-1321 ($p = 0.03$) or anti-PD-1 therapy ($p = 0.01$) (Fig. 5D). However, there was no significant difference in CD8+ T cell infiltration in the omentums of mice treated with CGX-1321 + anti-PD-1 therapy compared to either monotherapy (Fig. 5D). There was a significant decrease in B cells (B220+) in mice treated with CGX-1321 ($p = 0.0064$) and CGX-1321 + anti-PD-1 therapy ($p = 0.005$) compared to vehicle/control-treated mice (Fig. 5E). Furthermore, there was no difference NK cells (Fig. 5F) following treatments. These results confirm a CGX-1321-mediated CD8+ T cell response and highlighted a potential B cell response to CGX-1321 treatment.

4. Discussion

The interplay of Wnt/ β -catenin signaling and immune exclusion is increasingly complicated in ovarian cancer and pre-clinical ovarian cancer models. PORCNI and DKK1 play differing roles in the Wnt/ β -catenin pathway; PORCNI, CGX-1321, inhibits Wnt/ β -catenin signaling by impairing Wnt ligand biosynthesis, while DKK1i, DKN-01, promotes Wnt/ β -catenin signaling by blocking DKK1 negative regulation. When analyzing Wnt signaling activity, we found that treatment with DKN-01 increased Wnt signaling activity (measured via p-LRP6) (Fig. 3A), confirming results that our group had previously obtained [2]. Since DKK1 is a negative regulator of Wnt signaling, these results confirm that the inhibition of DKK1 activity causes Wnt signaling to go unregulated in ovarian cancer. Treatment with CGX-1321 alone did not decrease Wnt signaling as measured by p-LRP6; we speculate that this is

due to the inherent levels of baseline Wnt signaling in the ID8p53^{-/-} model. However, the addition of CGX-1321 to DKN-01 restored Wnt signaling inhibition (Fig. 3A). These results were supported by data obtained analyzing the change in Wnt-related gene expression in omental tumors where CGX-1321 and CGX-1321 + DKN-01 treatments resulted in an overall reduction in Wnt-related gene expression compared to mice treated with DKN-01 (Supplemental Fig. 1A).

Previously, our lab found that CGX-1321 did not decrease tumor burdens in the ID8p53^{-/-} model when given for 14 days [3], but herein we found that lengthening the course of CGX-1321 treatment to 31 days significantly decreased tumor burden, as assessed by both omental weights and ascites volumes (Figs. 2A–B). However, DKN-01 monotherapy was not sufficient to decrease tumor burden in the ID8p53^{-/-} model. This data supports our previous observation where DKN-01 had no effect on tumor growth in the ID8 parental model [2]. Additionally, our data showed that CGX-1321 monotherapy ablated tumor progression in MISIIR-Tag model when given for 8 weeks (Figs. 2C–D). These results imply that CGX-1321 has a more direct anti-tumor effect than DKN-01, which could be explained by DKN-01's role in immune landscape regulation in the TME. We did not observe the degree of synergism in reducing tumor burden (via omental weight) that was expected when treating with both agents. It is possible that enhanced tumor killing may occur if mice are first treated with DKN-01 to up-regulate Wnt signaling, thereby making the tumor more susceptible to treatment with CGX-1321 (Fig. 6).

Since DKN-01 monotherapy had minimal effects on tumor growth, we wanted to investigate its effects on the immune landscape in the omentum TME of the ID8p53^{-/-} model. Previously, our lab found that DKN-01 did not alter the immune landscape in the omentum TME of the ID8 parental model [2]. However, DKK1 over-expression in the ID8 parental model resulted in decreased anti-tumor immune cell infiltration (e.g. NK and CD8+ T cells) in omentum TME, suggesting DKK1 prevents immunosurveillance in ovarian cancer [2]. Additionally, previous reports have shown that down-regulation of β -catenin in MDSCs was necessary for their accumulation in the TME and resultant tumor growth due to their immunosuppressive effects [20,21]. In our current study, we found that DKN-01 did not have any significant effects on gene expression in immune-related pathways (Fig. 4A), but mice treated with DKN-01 had decreased expression of MDSC-related genes (Fig. 4B), with no significant changes in CD8+ T cell infiltration observed (Fig. 4B). Conversely, mice treated with CGX-1321 and CGX-1321 + DKN-01 had an increase in gene expression in immune pathways related to antigen presentation and leukocyte function (Fig. 4A), and significantly higher CD8+ T cell infiltration in the omental TMEs (Fig. 4C). Importantly, this supports the claim by our group, and others, that increased β -catenin levels are associated with T cell exclusion and tumor growth. In addition, these results suggest that each inhibitor is affecting the immune landscape in the TME differently, and further investigation is needed to characterize more immune cell populations and their function in response to treatments. The combination of DKN-01 with anti-PD1 may prove effective in improving the immune response and is an on-going area of research in our group.

