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PRINCIPAL INVESTIGATOR: Charles N. Landen, Jr., MD, MS

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22908

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Charles N. Landen, Jr., MD, MS

E-Mail: clanden@virginia.edu

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14. ABSTRACT

Despite a common outstanding response to primary therapy, most ovarian cancer patients will experience recurrence due to what is often microscopic undetectable disease. One possible cause of this is a chemoresistant population of cells with stem cell characteristics. We have examined one potential population in particular, the ALDH-positive population. We have shown that ALDH1A1-positive cells are more tumorigenic than ALDH1A1-negative cells, contribute to poor patient outcomes, and contribute to chemoresistance. These effects can be reversed by downregulating ALDH1A1 expression with nanoparticle-delivered siRNA. Additionally, we have shown that CSCs are clinically significant, in that chemoresistant tumors have increased density of ALDH and CD133 cells. Importantly, they do not seem to explain the entire story, as there are still many CSC-negative cells present at the conclusion of treatment. Specifically, endoglin (CD105) and hedgehog family members (Gli1 and Gli2) appear to play important roles in chemotherapy resistance, and when targeted enhance response to chemotherapy. To further identify other important players, we have further developed the patient-derived xenograft (PDX) model where patient samples are directly implanted into mice, and when formed, treated with chemotherapy. The treated tumors, like patient specimens, are enriched with ALDH1-positive cells. Further characterization of the surviving population is underway, in conjunction with separately-funded protocols.

15. SUBJECT TERMS
Ovarian Cancer, aldehyde dehydrogenase, ALDH1A1, cancer stem cell

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1. **INTRODUCTION:**

While most ovarian cancer patients initially respond to chemotherapy, most will ultimately recur and succumb to disease, suggesting that there is a subpopulation of cells within a heterogeneous tumor that has either inherent or acquired resistance to chemotherapy[1]. Recently subpopulations of cancer cells in solid tumors have been observed to have properties of stem cells, and therefore designated as "cancer stem cells" (CSC's) or tumor initiating cells (TIC's)[2,3]. The intent of this project is to characterize whether ovarian cells that express aldehyde dehydrogenase (ALDH1A1) have cancer stem cell properties, and if targeting ALDH1A1 would lead to a reversal of the chemoresistant properties. Characteristics of cancer stem cell that will be assessed include tumorigenicity experiments, evidence of multipotentiality, and enhanced resistance to chemotherapeutics. The effects of ALDH1A1 downregulation will be determined both *in vitro* and *in vivo*, using small interfering RNA (siRNA) encapsulated in nanoparticles that allow efficient *in vivo* delivery. If our hypotheses are confirmed, we will have identified a subpopulation of ovarian cancer cells that might survive initial chemotherapy and contribute to resistance, and furthermore may find a clinically feasible novel methodology to target these cells to improve outcomes in this devastating disease. If ALDH1 cells are not explaining the full population of chemoresistant cells, these studies will provide the opportunity to more fully characterize which cells are mediating survival of primary therapy.

2. **KEYWORDS:**

Ovarian cancer
Chemoresistance
Cancer Stem Cells
Aldehyde Dehydrogenase
Patient-Derived Xenografts
Hedgehog Pathway
Endoglin

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

The following were the Major Goals of the project

Task 1: Determine tumorigenicity of ALDH1A1 subpopulations
Task 2: Determine if ALDH1-positive cells survive chemotherapy in the tumor microenvironment.
Task 3: Target ALDH1 with siRNA *in vivo*
Task 4: Evaluate mechanisms of ALDH1-mediated chemoresistance

- **What was accomplished under these goals?**

Task 1: Determine tumorigenicity of ALDH1A1 subpopulations

The goal of task 1 was to determine the tumorigenicity of ALDH1A1 subpopulations. We first injected, in limiting dilutions, sorted ALDH1A1-positive and ALDH1A1-negative populations (based on the ALDEFLUOR assay) of previously-collected and stored primary ovarian cancer specimens. Viability of these cells based on PI exclusion appeared good. Unfortunately, tumors failed to grow in either population. Therefore we changed our initial focus to examine sorted populations from two cell lines with ALDH1A1 activity, A2780cp20 and SKOV3TRip2 (Figure 2B-D of appended manuscript).

We sorted ALDH1A1-positive and negative populations from the A2780cp20 cell line using the ALDEFLUOR assay and injected cells intraperitoneally into NOD-Scid mice in limiting dilutions to determine tumor initiating potential (for methods, see appended manuscript) {Landen, 2010 #4549}. As summarized in Table 1, ALDEFLUOR-positive cells exhibited increased tumorigenic potential, with 100% tumor initiation after injection of 100,000, 25,000, or 5,000 cells, and 1 tumor established after 1,000 cells injected. ALDEFLUOR-negative cells were also able to form tumors, although at a lower rate: two of 5 mice formed tumors after injection of 25,000 or 100,000 cells, and no tumors formed after injection of 5,000 or 1,000 cells. Mice were followed for 1 year after injection, and thorough necropsies were performed in remaining mice to confirm that tumors failed to develop. The TD50, or dose of cells required to permit tumor formation in 50% of animals, was 50-fold lower with ALDEFLUOR-positive cells.

Table 1. Tumorigenicity of ALDH1A1-positive and ALDH1A1-negative cells.

A2780cp20 cells injected IP	**1 mil**	**250k**	**100k**	**25k**	**5k**	**1k**	**Serial transplantation rate**
ALDEFLUOR-negative	5/5	4/5	2/5	2/5	0/5	0/5	0/5
ALDEFLUOR-positive			5/5	5/5	5/5	1/5	5/5

Perhaps more striking was the make-up of these tumors. One requirement of a tumor-initiating population is that they have the capacity to give rise to heterogeneous tumors, composed of both stem cell and non-stem cell populations, therefore demonstrating multipotent differentiating potential. This was noted in tumors that formed after injection of ALDEFLUOR-positive cells. In all 16 of these tumors, a strongly-positive ALDH1A1 population was noted in the minority of the sample, on average 4.7% of the tumor (range 2.4-6.1%, Figure 4A of appended manuscript). However, no ALDEFLUOR-positive cells were found in the tumors that formed after injection of ALDH1A1-negative cells (Figure 4B). This was confirmed with IHC (Figure 4C,D). This argues against the idea that tumors formed because of contamination with ALDEFLUOR-positive cells, or that ALDH1A1 expression is simply induced by the tumor microenvironment regardless of the capacity of the cells.

This difference in the capacity to generate ALDEFLUOR-positive cells was also noted *in vitro*. SKOV3TRip2 cells sorted into ALDEFLUOR-positive and negative populations were cultured separately, and the ALDEFLUOR assay performed on the different populations at 24, 48, and 72 hours (Figure 4E,F). Of the ALDEFLUOR-positive cells, the population gradually reverted to 75.3%, 54.2%, and 51.4% ALDEFLUOR-positive, respectively for each timepoint. However, the ALDEFLUOR-negative cells could not produce any ALDEFLUOR-positive cells.

Task 2: Determine if ALDH1-positive cells survive chemotherapy in the tumor microenvironment.

Although ALDH1 and other putative cancer stem cell populations have enhanced tumorigenicity, that does not necessarily mean that they have preferential survival in patient tumors. We utilized a unique cohort of patients in whom we have both primary and recurrent ovarian cancer specimens. We performed IHC on these for ALDH1, CD44, and CD133 to determine whether recurrent tumors, which are generally more chemoresistant, are predominantly composed of these populations. What we discovered was very interesting, and was published in Clinical Cancer Research[5]. Many recurrent tumors were indeed composed of a greater number of each of these CSC populations, most significantly in the case of CD133.

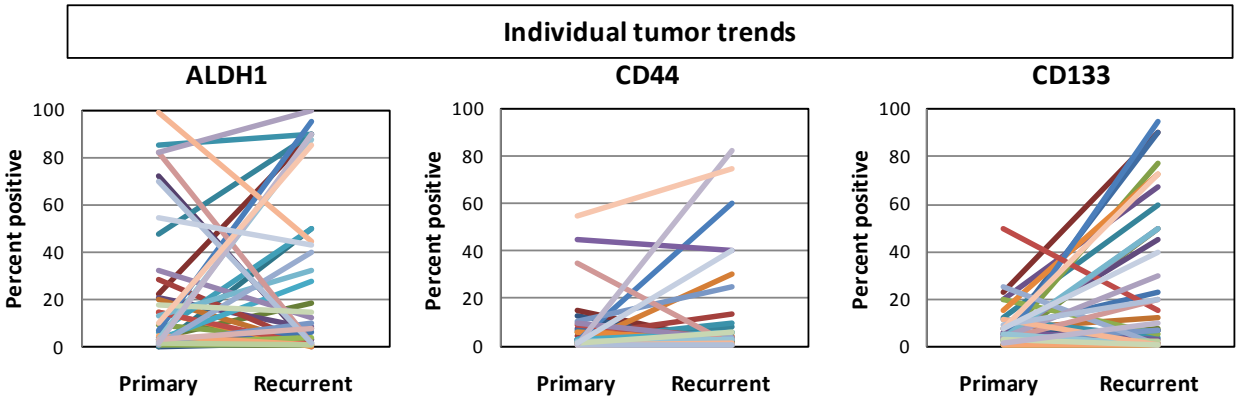


Figure 1. Patient tumors collected in the recurrent setting were more densely positive for CD133 cells compared to tumors collected at primary therapy from the same patient. Each line represents a patient.

Interestingly, many tumors actually had less of each population in the recurrent tumor, most notably in the case of ALDH1. But if the patients were stratified by the setting in which their tumors were collected, the difference was even more striking. Tumors collected immediate after receiving primary therapy, the time at which cells surviving would ultimately cause recurrent disease, were higher in both ALDH1 (2-fold) and CD133 (24-fold) cells. CD44 was higher, but not to a statistically significant degree. Tumors collected at first recurrence were very similar to their primary tumor. This is clinically consistent, because many patients will again have a positive response to chemotherapy when having a first recurrence. It is also consistent with the stem cell hypothesis, since surviving cancer stem cells would be expected to give rise to a heterogeneous tumor resembling the initial tumor.

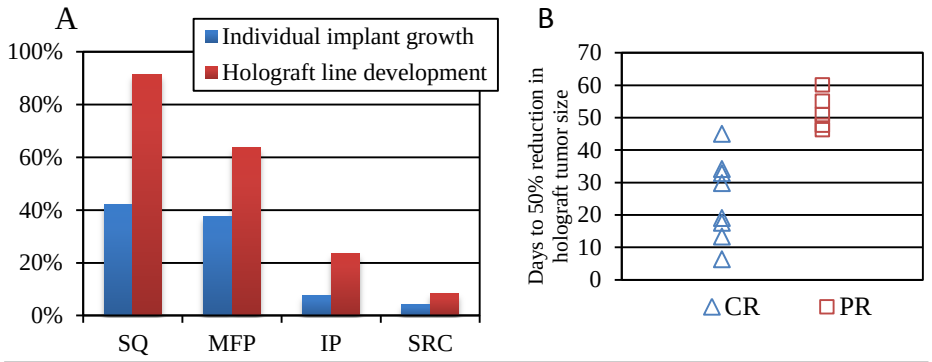
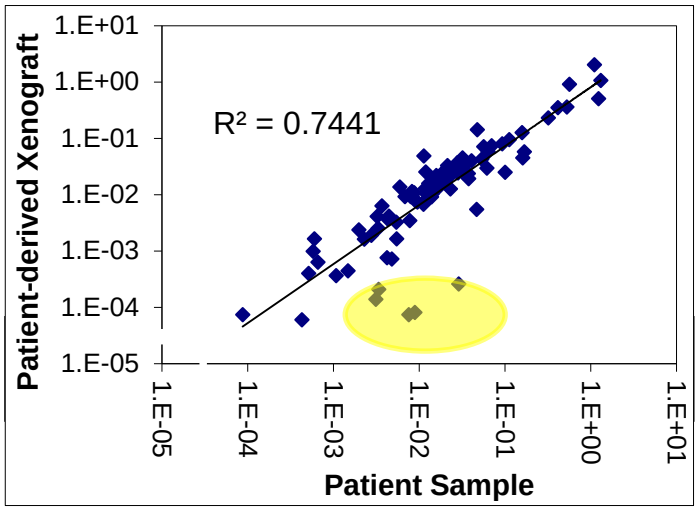


Figure 2. "Holografts" are efficiently established after SQ implantation (A), and response to chemo correlates with patient response (B).

To examine whether this is also noted in a setting where chemotherapy administration and tumor collection is more controlled, we have established protocols for development of primary xenografts in SCID mice. We first examined which sites of implantation are optimal for xenograft formation. We have implanted and compared growth in four sites: 1) subcutaneous, 2) subrenal capsule, 3) intraperitoneal, and 4) mammary fat pad. After attempts in 23 patients, these respective sites have yielded take rates (defined as at least one tumor formed that can be re-established and expanded) of 91.3%, 8.0%, 23.5%, and 63.6%, respectively (Figure 2A). To determine if the tumors are only composed of putative tumor initiating cells, we have performed immunohistochemistry for ALDH1A1, CD44, and CD133, and found that there is less than 10% variability between xenograft and patient tumors. They also retain the heterogeneity and histologic classification of patient tumors. Even mixed-histology tumors display both histologic subtypes in the growing holografts. Most importantly, these xenografts retain biologic tumor heterogeneity and respond to combined platinum/taxane therapy similarly to how patients respond from whom these matched tumors were obtained. Once tumors have been established, at least one is collected for banking purposes, but remaining mice are randomized to continued observation or treatment with combination carboplatin and paclitaxel. Mice are treated for 4 weeks (or until complete response), and response recorded based on traditional RESIST criteria. In the first 13 holografts established, patients who ultimately had only a partial response (PR) to primary therapy had a much slower tumor reduction (or no response at all) compared to patients who had a complete response (CR) ($p<0.001$, Figure 2B).

To further characterize the similarity of the PDX tumors to original patient samples, we have also performed a quantitative PCR array for 84 oncogenes that are recognized targets for therapy, on 4 pair of PDX tumors and patient tumors. There was not a significant difference in gene expression in 79 of the cancer drug target genes, with an overall R^2-value of .7441 (Figure 3). 5 genes had a decrease in expression in the PDX sample when compared to the patient specimen. These genes were PDGFRA, PDGFRB, FLT1, KDR and FLT4. All of these genes

would be expected to be decreased in the PDX tumor, since they are genes produced by the host, and the primers for qPCR are human-specific. If these genes are removed from the analysis and only tumor cell-specific gene expression is considered, the R^2-value increases to 0.8891. Therefore, while the PDX model may not be ideal for targeting proteins expressed by stromal cells, overall there is consistency in expression of targetable oncogenes, supporting use of the model for drug development.



In order to determine if ALDH1A1 and other putative cancer stem cells make up the majority of the xenograft tumors collected after chemotherapy, we performed IHC for these markers on treated tumors. We found that on average, there was a significant increase in ALDH1 and CD133-positive CSCs comprising treated tumors (Figure 2). CD44 was only increased in two tumors, and not significant overall. These are consistent with findings from patient tumors. However, it is important to note that treated tumors are not composed of ONLY these cells. Therefore we have subjected untreated and treated PDX tumors to RNASeq analysis, and in pairwise fashion examined the genes and pathways changing with chemotherapy treatment, either by enrichment of the surviving population, or induced by chemotherapy exposure. Initially 6 pair of tumors have been sequenced and analyzed (support for sequencing provided in a separate grant, not funded by this grant, but work is related).

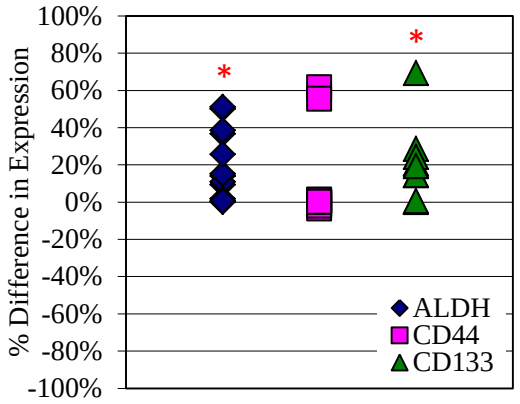


Figure 2. Xenografts with a significant response to carboplatin/paclitaxel therapy are enriched in ALDH and CD133-positive cells (*=p<0.05)

Initially, analysis of all 6 tumor pairs together only found 85 genes that were, on average, significantly different when comparing the 6 treated and untreated tumors. However, when subjected to pathway analysis with IPA software, some very interesting trends are apparent (Table 2). Several pathways are indeed significant altered among several tumors. These include EIF2 signaling (the #1 pathway in 4 of the 6 pair), mTOR signaling, antigen presentation, protein ubiquitination, mitochondrial dysfunction, glycolysis, and remodeling of epithelial adherens junctions.

Intriguingly, it was the same 4 tumors in which these pathways were altered, suggesting either a link between them, or duplication of family members leading to their reveal as important. In the other two pair, most of the pathways significantly altered were participants in the immune system. Therefore, not only are several pathways in common among the multiple pair, there appears to be a dichotomy, whereby one family of tumors may respond to chemo with

one set of pathways relating to metabolism and controls on translation/transcription/protein turnover, and the other through the immune system. Additional work is required to validate these findings, and identify ways to target the system to enhance chemotherapy response.

Table 1. Pathways significantly altered in PDX tumors treated with chemotherapy.

Tumor 106	
Ingenuity Canonical Pathways	fold increase
EIF2 Signaling	47.40
Regulation of eIF4 and p70S6K Signaling	15.40
mTOR Signaling	15.20
Antigen Presentation Pathway	9.85
Protein Ubiquitination Pathway	6.44
Mitochondrial Dysfunction	5.20
Remodeling of Epithelial Adherens Junctions	5.11
Atherosclerosis Signaling	4.92
RhoGDI Signaling	3.66
Clathrin-mediated Endocytosis Signaling	3.64

Tumor 108	
Ingenuity Canonical Pathways	fold increase
EIF2 Signaling	7.75
Mitochondrial Dysfunction	6.85
Protein Ubiquitination Pathway	6.12
Glycolysis I	5.69
mTOR Signaling	3.45
Aryl Hydrocarbon Receptor Signaling	2.95
Regulation of eIF4 and p70S6K Signaling	2.69
Superpathway of Serine and Glycine Biosynthesis I	2.69
4-hydroxyproline Degradation I	2.63
Cell Cycle: G1/S Checkpoint Regulation	2.60

Tumor 115	
Ingenuity Canonical Pathways	fold increase
Role of NFAT in Regulation of the Immune Response	3.26
Ephrin A Signaling	3.20
PKCÎ¸ Signaling in T Lymphocytes	2.59
Systemic Lupus Erythematosus Signaling	2.08
Axonal Guidance Signaling	2.08
Pentose Phosphate Pathway (Oxidative Branch)	2.03
G Protein Signaling Mediated by Tubby	2.00
Complement System	2.00
Calcium-induced T Lymphocyte Apoptosis	1.91
Antiproliferative Role of Somatostatin Receptor 2	1.87

Tumor 116	
Ingenuity Canonical Pathways	fold increase
EIF2 Signaling	70.90
mTOR Signaling	24.80
Regulation of eIF4 and p70S6K Signaling	23.90
Mitochondrial Dysfunction	9.90
Antigen Presentation Pathway	5.92
Complement System	4.61
Remodeling of Epithelial Adherens Junctions	4.56
Glutathione Redox Reactions I	4.10
Crosstalk between Dendritic Cells and Natural Killer Cells	3.89
Regulation of Actin-based Motility by Rho	3.69

Tumor 121	
Ingenuity Canonical Pathways	fold increase
EIF2 Signaling	64.90
Regulation of eIF4 and p70S6K Signaling	24.50
mTOR Signaling	21.00
Mitochondrial Dysfunction	7.67
RhoGDI Signaling	5.66
Protein Ubiquitination Pathway	5.62
Epithelial Adherens Junction Signaling	5.36
Remodeling of Epithelial Adherens Junctions	5.16
Glycolysis I	4.51
Antigen Presentation Pathway	4.11

Tumor 136	
Ingenuity Canonical Pathways	fold increase
Atherosclerosis Signaling	13.00
LXR/RXR Activation	12.10
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	8.83
Hepatic Fibrosis / Hepatic Stellate Cell Activation	8.37
Crosstalk between Dendritic Cells and Natural Killer Cells	7.76
Coagulation System	7.58
Dendritic Cell Maturation	7.45
B Cell Development	7.01
Inhibition of Matrix Metalloproteases	6.99
T Helper Cell Differentiation	6.37

Task 3: Target ALDH1 with siRNA *in vivo*

There are no known inhibitors of ALDH1A1 for *in vivo* studies. Therefore, after IACUC approval, we utilized a method for delivery of siRNA *in vivo* using DOPC nanoparticles. We and others {Gray, 2008 #4402;Halder, 2006 #3574;Kamat, 2006 #3491;Landen, 2005

#2798;Villares, 2008 #4401} have previously demonstrated delivery of siRNA incorporated into DOPC nanoliposomes to the tumor parenchyma with subsequent target downregulation. In this study nude mice were injected intraperitoneally with either SKOV3TRip2 or A2780cp20 cells and randomized to four treatment groups to begin 1 week after cell injection: 1) control siRNA in DOPC, delivered IP twice per week; 2) docetaxel 35 mg, delivered IP weekly (for SKOV3TRip2 model) or cisplatin 160 μg, delivered IP weekly (for A2780cp20 model); 3) ALDH1A1-siRNA in DOPC, IP twice per week; or 4) ALDH1A1-siRNA in DOPC plus docetaxel (for SKOV3TRip2) or cisplatin (for A2780cp20). After four weeks of treatment, mice were sacrificed and total tumor weight recorded. Immunohistochemical analysis confirmed reduced ALDH1A1 expression with ALDH1A1-siRNA/DOPC treatment compared to controls but not with chemotherapy alone. In SKOV3TRip2 xenografts (Figure 5F in appended manuscript) there was a non-significant reduction in tumor growth with docetaxel treatment of 37.0% ($p=0.17$) and with ALDH1A1 siRNA treatment of 25.0% ($p=0.38$) compared to control-DOPC. The observation that ALDH1A1 downregulation alone significantly decreased SKOV3TRip2 growth *in vitro* but was less pronounced *in vivo* suggests that tumor microenvironment factors such as supporting stromal cells may be able to protect cells from ALDH1A1 depletion. However, the combination of ALDH1A1 siRNA and docetaxel resulted in significantly reduced growth, by 93.6% compared to control siRNA ($p<0.001$), by 89.8% compared to docetaxel plus control siRNA ($p=0.003$), and by 91.4% compared to ALDH1A1 siRNA ($p=0.002$). In A2780cp20 (Figure 5G in appended manuscript), there was a similar non-significant reduction in tumor weight with cisplatin alone of 43.9% ($p=0.32$) and with ALDH1A1 siRNA treatment of 57.0% ($p=0.19$). These effects may be even less significant than the mean tumor weights suggest, given the presence of two especially large tumors in the control siRNA group. However, again combined therapy showed a sensitization to chemotherapy with ALDH1A1 siRNA, with combination therapy reducing growth by 85.0% compared to control siRNA ($p=0.048$), by 73.4% compared to cisplatin plus control siRNA ($p=0.013$), and by 65.3% compared to ALDH1A1 siRNA alone ($p=0.039$). Given the minimal effects of either single agent and the consistent finding of significant improvement with combined therapy, these data suggest a synergy between ALDH1A1 downregulation and both taxane and platinum chemotherapeutic agents, though formal dose-finding experiments would be required to definitively prove synergy.

Task 4: Evaluate mechanisms of ALDH1-mediated chemoresistance

We have sorted the A2780cp20 cell lines based on ALDH1A1 activity, as defined by the ALDEFLUOR assay. mRNA was extracted by the Trizol method, and submitted to our core facility for microarray analysis with the Illumina Human_12 chip. Differential expression of the populations is shown in Table 1.

Table 2. Differential expression ALDH-positive and –negative A2780cp20 cells

SYMBOL	ALDHneg mean	ALDHpos mean	Ratio Pos:Neg	T-test
OVEREXPRESSED				
ALDH1A1	2321.55	18392.72	7.92	0.0017
NSUN5C	68.08	193.72	2.85	0.0057

ZNF286A	70.46	145.51	2.07	0.0088
2-Sep	58.28	118.05	2.03	0.0078
PRRG4	103.39	209.32	2.02	0.0021
CD97	71.23	142.09	1.99	0.0007
TWIST2	76.32	149.70	1.96	0.0044
MAT2B	78.75	151.76	1.93	0.0024
AP1M2	72.74	137.81	1.89	0.0089
NDRG2	84.04	159.13	1.89	0.0090
C2CD2	132.93	251.12	1.89	0.0014
CDCA1	85.56	155.65	1.82	0.0052
C7orf28A	74.91	131.89	1.76	0.0026
ZNF714	287.74	486.13	1.69	0.0093
ZNF501	87.71	147.49	1.68	0.0085
TCF20	58.51	96.52	1.65	0.0006
KCNH2	65.48	104.66	1.60	0.0066
RAD51L1	84.59	133.86	1.58	0.0036
REDUCED EXPRESSION				
STRC	135.54	90.32	0.67	0.0019
ZNF3	231.49	153.44	0.66	0.0003
HOXB1	199.72	132.32	0.66	0.0053
ZFP37	219.24	144.25	0.66	0.0005
CHES1	887.74	581.44	0.65	0.0086
DAAM1	625.07	402.09	0.64	0.0088
ZMIZ2	318.61	204.68	0.64	0.0089
DKFZ	99.51	62.03	0.62	0.0097
FBXO2	325.58	202.91	0.62	0.0060
ALDH3A2	636.03	395.36	0.62	0.0089
DAAM1	596.23	368.13	0.62	0.0031
NOV	1011.84	614.10	0.61	0.0073
SFH	203.09	119.64	0.59	0.0067
SCARA3	217.30	127.41	0.59	0.0008
CGAO	102.98	60.02	0.58	0.0097
LIPC	291.32	166.38	0.57	0.0041
PKP4	366.28	208.85	0.57	0.0086
ZNF304	164.21	91.06	0.55	0.0042
AGPAT7	370.26	189.80	0.51	0.0052

In conjunction with this list, as well as genes identified in stem cell pathway analysis of patient primary/recurrent pair, two genes have been further characterized for their contribution to chemotherapy resistance.

First, the endoglin pathway was evaluated. Endoglin expression was intriguing, as it had previously only been known to be expressed in developing endothelial cells. Therefore, Western blot and qPCR were used to evaluate endoglin expression in multiple ovarian cancer lines. Anti-endoglin siRNAs were used to downregulate expression in ES2 and HeyA8MDR. In vitro, the effects of endoglin-knockdown individually and with chemotherapy were evaluated by MTT assay, cell-cycle analysis, alkaline comet assay, and γ-H2AX foci formation. In vivo, mice inoculated with ES2 or HeyA8MDR cell lines were administered chitosan-encapsulated anti-ENG siRNA or control siRNA with and without carboplatin. As described in the accompanying manuscript, endoglin was indeed highly expressed in at least 4 ovarian cancer cell lines (Figure 5). Inhibition of endoglin expression with siRNA significantly decreased cell viability (by 50%, $p<0.001$, and 84%, $p<0.001$, respectively), increased apoptosis, induced double-stranded DNA damage, and increased cisplatin sensitivity. In an orthotopic mouse model, anti-endoglin treatment decreased tumor weight in both ES2 and HeyA8MDR models when compared to control (41.2% reduction, $p=0.001$; and 35.6% reduction, $p=0.014$; respectively, Figure 6). Endoglin inhibition with carboplatin administration was associated with even greater response when compared to control (61.2% and 57.7% reduction, $p<0.001$ for both).

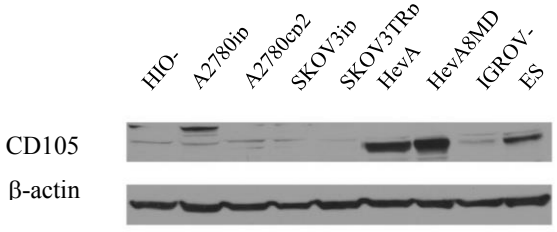


Figure 5. Expression of CD105 (endoglin) in ovarian cancer cell lines.

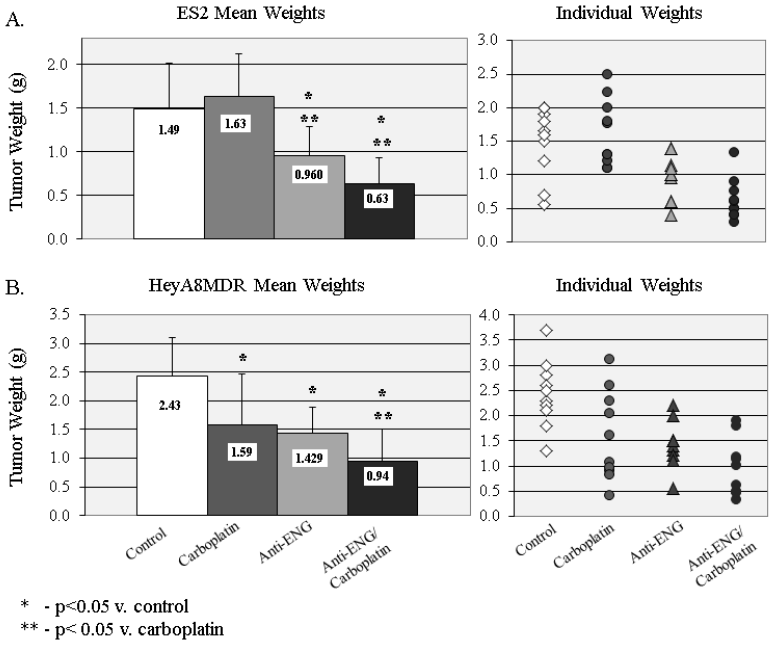


Figure 6. SiRNA-mediated downregulation of CD105 (endoglin) in orthotopic models of ovarian cancer – ES2 (A) and HeyA8MDR (B).

In parallel, the Hedgehog pathway was examined for its potential in chemotherapy resistance. The hedgehog (HH) pathway has been implicated in the formation and maintenance of a variety of malignancies, including ovarian cancer; however, it is unknown whether HH signaling is involved in ovarian cancer chemoresistance. The goal of this investigation was to determine the effects of antagonizing the HH receptor, Smoothened (Smo), on chemotherapy response in ovarian cancer. As reported in the accompanying manuscript, expression of HH pathway members was assessed in 3 pairs of parental and chemotherapy-resistant ovarian cancer cell lines (A2780ip2/A2780cp20, SKOV3ip1/SKOV3TRip2, HeyA8/HeyA8MDR) using qPCR and Western blot. Cell lines were exposed to increasing concentrations of two different Smo antagonists (cyclopamine, LDE225) alone and in combination with carboplatin or paclitaxel. Selective knockdown of Smo, Gli1 or Gli2 was achieved using siRNA constructs. Cell viability was assessed by MTT assay. A2780cp20 and SKOV3TRip2 orthotopic xenografts were treated with vehicle, LDE225, paclitaxel or combination therapy. Chemoresistant cell lines demonstrated higher expression (>2-fold, $p<0.05$) of HH signaling components compared to their respective parental lines. Smo antagonists sensitized chemotherapy-resistant cell lines to paclitaxel (Figure 7A), but not to carboplatin (data not shown). With treatment, cells had a profound G2 phase arrest (Figure 7B-C). LDE225 treatment also increased sensitivity of ALDH-positive cells to paclitaxel. A2780cp20 and SKOV3TRip2 xenografts treated with combined LDE225 and paclitaxel had significantly less tumor burden than those treated with vehicle or either agent alone. Increased taxane sensitivity appeared to be mediated by a decrease in P-glycoprotein (MDR1) expression. Selective knockdown of Smo, Gli1 or Gli2 all increased taxane sensitivity. Smo antagonists reverse taxane resistance in chemoresistant ovarian cancer models, suggesting combined anti-HH and chemotherapies could provide a useful therapeutic strategy for ovarian cancer

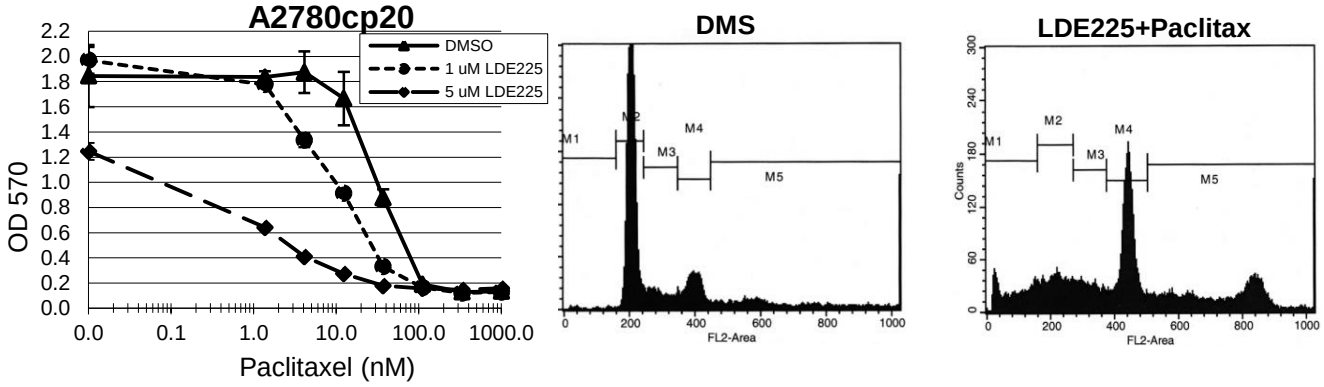


Figure 7. (A) Treatment of the chemoresistant cell line A2780cp20 with LDE225 sensitized cells to paclitaxel, and (B,C) led to a dramatic phase G2 arrest

- **What opportunities for training and professional development has the project provided?**
  - Funding allowed competitive job search and an argument for protected time to allow dedicated time for research as a physician scientist
  - Allowed structured mentorship from the project mentor, Dr. Ronald Alvarez
  - Allowed exposure to other physician scientists in ovarian cancer research, both new scholars and their mentors that provided valuable insight into projects and career development
  - Allowed support and structure to mentor an MD/PhD candidate during the PhD portion of their career, who has now chosen gynecologic oncology as his chosen field

- **How were the results disseminated to communities of interest?**
  - Through publication and presentation of data at invited meetings and lectures.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Nothing to Report, this is the final report

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Recognition of the ALDH-positive as a population with enhanced tumorigenitic and chemoresistance, and a target for therapy
  - An understanding of the mechanisms through which ALDH-positive cells may confer chemoresistance
  - Recognition of the Endoglin pathway as a mediator of chemoresistance and target for therapy
  - Recognition of the Hedgehog pathway as a mediator of chemoresistance and target for therapy
  - Allowed development of methods to establish patient-derived xenografts for testing novel therapeutics and tumor heterogeneity in ovarian cancer

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

- Demonstration that methods and pathways can be applicable to chemoresistance in other malignancies as well

- **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**
  - Nothing to Report
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - There was a delay in productivity during a change of institution
- **Changes that had a significant impact on expenditures**
  - Nothing to Report
- **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.**

    Published (funding acknowledged in all)

    - **Landen CN**, Goodman B, Katre AA, Steg AD, Nick AM, Stone RL, Miller LD, Mejia PV, Jennings NB, Gershenson DM, Bast RC, Jr., Coleman RL, Berestein G, and Sood AK. Targeting Aldehyde Dehydrogenase Cancer Stem Cells in Ovarian Cancer. *Molecular Cancer Therapeutics* 9(12): 3186-99, 2010.
    - Steg AD, Katre AA, Goodman B, Han HD, Nick AM, Stone RL, Coleman RL, Alvarez RD, Lopez-Berestein G, Sood AK, **Landen CN**. Targeting the Notch Ligand Jagged1 in Both Tumor Cells and Stroma in Ovarian Cancer. Clin Can Res, 17(17): 5674-85, 2011.
    - Steg AS, Bevis KS, Katre AA, Ziebarth A, Alvarez RD, Zhang K, Conner M, **Landen CN**. Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. Clin Can Res, 18(3):869-81, 2012.
    - Ziebarth AJ, **Landen CN Jr**, Alvarez RD. Molecular/genetic therapies in ovarian cancer: future opportunities and challenges. Clin Obstet Gynecol, 55(1):156-72, 2012.
    - Steg AS, Katre AA, Bevis KS, Ziebarth A, Dobbin ZC, Shah MS, Alvarez RD, **Landen CN**. Smoothened Antagonists Reverse Taxane Resistance in Ovarian Cancer. Mol Cancer Ther, 11(7): 1587-97, 2012.
    - Ziebarth AJ, Nowsheen S, Steg AS, Shah MM, Katre AA, Dobbin ZC, Han HD, Lopez-Berestein G, Sood AK, Conner MG, Yang ES, **Landen CN**. Endoglin (CD105) contributes to platinum resistance and is a target for tumor-specific therapy in epithelial ovarian cancer. Clin Can Res, 19(1): 170-82, 2013.
    - Chen H, **Landen CN**, Li Y, Alvarez RD, Tollefsbol TO. Epigallocatechin Gallate and Sulforaphane Combination Treatment Induce Apoptosis in Paclitaxel-Resistant Ovarian Cancer Cells through hTERT and Bcl-2 Down-regulation. Exp Cell Res, 319(5): 697-706, 2013.
    - Chen H, **Landen CN**, Li Y, Alvarez RD, Tollefsbol TO. Enhancement of Cisplatin-mediated Apoptosis in Ovarian Cancer Cells through Potentiating G2/M Arrest and p21 Upregulation by Combinatorial Epigallocatechin Gallate and Sulforaphane. J Oncol, 2013: 872957, 2013.
    - Schultz MJ, Swindall AF, Wright JW, Sztul ES, **Landen CN**, Bellis SL. ST6Gal-I sialyltransferase confers cisplatin resistance in ovarian tumor cells. J Ovar Res, 6(1): 25, 2013.
    - Erickson BK, Conner MG, **Landen CN Jr**. The Role of the Fallopian Tube in the Origin of Ovarian Cancer. Am J Obstet Gynecol, 209 (5): 409-14, 2013.
    - Dobbin ZA, **Landen CN**. The importance of the PI3K/AKT/mTOR pathway in the progression of ovarian cancer. *Int J Mol Sciences*, 14(4): 8213-27, 2013.

- **Landen CN** and Lengyl E. Summary of the 2013 American Association for Cancer Research (AACR) Annual Meeting. Gyn Onc, 130 (1): 6-8, 2013..
- Shah MS and **Landen CN**. Ovarian Cancer Stem Cells: Are They Real and Why are they Important? Gynecol Oncol, 132(2): 483-89, 2014. PMID 24321398
- Arend RC, Londoño-Joshi AL, Samant RS, Li Y, Conner M, Hidalgo B, Alvarez RD, **Landen CN**, Straughn JM, DJ Buchsbaum. Inhibition of Wnt/ß-catenin pathway by niclosamide: a therapeutic target for ovarian cancer. Gynecol Oncol. 134(1): 112-20, 2014. PMID:24736023
- Shah MM, Dobbin ZC, Nowsheen S, Wieglos M, Katre AA, Alvarez RD, Konstantinopoulos PA, Yang ES, **Landen CN**. An ex-vivo assay of XRT-induced Rad51 foci formation predicts response to PARP-inhibition in ovarian cancer. Gynecol Oncol, 134(2): 331-7, 2014. PMID 24844596.
- Steg AD*, Burke MR*, Amm HM, Katre AA, Dobbin ZC, Jeong DH, **Landen CN**. Proteasome inhibition reverses hedgehog inhibitor and taxane resistance in ovarian cancer. Oncotarget, Aug 30;5(16):7065-80, 2014. PMID 25216523.
- Dobbin ZC, Katre AA, Steg AD, Erickson BK, Shah MM, Alvarez RD, Conner MG, Schneider D, Chen D, **Landen CN**. Using heterogeneity of the patient-derived xenograft model to identify the chemoresistant population in ovarian cancer. Oncotarget. 5(18): 8750-64, 2014. PMID 25209969.
- Erickson BK, Kinde I, Dobbin ZC, Wang Y, Martin JY, Alvarez RD, Conner MG, Huh WK, Roden RBS, Kinzler KW, Papadopoulos N, Vogelstein B, Diaz LA, **Landen CN Jr**. Detection of Somatic TP53 Mutations in Tampons of Patients With High-Grade Serous Ovarian Cancer. Obstet Gynecol, 124(5): 881-5, 2014. PMID 25437714.
- Desai A, Xu J, Aysola K, Qin Y, Okoli C, Hariprasad R, Chinemerem U, Gates C, Reddy A, Danner O, Franklin G, Ngozi A, Cantuaria G, Singh K, Grizzle W, **Landen C**, Partridge EE, Rice VM, Reddy ES, Rao VN. Epithelial ovarian cancer: An overview. World J Transl Med. 2014 Apr 12;3(1):1-8. PMID: 25525571
- Chien J and **Landen CN**. Summary of the 2015 American Association for Cancer Research (AACR) Annual Meeting. Gynecol Oncol, 138(1):7-10, 2015.
- Schultz MJ, Holdbrooks AT, Chakraborty A, Grizzle WE, **Landen CN**, Buchsbaum DJ, Conner MG, Arend RC, Yoon KJ, Klug CA, Bullard DC, Kesterson RA, Oliver PG, O'Connor AK, Yoder BK, Bellis SL. The tumor-associated glycosyltransferase ST6Gal-I regulates stem cell transcription factors and confers a cancer stem cell phenotype. *Cancer Research*,76(13): 2978-88, 2016.

In preparation:

- Cornelison R, Dobbin ZC, Katre AA, Jeong DH, Petrova Y, Llaneza DC, Steg AD, Parsons L, Zhang Y, Schneider DA, and Landen CN. Targeting Rna-Polymerase I To Overcome Chemotherapy Resistance in Epithelial Ovarian Cancer. Clinical Cancer Research, in preparation

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

Abstracts:

- **Landen CN**, Goodman B, Nick AM, Armaiz-Pena G, Stone RL, Danes C, Shahzad M, Jennings N, Markman M, Gershenson DM, Cooper L, Bast, Jr RC, Coleman R, Sood AK. Isolation of potential ovarian tumor initiating cells by aldehyde dehydrogenase expression. *Proceedings of the American Association of Cancer Research,* 2009.
- **Landen CN**, Goodman B, Nick AM, Stone RL, Miller LD, Mejia PV, Jennings NB, Gershenson DM, Bast RC, Coleman RL, Lopez-Berestein G, and Sood AK. Targeted therapy against aldehyde dehydrogenase in ovarian cancer. *Proceedings of the American Association of Cancer Research*, 2010.
- Bevis KS, Steg AD, Katre AA, Ziebarth AA, Zhang K, Conner MG, **Landen CN**. The significance of putative ovarian cancer stem cells to recurrence. *Center for Clinical and Translational Science Annual Scientific Symposium,* 2010. §
- Bevis KS, Katre AA, Steg AD, Erickson BK, Frederick PJ, Backes TK, Zhang K, Conner MG, **Landen CN**. Examination of matched primary and recurrent ovarian cancer specimens supports the cancer stem cell hypothesis. *Proceedings of the 42nd Annual Society of Gynecologic Oncologists Meeting*, 2011.
- Ziebarth AA, Steg AD, Bevis KS, Katre AA, Alvarez RA, **Landen CN**. Targeting the Hedgehog pathway reverses taxane resistance in ovarian cancer. *Proceedings of the 42nd Annual Society of Gynecologic Oncologists Meeting*, 2011.
- Bevis KS, Katre AA, Steg AD, Erickson BK, Frederick PJ, Backes TK, Zhang K, Conner MG, **Landen CN**. Examination of matched primary and recurrent ovarian cancer specimens supports the cancer stem cell hypothesis. *Proceedings of the 42nd Annual Society of Gynecologic Oncologists Meeting*, 2011.
- Steg AD, Ziebarth AA, Katre A, **Landen CN Jr**. Targeting hedgehog reverses taxane resistance by Gli-dependent and independent mechanisms in ovarian cancer. *Proceedings of the American Association of Cancer Research*, 2011.
- Ziebarth A, Steg AD, Katre AA, Zhang K, Nowsheen S, Yang SH, Connor MG, Lopez-Berestein G, Sood AK, **Landen CN**. A novel role for the TGF-β co-receptor endoglin (CD105) in platinum resistant epithelial ovarian cancer. *43rd Annual Society of Gynecologic Oncologists Meeting*, 2012.
- Ziebarth A, Dobbin ZC, Katre AA, Steg AD, Alvarez RD, Conner MG, and **Landen CN**. Primary ovarian cancer murine xenografts maintain tumor heterogeneity and biologically correlate with patient response to primary chemotherapy. *43rd Annual Society of Gynecologic Oncologists Meeting*, 2012.
- Dobbin ZC, Katre AA, Ziebarth A, Shah MM, Steg AD, Alvarez RD, Conner MG, **Landen** CN. An Optimized Primary Ovarian Cancer Xenograft Model Mimics Patient Tumor Biology and Heterogeneity. *Ovarian Cancer: Prevention, Detection and Treatment of the Disease and its Recurrence*, Pittsburg, PA, 2012.
- Dobbin ZC, Katre AA, Ziebarth A, Shah MM, Steg AD, Alvarez RD, Conner MG, **Landen CN**. Use of an optimized primary ovarian cancer xenograft model

to mimic patient tumor biology and heterogeneity. *American Society of Clinical Oncology, 2012.*

- Kim KH, Bevis KS, Walsh-Covarrubias J, Alvarez RD, Straughn JM, **Landen CN**. Optimizing the Research Experience in Gynecologic Oncology Fellowships. *43rd Annual Society of Gynecologic Oncologists Meeting*, 2012.

- Dobbin ZC, Katre AA, Ziebarth A, Shah MM, Steg AD, Alvarez RD, Conner MG, **Landen** CN. An Optimized Primary Ovarian Cancer Xenograft Model Mimics Patient Tumor Biology and Heterogeneity. *Ovarian Cancer: Prevention, Detection and Treatment of the Disease and its Recurrence*, Pittsburg, PA, 2012.

- *Zimmerman J, Crittenden F, **Landen CN**, Alvarez RD, Brezovich I, Kuster N, Costa F, Barbault A, Pasche B. Amplitude Modulated Radiofrequency Electromagnetic Fields as a Novel Treatment for Ovarian Cancer. 34th Annual Meeting of the Bioelectromagnetics Society, Brisbane, Australia, 2012.*

- *Dobbin ZC, Katre AA, Ziebarth A, Shah MM, Steg AD, Alvarez RD, Conner MG, **Landen CN**. Use of an optimized primary ovarian cancer xenograft model to mimic patient tumor biology and heterogeneity. American Society of Clinical Oncology, 2012.*

- *Leath CA, Alvarez RA, **Landen CN**.* Determination of Potential Ovarian Cancer Stem Cells in Patients with High Grade Serous Cancer Undergoing Neoadjuvant Chemotherapy. *WRHR Scholars Research Symposium*, Philadelphia, PA, 2012.

- Walters C, Straughn J, Landen C, Estes J, Huh W, Kim K. Port-Site Metastases after Robotic Surgery for Gynecologic Malignancy. *43rd Annual Society of Gynecologic Oncologists Meeting*, 2013.

- Shah M, Nowsheen S, Katre A, Dobbin Z, Erickson B, Alvarez R, Konstantinopoulos P, Yang E, Landen C. Towards personalized PARP therapy: XRT-induced Rad51 predicts response to ABT-888 in ovarian cancer. *43rd Annual Society of Gynecologic Oncologists Meeting*, 2013.

- Ziebarth AJ, Nowsheen S, Steg AD, Shah MM, Katre AA, Dobbin ZC, Sood AK, Conner MG, Yang ES, and **Landen CN**. Endoglin (CD105) is a target for ovarian cancer cell-specific therapy through induction of DNA damage *Proceedings of the American Association of Cancer Research,* 2013.

- Erickson BK, Steg AD, Dobbin ZC, Katre AA, Alvarez RD, **Landen CN**. Examination of the chemoresistant subpopulation in ovarian cancer identifies DNA repair genes contributing to survival after primary therapy. *Proceedings of the American Association of Cancer Research,* 2013.

- Erickson BK, Dobbin ZC, Shim E, Alvarez RD, Conner MG, **Landen CN**. Identical TP53 mutations support a common origin for mixed histology epithelial ovarian cancer. *Proceedings of the American Association of Cancer Research,* 2013.

- Burke MR, Steg AD, Jeong DH, Dobbin ZC, **Landen CN.** GSI-1 synergizes with LDE225 in ovarian cancer cells by inhibiting the proteasome *Proceedings of the American Association of Cancer Research,* 2013.