Due to the fact that treatment with CGX-1321 promotes CD8+ T cell infiltration into the omentum TME, we wanted to determine if this effect would sensitize the tumors to ICB therapy. When investigating ICB therapy, anti-PD-1, in combination with CGX-1321, our results were consistent with single agent anti-PD-1 therapy in the clinic, finding that anti-PD-1 therapy did not decrease ovarian tumor burden in the ID8p53^{-/-} model (Figs. 5A–B). While CGX-1321 addition to anti-PD-1 therapy decreased tumor burden compared to anti-PD-1 monotherapy,

Fig. 4. CGX-1321 promotes CD8+ T cell levels, while DKN-01 decreases MDSC-related gene signatures in ID8p53^{-/-} model. (A) NanoString analysis of omentum samples from mice treated with CGX-1321, DKN-01, and DKN-01 + CGX-1321 (Combo) in ID8p53^{-/-} model. Heat map depicts changes in immune pathway scores compared to vehicle/control-treated mice. (B) MDSC-related gene expression changes in ID8p53^{-/-} model where mice were treated with vehicle/control, DKN-01, CGX-1321, or DKN-01 + CGX-1321 (Combo). Flow cytometry analysis was performed on omentums to determine the levels of infiltrating (C) cytotoxic T (Tc; CD8+) and (D) helper T (Th; CD4+) cells in omentum TME.

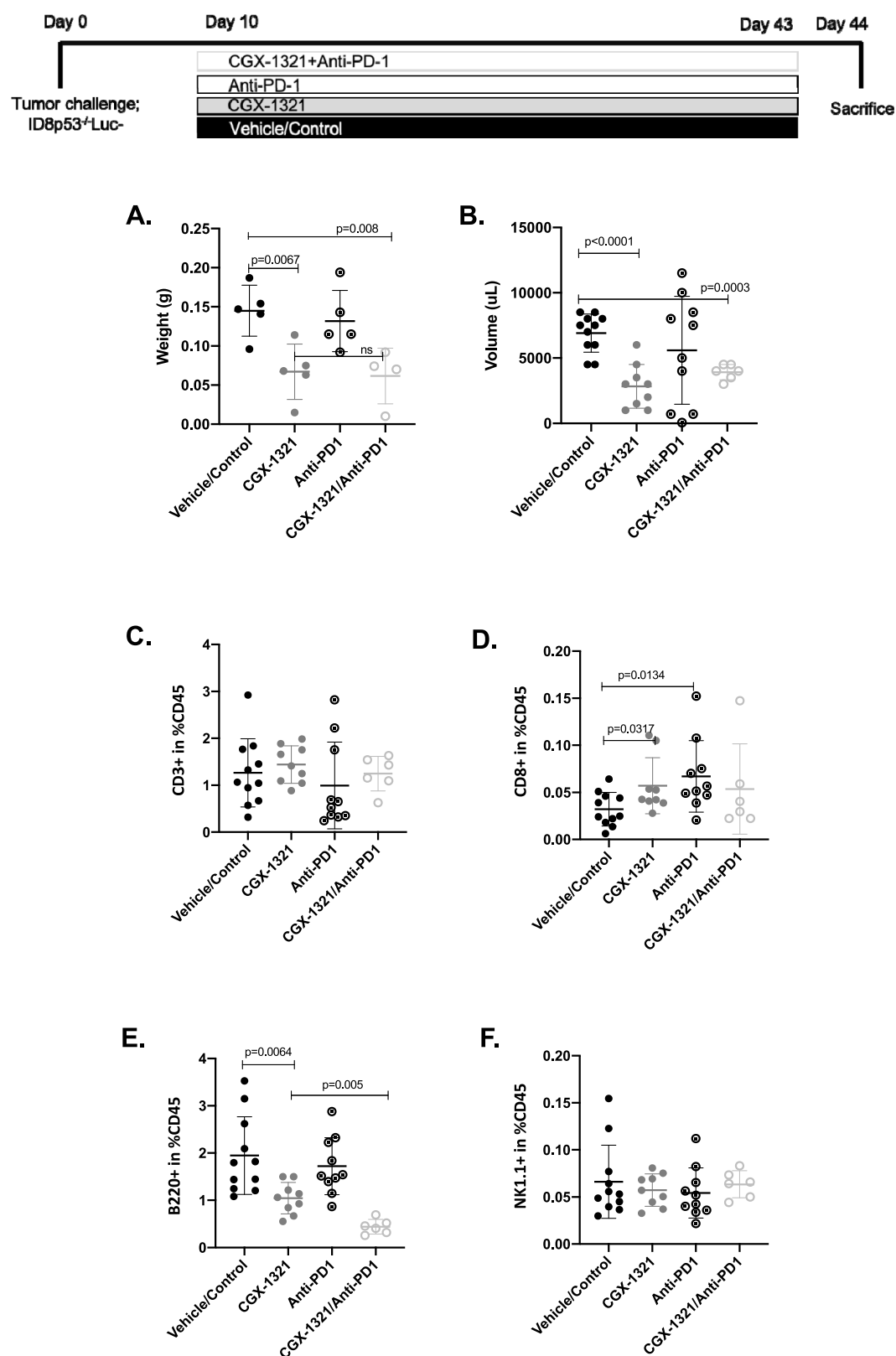


Fig. 5. The addition of anti-PD-1 does not enhance CGX-1321-mediated anti-tumor effects in the ID8p53^{-/-} model. ID8p53^{-/-} model was initiated and treatment with 1 mg/kg CGX-1321 (oral gavage; daily), 250 µg/mouse anti-PD-1 (intraperitoneal injection; every 3 days), and vehicle/control started 10-days post-tumor challenge. (A) Omental weights (g) and (B) ascites volumes (uL) were measured at day 44. Flow cytometry analysis was performed on omentums to determine the levels of infiltrating of (C) T (CD3+) cells, (D) cytotoxic T (Tc; CD8+) cells, (E) B (B220+) cells, and (F) natural killer (NK1.1+) cells in TME.

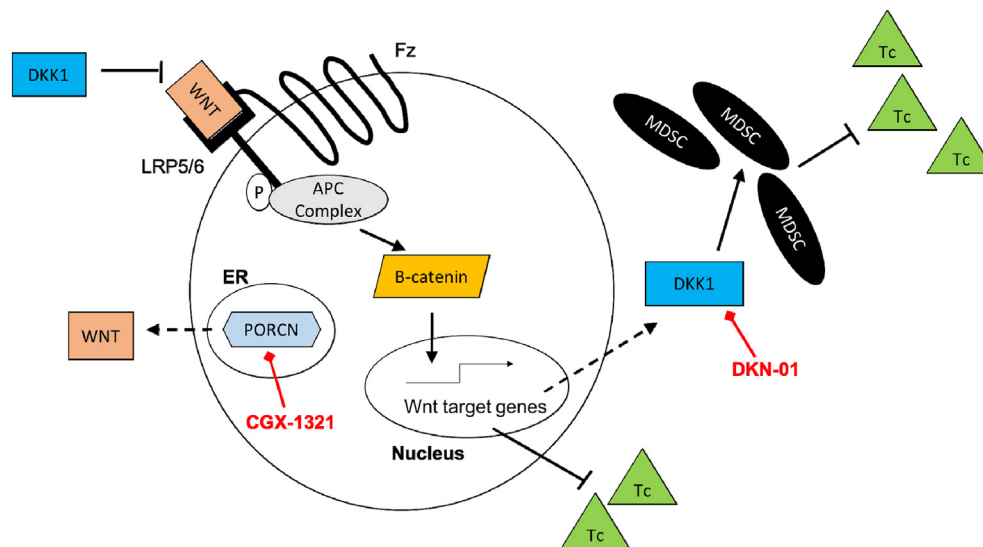


Fig. 6. Dual inhibition of PORCN and DKK1 inhibition in ovarian cancer. CGX-1321 inhibits Wnt/ β -catenin signaling by impairing Wnt ligand biosynthesis, thus decreasing tumor progression and increasing Tc cell infiltration into the TME. DKN-01 inhibits DKK1 causing a reduction in MDSC infiltration and increasing Tc cell infiltration into the TME. (Tc) cytotoxic T cell; (MDSC) myeloid-derived suppressor cell; (DKK1) dickkopf-1; (PORCN) porcupine; (Fz) frizzled receptor; (LRP5/6) low-density lipoprotein receptor-related protein 5/6; (ER) endoplasmic reticulum.

the combination was no better than CGX-1321 monotherapy (Figs. 5A–B). Similar to our results seen with DKN-01, CGX-1321 and CGX-1321 + anti-PD-1 treatment regimens caused an overall reduction in Wnt-related gene expression as compared to vehicle/control- and anti-PD-1-treated mice (Supplemental Fig. 1B).

One thing to consider in this study is only a single treatment regimen investigating the effects of CGX-1321 + anti-PD-1 therapy was tested, with treatments starting simultaneously. It has been established that a positive response to anti-PD-1 therapy requires a robust adaptive immune response within the TME at baseline [22], which is generally not the case in ovarian cancers. Moving forward, prior treatment with CGX-1321 might be required to provide an adaptive immune response to then sensitize tumors to anti-PD-1 therapy by enhancing CD8+ T cell infiltration within the tumor.

While the addition of CGX-1321 to anti-PD-1 therapy did not affect CD8+ T cell infiltration (Fig. 5D), the percentage of B cells (Fig. 5E) in omentum TME was further decreased when CGX-1321 was combined with anti-PD-1 therapy. To date, the data regarding the role of B cells in gynecologic cancers is extremely limited. In ovarian cancer, both pro- and anti-tumor B cell responses have been identified [23]. Specifically, a subset of regulatory B cells (Bregs) has been found to be immunosuppressive by secreting cytokines that convert cytotoxic T (Tc) cells to Tregs [24], and increased levels of these Bregs is negatively associated with patient survival [25]. Our data regarding B cells is limited given we did not utilize more specific markers to further distinguish specific B cell populations within our tumors, which will be critical in further studies to elucidate the contributions of B cells to tumor immunity in gynecologic cancers.

Prior to initiating our study, we anticipated to see enhanced tumor cell death and increased anti-tumor immune infiltration and function when CGX-1321 was combined with either DKN-01 and/or anti-PD-1 therapy. We found that the combination therapies did not enhance the anti-tumor effects of CGX-1321 monotherapy. Importantly, this was the first study to investigate PORCN and DKK1 inhibition in the context of ovarian cancer, and more in-depth investigation is needed to further characterize immune landscape in response to these treatments. Moving forward, we consider that the simultaneous inhibition of PORCN and DKK1 prior to ICB therapy (sequential therapy), will better sensitize ovarian tumors to this therapeutic regimen resulting in the most robust anti-tumor effects.

Author contribution

Conceptualization: RCA, JAW, TR, LAN. Data curation: JAW, AK, SP, AIL, WJT. Formal analysis: JAW, SP, CBS, LAN, RCA. Writing - original draft: JW. Writing - reviewing and editing: JAW, CBS, SP, LAN, RCA.

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Declaration of Competing Interest

RCA has participated in Advisory Boards for Leap Therapeutics, AstraZeneca, GSK, Merck, VBL Therapeutics, and Caris Life Sciences.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygyno.2020.10.031>.

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Review

Assessing Preclinical Research Models for Immunotherapy for Gynecologic Malignancies

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Simple Summary: Novel treatments in immunotherapy for gynecologic oncology have not successfully developed from preclinical research to clinical trials. Preclinical models used to investigate immunotherapy agents are summarized in order to enhance understanding of the inherent limitations and areas of improvement necessary to optimize this research. It is necessary to develop and utilize appropriate preclinical models whose outcomes can be translated to clinical practice in order to identify novel treatments to improve outcomes in patients with gynecologic malignancies.