- Jackson WP, Katre AA, Dobbin ZC, Steg AD, **Landen CN**. Pathway analysis of chemoresistance in ovarian cancer cell lines. *Proceedings of the American Association of Cancer Research*, 2013.
- Jimenez H, Zimmerman JW, **Landen CN**, Brezovich I, Chen D, Kuster N, Capstick M, Gong Y, Barbault A, Pasche B. Amplitude-Modulated Radiofrequency Electromagnetic Fields Inhibit Ovarian Cancer cell Growth has been accepted for Platform presentation. *Bioeletromagnetics Society*, 2013.
- Meredith R, Torgue J, Shen S, Banaga E, Bunch P, **Landen CN**. Phase I Trial of Intraperitoneal Alpha Radioimmunotherapy with ^{212}Pb-TCMC-trastuzumab. *12th International Congress of Targeted Anticancer Therapies*, Washington, DC, March 2014.
- Walters Haygood CL, Arend RC, Londono-Joshi A, Kurpad C, Katre AA, Conner MG, **Landen Jr. CN**, Straughn JM, Buchsbaum DJ. *Ovarian Cancer Ascites Stem Cell Population Compared to Primary Tumor. Annual Meeting of the Society of Gynecologic Oncologists*. Tampa, FL. March 2014.
- Erickson BK, Dobbin ZC, Kinde I, Martin JY, Wang Y, Roden R, Huh WK, Vogelstein B, Diaz LA, **Landen Jr CN**. Testing the Accuracy of Mutation detection for the Prevention of Ovarian Neoplasia: the TAMPON study. *Annual Meeting of the Society of Gynecologic Oncologists*. Tampa, FL. March 2014.
- Dobbin ZC, Katre AK, Shah MM, Erikson BK, Chen H, Alvarez RD, Conner MG, Chen D, and **Landen CN**. An ovarian patient-derived xenograft model to identify the chemoresistant population. *10th Biennial Ovarian Cancer Research Symposium*. Seattle, WA. September, 2014.
- Arend RC, Gangrade A, Walters Haygood CL, Kurpad C, Metge BJ, Samant RS, Li PK, Li Y, Bhasin D, **Landen CN**, Alvarez RD, Straughn JM, Buchsbaum DJ. Overcoming Platinum Resistance in Ovarian Cancer with Niclosamide. *10th Biennial Ovarian Cancer Research Symposium.* Seattle, WA. September, 2014.
- Garcia AA, Makker V, Spitz DL, Matei DE, Nick AM, **Landen CN**, Alvarez EA, Mendelson DS, Strother RM, Seon BK, Alvarez D, Adams BJ, Theuer CP, Gordon M. TRC105 (Anti-endoglin Antibody) in Combination with Bevacizumab (BEV) and as a Single Agent for Platinum Resistant Ovarian Cancer. *ESMO*. Madrid, Spain. September, 2014.
- Dobbin ZC, Katre AA, Jeong DJ, Erickson BK, Alvarez RD, Schneider DA, **Landen Jr CN**. Post-chemotherapy tumors in the PDX model identify ribosomal synthesis as a novel targeting strategy in ovarian cancer. *Annual Meeting of the Society of Gynecologic Oncologists*. Chicago, IL. March 2015.
- Kreitzburg KM, Dobbins Z, Katre A, Anwer T, Alvarez R, Landen CN, Yoon KJ. Developing targeted therapy for the treatment of drug-resistant ovarian cancer. *Proceedings of the American Association of Cancer Research*, 2015.

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

Presentations:

Invited

- "Ovarian Cancer Stem Cells: Clinically Significant or Experimental Phenomenon." 3rd Annual International Conference, Ovarian Cancer: Prevention, Detection and Treatment of the Disease and its Recurrence, Pittsburg, PA, 5/2012.
- "Promising Recent Advances in Ovarian Cancer Research". Foundation for Women's Cancer Survivor's Course, Washington, D.C., 10/2012
- "Patient-Derived Xenografts for discovery of de novo mediators of chemoresistance in ovarian cancer." Reproductive Scientist Development Program annual meeting, Boulder CO, 10/2013.
- "Meet the Expert: Managing Your First Lab." AACR / Marsha Rivkin Ovarian Cancer Research Symposium. Seattle, WA. 9/2014.
- "Fallopian tube origin in ovarian cancer." South Carolina Obstetrical and Gynecological Society, Hilton Head, SC, 9/2015.
- "Screening in Ovarian Cancer: Any Closer to the Holy Grail?" South Carolina Obstetrical and Gynecological Society, Hilton Head, SC, 9/2015.
- "The Fallopian Tube as Origin of Ovarian Cancer and Opportunistic Salpingectomy." 47th Annual OB/GYN Spring Symposium, Charleston, SC, 4/2016.
- "Update on Screening in Ovarian Cancer". 47th Annual OB/GYN Spring Symposium, Charleston, SC, 4/2016.

Invited Seminars from Other Institutions

- "Cancer Stem Cells: Clinically significant or an experimental phenomenon?" Felix Rutledge Society, MD Anderson Cancer Center, 5/2011.
- "Cancer Stem Cells: Clinically significant or an experimental phenomenon?" Hudson Alpha Lecture series, Huntsville, AL, 4/2013.
- "Cancer Stem Cells: Clinically significant or an experimental phenomenon?" Southern Cell Biology Research Symposium, Tuskegee University, 6/2013.
- "Development of the patient-derived xenograft model to identify de novo mediators of chemoresistance." University of Pittsburg, 3/2014.
- "IN SEARCH OF: The chemoresistant population in ovarian cancer." Department of Obstetrics and Gynecology Grand Rounds, University of Chicago, 4/2014.
- "Targeting of mediators of chemoresistance in ovarian cancer. Legyel Lab, University of Chicago, 4/2014.
- "Development of the patient-derived xenograft model to identify de novo mediators of chemoresistance." University of Virginia, 4/2014.

- "Development of the patient-derived xenograft model to identify de novo mediators of chemoresistance." Kansas University, 5/2014.
- "Identification and Targeting Mediators of Chemoresistance in Ovarian Cancer." Indiana University, 9/2015.

Presentations at Local Conferences

- "In search of... Ovarian Cancer Stem Cells." Program in Experimental Therapeutics, UAB, 1/2010.
- "What's New in Gynecologic Cancer Research." Progress in OB/GYN Annual Meeting, UAB, 2/2010.
- "Independent targeting of the Notch pathway in tumor cells and tumor stroma." Cancer Cell Biology seminar series, UAB, 5/2010.
- "Neoadjuvant Chemotherapy in Ovarian Cancer." Division of Gynecologic Oncology Grand Rounds, UAB, 7/2010.
- "Historical Vignettes in Obstetrics and Gynecology." Department of Obstetrics and Gynecology Grand Rounds, UAB, 7/2010.
- "Surgical Management of Gynecologic Malignancies." Department of Radiology Grand Rounds, UAB, 7/2010.
- "Ex vivo and animal models of cancer." Graduate School in Biomedical Sciences, Translational Research Course, UAB, 10/2013.
- "Development of the patient-derived xenograft model to identify de novo mediators of chemoresistance." Grand Rounds, Department of Hematology and Oncology, UAB, 5/2014.
- "Targeting Mediators of the Chemoresistance in Ovarian Cancer." Grand Rounds, Department of Obstetrics and Gynecology, UVA, 9/2014
- "Targeting Mediators of the Chemoresistance in Ovarian Cancer." Grand Rounds, Department of Pathology, UVA, 10/2014
- "Targeting Mediators of the Chemoresistance in Ovarian Cancer." Grand Rounds, UVA Cancer Center, UVA, 10/2014.

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

  None

- **Technologies or techniques**

  None

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

  None

- **Other Products**

  A cohort of ovarian cancer specimens, and corresponding clinical information, was collected during the course of this study, that have been retained for potential future use.

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
- **What individuals have worked on the project?**

***Example**:*

Name:	Charles N. Landen, Jr., MD, MS
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-2780-2444
Nearest person month worked:	19
Contribution to Project:	Dr. Landen is the PI on the proposal, overseeing all activities
Funding Support:	Detailed below in Other Support

Name:	Zachary Dobbin, MD, PhD
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	15
Contribution to Project:	Dr. Dobbin performed numerous experiments on this proposal during the course of his PhD thesis project
Funding Support:	None additional

Name:	Danielle, Llaneza, MS
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	Ms. Llaneza has performed experiments on this proposal, and oversought administrative management of the grant during the transition to UVA
Funding Support:	None additional

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

  - Listed below are the other granting agencies and awards that have provided to support to the PI during the course of the award:

Active

Co-Investigator, *Glycosylation-dependent mechanisms regulating ovarian tumor cell survival*. R01 GM111093, NIH/NIGMS, 4/1/2014 – 3/31/2017, $570,000 total direct.

Co-Investigator, *Development of a Novel Small Molecule PTP4A3 Inhibitor for the Treatment of Ovarian Cancer*. The Ivy Foundation, 3/1/2016 – 2/28/2017, 78,000 total direct.

Co-Investigator, *Developing ovarian cancer stem-like cell targeted therapy to prevent disease recurrence*, Ovarian Cancer Research Program Pilot Award, CDMRP Department of Defense, 9/1/2014 – 8/31/2016, $51,532 over 2 years.

Co-Investigator, *DNA repair enzyme tyrosyl-DNA phosphodiesterase I as novel therapeutic target for ovarian cancer treatment*, Ovarian Cancer Research Program Pilot Award, CDMRP Department of Defense, 9/30/2015 – 09/29/2017, $10,324 over 2 years.

Principal Investigator (Faculty Director), Molecular Assessments and Preclinical Studies (MAPS) Core Facility, UVA Cancer Center, 9/2015-current. $11,800/yr.

Prior

Principal Investigator, *Characterization and Targeting of the Aldehyde Dehydrogenase Subpopulation in Ovarian Cancer*, OC093443, Department of Defense Ovarian Academy Award, 7/1/2010 – 7/30/2016, $750,000 total direct.

Principal Investigator, *Nanoparticle delivery of siRNA to target chemoresistance in ovarian cancer*. Transdisciplinary Research Grant, UVA Cancer Center, 1/1/2015-12/31/2015, $100,000 total direct.

Co-Investigator, *Ribosome biogenesis, turnover and function as a therapeutic target for ovarian cancer*, Program Project Grant Pilot Fund, UAB Comprehensive Cancer Center, 8/1/2014 – 7/31/2015, $150,000.

Co-Investigator, *Using RPS25 to Target the Survival Pathway in Ovarian Cancer*, Faculty Development Award, UAB Comprehensive Cancer Center, 3/15/14 – 3/14/15, $40,000.

Principal Investigator, *Targeting Ribosomal RNA Synthesis for Treatment of Ovarian Cancer*, RSDP Seed Grant Program. 9/1/2014 – 8/31/2015, $25,000.

Co-Investigator, U54 pilot project: *BRCA1 Deficiency and Epithelial Ovarian Cancers.* Morehouse School of Medicine/Tuskegee University/University of Alabama Cancer Center Partnership. 9/1/2011-8/31/2014, $18,000.

Principal Investigator. *Identifying mediators of chemoresistance in ovarian cancer*. The Norma Livingston Foundation. 5/1/2012-4/30/2014. $50,000.

Principal Investigator. *Development of a Personalized Therapy Model in Cervical Cancer*. Pilot Project, SPORE in Cervical Cancer. 9/1/2012 – 8/31/2014. $30,000.

Co-Investigator, *Chemosensitization of Ovarian Cancer by Exploiting Novel and Safe Epigenetic Compounds*. College of Arts and Sciences Interdisciplinary Innovation Team Award (PI Trygve Tollefsbol). 10/1/2012-9/30/2014. $30,000.

Principal Investigator. *Detection of ovarian cancer-derived mutations in tampon extracts using Safe-SeqS*. The Laura Crandall Brown Foundation. 12/5/2012-12/4/2014. $50,000.

Co-Principal Investigator. Predicting response of ovarian cancers to PARP Inhibitors. The ROAR Foundation. 12/14/2012 – 12/13/2014. $50,000.

Principal Investigator, *105OC201: A Phase 2 Evaluation of TRC105 in the Treatment of Recurrent Ovarian Fallopian tube, or Primary Peritoneal Carcinoma*. Sponsor: TRACON Pharmaceuticals, Inc. 9/14/2011-9/9/2012. $19,385 in charges.

Principal Investigator, *Targeting Jagged in Ovarian Tumor Initiating Cells*, Research Scientist Development Program Phase II (through the Ovarian Cancer Research Fund), 7/1/2009-6/30/2012, $240,000 over 3 years.

Principal Investigator, *Examination of the true mediators of resistance in ovarian cancer*, Translational Research Intramural Grant, UAB CCTS and CCC, 4/1/2010 – 3/31/2011, $71,000 over 1 year.

- **What other organizations were involved as partners?**

Organization Name: University of Alabama at Birmingham

Location of Organization: Birmingham, AL

Partner's contribution to the project – THIS WAS THE INITIAL LOCATION OF THE AWARD, and therefore provided additional **Financial support, Facilities**, and **Collaborators**

7. **SPECIAL REPORTING REQUIREMENTS**

Not applicable

8. **APPENDICES:**

Appendix 1: **Landen CN**, Goodman B, Katre AA, Steg AD, Nick AM, Stone RL, Miller LD, Mejia PV, Jennings NB, Gershenson DM, Bast RC, Jr., Coleman RL, Berestein G, and Sood AK. Targeting Aldehyde Dehydrogenase Cancer Stem Cells in Ovarian Cancer. *Molecular Cancer Therapeutics* 9(12): 3186-99, 2010.

Appendix 2: Steg AS, Bevis KS, Katre AA, Ziebarth A, Alvarez RD, Zhang K, Conner M, **Landen CN**. Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. *Clin Can Res*, 18(3):869-81, 2012.

Appendix 3: Steg AS, Katre AA, Bevis KS, Ziebarth A, Dobbin ZC, Shah MS, Alvarez RD, **Landen CN**. Smoothened Antagonists Reverse Taxane Resistance in Ovarian Cancer. *Mol Cancer Ther*, 11(7): 1587-97, 2012.

Appendix 4: Ziebarth AJ, Nowsheen S, Steg AS, Shah MM, Katre AA, Dobbin ZC, Han HD, Lopez-Berestein G, Sood AK, Conner MG, Yang ES, **Landen CN**. Endoglin (CD105) contributes to platinum resistance and is a target for tumor-specific therapy in epithelial ovarian cancer. *Clin Can Res*, 19(1): 170-82, 2013.

Appendix 5: Dobbin ZC, Katre AA, Steg AD, Erickson BK, Shah MM, Alvarez RD, Conner MG, Schneider D, Chen D, **Landen CN**. Using heterogeneity of the patient-derived xenograft model to identify the chemoresistant population in ovarian cancer. *Oncotarget*. 5(18): 8750-64, 2014.

Appendix 6: Curriculum Vitae, Charles N. Landen, Jr, MD, MS

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Molecular Cancer Therapeutics

Targeting Aldehyde Dehydrogenase Cancer Stem Cells in Ovarian Cancer

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Therapeutic Discovery

Molecular Cancer Therapeutics

Targeting Aldehyde Dehydrogenase Cancer Stem Cells in Ovarian Cancer

Charles N. Landen Jr[1], Blake Goodman[2], Ashwini A. Katre[1], Adam D. Steg[1], Alpa M. Nick[2], Rebecca L. Stone[2], Lance D. Miller[3], Pablo Vivas Mejia[4,5], Nicolas B. Jennings[2], David M. Gershenson[2], Robert C. Bast Jr.[4], Robert L. Coleman[2,6], Gabriel Lopez-Berestein[4,6,7], and Anil K. Sood[2,6,7]

Abstract

Aldehyde dehydrogenase-1A1 (ALDH1A1) expression characterizes a subpopulation of cells with tumor-initiating or cancer stem cell properties in several malignancies. Our goal was to characterize the phenotype of ALDH1A1-positive ovarian cancer cells and examine the biological effects of ALDH1A1 gene silencing. In our analysis of multiple ovarian cancer cell lines, we found that ALDH1A1 expression and activity was significantly higher in taxane- and platinum-resistant cell lines. In patient samples, 72.9% of ovarian cancers had ALDH1A1 expression in which the percentage of ALDH1A1-positive cells correlated negatively with progression-free survival (6.05 vs. 13.81 months; $P < 0.035$). Subpopulations of A2780cp20 cells with ALDH1A1 activity were isolated for orthotopic tumor–initiating studies, where tumorigenicity was approximately 50-fold higher with ALDH1A1-positive cells. Interestingly, tumors derived from ALDH1A1-positive cells gave rise to both ALDH1A1-positive and ALDH1A1-negative populations, but ALDH1A1-negative cells could not generate ALDH1A1-positive cells. In an *in vivo* orthotopic mouse model of ovarian cancer, ALDH1A1 silencing using nanoliposomal siRNA sensitized both taxane- and platinum-resistant cell lines to chemotherapy, significantly reducing tumor growth in mice compared with chemotherapy alone (a 74%–90% reduction; $P < 0.015$). These data show that the ALDH1A1 subpopulation is associated with chemoresistance and outcome in ovarian cancer patients, and targeting ALDH1A1 sensitizes resistant cells to chemotherapy. ALDH1A1-positive cells have enhanced, but not absolute, tumorigenicity but do have differentiation capacity lacking in ALDH1A1-negative cells. This enzyme may be important for identification and targeting of chemoresistant cell populations in ovarian cancer. *Mol Cancer Ther; 9(12); 3186–99. ©2010 AACR.*

Introduction

Ovarian cancer was expected to be diagnosed in 21,550 women in 2009 and take the lives of 14,600 women (1). Although ovarian cancer is among the most chemosensitive malignancies at the time of initial treatment (surgery and taxane/platinum-based chemotherapy), most patients will develop tumor recurrence and succumb to chemoresistant disease (2). An understanding of the mechanisms mediating survival of subpopulations of ovarian cancer cells is necessary to significantly improve outcomes in this disease.

In many malignancies, a subpopulation of malignant cells termed cancer stem cells or tumor-initiating cells has been hypothesized to represent the most tumorigenic and treatment-resistant cells within a heterogeneous tumor mass. Defined by their enhanced ability to generate murine xenografts and give rise to heterogeneous tumors that are composed of both tumor-initiating cell and non-tumor-initiating cell populations, these cells may also be more chemoresistant and depend on unique biological processes compared with the majority of tumor cells (3, 4). In ovarian cancer, many of these properties have been identified in populations of CD44/c-kit–positive cells (5), CD133-positive cells (6–8), and Hoechst-excluding cells (the side population; ref. 9).

Among several markers that have been used to identify cancer stem cells, aldehyde dehydrogenase-1A1

Authors' Affiliations: [1]Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, Alabama; [2]Department of Gynecologic Oncology, U.T.M.D. Anderson Cancer Center, Houston, Texas; [3]Department of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, North Carolina; [4]Department of Experimental Therapeutics, U.T.M.D. Anderson Cancer Center, Houston, Texas; [5]The University of Puerto Rico Comprehensive Cancer Center, Rio Piedras, PR; and [6]Center for RNA Interference and Non-Coding RNA; [7]Department of Cancer Biology, U.T.M.D. Anderson Cancer Center, Houston, Texas

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Corresponding Author: Charles N. Landen Jr., Department of Obstetrics and Gynecology, The University of Alabama at Birmingham, 1825 University Blvd, 505 Shelby Bldg, Birmingham, AL 35294. Phone: 205-934-0473; Fax: 205-934-0474. E-mail: clanden@uab.edu

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(ALDH1A1) has been a valid marker among several malignant and nonmalignant tissues (10–20). It holds the attractive distinction of not only being a potential marker of stemness but potentially playing a role in the biology of tumor-initiating cells as well (10). ALDH1A1, 1 of 17 ALDH isoforms, is an intracellular enzyme that oxidizes aldehydes, serving a detoxifying role, and converts retinol to retinoic acid, mediating control on differentiation pathways. The ALDH1A1 population defines normal hematopoietic stem cells, being used to isolate cells for stem cell transplants in patients. Using the ALDEFLUOR assay, a functional flow cytometric assay that identifies cells with active ALDH1A1, tumor-initiating cell-enriched populations have been identified in multiple malignancies (20), including breast (11–14), colon (15, 16), pancreas (17), lung (18), and liver (19). Whether or not the ALDH1A1-active population is enriched for tumor-initiating cells has not been demonstrated for ovarian cancer. More importantly, although ALDH1A1 is implicated in chemoresistance pathways, it is not known whether targeting ALDH1A1 can sensitize resistant cells to chemotherapy and therefore represent a potential target for cancer stem cell–directed therapy. We sought to characterize expression of ALDH1A1 in ovarian cancer cell lines and patient samples, determine whether it contains tumor-initiating cell properties, and examine whether targeting ALDH1A1 sensitizes cells to chemotherapy in both *in vitro* and *in vivo* ovarian cancer models.

Materials and Methods

Cell lines and culture

The ovarian cancer cell lines SKOV3ip1, SKOV3-TRip2, HeyA8, HeyA8MDR, A2780ip2, A2780cp20, IGROV-AF1, and IGROV-cp20 (21, 22) were maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum (Hyclone). SKOV3TRip2 [taxane-resistant, a kind gift of Dr. Michael Seiden (23)] and HeyA8MDR were maintained with the addition of 150 nmol/L of paclitaxel. The HIO-180 SV40-immortalized, nontumorigenic cell line derived from normal ovarian surface epithelium was a kind gift of Dr. Andrew Godwin. All cell lines were routinely screened for *Mycoplasma* species (GenProbe detection kit) with experiments done at 70% to 80% confluent cultures. Purity of cell lines was confirmed with STR genomic analysis, and cells used were always less than 20 passages from the stocks tested for purity.

Whole genomic analysis

RNA was extracted from 3 independent collections of SKOV3ip1 and SKOV3TRip2 cells at 80% confluence with the RNeasy Mini kit (Qiagen). It was subjected to microarray analysis using the Illumina HumanRef-8 Expression BeadChip, which targets ~24,500 well-annotated transcripts. Microarray data were normalized by the cubic-spline method (24) using the Illumina BeadStudio software. The significance of differentially expressed genes was determined by Student's t test followed by correction for false discovery (25). A heat map was generated using Cluster 3.0 and Java TreeView software. The array data have been registered with GEO (accession #GSE23779) for public access.

Western blot analysis

Cultured cell lysates were collected in modified radioimmunoprecipitation assay lysis buffer with protease inhibitor cocktail (Roche) and subjected to immunoblot analysis by standard techniques (26) using anti-ALDH1A1 antibody (BD Biosciences) at 1:1,000 dilution overnight at 4°C, or anti–β-actin antibody (Sigma Chemical) at 1:2,000.

Immunohistochemical staining and clinical correlations

Immunohistochemical (IHC) analysis was done on formalin-fixed, paraffin-embedded samples, using standard techniques (26). For ALDH1A1, antigen retrieval was in citrate buffer for 45 minutes in an atmospheric pressure steamer, using anti-ALDH1A1 antibody (BD Biosciences) at 1:500 dilution in Cyto-Q reagent (Innovex Biosciences) overnight at 4°C. Primary antibody detection was with Mach 4 HRP polymer (Biocare Medical) for 20 minutes at room temperature, followed by diaminobenzidine incubation. After IHC staining, the number of tumor cells positive for ALDH1A1 was counted and expressed as a percentage of all tumor cells by an examiner blinded to clinical outcome. Patient samples were categorized as having low (<1%), intermediate (1%–20%), or high (21%–100%) ALDH1A1 expression. The IHC analysis was done on samples collected at primary debulking surgery on 65 untreated patients with stage III–IV, high-grade papillary serous adenocarcinoma; with institutional review board approval, clinical information was collected. Progression-free and overall survival were plotted with the Kaplan–Meier method for patients in each group of ALDH1A1 expression and compared with the log-rank statistic by using PASW 17.0.

For dual staining of ALDH1A1 and CD68 (for macrophages), staining for ALHD1A1 was done first as previously, followed by exposure to anti-CD68 antibody (1:4,000; Dako) and goat anti-mouse-AP (Jackson Immunoresearch). AP was developed with Ferangi Blue chromagen kit (Biocare Medical). For dual staining of ALDH1A1 and hypoxic tumor regions, mice bearing SKOV3TRip2 xenografts were injected with 60 mg/kg of Hypoxyprobe-1 reagent (HPI, Inc.). Tumor sections in FFPE were subjected to antigen retrieval as above, followed by exposure to fluorescein isothiocyanate (FITC)-conjugated anti-hypoxyprobe-1 mouse antibody (1:50) overnight at 4°C. This was detected with HRP-conjugated anti-FITC antibody (1:500, Jackson Immunoresearch) and DAB resolution. Endogenous murine

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IgG was then blocked with anti-mouse IgG F(ab')2 fragments (Jackson ImmunoResearch), and ALDH1A1 stained as above using AP-conjugated anti-mouse IgG and Ferangi Blue chromagen.

ALDEFLUOR assay and tumorigenicity in limiting dilutions

Active ALDH1A1 was identified with the ALDEFLUOR assay according to manufacturer's instructions (StemCell Technologies). The ALDH1A1-positive population was defined by cells with increased FITC signal, with gates determined by diethylaminobenzaldehyde (DEAB)-treated cells (DEAB being an inhibitor of ALDH1A1 activity). For tumorigenicity experiments, the ALDEFLUOR-positive population from A2780cp20 cells were sorted with a FACS Aria II flow cytometer (BD Biosciences) and reanalyzed to confirm at least 95% positivity. Collected cells were washed and resuspended in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS; Gibco) and injected intraperitoneally into NOD-SCID mice in limiting dilutions . Mice were followed for 1 year or until tumors formed, then sacrificed and tumor confirmed histologically. For flow cytometric analysis of these tumors, xenografts were dissociated mechanically with a scalpel, passed through a 70-mm filter to collect single-cell suspensions, with the remaining clumped cells incubated in 0.5 mg/mL of collagenase and 0.0369 mg/mL of hyaluronidase (Calbiochem) for 30 minutes at 37°C. These chemically digested cells were again filtered through a 70 μm filter, added to the initial collection and subjected to the ALDEFLUOR assay. ALDEFLUOR-positive cells or negative cells were then injected into additional mice ($n = 5$) to examine maintenance of tumorigenicity.

Primary xenograft development

With institutional IRB and IACUC approval, excess of freshly collected omental metastases from advanced stage ovarian cancer patients were acquired after tissue required for diagnosis and management had been sequestered. 3 to 4-mm^3 sections were cut and implanted subcutaneously on the dorsal aspect of NOD-SCID mice. Adjacent sections were submitted for histologic analysis to confirm tumor. Tumors were measured in 2 dimensions twice per week. After progressive growth was noted, mice with formed tumors were treated with vehicle or cisplatin (7.5 mg/kg weekly by intraperitoneal administration). Mice were treated for 8 weeks and then sacrificed, and tumors were harvested.

SiRNA downregulation *in vitro*

To examine downregulation of ALDH1A1 with siRNA, cells were exposed to 2.5 μg/mL of control siRNA (target sequence 5′-AATTCTCCGAACGTGTCACGT-3′; Sigma), or 1 of 3 tested ALDH1A1-targeting constructs (SASI_Hs01_00244055, 00244056, or 00303091; Sigma), at a 1:3 siRNA (μg) to Lipofectamine 2000 (μL) ratio. Lipofectamine 2000 and siRNA were incubated for 20 minutes at room temperature, added to cells in serum-free RPMI to incubate for 6 hours, followed by the addition of 15% FBS/RMPI thereafter. Transfected cells were grown at 37°C for 48 to 72 hours and then harvested for Western blot.

Assessment of cell viability with chemotherapy IC_{50} and cell-cycle analysis

To a 96-well plate, 2,000 cells per well were exposed to increasing concentrations of docetaxel or cisplatin in triplicates. Viability was assessed by 2-hour incubation with 0.15% MTT (Sigma) and spectrophotometric analysis at OD_{450} (optical density at 450 nm). For effects of siRNA on IC_{50}, cells were incubated with siRNA for 24 hours in 6-well plates and then replated in 96-well plates, and chemotherapy was administered after 12 hours to allow attachment. IC_{50} was determined by finding the dose at which the drug had 50% of its effect and calculated by the following equation: $IC_{50} = [(OD_{450max} - OD_{450min})/2) + OD_{450min}]$. Test of synergy was according to the Loewe additivity model (27) and calculated by the following equation: combination index (CI) = $[D_1/D_{x1}] + [D_2/D_{x2}]$ (where a CI of 1 suggests an additive effect, <1 suggests synergy, and >1 suggests antagonism). For cell-cycle analysis, cells were transfected with siRNA as described previously for 72 hours, trypsinized, washed in PBS, and fixed in 75% ethanol overnight. Cells were then centrifuged, washed twice in PBS, and reconstituted in PBS with 50 μg/mL of propidium iodide. Propidium iodide fluorescence was assessed by flow cytometry, and percentage of cells in each cycle was calculated by the cell-cycle analysis module for FlowJo.

Orthotopic ovarian cancer model and *in vivo* delivery of siRNA

For orthotopic therapy experiments using ovarian cancer cell lines, female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute and cared for in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care. For all *in vivo* experiments, trypsinized cells were suspended in HBSS and 10^6 cells injected intraperitoneally into 40 mice per experiment. After 1 week, mice were randomized to: a) control siRNA/DOPC, b) control siRNA/DOPC plus chemotherapy, c) ALDH1A1-targeting siRNA/DOPC, or d) chemotherapy plus ALDH1A1-targeting siRNA/DOPC. SiRNA/DOPC dose was 5 μg twice per week in a volume of 100 μL intraperitoneally. Chemotherapy doses were docetaxel 35 μg intraperitoneally weekly for SKOV3TRip2, or cisplatin 160 μg intraperitoneally weekly for A2780cp20. Mice were treated for 4 weeks before sacrifice and tumor collection. SiRNA was incorporated into 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) neutral nanoliposomes as previously described (28), lyophilized, and reconstituted in 0.9% saline for administration.

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Statistical analysis

Comparisons between treatment groups of tumor weight was carried out with the 2-tailed Student's *t* test, if tests of data normality were met. Those represented by alternate distribution were examined by Mann–Whitney *U* statistic. Differences between groups were considered statistically significant at $P < 0.05$. The number of mice per group ($n = 10$) was chosen as directed by a power analysis to detect a 50% decrease in tumor growth with β error of 0.2. Progression-free and overall survival in patients with 3 categories of ALDH1A1 staining were compared by plotting with the Kaplan–Meier method and assessing for statistical differences with the log-rank statistic, using PASW 17.0 software.

Results

Expression profiling of chemoresistant ovarian cancer cell lines

To discover genes mediating taxane resistance, expression profiling of parental SKOV3ip1 and taxane-resistant SKOV3TRip2 cells was done with microarray analysis using the Illumina HumanRef-8 Expression BeadChip. The SKOV3TRip2 cell line was previously generated through progressive exposure to paclitaxel (designated SKOV3TR; 23) and then passaged intraperitoneally in mice for 2 generations to select populations with enhanced tumorigenicity. Similarly, SKOV3ip1 were derived from SKOV3 parental cells to select for cells with enhanced tumorigenicity. We found 34 genes to be upregulated more than 10-fold in SKOV3TRip2 (Fig. 1), among which was ALDH1A1, with a 92.7-fold increase ($P = 0.0025$). Twenty genes were more than 10-fold increased in SKOV3ip1. SKOV3TRip2 cells were confirmed to have approximately 3,000-fold increased resistance to docetaxel, as measured by MTT IC_{50} (62.5 nmol/L vs. 0.02 nmol/L; Fig. 2A).

ALDH1A1 expression in ovarian cancer cell lines

To confirm an increase in ALDH1A1 expression/activity in SKOV3TRip2 and examine expression in other ovarian cancer cell lines, 4 pairs of parental and chemoresistant cell lines were examined: SKOV3ip1/SKOV3-TRip2; HeyA8/HeyA8MDR (multidrug resistant); A2780ip2/A2780cp20 (10-fold increased cisplatin resistance); and IGROV-AF1/IGROV-cp20 (5-fold increased cisplatin resistance). In addition, an immortalized, nontransformed cell line derived from normal ovarian surface epithelium, HIO-180, was examined. We found that expression of total ALDH1A1, as measured by Western blot analysis, was in each case higher in the chemoresistant cell line, with the exception of HeyA8/HeyA8MDR, in which ALDH1A1 was low to absent in both (Fig. 2B). To examine whether ALDH1A1 was not only present but also active, we subjected cells to flow cytometric analysis using the ALDEFLUOR assay. This functional assay predominantly identifies active ALDH1A1 by conversion of a chemical to a fluorochrome. The presence of a subpopulation of ALDH1A1-active cells could be readily identified in SKOV3TRip2 (58% of the total population) and A2780cp20 (2.2%) but not in their parental cell line (Fig. 2C). Furthermore, the strong shift in fluorescent signal in some cells suggests that there was not simply a general increase in expression in all cells but rather separate populations of ALDH1A1-positive and -negative cells. This was confirmed by immunohistochemistry, which showed distinct populations of ALDH1A1-positive or -negative cells in A2780cp20 and SKOV3TRip2 cells but not in the parental A2780ip2 and SKOV3ip1 cells in culture (Fig. 2D). Finally, we observed that this heterogeneous profile was maintained in tumors. After intraperitoneal injection of SKOV3TRip2 cells into nude mice and collection of the resulting orthotopic tumor implants, IHC staining of for ALDH1A1 showed both positive and negative ALDH1A1 subpopulations (Fig. 2E). To examine whether this heterogeneity in expression was due to differential expression in hypoxic regions, a tumor-bearing mouse was injected with hypoxyprobe reagent and sacrificed after 30 minutes. The tumor was costained with ALDH1A1 and antihypoxyprobe antibody. We found that the ALDH1A1-positive cells were not preferentially localized to hypoxic regions in the tumor, with only 1.5% of ALDH1A1-positive cells concurrently positive for hypoxyprobe and only 3.3% of hypoxyprobe-positive cells also positive for ALDH1A1 ($P < 0.01$; Fig. 2F).

ALDH1A1 expression in human ovarian cancer specimens

To determine the pattern of ALDH1A1 expression and possible correlations with chemoresistance in patients, we next examined ALDH1A1 expression in 65 untreated, high-grade papillary serous stage III–IV ovarian cancer patient specimens (patient characteristics in Table 1). We found a wide range of expression patterns (Fig. 3A). There was no ALDH1A1 in tumor cells in 27.1% of samples. ALDH1A1 expression was noted in 1% to 20% of cells in 44% of tumors, representing the largest cohort of expression patterns. As in xenografts from cell lines, expression was typically strong in some cells and negative in others, signifying distinct heterogeneity in the tumor. There was no distinct histologic pattern to the location of the positive cells (such as around vasculature or on the leading edge of the tumor), but positive cells did tend to cluster together. The remaining tumors (28.9%) all had between 21% and 100% staining, with 10% of all patients having strong ALDH1A1 expression in nearly 100% of their tumor cells. To confirm that ALDH1A1 expression was not being mistakenly identified in tumor-infiltrating macrophages, several snap-frozen samples were dual stained for ALDH1A1 and CD68. Although images are not as detailed as those from paraffin-embedded samples, dual staining clearly shows that the majority of macrophages (blue) are ALDH1A1 negative and therefore the heterogeneous ALDH1A1 positivity in tumors is not simply due to detection of macrophage infiltration (Fig. 3B).

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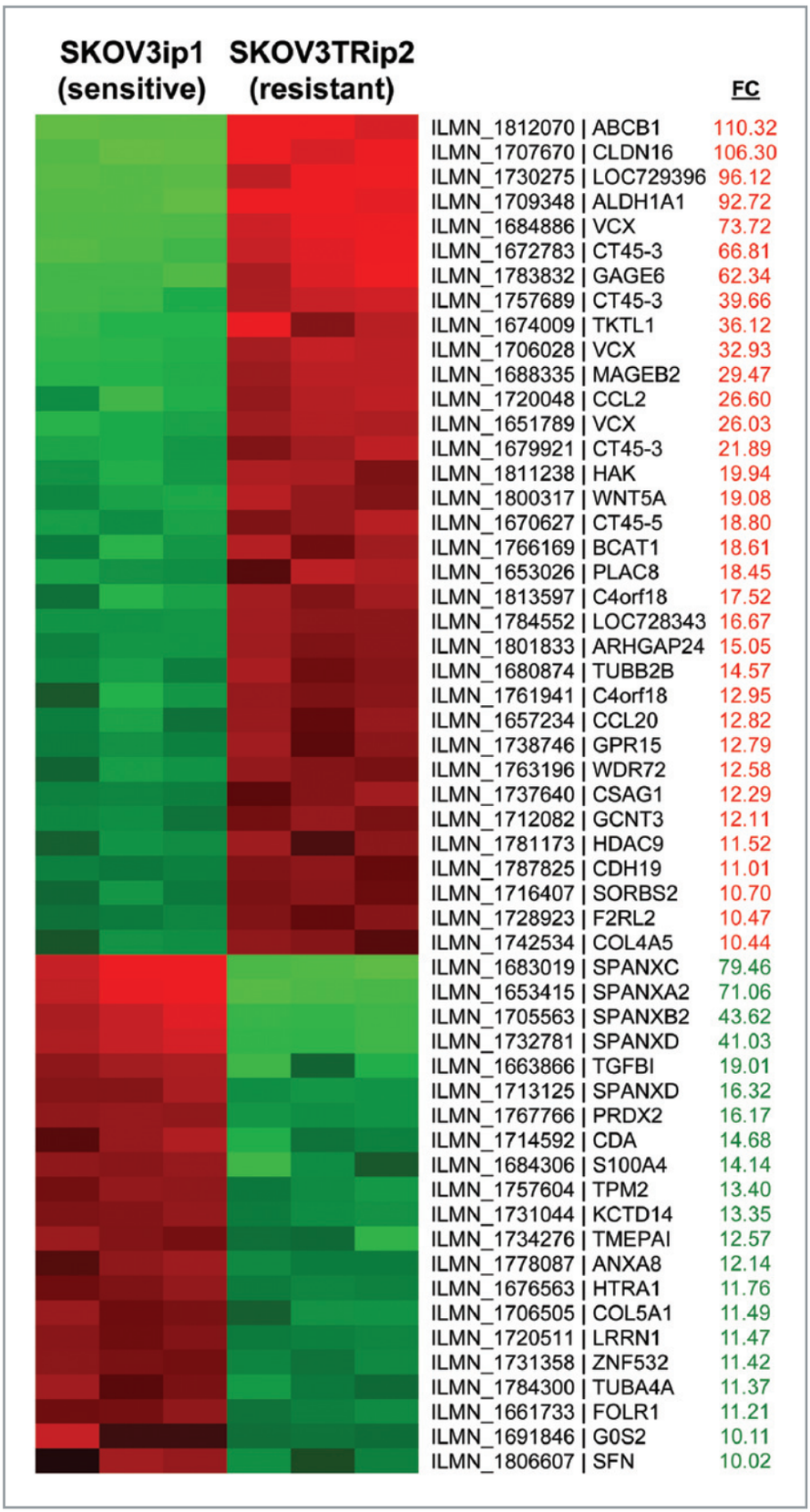

Figure 1. Comparison of whole genome expression profiling between SKOV3TRip2 and SKOV3ip1 cell lines. Total RNA from the SKOV3TRip2 and SKOV3ip1 cell lines were subjected to whole genome expression profiling using the Illumina platform. The genes with a greater than 10-fold increase in SKOV3TRip2 are shown in red, whereas those with a greater than 10-fold increase in SKOV3ip1 are shown in green. FC, fold change.

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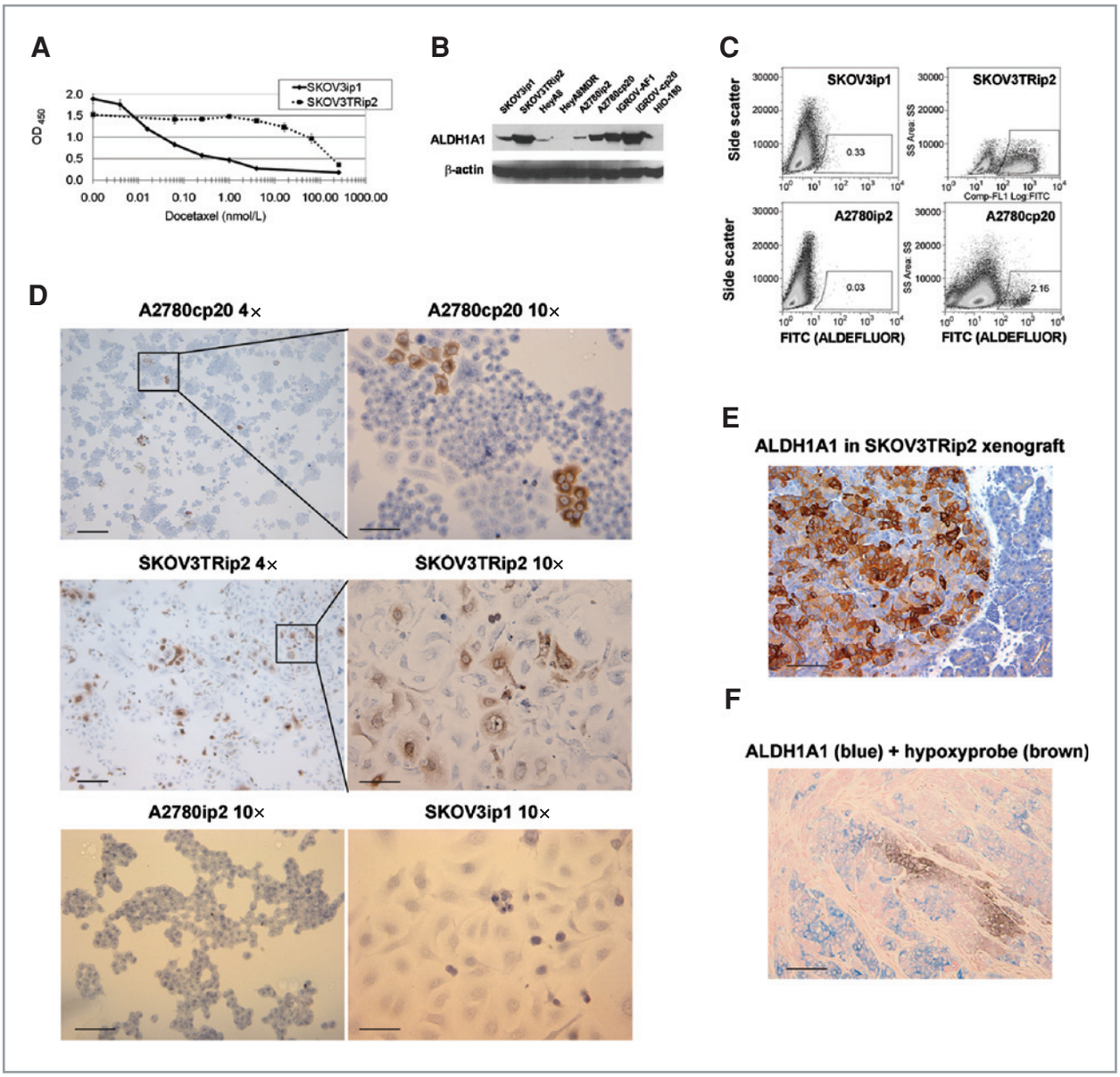

Figure 2. ALDH1A1 expression in ovarian cancer cell lines. A, as measured with the MTT viability assay, the SKOV3TRip2 ovarian cancer cell line has a docetaxel IC_{50} approximately 3,000-fold higher than that of its parental SKOV3ip1 cell line. B, expression of ALDH1A1 by Western blot in 4 pairs of chemosensitive and chemoresistant ovarian cancer cell lines and the nontransformed HIO-180 normal ovarian surface epithelium line. In all cases except HeyA8/HeyA8MDR, in which both lines had minimal expression, the chemoresistant line had increased ALDH1A1 expression. C, as measured by the ALDEFLUOR assay, the A2780cp20 (cisplatin resistant) and SKOV3TRip2 (taxane resistant) also contained a higher percentage of cells with functional ALDH1A1. D, this was confirmed by the IHC analysis for ALDH1A1 on these cell lines *in vitro* in which individual cells appeared either negative or strongly positive, demonstrating heterogeneity of ALDH1A1 expression in the cell line population. A low-power (4×) view gives an appreciation for the distinct colonies of ALDH1A1-positive cells, whereas examination at high power (10×) shows the definitive ALDH1A1-positive or -negative nature of individual A2780cp20 and SKOV3TRip2 cells but an absence of ALDH1A1 in parental A2780ip2 and SKOV3ip1 lines. E, this heterogeneity is also present in tumor xenografts, as seen by the IHC analysis for ALDH1A1 in SKOV3TRip2 tumors grown in mice (intraperitoneal location is confirmed by the presence of normal pancreatic tissue on the right side of the slide). F, ALDH1A1 expression is not limited to hypoxic cells, as shown in xenografts collected from mice given the hypoxyprobe reagent and subjected to the co-IHC analysis for ALDH1A1 (in blue) and the hypoxyprobe by-product (in brown). Scale bars represent 50 μm in 10× views, 100 μm in 4× views (E, F).

Correlation of ALDH1A1 expression with clinical outcomes

To determine whether ALDH1A1 expression correlated with clinical outcomes, we compared progression-free survival and overall survival from patient samples described earlier (and in Table 1) in cohorts with no ALDH1A1 expression, 1% to 20% expression, and greater than 20% expression, as this grouping allowed similar

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Table 1. Characteristics of patients tested for ALHD1A1 expression ($n = 65$)

Characteristic	Percentage or average (range)
Age at diagnosis	62.2 (34–89)
Caucasian race	71%
Pretreated with chemotherapy	0%
Stage	
III	74%
IV	26%
Ca125	3,071 (161–9,600)
Ascites	87%
Optimal debulking	74%
Papillary serous histology	100%
Platinum/taxane primary therapy	96%
Progression-free survival, mo	14.2 (1.7–108)
Overall survival, y	2.5 (0.2–11.8)
ALDH1A1 staining	
Absent	27.1%
1%–20% of cells	44.0%
21%–100% of cells	28.9%

Abbreviation: Ca125, cancer antigen 125

numbers between groups. Patients with greater than 20% ALDH1A1-positive cells had a shorter median progression-free survival (6.1 months) than those with 1% to 20% ALDH1A1-positive cells (8.2 months) or those with no ALDH1A1-positive cells (13.8 months), which was statistically significant according to the log-rank test ($P = 0.035$; Fig. 3C). Overall survival, which reflects resistance to multiple chemotherapeutic agents used in the recurrent setting, showed a trend toward a poor outcome with increasing ALDH1A1 expression (median overall survival 1.09 vs. 1.84 vs. 2.32 years), but the trend was not statistically significant ($P = 0.33$; Supplementary Fig. 1).

Preferential survival of ALDH1A1-positive cells with cisplatin treatment

To determine whether the ALDH1A1-positive cells have preferential survival in the tumor microenvironment with platinum treatment, we established mouse xenografts from primary patient samples by subcutaneously implanting a freshly collected tumor specimen into NOD-SCID mice. A subcutaneous rather than orthotopic model was used so that tumor growth and response could be accurately measured. Once tumors were established and growing, and achieved a size of approximately 1 cm^3, intraperitoneal administration of 7.5 μg/kg of cisplatin weekly was initiated whereas only vehicle was administered to controls (Fig. 3D). When tumors grew to a size of 2 cm^3 in controls, having remained stable with cisplatin treatment, they were harvested and sections stained for ALDH1A1 expression. Baseline expression of ALDH1A1 in the implanted tumor was seen in approximately 1% of cancer cells and similar levels were found in growing xenografts in untreated mice (Fig. 3E). A significant increase in the percentage of ALDH1A1-positive cells was, however, noted in cisplatin-treated xenografts to 38% ($P < 0.001$; Fig. 3E). Consistent with this, the ALDEFLUOR assay on the dissociated tumor showed that 0.6% of cells from untreated tumors were ALDEFLUOR positive whereas 17.6% of cells from cisplatin-treated tumors were ALDEFLUOR positive. Because the treated xenograft in this case did not regress, but rather remained stable in size, cisplatin exposure may have induced ALDH1A1 expression in surviving cells in addition to preferential killing of ALDH1A1-negative cells.