Abstract: Gynecologic malignancies are increasing in incidence, with a plateau in clinical outcomes necessitating novel treatment options. Immunotherapy and modulation of the tumor microenvironment are rapidly developing fields of interest in gynecologic oncology translational research; examples include the PD-1 (programmed cell death 1) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) axes and the Wnt pathway. However, clinical successes with these agents have been modest and lag behind immunotherapy successes in other malignancies. A thorough contextualization of preclinical models utilized in gynecologic oncology immunotherapy research is necessary in order to effectively and efficiently develop translational medicine. These include murine models, in vitro assays, and three-dimensional human-tissue-based systems. Here, we provide a comprehensive review of preclinical models for immunotherapy in gynecologic malignancies, including benefits and limitations of each, in order to inform study design and translational research models. Improved model design and implementation will optimize preclinical research efficiency and increase the translational value to positive findings, facilitating novel treatments that improve patient outcomes.

Keywords: gynecologic oncology; translational research; preclinical models; immunotherapy; cancer



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1. Introduction

An Unmet Need for Novel Treatments in Gynecologic Oncology: The Potential of Immunotherapy

The increasing incidence and plateau in outcomes for gynecologic malignancies indicate a significant need for novel treatment options. Uterine cancers are the third most common malignancy for women in the United States and are increasing in incidence and prevalence. These cancers, when recurrent or advanced-stage, exhibit limited responses to existing treatments. Patients with advanced-stage and recurrent cervical cancers have a similarly poor prognosis: survival rates are largely unchanged over the last 50 years [1]. In OC (ovarian cancer), over 70% of women present at an advanced stage, and the majority will experience recurrence and die of disease [2–4]. OC is the most deadly gynecologic malignancy, with 13,940 deaths estimated for 2020 and a five-year overall survival rate of approximately 39% [1]. These statistics indicate an unmet need for landscape-changing therapies for gynecologic malignancies.

The potential for immunotherapy in gynecologic oncology is based on successes in renal cell carcinoma, non-small-cell lung carcinoma, and melanoma [5]. However, the field

has yet to hit any “home runs”. There is interest in harnessing the enhanced sensitization of the adaptive immune system and modification of the TME (tumor microenvironment) to promote a more robust immune response. In particular, immunotherapy holds promise in changing the landscape in poor-prognostic cancer types such as ovarian epithelial carcinoma [5,6] and advanced uterine and cervical cancers. However, gynecologic cancers are characterized by many features of a “cold” system (low tumor mutational burden, poor antigenicity, low immunoscore, and lack of T-cell killing): these features make immunotherapy more challenging [7]. Thus, there is a need for an enhanced understanding of the factors contributing to these characteristics in order to identify treatment strategies.

Preclinical models are necessary for the preliminary therapeutic development of effective agents. However, preclinical immunotherapy research in gynecologic oncology has thus far shown limited translational value to the clinical setting. This may be in part due to the inherent limitations of currently used models in accurately representing the human immune system and TME. Without a thorough contextualization of these limitations, research risks premature drug acceptance and attempts at translation to human studies. In so doing, researchers may under-recognize the complexity of immune pathways and TME, setting themselves and patients up for failure. This review will provide an overview of preclinical gynecologic cancer models for immunotherapy research in order to improve study design, data collection, and analysis. We will interpret the unique aspects of each methodology with a focus on translational applicability to the clinical setting, including limitations of each model that may preclude meaningful outcomes for immunotherapy agents in human trials.

2. Targeted Molecular Therapies in Gynecologic Malignancies

Targeted molecular therapies have shown promise in the treatment of gynecologic malignancies and offer valuable insight into the unexpected complexity in translation from preclinical to clinical studies. An example is bevacizumab, a human monoclonal antibody targeting VEGF (vascular endothelial growth factor), originally investigated for its angiogenic influence. However, its roles in vascular permeability and cell migration support the complexity of anti-VEGF agents’ impact on the TME [8]. When added to standard of care treatment, anti-VEGF therapy improves overall and progression-free survival for OC patients with poor prognosis [9–11]. In cervical cancer, the addition of bevacizumab to chemotherapy in metastatic or recurrent disease improves overall survival [12]. Anti-VEGF agents provide an example of TME-directed therapy, and studies are ongoing regarding optimal use, particularly as it relates to its potential in causing hypoxia, which could sensitize cells to ICB (immune checkpoint blockade).

Similarly, PARP (poly ADP-ribose polymerase) inhibitors were originally intended as a targeted treatment for patients with germline BRCA mutations and deficiencies in homologous recombination pathways, inducing “synthetic lethality” to tumor cells via inhibition of single-strand DNA repair [13]. Patients with BRCA mutations on maintenance PARP inhibitors after standard of care primary treatment for OC experienced a 70% lower risk of progression or death [14]; in addition, patients without these mutations showed improved progression-free survival when PARP was used as maintenance therapy after a platinum-sensitive recurrence [15]. As a result, PARP inhibitor use has expanded to consideration as part of primary treatment and/or maintenance in many, if not all, patients with advanced OC [16,17], and phase I and II clinical trials are ongoing to investigate the role of PARP inhibition in endometrial cancers [18]. Recently, PARP inhibitors have been shown to activate anti-tumor immune responses via the STING (stimulator of interferon genes) pathway, potentially sensitizing tumors to ICB and expanding their role in the treatment of gynecologic malignancies [19]. Research is also ongoing regarding chemokine CCL5, which is epigenetically silenced in OC to enable immune cloaking [20], as well as preclinical and clinical investigation of the PI3K/AKT/mTOR pathway [21]. These examples show that the development of effective novel agents for gynecologic malignancies

relies on understanding cancer pathophysiology at the molecular, cellular, and TME levels, underscoring the importance of effective preclinical studies.

2.1. Immunotherapy in Gynecologic Malignancies

At its foundation, immunotherapy seeks to create a durable immune response that acts against cancer cells, both eliminating existing disease and preventing recurrence. Cancer evasion from the immune system occurs at multiple levels, and harnessing the cancer-immunity cycle similarly may require a multi-step approach [22]. The main components of this process in gynecologic malignancy research include improving antigen detection and activation of T-cell responses, as well as manipulating the TME to promote a robust local immune response against tumor cells [23]. For example, CAR-T (chimeric antigen receptor-modified T-cell) therapy aims to genetically construct T cells to specifically target tumor antigens [24]. Clinical trials are ongoing to assess the safety and efficacy of CAR-T; however, its use is still investigational in gynecologic malignancies, as is the use of epigenetic modulators such as HDAC (histone deacetylase) and DNA methyltransferase inhibitors. Vaccination against either specific tumor antigens or whole tumor cells has been limited by an inability to recruit sufficient T-cell responses and, despite extensive preclinical research, has not shown durable responses in clinical trials [25]. The challenges faced by these approaches—successful in other cancers—in reliably stimulating a clinical response indicate the complexity of immunotherapy in gynecologic malignancies.

Assessing the TME in gynecologic malignancies, the presence of TILs (tumor-infiltrating lymphocytes) has been associated with improved progression-free and overall survival in patients with advanced OC [26]. TILs have been associated with increased local levels of immune chemokines, implying a multifaceted, robust immune response against the tumor, and tumors with increased levels show improved responses to immunotherapy and improved survival outcomes [27]. As a result, there is significant interest in manipulating the TME for targeted immunotherapy. An example of a tumor cellular factor impacting the TME and local immune response is PD-L1 (programmed cell death ligand 1), which interacts with PD-1 (programmed cell death 1) receptors on cytotoxic T cells. PD-L1 has been suggested to suppress CD8⁺ T cells; overexpression on tumor cells confers a poorer prognosis for patients [28]. PD-1 interactions with target ligands PD-L1 and PD-L2 also result in net decreased proliferation and survival of T cells, decreased local cytokine release, and enhanced regulatory T-cell activity, contributing to local suppression of the immune response [29]. CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) interactions represent another mechanism of immune modulation under investigation. CTLA-4 activation promotes the immunosuppression of CD8⁺ T cells and is influenced by other T-cell receptor pathways; antibody-mediated blockade has been shown to stimulate anti-tumor immune responses [30]. Increased CTLA-4 expression results in a net inhibitory response to the T cell itself, leading to decreased T-cell survival, decreased local cytokine release (namely IL-2 [29]), and a more “pro-tumor” microenvironment. These preclinical discoveries have had translational applicability: clinical trials in both PD-1/PD-L1 and CTLA-4 immune checkpoint inhibition have been promising [31]. Anti-PD-1 treatment has shown encouraging and durable responses, including complete responses, for recurrent vulvar, vaginal, and cervical cancers [32]. Studies assessing treatments with both agents aim to alter the cancer-immunity cycle at two separate points for a synergistic impact [22]. Clinical trials have shown positive overall response rates (ORR) and modestly improved progression-free survival in epithelial ovarian, primary peritoneal, or fallopian tube cancers with poor prognosis, as well as anti-tumor effects in recurrent endometrial cancer [33,34].

The PI3K/AKT/mTOR signaling pathway is of increasing interest in gynecologic oncology. Originally investigated for its role in cell cycle progression, it also has been implicated in angiogenesis and hypoxia tolerance, facilitating tumor growth [35]. Activating mutations in this pathway (e.g., *PIK3CA*) are common in gynecologic malignancies [36,37]. PI3K overexpression is also implicated in oncogenesis, particularly in cervical cancer, and pathway inhibition has been shown to stimulate tumor cell apoptosis [38]. The

PI3K/AKT/mTOR signaling pathway is also under investigation for its influence on the TME: recent studies suggest its role in T-cell recruitment and macrophage polarization [39]. Numerous ongoing phase I/II clinical trials aim to assess the role of targeting this component, both individually and in combination with hormonal therapy [40,41]; however, due to the pathway's inherent complexity and diverse influences, more preclinical research is necessary to enhance our mechanistic understanding and inform effective use in the clinical setting.

2.2. Utilization of Targeted Therapies in Priming Responses to Immunotherapy in Gynecologic Malignancies

Combinations of immune checkpoint inhibitors with PARP inhibitors, chemotherapy, and anti-angiogenic therapies are an active area of research. Anti-VEGF agents are now postulated to positively impact T-cell trafficking and PD-L1 expression, supporting a possible role in the TME. Similarly, PARP inhibitors may enhance TME interferon pathways and immune cell infiltration. For example, combination treatment with PARP inhibitor and PD-1 inhibitor indicates an ORR of 18% with a disease control rate of 65%, improved from ORR with monotherapy [42–46]. Similarly, PARP inhibition with an anti-PD-L1 agent showed a 68% ORR, with six complete responses [47]. Anti-VEGF and anti-PD-L1 combination therapy also improved ORR in recurrent OC compared to anti-PD-L1 alone, suggesting a possible synergy [48–50]. In patients with advanced endometrial cancer, anti-PD-1/anti-CTLA-4 combination therapy demonstrated a survival benefit compared to chemotherapy, contributing to FDA approval for the regimen [51]. Combination anti-CTLA-4 and PARP inhibition has also demonstrated survival benefits in preclinical models [52]. Despite these clinical trial results, no preclinical data regarding the timing of administration have been published. Preclinical research into the impact of medication timing (e.g., sequential or concurrent) could inform and enhance this observed synergy. In addition to these efforts, there is active research using other agents, such as TGF β , epigenetic therapy, and the Wnt signaling pathway [53].