Tumor-initiating capacity of ALDH1A1-positive ovarian cancer cells

In breast and other cancers, the ALDH1A1-active cancer cells have been shown to represent a tumor-initiating population (10–19). To determine whether this were the case in ovarian cancer, we sorted ALDH1A1-positive and -negative populations from the A2780cp20 cell line using the ALDEFLUOR assay and injected cells intraperitoneally into NOD-SCID mice in limiting dilutions to determine tumor-initiating potential. As summarized in Table 2, ALDEFLUOR-positive cells exhibited increased tumorigenic potential, with 100% tumor initiation after the injection of 100,000, 25,000, or 5,000 cells, and 1 tumor was established after the injection of 1,000 cells. ALDEFLUOR-negative cells could form tumors, although at a lower rate: 2 of 5 mice formed tumors after the injection of 25,000 or 100,000 cells and no tumors formed after the injection of 5,000 or 1,000 cells. Mice were followed for 1 year after injection and thorough necropsies were performed in remaining mice to confirm that tumors failed to develop. The TD_{50}, or dose of cells required to permit tumor formation in 50% of animals, was 50-fold lower with ALDEFLUOR-positive cells. Perhaps, more striking was the makeup of these tumors. One requirement of a tumor-initiating population is that it has the capacity to give rise to heterogeneous tumors, composed of both stem cell and non–stem cell populations, therefore

Table 2. Tumorigenicity of ALDEFLUOR-positive and negative cells

A2780cp20 cells injected intraperitoneally	1,000,000	250,000	100,000	25,000	5,000	1,000	Serial transplantation rate
ALDEFLUOR negative	5/5	4/5	2/5	2/5	0/5	0/5	0/5
ALDEFLUOR positive			5/5	5/5	5/5	1/5	5/5

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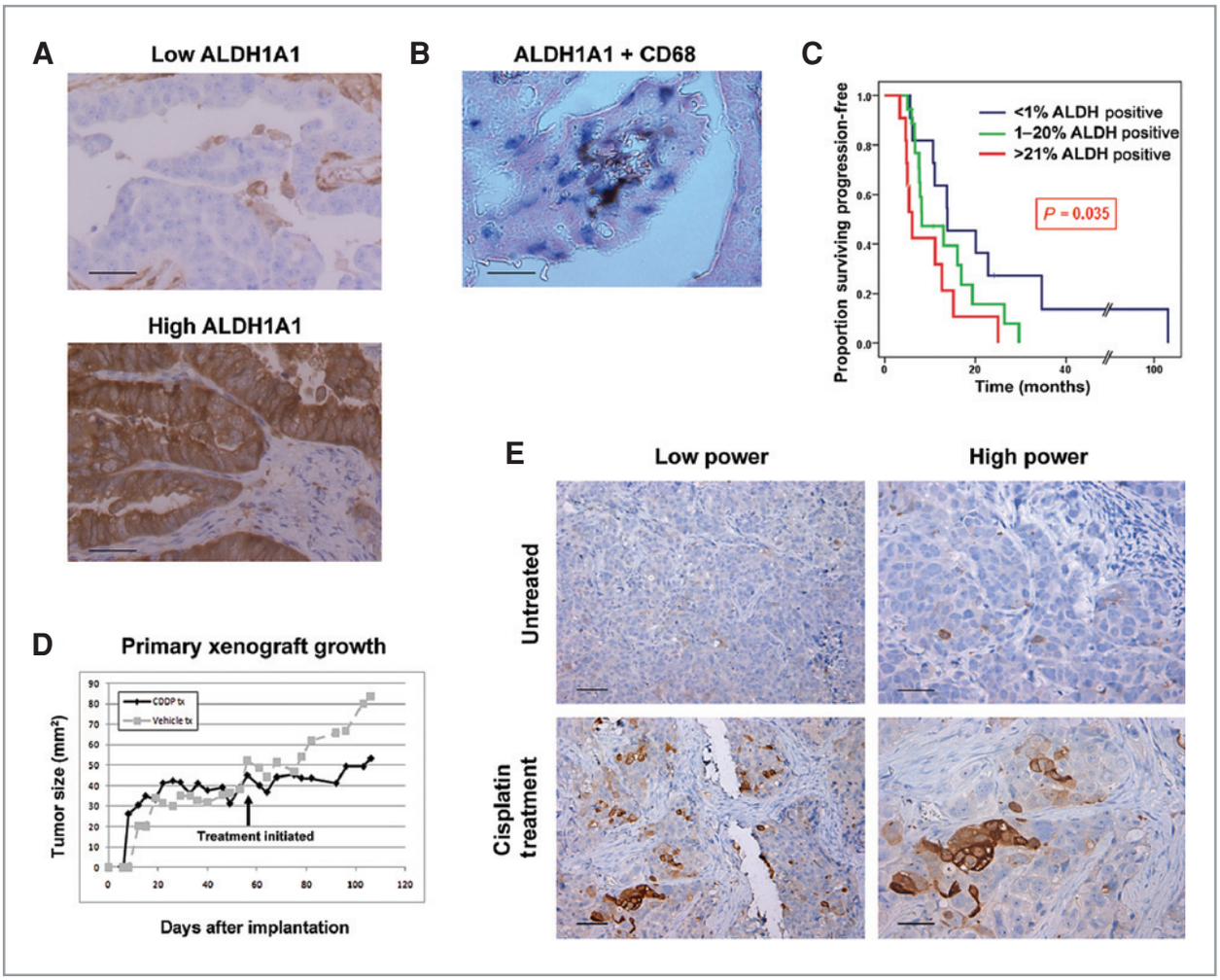

Figure 3. ALDH1A1 expression in ovarian cancer patients. ALDH1A1 was assessed by the IHC analysis in 65 high-grade stage III–IV papillary serous ovarian cancer patients. A, several expression patterns were seen, including absent, spotty (e.g., Low ALDH), and diffuse (High ALDH) staining. Consistent with staining in cell lines, both strongly positive and negative populations were noted. B, to confirm the spotty ALDH1A1 pattern was not identifying infiltrating macrophages, the co-IHC analysis on frozen tissue for ALDH1A1 (brown) and CD68 (a pan-macrophage marker, blue) was done. C, patients were stratified into less than 1%, 1%–20%, and greater than 20% ALDH1A1 expression, and progression-free and overall survival was plotted by the Kaplan–Meier method and tested for statistical significance by the log-rank test. There was a significantly shorter progression-free survival in patients with increasing ALDH1A1 expression. D, mice with established primary subcutaneous xenografts were treated with vehicle or cisplatin for 5 weeks. E, tumors from these mice were harvested and subjected to IHC analysis for ALDH1A1. Tumors treated with cisplatin showed a significant increase in the number of ALDH1A1-positive tumors cells. Magnification at low and high powers is shown. Scale bars represent 50 mm in panels A, B, and High-power images of E, and 100 mm in Low-power images of E.

demonstrating multipotent differentiation potential. This was noted in tumors that formed after the injection of ALDEFLUOR-positive cells. In all 16 of these tumors, a strongly positive ALDH1A1 population was noted in the minority of the sample, on average 4.7% of the tumor (range 2.4%–6.1%; Fig. 4A). However, no ALDEFLUOR-positive cells were found in the tumors that formed after the injection of ALDH1A1-negative cells (Fig. 4B). This was confirmed by the IHC analysis (Fig. 4C and D). This argues against the idea that tumors are formed because of contamination with ALDEFLUOR-positive cells or that ALDH1A1 expression is simply induced by the tumor microenvironment regardless of the capacity of the cells.

This difference in the capacity to generate ALDEFLUOR-positive cells was also noted *in vitro*. SKOV3TRip2 cells sorted into ALDEFLUOR-positive and -negative populations were cultured separately, and the ALDEFLUOR assay was done on the different populations at 24, 48, and 72 hours (Fig. 4E and F). Of the ALDEFLUOR-positive cells, the population gradually reverted to 75.3%, 54.2%, and 51.4% ALDEFLUOR-positive cells, respectively, for each time point. However, the ALDEFLUOR-negative cells could not produce any ALDEFLUOR-positive cells.

To confirm that the ALDEFLUOR-positive cells from tumors maintained tumorigenicity, these populations

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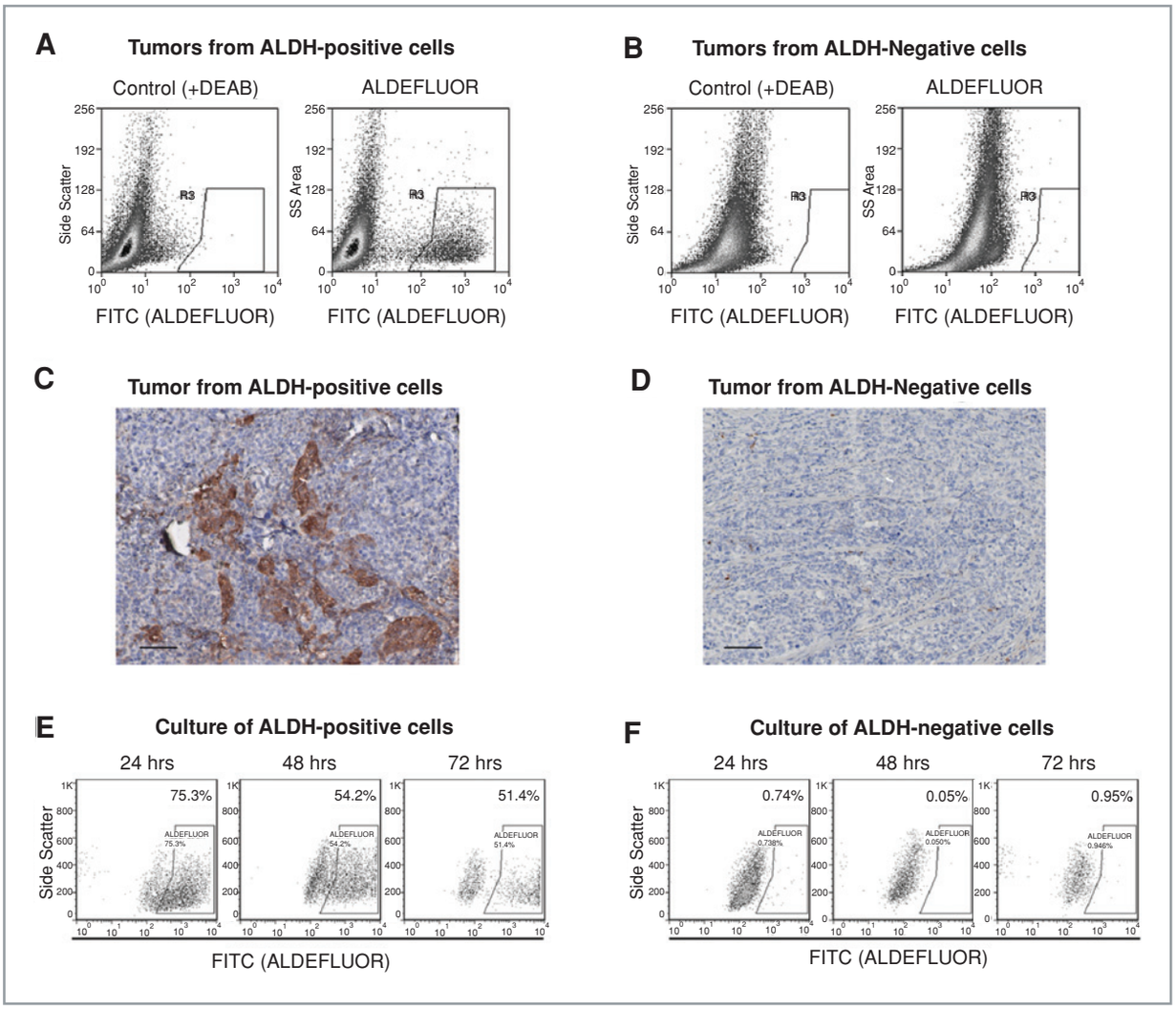

Figure 4. ALDH1A1 populations in A2780cp20 xenografts. Intraperitoneal tumors that developed after the injection of ALDEFLUOR-positive or -negative A2780cp20 cells were assessed for ALDH1A1 composition. A, tumors that formed after the injection of purely ALDEFLUOR-positive cells showed both ALDEFLUOR-positive and -negative populations and recapitulated the tumor-initiating cells phenotype of having a small (2.4%–6.1%) percentage of ALDEFLUOR-positive cells. B, interestingly, tumors that formed after the injection of purely ALDEFLUOR-negative cells contained only ALDEFLUOR-negative cells, showing an absence of capacity for differentiation, at least in terms of ALDEFLUOR positivity. C and D, this expression discrepancy was also noted on the immunohistochemical analysis for ALDH1A1 from these samples. Scale bars represent 100 μm. Similarly, *in vitro*, SKOV3TRip2 ALDEFLUOR-positive cells give rise to both ALDEFLUOR-positive and -negative cells, (E) reestablishing baseline levels at 48 hours, whereas ALDEFLUOR-negative cells cannot give rise to ALDEFLUOR-positive cells (F).

were sorted and reinjected intraperitoneally into mice and continued to form tumors at 100% rate with 25,000 cells injected. However, ALDEFLUOR-negative cells from the tumors forming after ALDEFLUOR-negative cells were injected did not form tumors. Taken together, these studies show that ALDEFLUOR-positive cells have increased but not absolute tumorigenicity, but they do have a differentiation capacity and maintenance of the tumorigenic phenotype that is absent in ALDEFLUOR-negative cells.

In an effort to determine whether ALDEFLUOR-positive cells, freshly collected from ovarian cancer patients, have similar tumorigenicity, we have sorted ALDEFLUOR-positive and -negative cells from 5 separate ovarian cancer patients, dissociating tumors metastatic to the omentum at the time of primary debulking surgery. In this cohort, 1.5% to 17.8% of cells were ALDEFLUOR positive. A total of 25,000 ALDEFLUOR-positive cells, 100,000 ALDEFLUOR-negative cells, or 100,000 unsorted cells were injected intraperitoneally into 5 mice per group per patient. Unfortunately, no tumors formed in any mice, highlighting the difficulty of tumorigenicity studies in primary ovarian cancer samples dissociated to single cell suspensions.

To preliminarily determine whether there is an overlap between the ALDEFLUOR-positive population and other

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markers of putative stem cells in ovarian cancer, these 5 samples were also profiled for CD44, c-kit, and CD133. We were not able to identify a convincing positive c-kit population from any sample. CD133-positive cells made up an average of 3.1% of total tumor cells (range, 0.6%–5.7%) and were greater than 80% of ALDEFLUOR positive in all 5 samples (mean, 86.7%; range, 81.5%–100%). CD44 was more commonly expressed, representing an average of 45.7% of tumors (but with a very broad range of 2.4%–98.2%). Of the CD44-positive cells, 75.4% were also ALDEFLUOR positive (range, 46.6%–88.8%). Similarly, the SKOV3TRip2 line has 82% CD44-positive cells, and of these, 74% were ALDEFLUOR positive. Although a great number of samples will need to be examined to fully delineate whether multiple marker–positive cells can more accurately define the most pure tumorigenic cell, there is certainly overlap in marker expression. There are both double-positive CD44/ALDEFLUOR and CD133/ALDEFLUOR-positive populations that may prove more discerning as cancer stem cell populations, and ongoing studies could assess this distinction. Interestingly, the A2780cp20 cell line is completely negative for CD44 and the HeyA8 cell line is negative for ALDH1A1/ALDEFLUOR, despite the fact that both are highly tumorigenic. This highlights the fact that these cannot be the sole mediators of tumorigenicity in mice.

Downregulation of ALDH1A1 sensitizes ovarian cancer cells to chemotherapy

Given the association of ALDH1A1 expression with chemoresistant cell lines and a decreased progression-free survival in ovarian cancer patients, we asked whether downregulation of ALDH1A1 could sensitize resistant cells to chemotherapy. Two different siRNA constructs were identified that reduced ALDH1A1 expression by greater than 80% (Fig. 5A). Reduction in the ALDEFLUOR population was confirmed (Fig. 5B). SKOV3TRip2 or A2780cp20 cells were exposed to ALDH1A1-targeting siRNA (ALDH1A1 siRNA) or control siRNA for 24 hours before replating and adding increasing concentrations of docetaxel or cisplatin, respectively. Cell viability 4 days after the addition of chemotherapy was assessed with the MTT assay. In SKOV3TRip2 cells, siRNA-ALDH1A1 alone reduced viability by 49% (Fig. 5C; $P < 0.001$). Downregulation of ALDH1A1 also reduced the docetaxel IC_{50} from 178 to 82 nmol/L. In A2780cp20, the effects of ALDH1A1 downregulation alone were modest (Fig. 5D; reduced viability by 15.9%, $P = 0.040$) but sensitization to cisplatin was considerable, with a decrease in the IC_{50} from 5.1 to 2.0 μmol/L. Tests for synergy suggest moderate synergy in each cell line (CI = 0.82 for SKOV3TRip2 and 0.75 for A2780cp20). The contrasting effects of ALDH1A1-siRNA alone are consistent with the number of ALDH1A1-active cells in these cell lines, with SKOV3TRip2 cell lines having 50% to 60% of ALDEFLUOR-positive cells and A2780cp20 having just 2% of 3%. To determine how ALDH1A1 downregulation alone may affect cell growth, cell-cycle analysis was done in a separate experiment. We found that ALDH1A1 downregulation induced an accumulation of SKOV3TRip2 cells in S and G_2 phases ($P < 0.001$; compared with control siRNA) but had only minimal effects on the cell cycle of A2780cp20 cells (Fig. 5E).

There are no known inhibitors of ALDH1A1 for *in vivo* studies. Therefore, we used a method for delivery of siRNA *in vivo*, using DOPC nanoparticles. We and others (28–32) have previously shown delivery of siRNA incorporated into DOPC nanoliposomes to the tumor parenchyma with subsequent target downregulation. In this study, nude mice were injected intraperitoneally with either SKOV3TRip2 or A2780cp20 cells and randomized to 4 treatment groups to begin 1 week after cell injection: a) control siRNA in DOPC, delivered intraperitoneally twice per week; b) docetaxel 35 mg, delivered intraperitoneally weekly (for SKOV3TRip2 model) or cisplatin 160 μg, delivered intraperitoneally weekly (for A2780cp20 model); c) ALDH1A1-siRNA in DOPC, intraperitoneally twice per week; or d) ALDH1A1-siRNA in DOPC plus docetaxel (for SKOV3TRip2) or cisplatin (for A2780cp20). After 4 weeks of treatment, mice were sacrificed and total tumor weight recorded. The IHC analysis confirmed reduced ALDH1A1 expression with ALDH1A1-siRNA/DOPC treatment compared with controls but not with chemotherapy alone (Supplementary Fig. 2; too little tissue was available to examine with the ALDEFLUOR assay). In SKOV3TRip2 xenografts (Fig. 5F), there was a nonsignificant reduction of 37.0% in tumor growth with docetaxel treatment ($P = 0.17$) and of 25.0% with ALDH1A1 siRNA treatment ($P = 0.38$) compared with control siRNA/DOPC. The observation that ALDH1A1 downregulation alone significantly decreased SKOV3TRip2 growth *in vitro* but was less pronounced *in vivo* suggests that tumor microenvironment factors such as supporting stromal cells may be able to protect cells from ALDH1A1 depletion. However, the combination of ALDH1A1 siRNA and docetaxel resulted in significantly reduced growth by 93.6% compared with control siRNA ($P < 0.001$), by 89.8% compared with docetaxel plus control siRNA ($P = 0.003$), and by 91.4% compared with ALDH1A1 siRNA ($P = 0.002$). In A2780cp20 (Fig. 5G), there was a similar nonsignificant reduction of 43.9% in tumor weight with cisplatin alone ($P = 0.32$) and of 57.0%with ALDH1A1 siRNA treatment ($P = 0.19$). These effects may be even less significant than the mean tumor weights suggest, given the presence of 2 especially large tumors in the control siRNA group. However, again combined therapy showed a sensitization to chemotherapy with ALDH1A1 siRNA, with combination therapy reducing growth by 85.0% compared with control siRNA ($P = 0.048$), by 73.4% compared with cisplatin plus control siRNA ($P = 0.013$), and by 65.3% compared with ALDH1A1 siRNA alone ($P = 0.039$). Given the minimal effects of each single agent and the consistent finding of significant improvement with combined therapy, these data suggest a synergy between

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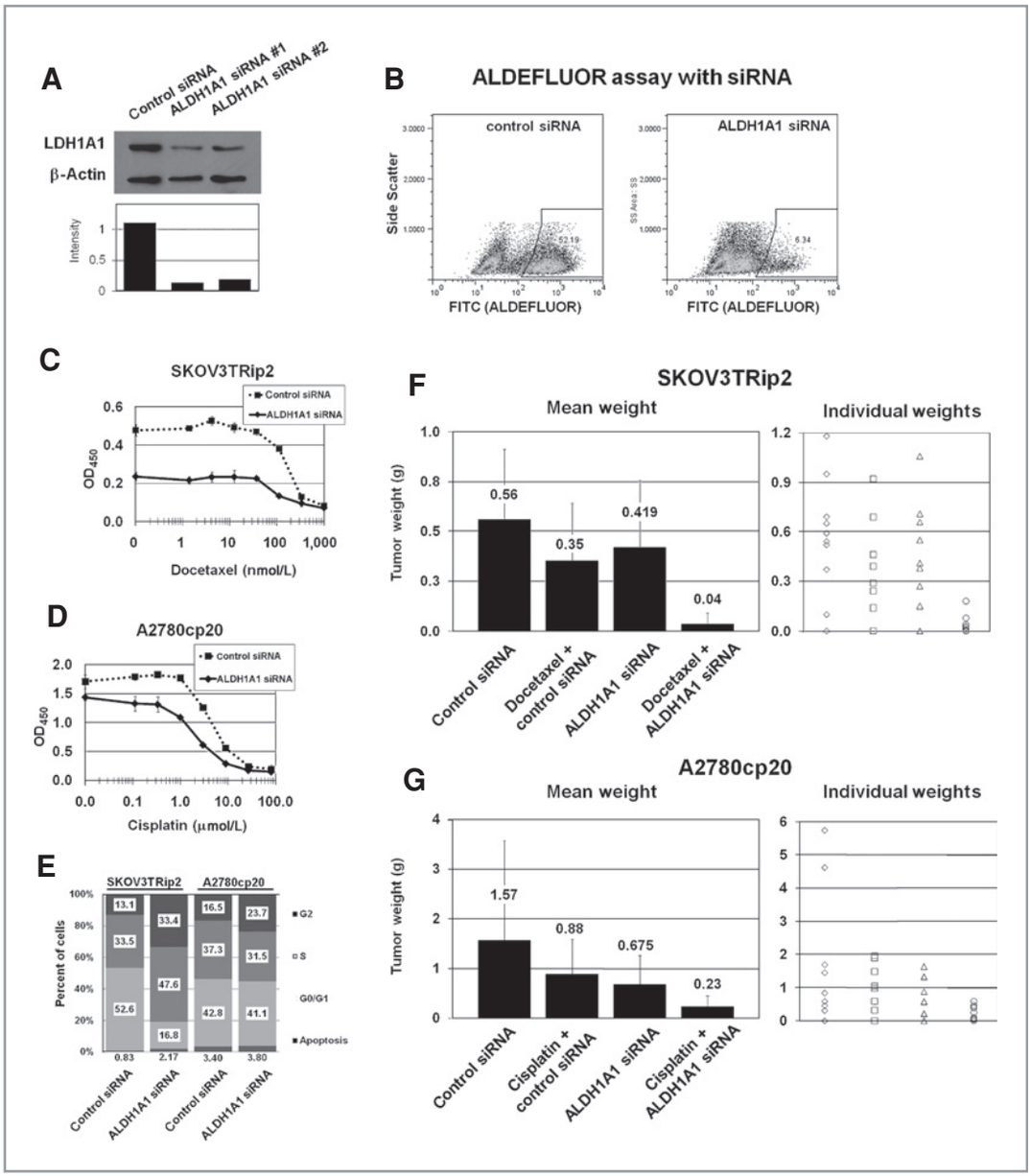

Figure 5. Efficacy of ALDH1A1 downregulation with siRNA *in vitro* and *in vivo*. Identification of siRNA constructs that decrease ALDH1A1 expression was confirmed by Western blotting (A) and by flow cytometry (B) using the ALDEFLUOR assay in the SKOV3TRip2 cell line. C, downregulation of ALDH1A1 with siRNA 48 hours prior to the treatment of SKOV3TRip2 cells with increasing doses of docetaxel showed a sensitization effect, decreasing IC_{50} from 178 to 82 nmol/L. siRNA alone also showed an effect, with decreased viability by 49%. D, in the A2780cp20 cell line, downregulation of ALDH1A1 alone had minimal effect but sensitized cells to cisplatin, decreasing IC_{50} from 5.1 to 2.0 μmol/L. E, cell-cycle analysis shows that ALDH1A1 downregulation induces accumulation of cells in S and G_2 phases in SKOV3TRip2, with little effect on A2780cp20. F, *in vivo*, mice injected intraperitoneally with SKOV3TRip2 cells were treated with ALDH1A1-siRNA incorporated in DOPC nanoparticles, docetaxel/control siRNA in DOPC, or the combination and compared with mice treated with control siRNA in DOPC. Mice treated with either of the single agents had minimal effect, but the combination showed a significant reduction compared with treatment with control siRNA (94% reduction in tumor growth; $P < 0.001$) or either of the single agents (90%–91% reduction; $P < 0.005$). G, similarly, mice injected with A2780cp20 cells showed a minimal, nonsignificant reduction in growth with cisplatin or ALDH1A1-siRNA in DOPC, but combination therapy was statistically superior to either of the single agents (65%–73% reduction; $P < 0.04$) or control siRNA (85% reduction; $P = 0.048$). Mean tumor weight and individual tumor sizes are presented.

ALDH1A1 downregulation and both taxane and platinum chemotherapeutic agents, though formal dose-finding experiments would be required to definitively prove synergy.

Discussion

We have found that ALDH1A1 expression and activity are increased in chemoresistant ovarian cancer cell lines

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and in *in situ* primary ovarian cancer xenografts treated with cisplatin. Expression of ALDH1A1 is frequent in ovarian tumors, and patients with low ALDH1A1 expression levels have a more favorable outcome than those with more ALDH1A1-positive cells. ALDEFLUOR-positive cells have increased (but not absolute) tumorigenicity compared with ALDEFLUOR-negative cells and have a differentiating capacity that is not present in the ALDEFLUOR-negative population. Most important, downregulation of ALDH1A1 expression sensitized normally chemoresistant tumors to both docetaxel and cisplatin both *in vitro* and in an orthotopic mouse model of ovarian cancer.

The search for tumor-initiating cells in ovarian cancer has resulted in observations that the $CD44^+/c\text{-}kit^+$ population has an approximately 5,000-fold increase in tumorigenicity, with tumors forming after the injection of as few as 100 cells from primary tumor, xenograft, or spheroid heterogeneous populations (5), and that the $CD133^+$ population has approximately 20-fold increased tumorigenicity, with tumor formation with as few as 100 to 500 cells from murine xenografts, and tumor formation 4 times faster with $CD133^+$ cells (7). Furthermore, the increased tumorigenicity of $CD133^+$ cells can be inhibited by interfering with binding between CD44 and its ligand hyaluronic acid (6). Other investigators have found equal rates of tumor formation among $CD133^+$ and $CD133^-$ cells from the A2780 cell line, but a faster growth rate in $CD133^+$ cells (8). The side population (SP) cells from the MOVCAR cell line also formed tumors more frequently and appeared 3 to 4 weeks sooner than tumors derived from non-SP cells (9). In all of these studies, as in ours, the tumors resulting from the putative tumor-initiating cell population contained both tumor-initiating cell and non-tumor-initiating cell populations, demonstrating multipotentiality. Interestingly, we have seen that cells comprising tumors formed from ALDH1A1-negative cells lack the capacity to generate ALDH1A1-positive cells and do not continue to propagate tumors over multiple generations, suggesting that their multipotentiality is limited. This lack of differentiating capacity has also been noted in ALDEFLUOR-negative cells from breast cancer cell lines (33).

The most appropriate source of tumor cells for tumorigenicity experiments is of some debate. Although it is desirable to use samples freshly collected from primary tumors, sorting these samples and establishing primary xenografts have proven problematic. Ovarian cancer xenografts and cells lines have traditionally been challenging to establish from primary samples. All previously reported studies of ovarian tumor-initiating cells have used selected cells of some sort, either from xenografts of varying generations or from cells grown in differentiation-inhibiting media (to form tumor spheres), to serve as a compromise between freshly collected specimens and cell lines. However, those cells that form tumors in mice even in the first generation almost certainly represent some select portion of the original tumor. That these xenografts still contain only a small percentage of tumor-initiating cells speaks either to the appropriateness of this approach or to the testament that the tumor-forming cells are multipotent, give rise to tumor-initiating cell-negative populations, and remain relatively rare. Use of cell lines is often discouraged because of their homogenous nature. But clearly, even within cell lines, there is heterogeneity in ALDH1A1 expression, as shown by the detection of distinct populations by flow cytometric and IHC analyses (Fig. 2). Distinct ALDEFLUOR-positive and -negative populations have also been found in several breast cancer cell lines, with ALDEFLUOR-positive cells having increased tumorigenicity and differing molecular signatures (33). Therefore, our finding that the ALDEFLUOR-positive population in cell lines has increased tumorigenicity may reflect the more aggressive phenotype of ALDH1A1-active cells but does not represent proof that this population is important to *in situ* ovarian cancers. Evidence that patients with increasing ALDH1A1 expression have poor outcomes suggests this association, but additional tumorigenicity experiments from freshly collected tumors would more appropriately define the ALDEFLUOR population as clinically significant tumor-initiating cells.

The importance of tumorigenicity in defining cancer stem cells has also been debated. Although tumor formation with 100 to 500 ALDEFLUOR-positive cells and a lack of tumor formation with the injection of 10^5 ALDEFLUOR-negative cells definitely reflect an aggressive phenotype, the biologic processes required for xenograft formation-–survival under stressful experimental conditions, adhesion, time to proliferation, and variations in host immunocompetence-–may not reflect the true population that cancer stem cell research seeks to identify. Our ultimate goal should be to identify the subpopulations in parent tumors that survive chemotherapy and therefore are more likely to cause recurrence. Stem cells that survive chemotherapy should exhibit chemoresistance to be clinically relevant. In breast cancer, for example, the $CD44^+/CD24^-$ population is highly tumorigenic. However, Tanei et al., who studied tissue obtained before and after neoadjuvant chemotherapy, found that despite a positive response to treatment, the proportion of $CD44^+/CD24^-$-negative cells was unchanged. In these samples, however, the ALDH1A1-positive population was significantly increased (34).

ALDH1A1 has previously been proposed to play a role in chemoresistance, having been noted to be higher in proteomic profiling of IGROV platinum-resistant ovarian cancer cells (35), in genomic profiling of multidrug-resistant gastric carcinoma (36), and in cells resistant to cyclophosphamide (37, 38), oxazaphosphorines (39), and now docetaxel and cisplatin. ALDH1A1 oxidizes many intracellular aldehydes into carboxylic acids (40), detoxifying many of the free oxygen radicals generated by chemotherapeutic agents. It stands to reason that a stem cell population should be resistant to multiple chemotherapeutic

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agents rather than being specific to one class. This also follows clinically, in that most ovarian cancer patients who develop resistance to platinum agents have resistance to multiple agents (2). ALDH1A1 has been shown to be associated with BRCA1 in breast cancer, in that knockdown of BRCA1 increases the ALDEFLUOR population and ALDEFLUOR-positive cells preferentially contain BRCA1 loss of heterozygosity (41). These findings could also be important to BRCA-mediated ovarian cancer. Despite this body of evidence for the importance of ALDH1A1, it is not fully understood whether any of the additional ALDH1 isoforms are important to stem cell biology. In our study, ALDH1A1 can be specifically identified with isotype-specific antibodies (as used for the IHC analysis and Western blotting). However, the more important and consistently used identifier of a stem cell population is the ALDEFLUOR assay, which, although primarily dependent on ALDH1A1, may also identify ALDH1A2 and ALDH1A3 isotypes [(42) and unpublished data by Stem Cell Technologies]. As a therapeutic agent, we have seen positive effects by targeting ALDH1A1 with siRNA, but to maximize the efficacy of therapeutics, the contribution of these additional isotypes will need to be defined with additional studies.

Although our finding of a poor outcome in patients with high ALDH1A1 expression agrees with similar investigations in breast cancer (12, 13) and ovarian cancer (20), one interesting report found that a high ALDH1A1 expression level actually confers a positive prognosis in ovarian cancer (43). This cohort also contained patients with absent, scattered, and diffuse staining. However, this cohort included patients with stage I and II disease and low-grade tumors, and ALDH1A1 expression was higher in these patients [confirming findings from a previous report (44)]. Furthermore, with multivariate analysis, only stage correlated with survival; ALDH1A1 expression no longer predicted outcomes. In ovarian cancer, there is a well-recognized dichotomy in carcinogenesis and pathobiology (45), whereby low-grade tumors (which are more often diagnosed at stage I or II) are paradoxically more chemoresistant but have prolonged survival due to slow growth. Given these collective data, and the several mechanisms by which ALDH1A1 has been shown to contribute to chemoresistance, it may be that ALDH1A1 is more frequently expressed in low-grade tumors but participates in chemoresistance to both high-grade and low-grade subtypes.

We have shown that the ALDH1A1-positive population has properties of cancer stem cells, is associated with taxane and platinum resistance, and can be resensitized to chemotherapy with downregulation of ALDH1A1 *in vitro* and *in vivo*. Therefore, ALDH1A1 is not just a marker of an aggressive population but also a mediator of the phenotype and a viable target for therapy. As better models are developed to more purely define the true chemoresistant population in *de novo* patient tumors, the ALDH1A1 population, either alone or in combination with other markers and mediators of resistance, may represent a population that must be targeted to achieve increased response rates and survival in ovarian cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Therapy: Clinical

Clinical Cancer Research

Stem Cell Pathways Contribute to Clinical Chemoresistance in Ovarian Cancer

Adam D. Steg[1], Kerri S. Bevis[1], Ashwini A. Katre[1], Angela Ziebarth[1], Zachary C. Dobbin[1], Ronald D. Alvarez[1], Kui Zhang[2], Michael Conner[3], and Charles N. Landen[1]

Abstract

Purpose: Within heterogeneous tumors, subpopulations often labeled cancer stem cells (CSC) have been identified that have enhanced tumorigenicity and chemoresistance in *ex vivo* models. However, whether these populations are more capable of surviving chemotherapy in *de novo* tumors is unknown.

Experimental Design: We examined 45 matched primary/recurrent tumor pairs of high-grade ovarian adenocarcinomas for expression of CSC markers ALDH1A1, CD44, and CD133 using immunohistochemistry. Tumors collected immediately after completion of primary therapy were then laser capture microdissected and subjected to a quantitative PCR array examining stem cell biology pathways (Hedgehog, Notch, TGF-β, and Wnt). Select genes of interest were validated as important targets using siRNA-mediated downregulation.

Results: Primary samples were composed of low densities of ALDH1A1, CD44, and CD133. Tumors collected immediately after primary therapy were more densely composed of each marker, whereas samples collected at first recurrence, before initiating secondary therapy, were composed of similar percentages of each marker as their primary tumor. In tumors collected from recurrent platinum-resistant patients, only CD133 was significantly increased. Of stem cell pathway members examined, 14% were significantly overexpressed in recurrent compared with matched primary tumors. Knockdown of genes of interest, including endoglin/CD105 and the hedgehog mediators Gli1 and Gli2, led to decreased ovarian cancer cell viability, with Gli2 showing a novel contribution to cisplatin resistance.

Conclusions: These data indicate that ovarian tumors are enriched with CSCs and stem cell pathway mediators, especially at the completion of primary therapy. This suggests that stem cell subpopulations contribute to tumor chemoresistance and ultimately recurrent disease. *Clin Cancer Res; 18(3); 869–81. ©2011 AACR.*

Introduction

Ovarian cancer is the leading cause of death from a gynecologic malignancy. Although ovarian cancer is among the most chemosensitive malignancies at the time of initial treatment (surgery and taxane/platinum-based chemotherapy), most patients will ultimately develop tumor recurrence and succumb to chemoresistant disease (1). Evaluation of multiple chemotherapy agents in several combinations in the last 20 years has yielded modest improvements in progression-free survival but no increases in durable cures. This clinical course suggests that a population of tumor cells has either inherent or acquired resistance to chemotherapy that allows survival with initial therapy and ultimately leads to recurrence. Targeting the cellular pathways involved in this resistance may provide new treatment modalities for ovarian cancer.

In several hematologic and solid tumors, subpopulations of cells termed cancer stem cells (CSC) or tumor-initiating cells (TIC) have been identified as representing the most tumorigenic and treatment-resistant cells within a heterogeneous tumor mass. Usually defined by their enhanced ability to generate murine xenografts and give rise to heterogeneous tumors that are composed of both CSC and non-CSC populations, these cells may also be more chemoresistant and depend on unique biologic processes compared with the majority of tumor cells (2, 3). In ovarian cancer, many of these properties have been identified in populations of CD44-positive cells (4, 5), CD133-positive cells (6–8), Hoechst-excluding cells (the side population; ref. 9), and aldehyde dehydrogenase (ALDH1A1)-positive cells (10–13) and are associated with poor clinical

Authors' Affiliations: Departments of [1]Obstetrics and Gynecology, [2]Biostatistics, and [3]Pathology, University of Alabama at Birmingham, Birmingham, Alabama

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Corresponding Author: Charles N. Landen, Department of Obstetrics and Gynecology, The University of Alabama at Birmingham, 1825 University Boulevard, 505 Shelby Building, Birmingham, AL 35294. Phone: 205-934-0473; Fax: 205-934-0474; E-mail: clanden@uab.edu

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Translational Relevance

Most patients with ovarian cancer will have an excellent response to initial surgical debulking and chemotherapy, but about 75% of patients will later recur and succumb to disease. Primarily on the basis of *ex vivo* models, subpopulations of cancer cells, often described as cancer stem cells, have been hypothesized to represent the most tumorigenic and treatment-resistant cells within a heterogeneous tumor mass. Using a unique cohort of matched primary/recurrent ovarian tumors, we have shown that the expression of putative cancer stem cell markers ALDH1A1, CD44, and CD133 and several additional mediators of stem cell pathways are upregulated in recurrent, chemoresistant disease compared with primary tumor. Further development revealed novel mechanisms of the TGF-β coreceptor endoglin (CD105) and the Gli2 hedgehog transcription factor in platinum resistance. Our findings highlight the importance of stem cell pathways in ovarian cancer recurrence and chemoresistance and show that therapies targeting these pathways may reverse platinum resistance in ovarian cancer.

outcomes. It is acknowledged that these markers are not identifiers of pure populations with all capabilities of conventional stem cells but rather enrich for a population with some stem cell properties.

Whether or not these populations actually have preferential survival in *de novo* tumors and thus contribute to recurrent disease is not known. An increased density of these populations in recurrent or chemoresistant tumors would suggest their importance to the clinical course of ovarian cancer and suggest that these populations would have to be targeted to achieve durable cures. In the current study, we used a unique cohort of matched primary/recurrent ovarian cancer specimens to determine whether putative CSC subpopulations comprise a larger percentage of recurrent tumors and to examine other known mediators of stem cell biology that might correlate with contributors to recurrence. In addition, novel genes were revealed to be highly expressed in recurrent samples, specifically endoglin (CD105) and the Hedgehog mediator Gli2, and were targeted in validation studies to confirm that stem cell pathway members represent novel therapeutic targets in ovarian cancer.

Methods

Immunohistochemical staining and clinical correlations

Immunohistochemical (IHC) analysis was conducted using standard techniques (14) on samples collected from matched primary and recurrent tumors taken from 45 patients with ovarian adenocarcinoma, and with Institutional Review Board approval, clinical information was collected. Pathology was confirmed and formalin-fixed, paraffin-embedded (FFPE) slides were cut at 5 or 10 μm. Antigen retrieval was carried out in citrate buffer (pH 6.0) for 45 minutes in an atmospheric pressure steamer. Slides were then stained using antibodies against ALDH1A1 (Clone 44; BD Biosciences), CD44 (Clone 2F10; R&D Systems), or CD133 (Clone C24B9; Cell Signaling Technology) at 1:500 dilution in Cyto-Q reagent (Innovex Biosciences) overnight at 4°C. Primary antibody detection was achieved with Mach 4 HRP polymer (Biocare Medical) for 20 minutes at room temperature, followed by 3,3′-diaminobenzidine (DAB) incubation. After IHC staining, the number of tumor cells positive for ALDH1A1, CD44, or CD133 were counted by two independent examiners (and a third if there was >20% discrepancy) blinded to the setting in which the tumor was collected (primary or recurrent) and expressed as a percentage of all tumor cells. To be consistent with prior identification of putative CSCs identified through surface expression with flow cytometry, in the case of CD44 and CD133, only strong expression at the surface membrane was considered positive. Intensity was not scored separately, staining was considered only positive or negative, with the primary endpoint percentage of positive tumor cells across the entire slide. The average number of positive cells for each marker among the 45 primary samples was compared with the average among recurrent samples, with additional subgroup analyses conducted as described in the Results section. A subgroup analysis of IHC staining using an antibody against endoglin (Sigma) was also conducted.

Laser capture microdissection

Ten-micrometer thick FFPE sections were prepared from 12 matched pairs of samples from patients with ovarian adenocarcinoma, in whom the recurrent tumors had been collected within 3 months of completion of primary therapy. Sections were rapidly stained with hematoxylin and eosin. Three to five thousand tumor epithelial cells were microdissected from each sample using a PixCell II Laser Capture Microdissection system (Arcturus Engineering). Care was taken to ensure that no stromal cells were collected (see Supplementary Fig. S1). RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems) optimized for FFPE samples.

RT^2 profiler PCR array

RNA extracted from microdissected samples was converted to cDNA and amplified using the RT^2 FFPE PreAMP cDNA Synthesis Kit (SABiosciences). Quality of cDNA was confirmed with the Human RT^2 RNA QC PCR Array (SABiosciences), which tests for RNA integrity, inhibitors of reverse transcription and PCR amplification, and genomic and general DNA contamination (15). Gene expression was then analyzed in these samples using the Human Stem Cell Signaling RT^2 Profiler PCR Array (SABiosciences), which profiles the expression of 84 genes involved in pluripotent cell maintenance and differentiation (16). Functional gene groupings consist of the Hedgehog, Notch,

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TGF-β, and Wnt signaling pathways. PCR amplification was conducted on an ABI Prism 7900HT sequence detection system, and gene expression was calculated using the comparative C_T method as previously described (17).

Cell lines and culture

The ovarian cancer cell lines A2780ip2, A2780cp20, ES2, HeyA8, HeyA8MDR, IGROV-AF1, OvCar-3, and SKOV3ip1 (18–27) were maintained in RPMI-1640 medium supplemented with 10% FBS (Hyclone). All cell lines were routinely screened for *Mycoplasma* species (GenProbe detection kit; Fisher) with experiments carried out at 70 to 80% confluent cultures. Purity of cell lines was confirmed with short tandem repeat genomic analysis, and only cells less than 20 passages from stocks were used in experiments.

RNA extraction from cell lines

Total RNA was isolated from ovarian cancer cell lines using TRIzol reagent (Invitrogen) per manufacturer's instructions. RNA was then DNase treated and purified using the RNEasy Mini Kit (QIAGEN). RNA was eluted in 50 μL of RNase-free water and stored at −80°C. The concentration of all RNA samples was quantified by spectrophotometric absorbance at 260/280 nm using an Epoch microplate spectrophotometer (BioTek Instruments).

Reverse transcription and quantitative PCR

Prior to reverse transcription, all RNA samples were diluted to 20 ng/μL using RNase-free water. The cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA samples were analyzed using quantitative PCR. Primer and probe sets for *ABCG2* (Hs01053790_m1), *ALDH1A1* (Hs00946916_m1), *CD44* (Hs01075861_m1), *CD133* (Hs01009259_m1), *GLI1* (Hs00171790_m1), *GLI2* (Hs00257977_m1), and *RPLP0* (Hs99999902_m1; housekeeping gene) were obtained from Applied Biosystems; primers for *endoglin* (*ENG*; PPH01140F) were obtained from SABiosciences and used according to manufacturer's instructions. PCR amplification was conducted on an ABI Prism 7900HT sequence detection system, and gene expression was calculated using the comparative C_T method.

siRNA transfection

To examine knockdown of endoglin, Gli1, or Gli2 with siRNA, cells were exposed to control siRNA (target sequence: 5′-UUCUCCGAACGUGUCACGU-3′; Sigma), one of 2 tested endoglin-targeting constructs (ENG_A siRNA: 5′-CAAUGAGGCGGUGGCAAU-3′ or ENG_B siRNA: 5′-CAGAAACAGUCCAUUGUGA-3′; Sigma), one of 2 tested Gli1-targeting constructs (GLI1_A siRNA: 5′-CUACUGAUACUCUGGGAUA-3′ or GLI1_B siRNA: 5′-GCAAAUAGGGCUUCACAUA-3′), or one of 2 tested Gli2-targeting constructs (GLI2_A siRNA: 5′-CGAUUGACAUGCGACACCA-3′ or GLI2_B siRNA: 5′-GUACCAUUACGAGCCUCAU-3′) at a 1:3 siRNA (pmol) to Lipofectamine 2000 (μL) ratio. Lipofectamine and siRNA were incubated for 20 minutes at room temperature, added to cells in serum-free RPMI to incubate for 6 to 8 hours, followed by 10% FBS/RMPI thereafter. Transfected cells were grown at 37°C for an additional 48 hours and then harvested for quantitative PCR or Western blot analysis.

Western blot analysis

Cultured cell lysates were collected in modified radioimmunoprecipitation assay (RIPA) lysis buffer with protease inhibitor cocktail (Roche) and subjected to immunoblot analysis by standard techniques (14) using anti-endoglin antibody (Sigma) at 1:500 dilution overnight at 4°C; or anti-β-actin antibody (Clone AC-15, Sigma) at 1:20,000 dilution for 1 hour at room temperature, which was used to monitor equal sample loading. After washing, blots were incubated with goat anti-rabbit (for endoglin) or goat anti-mouse (for β-actin) secondary antibodies (Bio-Rad) conjugated with horseradish peroxidase. Visualization was conducted by the Enhanced Chemiluminescence Method (Pierce Thermo Scientific).

Assessment of cell viability and cell-cycle analysis following siRNA-mediated knockdown

For effects of siRNA-mediated downregulation on cell viability, cells were first transfected with siRNA (5 μg) for 24 hours in 6-well plates (2.5×10^5 cells per well), trypsinized, and then replated on a 96-well plate at 2,000 cells per well. After 4 to 5 days, cell viability was assessed by optical density measurements at 570 nm using 0.15% MTT (Sigma) in PBS. For cell-cycle analysis, 5×10^5 cells in a 60-mm dish were transfected with siRNAs and then cultured in RPMI/10% FBS at 37°C for an additional 48 hours. Cells were then trypsinized, washed in PBS, and fixed in 100% ethanol overnight. Cells were then centrifuged, washed in PBS, and resuspended in PBS containing 0.1% Triton X-100 (v/v), 200 μg/mL DNase-free RNase A, and 20 μg/mL propidium iodide (PI). PI fluorescence was assessed by flow cytometry, and the percentage of cells in sub-G_0, G_0–G_1, S, and G_2–M phases was calculated by the cell-cycle analysis module for Flow Cytometry Analysis Software (FlowJo v.7.6.1). For effects of siRNA-mediated downregulation on cisplatin IC_{50}, cells were first transfected with siRNA (5 μg) in 6-well plates, trypsinized, and then replated on a 96-well plate at 2,000 cells per well, followed by addition of chemotherapy after attachment. IC_{50} was determined by finding the dose at which the drug had 50% of its effect, calculated by the equation $[(OD_{570max} - OD_{570min})/2) + OD_{570min}]$.

Statistical analysis

Comparisons of continuous variables were made using a two-tailed Student *t* test, if assumptions of data normality were met. Those represented by alternate distribution were examined using a nonparametric Mann–Whitney *U* test. Differences between groups were considered statistically significant at $P < 0.05$. Error bars represent SD unless otherwise stated.

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Results

ALDH1A1, CD44, and CD133 expression in primary human ovarian cancer specimens

We identified a cohort of 45 patients with either papillary serous or endometrioid high-grade ovarian cancer for whom tumor specimens were collected at primary therapy and at the time of recurrent disease. The clinical characteristics of these patients are described in Supplementary Table S1 and represent the typical clinical profiles of patients with ovarian cancer. All patients were initially treated with combination platinum (either cisplatin or carboplatin) and taxane (either paclitaxel or docetaxel) by intravenous infusion. We first examined baseline expression of ALDH1A1, CD44, and CD133, the markers most consistently showing a putative CSC population in ovarian cancer. The percentage of positive ALDH1A1, CD44, and CD133 cells in primary samples averaged 23.4%, 6.2%, and 7.1%, respectively (Fig. 1A). Representations of high and low distribution patterns are shown in Fig. 1B and for CD44 and CD133 high-power views in Fig. 1C. For all 3 proteins examined, staining was typically strong in some cells and negative in others, rather than having a range of intensity across all tumor cells, signifying distinct heterogeneity within the tumor. There was no distinct pattern to the location of the positive cells (such as around vasculature, or on the leading edge of the tumor) but positive cells did tend to cluster together. Staining was appropriately noted intracellularly for ALDH1A1 and on the cell membrane for CD44 and CD133. Interestingly, CD133 expression was usually noted at cell–cell borders rather than circumferentially, suggesting a polarity to expression and possible participation in cell–cell interactions (Fig. 1C).

Change in expression of ALDH1A1, CD44, and CD133 from primary to recurrent ovarian cancer

To determine whether recurrent ovarian tumors have altered expression of ALDH1A1, CD44, and CD133, we compared the average number of positive cells for each marker among the 45 primary samples to that of the recurrent samples taken from the same patients (Fig. 1D). There was a modest increase in ALDH1A1-positive cells (from 23.4% to 29.2%, $P = 0.28$) and CD44-positive cells (from 6.2% to 11%, $P = 0.11$); however, CD133-positive cells were significantly higher (from 7.1% to 29.6%, $P = 0.0004$) in recurrent than in primary samples. To appreciate the change in each subpopulation for each patient, in addition to the mean of the entire group, the change for each tumor is graphically presented in Fig. 1E. For ALDH1A1 and CD44, both increases and decreases were noted for different patients. However, for CD133, the change was almost always an increase. The percentage of CD133-positive cells increased by more than 2-fold in 58% of recurrent samples than in matched primary samples.

Subgroup analysis of ALDH1A1, CD44, and CD133 based on setting of recurrent tumor collection

If the CSC hypothesis is clinically significant, then surviving cells would be expected to give rise again to both resistant CSCs and differentiated chemosensitive cells. Clinically this is seen as most patients will again have a response to treatment at first recurrence. Therefore, we examined the pairs on the basis of when their recurrent tumor was collected: (i) in patients who were clinically without evidence of disease but had other indications for surgery conducted within 3 months of completion of primary therapy, termed persistent tumor; (ii) in patients who recurred more than 6 months after completion of primary therapy and had tumors collected prior to second-line chemotherapy, termed untreated recurrence; and (iii) in the setting of recurrent, chemoresistant disease, termed treated recurrence. Among persistent tumors, there was an even more pronounced increase in ALDH1A1-positive cells (from 29.7% to 54.9%, $P = 0.018$), CD44-positive cells (from 8.3% to 21.2%, $P = 0.16$), and CD133-positive cells (from 6.6% to 53.9%, $P = 0.001$; Fig. 2A). In contrast, samples collected at first recurrence before initiating secondary therapy were composed of similar percentages of each marker as their primary tumor (Fig. 2B), suggesting that the tumor was repopulated with marker-negative differentiated cells. In tumors collected from recurrent platinum-resistant patients, only CD133 was significantly increased in expression (from 6.3% to 34.5%, $P = 0.027$; Fig. 2C). The percentage of CD133-positive cells increased by more than 2-fold in 50% of treated recurrence samples than in matched primary.

Table 1 illustrates the changes in ALDH1A1, CD44, and CD133 staining from primary to persistent tumor in individual patients. Overall, the percentage of ALDH1A1-, CD44-, and CD133-positive cells increased by more than 2-fold in 64%, 67%, and 89% of persistent tumor specimens, respectively, than in matched primary samples. While the expression of at least 2 of the 3 markers was elevated in the majority of specimens, only 4 patients had increased expression of all 3 markers. This suggests that certain mediators may be more active than others in different patients, and there may be other markers of treatment-resistant cells yet to be identified.

Expression of genes involved in human stem cell signaling is increased in recurrent compared with matched primary ovarian tumors

Building on the model that tumor samples present at the completion of primary therapy represent the cells responsible for recurrent disease and are therefore most relevant for study, we laser capture microdissected tumor cells from the 12 patients with persistent tumor analyzed above (Supplementary Fig. S1). Gene expression of putative CSC markers (*ALDH1A1*, *CD44*, *CD133*, and *ABCG2*) as well as 84 genes involved in pluripotent cell maintenance and differentiation was analyzed in these matched samples by qPCR or qPCR array. As shown in Table 2, expression of *ALDH1A1* (2.5-fold, $P = 0.23$) and *CD44* (4.1-fold, $P = 0.0023$) was

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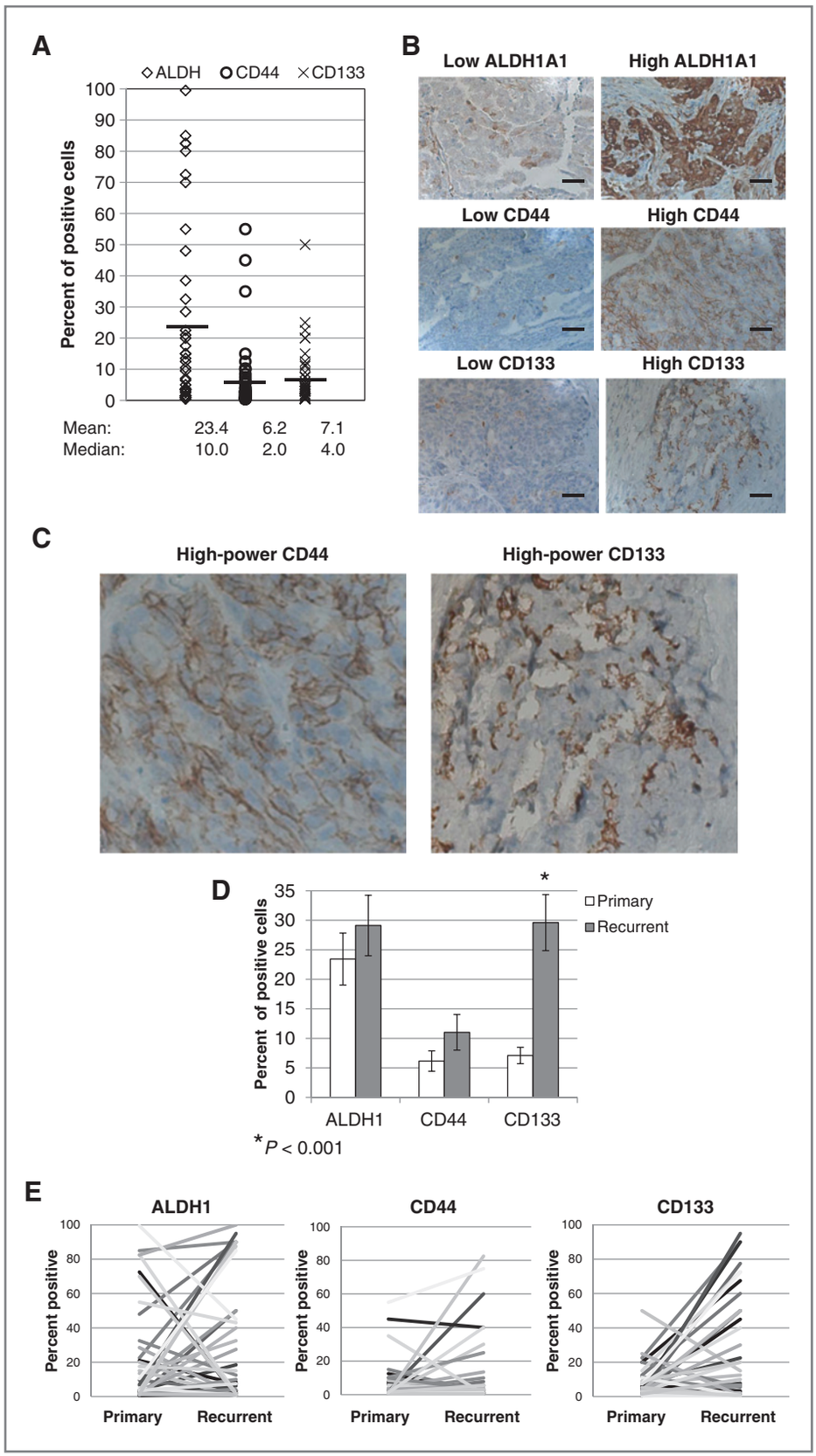

Figure 1. Change in expression of ALDH1A1, CD44, and CD133 from primary to recurrent ovarian cancer. A, ALDH1A1, CD44, and CD133 expression in 45 high-grade ovarian adenocarcinomas was examined using immunohistochemistry. The estimated percentage of positive cells for each sample, with mean (black bars) and median are shown. B, for all 3 proteins examined, staining was heterogeneous, rather than diffusely positive. Examples of high and low frequency expression for each are shown (black bar, 100 μm). C, a higher magnification of CD44 and CD133 expression in primary ovarian cancer specimens, showing cell surface expression. D, the average number of positive cells for ALDH1A1, CD44, and CD133 among the 45 primary samples was compared with the average among matched recurrent samples. Only CD133 was significantly higher in recurrent samples. Error bars represent SEM. *, $P < 0.001$. E, to evaluate the change in each subpopulation for each patient, in addition to the mean of the entire group, the change for each tumor is shown in individual graphs.

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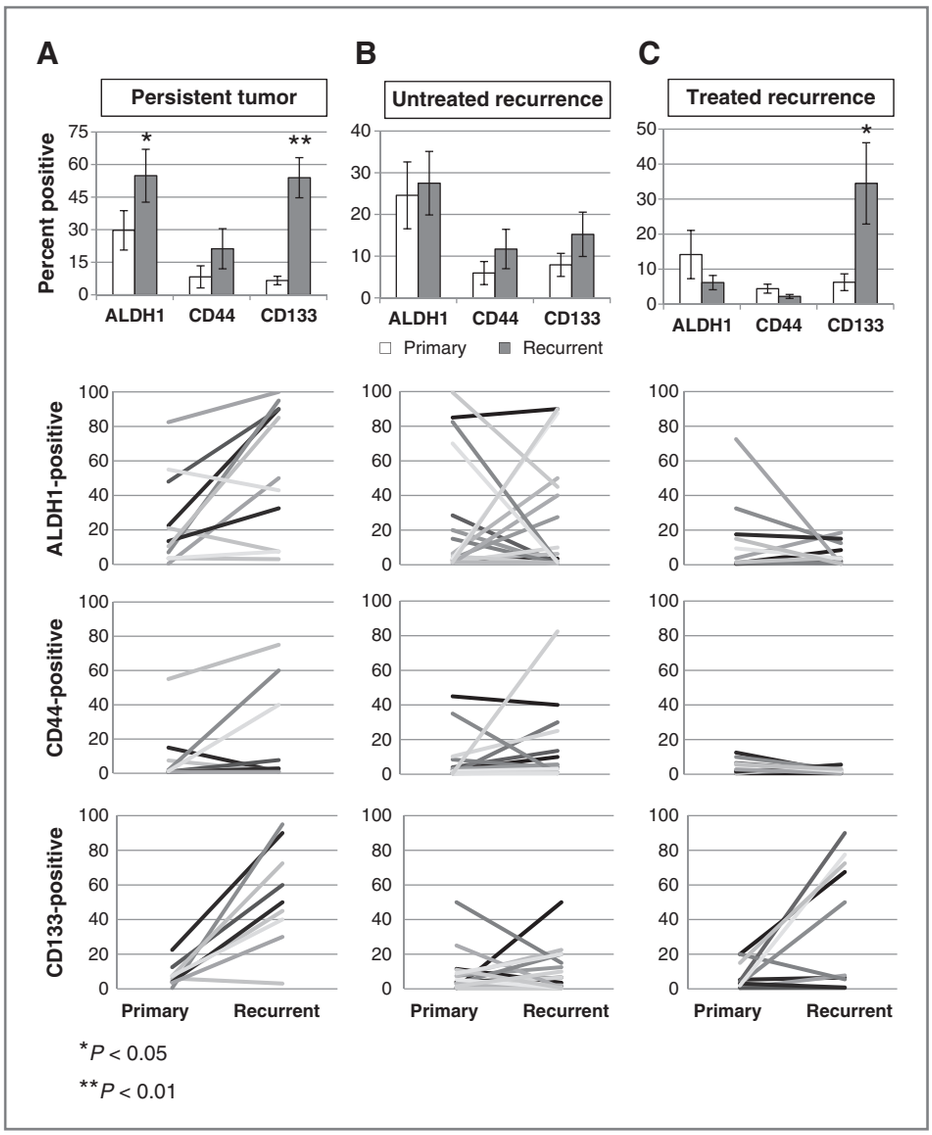

Figure 2. Subgroup analysis of ALDH1A1, CD44, and CD133 based on setting of recurrent tumor collection. Expression of ALDH1A1, CD44, and CD133 was broken down into subcategories based on the setting in which the recurrent tumor was retrieved. A, ALDH1A1, CD44, and CD133 expression was higher in samples collected immediately after the completion of primary therapy (persistent tumor; $n = 12$). B, samples collected at first recurrence before initiating secondary therapy (untreated recurrence; $n = 20$) were composed of similar percentages of each marker. C, in tumors collected from recurrent, platinum-resistant patients (treated recurrence; $n = 13$), only CD133 was increased in expression. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$.

elevated in persistent tumors compared with matched primary samples, similar to IHC analysis. Expression of breast cancer resistance protein (*ABCG2*/*BCRP*), a well-characterized drug efflux transporter that has been associated with stem cell phenotype (9, 28), was also increased in persistent tumors (7.7-fold, $P = 0.0163$). Attempts to optimize experimental conditions to examine BCRP by immunohistochemistry failed and therefore we could not validate this increase at the protein level. CD133 mRNA expression was virtually undetectable in both primary and persistent tumor samples. This suggests that increased CD133 protein expression in recurrent tumors noted by immunohistochemistry may be due to posttranscriptional or posttranslational regulation.

Of the 84 genes examined by the Human Stem Cell Signaling RT^2 Profiler Array (16), we found that 12 of these genes (14%) were significantly increased in persistent compared with matched primary tumor. Members of the TGF-β superfamily signaling pathway (*ENG*, *ZEB2*, *LTBP4*, *TGFBR2*, *RGMA*, *ACVR1B*, and *SMAD2*) were most commonly significantly increased as well as members of the Hedgehog (*GLI1* and *GLI2*), Notch (*PSEN2*), and Wnt (*FZD9* and *BCL9L*) pathways. Of particular interest, the TGF-β coreceptor endoglin (*ENG*) was, on average, 3.77-fold ($P = 0.0023$) higher in persistent tumors and more than 2-fold higher in 9 of the 12 samples. All of the tumors, either primary or recurrent, expressed endoglin. This protein is a recognized marker for angiogenesis, primarily expressed on endothelial cells (29, 30), but increased expression specific to tumor cells in our laser-microdissected tissues suggest that it may play a role in tumor cell chemoresistance and could be targeted for therapy. IHC staining of these specimens for endoglin expression confirmed that recurrent tumors had a greater density of

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Table 1. Changes in ALDH1A1, CD44, and CD133 staining from primary to persistent ovarian tumor

Patient	ALDH1A1[a]	CD44[a]	CD133[a]
502	↑	↓	↑
505	↑	NM	NM
510	↓	↓	↑
511	↑	↑	↑
522	NC	↑	NC
525	↑	↑	↑
535	↑	↑	↑
540	NC	NC	↑
544	↑	NM	NM
548	↑	↑	↑
549	NC	↑	↑

Abbreviations: NC, density of cells did not change by more than 2-fold; NM, not measured because of insufficient tumor.
[a]An increase or decrease more than 2-fold designated by arrow.

endoglin positivity than in the matched primary tumor and that expression was definitively present in tumor cells not just the in vasculature (Fig. 3A). In addition, endoglin and CD133 expression significantly correlated ($r = 0.62$, $P = 0.006$), as did Gli1 and CD133 expression ($r = 0.54$, $P = 0.022$), suggesting that the increase in CD133 positivity observed in recurrent compared with matched primary tumors is accompanied by an increase in markers of stem cell signaling.

Endoglin is expressed in ovarian cancer cell lines and its downregulation leads to decreased cell viability

To further explore the potential role of endoglin in ovarian cancer, we first examined gene expression in cell lines. These included ES2, IGROV-AF1, OvCar-3, SKOV3ip1 and 2 pairs of parental and chemoresistant ovarian cancer cell lines: A2780ip2/A2780cp20 (20-fold increased cisplatin resistance and 10-fold increased taxane resistance) and HeyA8/HeyA8MDR (500-fold taxane resistant). As shown in Fig. 3B, mRNA expression of endoglin was prominent in ES2, HeyA8, and HeyA8MDR cells. Minimal expression of endoglin was detected in the A2780ip2, A2780cp20, IGROV-AF1, OvCar-3, and SKOV3ip1 cell lines. Protein expression was assessed by Western blot and correlated with mRNA quantification (data not shown).

To determine whether endoglin might be a target for tumor-specific therapy, 2 different siRNA constructs (ENG_A siRNA and ENG_B siRNA) were identified with variable efficacy in reducing endoglin expression (95%–99% reduction with construct A, 50% reduction with construct B), as determined by Western blot (Fig. 3C). ES2 and HeyA8MDR cells transiently transfected with these

Table 2. Quantitative PCR analysis of putative CSC markers and stem cell pathways in matched primary/persistent ovarian cancers (n=12)

Gene name (symbol)	Signaling pathway	Mean		No. of decreased	No. of increased
		Fold change[a]	P[b]	>50%	>2-fold
Putative CSC markers					
Aldehyde dehydrogenase 1A1 (*ALDH1A1*)		2.46	0.2343	3	6
CD44 molecule (*CD44*)		4.08	0.0023	2	9
Prominin 1 (*PROM1/CD133*)		1.11	0.8877	4	5
ATP-binding cassette, sub-family G, member 2 (*ABCG2/BCRP*)		7.65	0.0163	1	5
Human Stem Cell Signaling RT2 Profiler PCR Array					
Endoglin (*ENG*)	TGF-β	3.77	0.0023	0	9
Zinc-finger E-box–binding homeobox 2 (*ZEB2*)	TGF-β	3.66	0.0062	1	9
Presenilin 2 (*PSEN2*)	Notch	3.30	0.0071	0	7
GLI family zinc finger 1 (*GLI1*)	Hedgehog	10.21	0.0076	1	10
GLI family zinc finger 2 (*GLI2*)	Hedgehog	7.61	0.0111	2	9
Latent transforming growth factor-β binding protein 4 (*LTBP4*)	TGF-β	4.69	0.0146	1	9
Transforming growth factor-β receptor II (*TGFBR2*)	TGF-β	2.76	0.0190	0	8
RGM domain family, member A (*RGMA*)	TGF-β	7.84	0.0204	2	9
Activin A receptor, type IB (*ACVR1B*)	TGF-β	2.20	0.0275	0	4
Frizzled homolog 9 (*FZD9*)	Wnt	10.43	0.0393	2	8
SMAD family member 2 (*SMAD2*)	TGF-β	1.79	0.0435	1	6
B-cell CLL/lymphoma 9-like (*BCL9L*)	Wnt	2.06	0.0463	1	6

[a]Persistent compared with primary tumor.
[b]Calculated using paired Student *t* test.

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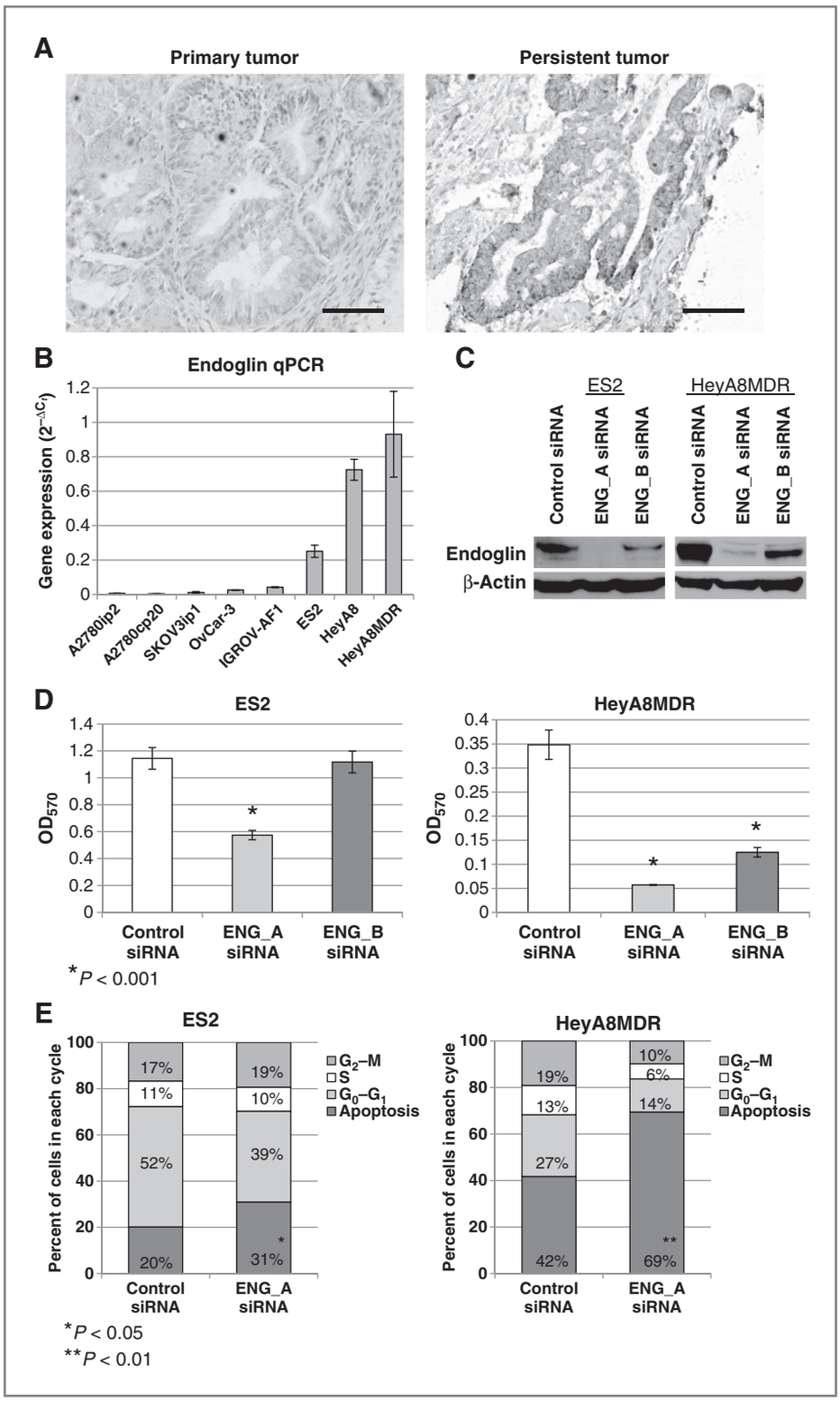

Figure 3. Endoglin is expressed in persistent ovarian tumor and ovarian cancer cell lines, and its downregulation leads to decreased cell viability. A, matched primary/persistent ovarian tumor pairs ($n = 12$) were subjected to IHC analysis of endoglin to evaluate changes in expression. Persistent tumors were found to have a higher density of endoglin staining than in primary specimens. Representative histologic sections are shown for a matched pair (black bar, 100 μm). B, mRNA expression of endoglin was quantified in 8 different ovarian cancer cell lines using quantitative PCR. Gene expression is shown as $\log_2$ transformed ΔC_T values [difference between the C_T value of the gene of interest (*endoglin*) and that of the housekeeping gene (*RPLP0*)]. C, downregulation of endoglin in ES2 and HeyA8MDR cells using 2 different siRNA constructs was determined by Western blot analysis. β-Actin was used as a loading control. D, ES2 and HeyA8MDR cells transiently transfected with anti-endoglin siRNAs had decreased viability as determined by MTT assay. E, cell-cycle analysis (PI staining) revealed that downregulation of endoglin led to an accumulation of both ES2 and HeyA8MDR cells in the sub-G_0 or apoptotic fraction. Data are representative of 3 independent experiments. *, $P < 0.001$.

endoglin-targeting siRNAs showed a significant reduction in viability, as determined by MTT assay (Fig. 3D). This effect on viability correlated with the degree of endoglin downregulation, as ENG_A siRNA reduced cell viability by 50% to 84% (in ES2 and HeyA8MDR, respectively, $P < 0.001$), whereas ENG_B siRNA had no effect on ES2 and a

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64% reduction in HeyA8MDR ($P < 0.001$). The variability in effects on the 2 cell lines may reflect their dependency on endoglin, as HeyA8MDR cells have 3.7-fold higher endoglin expression than ES2 cells. In addition, ES2 cells may have compensatory pathways active at a baseline that reduce their dependency on endoglin. Additional studies will be required to fully elucidate these mechanisms.

To determine the mechanism by which endoglin downregulation may affect cell viability, cell-cycle analysis was conducted in a separate experiment. ES2 and HeyA8MDR cells were exposed to control or anti-endoglin siRNA (ENG_A), allowed to grow for a total of 72 hours, and examined for DNA content by PI staining (Fig. 3E). In both ES2 and HeyA8MDR, endoglin knockdown resulted in a significant accumulation of cells in the sub-G_0/apoptotic fraction compared with cells transfected with control siRNA (from 20% to 31%; $P < 0.05$ and from 42% to 69%; $P < 0.01$, respectively).

Targeting of Gli1 and Gli2 in ovarian cancer cells

Analysis of stem cell genes upregulated in recurrent tumors reveals both primary mediators of the Hedgehog pathway to be increased after chemotherapy (Table 2). The Hedgehog pathway has previously been implicated in the survival of CSCs (31). To validate its targetability in ovarian cancer, we first examined gene expression of *GLI1* and *GLI2* in the same cell lines as mentioned above. As shown in Fig. 4A, there was no correlation between *GLI1* and *GLI2* expression among the cell lines examined, although all cell lines expressed *GLI1*, *GLI2*, or both. Of note, A2780cp20 cells were found to express *GLI1* 2.05-fold higher and *GLI2* 1.40-fold higher ($P < 0.001$) than their parental line (A2780ip2), suggesting that these Hedgehog pathway members may be involved in mediating platinum resistance.

A2780cp20 ($Gli1^+/Gli2^+$) and ES2 ($Gli1^-/Gli2^+$) cells were subsequently used for examining the biologic effects of Gli1/2 knockdown. Downregulation of Gli1/2 in these cell lines was achieved using 2 different siRNA constructs as confirmed by quantitative PCR (Fig. 4B). Importantly, each siRNA construct showed selectivity for the *GLI* gene to which it was designed against (i.e., *GLI1* siRNAs had no effect on *GLI2* expression and *GLI2* siRNAs had no effect on *GLI1* expression). As shown in Fig. 4C, knockdown of Gli1 or Gli2 alone significantly decreased A2780cp20 cell viability [by up to 65% ($P < 0.001$) and 61% ($P < 0.001$), respectively], whereas in ES2 cells, knockdown of Gli2, but not Gli1, significantly reduced cell viability (by up to 82%, $P < 0.001$). The lack of an effect of *GLI1* downregulation on ES2 cells would be expected as these cells have little to no detectable *GLI1* expression. Interestingly, an increased sensitivity to cisplatin was observed in both A2780cp20 and ES2 cell lines after knockdown of Gli2, but not Gli1 (Fig. 4C). Cisplatin IC_{50} decreased from 4 to 0.8 μmol/L (5.0-fold change) in A2780cp20 cells and from 0.7 to 0.15 μmol/L (4.7-fold change) in ES2 cells. Taken with the demonstration of increased Gli2 expression in samples collected immediately after platinum-based chemotherapy (Table 2), these data make a compelling argument that Gli2 plays a role in platinum resistance, which can be at least partially overcome with Gli2 downregulation. However, Gli1 only appears to contribute to absolute viability, with no platinum-sensitizing effects.

To determine the mechanism by which Gli1/2 downregulation may affect cell viability and/or platinum sensitivity, cell-cycle analysis was conducted in a separate experiment. A2780cp20 cells were exposed to control, anti-Gli1 (GLI1_B), or anti-Gli2 (GLI2_B) siRNA, allowed to grow for a total of 72 hours, and examined for DNA content by PI staining. As shown in Fig. 4D, downregulation of Gli1 had little effect on the cell-cycle distribution of A2780cp20 cells, with a modest accumulation in the sub-G_0 or apoptotic fraction compared with control siRNA (8%–12%, $P < 0.05$). This suggests that the observed decrease in cell viability following Gli1 knockdown may be due to mechanisms independent of the cell cycle. Alternatively, downregulation of Gli2 had a greater impact, with a 4-fold increase (8%–32%, $P < 0.001$) in induction of apoptosis than in control siRNA. This further suggests that Gli2 plays a critical role in ovarian cancer cell survival.

Discussion

We have found that recurrent tumors are more densely composed of putative CSCs as characterized by ALDH1A1, CD44, and CD133 than their matched primary ovarian cancer specimens, suggesting that their expression is clinically significant and may correlate with residual chemoresistant populations that must be present at the end of primary therapy. Presumably targeting these populations with some other treatment modality would be required to achieve durable cures in patients with ovarian cancer. In addition, we identified several genes from a large panel of 84 genes involved in stem cell biology to be significantly overexpressed in recurrent patient samples, further suggesting that resistant tumors are enriched with genes involved in stem cell pathways. With this methodology, the TGF-β coreceptor endoglin was found to be overexpressed in residual tumor cells and thus important to the chemoresistant cancer cell population. This represents a previously unrecognized function of this gene as a mediator of survival in tumor cells, in addition to its known role in angiogenesis. Moreover, the Hedgehog transcription factor Gli2 was also overexpressed and functional in the chemoresistant population and, with correlative *in vitro* data, was found to play a novel role in platinum resistance.

It is hypothesized that CSCs may be responsible for tumor initiation or recurrent disease. There are many facets of this hypothesis that are still under debate, including what level of stemness such populations may have, how best to identify the true stem cell population, and whether these marker-defined cells are also the ones surviving initial chemotherapy (32). However, there clearly are subpopulations within a heterogeneous tumor that have more aggressive, chemoresistant features than others in *ex vivo* and now *de novo* models (2, 33). This is clinically evident in the

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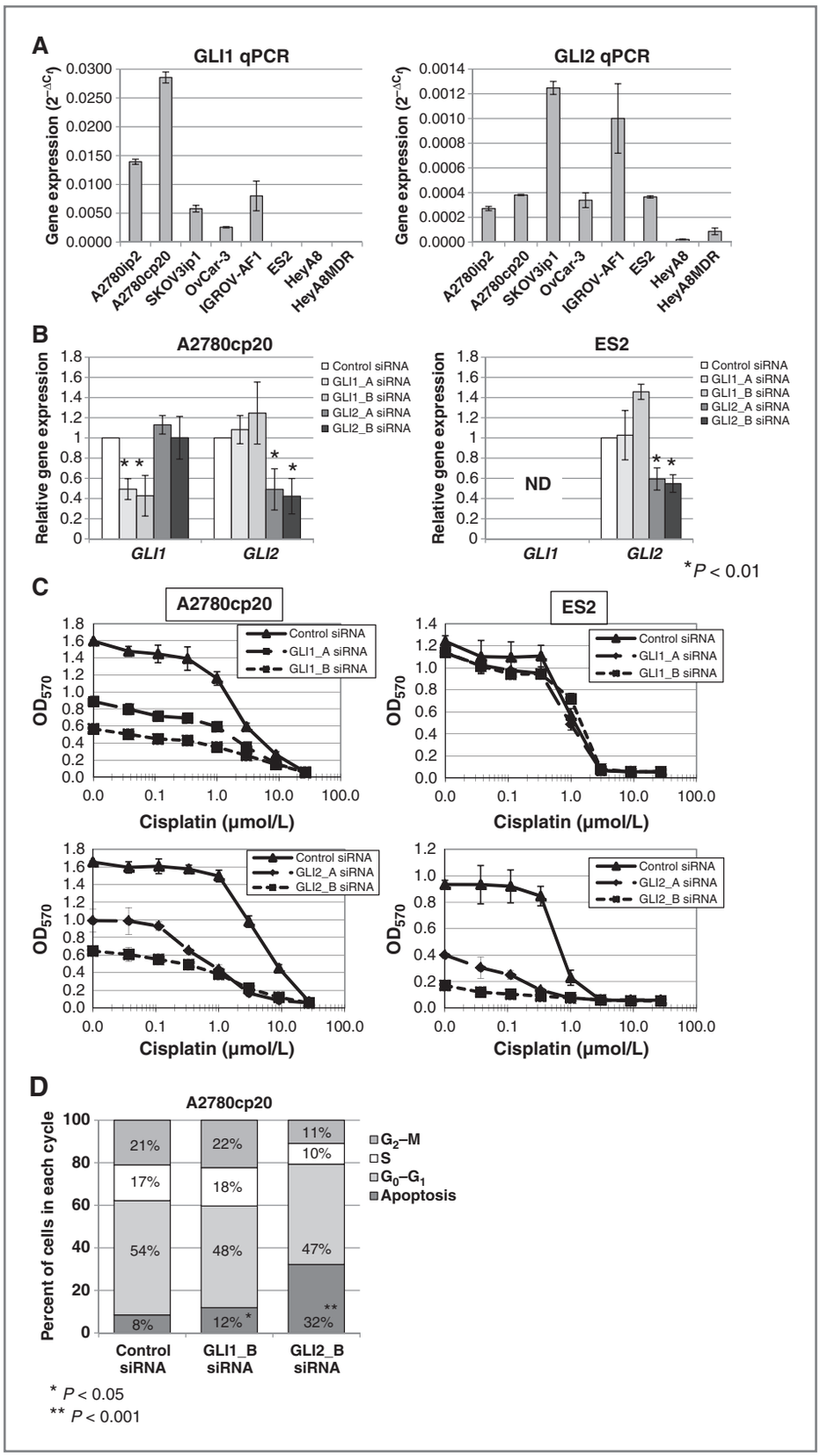

Figure 4. Downregulation of Gli1/2 leads to decreased cell viability and downregulation of Gli2, but not Gli1, sensitizes ovarian cancer cells to cisplatin *in vitro*. A, mRNA expression of *GLI1* and *GLI2* was quantified in 8 different ovarian cancer cell lines using quantitative PCR (qPCR). Gene expression is shown as $\log_2$ transformed ΔC_T values. B, downregulation of Gli1/2 in A2780cp20 and ES2 cells using 2 different siRNA constructs was determined by quantitative PCR. Each siRNA construct showed selectivity for the *GLI* gene to which it was designed against. ND, not detectable; *, $P < 0.01$. C, knockdown of GLI1 or GLI2 alone diminished A2780cp20 cell viability, whereas only knockdown of GLI2 diminished ES2 cell viability as determined by MTT assay. Increased sensitivity to cisplatin (CDDP) was noted in A2780cp20 and ES2 cells transfected with *GLI2* siRNAs, but not *GLI1* siRNAs. D, cell-cycle analysis (PI staining) of A2780cp20 cells exposed to control siRNA, *GLI1* siRNA, or *GLI2* siRNA for a total of 72 hours. Downregulation of Gli2 and, to a lesser extent Gli1, led to an accumulation of cells in the sub-G_0 or apoptotic fraction. Data are representative of 3 independent experiments.

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observation that patients often have outstanding initial responses to chemotherapy, suggesting that the majority of primary tumor is actually chemosensitive. It is important to note that although we do see an increase in these populations, recurrent tumors are not completely composed of these cells. This indicates that either additional chemoresistant populations are yet to be identified, or these cells have such differentiating capacity that they rapidly produce marker-negative cells, or both. An additional limitation of our analysis is the specific examination of stem cell pathways. Other pathways almost certainly play important roles in mediating survival of the therapy-resistant population; one example being altered DNA repair mechanisms. Recent evidence suggests that ovarian cancers can arise from specific defects in DNA repair pathways, and that inhibitors of the proteins involved in these pathways, such as PARP, could be used to reverse chemoresistance (34), It is reasonable to postulate that CSCs, like normal stem cells, would have enhanced mechanisms of DNA repair, allowing for survival with prolonged exposures to DNA-damaging insults. Analysis of RNA from FFPE samples showed that the extract was of quality appropriate for qPCR analysis, but not enough samples had sufficient quality for full microarray analysis, which could be used in future studies to examine the role of DNA repair or other pathways in mediating chemoresistance. Further characterization of the recurrent chemoresistant tumors with evolving high-throughput methods that can be conducted on FFPE samples, or identification of a cohort of patients with snap frozen tumors, would be required to fully characterize this aggressive population.

Whether the chemoresistant population is composed of predominantly cancer cells with stem cell biology or not, we propose a model of how such a population may comprise the overall tumor during different clinical settings. Because most patients have an initial positive response to chemotherapy, the presenting tumor must be composed of mostly therapy-sensitive cells (TSC), with a small component of therapy-resistant cells (TRC). Treatment selectively kills TSCs, resulting in predominantly TRCs, but in a small enough volume that they are not clinically detectable (persistent tumor). Therefore, the patient is observed, but in about 75% of cases, tumors will recur 18 to 24 months after completion of therapy (with an untreated recurrent tumor). Because of the differentiation capacity of the resistant cells, this tumor has become repopulated with CSC marker–negative differentiated cells and is again heterogeneous, with a significant portion of chemosensitive cells. This would seem to be the case, given the observed 50% response rate seen in patients receiving second-line chemotherapy. However, either because of genetic changes in genetically unstable tumor cells or further selective growth of the therapy-resistant population, ultimately the TRCs dominate, patients get no further response with multiple agents and succumb to tumor burden (treated recurrent tumor). The observed increase in CSC marker staining, particularly ALDH1A1 and CD133, in samples collected immediately at the completion of primary therapy suggests these cells have preferential survival and can go on to give rise to recurrent disease. These cells may represent a population that could be targeted to achieve increased response rates and survival in patients with ovarian cancer.

It is an interesting finding that $CD44^+$ cells were less dense in recurrent tumors than in CD133 and ALDH1, despite multiple studies showing that $CD44^+$ cells have CSC properties. Many of these studies have used CD44 in combination with other markers, such as c-kit (4), MyD88 (5), CD133 (6), and CD24 (35). It is for this reason that we examined CD44 by itself as potentially important, but at the same time may have introduced a limitation by not being able to evaluate dual-positive populations. It is yet to be determined the degree of crossover between individual markers. Likely, the combination of markers will identify a more aggressive population than either alone, as previously shown with CD133 and ALDH1 (11), but it is unknown whether such combinations then exclude other aggressive populations. This disparity, however, highlights the limitations in defining the key population by marker status alone, instead relying on clinical behaviors such as resistance to chemotherapy.

Recent studies have shown that developmental pathways (such as Notch, Wnt, Hedgehog, and TGF-β) play an important role in the self-renewal and maintenance of CSCs and that inhibiting these pathways may provide useful therapeutic strategies both alone and in combination with traditional chemotherapies (36, 37). In our study, genes identified as being significantly overexpressed in persistent tumors included endoglin (a member of the TGF-β superfamily) and the primary mediators of hedgehog transcription, *GLI1* and *GLI2*, among others (Table 2). The most significant and consistent increase in expression from primary to persistent tumor occurred in endoglin (CD105), a TGF-β coreceptor. This molecule interacts with TGF-β receptor II [TGFBR2, which was also significantly increased in persistent tumors (2.76-fold, $P = 0.0190$)], both dependently and independently of the TGF-β ligand (38). This interaction subsequently promotes gene transcription mediated by the Smad family of transcription factors (Smad2 and 4). In contrast, a proteolytically cleaved, secreted form of endoglin, known as soluble endoglin (Sol-Eng) appears to inhibit TGF-β signaling by scavenging circulating TGF-β ligands (39). Endoglin is a well-described marker of angiogenesis whose expression is turned on in growing/sprouting endothelial cells (such as those supplying vasculature to tumors). This characteristic of endoglin has made it a desirable target for antiangiogenic cancer therapy, with monoclonal antibodies being developed for future clinical use (29, 30). Previous studies have shown that endoglin expression in the stroma of ovarian tumors is associated with poor survival (40, 41), but the role of this receptor in cancer cell biology remains largely unexplored. On the basis of our data, it appears that endoglin plays a role in ovarian cancer chemoresistance and recurrence. Moreover, endoglin appears to be important for continued ovarian cancer cell survival as evidenced by our *in vitro* data. In a study conducted by Li and colleagues, it was shown that endoglin

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prevents apoptosis in endothelial cells undergoing hypoxic stress, either in the presence or absence of TGF-β ligand (42). It could be speculated that endoglin serves a similar antiapoptotic function in tumor epithelial cells and thereby promotes ovarian cancer cell survival. Whether this is due to the promotion of TGF-β signaling or through a TGF-β–independent mechanism remains to be determined. Taken together, these data suggest that inhibiting endoglin could be used to target both the tumor and its developing vasculature, thereby having a potentially greater therapeutic benefit. Additional studies will determine the viability of endoglin as a therapeutic target, as antibodies have been developed that disrupt the interaction of endoglin and TGF-β receptor II (43, 44).

Previous studies have implicated hedgehog signaling in multidrug resistance (45, 46); however, the role of this pathway in resistance to platinum-based compounds remains largely unexplored. While both Gli1 and Gli2 appeared to mediate ovarian cancer cell survival *in vitro*, only downregulation of Gli2 sensitized cells to cisplatin in a synergistic fashion, with a 5-fold reduction in IC_{50} concentrations in two different cell lines. It is suggested that the mechanism underlying this sensitization involves apoptosis. Inhibition of apoptosis is known to mediate cisplatin resistance (47), and Gli2 has previously been shown to serve an antiapoptotic function through transcriptional regulation of apoptotic inhibitor molecules (48–50). In our study, we found that downregulation of Gli2 alone induced apoptosis, and this may have contributed to the increased sensitivity of ovarian cancer cells to cisplatin *in vitro*. Interestingly, downregulation of Gli1 had no effect on cisplatin toxicity. Future studies on the link between Gli2, apoptosis, and cisplatin resistance are warranted.

Collectively, the data presented in this study show that cells with stem cell properties enrich recurrent ovarian tumors, especially in their more chemoresistant forms. The varied density of these subpopulations in different clinical scenarios provides insight into the dynamic heterogeneity during the typical natural history of ovarian cancer progression. Additional stem cell pathways contribute to the continued survival and chemoresistance of ovarian cancer, and targeting these pathways may be necessary to achieve durable clinical response in this disease. In addition, the TGF-β coreceptor endoglin (CD105) and the Hedgehog mediator Gli2 were found to be overexpressed in recurrent ovarian tumors and are promising targets in overcoming chemoresistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Endoglin (CD105) contributes to platinum resistance and is a target for tumor-specific therapy in epithelial ovarian cancer

Angela Ziebarth, Somaira Nowsheen, Adam D. Steg, et al.

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Endoglin (CD105) contributes to platinum resistance and is a target for tumor-specific therapy in epithelial ovarian cancer

Angela J. Ziebarth[1], Somaira Nowsheen[2], Adam D. Steg[1], Monjri M. Shah[1], Ashwini A. Katre[1], Zachary C. Dobbin[1], Hee-Dong Han[3], Gabriel Lopez-Berestein[3,4,5], Anil K. Sood[4,5,6], Michael Conner[7], Eddy S. Yang[2], and Charles N. Landen[1*]

[1] Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, AL 35294
[2] Department of Radiation Oncology, University of Alabama at Birmingham, Birmingham, AL 35294
[3] Center for RNA Interference and Non-Coding RNA, [4]Departments of Experimental Therapeutics, [5]Cancer Biology, and [6]Gynecologic Oncology & Reproductive Medicine, U.T.M.D. Anderson Cancer Center, Houston, TX 77030
[7] Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294

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*Correspondence: Charles N. Landen, Jr., MD, MS
Associate Professor
Department of Obstetrics and Gynecology
The University of Alabama at Birmingham
176F RM 10250
619 19th Street South
Birmingham, AL 35249
Work: 205-934-4986
Fax: 205-975-6174
clanden@uab.edu

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Statement of Translational Relevance: Ovarian cancer remains the most lethal gynecologic malignancy, largely due to its high rate of chemoresistant recurrence. Endoglin (CD105) is overexpressed on tumor-associated endothelial cells and is a target for anti-angiogenic therapy, but expression on tumor cells has only been recently demonstrated. In the current study, we demonstrate that endoglin is actually predominantly expressed in the cytoplasm of malignant cells, and downregulating endoglin promotes apoptosis, induces DNA damage, and sensitizes cells to platinum therapy *in vitro* and *in vivo*. This occurs through effects on numerous DNA repair genes, most prominently BARD1. The novel demonstration of efficacy in targeting tumor cells themselves, in addition to the previously-recognized effects of targeting vasculature, make this therapeutic an attractive mechanism to target both compartments of the tumor microenvironment.

Abstract:

Purpose: Endoglin (ENG, CD105) is a membranous protein overexpressed in tumor-associated endothelial cells, chemoresistant populations of ovarian cancer cells, and potentially stem cells. Our objective was to evaluate the effects and mechanisms of targeting endoglin in ovarian cancer.

Experimental Design: Global and membranous endoglin expression was evaluated in multiple ovarian cancer lines. *In vitro,* the effects of siRNA-mediated endoglin knockdown with and without chemotherapy were evaluated by MTT assay, cell-cycle analysis, alkaline comet assay, γ-H2AX foci formation, and qPCR. In an orthotopic mouse model, endoglin was targeted with chitosan-encapsulated siRNA with and without carboplatin.

Results: Endoglin expression was surprisingly predominantly cytoplasmic, with a small population of surface-positive cells. Endoglin inhibition decreased cell viability, increased apoptosis, induced double-stranded DNA damage, and increased cisplatin sensitivity. Targeting endoglin downregulates expression of numerous DNA repair genes, including BARD1, H2AFX, NBN, NTHL1, and SIRT1. BARD1 was also associated with platinum resistance, and was induced by platinum exposure. *In vivo*, anti-endoglin treatment decreased tumor weight in both ES2 and HeyA8MDR models when compared to control (35-41% reduction, $p<0.05$). Endoglin inhibition with carboplatin was associated with even greater inhibitory effect when compared to control (58-62% reduction, $p<0.001$).

Conclusions: Endoglin downregulation promotes apoptosis, induces significant DNA damage through modulation of numerous DNA repair genes, and improves platinum sensitivity both *in vivo* and *in vitro.* Anti-endoglin therapy would allow dual treatment of both tumor angiogenesis

and a subset of aggressive tumor cells expressing endoglin and is being actively pursued as therapy in ovarian cancer.

Introduction

Epithelial ovarian carcinoma (EOC) remains the most lethal gynecologic malignancy.[1] While initial response to first-line therapy (consisting of surgical cytoreduction and combination platinum/taxane therapy) is usually effective, the majority of patients will ultimately recur with chemotherapy-resistant cancer and succumb to disease. This emphasizes the need for novel therapies aimed at targeting the population of cancer cells most resistant to initial therapy.

Endoglin (ENG) is a 180kDa disulfide-linked homodimer transmembrane protein most prominently expressed on proliferating endothelial cells. It is a well-characterized angiogenic marker that is upregulated during angiogenesis, and is overexpressed in vascular endothelium in malignancies including ovarian, leukemia, gastrointestinal stromal tumors (GIST), melanoma, and laryngeal cancers, but is rarely expressed in non-endothelial cells.[2-3] It is a co-receptor of TGFBR2 that binds TGF-B and is an important mediator of fetal vascular/endothelial development.[4] Recently, anti-angiogenic agents have received extensive attention as new therapeutic modalities, and CD105 has become an additional target by which intratumoral angiogenesis may be targeted.[5-6] However, endoglin may serve in a capacity beyond angiogenesis alone. Studies in GIST[7] and breast cancer[8] suggest that endoglin is upregulated not only in tumor endothelial cells, but also in actual tumor cells, and is associated with poor prognosis. Soluble endoglin has also been noted in ovarian cancer ascites,[9] and increased endoglin expression in ovarian cancer endothelial cells is associated with poor prognosis.[10] Additionally, we have recently shown that while endoglin is rarely expressed in primary ovarian cancer cells, it is frequently expressed in recurrent platinum-resistant tumor cells, as compared to the primary untreated tumor.[11] These findings suggest a broader role of endoglin in tumor cell biology beyond that of endothelial expression alone. The goal of our current study is to evaluate

the effects of targeting tumor-specific endoglin in ovarian cancer both *in vitro* and *in vivo* and explore the mechanisms by which endoglin may contribute to chemoresistance.

Methods and Materials

Evaluation of endoglin expression in ovarian cancer cell lines. Multiple ovarian cancer cell lines were evaluated for the presence of endoglin, including HeyA8, HeyA8MDR, ES2, A2780ip2, A2780cp20, A2780cp55, SKOV3ip1, SKOV3TRp2, IGROV-AF1, and HIO-180. Cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). The taxane-resistant cell line HeyA8MDR was maintained in the same media with the addition of 150 ng/ml of paclitaxel. Cell lines were routinely screened for Mycoplasma (GenProbe detection kit; Fisher, Itasca, IL) and all experiments performed on 70-80% confluent cultures. Cells less than 20 passages from confirmation of genotype by STR analysis were used.

Cell lysates were collected in modified radioimmunoprecipitation assay lysis buffer with protease inhibitor cocktail (Roche, Manheim, Germany). Immunoblot analysis was performed using rabbit anti-endoglin antibody (Sigma, St. Louis, MO) at 1:500 dilution overnight at 4°C. A loading control was performed with mouse anti-β-actin antibody (Clone AC-15, Sigma) at 1:20,000 dilution for 1 hour at RT. After washing, membranes were incubated in HRP-conjugated goat anti-rabbit (for Endoglin) or goat anti-mouse (for β-actin) secondary antibodies (Bio-Rad, Hercules, CA). Visualization was performed by enhanced chemiluminescence (Pierce Thermo Scientific, Rockford, IL).

Immunohistochemistry. Cell lines in culture were washed with ice cold PBS twice, then fixed by applying 100% ice cold methanol for 10 min. Cells were rehydrated with PBS. Endogenous

peroxidase was blocked with 3% H2O2 in methanol for 15min at RT. The slides were incubated in 10% normal goat serum for 1 hr at RT. The primary anti-endoglin antibody (Sigma HPA011862) was diluted in 10% normal goat serum at 1:50. The slides were kept at 4°C overnight. Biotin-labeled secondary antibody was applied on cells at the concentration of 1:2000 for 1hr at RT, followed by avidin-biotin peroxidase buffer. DAB (3,3'-diaminobenzidine) was used as chromophore to detect the staining. To visualize endoglin expression in tumor sections, formalin-fixed paraffin-embedded tissue was cut in sections of 5uM thickness. Slides were warmed for 15 minutes and sequentially deparaffinized. Antigen retrieval was carried out in Citrate buffer (pH6.0) in a pressure cooker at high pressure for 5 min. Endogenous peroxidase was quenched by 3% H2O2 in methanol for 15 min. Slides were incubated in 10% normal goat serum for 1hr at RT. Slides were then incubated (4°C, Overnight) in antibody against endoglin (Sigma HPA011862) in 10% normal goat serum at 1:200 dilution. Detection was carried out using biotin labeled secondary antibody against rabbit at dilution of 1:2000 incubated at RT for 1 hr, followed by avidin-biotin peroxidase buffer. DAB (3,3'-diaminobenzidine) was used as chromophore.

Flow cytometry. After trypsinisation and centrifugation, the cell pellet was washed and resuspended in washing buffer (PBS containing 2% FBS and 0.1% sodium azide).$1x10^7$ cells were resuspended in 50µls of 10% goat buffer for 1hr kept on ice. Cells were incubated in antibody against endoglin 1:100 (Sigma HPA011862) in 10% goat serum for 1hr on ice. Alexa-488-conjugated anti rabbit antibody was applied on cells for 30 minutes and incubated on ice. The cells were washed twice in PBS and analyzed by FACS.