Preclinical research has the opportunity to identify these interactions, enabling molecular targeting and synergistic treatment, but it requires accurate and reliable models. This review will discuss current and emerging preclinical models (Table 1), aiming to facilitate a better understanding of the advantages and limitations of each methodology in order to better inform effective clinical trial design for immunotherapy in gynecologic cancers.

Table 1. Promising preclinical models utilizing immunotherapy interventions in gynecologic malignancies.

Model	Description	Immunotherapy	References
Syngeneic	ID8	Murine ovarian cancer	Vaccinia virus (VV) + anti-PD-1 CXCR4 agonist (AMD3100) + anti-PD-1 [54,55]
	ID8p53 ^{-/-}	CRISPR/Cas9 TP53 murine ovarian cancer	Carboplatin + STING agonist + anti-PD-1 [56]
Humanized	SKOV-3 IGROV-1, OVCAR-5, and OVCAR-8	Human ovarian cancer	CAR-T cells Anti-CCR4 [57,58]
	SiHa	Human cervical cancer	CRISPR/Cas9 HPV + anti-PD-1 [59]
Organoids	Patient derived	Human ovarian cancer	Anti-PD-1 [60]

3. Preclinical Models of Gynecologic Malignancies

3.1. Patient-Derived Xenograft (PDX) Models

Patient-derived xenograft (PDX) models utilize implantation of patient tumor tissue or ascites tumor cells into mice and attempt to replicate a human tumor. PDX models are commonly used to study tumor biology, resistance to chemotherapy, clinical correlations

and applications, as well as challenges and limitations [61]. For ovarian and fallopian tube cancers, xenografts develop similar metastatic patterns to humans when implanted in the peritoneal cavity and have been utilized to assess responses to chemotherapy regimens [62]. Liu et al. generated PDX models from tumor cells isolated from the ascites or pleural fluid of patients, providing a platform for proof-of-concept for efficacy and biomarker studies [63]. Depreeuw et al. characterized 24 endometrial cancer PDX models by engrafting fresh tumor tissues from patients and evaluated PI3K and MEK inhibitor treatments based on tumor mutations [64]. Other studies have used PDX models to monitor invasion and metastasis patterns [65], including orthotopic luciferase-tagged xenografts for endometrial cancer and models for cervical dysplasia and cancer [66–68]. From a translational standpoint, PDX models hold promise for high-throughput drug testing for patient-specific tumors, based on the rapid uptake of small quantities of human tumor tissue and high rates of engraftment; however, this application is still in its infancy. Although the advantage of studying human cells and tissues in vivo is significant, there are notable limitations. First, there may be genomic instability of these tumors; this complicates the ability to accurately model treatment responses in humans and may represent a potential confounder [69,70]. Human stroma is also replaced by murine stroma over time, limiting the application in investigating the TME [71]. This is of particular importance when targeting angiogenesis. Anti-VEGF agent bevacizumab, used clinically in gynecologic oncology, is often also used in preclinical models. However, this is a human monoclonal antibody: when murine stroma replaces human xenograft tissue, such agents may not act effectively against murine angiogenic factors [72]. This may allow for tumor support and growth that does not authentically represent human growth patterns: cross-species targeted therapy has been suggested as a possible intervention [73]. As the impact of anti-VEGF agents on TME interactions has expanded, the importance of accurately recognizing potential confounders such as host stroma angiogenesis must not be overlooked. Lastly, using immunodeficient host mice inherently limits the translatability of research on the complex TME and immune system interactions under consideration [62,71].

3.2. Syngeneic Murine Models

Syngeneic mouse models for gynecologic cancer utilize in vitro tumorigenesis with subsequent tissue reimplantation into mice [74,75]. The ID8 parental murine OC line provided valuable insight into tumor pathophysiology, including angiogenesis and the importance of VEGF in tumor growth [76]. Preclinical studies using this model have been used to investigate novel treatments, including the synergistic effects of vaccinia virus (VV) and anti-PD-1 therapy and C-X-C chemokine receptor type 4 (CXCR4) antagonist AMD3100 and anti-PD-1 combination therapy [54,55]. The syngeneic ovarian OV2944-HM-1 cell line (also known as HM-1) has also been used to assess immune cell recruitment and interactions, specifically MDSCs (myeloid-derived suppressor cells) and its influence on immune escape [77–79]. Notably, HM-1 may be subcutaneously injected into mice, compared to largely intraperitoneal ID8 models. As illustrated by experiments by Horikawa et al. regarding anti-VEGF therapy resistance, this heterogeneity in models provides a useful opportunity to compare tumor growth and microenvironment patterns within ovarian cancer models [80]. In endometrial carcinoma, a syngeneic model found that the PoleP286R allele in the endometrium enhances anti-tumor immune response and tumor regression, suggesting that POLE-driven cancers may be more sensitive to ICB [81]. However, these findings have not been translatable in clinical trials.