Endoglin Downregulation by siRNA transfection: In order to determine the effects of endoglin downregulation in ovarian cancer cells, transient knockdown was accomplished with anti-endoglin siRNA. Lipofectamine 2000 (Invitrogen) transfection was performed on Hey8MDR and ES2 cell lines using control siRNA (target sequence: 5'-UUCUCCGAACGUGUCACGU-3', Sigma) lacking known human or mouse targets, or one of two different Endoglin-targeting constructs (5'-CAAUGAGGCGGUGGCAAU-3' ["ENG_A"} or 5'-CAGAAACAGUCCAUUGUGA-3' ["ENG_B"], Sigma). These anti-human sequences have no more than 8 consecutive bp homology with murine CD105 (by BLASTN) and therefore should not affect murine endoglin expression. Lipofectamine was added to 5µg siRNA at a 3:1 v/v ratio (or as otherwise specified, as in Figure 1E) were incubated for 20 min at RT, added to cells in serum-free RPMI to incubate for 12 hours in 6- well plates, then maintained in 10% FBS/RPMI for an additional 12 hours, trypsinized and re-plated on a 96-well plate at a concentration of 2,000 cells per well. Cells were treated with vehicle or increasing doses of carboplatin or paclitaxel to generate an IC 50 curve. After 5 days, cells were washed and incubated with MTT reagent (Sigma) for 2 hours at 37°C. Media was then removed, cells dissolved in DMSO, and optical density measurements at 570 nm read with a spectrophotometer. The IC50 was the chemotherapy concentration giving the OD_{IC50} reading, calculated by the formula $OD_{IC50} = [(OD_{MAX} - OD_{MIN})/2 + OD_{MIN}]$. Assays were repeated in triplicate.

Apoptosis analysis. Analysis of apoptosis was performed with the Annexin V assay combined with propidium iodide (PI, eBiosciences #88-8005-74). ES2 and HeyA8MDR cells were transfected with either control siRNA or anti-endoglin siRNA in serum-free RPMI growth media for 12 hours, followed by maintenance in 10% FBS/RMPI. Cells were trypsinized 96 hours

following transfection, washed twice in PBS, and then resuspended in 200μL 1x binding buffer containing 5μL of Annexin V. 10μL of PI was added, cells were incubated for 10 minutes at RT in the dark. Fluorescent signal (FITC and PI) in cells were analyzed by FACS and data were analyzed with FlowJo v.7.6.1 (Ashland, OR).

Alkaline comet assay. ES2 cells (n=400,000 in 6-well plate) were transfected with endoglin and control siRNA. Twenty-four hours following transfection, cells were exposed to cisplatin without supplemental SVF at a concentration of 1μM (the approximate IC80 level for this line) for either 1 or 4 hours, carefully rinsed to remove the drug, and cultured in regular media. Vehicle or control siRNA were included in all experiments. At the indicated time points, cells were collected and subjected to alkaline comet assay according to the manufacturer's instructions (catalog # 4250-050-K; Trevigen). Briefly, cells were combined with low melting agarose onto CometSlides (Trevigen). After lysis, cells were subjected to electrophoresis and stained with SYBR green. Subsequently, cells were visualized using fluorescent microscopy (Carl Zeiss, Thornwood, NY). At least 200 comet images were analyzed for each time point using Comet Score software (version 1.5; TriTek Corp.). The number of tail-positive cells with small and large nuclei was manually counted by an examiner blinded to treatment group, and expressed as a percentage of all cells evaluated. Experiments were repeated in triplicate.

γ-H2AX foci formation. ES2 cell lines were cultured and seeded on sterile cover slips. Twenty-four hours following transfection with control or anti-endoglin siRNA, cells were exposed to 1μM cisplatin for either 1 or 4 hours, carefully rinsed to remove the drug, and cultured in regular media. Following the treatment period, IHC was performed as previously described[12-13] with

slight modification for foci staining. Briefly, cells were rinsed in phosphate buffered saline (PBS) and incubated for 5 minutes at 4°C in ice-cold cytoskeleton buffer (10mM Hepes/KOH, pH 7.4, 300mM sucrose, 100mM NaCl, 3mM MgCl2) supplemented with 1mM PMSF, 0.5mM sodium vanadate and proteasome inhibitor (Sigma, 1:100 dilution) followed by fixation in 70% ethanol for 15 minutes. The cells were blocked and incubated with primary antibody (1:500 dilution, anti-phosphoH2AX Ser139, Millipore, catalog # MI-07-164). The secondary antibody was anti-rabbit Alexa Fluor 488–conjugated antibody (1:2000 dilution; Invitrogen). DAPI (Invitrogen, catalog # D21490) was used for nuclear staining. The cover slips were subsequently mounted onto slides with mounting media (Aqua poly mount, Polysciences, Inc. catalog # 18606) and analyzed via fluorescence microscopy (Carl Zeiss, Thornwood, NY). Positive and negative controls were included on all experiments. A total of 500 cells were assessed. For foci quantification, cells with greater than 10 foci were counted as positive according to the standard procedure. Experiments were repeated in triplicate.

RNA extraction from cell lines. Total RNA was isolated from ovarian cancer cell lines using Trizol reagent (Invitrogen, Carlsbad, CA) per manufacturer's instructions. RNA was then DNase treated and purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA was eluted in 50 μL of RNase-free water and stored at -80°C. The concentration of all RNA samples was quantified by spectrophotometric absorbance at 260/280 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT).

DNA repair qPCR array. ES2 and HeyA8 cells in culture were exposed to siRNA against endoglin in Lipofectamine 2000 as described above. After 48 hours, cells were collected and

mRNA extracted. Two replicates per cell line were performed. These four samples were then subjected to a quantitative PCR array consisting of 84 genes from DNA damage/repair pathways (plus additional housekeeping genes; the RT2 Profiler PCR Array Human DNA Damage Signaling Pathway, SA Biosciences Cat# PAHS-209Z, performed per manufacturer's instructions). Briefly, extracted RNA was converted to cDNA and amplified using the RT^2 FFPE PreAMP cDNA Synthesis Kit (SABiosciences, Frederick, MD). Quality of cDNA was confirmed with the Human RT^2 RNA QC PCR Array (SABiosciences). Gene expression was analyzed using the Human DNA Damage Signaling Pathway RT^2 Profiler PCR Array (SABiosciences), which profiles the expression of 84 genes involved in pluripotent cell maintenance and differentiation[14]. Functional gene groupings consist of the ATM/ATR signaling, nucleotide excision repair, base-excision repair, mismatch repair, double strand break repair, apoptosis, and cell cycle checkpoint regulators. PCR amplification was performed on an ABI Prism 7900HT sequence detection system and gene expression was calculated using the comparative C_T method[15].

Reverse transcription and quantitative PCR. Extracted RNA samples were diluted to 20 ng/μL using RNase-free water. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA samples were analyzed using quantitative PCR. Primer and probe sets for *ENG* (PPH01140F) *ATM* (PPH00325C), *BARD1* (PPH09451A), *DDIT3* (PPH00310A), *H2AFX* (PPH12636B), *NBN* (PPH00946C), *NTHL1* (PPH02720A), *PPP1R15A* (PPH02081E), *SIRT1* (PPH02188A), *ATP7B* (PPH06148A), and *RPLP0* (Hs99999902_m1, housekeeping gene) were obtained from SABiosciences and used according to manufacturer's instructions. PCR amplification was performed on an ABI Prism

7900HT sequence detection system and gene expression was calculated using the comparative C_T method.

Orthotopic Mouse Model. Female athymic nude mice (nu-nu) were obtained from the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). Mice were cared for in accordance with American Association for Accreditation of Laboratory Animal Care guidelines, the United States Health Services Commissioned Corps "Policy on Human Care and Use of Laboratory Animals," and University of Alabama at Birmingham Institutional Animal Care and Use Committee policies. ES2 tumors were established by intraperitoneal (IP) injection of $1x10^6$ cells suspended in 200μL of serum free RPMI media. Hey8MDR tumors were established in a similar way, using $5x10^5$ cells. To evaluate the effectiveness of endoglin-targeted therapy *in vivo,* siRNA was incorporated into chitosan nanoparticles as previously described.[16-17] Therapy was initiated 1 week after tumor cell injection. Mice were randomized to one of four treatments (n=10 per group): a) control siRNA alone (150 ug/kg twice weekly injected IV), b) control siRNA with IP carboplatin (160 mg), c) anti-endoglin siRNA (150 ug/kg twice weekly) alone, or d) anti-endoglin siRNA with carboplatin. All treatments were suspended in 100 μL 0.9% normal saline (NS). Mice were monitored for adverse effects, and all treatment groups sacrificed when control mice became uncomfortable with tumor burden. ES2 tumors behaved aggressively, and were harvested following 2 weeks of treatment. Hey8MDR tumors were harvested after 3 weeks of therapy. Mouse weight, ascites volume, tumor weight and distribution of tumor were recorded. Representative tumor samples were obtained from 5 mice in each treatment group, formalin-fixed, paraffin-embedded, and cut into 5 micron sections for evaluation of Proliferating Cell Nuclear Antigen (PCNA), Terminal deoxynucleotidyl transferase

mediated dUTP Nick End Labeling assay (TUNEL), γ H2AX (phosphorylation of Histone 2A protein) and 53BP1 (a mediator of the DNA damage checkpoint).

Tumor PCNA Immunohistochemistry and TUNEL. Sections were deparaffinized and re-hydrated, and antigen retrieval was performed with citrate buffer (pH 6.0) in pressure cooker for 5 minutes. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide solution in methanol for 15 minutes. Sections were blocked with CytoQ immune diluent and block and probed with PCNA primary antibody (PCNA-PC10, Cell signaling Technology, 1:5000 dilution) at 4°C overnight. Sections were washed and incubated with the Mach 3 mouse HRP polymer system. After rinsing, the sections were incubated with DAB chromophoric solution (Scytek Labs, Utah, USA) for 5 min at room temperature, then counterstained with Gill's hematoxylin (Ricca chemicals). Four 40x microscopic fields were counted from each section, averaged over 5 mice in each treatment group, and expressed as a percentage of the total number of tumor cells. Apoptosis was determined by TUNEL assay with a colorimetric apoptotic cell detection kit (Promega), per manufacturer's instruction. As with PCNA IHC, 4 microscopic fields at 40x magnification were evaluated from each section. Stained cells were recorded as a percentage of the total number of tumor cells.

Tumor γH2AX and 53BP1 IHC. Formalin fixed tissues were heated at 60°C for 1hr and rehydrated according to standard protocol. Subsequently, the tissues were permeabilized in 0.5% Triton X-PBS for 10 min, blocked in 2% BSA-0.1% Triton-X-PBS for 1 hr, and incubated with primary antibodies (1:500 dilution, anti phospho H2AX Ser139, Millipore, catalog # MI-07-164; 1:500 dilution, anti-53BP1, Novus Biologicals, catalog # NB100-304). The secondary antibody

was anti-rabbit Alexa Fluor 488–conjugated antibody (1:2000 dilution; Invitrogen). DAPI (Invitrogen, catalog # D21490) was used for nuclear staining. The slides were subsequently mounted using mounting media (Aqua poly mount, Polysciences, Inc. catalog # 18606) and analyzed via fluorescence microscopy (Carl Zeiss, Thornwood, NY). Positive and negative controls were included on all experiments. A total of 500 cells were assessed. For foci quantification, cells with greater than 10 foci were counted as positive according to the standard procedure. Experiments were repeated in triplicate. Data show the mean and SEM.

Statistics. Analysis of normally distributed continuous variable was performed using a two-tailed Student's t-test. Those data with alternate distribution were examined using a nonparametric Mann-Whitney U test. A $p<0.05$ was considered statistically significant.

Results

Effects of endoglin downregulation on cell viability and platinum sensitivity. Endoglin is expressed by multiple ovarian cancer cell lines (Figure 1A), most prominently in HeyA8, HeyA8MDR, and ES2 cells. Weak expression was detected in the HIO-180, A2780ip2, A2780cp20, SKOV3ip1, SKOV3TRp2, and IGROV-AF1 cell lines. This was previously demonstrated at the mRNA level by quantitative PCR[11]. To confirm that expression was predominantly at the cell surface, consistent with its function as a co-receptor for TGFβ, we performed immunohistochemistry on the ES2 and HeyA8MDR cell lines. Surprisingly, the predominant staining was noted n the perinuclear cytoplasm (Figure 1B). This was confirmed by flow cytometry, where interestingly not only was membranous staining rare, but there was a very distinct separate population with 100-fold fluorescent intensity (rather than a global shift among

all cells), consistent with a separate small population of cells with strong endoglin surface expression (Figure 1C). This population represented 6.0% of HeyA8MDR and 5.4% of ES2 cells. On close examination of IHC on cultured cells, a minority of the cells could be seen to have strong membranous expression of CD105 (arrows, Figure 1B). A separate endoglin-positive population has previously been noted in renal cell carcinoma cells, which did exhibit stem-cell properties.[18] However, these data are conclusive that the majority of endoglin expression in ovarian cancer is cytoplasmic, suggesting a role other than just as a co-receptor for TGF-beta.

To determine whether siRNA-mediated downregulation of endoglin had significant effects on viability and chemosensitivity, two different siRNA constructs (ENG_A siRNA and ENG_B siRNA) were examined. Both effectively reduced endoglin expression at 48 hours at the mRNA (Figure 1D) and protein level[11]). Both were previously shown to reduce cell viability[11]. To determine the mechanism by which endoglin knockdown reduced viability, evaluation of apoptosis was performed by the TUNEL assay. Annexin V/PI co-fluorescent staining performed 48 hours following transfection indicated significantly fewer viable cells in those treated with anti-endoglin siRNA than those treated with control siRNA (47.2% vs. 65.1%, $p<0.05$). A sample flow cytometry plot and a graph of average over three experiments are shown in Figure 1D. Those treated with anti-endoglin siRNA had increased percentages of cells in both early apoptosis (21.5% vs. 17.9%, $p<0.05$) and late apoptosis (18.9% vs. 12.0%, $p<0.05$). Effects were more pronounced when combined with cisplatin. In order to determine whether Endoglin knockdown had an effect on viability in combination with chemotherapy, cells were exposed to siRNA, then re-plated after 24 hours, and incubated with increasing concentrations of cisplatin or paclitaxel. Because endoglin downregulation alone was associated with substantial cell death in the HeyA8MDR model, knockdown was performed with several dilutions of siRNA in an effort

to more clearly delineate effects on platinum sensitivity. In both ES2 (normal IC50 for cisplatin = 0.7μM) and HeyA8MDR (normal IC50 for cisplatin = 0.65μM) models, increased cisplatin chemosensitivity was noted (up to 4-fold and 2-fold reduction in IC50, respectively, Figure 1E). Similar experiments were performed with paclitaxel, which did not show an increased sensitization with endoglin downregulation (data not shown).

Downregulation of endoglin induces DNA damage *in vitro*. Because platinum toxicity is mediated primarily through induction of DNA damage, we evaluated whether the enhanced cisplatin sensitivity from endoglin knockdown was a result of increased DNA damage. DNA damaging agents can induce both single-stranded breaks (SSBs) and double stranded breaks (DSBs) which can lead to initiation of apoptotic pathways. DNA damage in the ES2 line was first assessed via an alkaline comet assay, which detects both SSB and DSB. As quantified in Figure 2A, increased DNA damage over 24 hours was observed with cisplatin, endoglin downregulation with siRNA, and the combination (although combination therapy was not significantly increased compared to either single-agent treatment). A representative section demonstrating common effects on nearly all cells is shown (Figure 2B). Because a long comet tail can be the result of either DNA damage without death or apoptosis-associated DNA release, the nucleus size was also quantified. A small nucleus would be associated with apoptosis, whereas a long comet tail associated with a normal (larger) nucleus would indicate just DNA damage. As shown in Figure 2C for cells treated for 24 hours, those cells with a long tail present predominantly still had a large nucleus. Because most toxic effects on viability noted previously were assessed at 48 hours or longer, this DNA damage may be a precursor to apoptosis

induction. But it does demonstrate that DNA damage is the inciting event, rather than a result of apoptosis triggered by other mechanisms.

To further characterize the specific nature of DNA damage, development of foci of activated γ-H2AX was performed (Figure 2D). ES2 cells were employed, due to the rapid toxicity and cell death noted with endoglin downregulation with HeyA8. Phosphorylation of the histone protein H2AX on serine 139 (γ-H2AX) occurs at sites flanking DNA DSBs. The phosphorylation of thousands of H2AX molecules forms a focus in the chromatin flanking the DSB site that can be detected *in situ*. A higher proportion of cells with persistent γ-H2AX foci was noted with endoglin downregulation, to an even greater extent than cisplatin alone. The combination of cisplatin and endoglin downregulation induced more DSB repair than either agent alone. Collectively, these data suggest that a primary mechanism of DNA damage after endoglin downregulation is through induction of double-strand breaks in DNA.

Endoglin-targeting DNA damage is through effects on multiple mediators of DNA repair. In order to determine the mechanism by which downregulation of endoglin induces DNA damage, we first subjected both ES2 and HeyA8MDR cells treated with control siRNA or endoglin-siRNA for 48 hours to a qPCR-based array of 84 genes participating in DNA damage and repair pathways. This exploratory analysis found multiple genes that were either downregulated or upregulated in response to decreased endoglin, some of which were only associated with changes in one cell line (Supplemental Table 1). Select genes were then chosen for confirmatory assessment with qPCR (Figure 3). Genes for these analyses were selected based on the degree to which they were altered, the associated p-value, and whether the change was noted in both cell lines. With endoglin downregulation, significant concurrent downregulation

was noted by qPCR in H2AFX (36-43%), BARD1 (47-71%), NBN (38-41%), NTHL1 (39-53%), and SIRT1 (34-49%). A significant induction of mRNA was noted in DDIT3 (1.9-2.6-fold) and PPP1R15A (1.27-1.74-fold). There was no single DNA repair pathway subclass that comprised all affected genes, but consistent with data from the γ-H2AX assay, most were participants in either the double stranded break repair (BARD1, H2AFX, NBN) or nucleotide excision repair (SIRT1, NTHL1).

The downregulation of BARD1 was particularly interesting. BARD1 is an oncogenic regulator of BRCA1, and downregulation would be expected to result in export of BRCA1 from the nucleus and impairment of DNA repair. Furthermore, BARD1 was noted to be significantly *upregulated* in chemoresistant tumor samples from patients, compared to their primary tumors.[11] BARD1 expression is prominent in ES2 and HeyA8MDR, which follows if it is under transcriptional regulation by endoglin. Therefore, we examined BARD1 induction in response to platinum treatment in a progressively platinum-resistant triad of cell lines derived from A2780: A2780ip2 (which generates IP tumors more consistently than the parental line but is chemosensitive), A2780cp20 (having a platinum IC50 of 20μM), and A2780cp55 (with an IC50 of 55μM). The A2780cp20 and cp55 lines are stably platinum-resistant, and not chronically maintained in platinum. BARD1 expression is minimal in the parental A2780ip2 line, but increases at baseline ("Untreated") with each degree of platinum resistance (Figure 3B). Additionally, when exposed to an IC50 concentration of carboplatin, BARD1 mRNA production is significant increased in both A2780ip2 and A2780cp20. Levels were unchanged with carboplatin exposure in A2780cp55, likely due to its high baseline expression. A significant reduction in BARD1 with endoglin downregulation and an induction of BARD1 in response to platinum exposure strongly implicate this gene and its control on BRCA1 as a major mechanism

through which endoglin downregulation may lead to DNA damage, apoptosis, and sensitivity to platinum.

In addition to enhanced DNA repair mechanisms, a major mechanism of platinum resistance is through increased export of platinum agents through copper transporters such as ATP7B.[19] Therefore we also examined the effects of endoglin downregulation on ATP7B by qPCR. SiRNA-mediated targeting of endoglin resulted in a significant downregulation of ATP7B (by 20-24%, $p<0.05$, Figure 3C). While significant, this was not to the same extent many DNA repair genes were induced or activated.

Evaluation of tumor growth with anti-endoglin treatment in an orthotopic murine model. In order to determine if endoglin downregulation was an effective therapy *in vivo*, an orthotopic murine model was utilized using human specific anti-endoglin siRNA delivered within a chitosan nanoparticle. Chitosan (CH) is a natural nanoparticle that has been previously demonstrated to result in efficient delivery of siRNA to tumor after IV administration, with subsequent protein downregulation and gene-specific modulation.[16, 20-22] Because the siRNA delivered is specific to the human endoglin mRNA, any observed effect would be expected to be due to targeting the tumor cells, rather than the vasculature, which would require murine-specific siRNA. ES2 and HeyA8MDR cells were injected IP, and treatment was started 1 week later with a) control siRNA-CH alone, b) control siRNA-CH plus carboplatin, c) anti-endoglin siRNA-CH alone, or d) anti-endoglin siRNA-CH plus carboplatin. Carboplatin was used instead of cisplatin because of its preferable side-effect profile *in vivo*, which has led to its choice as standard of care in ovarian cancer patients. Tumors demonstrated reduced growth both with endoglin downregulation alone and in combination with platinum. In the ES2 model (Figure 4A), mice

treated with carboplatin had similar tumor burden to control (p=0.555), an expected result due to the highly platinum-resistant nature of the ES2 cell line, which is derived from a patient with clear cell carcinoma. Mice treated with anti-endoglin siRNA alone had a significantly reduced tumor weight, by 35.6% (p=0.014). Combined END-siRNA-CH with carboplatin was more effective that either agent alone, with a 57.7% reduction in tumor weight compared to control (p<0.001). Furthermore, combination therapy was more effective than siRNA-endoglin-CH alone, with an additional 34.3% reduction (p=0.033) In the HeyA8MDR model (Figure 4B), mice treated with carboplatin, endoglin-siRNA-CH, or combination therapy had significantly less tumor weight when compared to control (34% reduction p=0.027, 41.2% reduction p=0.002, and 61.2% reduction p<0.01, respectively). Those treated with carboplatin and control siRNA-CH had similar tumor burden reduction as those treated with endoglin-siRNA-CH (p=0.628). Combination therapy was again more effective than either single-agent carboplatin (additional 40.6% reduction, p=0.069), or endoglin-siRNA alone (34%, p=0.048). In the resected tumors, reduced expression of endoglin was confirmed with immunohistochemistry, in both groups of tumors treated with endoglin-siRNA-CH. Representative sections are pictured (Figure 4C). With both models, there was not a significant difference in mouse weight in any group. The distribution of tumor was also similar in all groups, suggesting there was not a significant effect on particular site of growth, adhesion, or migration.

Endoglin downregulation induces DNA damage and apoptosis *in vivo*. Our *in vitro* findings suggest a role of DNA damage and apoptosis following endoglin downregulation. To validate these findings *in vivo*, tumors from each treatment group described above were examined for proliferation, apoptosis, and induction of DNA damage. PCNA IHC was performed and revealed

no significant differences in percentage of PCNA positive cells, with approximately half of cells being positive in each treatment group (Figure 5A). A lack of effect on progression through the cell cycle and proliferation may explain why combination with taxanes was not synergistic with endoglin downregulation *in vitro*. TUNEL assay was performed to evaluate to detect differences in apoptosis between treatment groups. Control, carboplatin and anti-endoglin siRNA groups were not significantly different. However, the cohort receiving combination therapy had a significantly higher percent of apoptotic cells when compared to control ($p<.001$, Figure 5B). This increase, though statistically significant, is relatively small, which may be due to clearance of dead cells over the course of the 4-week experiment. To determine if DNA damage was still noted in the tumors collected at completion of therapy, fluorescent IHC was performed to evaluate for γ-H2AX as an indicator of *in vivo* DSB. A significantly higher amount of DNA damage was detected in both treatment groups receiving anti-endoglin treatment than either control or single-agent carboplatin treatment (Figure 5C). Additionally, 53BP1 is a mediator of DNA damage response and a tumor suppressor whose accumulation on damaged chromatin promotes DNA repair and enhances DNA damage response signaling. A significantly higher number of 53BP1-positive cells was noted in both cohorts that received anti-endoglin treatment when compared to either control or single-agent platinum (Figure 5D). These data are consistent with *in vitro* studies demonstrating that endoglin downregulation alone leads to DNA damage and apoptosis.

Discussion

Endoglin is overexpressed in solid tumor vasculature and is a reliable marker of angiogenesis.[5] Multiple anti-angiogenic therapies have been studied in ovarian cancer, and anti-

endoglin therapy has been proposed for several cancers in which increased endothelial endoglin expression has been noted.[23] However, to date, few studies address the expression of endoglin on tumor cells and its potential role in cancer progression. Building off our previous findings that Endoglin is increase in recurrent samples when compared to matched primary tumors[11], we have demonstrated that endoglin expression is highly expressed in many ovarian cancer cell lines, and that downregulation results in induction of cell death through induction of DNA damage and a synergistic killing effect with platinum agents both *in vitro* and *in vivo*. These novel findings demonstrate that therapeutics targeting endoglin may affect both the vasculature and malignant cells within the tumor microenvironment.

The primary canonical role of endoglin is as a co-receptor for TGF-beta.[24-26] As such, its expression on endothelial cells is primarily on the cell membrane.[27] However, we interestingly found endoglin expression in ovarian cancer cells was predominantly cytoplasmic, and clustered together in the perinuclear region of the cell. This would suggest that endoglin either has a separate TGF-beta-independent function dependent on nuclear proximity, or trafficking to the cell membrane is an important component of its regulation. Only a small (5-6%), but well-defined population had surface expression. This distinct population would be consistent with a cancer stem cell-like population, as has been previously described in endoglin-positive renal cell carcinoma[18]. Endoglin-positive meningioma cells have similar increased tumorigenicity and capacity to differentiate into adipocytes and osteocytes.[28]

Henriksen et al. evaluated endoglin expression in primary ovarian cancer cells and found that high tumor cell endoglin staining correlated with short overall survival.[29] Another group has shown that cells from cultured ascites that progressed towards a mesenchymal phenotype were high in endoglin.[30] We identified endoglin as a potential target for therapeutics through a screen

of stem cell pathways overexpressed in recurrent ovarian cancer samples. Among members of the TGF-β, Notch, Wnt, and Hedgehog pathways, endoglin was most significantly and consistently overexpressed in recurrent ovarian cancer samples when compared to their matched primaries, suggesting a role in chemoresistance.[11] We specifically examined stem cell pathways to address the question of whether the cancer stem cell population may be responsible for surviving initial chemotherapy. Endoglin has previously been implicated in stem cell biology, having originally been described on hematopoietic progenitor cells[31], and later demonstrated to identify precursor cells capable of tissue-specific differentiation[32-33].

It makes sense that cells with prolonged survival, such as stem/progenitor cells, would reply on pathways to mediate DNA damage. Because of the association noted with increased endoglin expression in platinum (and taxane)-resistant recurrent ovarian cancers,[11] and the contribution of enhanced DNA repair for platinum resistance,[19] we further examined the contribution of endoglin to DNA repair. We have found a previously unknown contribution of endoglin to expression of numerous DNA repair genes. These encompass several subtypes of DNA repair, predominantly double stranded break repair (BARD1, H2AFX, NBN), but also nucleotide excision repair (SIRT1, NTHL1), and cell cycle arrest (DDIT3, PPP1R15A), which may be a reactionary process in order to accomplish DNA repair. Recently BARD1 has been implicated in ovarian cancer pathogenesis for its interaction with BRCA1 and 2. BARD1 and BRCA1 interact with each other through their amino terminal RING finger domains. This interaction is required for BRCA1 stability, as well as for nuclear localization. The BRCA1-BARD1 complex serves as an E3 ubiquitin ligase, which has been noted to have critical activity in both the cell cycle check point through H2AX, NPM and γ-tubulin and in DNA fragmentation.[34-35] Additionally, patients with mutations of both BARD1 and BRCA2 have a

substantially increased risk for development of both breast and ovarian cancer. While BARD 1 has been found to interact and co-localize with BRCA1 at the spindle poles in early mitosis, it also interacts with BRCA2 at late mitosis in the midbody. Therefore BARD1 has been found to sequentially link the function of these[36] two proteins. In our analysis, BARD1 expression was reduced by 50-75% and H2AX expression was reduced 35-50% following endoglin knockdown. endoglin-mediated downregulation of BARD1 and its subsequent effects on BRCA1 and 2 and H2AX may therefore explain why we found substantial decreased cell viability, DNA damage and increased apoptosis.[34]

Silent Information Regulator Type 1 (SIRT1) is a nicotinamide adenine dinucleotide-dependent class III histone deacetylase (HDAC). SIRT1 has is associated with longevity and has been found to act primarily by inhibiting cellular senescence. SIRT1 is up-regulated in tumor cell lines and human tumors, and may be involved in tumorigenesis.[36] It has also been found to be over-expressed in chemoresistant tumors of cancer patients. SIRT1 inhibition leads to decrease in MDR1 expression and increase in drug sensitivity in ovarian cancer cell lines.[37] Our research suggests that Endogin knockdown was associated with a 30-50% reduction in SIRT1. This inhibition may help account for the increased platinum sensitivity we found with endoglin downregulation.

In regards to therapeutic development in cancer patients, delivery of siRNA constructs has the potential to offer long duration of target inhibition as well as reduced toxicity compared other approaches.[16, 20, 38-44] However, development of a delivery modality for siRNA constructs remains the rate-limiting step in translational research. Early delivery modalities included delivery of "naked" siRNA. Later attempts included high-pressure siRNA injections and intratumoral injections, neither of which has demonstrated substantial success. The development

of chitosan encapsulation and nanoliposomes to deliver siRNA has become widely accepted in translational studies and is and promising as a therapeutic modality as modifications to enhance in vivo delivery progress.[22] SiRNA mediated therapeutics are being used in ongoing trials with patients with macular degeneration, AIDS, malignant melanoma, acute renal failure, hepatitis B, and now in cancer patients, where phase I trials are in development. One particular advantage of siRNA-based therapeutics over conventional treatment modalities would apply to endoglin-based targeting. If indeed the cytoplasmic portion of endoglin is important to chemoresistance, downregulation of production at the mRNA level may be more effective than antibody-based targeting currently aimed at inhibiting angiogenesis.[45-46]

Because of the rarity of endoglin expression in normal tissues, anti-endoglin therapy has the potential to offer tumor-directed therapy in addition to anti-angiogenic therapy. Anti-endoglin therapy is being explored as a therapeutic in several cancers as an anti-angiogenic agent. In ovarian cancer, endoglin-targeted therapies may offer the additional advantage of targeting tumor cells overexpressing endoglin, including platinum-resistant tumors. Its effects on BRCA1 and 2 and H2AX through BARD1 downregulation, and its association with SIRT1 downregulation contribute to DNA damage repair and enhancement of platinum sensitivity. Our data strongly suggest that endoglin-targeted therapy has the potential to improve platinum sensitivity through induction of DNA damage and should be actively pursued as a potential therapy in the treatment of ovarian cancer.

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FIGURE LEGENDS

FIGURE 1. A) Endoglin expression in multiple ovarian cancer cell lines, as measured by Western blot. B) As assessed by IHC, endoglin expression is predominantly cytoplasmic, though some cells with strong membranous staining are noted (arrows). C) A small but distinct endoglin-positive population is seen by flow cytometry. D) Endoglin was effectively downregulated with siRNA. By TUNEL assay, Annexin V/PI co-fluorescence demonstrate a decrease in viable cells, and an increase in both early and late apoptosis, both alone and in combination with cisplatin. E) Cells treated with increasing doses of cisplatin after endoglin downregulation were also assessed by MTT, with the OD570 reflecting the absorbance produced by viable cells. Endoglin downregulation resulted in a significant reduction in cell viability, and increased cisplatin chemosensitivity about 4-fold in ES2 model and 2-fold in HeyA8MDR. Lines denoting the calculated IC50 for control and endoglin-siRNA treatment are shown (grey lines).

FIGURE 2. ES2 cells were evaluated for DNA damage after endoglin targeting. SiRNA-mediated endoglin downregulation induces significant persistent DNA damage, as indicated by alkaline comet assay mean tail moment (A), and visually at 24 hours (B, Original magnification, ×100). This is not a result of immediate apoptosis, as demonstrated by a predominance of large nuclei despite a prominent comet tail (C). Downregulation also induces activation of γ-H2AX foci, a specific measure of double-stranded DNA damage (D). The combination of endoglin downregulation and cisplatin on induction of γ-H2AX foci was greater than either agent alone. Error bars represent SEM.

FIGURE 3. A) ES2 and HeyA8MDR cells were exposed to endoglin-targeting siRNA or control siRNA, mRNA extracted 48 hours later, and subjected to quantitative PCR for selected genes. Each collection was performed in triplicate, and the mean change over housekeeping gene presented. Significant decreases were noted in H2AFX, BARD1, NBN, NTHL1, and SIRT1. Induction of DDIT3 and PPP1R15A was also significant. B) BARD1 mRNA was assessed by qPCR in a triad of progressively platinum-resistant A780 cell lines, and noted to be significantly increased in A2780cp55 at baseline, and in A2780ip2 and A2780cp20 with exposure to carboplatin. C) The copper transporter ATP7B was also modestly, but significantly, reduced with endoglin downregulation.

FIGURE 4. An orthotopic murine model using ES2 and HeyA8MDR cell lines was employed to evaluate treatment with control siRNA-CH alone, control siRNA-CH with carboplatin, anti-endoglin siRNA-CH alone, or anti-endoglin siRNA-CH plus carboplatin. A) In the ES2 model, carboplatin was ineffective, as expected given the platinum-resistant nature of the ES2 cell line. Mice treated with anti-endoglin siRNA-CH alone and combined with carboplatin demonstrated less tumor burden when compared to control or carboplatin alone. Those treated with both anti-endoglin siRNA-CH and carboplatin also demonstrated reduced tumor burden when compared to those endoglin-siRNA-CH alone ($p=0.03$). B) In the HeyA8MDR model, tumors were smaller in mice treated with carboplatin or anti-endoglin siRNA-CH alone, and again combination therapy was more effective than either agent alone ($p<0.05$). C) By qualitative assessment with IHC, endoglin expression was reduced in the tumors treated with endoglin-siRNA-CH therapy.

FIGURE 5. Tumors from each treatment group in our orthotopic mouse model were collected and analyzed by PCNA immunohistochemistry, TUNEL assay, γ-H2AX IHC and 53BP1 IHC. A) There were no significant differences in PCNA IHC, with approximately half of cells being positive. B) There was a significant increase in apoptosis in the cohort receiving combination therapy when compared to control as demonstrated by TUNEL assay. C) Fluorescent IHC was performed to evaluate for γ-H2AX as an indicator of DNA damage. There was a significantly higher amount of DNA damage in both treatment groups receiving anti-endoglin treatment when compared to control or single-agent carboplatin. D) Lastly, 53BP1 is a key protein in the DNA damage checkpoint that was evaluated by IHC. A significantly higher amount of 53BP1 was noted in both cohorts that received anti-endoglin treatment when compared to either control or single-agent carboplatin.

Figure 1.

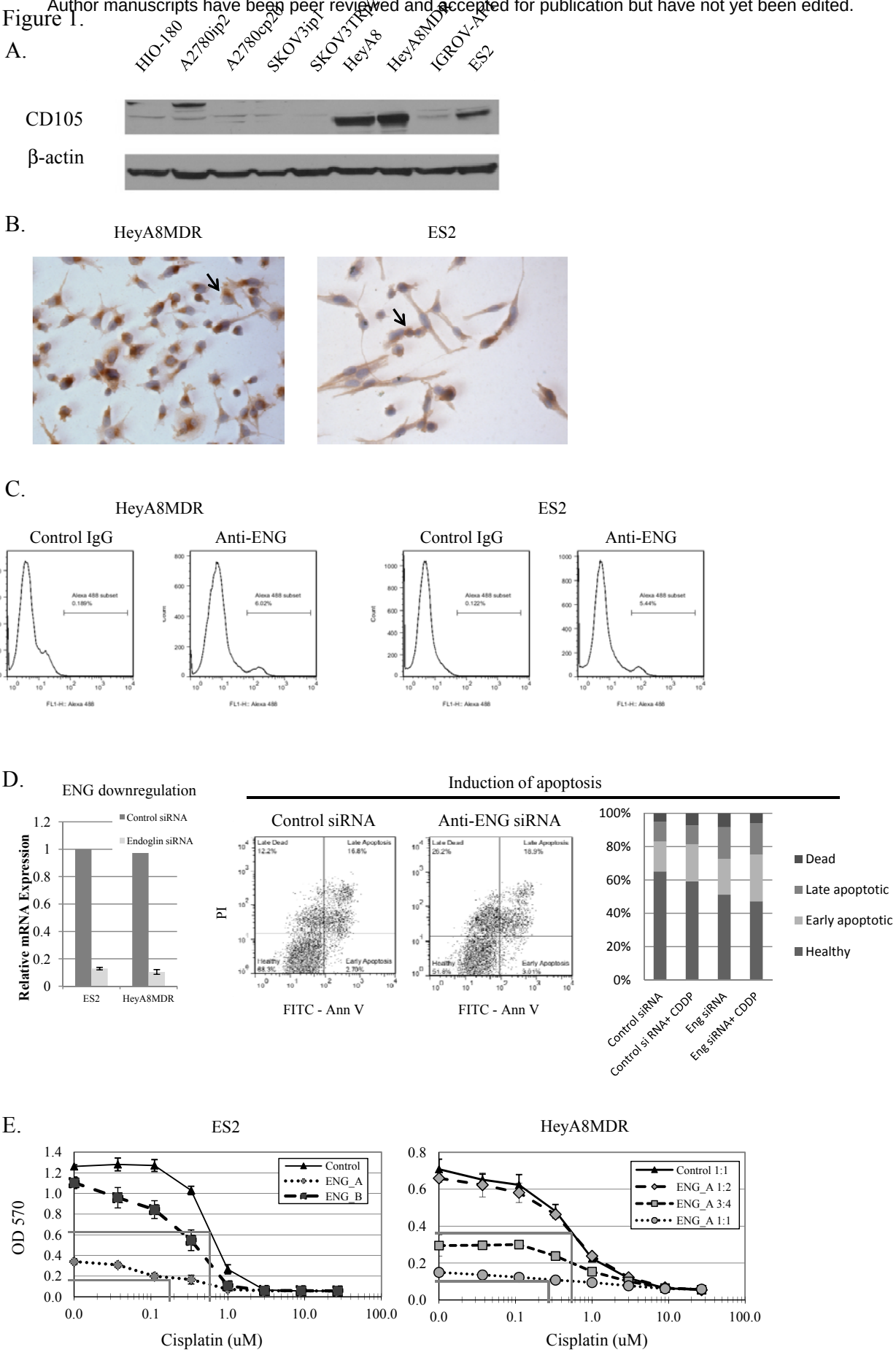


Figure 2.

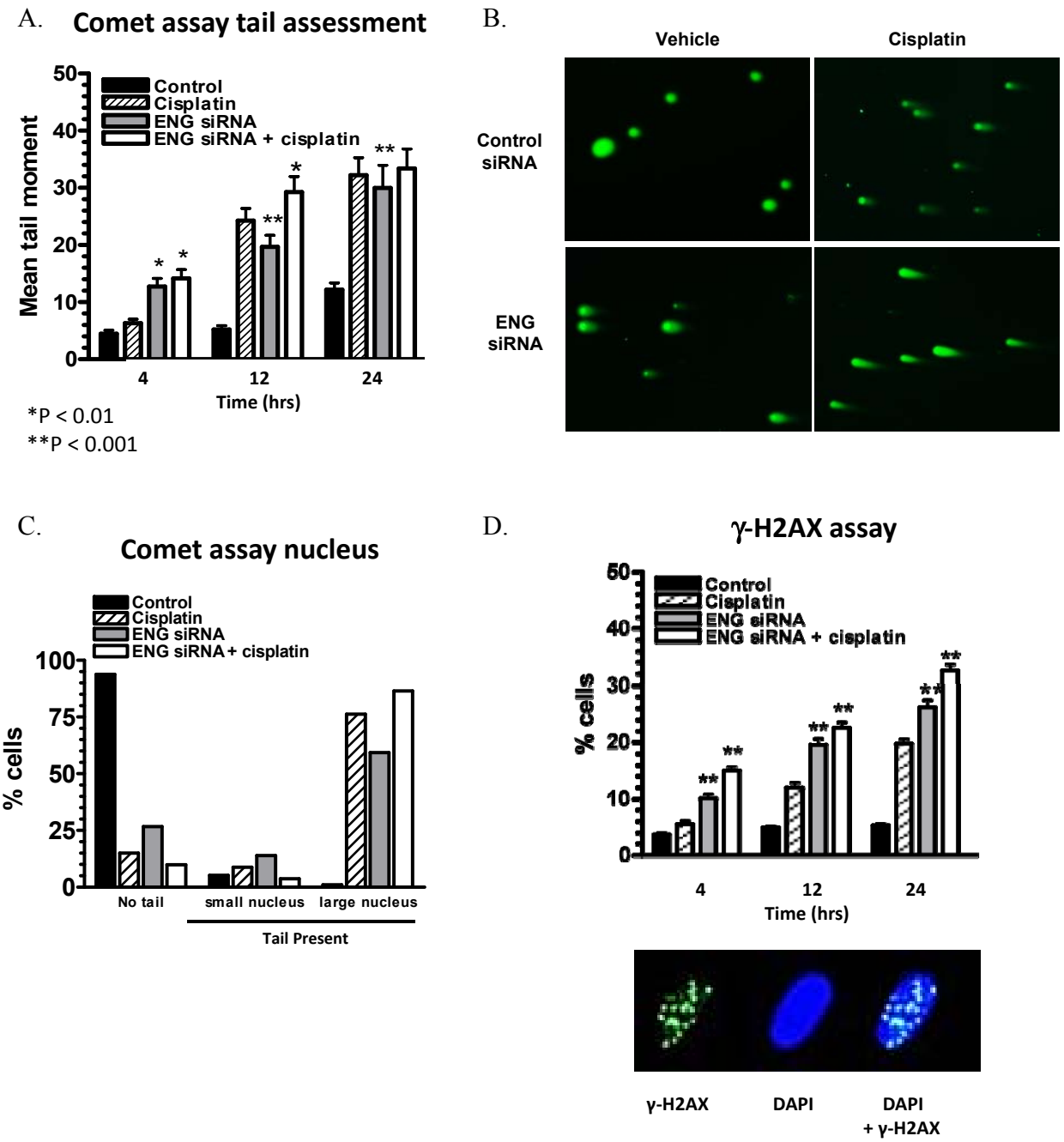


Figure 3.

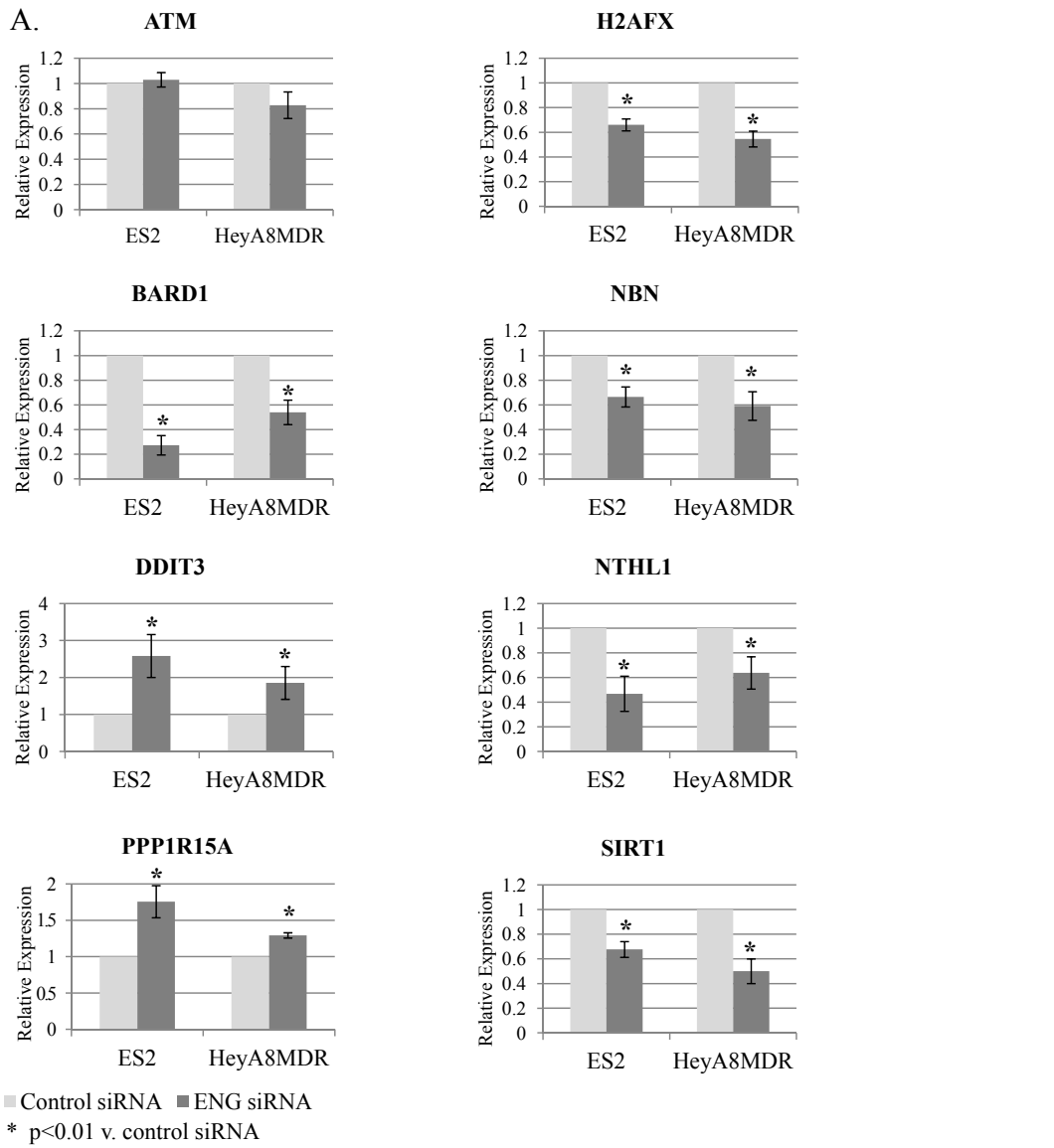
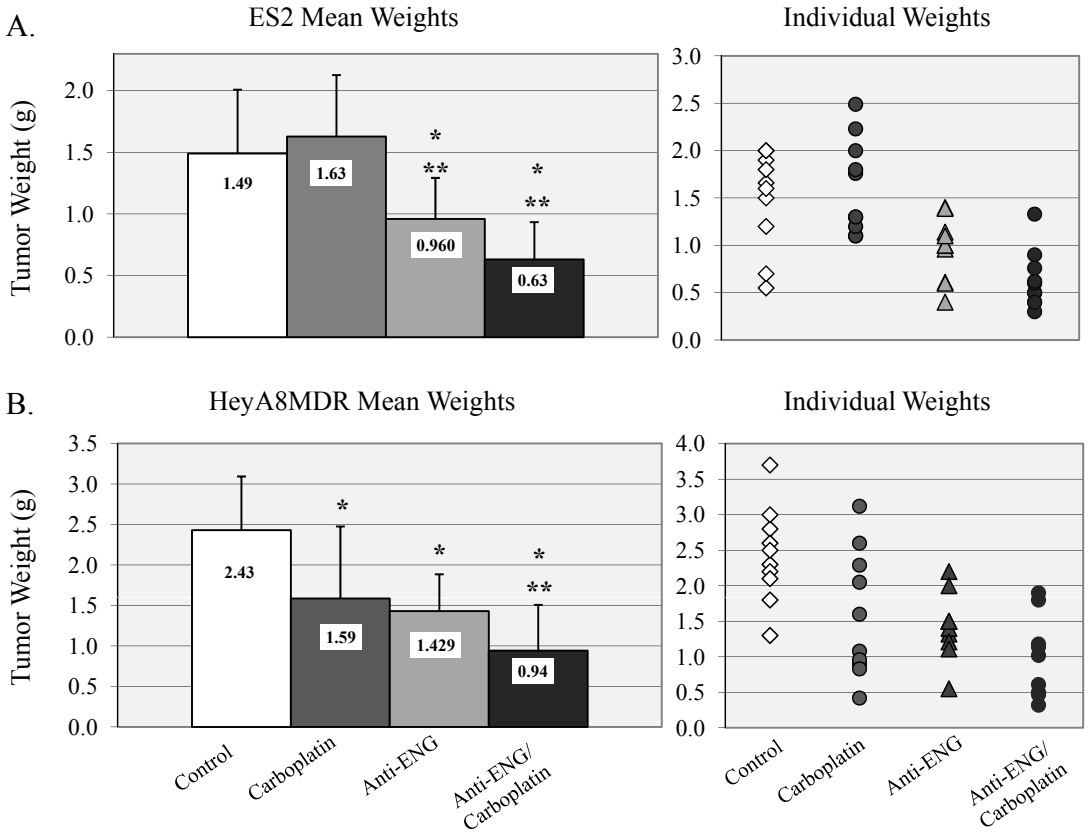


Figure 4.



* - p<0.05 v. control
** - p< 0.05 v. carboplatin

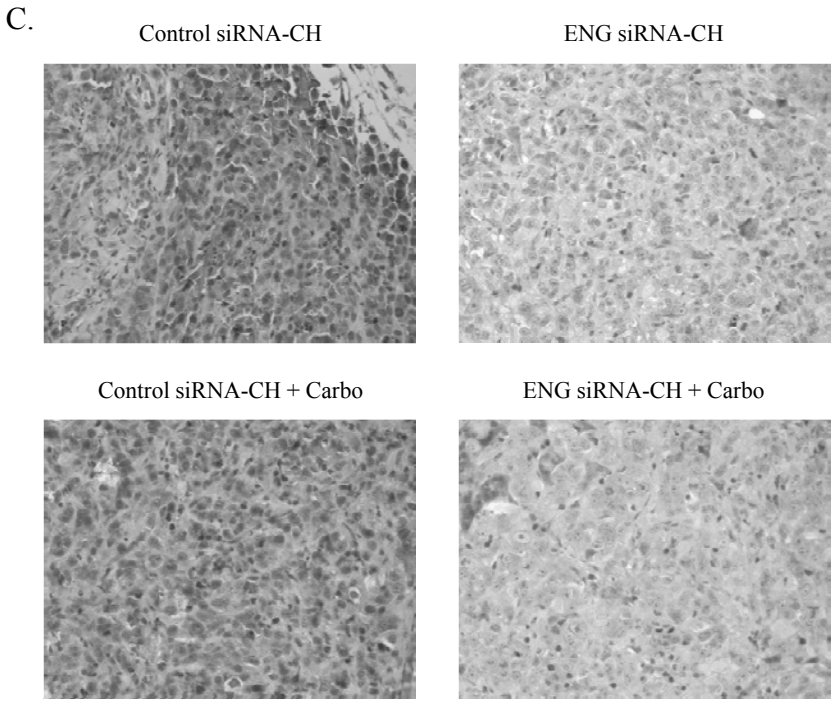
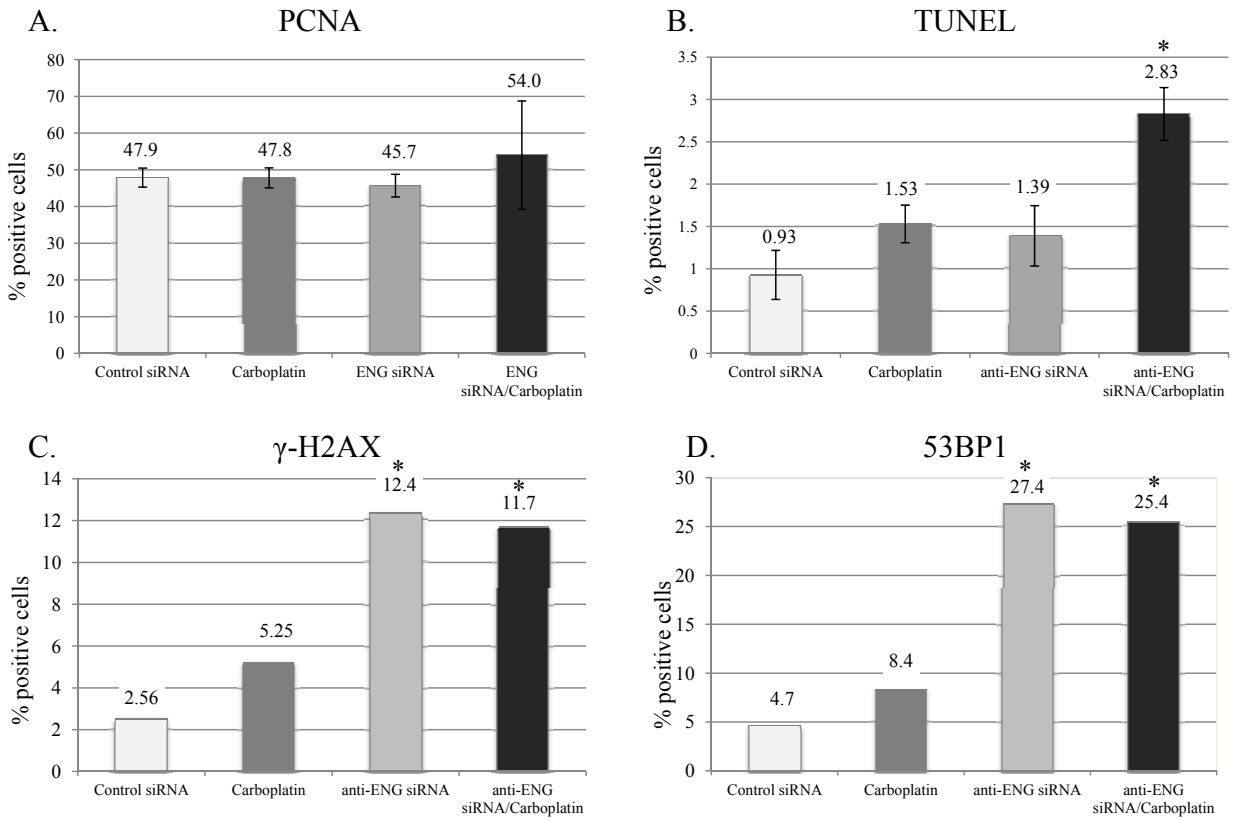


Figure 5.



* $P < 0.01$ compared to control

Preclinical Development

Molecular Cancer Therapeutics

Smoothened Antagonists Reverse Taxane Resistance in Ovarian Cancer

Adam D. Steg, Ashwini A. Katre, Kerri S. Bevis, Angela Ziebarth, Zachary C. Dobbin, Monjri M. Shah, Ronald D. Alvarez, and Charles N. Landen

Abstract

The hedgehog (HH) pathway has been implicated in the formation and maintenance of a variety of malignancies, including ovarian cancer; however, it is unknown whether HH signaling is involved in ovarian cancer chemoresistance. The goal of this study was to determine the effects of antagonizing the HH receptor, Smoothened (Smo), on chemotherapy response in ovarian cancer. Expression of HH pathway members was assessed in three pairs of parental and chemotherapy-resistant ovarian cancer cell lines (A2780ip2/A2780cp20, SKOV3ip1/SKOV3TRip2, HeyA8/HeyA8MDR) using quantitative PCR and Western blot analysis. Cell lines were exposed to increasing concentrations of two different Smo antagonists (cyclopamine, LDE225) alone and in combination with carboplatin or paclitaxel. Selective knockdown of Smo, Gli1, or Gli2 was achieved using siRNA constructs. Cell viability was assessed by MTT assay. A2780cp20 and SKOV3TRip2 orthotopic xenografts were treated with vehicle, LDE225, paclitaxel, or combination therapy. Chemoresistant cell lines showed higher expression (>2-fold, $P < 0.05$) of HH signaling components compared with their respective parental lines. Smo antagonists sensitized chemotherapy-resistant cell lines to paclitaxel, but not to carboplatin. LDE225 treatment also increased sensitivity of ALDH-positive cells to paclitaxel. A2780cp20 and SKOV3TRip2 xenografts treated with combined LDE225 and paclitaxel had significantly less tumor burden than those treated with vehicle or either agent alone. Increased taxane sensitivity seems to be mediated by a decrease in P-glycoprotein (MDR1) expression. Selective knockdown of Smo, Gli1, or Gli2 all increased taxane sensitivity. Smo antagonists reverse taxane resistance in chemoresistant ovarian cancer models, suggesting combined anti-HH and chemotherapies could provide a useful therapeutic strategy for ovarian cancer. *Mol Cancer Ther; 1–11. ©2012 AACR.*

Introduction

Ovarian cancer is the leading cause of death from a gynecologic malignancy. Although ovarian cancer is among the most chemosensitive malignancies at the time of initial treatment (surgery and taxane/platinum-based chemotherapy), most patients will develop tumor recurrence and succumb to chemoresistant disease (1). Evaluation of multiple chemotherapy agents in several combinations in the last 20 years has yielded modest improvements in progression-free survival, but no increase in durable cures. This clinical course suggests that a population of tumor cells has either inherent or acquired resistance to chemotherapy that allows survival with initial therapy and ultimately leads to recurrence. Targeting the cellular pathways involved in this resistance may provide new treatment modalities for ovarian cancer.

The Hedgehog (HH) pathway plays an important role in cell growth and differentiation during embryonic development (2). There are 3 known mammalian HH ligands—Sonic, Indian, and Desert. These ligands are secreted peptides that bind to the transmembrane Patched (Ptch) receptor. In the absence of HH ligand, Ptch serves as a negative regulator of Smoothened (Smo), a G-protein–coupled receptor. In the presence of HH ligand, Ptch repression of Smo is abolished, leading to downstream activation of the Gli family of transcription factors (Gli1, refs. 2, 3). Gli transcription factors translocate from the cytoplasm to the nucleus, where they bind DNA and activate transcription of HH target genes, including *PTCH1* and *GLI1*, the expression of which are frequently measured to evaluate the presence or absence of HH pathway activity (3, 4). Gli homologues have distinct, but overlapping functions; Gli1 serves only as a transcriptional activator, whereas Gli2 and Gli3 are capable of both activating and repressing HH gene transcription.

Recent reports have implicated HH signaling in multiple malignancies (5, 6), including ovarian cancer (7–9), and suggest this pathway may be especially important in maintaining the subpopulation of cancer cells with stem

Authors' Affiliation: Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, Alabama

Corresponding Author: Charles N. Landen, Department of Obstetrics and Gynecology, The University of Alabama at Birmingham, 1825 University Boulevard, 505 Shelby Building, Birmingham, AL 35294. Phone: 205-934-0473; Fax: 205-934-0474; E-mail: clanden@uab.edu

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cell properties (10, 11) as well as conferring resistance to chemotherapies (12, 13). Inhibition of the HH signaling pathway, therefore, has become a desirable therapeutic strategy for the treatment of various cancers. Cyclopamine, a steroidal alkaloid derived from the lily plant *Veratrum californicum*, was the first compound identified that inactivates HH signaling by antagonizing Smo function (14–16). Since this discovery, pharmaceutical companies have synthesized more selective Smo antagonists, including NVP-LDE225 (17), which is currently being investigated in clinical trials (11).

The effects of Smo antagonists, both alone and in combination with chemotherapies, remains an active area of study in cancer research. Examination of combination effects is potentially important, given the hypothesized role of stem cell pathways in chemoresistance. However, the mechanisms by which HH inhibition might sensitize cells to chemotherapy, and whether such an approach would be effective in ovarian cancer, are not known. In our study, we sought to determine the effects of Smo antagonists on the viability of ovarian cancer cells, both alone and in combination with chemotherapy. We show that Smo antagonists have activity alone, but more dramatically can reverse taxane resistance in ovarian cancer, both *in vitro* and *in vivo*, through modulation of the multidrug resistance mediator, P-glycoprotein (MDR1). These findings provide new insight into HH signaling, its contribution to an aggressive subpopulation of cells, and new opportunities for clinical development.

Materials and Methods

Reagents and cell culture

Cyclopamine was purchased from Toronto Research Chemicals and dissolved in 95% ethanol to create a 10 mmol/L stock solution. NVP-LDE225 (LDE225) was kindly provided by Novartis Pharma AG and dissolved in dimethyl sulfoxide (DMSO) to create a 10 mmol/L stock solution. The ovarian cancer cell lines A2780ip2, A2780cp20, HeyA8, HeyA8MDR, SKOV3ip1, and SKOV3TRip2 (18–23) were maintained in RPMI-1640 medium supplemented with 10% FBS (Hyclone). A2780cp20 (platinum- and taxane-resistant), HeyA8MDR (taxane-resistant), and SKOV3TRip2 (taxane-resistant, a kind gift of Dr Michael Seiden; ref. 24) were generated by sequential exposure to increasing concentrations of chemotherapy (25). HeyA8MDR and SKOV3TRip2 were maintained with the addition of 150 ng/mL of paclitaxel. All cell lines were routinely screened for *Mycoplasma* species (GenProbe detection kit; Fisher) with experiments done at 70% to 80% confluent cultures. Purity of cell lines was confirmed with STR genomic analysis, and only cells less than 20 passages from stocks were used in experiments.

RNA extraction and reverse transcription

Total RNA was isolated from ovarian cancer cell lines using TRIzol reagent (Invitrogen) per manufacturer's instructions. RNA was then DNase treated and purified using the RNeasy Mini Kit (QIAGEN). RNA was eluted in 50 μL of RNase-free water and stored at −80°C. The concentration of all RNA samples was quantified by spectrophotometric absorbance at 260/280 nm using an Eppendorf BioPhotometer plus. Before cDNA synthesis, all RNA samples were diluted to 20 ng/μL using RNase-free water. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA samples were analyzed using quantitative PCR (qPCR).

Quantitative PCR

Primer and probe sets for *Desert HH* (Hs0036806_m1), *GLI1* (Hs00171790_m1), *GLI2* (Hs00257977_m1), *Indian HH* (Hs00745531_s1), *MDR1* (Hs00184500_m1), *PTCH1* (Hs00181117_m1), *SMO* (Hs00170665_m1), *Sonic HH* (Hs), and *RPLP0* (Hs99999902_m1; housekeeping gene) were obtained from Applied Biosystems and used according to manufacturer's instructions. PCR amplification was conducted on an ABI Prism 7900HT sequence detection system and gene expression was calculated using the comparative C_T method as previously described (26). Briefly, this technique uses the formula $2^{-\Delta\Delta C}{}_T$ to calculate the expression of target genes normalized to a calibrator. The cycling threshold (C_T) indicates the cycle number at which the amount of amplified target reaches a fixed threshold. C_T values range from 0 to 40 (the latter representing the default upper limit PCR cycle number that defines failure to detect a signal).

Western blot analysis

Cultured cell lysates were collected in modified radioimmunoprecipitation assay lysis buffer with protease inhibitor cocktail (Roche) and subjected to immunoblot analysis by standard techniques (25) using anti-Gli1 antibody (Cell Signaling Technology) at 1:1,000 dilution overnight at 4°C, anti-Smo antibody (LifeSpan Biosciences) at 1:1,000 dilution overnight at 4°C, or anti-β-actin antibody (AC-15, Sigma-Aldrich) at 1:20,000 dilution for 1 hour at room temperature (RT), which was used to monitor equal sample loading. After washing, blots were incubated with goat anti-rabbit (for Gli1 and Smo) or goat anti-mouse (for β-actin) secondary antibodies (Bio-Rad) conjugated with horseradish peroxidase. Visualization was done by the enhanced chemiluminescence method (Pierce Thermo Scientific).

siRNA transfection

To examine downregulation of Smo, Gli1, or Gli2 individually with siRNA, cells were exposed to control siRNA (target sequence: 5′-UUCUCCGAACGUGUCACGU-3′, Sigma-Aldrich), one of 2 tested Smo-targeting constructs (siRNA1: 5′-GAGGAGUCAUGACUCUGUUCUCCAU-3′ or siRNA2: 5′-UGACCUCAAUGAGCCCUCAGCU-GAU-3′; Invitrogen), one of 2 tested Gli1-targeting constructs (siRNA1: 5′-CUACUGAUACUCUGGGAUA-3′ or siRNA2: 5′-GCAAAUAGGGCUUCACAUA-3′;

Sigma-Aldrich), or one of 2 tested Gli2-targeting constructs (siRNA1: 5′-GACAUGAGCUCCAUGCUCA-3′ or siRNA2: 5′-CGAUUGACAUGCGACACCA-3′; Sigma-Aldrich) at a 1:3 siRNA (μg) to Lipofectamine 2000 (μL) ratio. Lipofectamine and siRNA were incubated for 20 minutes at RT, added to cells in serum-free RPMI to incubate for up to 8 hours, followed by 10% FBS/RMPI thereafter. Transfected cells were grown at 37°C for 48 to 72 hours and then harvested for qPCR or Western blot analysis.

Assessment of cell viability and cell-cycle analysis

To a 96-well plate, 2,000 cells/well were exposed to increasing concentrations of cyclopamine or LDE225, alone or in combination with carboplatin or paclitaxel, in triplicate. Viability was assessed with 0.15% MTT (Sigma-Aldrich). For effects of siRNA-mediated downregulation on paclitaxel IC_{50}, cells were first transfected with siRNA (5 μg) for 24 hours in 6-well plates, then trypsinized and replated at 2,000 cells per well, followed by addition of chemotherapy after attachment. IC_{50} of the agent of interest was determined by finding the dose at which the drug had 50% of its effect, calculated by the equation $[(OD450_{MAX} - OD450_{MIN})/2) + OD450_{MIN}]$. For cell-cycle analysis, cells were treated with vehicle alone, paclitaxel alone, LDE225 alone, or combined LDE225 and paclitaxel for 72 hours, trypsinized, and fixed in 100% ethanol overnight. Cells were then centrifuged, washed in PBS, and resuspended in PBS containing 0.1% Triton X-100 (v/v), 200 μg/mL DNase-free RNase A, and 20 μg/mL propidium iodide (PI). PI fluorescence was assessed by flow cytometry and the percentage of cells in sub-G_0, G_0–G_1, S-, and G_2–M phases was calculated by the cell-cycle analysis module for Flow Cytometry Analysis Software (FlowJo v.7.6.1).

ALDEFLUOR assay

Active aldehyde dehydrogenase (ALDH) was identified with the ALDEFLUOR assay according to manufacturer's instructions (StemCell Technologies). The ALDH-positive population was defined by cells with increased FITC signal absent in DEAB-treated cells, as previously described (27). ALDEFLUOR-positive and -negative populations from SKOV3Trip2 cells were sorted with a FACS Aria II flow cytometer (BD Biosciences), and collected cells were seeded onto a 96-well plate at a concentration of 2,000 cells/well. After overnight attachment, cells were then exposed to either DMSO or 5 μmol/L LDE225, alone or in combination with increasing concentrations of paclitaxel. Viability was assessed with 0.15% MTT (Sigma-Aldrich).

Orthotopic ovarian cancer model

For orthotopic therapy experiments using ovarian cancer cell lines, female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute (Frederick, MD, USA) after Institution Animal Care and Use Committee approval of protocols, and cared for in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care. For all *in vivo* experiments, trypsinized cells were resuspended in 10% FBS-containing RPMI, washed with PBS, and suspended in serum-free HBSS at a concentration of 5×10^6 cells/mL, and 1×10^6 cells (A2780cp20 or SKOV3TRip2) were injected IP in 200 μL into 40 mice per experiment. After 1 week, mice ($n = 10$ per group) were randomized to treatment with (a) vehicle alone (0.5% methyl cellulose/0.5% Tween 80 in sterile water), (b) vehicle plus paclitaxel 75 μg, (c) LDE225 alone (60 mg/kg), or (d) combined LDE225 and paclitaxel. Vehicle and LDE225 were administered by gavage once daily and paclitaxel was administered i.p. weekly. Mice were treated for 4 weeks (A2780cp20) or 6 weeks (SKOV3TRip2, which grow more slowly) before sacrifice and tumor collection. All tumors were excised and weighed in total.

Statistical analysis

Comparisons of gene expression, cell viability, PI fluorescence, and mean tumor weight were analyzed using a 2-tailed Student *t* test, if assumptions of data normality were met. Those represented by alternate distribution were examined using a nonparametric Mann–Whitney *U* test. Differences between groups were considered statistically significant at $P < 0.05$. Error bars represent standard deviation unless otherwise stated. Number of mice per group ($n = 10$) was chosen as directed by a power analysis to detect a 50% decrease in tumor growth with β error of 0.2.

Results

Expression of HH pathway members in chemosensitive and chemoresistant ovarian cancer cell lines

We first examined mRNA expression of HH ligands [Sonic (*SHH*), Indian (*IHH*), Desert (*DHH*)], receptors (*PTCH1*, *SMO*), and transcription factors (*GLI1*, *GLI2*) in 3 pairs of parental and chemoresistant ovarian cancer cell lines: A2780ip2/A2780cp20 (20-fold increased cisplatin resistance and 10-fold increased taxane resistance), HeyA8/HeyA8MDR (500-fold taxane resistant), and SKOV3ip1/SKOV3TRip2 (1000-fold taxane resistant). As shown in Fig. 1A, mRNA levels of *SHH* were significantly higher in A2780cp20 (17.4-fold, $P < 0.05$) and SKOV3-TRip2 (2.4-fold, $P < 0.05$) cells compared with parental. *IHH* was also higher (3.5-fold, $P < 0.05$) in SKOV3TRip2 cells with *DHH* expression remaining unchanged or decreased in chemoresistant cell lines compared with parental. mRNA levels of *PTCH1* were significantly higher (2.1-fold, $P < 0.05$) in SKOV3TRip2 compared with parental SKOV3ip1 cells; however, no significant changes in *SMO* expression were observed between chemoresistant and chemosensitive cell lines (Fig. 1B). Protein expression of Smo was confirmed in all cell lines tested and did not always correlate with expression at the mRNA level (Fig. 1C). *GLI1* mRNA expression was significantly

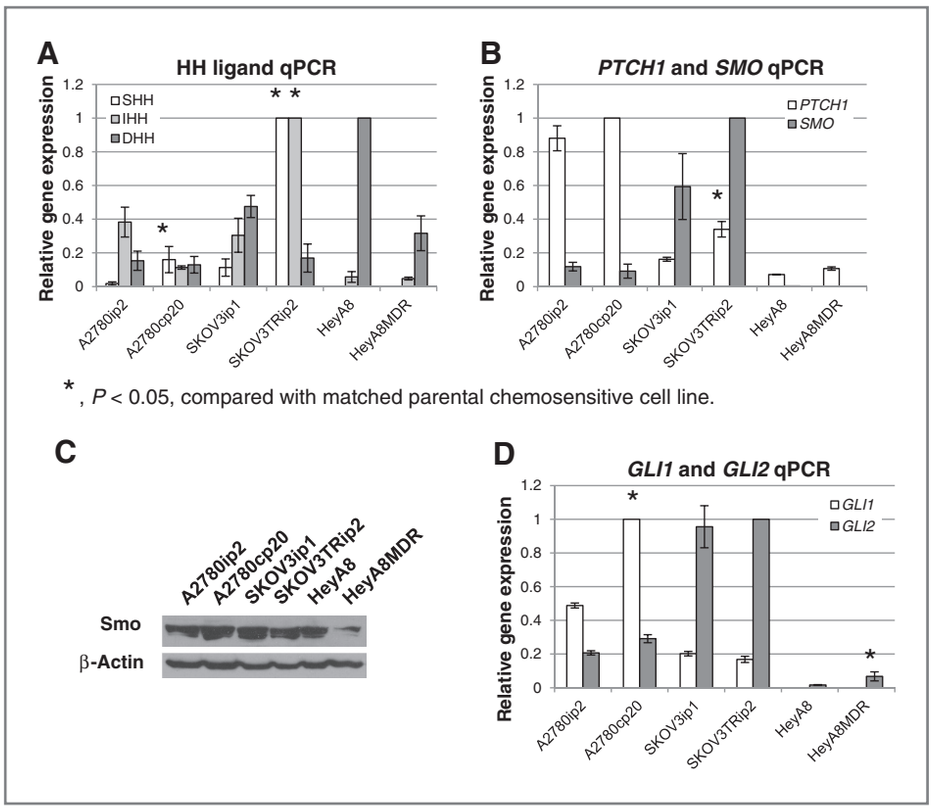

Figure 1. Expression of HH signaling components in chemosensitive and chemoresistant ovarian cancer cell lines. Gene expression was calculated relative to the sample/cell line with the highest expression of a particular gene. A, mRNA expression of HH ligands, Sonic (*SHH*), Indian (*IHH*), and Desert (*DHH*). B, mRNA expression of HH receptors, *PTCH1* and *SMO*. C, protein expression of Smo was also measured using Western blot analysis. β-Actin was used as a loading control. D, mRNA expression of HH transcription factors, *GLI1* and *GLI2*. Data are representative of 3 independent experiments. *, $P < 0.05$, compared with parental chemosensitive cell line.

higher (2.0-fold, $P < 0.05$) in A2780cp20 compared with parental A2780ip2 cells and *GLI2* mRNA expression was significantly higher (4.1-fold, $P < 0.05$) in HeyA8MDR compared with parental HeyA8 cells, although at very low levels in both (Fig. 1D). These results show that HH signaling is often higher in chemoresistant matched ovarian cancer cell lines.

Smo antagonists diminish cell viability and HH gene expression in ovarian cancer cell lines

Having observed Smo expression (both mRNA and protein) in both chemosensitive and chemoresistant ovarian cancer cell lines, we next examined response to the Smo antagonists cyclopamine and LDE225 among these cell lines. The chemical structure of LDE225 is shown in Fig. 2A. As shown in Table 1, cyclopamine IC_{50}s varied from 7.5 μmol/L (A2780ip2) to 19 μmol/L (SKOV3TRip2) and LDE225 IC_{50}s varied from 7.5 μmol/L (A2780cp20) to 24 μmol/L (SKOV3ip1). Interestingly, chemoresistant cell lines were more sensitive (up to 2.25-fold, $P < 0.05$) to LDE225 compared with their chemosensitive counterparts. Chemoresistant cell lines were also more sensitive to LDE225 than cyclopamine. To confirm that decreased cell viability was associated with diminished HH pathway activity, A2780cp20 cells were exposed to increasing concentrations of LDE225 (1, 5, and 10 μmol/L) for 72 hours and gene expression of HH target genes *PTCH1*, *GLI1*, and *GLI2* was analyzed by qPCR. A dose-dependent decrease in the expression of all 3 genes was observed with a maximum reduction of 39%, 43%, and 32% ($P < 0.05$), respectively, after exposure to 10 μmol/L LDE225 (Fig. 2B). Protein expression of the HH transcriptional activator Gli1 was also reduced in a dose-dependent manner after LDE225 treatment (Fig. 2C). Taken together, these data show the efficacy and HH-specific activity of LDE225 in multiple chemoresistant cell lines.

Smo antagonism reverses taxane resistance in chemoresistant ovarian cancer cell lines both *in vitro* and *in vivo*

Having observed increased expression of HH signaling components and response to Smo antagonists in chemoresistant ovarian cancer cell lines, we sought to determine whether targeting the HH pathway could increase sensitivity to carboplatin and paclitaxel, chemotherapy agents most commonly used in the treatment of ovarian cancer. Neither cyclopamine nor LDE225 affected response to carboplatin among the chemoresistant cell lines examined (data not shown). However, as shown in Table 1, both Smo antagonists significantly increased the sensitivity of all 3 chemoresistant cell lines to paclitaxel (by up to 27- and 20-fold, respectively; $P < 0.05$). Increased sensitivity to paclitaxel after combination with cyclopamine or LDE225 even occurred at low doses that were not effective alone (5 μmol/L cyclopamine, Fig. 3A and 1 μmol/L

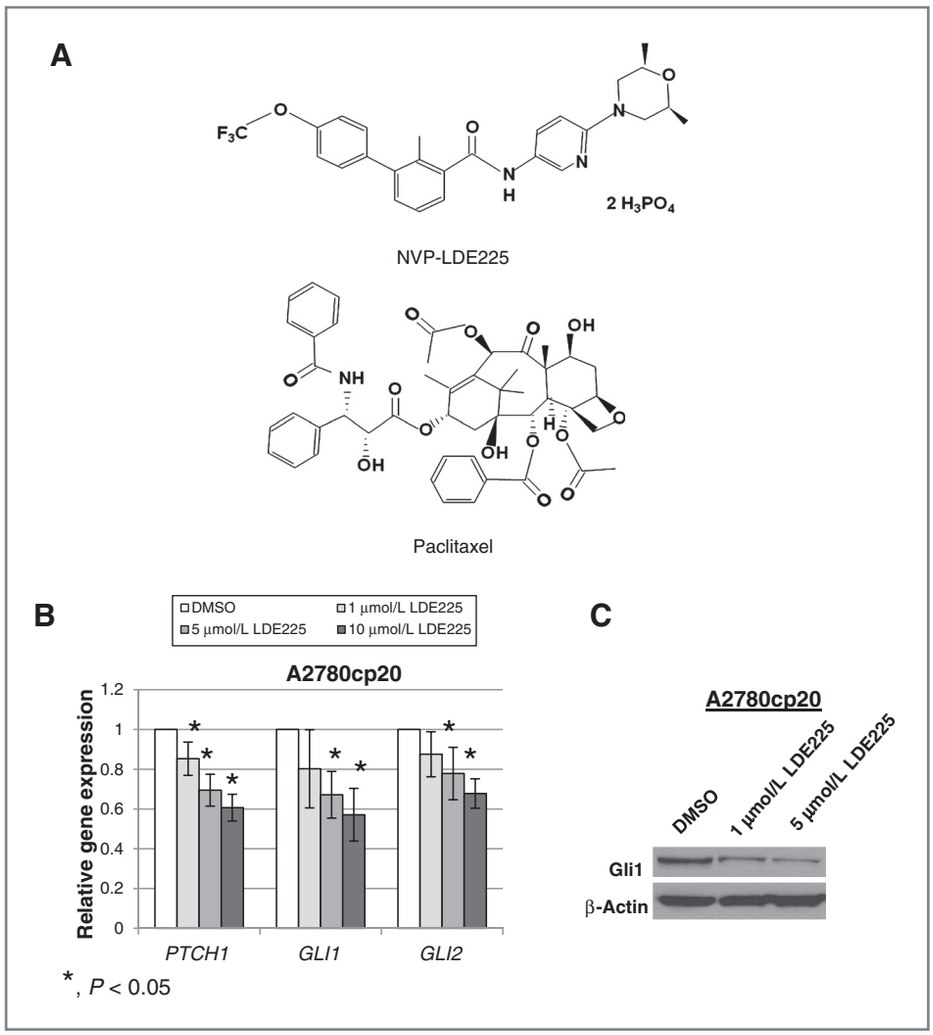

Figure 2. LDE225 reduces HH pathway activity in chemoresistant ovarian cancer cells. A, chemical structures of NVP-LDE225 and paclitaxel. B, gene expression of *PTCH1*, *GLI1*, and *GLI2* was examined in A2780cp20 cells after exposure to increasing concentrations of LDE225 using qPCR. *, $P < 0.05$, compared with DMSO vehicle control. C, protein expression of Gli1 in A2780cp20 cells after exposure to increasing concentrations of LDE225 was measured using Western blot analysis to confirm mRNA results. β-Actin was used as a loading control. Data are representative of 3 independent experiments.

LDE225, Fig. 3B). To determine the mechanism by which Smo antagonism combined with paclitaxel affects cell growth, we carried out cell-cycle analysis on A2780cp20 cells that were treated with DMSO alone (vehicle control), paclitaxel alone (30 nmol/L), LDE225 alone (5 μmol/L), or combined paclitaxel and LDE225 for 72 hours. As shown in Fig. 3C, combination treatment resulted in a greater accumulation of cells in the sub-G_0/apoptotic, S-, and G_2–M phases compared with control or either treatment alone. These data suggest that LDE225 enhances cell-cycle

Table 1. Ovarian cancer cell line response to Smo antagonists, alone and in combination with paclitaxel

	Mean IC_{50}, μmol/L		Mean paclitaxel IC_{50}, nmol/L			
Cell line	**Cyclopamine**	**LDE225**	**Control**	**w/Cyclopamine (5 μmol/L)**	**w/LDE225 (5 μmol/L)**	***P***
A2780ip2	7.5	12	4	1.5	2.6	NS
A2780cp20	10	7.5	30	1.3	1.5	<0.05
SKOV3ip1	14	24	6	3	5.5	NS
SKOV3TRip2	19	12	400	15	120	<0.05
HeyA8	12	18	7	4.2	6.5	NS
HeyA8MDR	13	8	650	50	115	<0.05

Abbreviation: NS, not significant.

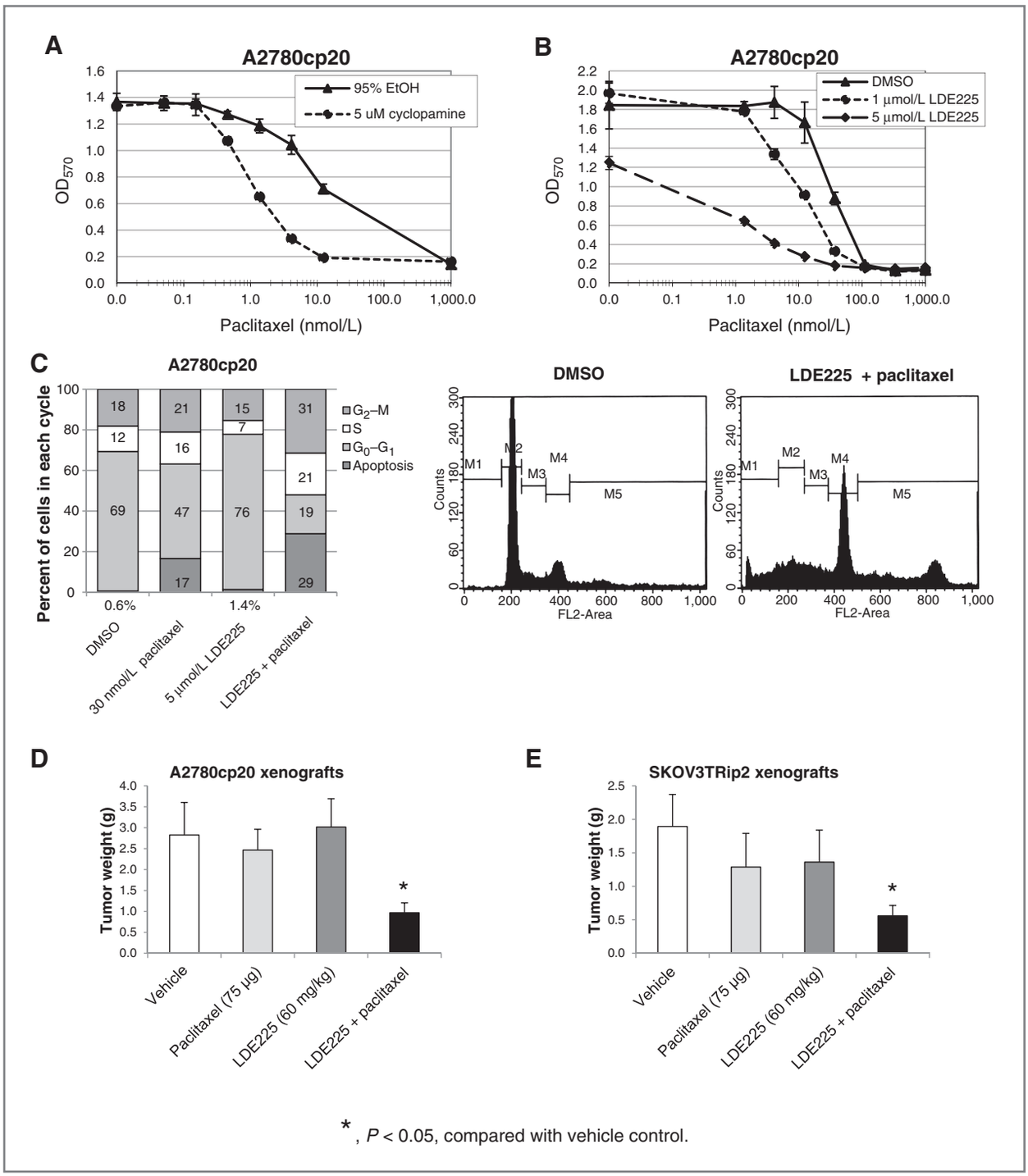

Figure 3. Smo antagonism reverses taxane resistance in chemoresistant ovarian cancer cell lines both *in vitro* and *in vivo*. A, A2780cp20 cells were exposed to either 95% ethanol (EtOH, vehicle control) or cyclopamine (5 μmol/L) in combination with increasing concentrations of paclitaxel. Cell viability was determined by MTT assay. B, A2780cp20 cells were exposed to either DMSO (vehicle control) or LDE225 (1 and 5 μmol/L) in combination with increasing concentrations of paclitaxel. Cell viability was determined by MTT assay. C, cell-cycle analysis was conducted on A2780cp20 cells treated with DMSO alone, paclitaxel alone, LDE225 alone, or combined paclitaxel and LDE225 using propidium iodide (PI) staining. Representative histograms of DMSO- and combination-treated cells are shown on the right. Data are representative of 3 independent experiments. D, mice injected intraperitoneally with A2780cp20 cells were treated with vehicle alone, paclitaxel alone, LDE225 alone, or combined paclitaxel + LDE225. E, mice injected intraperitoneally with SKOV3TRip2 cells were treated with either vehicle alone, paclitaxel alone, LDE225 alone, or combined paclitaxel + LDE225. For both xenograft models, mice treated with the combination paclitaxel + LDE225 showed a significant reduction in tumor weight compared with treatment with vehicle alone. Mean tumor weights with standard error are presented. *, $P < 0.05$, compared with vehicle control.

arrest and cell death induced by the microtubule-stabilizing effects of paclitaxel.

To determine if LDE225 can similarly reverse taxane resistance *in vivo*, an orthotopic mouse model using chemoresistant cell lines was used. Nude mice were injected intraperitoneally with either A2780cp20 or SKOV3TRip2 cells and randomized to 4 treatment groups: (a) vehicle alone, (b) paclitaxel alone (75 μg weekly), (c) LDE225 alone (60 mg/kg daily), or (d) combined paclitaxel and LDE225. When control mice started to become moribund with tumor burden, all mice were sacrificed and total tumor weights recorded. In the A2780cp20 model (Fig. 3D), there was no significant reduction in tumor growth with either paclitaxel or LDE225 alone. However, the combination of paclitaxel and LDE225 resulted in significantly reduced tumor weight, by 65.7% compared with vehicle alone ($P = 0.028$). This represented a 60.7% reduction compared with paclitaxel alone ($P = 0.014$) and a 68% reduction compared with LDE225 alone ($P = 0.010$), again showing synergy of paclitaxel and LDE225. Similar results were observed in SKOV3TRip2 xenografts (Fig. 3E). Neither paclitaxel nor LDE225 alone had a statistically significant impact on tumor growth, whereas combination treatment significantly reduced tumor weight, by 70.4% compared with vehicle alone ($P = 0.015$). This represented a 56.6% reduction compared with paclitaxel alone ($P = 0.18$) and a 58.8% reduction compared with LDE225 alone ($P = 0.13$), although neither was statistically significant.

LDE225 sensitizes chemoresistant ovarian cancer cells to paclitaxel by downregulating MDR1 expression and sensitizes both ALDH-negative and -positive ovarian cancer cells to paclitaxel

The primary mediator of taxane resistance in general, and in the chemoresistant cell lines examined in this study (27), is the expression of the drug efflux protein, P-glycoprotein (ABCB1/MDR1). To identify the mechanism underlying taxane sensitization after Smo antagonism, we next examined whether LDE225 could modulate *MDR1* gene expression. In A2780cp20 cells exposed to LDE225 alone, paclitaxel alone, and combined LDE225 + paclitaxel for 72 hours, it was observed that LDE225 decreased *MDR1* expression (by up to 49.2%, $P < 0.05$), whereas paclitaxel actually led to a compensatory *increase* in *MDR1* expression (2.88-fold, $P < 0.05$) compared with vehicle control (Fig. 4A). This compensatory increase in *MDR1* was alleviated by LDE225 in a dose-dependent manner (up to a 59.9% decrease, $P < 0.05$), showing that this compound increases sensitivity to paclitaxel, at least in part, by downregulating *MDR1*. Similar results were observed in SKOV3TRip2 cells (Fig. 4B); LDE225 decreased *MDR1* expression both alone (by up to 36.4%, $P < 0.05$ compared with vehicle control) and in combination with paclitaxel (by up to 50.8%, $P < 0.05$ compared with paclitaxel alone). In this cell line, a compensatory increase in *MDR1* was not observed with paclitaxel alone, likely because *MDR1* is already expressed at extremely high levels (140-fold more than in A2780cp20) in this 1,000-fold taxane-resistant cell line (27). To determine if similar modulation of *MDR1* occurs *in vivo*, RNA isolated from A2780cp20 tumors (from Fig. 3D) was examined. In agreement with the *in vitro* data, LDE225 alone significantly reduced *MDR1* expression (by 35.2%, $P < 0.05$) and paclitaxel alone significantly increased *MDR1* expression (2.55-fold, $P < 0.05$) compared with vehicle control (Fig. 4C). In addition, combination treatment significantly reduced *MDR1* expression compared with paclitaxel alone (by 48.8%, $P < 0.05$), blunting this compensatory rise.

In addition to our examination of MDR1 expression after LDE225 treatment, we also examined βIII-tubulin and stathmin, proteins that have been associated with microtubule regulation and resistance to taxanes (28). It was found that neither of these proteins was affected by LDE225 treatment *in vitro* (as determined by Western blot analysis, data not shown). Taken together, these data support a mechanism whereby LDE225 causes the downregulation of *MDR1* expression, which then leads to increased uptake of paclitaxel within chemoresistant cells, rather than potentiating the microtubule stabilizing effect of this compound.

We have previously shown that ALDH activity is associated with enhanced tumorigenicity and chemoresistance in ovarian cancer, and may define one of potentially many cancer cell populations with stem cell-like features (27, 29). To determine whether cancer stem cells (CSCs) might play a role in taxane sensitization after LDE225 treatment, we collected ALDH-negative and -positive cell populations from the SKOV3TRip2 cell line, and exposed them to combined LDE225 and paclitaxel. As shown in Fig. 4D, it was found that ALDH-negative and -positive SKOV3TRip2 cells showed a similar decrease in viability after LDE225 treatment alone (21.4% vs. 16.8%, respectively), compared with DMSO control. In addition, sensitivity to paclitaxel (as determined by IC_{50}) was similarly increased after combination treatment in ALDH-negative and -positive cells (5.1-fold vs. 4.0-fold change in IC_{50}, respectively). These results indicate that the more tumorigenic ALDH-positive cells are just as susceptible to LDE225 treatment as ALDH-negative cells, and that HH inhibition can sensitize both populations to taxane therapy. Whether other putative CSC populations such as CD133, CD44, and the side population, with which there is some (but not complete) crossover with the ALDH population (30), can also be sensitized to taxanes will be the subject of future investigations.

Knockdown of Smo diminishes HH pathway activity, reduces viability, and reverses taxane resistance in ovarian cancer cells

To determine whether LDE225 reverses taxane resistance through inhibition of Smo alone or off-target effects, we selectively targeted HH pathway members using siRNAs and observed effects on HH pathway activity and paclitaxel response. As shown in Fig. 5A, knockdown

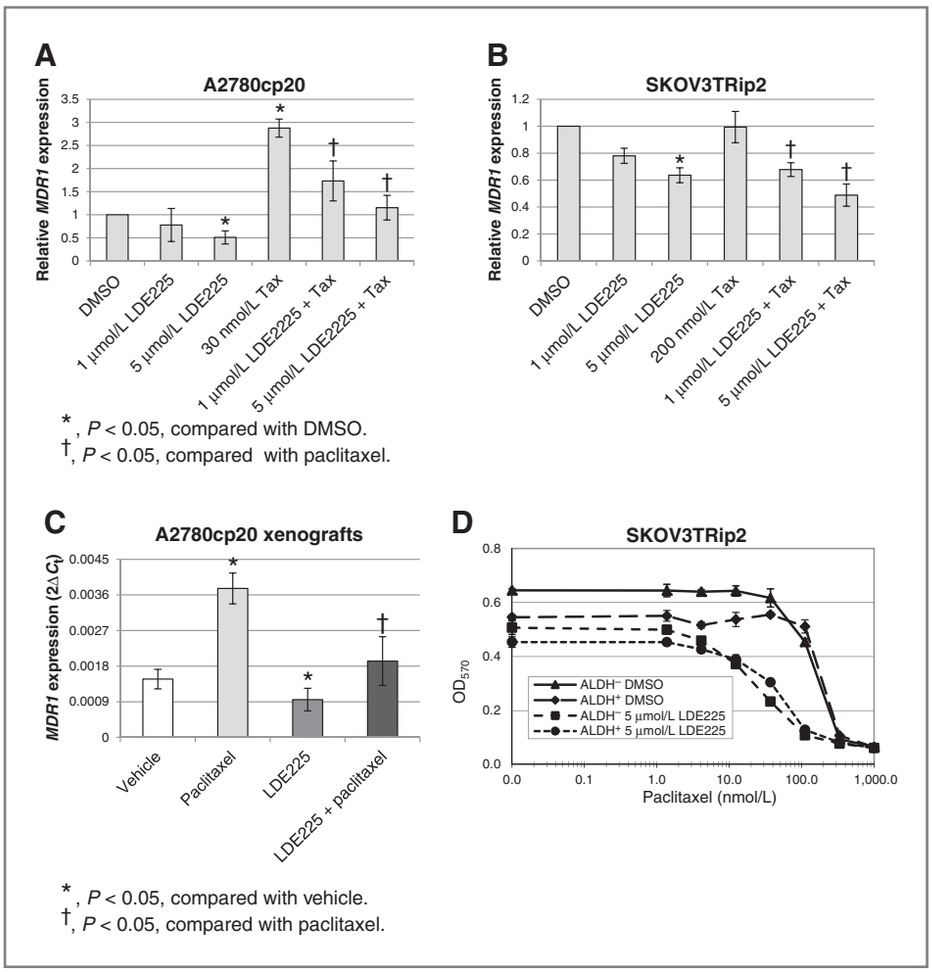

Figure 4. LDE225 sensitizes chemoresistant ovarian cancer cells to paclitaxel by downregulating MDR1 expression and sensitizes both ALDH-negative and -positive ovarian cancer cells to paclitaxel. A, A2780cp20 cells were exposed to DMSO, LDE225 (1 or 5 μmol/L), paclitaxel (Tax, 30 nmol/L), or combined LDE225 + paclitaxel for 72 hours and examined for *MDR1* gene expression. *, $P < 0.05$, compared with DMSO; †, $P < 0.05$, compared with paclitaxel alone. B, SKOV3TRip2 cells were exposed to DMSO, LDE225 (1 or 5 μmol/L), paclitaxel (Tax, 200 nmol/L), or combined LDE225 + paclitaxel for 72 hours and examined for *MDR1* gene expression. *, $P < 0.05$, compared with DMSO; †, $P < 0.05$, compared with paclitaxel alone. Data are representative of 3 independent experiments. C, A2780cp20 xenografts ($n = 5$ per group) treated with vehicle alone, paclitaxel alone, LDE225 alone, or combined LDE225 + paclitaxel were resected after 4 weeks of therapy and examined for *MDR1* gene expression. Mean expression with SE are presented. *, $P < 0.05$, compared with vehicle; †, $P < 0.05$, compared with paclitaxel alone. D, SKOV3TRip2 cells were sorted into aldehyde dehydrogenase–negative (ALDH−) and -positive (ALDH+) populations, using the ALDEFLUOR assay, and then exposed to either DMSO or 5 μmol/L LDE225, both alone and in combination with increasing concentrations of paclitaxel. Cell viability was determined by MTT assay.

of Smo was achieved both at the mRNA and protein level. As expected, this downregulation led to a significant decrease in HH target genes *PTCH1* (66.6%, $P < 0.01$), *GLI1* (86.5%, $P < 0.01$), and *GLI2* (62.0%, $P < 0.01$). Individual knockdown of HH mediators Smo, Gli1, or Gli2 using 2 distinct siRNA constructs for each gene led to increased sensitivity to paclitaxel (Fig. 5B–D). In particular, Smo knockdown decreased paclitaxel IC_{50} by up to 11.7-fold; Gli1 knockdown, up to 3.5-fold; and Gli2 knockdown, up to 5.9-fold. In agreement with cyclopamine and LDE225 biologic effects, knockdown of Smo, Gli1, or Gli2 alone significantly decreased cell viability (by up to 73.5%, 57.6%, and 26.5%, respectively, $P < 0.01$) compared with control siRNA. Collectively, these data suggest that HH signaling promotes ovarian cancer cell survival and mediates taxane resistance.

Discussion

In this study, we found that HH pathway signaling components are overexpressed in chemoresistant ovarian cancer cells. Moreover, targeting the HH pathway decreased ovarian cancer cell viability and sensitized chemoresistant ovarian cancer cells to paclitaxel therapy through decreased *MDR1* expression. The participation of HH signaling in ovarian cancer cell survival and chemotherapy resistance makes it an attractive target for therapy, especially because most patients with ovarian

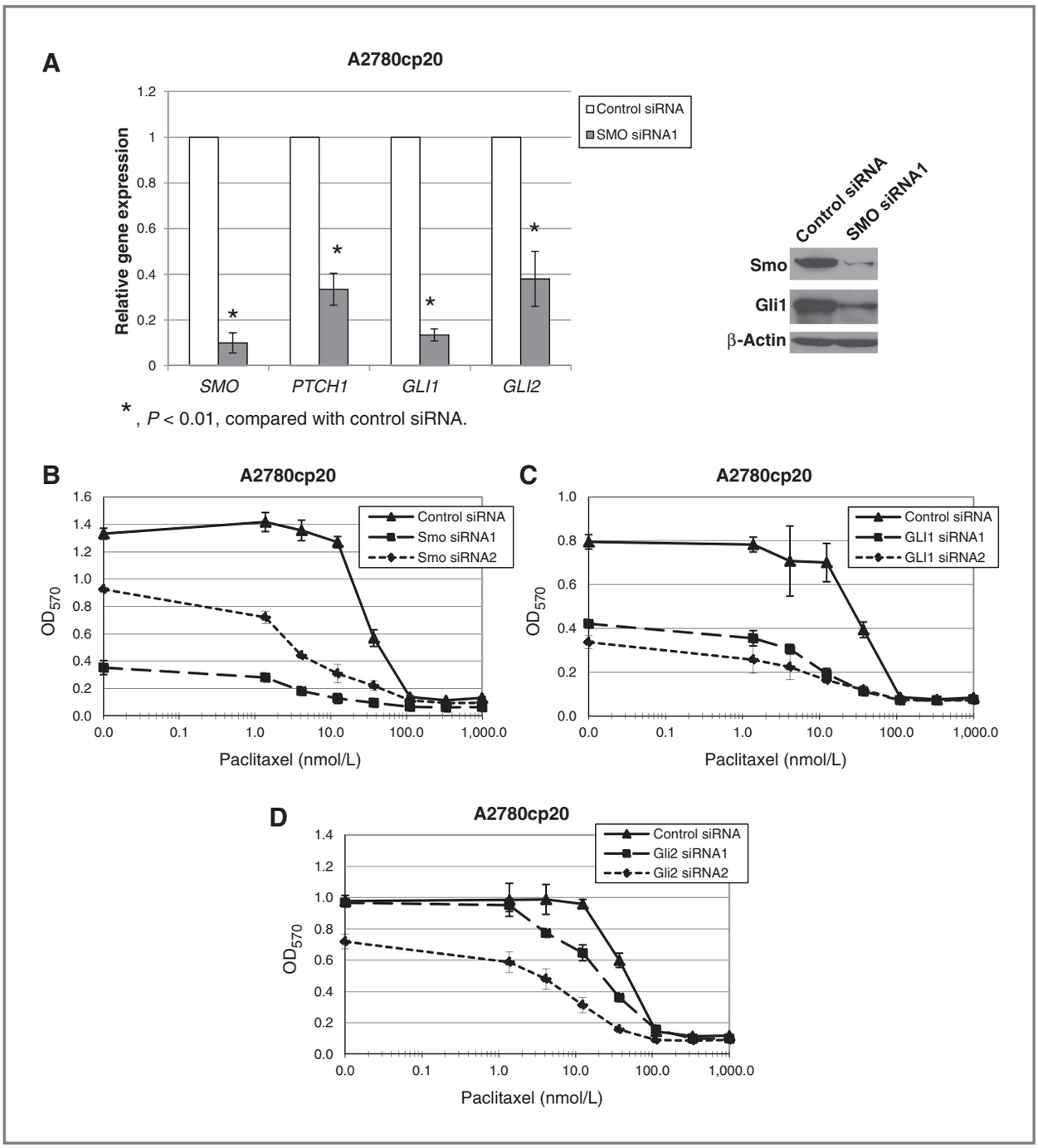

Figure 5. Knockdown of Smo diminishes HH pathway activity, reduces viability, and reverses taxane resistance in ovarian cancer cells. A, A2780cp20 cells were exposed to either control or Smo siRNA for 72 hours and examined for mRNA expression of HH pathway mediators *SMO*, *PTCH1*, *GLI1*, and *GLI2*. *, $P < 0.01$, compared with control siRNA. Protein expression of Smo and Gli (inset) was also measured using Western blot analysis to confirm mRNA results. β-Actin was used as a loading control. A2780cp20 cells were transfected with either control siRNA or 2 distinct siRNA constructs designed against Smo (B), Gli1 (C), or Gli2 (D) and exposed to increasing concentrations of paclitaxel. Cell viability was determined by MTT assay. Data are representative of 3 independent experiments.

cancer develop tumor recurrence and succumb to chemoresistant disease.