Genetic editing in syngeneic murine models, via the CRISPR (clustered regularly interspaced short palindromic repeats) and Cas9 (CRISPR-associated nuclease 9) (CRISPR–cas9) system, further develops the syngeneic murine model by approximating genetic mutations noted in human tumors. The system creates specific “knockdown” models that eliminate tumor suppressor genes or promote oncogenes, better replicating the genetic expression of the tumor of interest [82]. For example, OC frequently harbors a *p53* mutation. Via CRISPR/cas9 editing, *p53* knockout murine models can be created and are often used

in preclinical research. Ghaffari et al. investigated the effects of carboplatin, STING agonist, and anti-PD-1 therapy combinations in the ID8 model harboring *p53* knockout (ID8p53^{-/-}) [56]. Treatment with STING agonist modulated the TME towards a T_H1-type immune response, and combination carboplatin + STING agonist + anti-PD-1 resulted in the best survival [56]. This technology can also be used to study the impact of various tumor mutations on the TME, as assessed in a panel of knockdown models developed by Iyer et al.; this team has also worked on developing syngeneic murine organoids using similar technology [83,84]. In cervical cancer, CRISPR/Cas9 targeting of HPV (human papilloma virus) and anti-PD-1 had synergistic anti-tumor effects [59]. Such studies provide examples of the disease mechanism processes that can be elucidated via syngeneic mouse models. However, they are inherently limited by being purely murine models without human cells. This prevents their translatability to human responses and TME.

3.3. Transgenic Murine Models

Transgenic murine models, namely the *MISIIR-Tag* mouse lineage, were developed to more accurately replicate the progression of OC. These mice form poorly differentiated, bilateral ovarian tumors that metastasize to the omentum and form ascites, similarly to human ovarian cancers [85]. This lineage is used in preclinical research of both chemotherapy and novel immunotherapy agents. However, the female mice from this model are infertile, posing a challenge in establishing a stable line [85]. In cervical cancer, K14E6/K14E7 double transgenic mice have been used to study recurrence and resistance to progestin therapy [86]. In endometrial cancer, transgenic mice with PTEN (Phosphatase and tensin homolog), *p53*, and mitogen-inducible gene 6 (MIG-6) knockouts have been developed to investigate common mechanisms in the development of that malignancy [87]. Fluorescence technology has been incorporated to allow in vivo monitoring of tumor progression in preclinical models [88], although recent research shows that fluorescent proteins may interfere with the immune response [89,90]. Overall, transgenic mice are useful in studying gynecologic malignancy development, especially with respect to recognized genetic mutations, but they are also limited by the genetic homogeneity inherent to the model's development.

3.4. Humanized Murine Models

Humanized mice represent an in vivo model with significant potential in gynecologic oncology immunotherapy research. Immunodeficient mice are engrafted with human tumors and injected with human hematopoietic stem cells. Similar to PDX models, tumor growth follows a process faithful to that seen in humans. However, humanized mice also attempt to incorporate the immune response, including B and T cells [91], and therefore have the potential to recreate the TME while allowing systemic observation and manipulation, with improved fidelity to humans [92]. Humanized mice have been utilized in OC: a model developed with CAR-T cells demonstrated complete tumor regression [57], and monoclonal antibody immunotherapy in a separate model showed that anti-CCR4 enhanced the anti-tumor immune response by hindering the infiltration of Tregs into the TME [58]. Unfortunately, endometrial cancer humanized mouse models have not yet successfully been established [87], representing an area of unmet need.

The humanized mouse model has limitations that may prevent faithful representation of the TME. These include Graft Versus Host Disease due to persistent mouse immune response to human immune cells, difficulties in transgenic HLA (human leukocyte antigen) expression, and durable production of full human hematopoietic cell lines [93]. Additionally, this model requires significant financial investment: each mouse can cost hundreds of dollars.

4. Shifting Away from Murine Models

4.1. Co-Culture and Two-Dimensional (2-D) Models

Murine model limitations have opened the door to the optimization of more clinically relevant platforms. Co-culture and 3-D models are increasingly popular ways to study patient-derived tumor characteristics, including how cancer cells interact and communicate

with the surrounding environment to regulate disease progression. These models aim to bridge the gap between oversimplified cell cultures and animal models.

Two-dimensional co-culture models plate one cell type into an adherent cell culture dish with the consecutive plating of a different cell population. These models have been used to study the T-cell killing of cancer cells. For example, a TICS (tumor-immune co-culture system) studying the human OC intrinsic mechanisms regulating lymphocyte activation in response to ICB identified a cross-resistance model, suggesting that acquired chemotherapy resistance may confer ICB resistance and that agents restoring HLA expression represent a partner for immunotherapy in chemotherapy-relapsed OC patients [94]. Another example of a co-culture model is the transwell assay. This method can incorporate multiple cell types and is often used to assess the secretion of soluble factors, cell migration, and invasion through an extracellular matrix. For example, in OC-involved omental tissue, transwell assays have been used to identify potential targets to limit tumor cell invasion and migration [95,96]. The transwell system showed that gamma-delta T lymphocytes on human OC stem-like cells (SK-OV-3) can efficiently kill cancer stem cells through IL-17 production, highlighting a promising immunotherapy [97]. In endometrial cancer, the transwell model showed that adiponectin from mesenchymal stem cells enhanced cancer proliferation [98]. Co-culture models have demonstrated that, when grown together, human cervical epithelial and fibroblast cells increase local chemokine expression [99]; the model itself, in this case, provided insight into the complexity of the TME and local inflammatory pathways. Immunotherapy works well in a “hot” TME, and the increase in the expression of certain chemokines suggests sensitivity to immunotherapy. Co-culture models, therefore, show potential via their ability to reconstruct components of the human TME and isolate interactions involved in ICB.