Currently, it has not been shown what role HH signaling might play in mediating ovarian cancer chemoresistance, a persistent obstacle in the treatment of this disease. Although the clinical behavior of ovarian cancer suggests that most cancer cells are initially sensitive to chemotherapy, they subsequently either develop resistance or

contain a population of cells that are inherently resistant. The latter hypothesis is consistent with what has become known as tumor initiating cells or CSCs. These CSCs are commonly believed to have enhanced tumorigenicity, differentiation capacity, and resistance to chemotherapy in comparison with non-CSCs. It is because of these features that CSCs have been examined for molecular pathways and markers that could be targeted for therapeutic purposes. Recent studies have suggested that developmental pathways, including HH, play important roles in the maintenance of CSCs (10/11) and that inhibiting these pathways may provide enhanced chemosensitivity when combined with traditional chemotherapies. In our study, we sought to define a role for HH signaling in ovarian cancer chemoresistance. Both *in vitro* and *in vivo*, we observed significant sensitization to paclitaxel after Smo antagonism (LDE225) in taxane-resistant ovarian cancer cells. This sensitization was also present in ALDH-positive cells, a subpopulation of cancer cells with enhanced tumorigenicity and chemoresistance. The mechanism underlying this sensitization seems to involve downregulation of P-glycoprotein (ABCB1/MDR1), a well-characterized mediator of multidrug resistance. By downregulating *MDR1* expression, uptake of paclitaxel by cancer cells would be increased, resulting in a greater response to the chemotherapeutic agent. This mechanism would explain why Smo antagonists did not sensitize chemoresistant cells to carboplatin, because this compound is not a substrate for the P-glycoprotein drug efflux pump. In addition, this model of HH inhibition and chemosensitization agrees with a previous study done by Sims-Mourtada and colleagues, in which it was showed that cyclopamine sensitized prostate cancer cells to a variety of chemotherapy agents *in vitro* (including the taxane docetaxel), through modulation of MDR1 expression (12). The observation that Smo antagonism did not sensitize cells to platinum therapy highlights the specificity of this effect.

Previous studies have showed aberrant expression of the HH pathway in primary specimens of ovarian cancer compared with normal ovarian epithelium (7–9), including a study that found elevated Gli1 expression is associated with decreased survival (9). These studies have also showed decreased ovarian cancer cell growth/viability after treatment with the Smo antagonist cyclopamine, results that our study supports. We have previously shown that *GLI1* and *GLI2* mRNA levels were significantly higher in cancer cells isolated from persistent/chemoresistant tumors compared with those isolated from matched primary tumors (29). Smo expression was also increased (3.7-fold) in persistent tumors; however, this increase was not statistically significant. Patients from whom persistent tumors were obtained had failed both taxane and platinum chemotherapies, making it difficult to determine whether this increase in HH pathway genes is a taxane-specific effect. The *in vitro* data presented in this study, however, would suggest that Smo, as well as Gli1 and Gli2, are associated with taxane resistance. In our initial experiments examining the effects of targeting HH alone, either with Smo antagonists or RNAi, ovarian cancer cell viability was significantly decreased *in vitro*, indicating that the HH pathway is important for ovarian cancer survival. However, this effect did not seem to translate to our xenograft models, in which the Smo antagonist LDE225 had no significant impact on tumor growth when used alone, even in models with relatively high Gli1 expression. These findings suggest that survival pathways are activated in the murine tumor microenvironment that allows resistance to HH antagonist monotherapy. Given the recognized importance of crosstalk between the tumor stromal cells and malignant cells in the HH pathway (6), and the failure of this model to target both murine and human compartments, more efficacy may be noted with monotherapy in humans.

Collectively, the data presented in this study show that increased expression of HH signaling components is associated with taxane resistance, which can be overcome by targeting multiple effectors of the HH signaling pathway. With the ability to identify subsets of patients with cancer with HH pathway overexpression, antagonism of HH signaling in combination with taxane therapy could ultimately provide a useful therapeutic strategy for recurrent, chemoresistant ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.D. Steg, C.N. Landen
Development of methodology: A.D. Steg, C.N. Landen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.D. Steg, A.A. Katre, K.S. Bevis, A. Ziebarth, Z. C. Dobbin, M.M. Shah, C.N. Landen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.D. Steg, C.N. Landen
Writing, review, and/or revision of the manuscript: A.D. Steg, A. Ziebarth, R.D. Alvarez, C.N. Landen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.A. Katre, Z.C. Dobbin, C.N. Landen
Study supervision: C.N. Landen

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Using heterogeneity of the patient-derived xenograft model to identify the chemoresistant population in ovarian cancer

Zachary C. Dobbin[1,2], Ashwini A. Katre[1], Adam D. Steg[1], Britt K. Erickson[1], Monjri M. Shah[1], Ronald D. Alvarez[1], Michael G. Conner[3], David Schneider[4], Dongquan Chen[5] and Charles N. Landen[6]

[1] Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of Alabama at Birmingham

[2] NIH Medical Scientist Training Program, University of Alabama at Birmingham

[3] Department of Pathology, University of Alabama at Birmingham

[4] Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham

[5] Division of Preventative Medicine, Department of Medicine, University of Alabama at Birmingham

[6] Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, The University of Virginia, Charlottesville, VA

***Correspondence to**: Charles N. Landen Jr, **email**: clanden@virginia.edu*

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ABSTRACT

A cornerstone of preclinical cancer research has been the use of clonal cell lines. However, this resource has underperformed in its ability to effectively identify novel therapeutics and evaluate the heterogeneity in a patient's tumor. The patient-derived xenograft (PDX) model retains the heterogeneity of patient tumors, allowing a means to not only examine efficacy of a therapy, but also basic tenets of cancer biology in response to treatment. Herein we describe the development and characterization of an ovarian-PDX model in order to study the development of chemoresistance. We demonstrate that PDX tumors are not simply composed of tumor-initiating cells, but recapitulate the original tumor's heterogeneity, oncogene expression profiles, and clinical response to chemotherapy. Combined carboplatin/paclitaxel treatment of PDX tumors enriches the cancer stem cell populations, but persistent tumors are not entirely composed of these populations. RNA-Seq analysis of six pair of treated PDX tumors compared to untreated tumors demonstrates a consistently contrasting genetic profile after therapy, suggesting similar, but few, pathways are mediating chemoresistance. Pathways and genes identified by this methodology represent novel approaches to targeting the chemoresistant population in ovarian cancer

INTRODUCTION

Although most ovarian cancer patients present with advanced-stage disease, response to front-line platinum-based chemotherapy is high, on the order of 75%. The combination of surgery and adjuvant chemotherapy will allow remission in most patients, and about 40% of advanced stage patients will live at least 5 years [1]. However, absolute cures are uncommon, with 80% of patients eventually having a recurrence [2]. The clinical profile of high rates of positive responses yet high recurrence rates suggests the presence of a subpopulation of cells within the heterogeneous tumor that survives initial chemotherapy, to lie dormant and eventually regrow with chemoresistant disease. Only by targeting this subpopulation can we achieve durable cures [3, 4].

Pre-clinical models used in drug discovery have predominately used clonal ovarian cancer cell lines, which cannot account for tumor heterogeneity, and evolve though selective growth and time to become very different from tumors growing in patients. Recently some of the most commonly used ovarian cell lines used were reported to have profiles more like endometrioid than papillary serous carcinoma, as defined by TCGA expression profiling[5]. Studying tumors preclinically that more closely resemble human tumors may increase the likelihood that

Table 1: Patient demographics of implanted and growing patient-derived xenograft (PDX) lines

Characteristic			Percent or Average (range)
Age at diagnosis			61.7 (47-87)
Stage			
		Stage IIIC	83%
		Stage IV	17%
Race			
		Caucasian	76%
		African American	24%
Procedure			
	Tumor Reductive Surgery	Optimal TRS	52%
		Suboptimal TRS	45%
	Laparoscopic Biopsy prior to neoadjuvant chemotherapy		3%
Histology			
		Papillary Serous Adenocarcinoma	79%
		Endometroid	3%
		Mixed Epithelial	9%
		Mucinous	3%
		Extra-ovarian in origin	6%
Chemotherapy Treatment			
		Carboplatin	4%
		Carboplatin/Avastin	4%
		Carboplatin/Paclitaxel	56%
		Carboplatin/Paclitaxel/Avastin	7%
		Carboplatin/Taxotere	19%
		Cisplatin/Docetaxel	4%
		Cisplatin/Paclitaxel	4%
		Cisplatin/Taxotere	4%

medications effective in preclinical studies are effective in clinical trials. The patient-derived xenograft (PDX) model, whereby tumors are collected from patients and immediately implanted into mice, has recently been characterized and may allow such an advantage[6-8].

We set out to further characterize the PDX model and determine whether the heterogeneity seen in ovarian cancer is recapitulated, in order to explore the cell populations responsible for chemoresistance. One potential subpopulation with chemotherapy resistance is the cancer stem cell (CSC) population. CSC's have been shown to have increased tumorigenicity in mice, chemotherapy resistance, and are enriched in recurrent ovarian cancer [9-11]. In developing and characterizing the PDX model our goals were to 1) optimize methods to allow a high success rate of implantation, 2) examine retention of heterogeneity, 3) determine if PDX tumors respond to chemotherapy similarly to patient tumors, 4) assess whether treatment with chemotherapy results in survival of just CSC populations, and 5) identify pathways that are amplified in resistant tumors. We demonstrate that the PDX model can be established with a high success rate, have similar expression profiles and biologic activities as patient tumors, and can be used as a model to identify the chemoresistant population.

RESULTS

Implantation success rate and establishment of the ovarian PDX model

Here we report outcomes on the first 34 patient samples implanted into SCID mice. Demographics for patients from whom tumors were collected are presented in Table 1. All patients had stage IIIC or IV high-grade epithelial ovarian cancers, and tumors were collected prior

to any chemotherapy.

Tumor collected and implanted into mice was either from an omental metastasis or peritoneal implant, since they are plentiful, composed of grossly-identifiable tumor, and most relevant to recurrent disease.

Different sites of implantation in the mouse were tested to identify the best location for growth. Subcutaneous (SQ) and mammary fat pat (MFP) sites were tested as their location allows for tumor growth to be monitored with caliper-measurements. Intraperitoneal (IP) injection was examined, to provide an orthotopic location for model establishment. The subrenal capsule (SRC) was evaluated given previous reports of high take rates in this site [12]. Implantation for all 4 sites was conducted as described in the methods. Therefore both site and method of processing were controlled for each patient. The rates for PDX tumor development in each site, including individual implants are presented in Figure 1A. In the first 34 patients, a PDX line was established in 85.3% of SQ implants. This is compared to 63.64% in the MFP, 22.2% IP, and 8.3% in the SRC. SQ xenografts almost always visually disappeared in the weeks after implantation before regrowing and being detectable at a mean of 78.4 days after implantation (range 17-174 days, Figure 1C) compared to 77.3 days for the MFP (range 29 to 129 days, NS). The success of a PDX being established is highest in the SQ site in part due to the increased number of implants per patient. Based on this data, and subsequent

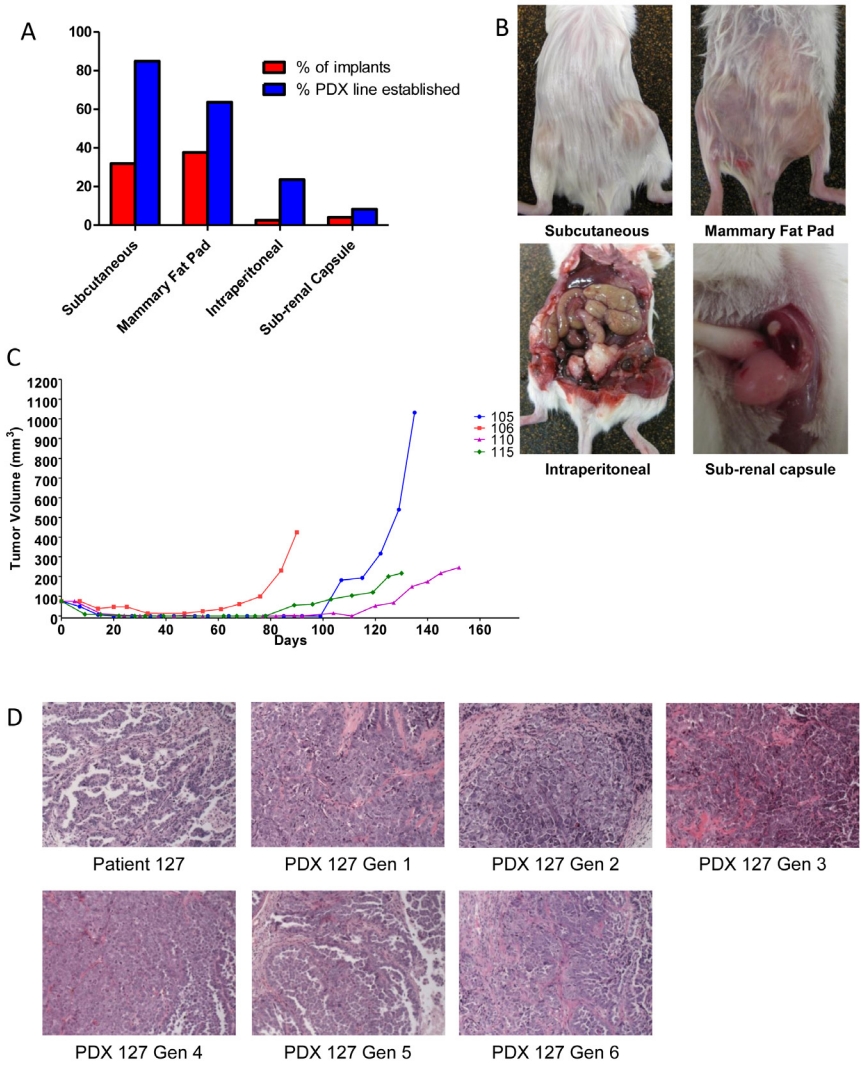

Figure 1: Take rates of different sites for implantation and maintenance PDX histology . (A) Tumors were implanted subcutaneously (SQ), in the mammary fat pad (MFP), intraperitoneal (IP), or sub-renal capsule. The success of implantation was similar comparing SQ to MFP, however more PDX lines were established from SQ implant due to number of implants. IP and SRC implants are not effective for establishing a PDX line. (B)Representative pictures of implanted tumors at either SQ, MFP, IP, or SRC. (C) After implantation, tumor volume decreased to an undetectable size then re-grew after a dormancy period. This implicate the small population of tumorigenic cells survive and re-capitulate the tumor after implantation. Representative growth chart showed of 4 different PDX lines after implantation. (D) Histology of the original tumor is maintained throughout subsequent generations. Patient 127 had a histology of papillary serous adenocarcinoma that has been maintained for 6 generations in the corresponding PDX.

studies showing similar expression profiles in tumors from the SQ site and original patient tumors (described below), continued development of the PDX model was done in the SQ site. PDX tumors were examined for histologic characteristics by a gynecologic pathologist. In all cases and in up to six generations of reimplantation, the original histology was maintained (Figure 1D). Interestingly, in the few cases where a mixed epithelial-type ovarian cancer was implanted, both histologies were present in each of the subsequent PDX generations.

Heterogeneity of PDX tumors

One potential advantage of the PDX model is that it may maintain patient heterogeneity, as opposed to the clonality that ultimately characterizes cell lines. However, a growing body of evidence suggests that certain cell subpopulations have enhanced ability to initiate tumors, often termed tumor-initiating cells (TIC's) or sometimes CSC's if additional attributes are demonstrated [10]. We examined whether resulting PDX tumors maintained tumor heterogeneity from a tumor-initiating cell standpoint.

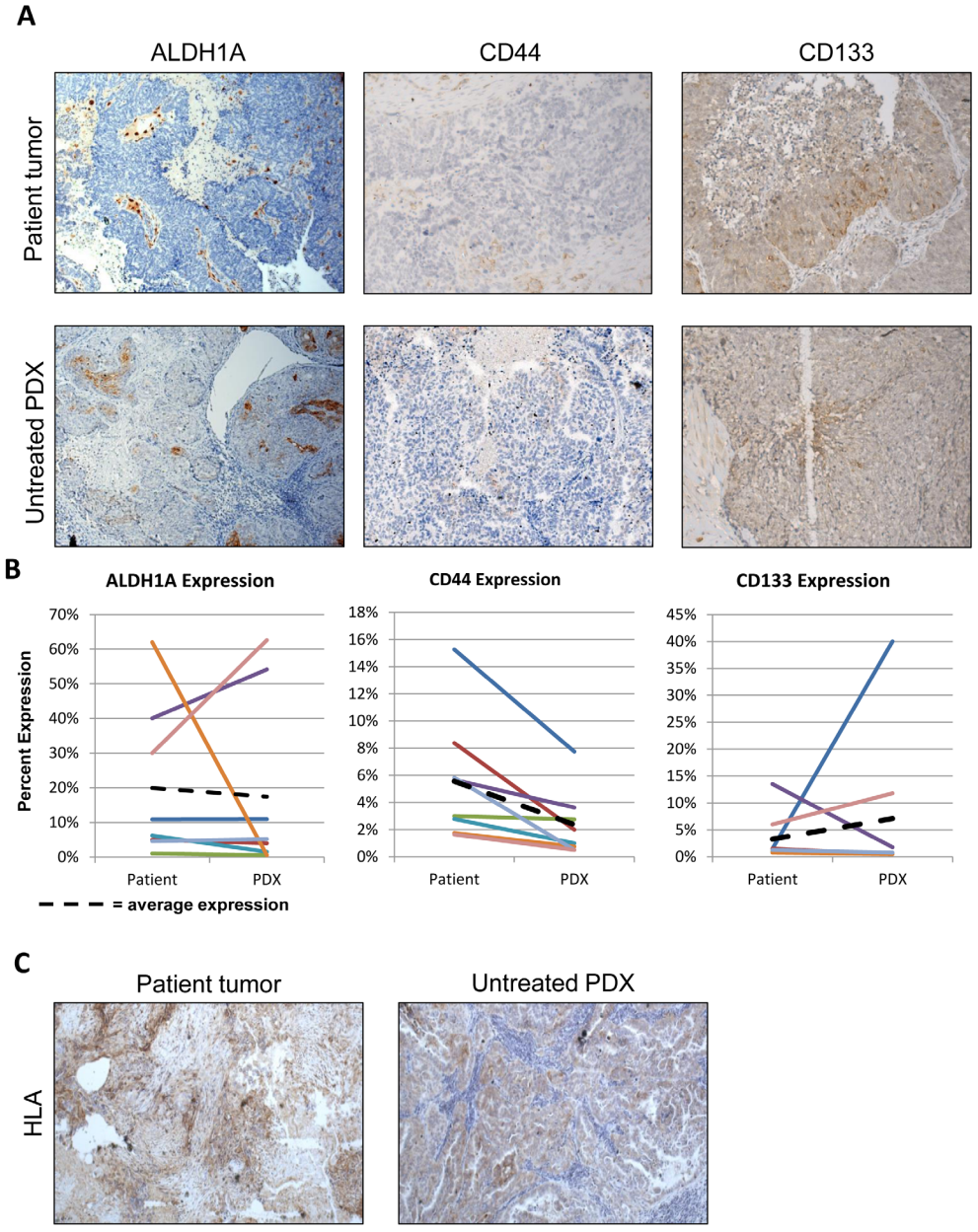

Figure 2: Establishment of the PDX line does not enrich for the tumorigenic cell population and human stroma is replaced in the implanted PDX. (A) Representative staining for ALDH1A1, CD133, and CD44 on the patient sample and untreated PDX. (B) Quantification of change in expression of ALDH1A1, CD133, and CD44 between the patient sample and the untreated PDX. Only CD44 had a significant decrease in expression (p-value <0.05). ALDH1A1 and CD133 had no significant change in expression. (C) Human HLA expression in patient and untreated PDX tumors, demonstrating replacement of human stroma with murine cells.

PDX tumors and original patient tumors were subjected to IHC for the TIC markers ALDH1A1 [11, 13, 14], CD133 [15-17], and CD44 [18, 19]. For ALDH1A1, CD44, and CD133, the patient samples averaged expression of 19.95%, 5.56%, and 3.27% respectively. The PDX tumors had similar expression of ALDH1A1 and CD133 at 17.4%, and 7.1% respectively (p=0.80 and 0.49, Figure 2A, 2B). There was a significant change in expression of CD44, but it was actually a decrease, from 5.54% to 2.36% (p=0.014). If TICs in ovarian cancer are indeed the cells mediating xenograft formation, these data suggest that they subsequently differentiate into marker-positive and -negative cells and recapitulate tumor heterogeneity, in keeping with the CSC hypothesis[10, 20].

Related to heterogeneity, the human/murine component of PDX tumor would have implications to the biologic relevance of this model. IHC for human HLA antigen was conducted to identify the species-specific composition of the PDX tumor. Interestingly, all stromal cells in the PDX tumors were of murine origin (Figure 2C). This was consistent across 100% of the tumor specimen, and in all of the first 15 PDX tumors established.

Biological and clinical characterization of PDX tumors

To begin to evaluate the biologic characteristics of PDX tumors compared to original patient tumors, we examined oncogenic expression, proliferation, and response to chemotherapy. Weroha *et al* have previously demonstrated similar amplification and deletion patterns

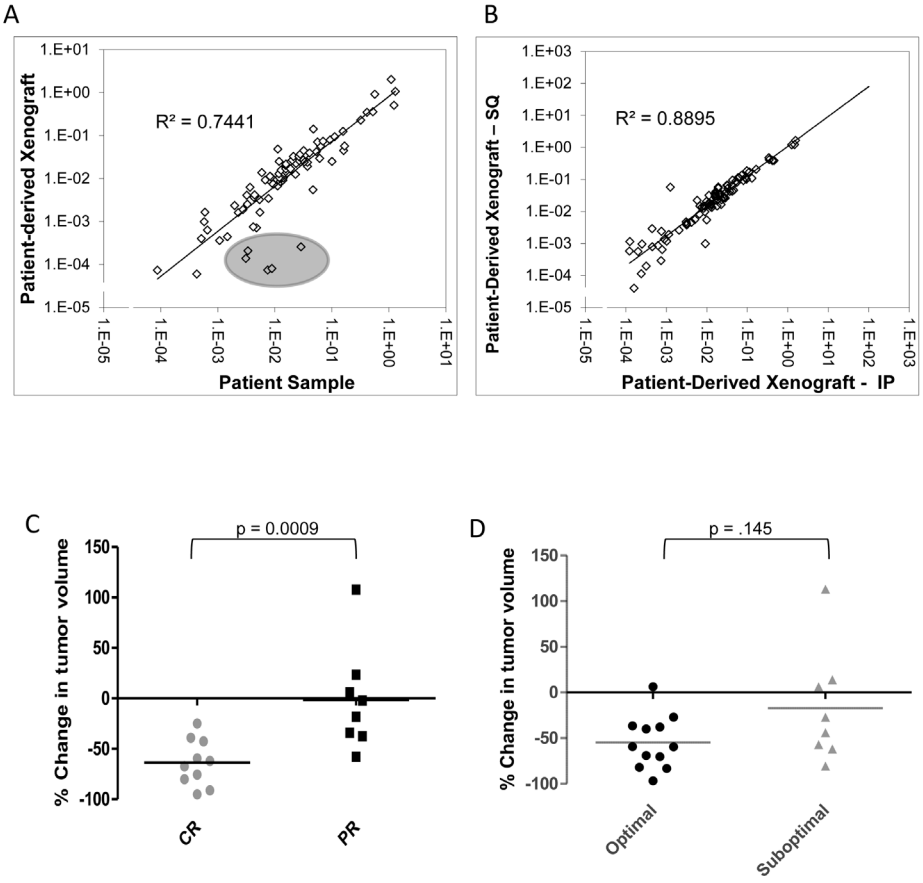

Figure 3: Cancer drug targets are maintained in the PDX line and the PDX response to treatment correlates to the patient's response to primary chemotherapy. (A) The SABiociences RT^2 qPCR array for cancer drug targets was run on the patient's tumor and their matched untreated PDX tumor. Differences in relative gene expression for each target was calculated and the $2^{\Delta Ct}$ value was determined. Most of the 84 cancer drug target genes had similar expression in the PDX and the original patient sample. 5 gene were down-regulated in the PDX sample, though all 5 are related to VEGF and PDGF signaling (circled in grey). (B) The SABiosciences RT^2 qPCR array for cancer drug targets was run on matched subcutaneous PDX tumors and intraperitoneal PDX tumors. Differences in relative gene expression for each target was calculated and the $2^{\Delta Ct}$ value was determined. All 84 cancer drug target genes showed a strong correlation between the IP and SQ PDX tumors(C) PDX lines were treated with combination carboplatin and paclitaxel IP weekly. The percent change in tumor volume at 30 days was compared to the patient's response to primary therapy. PDX lines with the greatest decrease in volume significantly correlated to patients with a complete response to therapy (p=0.0009) (D) Classifying reduction in tumor volume by outcome of tumor reductive surgery (optimal debulking vs suboptimal) shows a trend towards PDX with the greatest reduction in volume correlating to optimal debulking for the patient (p-value = NS).

between PDX and patient tumors using aCGH [6]. To characterize whether expression of key oncogenes are similarly expressed in PDX tumors, an RT^2 PCR array on four pair of patient samples and matched PDX tumors was used. This array quantifies mRNA levels of 84 genes that are recognized targetable oncogenes[21]. There was a strong correlation of expression in 79 of the cancer drug targets, with an overall R^2-value of .744 (Figure 3A). This correlation was also present in individual samples (Supplemental Figure 1). The five genes that exhibited the poorest correlation had expression in the patient with near-zero mRNA expression in the PDX. These genes were platelet-derived growth factor receptor, alpha and beta polypeptide (PDGFRA, PDGFRB) and vascular endothelial growth factor receptor one, two, and three (VEGFR1, VEGFR2, VEGF3). These genes were expected to be decreased in the PDX tumor, since they are produced by the host, and the primers are human-specific. Therefore, there is strong consistency in expression of targetable oncogenes intrinsic to malignant cells, despite the fact that these tumors are growing in the subcutaneous compartment. In addition, we profiled the genetic difference of oncogene expression using the RT^2 PCR array comparing PDX tumors from the IP location versus the SQ implant. There was a strong correlation of expression among the 84 genes in the oncogene drug target array, with an overall R^2-value of .8895 (Figure 3B). This indicates that the SQ tumor has similar expression to a tumor growing in the orthotopic location.

While expression at the single-gene level is important, biologic similarity regarding response to treatment is equally important. Mice with measurable tumors from 19 PDX models were treated with IP carboplatin (90 mg/kg/week) and paclitaxel (20 mg/kg/week) in combination for 4 weeks. After 4 weeks, percent-reduction in tumor volume was calculated and compared to the patient's response to therapy, categorized as complete (CR, no evidence of disease at completion of 6 cycles of primary chemotherapy) or partial (PR, residual disease present at completion of 6 cycles of primary therapy). Patients that had a CR to therapy had an average reduction in volume of 63.73% (range 95.04% to 24.87%) compared to an average reduction of just 1.53% (range 57.77% reduction to 107.9% increase) in patients that had a PR ($p = 0.0009$, Figure 3C). There was also a differential, but not significant, response between patients who had an optimal or suboptimal tumor reductive surgery (Figure 3D). While not definitive, this suggests that patients presenting with disease unable to be optimally debulked are more aggressive and resistant to chemotherapy.

Biologic mediators of chemotherapy resistance in the PDX

With evidence showing that the PDX model accurately replicates the biology and clinical properties of the original patient tumor, we sought to explore differences between matched untreated and treated tumors. Mice were treated as described above, with tumors harvested 6 days after the 4th weekly dose, to minimize acute tumoral effects that might occur after chemotherapy administration. Ki-67 was examined to measure proliferation, and was not significantly different in untreated PDX tumors compared to the original patient tumor (Figure 4A,B). However, treated tumors had significant decrease in Ki-67 positivity (33.6% compared to 64.9% in untreated tumors $p=0.0013$). Examining the trend of each tumor individually (Figure 4C), two pair actually showed an increase in Ki-67, one of which had a 107% increase in tumor size on therapy, but the other with a 70.9% reduction. Despite these aberrations, the overall decrease in proliferating fraction suggests that dormancy is either being induced by chemotherapy, or some cells are already in a dormant state at presentation, and have intrinsic resistance to chemotherapy.

For analysis of which subpopulations have enhanced survival with chemotherapy, we assessed the density of the CSC populations expressing ALDH1A1, CD44, and CD133. If these populations were important to survival in the presence of chemotherapy, they should be more densely present after treatment, as noted in human specimens [11]. Treatment resulted in the significant enrichment of ALDH1A1-positive cells (increased from 16.2 to 36.1%, $p=0.002$) and CD133-positive cells (increased from 9.5% to 33.8%, $p=0.011$) (Figure 4D). Mean CD44 expression increased, but this was driven by two samples, and was not significant. These data suggests treated tumors are enriched in CSC populations.

Differential expression of genes due to chemotherapy treatment

Although cells with CSC properties were increased in treated specimens, they did not make up the entirety of the tumor. To globally examine which other genes and pathways are significantly altered during chemotherapy treatment, RNA-Seq was conducted on 6 pairs of treated and untreated PDX tumors. Across all six pairs, 299 genes were found to be significantly differentially expressed in the treated PDX samples compared to untreated (Supplementary Table 1), 137 of which have known roles in cancer. The top up-regulated genes and down-regulated genes are in Table 2. When principal component analysis was performed, an interesting trend emerged. Four of the samples clustered together, and the remaining two were separated in the 3D space. All the treated samples showed a

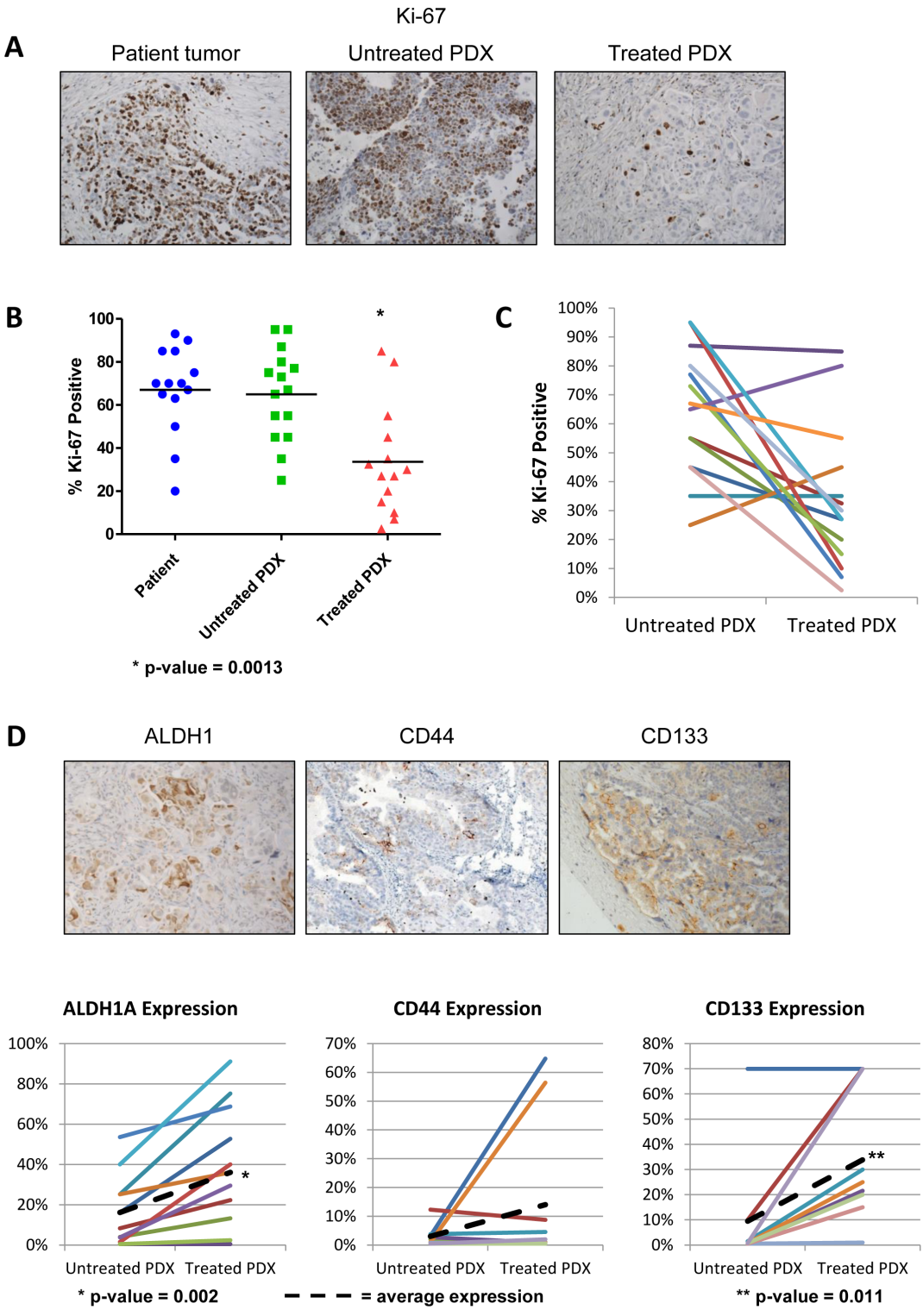

Figure 4: Chemotherapy treatment reduces proliferation and enriches the PDX for cancer stem cells. Tumor cell proliferation was quantified using the Ki67 marker on original patient samples, untreated PDX samples, and chemotherapy treated PDX samples. Change in cancer stem cell marker expression was analyzed after chemotherapy treatment. (A) Representative IHC of Ki67 staining in the patient sample, untreated PDX, and treated PDX. (B) On average, proliferation decreases with chemotherapy treatment in all PDX lines tested. There is no significant change in proliferation between the patient and the untreated PDX. (C) Proliferation rates for each treated and matched untreated pair show that the majority of tumors have a reduced proliferation rate after chemotherapy treatment (D) Representative IHC of CSC markers ALDH1A1, CD133, and CD44 of PDX treated with carboplatin and paclitaxel for 4 weeks. (E) In the treated PDX, expression of ALDH1A1 and CD133 are significantly increased (p-value = 0.0023 and p-value = 0.011 respectively).

Table 2: RNAseq analysis on PDX comparing 6 pairs of treated versus untreated samples.

Top Canonical Pathways	P-Value
Protein Kinase A Signaling	3.58E-05
GNRH Signaling	2.74E-04
Sphingosine-1-phosphate signaling	5.4E-04
α-Adrenergic signaling	9.39E-04
Cholecystokinin/Gastrin-mediated signaling	1.91E-03
Molecular and Cellular Functions	
Lipid Metabolism	1.33E-04 to 3.80E-02
Molecular Transport	1.33E-04 to 3.80E-02
Small Molecule Biochemistry	1.33E-04 to 3.80E-02
Cell Morphology	1.64E-04 to 3.80E-02
Cellular Assembly and Organization	1.64E-04 to 3.75E-02
Top Up-Regulated Molecules	Fold-Change Expression
ZNF750	2.441
ACP5	2.294
HIST2H2BE	2.141
CPEB3	2.117
DNM3	2.028
MPC1	1.980
ABCG1	1.938
MGLL	1.924
TLR5	1.884
Top Down-Regulated Molecules	Fold-Change Expression
APOC1	-2.488
GPHA2	-2.262
POLR3G	-1.862
TES	-1.759
PLCE1	-1.738
PUS7	-1.618
ARNT2	-1.607
MECOM	-1.570
CKAP4	-1.564
KLF5	-1.554

shift in the same direction away from their untreated PDX pair (Figure 5). This indicates that while the majority of genes are similar before and after treatment, all six tumors were affected similarly by therapy. IPA pathway analysis identified 5 major pathways that were significantly altered with treatment and key changes in molecular and cellular function (Table 2). Changes in these biological functions and pathways are consistent with the visualized phenotype of tumors responding to chemotherapy and reorganizing cellular function to adapt for survival.

DICUSSION

We demonstrated the feasibility of an ovarian PDX model that closely models the heterogeneity of the original patient's tumor and maintains clinical relevance. Ovarian PDX tumors form at a high rate when placed in the subcutaneous location. Growing tumors recapitulate the heterogeneity of the original patient tumor, and are not composed of just TICs, though the stromal component is murine. The PDX tumors have similar oncogene expression as the patient tumor, and respond

to chemotherapy in a similar manner as the patients from which they were harvested. These similarities make the PDX model an attractive platform for pre-clinical testing of therapies that will hopefully correlate with a clinical response better than noted in cell lines. Finally, using this model has allowed identification of pathways mediating survival after chemotherapy that are attractive targets for future study.

In most malignancies, preclinical studies have primarily utilized cell lines to assess novel therapies and biologic processes. Cell lines are still ideal for carefully controlled studies on mechanisms and pathways. However, in terms of translating results to the clinic, these models have underperformed [22]. The clonal nature of cell lines limits the ability to study both intratumoral and interpatient heterogeneity [8, 23]. In addition, new genomic studies indicate that commonly-used ovarian cancer cell lines do not accurately represent high-grade serous ovarian cancers when compared to profiling performed on the TCGA dataset[5].

Development of PDX models have been demonstrated in a few malignancies, including ovarian, colorectal, medulloblastoma, pancreatic, breast, and non-small cell lung cancers [6, 24-29], and have consistently been found to be similar to patient samples. One well-established program in pediatric malignancies has demonstrated prediction of response in the clinic is higher when the PDX model is used [30]. However, there are drawbacks to the model. The time for PDX tumors to grow is variable, but usually on the order of months, making experiments slow and expensive. Historically, rates for success of PDX establishment have been low, with the most successful models having 37% establishment rate [28, 31, 32], until Weroha's recent report of 74% overall success in ovarian cancer[6]. In this study, we had 85.29% success rate of establishing a PDX in the first 34 patients we implanted in the SQ. We believe the higher success rate is due to several factors. Given similar success of Weroha's report, this may be disease-specific. Strong working relationships with clinicians and pathologists allow for implantation within one hour of removal. We used two different processing methods that could be directly compared - one where solid tumors were implanted (SQ and SRC), and one where tumors were dissociated (MFP and IP). With both methods, the take rate was more dependent on the site implanted than the processing method. A crucial factor is the starting material. Other groups have reported that higher engraftment rates are associated with more aggressive tumors [6, 8, 29]. Instead of using the primary tumor from the ovary, we have implanted omental or peritoneal metastatic implants. The reasons for this are both biologic and practical. From a practical standpoint, omental implants are easily distinguished from normal tissue, reducing the risk of implanting normal tissue. A portion of "tumor" taken from the ovary, a complex tissue with normal solid components, may more likely be misinterpreted grossly as tumor, when in fact was benign. Because the omentum is well-vascularized, tumors are very "healthy", giving additional confidence that the portion implanted is not necrotic. Finally, it has been demonstrated that other factors produced in the omental microenvironment are

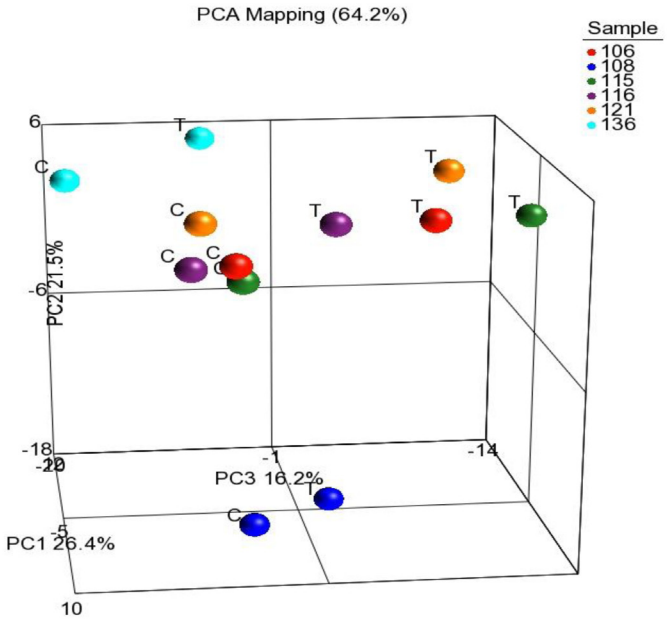

Figure 5: RNAseq comparing the treated PDX lines to the untreated PDX lines. Principal component analysis of genes expression in the treated and untreated PDX tumors. While matched treated and untreated PDX tumors clustered together, most treated PDX tumors had change of expression in the same direction indicating a small subset of genes responding to chemotherapy.

pro-tumorigenic, and are likely implanted with these tumors[33]. The biologic rationale for using metastatic implants is that these sites are more relevant to the portions of tumors that recur. Therefore it may be more clinically relevant to characterize the metastatic site.

The site of implantation is an important consideration as there are benefits and drawbacks from using an orthotopic or heterotopic site. Heterotopic locations allows for easier monitoring of the tumor while orthotopic preserves the appropriate microenvironment [24]. However, in developing this model, use of the intraperitoneal orthotopic location had practical limitations of lower engraftment rates and difficulty in assessment of growth. In several instances mice become moribund with ascites before there was appreciable tumor volume, even when following with micro-CT imaging. This limits the ability to measure response to a therapy, and provides less tissue for analysis and propagation into the next generation of PDX. However, the Weroha study demonstrated an ability for high take rate using the intraperitoneal injection with large volumes of tumor-cells [6]. Like our study, their mice also demonstrated development of ascites but by using ultrasound, were able to more accurately follow tumor progression then using a micro-CT. By using the heterotopic location, tumor growth can be easily monitored for establishment, growth, and response to therapy [8]. However, biologic relevance has to be demonstrated. With our findings that subcutaneous tumors have similar oncogene expression profiles to patient tumors and the orthotopic intraperitoneal PDX tumors, and respond to chemotherapy similarly, the subcutaneous model appears relevant. This information helps alleviate the primary concern of not using the orthotopic location and provides a mechanism for decreasing the technical complexity of establishing and using a PDX model. While in our hands, not enough intraperitoneal tumors developed to evaluate their correlation to the clinical response, based on our oncogene data comparing SQ and IP tumors and the Weroha study, it appears both models are equivalent. Not enough intraperitoneal tumors developed to demonstrate whether they would be equivalent, or superior, to the subcutaneous model. While previous groups have reported a high rate of success using the subrenal-capsule for tumor establishment [12], we did not see these successes. The ultimate proof of the importance of location in the PDX model will require testing numerous compounds, and relating the response in PDX tumors to responses in patients. PDX models in other malignancies have demonstrated a similar response rate between mice and the corresponding clinical trial [34-36]. Such studies in ovarian cancer are ongoing. But our analysis of the oncogene expression profiles, and their consistent similarity to patient tumors (Figure 3A), suggest that differences in targetable oncogenes between orthotopic PDX tumors and patient tumors are minimal.

We also demonstrate that the ovarian PDX model maintains the heterogeneity of the original patient tumor, at least from a TIC standpoint. Studies of CSC and TIC populations have shown that some cells are more capable of forming xenograft tumors than other[37]. Our analysis of density of ALDH1A1, CD44, and CD133 cells, the most consistent markers of TICs in ovarian cancer, demonstrates that PDX tumors are not only composed of these subpopulations (Figure 2B). It is possible that these subpopulations are the drivers of tumor formation, but as they grow they produce differentiated tumors with both CSC and non-CSC populations. This in fact would be predicted by the CSC model.

Potential limitations to the PDX model in ovarian cancer have been identified through our analysis. We saw that of 84 oncogenes examined, 5 were under-expressed in PDX tumors: receptors for platelet-derived growth factors and VEGF receptors. The fact that all members of these receptor families strengthens the validity of the association. Analysis of the species making up tumor stroma showed it to be composed purely of murine origin. The reduced content of human stromal genes is expected [38] as a result of the replacement of the human stroma with mouse stromal cells after implantation. Prior reports in pancreatic cancer have suggested that human stromal cells are maintained for several generations[39], although Weroha *et al* also found that IP ovarian PDX tumors had murine stroma. Whether murine stroma impacts the validity of the model will depend on the specific agent and pathway targeted.

The heterogeneity demonstrated in ovarian PDX tumors makes it uniquely positioned to investigate the key clinical problem of chemoresistance and recurrence. Ovarian cancer has a high rate of response to primary chemotherapy followed by an equally high rate of recurrence. One hypothesis is that this population is the same as the tumorigenic CSC population. While we have seen an increase in CSC density in the treated PDX tumors, and previously in treated patients[11], the persistent/recurrent tumors were by no means completely composed of these populations. Either the CSC populations had already begun to give rise to repopulating daughter cells negative for the CSC marker, or (more likely) other chemoresistant populations exist that cannot be identified by ALDH1A1, CD44, or CD133 alone. Going beyond CSCs, we have shown that surviving tumors have more cells in dormancy, decreasing from a baseline of 65% to 34%. RNA-seq analysis resulted in 299 genes being significantly different between the treated and untreated tumors with principal component analysis indicating that the changes in gene expression represent a small subset of the entire genetic makeup of the tumor (Figure 5, Supplementary Table 1). Most remarkable and encouraging is that the changes were similar in all pairs tested, providing hope that there may be common pathways to be targeted in most patients. One of the top up-regulated genes was ABCG1 (BCRP1), a member

of the White family of ATP-Binding cassette (ABC) transporters. Expression of ABCG1 has been shown to identify a side population of cancer cells that demonstrate CSC properties and chemoresistance [40]. Interestingly, one of the top activated pathways identified by IPA Ingenuity pathway analysis was Sphingosine-1-phosphate signaling. This pathway has been shown to protect oocytes from apoptosis induced by chemotherapeutic agents *in vitro* and *in vivo* [41, 42]. Taken together, the enrichment of CSC markers in the treated population, decrease in cell proliferation, and increase in genes and signaling pathways predicted to play a role in chemoresistance, it appears that treatment of the ovarian PDX results in the survival of a cell population that is chemoresistant to primary therapy. The global analysis by RNAseq provides a snapshot of possible pathways that are responsible for the development of chemoresistance. These will be important targets for therapy in future studies. With the development of an ovarian PDX model that recapitulates the clinical response and the heterogeneity of ovarian cancer, investigators are positioned to more effectively evaluate novel therapeutics and use the model to improve our understanding of the mechanisms of chemotherapy resistance. Hopefully targeting these pathways will sensitize cells to chemotherapy and lead to more durable cures.

CONCLUSION

Development of an ovarian PDX model to study *de novo* chemotherapy resistance provides a unique use of the xenograft model beyond testing pre-clinical compounds, allowing for possible novel understandings of tumoral responses to therapy that may lead to new strategies for targeting the residual survival population after primary therapy.

MATERIALS AND METHODS

Collection and Implantation of tumor specimens

Under IRB and IACUC approval, patients with suspected ovarian cancer that were being treated by the Division of Gynecologic Oncology at UAB were consented for this study. At the time of primary tumor reductive surgery, a specimen from an omental metastasis or peritoneal implant that was not required for pathologic diagnosis was collected and transported to the laboratory for processing. Specimens were sectioned and a portion submitted for formalin-fixed-paraffin embedding; placed in RNAlater (Qiagen, Frederick, MD); snap frozen in liquid nitrogen , and slow freezing in Optimal Cutting Temperature (OCT) Medium, and stored at -80°C. Remaining tumor was isolated for implantation into SCID mice (NCI-Frederick, Frederick MD) into four sites: subcutaneous (SQ), subrenal capsule (SRC), intraperitoneally (IP), and mammary fat pad (MFP). To discover the optimal site for tumor growth, of the first 22 patients, 22 were implanted SQ and MFP, 18 IP, and 12 SRC. When enough tumor was available, all four sites were implanted to allow direct comparison of growth rates. After it was evident that the subcutaneous implantation site was optimal, an additional 11 patients had tumors implanted only SQ.

For SQ implants, 5mm^2 tumor pieces (n=20 per patient) adjacent to the slice used for confirmation of histology were sectioned. 5 mice were implanted with four tumors each. The dorsal surface of the mouse was shaved and prepped with betadine solution. A 1cm midline incision was made and with blunt dissection, four pockets were created in four quadrants of the flank of the SCID mouse. One 5mm^2 tumor implant was placed in each quadrant and the incision was closed with staples.

For SRC implantation, five 3mm^2 tumor sections were prepared for implantation into five mice, one kidney per mouse. An incision was made in the body wall along the long axis of the kidney. The kidney was gently exposed through the incision, a 4 mm incision was made in the renal capsule, and an implant was inserted. The kidney was gently placed back into the body cavity and incision was closed with chromic gut sutures. For both SQ and SRC implantation, mice were anesthetized using isoflorane with 5% for induction of anesthesia and 1.5% for maintenance. Mice were administered carprofen (7mg/kg, Pfizer) prior to incision to reduce post-operative pain.

For injection into the MFP and IP sties, an adjacent portion of tumor was manually dissociated until fine enough to pass through a 21g needle. Prior to injection, the suspension was added to an equal volume of BD Matrigel (BD Biosciences, Cat#356234), mixed, and injected intraperitoneally (500,000 cells) or into bilateral MFPs (250,000 cells). Five mice were injected IP, and five mice had cells injected into the left and right MFP.

Treatment of PDX lines with chemotherapy

Once SQ or MFP tumors reached 500 mm^2 in volume, chemotherapy treatment was initiated in mice from 21 patients. Mice were injected IP with 90 mg/kg of carboplatin and 20 mg/kg of paclitaxel weekly or with vehicle, doses which approximate the maximal tolerated dose used in weekly dose-dense schedule of carboplatin and paclitaxel in patients. Tumors were measured biweekly using calipers. Volume of tumor was calculated using the formula (Length x Width^2)/2. After 5 weeks of treatment (4 weekly doses, then one week after last chemotherapy dose in order to minimize acute tumor effects of chemotherapy), mice were euthanized by CO_2 asphyxiation and cervical dislocation. Samples of treated and mice treated with vehicle were stored for

future analysis. Any remaining tumor was reimplanted for maintenance of the PDX.

Immunohistochemistry of patient samples and tumors from PDX tumors

Samples in FFPE were cut into 5 µm sections and placed on positively-charged slides. Hematoxylin and eosin stained tissue was analyzed by a gynecologic pathologist to confirm histology. For IHC of ALDH1A1, CD133, CD44, Ki-67 and human-HLA, slides were deparaffinized and rehydrated. Antigen retrieval was with 10 mM sodium citrate at pH 6.0 under pressure . Slides were washed in PBS. Endogenous peroxidases were blocked with 3% H_2O_2 in methanol. For ALDH1A1, CD133, and CD44, slides were blocked with Ctyo-Q immune-diluent (Innovex Biosciences Cat#NB307) followed by primary antibody incubation in Ctyo-Q immune diluent. Antibody concentrations were as follows: ALDH1A1 – 1:500 (BD Biosciences, Cat#611195) CD133 – 1:500 (Cell Signaling, Cat#3663S), CD44 – 1:500 (Cell Signaling, Cat# 3570S). After primary antibody, slides were washed in PBS. Primary antibody detection was achieved with Mach 4 HRP polymer (Biocare Medical), followed by 3,3'-diaminobenzidine incubation. Slides were counterstained with Gill's Hematoxylin then washed in water and PBS. Slides were sealed with Universal Mount (Open Biosystems, Cat#MBI1232). For Ki-67 (Abgent cat# AJ1427b) and human HLA (Proteintech Group Cat#15240-1), primary antibodies were used at concentrations of 1:200 in 10% normal goat serum. After incubation, slides were washed and blocked with 5% goat serum in 1X PBS. Primary antibody detection was visualized using an anti-rabbit HRP secondary at 1:500 in 5% goat serum (Vector Labs, Cat# PI-1000) and DAB substrate. Slides were counterstained as described above.

Scoring of IHC for TIC makers and Ki67

Two examiners (AK and CNL) visually estimated the percent of cancer cells staining for ALDH1A1, CD133, CD44, and Ki-67. A 3rd examiner (MGC) was included if there was a >20% discrepancy. The examiners were blinded to the experimental condition for each slide, and a 4th investigator (ZCD) averaged the scores for each specimen and decoded samples for analysis. To be consistent with prior identification of CSCs with flow cytometry, for CD133 and CD44 only expression at the surface membrane was considered. The average number of positive tumor cells for each marker was compared between the untreated PDX tumor and the patient's tumor, and between the treated and untreated PDX, with Student's t-test.

RT2-qPCR Arrays

RNA extracted from stored samples was converted to cDNA and amplified using the RT^2 First Strand cDNA Synthesis Kit (SABiosciences). Gene expression was then analyzed using the Cancer Drug Targets RT^2 Profiler PCR Array (SABiosciences), which profiles the expression of 84 genes that are potential oncogenic targets for anticancer therapeutics [21]. PCR amplification was conducted on an ABI Prism 7900HT and gene expression was calculated using the comparative C_T method as previously described [43].

High throughput sequencing of untreated and treated PDX tumors

Sample preparation, raw data prepressing, quality control were conducted in UAB Genomics Core and preliminary analysis was conducted in the UAB Biostatics Core. For RNA-seq, total RNA quality was assessed and the rRNA depleted and concentrated. The RNA-Seq libraries were prepared, validated and quantified. The raw fastq files were aligned to human genome hg19 of a local instance of Partek Flow software package (Saint Louis, MO). Pre-alignment was conducted to determine if trimming is needed based on reads quality score. Aligner STAR was used for best recovery[44]. The BAM files were loaded into Partek Genomics Suite 6.6 (Saint Louis, MO) for further analysis [45]. The reads per kilobase of exon model per million mapped reads (RPKM)-normalized reads were calculated and the expression levels of genes were estimated [46]. Additional filter was applied to exclude genes of low expression. The differential expressions were determined by using paired t-test [47]. Further functional analysis was conducted by using Ingenuity Pathway Analysis (IPA, Redwood City, CA).

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Conflict of Interest Statement

The authors have no conflict of interests to disclose.

Editorial Note

This paper has been accepted based in part on peer-review conducted by another journal and the author's response and revisions as well as expedited peer-review in Oncotarget.

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CURRICULUM VITAE

CHARLES N. LANDEN, JR., M.D., M.S.

PRESENT POSITION

Primary Appointment: Associate Professor, Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University of Virginia, Charlottesville, VA

Secondary Appointments: Associate Leader, Women's Oncology Program, UVA Cancer Center
Associate Professor, Department of Pathology
Graduate Faculty, UVA Graduate School
Member, UVA Cancer Center

OFFICE ADDRESS

Division of Gynecologic Oncology
University of Virginia
PO Box 800712
Charlottesville, VA 22908
Admin Office: (434) 243-9414
Admin Office Fax: (434) 982-1840
Email: clanden@virginia.edu

LAB ADDRESS

345 Crispell Dr.
Carter Harrison Bldg, Rm B704
Charlottesville, VA 22908
Lab Office Phone: (434) 243-6131
Lab Phone: (434) 924-5390

EDUCATION

Degree-Granting Education

University of North Carolina at Chapel Hill, Chapel Hill, NC, B.S., Biology, 1992

University of North Carolina School of Medicine, Chapel Hill, NC, M.D.,1998

University of Texas Graduate School of Biomedical Sciences, Houston, TX, M.S., 2005

Postgraduate Training

Resident, Obstetrics and Gynecology, Medical University of South Carolina, Charleston, SC
William Creasman (Chairman) and Peter Van Dorsten (Program Director), 7/1998 – 6/2002

Postdoctoral fellow, Microbiology and Immunology, Medical University of South Carolina, Charleston, SC
James Norris (Chairman), 7/2002 – 6/2003

Fellow, Gynecologic Oncology, MD Anderson Cancer Center, Houston, TX
David Gershenson (Chairman) and Diane Bodurka (Program Director), 7/2003 – 6/2007

PRIMARY FACULTY APPOINTMENTS

Clinical Instructor, Department of Obstetrics and Gynecology University of South Carolina School of Medicine	7/2002 – 6/2003
Assistant Professor, Research, Department of Gynecologic Oncology University of Texas MD Anderson Cancer Center	7/2007 – 6/2009
Assistant Professor, Department of Obstetrics and Gynecology University of Alabama at Birmingham	6/2009 – 10/2012
Associate Professor, Department of Obstetrics and Gynecology University of Alabama at Birmingham	10/2012 – 7/31/2014
Associate Professor, Departments of Obstetrics and Gynecology; and Pathology, University of Virginia	8/1/2014 – current

BOARD CERTIFICATION

Board certified, American Board of Obstetrics and Gynecology, 12/2003
Board certified, Gynecologic Oncology, 4/2011

LICENSES

Current:	Virginia	Avail on request	7/22/2014-current
	DEA	Avail on request	3/25/2005-current
Inactive:	South Carolina	21944	2002-2004
	Texas	M0466	2/04/2005-8/31/2010
	Alabama	29546	5/27/2009-7/31/2014

HONORS AND AWARDS

Medical School – *University of North Carolina School of Medicine, Chapel Hill, NC*
Dean's List, 1994-1996
Class Co-President, University of North Carolina School of Medicine, 1994-1996
University of North Carolina School of Medicine Merit Scholarship, 1995
NIH Summer Research Fellowship, University of North Carolina SOM, 1995

Residency – *Medical University of South Carolina, Charleston, SC*
First Place, Resident Oral Presentations, District IV Annual Meeting, ACOG, 2000
Donald F. Richardson Memorial Prize Paper Award, ACOG, 2001
Golden Apple Nominee, Medical University of South Carolina, 2002
Outstanding Resident Teaching Award, MUSC, Dept of OB/GYN, 1999 and 2001
First Place, Resident Oral Presentations, SC/GA Section, ACOG, 2001
Ortho-McNeil Award for Best Resident in Laparoscopy, MUSC, 2002
Resident Research Award for Outstanding Research in Women's Health, MUSC, 2002

Fellowship – *University of Texas MD Anderson Cancer Center, Houston, TX*
J.G. Moore Award, Best Presentation by Fellow or Resident, WAGO, 2004
Del and Dennis McCarthy Award for Advances in Surgery, MDACC, 2004
ASCO Fellows Travel Grant, 2005
AACR Scholar-in-Training Award, 2005
Trainee Excellence Award, MD Anderson Alumni and Faculty Association, 2005
Gynecologic Cancer Foundation / Carol's Cause Outstanding Paper Award, 2006
The Diane Denson Tobola Fellowship in Ovarian Cancer Research, MD Anderson Alumni and Faculty Assn, 2007
Bristol-Myers Squibb Poster Award in Clinical/Translational Research, MD Anderson Trainee Research Day, 2007

Faculty – *University of Texas MD Anderson Cancer Center, Houston, TX*
Listing in "Guide to America's Top Obstetricians and Gynecologists", 2007-2009
Reproductive Scientist Development Program Phase I Scholar, 2007-2009
Julie and Ben Rogers Award for Excellence Nominee, 2008

Faculty – *University of Alabama at Birmingham, Birmingham, AL*
Listing in "Best Doctors in America", 2010-2014
Reproductive Scientist Development Program Phase II Scholar, 20010-2012
John R. Durant Award for Excellence in Cancer Research, UAB CCC, 2009
Ovarian Cancer Academy Award Scholar, Department of Defense, 2010-2015
CCTS Scientific Symposium award, 1st place, UAB CCTS, 2010
Listing in Cambridge Who's Who among Executives and Professions in Research, Medicine, and Healthcare, 2011

Faculty – *University of Virginia, Charlottesville, VA*
Excellence in Reviewing, *Gynecologic Oncology* Case Report, 2014
Excellence in Reviewing, *Gynecologic Oncology*, 2014
Donald Swartz Travel Award, AAOGF/AGOS, 2014
NIH CBSS Study Section, Invited reviewer, 2014
NCI Special Emphasis Panel, ZCA1 RPRB – C: SPORE proposals; Invited reviewer, 2015
NIH CBSS Study Section, Standing member, 2015-2020

EXPERIENCE/SERVICE

PROFESSIONAL SOCIETY MEMBERSHIPS

American College of Obstetricians and Gynecologists – Fellow
Society of Gynecologic Oncology – Full Member
American Association for Cancer Research – Full Member
American Society of Clinical Oncology – Full Member
NRG / Gynecologic Oncology Group – Member
The Gynecology and Obstetrics Society, Medical University of South Carolina, 2002-present
UAB Griffin Society, University of Alabama at Birmingham, 2009-2014
The Felix Rutledge Society, MD Anderson Cancer Center, 2007-present

PROGRAMS

Director of Resident Research, Dept of OB/GYN, UAB, 2010-2014
Co-Director, Molecular Tumor Board (Precision Medicine initiative), UAB Comprehensive Cancer Center, 2013-14
Co-Leader, Women's Oncology Program, University of Virginia Cancer Center
Co-Director, Resident Research, Dept of OB/GYN, UVA, 2015-current
Faculty Director, Molecular Assessments and Preclinical Studies (MAPS) core facility, UVA, 2015-current.

COMMITTEES

National / International

Marketing and Publications Committee, Society of Gynecologic Oncologists (SGO), 2009-2011
Annual Meeting Program Committee, Society of Gynecologic Oncologists (SGO), 2010-2011
Awards Committee, Foundation for Women's Cancer (FWC), 2010-2013
Education Committee, Society of Gynecologic Oncologists (SGO), 2011-2016
Website Development Task Force, Foundation for Women's Cancer (FWC), 2011
Board of Directors, Foundation for Women's Cancer (FWC), 2011-2016
Outcome Research Institute, Society of Gynecologic Oncology (SGO), 2014-2016
Annual Meeting Program Committee, Society of Gynecologic Oncologists (SGO), 2014-2015
Translational Research Workgroup, Foundation for Women's Cancer, 2016-current

Regional / Institutional

Clinical Ethics Committee, MUSC, 1998-2003
Academic Program coordinator, MUSC, 2001-02
Fellowship Admissions Committee, MDACC, Dept of Gynecologic Oncology, 2005-2006
Fellowship Planning Committee, MDACC, Dept of Gynecologic Oncology, 2005-2006
Steering Committee Member, Comprehensive Cancer Center School of Medicine Strategic Plan, UAB, 2011
Biorepository Development Committee, UAB School of Medicine, 2011-14
Endowed chair in Pathology Search Committee, UAB, 2011-14
Resident Selection Committee, UAB, 2011-14
Clinical Trial Audits, UAB, 2011
Tissue Committee, UAB, 2012-14
Board of Directors, Norma Livingston Foundation, Birmingham, AL, 2012-14
OB/GYN Fund for Excellence in Education, Advisory Committee, UAB, 2012-14
Entering Mentoring Review Group, UAB, 2013-14
Institutional representative, LCME site review, UAB, 2014
Pathobiologist faculty search committee, UAB, 2014
Hematology/Oncology Division Director search committee, UAB, 2014
Protocol Review Committee, UVA Cancer Center, 2014-current
Biorepository and Tissue Research Facility (BTRF) Advisory Board, UVA, 2014-current
School of Medicine Committee on Women, Steering Committee, UVA, 2014-current
Anderson Committee, UVA, 2015-current

Chair, Peyton Taylor Endowed Scholarship Program, 2015-current
Dean's Research Advisory Committee, 2016-current

GRANT REVIEWER SERVICE

National / International

Ontario Institute for Cancer Research, Cancer Research Fund Translational Panel, Scientist Reviewer, 2009, '10. '11
External Reviewer, Ovarian Cancer Action Research Centre Quinquennial Review, 2011-12
CDMRP DOD Ovarian Cancer Research Program, Pathobiology Panel, Scientist Reviewer, 2010, '11, '12, '15
Foundation for Women's Cancer (FWC) Research Grants/Awards Committee, Grant Reviewer, 2010-2013
Ovarian Cancer Research Fund, Ann Schreiber Research Training Program of Excellence, Grant Reviewer, 2012
Target Ovarian Cancer, Grant Reviewer, 2013
NIH CBSS Study Section, Invited reviewer, 2014
NCI Special Emphasis Panel, ZCA1 RPRB – C: SPORE proposals; Invited reviewer, 2015
NIH SBIR Panel, ZRG1 OTC-B: Cancer diagnostics and Treatment; Invited reviewer, 2015
CDMRP DOD Ovarian Cancer Pilot Grant, Reviewer, 2015.
NIH CBSS Study Section, Standing member, 2015-2020
Ovarian Cancer Translational Research Initiative, Toronto, Ontario, Canada, 2016

Regional / Institutional

Ovarian Cancer Pilot Grant, University of Alabama at Birmingham Comprehensive Cancer Center, 2010
AAAS Research Competitiveness Program, Connecticut Bioscience Innovation Fund, Reviewer, 2014
Tina's Wish Research Grant, The Honorable Tina Brozman Foundation for Ovarian Cancer Research, 2014
UVA Cancer Center Transdisciplinary Project Grant, 2014
Peyton Taylor Endowed Scholarship Program, Chair, 2015-current

JOURNAL SERVICE

Editorial Board

Gynecologic Oncology
Gynecologic Oncology Case Report

Ad-hoc Reviewer

JAMA
Journal of Clinical Oncology
Cancer Research
Clinical Cancer Research
Molecular Cancer Therapeutics
Oncogene
Cancer
PLOS One
Genes and Cancer
Journal of Clinical Investigation
Oncotarget
Molecular Carcinogenesis
American Journal of Obstetrics and Gynecology
Expert Opinion on Therapeutic Targets
Cancer Letters
Tumor Biology
Neoplasia
Journal of Obstetrics and Gynaecology Research
British Journal of Cancer

TRAINING COURSES TAKEN

Ethics Consult Service, Medical University of South Carolina, 1998-2003
AACR Molecular Biology in Clinical Oncology Workshop, Aspen, Colorado, 7/2004
Stem Cell Training Workshop, Stem cell Technologies, Inc., Vancouver, BC, Canada, 9/2007
Clinical and Translational Science Training Program, UAB CTSA, 2011

CLINICAL TRIALS

105OC201: A Phase 2 Evaluation of TRC105 in the Treatment of Recurrent Ovarian Fallopian tube, or Primary Peritoneal Carcinoma. Role: Principal Investigator. Sponsor: TRACON Pharmaceuticals, Inc. 9/14/2011-9/9/2012.

UAB 0801: Phase I of Intraperitoneal ^{212}Pb-TCMC-trastuzumab for HER-2 expressing malignancy. Role: Co-Investigator. Sponsor: Areva Med LLC 2/23/2011-7/31/2014.

UAB 1357: Phase IB/II trial of LDE225 and Weekly Paclitaxel in Recurrent Platinum Resistant Ovarian Adenocarcinoma. Role: Co-Investigator. Sponsor: Novartis. 11/2013-7/31/2014.

TEACHING/MENTORSHIP

Courses/Programs

Gross Anatomy Teacher's Assistant, UNC School of Medicine, 1994-1995
Small group preceptor, Medical Ethics course, MUSC, 2002-2003
Angiogenesis section lecturer, Cancer Biology course, UAB Graduate School, 2010-2014
Director of Resident Research, Dept of OB/GYN, UAB, 2010-2014
Small Group leader, New Student Discussion Group (NSDG), UAB School of Medicine, 2012
Course Co-Director, Translational Cancer Research, UAB Graduate School, 2012-13
Small group preceptor, Research Ethics BIMS 7100, 2016

Graduate Student Mentorship

Primary Advisorship

Zachary Dobbin, MD/PhD candidate. *Development and validation of a primary xenograft model in ovarian cancer*. **Primary advisor**, UAB, 2011-2014. **Successful defense** 9/10/2014. **AWARDS**: Best poster presentation, UAB Medical Student Research Day, 2012. AACR Scholar-in-Training Award, AACR, 2013. Best poster presentation, UAB Medical Student Research Day, 2014.

Kelly Kreitzburg, PhD candidate. *Targeting phingosine-1-phosphate for the treatment of drug-resistant ovarian cancer.* Lab rotation and PhD mentor, 2013-2014 (Mentor change after I left the institution). **AWARDS**: 3rd place, UAB CCC Research Day, 2014.

Samantha Sherwood, PhD candidate. The role of the sphingosine 1-phosphate pathway in ovarian cancer growth and chemotherapy resistance. **Primary advisor**, UVA, 2015-2016 (Candidate decided to withdraw from graduate school). **AWARDS:** Recipient, NIH Training Grant, 2015-2016.

Robert Cornelison, PhD candidate. Targeting ribosomal machinery to overcome chemoresistance in ovarian cancer. Primary Advisor, UVA, starting 8/2016.

Secondary Advisorship

Amanda Debrot, PhD candidate. *Examination of stem cells in fallopian tubes.* Lab rotation, UAB, 2010.

Katie Jo Rohn, PhD candidate. *Development of an assay for Jagged1 inhibitors*. Lab rotation, UAB, 2011.

Hugo Jiminez, PhD candidate. *Examination of Notch-independent effects of Jagged1 signaling in ovarian cancer chemoresistance*. Lab rotation, UAB, 2011-12.

William Jackson, PhD candidate. *Targeting SPANX in taxane-resistant ovarian cancer*. Lab rotation, 2012.

Ann Hanna, PhD candidate. *Induction of dormancy by NR2F1 in ovarian cancer*. Lab rotation, 2013.

Marissa A. Gonzales, PhD candidate. Inhibition of the sphingosine-1-phosphate pathway on chemoresistant ovarian cancer cells. UVA, 2015.

Postdoctoral fellow mentorship

Adam Steg, PhD. *The role of Jagged1 and Sonic Hedgehog in ovarian cancer growth and chemoresistance*. UAB, 12/2009-7/2014.

Dae Hoon Jeong, MD, PhD. Associate Professor, Inje University, Busan, South Korea. Visiting Research Assistant, *Combined efficacy of hedgehog and proteasome targeting in ovarian cancer.* UAB, 2012-13.

Huaping Chen, PhD. *The role of microRNAs on epigenetic mediation of chemotherapy resistance in ovarian cancer*. 7/2013-7/2014.

Graduate Student Advisor Committee Member

Patrick Garcia, PhD candidate. *Development of an animal model of pancreatic cancer for therapeutic intervention*. 2010-2014.

Huaping Chen, PhD candidate. *Epigenetic targeting of ovarian cancer*. 2011-2013.

Matt Schultz, PhD candidate. *The role of STGal-I in the ovarian tumor cell phenotype.* 2012-2014.

Hugo Jiminez, PhD candidate. *Amplitude Modulated Radiofrequency Electromagnetic Fields as a Novel Treatment for Ovarian Cancer*. 2012-2014.

Monicka Wieglos, PhD candidate. *Mechanisms of PARP sensitization in HER2-positive breast cancer*. 2012-2015

Alice Weaver, MD-PhD candidate. *Targeting DNA damage repair mechanisms in HPV-driven head and neck cancers*. 2013-2015.

Ashley Conoway, PhD candidate. *The role of Tdp-1 in DNA damage and repair*. 2014-2015.

Kelly Kreitzburg, PhD candidate. *Targeting the sphingosine 1-phosphate pathway in drug-resistant ovarian cancer*. 2014-2016.

Kiley Anderson, PhD candidate. *SAS1B as a target in cancer*. 2014-current.

Annie Carlton, PhD candidate. *Identifying the role of CBF-beta in ovarian cancer*. 2015-current.

Alexandra Harris, PhD candidate. *The Contribution of Lymphatic Activation to Triple-Negative Breast Cancer Therapeutic Response.* 2016-current.

Camille M. Lewis, PhD candidate. *The Importance of Cellular E6AP in HPV E6 Function and Viral Life Cycle.* 2016-current.

Gynecologic Oncology Fellow Mentorship

Kerri Bevis, MD. Fellow in Gynecologic Oncology. *Examination of Stem Cell Markers in Matched Primary and Recurrent Ovarian Cancer*. UAB, 7/2009-6/2012. **AWARDS:** UAB CCTS Scientific Symposium award, 1st place, 2010; Featured poster, SGO Annual Meeting, 2011.

Angela Ziebarth, MD. Fellow in Gynecologic Oncology. *Targeting Endoglin (CD105) improves platinum sensitivity in epithelial ovarian cancer* and *The ubiquitin ligase EDD as a mediator of platinum resistance target for therapy in epithelial ovarian cancer*. UAB, 7/2010-6/2013. **AWARDS:** Featured poster, SGO Annual Meeting, 2011.

Monjri Shah, MD. Fellow in Gynecologic Oncology. *Functional assessment of defects in homologous recombination as a predictor of response to PARP inhibitors.* UAB, 7/2011-6/2014.

Erickson, Britt, MD. Fellow in Gynecologic Oncology. *Detection of somatic TP53 mutations in tampons of patients with high-grade serous ovarian cancer*. UAB, 7/1/2012-2015.

Arend, Rebecca, MD. Fellow in Gynecologic Oncology. *WNT/ß-Catenin Pathway as a Target for the Treatment of Ovarian Cancer.* UAB, 1/1/2014-6/30/2015. **AWARDS**: WeRoc/OchO Ovarian Cancer Research Grant, Foundation for Women's Cancer, 2014, $50,000.

OBGYN Resident Mentorship

Gretchen Zsebik, MD. Resident in Obstetrics and Gynecology. *Outcomes in Ovarian Cancer Patients Managed by General Gynecologists* and *Management of Complex Pelvic Masses Using the OVA1 Test: A Decision Analysis*, UAB, 2010-2012. **AWARDS:** Featured poster/Oral presentation, SGO Annual Meeting, 2011.

Jovana Martin, MD. Resident in Obstetrics and Gynecology. *Detection of ovarian cancer cells in the vagina: a pilot feasibility study.* UAB, 9/2011-6/2013. **AWARDS:** Best presentation, PGY2 Resident research Day, 2011.

Blake Porter, MD. Resident in Obstetrics and Gynecology. *Physician Adherence to the US Dept of Health and Human Services Physical Activity Guidelines: A Randomized Control Trial.* UAB, 9/2011-6/2013.

Medical Student Mentorship

Nick Nolte, MS3. *The contribution of claudin-16 to taxane resistance in ovarian cancer*. UAB, 2010.

Mata Burke, MS1. 1) *Combined Hedgehog and Notch targeting in ovarian cancer*. UAB, 2011, 2012. **AWARD**: 1st Place, Oral presentation Short Term Research Category, Medical Student Research Day, 10/25/2011, invited to present at National Student Research Forum 4/2012.
2) *Proteasome Inhibition Synergizes with Hedgehog Inhibition and Reverses Taxane Resistance in Ovarian Cancer*. **AWARD**: Honorable mention, Medical Student Research Day, 10/29/2013.

John Ogorek, MS3. *Patient characteristics of ovarian cancer managed initially managed by general OB/GYN physicians*. UAB, 2012.

Tooba Anwer, MS1. *Examination of the effect of targeting the Sphingosine-1-phosphate pathway in overcoming chemoresistance in ovarian cancer*. UAB/NIH Medical Student Summer Research Project Program, 2014.

Undergraduate Student Mentorship

Lindy Pence, Sophomore, Wofford College. *Inhibition of the mTOR/PI3K Pathways to Enhance Sensitivity of Ovarian Cancer Cells to Chemotherapy Treatment.* Summer in Biomedical Science (SIBS) Undergraduate Research Program, 2012. **AWARDS**: 2st Place, Life Sciences Division, UAB Summer Research Expo, 2012.

Jacqueline Upp, Junior, University of Alabama at Birmingham. *Racial disparities in ovarian cancer*. 2013. Summer Research Partnership, Morehouse-Tuskegee-UAB CCC Partnership.

Technician Direct Supervision

Guillermo Armaiz-Pena, PhD. *Mediators of Stress-Induced Cancer Progression*. MDACC, 2004-2005.

Blake Goodman, B.S. *Examination of Tumor Initiating Cells in Ovarian Cancer*. MDACC, 2008-2009.

Ashwini Katre, MS. C*hemoresistance mechanisms of ALDH1 in ovarian cancer*. UAB, 2010-present.

RESEARCH FUNDING

Active

Co-Investigator*, Glycosylation-dependent mechanisms regulating ovarian tumor cell survival*. R01 GM111093, NIH/NIGMS, 4/1/2014 – 3/31/2017, $570,000 total direct.

Co-Investigator, *Development of a Novel Small Molecule PTP4A3 Inhibitor for the Treatment of Ovarian Cancer.* The Ivy Foundation, 3/1/2016 – 2/28/2017, 78,000 total direct.

Co-Investigator, *Developing ovarian cancer stem-like cell targeted therapy to prevent disease recurrence*, Ovarian Cancer Research Program Pilot Award, CDMRP Department of Defense, 9/1/2014 – 8/31/2016, $51,532 over 2 years.

Co-Investigator, *DNA repair enzyme tyrosyl-DNA phosphodiesterase I as novel therapeutic target for ovarian cancer treatment,* Ovarian Cancer Research Program Pilot Award, CDMRP Department of Defense, 9/30/2015 – 09/29/2017, $10,324 over 2 years.

Principal Investigator (Faculty Director), Molecular Assessments and Preclinical Studies (MAPS) Core Facility, UVA Cancer Center, 9/2015-current. $11,800/yr.

Prior

Principal Investigator, *Characterization and Targeting of the Aldehyde Dehydrogenase Subpopulation in Ovarian Cancer*, OC093443, Department of Defense Ovarian Academy Award, 7/1/2010 – 7/30/2016, $750,000 total direct.

Principal Investigator, *Nanoparticle delivery of siRNA to target chemoresistance in ovarian cancer*. Transdisciplinary Research Grant, UVA Cancer Center, 1/1/2015-12/31/2015, $100,000 total direct.

Co-Investigator, *Ribosome biogenesis, turnover and function as a therapeutic target for ovarian cancer*, Program Project Grant Pilot Fund, UAB Comprehensive Cancer Center, 8/1/2014 – 7/31/2015, $150,000.

Co-Investigator, *Using RPS25 to Target the Survival Pathway in Ovarian Cancer*, Faculty Development Award, UAB Comprehensive Cancer Center, 3/15/14 – 3/14/15, $40,000.

Principal Investigator, *Targeting Ribosomal RNA Synthesis for Treatment of Ovarian Cancer*, RSDP Seed Grant Program. 9/1/2014 – 8/31/2015, $25,000.

Co-Investigator, U54 pilot project: *BRCA1 Deficiency and Epithelial Ovarian Cancers.* Morehouse School of Medicine/Tuskegee University/University of Alabama Cancer Center Partnership. 9/1/2011-8/31/2014, $18,000.

Principal Investigator. *Identifying mediators of chemoresistance in ovarian cancer*. The Norma Livingston Foundation. 5/1/2012-4/30/2014. $50,000.

Principal Investigator. *Development of a Personalized Therapy Model in Cervical Cancer*. Pilot Project, SPORE in Cervical Cancer. 9/1/2012 – 8/31/2014. $30,000.

Co-Investigator, *Chemosensitization of Ovarian Cancer by Exploiting Novel and Safe Epigenetic Compounds*. College of Arts and Sciences Interdisciplinary Innovation Team Award (PI Trygve Tollefsbol). 10/1/2012-9/30/2014. $30,000.

Principal Investigator. *Detection of ovarian cancer-derived mutations in tampon extracts using Safe-SeqS*. The Laura Crandall Brown Foundation. 12/5/2012-12/4/2014. $50,000.

Co-Principal Investigator. Predicting response of ovarian cancers to PARP Inhibitors. The ROAR Foundation. 12/14/2012 – 12/13/2014. $50,000.

Principal Investigator, *105OC201: A Phase 2 Evaluation of TRC105 in the Treatment of Recurrent Ovarian Fallopian tube, or Primary Peritoneal Carcinoma*. Sponsor: TRACON Pharmaceuticals, Inc. 9/14/2011-9/9/2012. $19,385 in charges.

Principal Investigator, *Targeting Jagged in Ovarian Tumor Initiating Cells*, Research Scientist Development Program Phase II (through the Ovarian Cancer Research Fund), 7/1/2009-6/30/2012, $240,000 over 3 years.

Principal Investigator, *Examination of the true mediators of resistance in ovarian cancer,* Translational Research Intramural Grant, UAB CCTS and CCC, 4/1/2010 – 3/31/2011, $71,000 over 1 year.

Principal Investigator, *Targeting the Notch pathway in Ovarian Cancer Initiating Cells*, Sarah Biedenharn/Gynecologic Cancer Foundation Ovarian Cancer Research Grant, 7/1/2009-6/30/2010, $50,000 over 1 year.

Principal Investigator, *Characterization and therapeutic targeting of ovarian cancer stem cells*, 5P50 CA083639, Career Development Award, Ovarian Cancer SPORE at MDACC, 9/1/2007 – 8/31/2009, $150,000 over 2 years.

Principal Investigator, *The role of the alpha v beta 3 integrin in signaling and as a target in human ovarian cancer*, NIH #5K12 HD00849: Reproductive Scientist Development Program (RSDP) Phase I, 7/1/2007-6/30/2009, $262,000 over 2 years.

Principal Investigator, *Characterization of ovarian cancer xenografts*, HERA Foundation Investigator Award, 5/1/2008-4/31/2009, $30,000.

Principal Investigator, *The role of EphA2 in ovarian cancer*. Bettyann Asche-Murray Fellowship Award, M.D. Anderson Cancer Center, 7/1/2005-6/30/2007, $10,000.

Patents Granted and Pending

Delivery of siRNA by neutral lipid compositions, MDACC, United States, 60/671,641, 4/15/2005, Filed.

PUBLICATIONS

1. Kay MA, Rothenberg S, **Landen CN**, Bellinger DA, Leland F, Toman C, Finegold M, Thompson AR, Read MS, Brinkhous KM, Woo SLC. In Vivo Gene Therapy of Hemophilia B: Sustained partial correction in Factor IX-deficient dogs. *Science* 262:117-119, 1993. PMID: 8211118
2. Kay MA, **Landen CN**, Rothenberg SR, Taylor LA, Leland F, Wiehle S, Fang B, Bellinger D, Finegold M, Thompson AR, , Read M, Brinkhous KM, Woo SLC. In Vivo hepatic gene therapy: Complete albeit transient correction of factor IX deficiency in hemophila B dogs. *Proc Natl Acad Sci* USA 91:2353-2357, 1994. PMID: 8134398
3. Fang B, Eisensmith RC, Wang H, Kay MA, Cross RE, **Landen CN**, Gordon G, Bellinger DA, Read MS, Hu PC, Brinkhous KM, Wood SLC. Gene therapy for hemophilia B.: Host immunosupression prolongs the therapeutic effect of adenovirus-mediated factor IX expression. *Hum Gene Ther* 6:1039-1044, 1995. PMID: 7578416
4. Liles D, **Landen CN**, Monroe DM, Lindley CM, Read MS, Roberts HR, Brinkhou KM. Extravascular administration of factor IX: Potential for replacement of therapy of canine and human hemophilia B. *Throm Haemo* 77(5):994-948, 1997. PMID: 9184407
5. **Landen CN Jr,** Zhang P, Young RC**.** Differing mechanisms of inhibition of calcium rises in human uterine myocytes by indomethacin and nimesulide. *Am J Obstet Gynecol* 1984(6):1100-1103, 2001. PMID: 11349169
6. Vermillion ST, **Landen CN**. Prostaglandin inhibitors as tocolytic agents. Sem Perinatol 25(4):256-262, 2001. PMID: 11561913
7. Mathur SP, **Landen CN**, Datta SM, Hoffman MC, Mathur RS, Young RC. Insulin-like growth factor-II in gynecologic cancers: A preliminary study. *Am J Reprod Immunol* 49(2):113-119, 2003. PMID: 12765350
8. **Landen CN Jr,** Mathur SP, Richardson MS, Creasman WT. Expression of cyclooxygenase-2 in cervical, endometrial, and ovarian malignancies. *Am J Obstet Gynecol* 188(5):1174-1176, 2003. PMID: 12748469
9. **Landen CN Jr,** Younger NO, Collins Sharp BA, Underwood PB. Cancer patients' satisfaction with physicians: PMH-SPQ-MD questionnaire results. *Am J Obstet Gynecol* 188(5):1177-1179, 2003. PMID: 12748470
10. Thaker PH, Deavers M, Celestino J, Thornton A, Fletcher MS, **Landen CN**, Kinch MS, Kiener PA, Sood AK. EphA2 espression is associated with aggressive features in ovarian carcinoma. Clin Cancer Res 10(15): 5145-50, 2004. PMID: 15297418
11. **Landen CN,** Klingelhutz A, Coffin JE, Sorosky JI, Sood AK. Genomic instability is associated with lack of telomerase activation in ovarian cancer. *Cancer Biol Ther*. 3(12): 1250-53, 2004. PMID: 15477760
12. **Landen CN Jr,** Chavez-Reyes A, Bucana C, Schmandt R, Deavers MT, Lopez-Berestein G, Sood AK. Therapeutic EphA2 gene targeting in vivo using neutral liposomal siRNA delivery. Cancer Research 65(15): 6910-18, 2005. PMID: 1606175
13. Imai M, **Landen C**, Ohta R, Cheung NK, Tomlinson S. Complement-mediated mechanisms in anti-GD2 monoclonal antibody therapy of murine metastatic cancer. *Cancer Res* 65(22): 10562-10568, 2005. PMID: 16288049
14. **Landen CN,** Kinch MS, Sood AK. EphA2 as a target for ovarian cancer therapy. *Expert Opin Ther Targets* 9(6): 1179-1187, 2005. PMID: 16300469
15. Halder J, **Landen CN Jr**, Lutgendorf SK, Li Y, Jennings NB, Fan D, Nelkin GM, Schmandt R, Schaller MD, Sood AK. Focal adhesion kinase silencing augments docetaxel-mediated apoptosis in ovarian cancer cells. *Clin Cancer Res* 11(24): 8829-36, 2005. PMID: 16361572
16. Sood AK, Bhatty R, Kamat AA, **Landen CN**, Han L, Thaker PH, Li Y, Gershenson DM, Lutgendorf S, Cole SW.. Stress hormone mediated invasion of ovarian cancer cells. *Clin Cancer Res* 12(2):369-75, 2006 PMID: 16428474
17. Han LY, **Landen CN Jr**, Kamat AA, Lopez A, Bender DP, Mueller P, Schmandt R, Gershenson DM, Sood AK.. Preoperative serum tissue factor (TF) levels are an independent prognostic factor in patients with ovarian carcinoma. *J Clin Oncol* 24(5): 755-61, 2006. PMID: 16380413

18. Kamat AA, Fletcher M, Gruman LM, Mueller P, Lopez A, **Landen CN Jr**, Han L, Gershenson DM, Sood AK. The clinical relevance of stromal matrix metalloproteinase expression in ovarian cancer. *Clin Cancer Res* 12(6): 1707-14, 2006. PMID: 16551853

19. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, Armaiz-Pena G, Bankson JA, Ravoori M, Merritt WM, Lin YG, Mangala LS, Kim TJ, Coleman RL, **Landen CN**, Li Y, Felix E, Sanguino AM, Newman RA, Lloyd M, Gershenson DM, Kundra V, Lopez-Berestein G, Lutgendorf SK, Cole SW, Sood AK. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nature Medicine* 12(8):939-44, 2006. PMID: 16862152

20. Halder J, Kamat AA, **Landen CN Jr**, Han LY, Lutgendorf SK, Lin YG, Merritt WM, Jennings NB, Chavez-Reyes A, Coleman RL, Gershenson DM, Schmandt R, Cole SW, Lopez-Berestein G, Sood AK. Focal adhesion kinase targeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. *Clin Cancer Res* 12(16): 4916-24, 2006. † PMID: 16914580

21. Han LY, **Landen CN**, Trevino JG, Halder J, Lin YG, Kamat AA, Kim TJ, Merritt WM, Coleman RL, Gershenson DM, Shakespeare WC, Wang Y, Sundaramoorth R, Metcalf CA 3rd, Dalgarno DC, Sawyer TK, Gallick GE, Sood AK. Anti-angiogenic and anti-tumor effects of Src inhibition in ovarian carcinoma. *Cancer Res* 66(17): 8633-8639, 2006. PMID: 16951177

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* Equal contribution † Selected for Cover Art and/or Issue Highlights

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121. Landen CN. Novel methods for Screening in Ovarian Cancer. *South Atlantic Association of Obstetricians and Gynecologists*, Hot Springs, VA, Jan 2017.

§ Selected for Meeting Award

BOOK CHAPTERS

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4. **Landen CN**. Biologic and Targeted Therapies, in *Society of Gynecologic Oncologists Chemotherapy Handbook*, under construction.

CONFERENCES AND SYMPOSIA

Conference organization

Chair, Program Committee, UAB Comprehensive Cancer Center Annual Research Retreat, "Personalized Cancer Care". October, 2014.

Session Director, 9th annual Cancer Molecular Therapeutics Research Association meeting, "How Clinical Data is Changing Drug Discovery". July, 2016.

Presentations at National or International Conferences

Invited

Differing mechanisms of inhibition of calcium rises in human uterine myocytes by indomethacin and nimesulide. The Donald F. Richardson Prize Paper Award Presentation. 50th Annual Clinical Meeting of the American College of Obstetricians and Gynecologists, 5/2002.

Neuroendocrine modulation of STAT3 in ovarian cancer. Western Association of Gynecolgic Oncologic Oncologists, 5/2004.

Genomic Instability is Associated with Lack of Telomerase Activation in Ovarian Cancer. 6th International Conference on Ovarian Cancer, MD Anderson Cancer Center, 12/2005.

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Targeting the αvβ3 integrin with a fully humanized antibody in ovarian cancer. 37th Annual Society of Gynecologic Oncologists Meeting, Palm Springs, CA, 3/2006.

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Perioperative factors associated with survival after secondary cytoreduction in patients with recurrent epithelial ovarian cancer. Society of Gynecologic Oncologists Winter Meeting, Beaver Creek, CO, 2/2007.

Trial-based review of Management of Ovarian Cancer. 39th Annual Meeting of the Society of Gynecologic Oncologists, Tampa, Fl, 3/2008.

Targeting the αvβ3 integrin in ovarian cancer. Society of Gynecologic Investigation Annual Meeting, San Diego, CA, 3/2008.

Trial-based review of Management of Ovarian Cancer. 40th Annual Meeting of the Society of Gynecologic Oncologists, San Antonio, TX, 3/2009.

"Nanoparticle Delivery Systems for siRNA Therapy." 3rd Annual Symposium on Ovarian Cancer Research, Medical University of South Carolina, 5/2009.

"Independent targeting of the Notch pathway in tumor cells and tumor stroma." Reproductive Scientist Development Program Retreat, 10/2010.

"Establishing successful collaborations in research." Southeastern Medical Scientist Symposium, Emory University, Atlanta, GA, 9/2011.

"Ovarian Cancer Stem Cells: Clinically Significant or Experimental Phenomenon." 3rd Annual International Conference, Ovarian Cancer: Prevention, Detection and Treatment of the Disease and its Recurrence, Pittsburg, PA, 5/2012.

"Promising Recent Advances in Ovarian Cancer Research". Foundation for Women's Cancer Survivor's Course, Washington, D.C., 10/2012

"Patient-Derived Xenografts for discovery of *de novo* mediators of chemoresistance in ovarian cancer." Reproductive Scientist Development Program annual meeting, Boulder CO, 10/2013.

"Meet the Expert: Managing Your First Lab." AACR / Marsha Rivkin Ovarian Cancer Research Symposium. Seattle, WA. 9/2014.

"Fallopian tube origin in ovarian cancer." South Carolina Obstetrical and Gynecological Society, Hilton Head, SC, 9/2015.

"Screening in Ovarian Cancer: Any Closer to the Holy Grail?" South Carolina Obstetrical and Gynecological Society, Hilton Head, SC, 9/2015.

"The Fallopian Tube as Origin of Ovarian Cancer and Opportunistic Salpingectomy." 47th Annual OB/GYN Spring Symposium, Charleston, SC, 4/2016.

"Update on Screening in Ovarian Cancer". 47th Annual OB/GYN Spring Symposium, Charleston, SC, 4/2016.

"Collaborative efforts to discover mediators of chemoresistance in ovarian cancer". Annual Meeting of the Research Scientist Development Program , Chicago, IL, 10/2016

"Novel methods for Screening in Ovarian Cancer". South Atlantic Association of Obstetricians and Gynecologists, Hot Springs, VA, 1/2017.

Discussant

Molecular Therapeutics of Cancer Research Conference, Princeton, NJ, 7/2010.

"KLF6-SV1 is a Novel Uterine Leiomyosarcoma Gene: From Transgenic Mouse Model to Human Disease." 42nd Annual Meeting of the Society of Gynecologic Oncologists, Orlando, FL, 2011.

Moderator

Focused Plenary Session III, Translational and Basic Science, 42nd Annual Meeting of the Society of Gynecologic Oncologists, Orlando, FL, 2011.

Scientific Plenary V. Annual Meeting of the Society of Gynecologic Oncologists, Chicago, IL, March 2015.

Invited Seminars from Other Institutions

"Therapeutic targeting of EphA2 in ovarian cancer." Dept of Obstetrics and Gynecology Grand Rounds, Washington University, St. Louis, MO, 4/2007.

"In search of: Ovarian Cancer Stem Cells." Lineberger Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, 10/2008.

"Translating Discovery to the Patient in Ovarian Cancer." Dept of Obstetrics and Gynecology Grand Rounds, University of North Carolina at Chapel Hill, Chapel Hill, NC, 10/2008.

"In search of: Ovarian Cancer Stem Cells." Hellen Diller Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, 10/2008.

"Update on screening and genetic susceptibility in gynecologic cancers." The Gynecologic and Obstetrics Society, Medical University of South Carolina, 5/2011.

"Cancer Stem Cells: Clinically significant or an experimental phenomenon?" Felix Rutledge Society, MD Anderson Cancer Center, 5/2011.

"Cancer Stem Cells: Clinically significant or an experimental phenomenon?" Hudson Alpha Lecture series, Huntsville, AL, 4/2013.

"Cancer Stem Cells: Clinically significant or an experimental phenomenon?" Southern Cell Biology Research Symposium, Tuskegee University, 6/2013.

"Development of the patient-derived xenograft model to identify de novo mediators of chemoresistance." University of Pittsburg, 3/2014.

"IN SEARCH OF: The chemoresistant population in ovarian cancer." Department of Obstetrics and Gynecology Grand Rounds, University of Chicago, 4/2014.

"Targeting of mediators of chemoresistance in ovarian cancer. Legyel Lab, University of Chicago, 4/2014.

"Development of the patient-derived xenograft model to identify de novo mediators of chemoresistance." University of Virginia, 4/2014.

"Development of the patient-derived xenograft model to identify de novo mediators of chemoresistance." Kansas University, 5/2014.

"Identification and Targeting Mediators of Chemoresistance in Ovarian Cancer." Indiana University, 9/2015.

Presentations at Local Conferences

"Patient Satisfaction with Their Physician," Camp Bluebird Retreat for Cancer Survivors, 5/2000.

"Telomerase and Microsatellite Instability in Ovarian Cancer." Dept of Gynecologic Oncology Grand Rounds, MD Anderson Cancer Center, 2/2004.

"Neuroendocrine modulation of STAT3 in Ovarian Cancer." Dept of Gynecologic Oncology Grand Rounds, MD Anderson Cancer Center, 4/2004.

"Therapeutic silencing of EphA2 by in vivo liposomal siRNA delivery." Dept of Gynecologic Oncology Grand Rounds, MD Anderson Cancer Center, 4/2005.

"Therapeutic targeting of EphA2 in ovarian cancer." Dept of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX, 4/2005.

"Trial-based review of Management of Ovarian Cancer." Dept of Gynecologic Oncology, MD Anderson Cancer Center, 11/2007.

"Cancer Stem Cells in Epithelial Ovarian Cancer." Dept of Cancer Biology, MD Anderson Cancer Center, 4/2008.

"Important Aspects of Mentorship." Dept of Gynecologic Oncology Grand Rounds, MD Anderson Cancer Center, 5/2008.

"In search of... Ovarian Cancer Stem Cells." Program in Experimental Therapeutics, UAB, 1/2010.

"What's New in Gynecologic Cancer Research." Progress in OB/GYN Annual Meeting, UAB, 2/2010.

"Independent targeting of the Notch pathway in tumor cells and tumor stroma." Cancer Cell Biology seminar series, UAB, 5/2010.

"Neoadjuvant Chemotherapy in Ovarian Cancer." Division of Gynecologic Oncology Grand Rounds, UAB, 7/2010.

"Historical Vignettes in Obstetrics and Gynecology." Department of Obstetrics and Gynecology Grand Rounds, UAB, 7/2010.

"Surgical Management of Gynecologic Malignancies." Department of Radiology Grand Rounds, UAB, 7/2010.

"Ex vivo and animal models of cancer." Graduate School in Biomedical Sciences, Translational Research Course, UAB, 10/2013.

"Development of the patient-derived xenograft model to identify de novo mediators of chemoresistance." Grand Rounds, Department of Hematology and Oncology, UAB, 5/2014.

"Targeting Mediators of the Chemoresistance in Ovarian Cancer." Grand Rounds, Department of Obstetrics and Gynecology, UVA, 9/2014

"Targeting Mediators of the Chemoresistance in Ovarian Cancer." Grand Rounds, Department of Pathology, UVA, 10/2014

"Targeting Mediators of the Chemoresistance in Ovarian Cancer." Grand Rounds, UVA Cancer Center, UVA, 10/2014.

Courses Organized or Presented

Oncology Topics. America's OB/GYN Board Review Course. Charlotte, NC, September 2014.