4.2. Three-Dimensional (3-D) Models

Some co-culture models utilize tumor and stromal cells in a matrix to create a 3-D system, improving traditional 2-D methods by incorporating tissue architecture into these models and allowing investigation of the role of drug penetrance and distribution [100]. These models allow researchers to observe and manipulate a medium approximating the *in vivo* condition. Benefits include having human stromal and immune components in conjunction with tumor cells to approximate the TME, allowing research into cellular interactions. This is potentially advantageous for immunotherapy, where complex molecular signaling and its impact on the TME are of particular interest. The 3-D multi-cellular spheroid model can represent *in vivo* micrometastasis via the creation of a matrix incorporating human stromal cells or replicate ascites via the “hanging drop” method [95,101–103], particularly pertinent in OC. The spheroid structure allows approximation of *in vivo* physiology, incorporating consideration of 3-D growth components such as drug penetration, metabolism, chemokine interactions, and O₂ diffusion [104]. Human immune cells can be included, increasing their potential in immunotherapy research, as demonstrated by Wan et al. and their work on PD-1/PD-L1 ICB, incorporating NK cells into patient-derived organoids [60]. Shigeta et al. have used the organoid model to investigate PI3K/AKT pathways in ovarian clear cell carcinoma, demonstrating its utility in high-throughput treatment for rare cancers [105]. In endometrial cancers, 3-D spheroids were used to analyze drug efficacy, cell viability, and proteomics [106]. Studies have also used patient-derived endometrial cancer organoids as a high-fidelity 3-D model preserving the TME and tumor–stromal interactions [107]. These models utilize limited quantities of human tissue to replicate the original physiology.

4.3. Models in Development

Moving forward, one of the most promising approaches to reproducing tumor structure and microenvironment is tumor tissue engineering (TTE) bioreactor systems. Bioreactors are closed systems that allow controlled experimental conditions, typically including a tank, pumps, and sensors, with human tumor tissue, scaffolds, and other components of the TME [108]. Scaffolds are a key component, functioning as a bioactive extra-cellular matrix-

providing mechanical support for cells in order to promote adhesion and/or motility, stimulate required differentiation, and maintain cell functionality while incorporating perfusion and gas exchange components to assess cellular growth and treatment response [108]. A perfusion-based bioreactor system using TNBC (triple-negative breast cancer) patient tissue found that ICB increased immune activation and proliferation followed by cancer cell death, highlighting the use of bioreactors for the preclinical evaluation of conventional and immune-mediated treatments [109]. OC bioreactors are in development but remain investigational [110].

When compared to murine models that may require immunosuppression or incorporate murine tissue responses, co-culture systems may also be helpful for immunotherapy by limiting potential confounders. These models also use human tissue directly. Financially, co-culture systems may be a feasible preliminary research model for novel therapeutics targeting the TME prior to attempting more complex and resource-heavy in vivo studies. However, these systems are inherently limited by being in vitro models. Additionally, these models are synthetically constructed, and incorporation of all components of the TME must therefore be consciously performed. As the roles of fibroblasts, vascular tissue, and other cells are recognized, more simplistic systems may not replicate in vivo complexity; in addition, potentially valuable TME factors that are not yet recognized may be overlooked. The scale of co-culture models—either as droplets or in vitro matrices—also limits the observation and manipulation of larger tissue growth. The perfusion-based bioreactor, in particular, lacks vascular factors [111] that may play an important role in tumor growth and immunotherapy, and each unit has a short time-span of viability. Overall, the utility and translational value have yet to be fully defined for these models, but their potential represents an actively developing area for preclinical tissue modeling.

5. Conclusions

Despite the significant need for novel therapies in gynecologic oncology treatment, breakthroughs have been modest. Immunotherapy is an emerging field with multiple pathways under active investigation, but rather than attempting numerous permutations of new agents and regimens, a thorough understanding of expected outcomes and limitations in preclinical models will improve the efficiency of investments in preclinical research and contribute to meaningful clinical trial outcomes. However, this requires careful study development and a thorough understanding of each model and its ideal role in preclinical immunotherapy research, in order to effectively analyze and interpret findings. As seen in this review, each model (Table 2) has characteristics that influence its translational applicability: effective study design will require multi-disciplinary pharmaceutical, researcher, and clinician collaboration. Utilizing appropriate preclinical models will be integral to identifying the interventions with the highest translational potential and developing impactful, efficient science that will improve patient outcomes in gynecologic malignancies.

Table 2. Summary of preclinical models used in gynecologic oncology research.

Model	Description	References
Syngeneic	ID8 ID8p53 ^{-/-} HM-1 Murine ovarian cancer	[54–56,76,78,79,83]
	Conditional <i>Pole</i> ^{P286R/+} knock-in mice Murine endometrial	[81]
Transgenic	<i>MISIIR-Tag</i> Spontaneous murine ovarian cancer	[85]
	K14E6/K14E7—double transgenic Murine cervical cancer	[86]
	Endometrial	[87]
Humanized	Ovarian	[57,58,91–93]
	SiHa Cervical	[59]
Organoid/3-D	Ovarian Organoids	[84,95,101]
	Ovarian Spheroids/3-D	[60,102–104]
	Endometrial	[106,107]

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