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TITLE: Characterization and Targeting of the Aldehyde Dehydrogenase Subpopulation in Ovarian Cancer

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> 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. Importantly, 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. Thus they likely represent at least part of the chemoresistant population within a heterogeneous tumor. Importantly, they do not seem to explain the entire story, as there are still many CSC-negative cells present at the conclusion of treatment. Additional studies will be performed to determine which other cell types may be present in chemoresistant tumors, and which pathways or mechanisms may be mediating this resistance.					
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**Characterization and targeting of the ALDH subpopulation in ovarian cancer**  
**Charles N. Landen, Jr., MD, MS**  
**University of Alabama at Birmingham, Birmingham, AL**  
**Ovarian Cancer Academy OC093443 2010-2011 Annual Report**

**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<sup>1</sup>. 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)<sup>2-3</sup>. 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.

**BODY:**

Task 1: Determine tumorigenicity of ALDH1A1 subpopulations

The goal of task 1 was to determine the tumorigenicity of ALDH1A1 subpopulations. In last year’s annual report, we described results published in *Molecular Cancer Therapeutics*<sup>4</sup> showing tumorigenicity of ALDH1A1-positive cells compared to ALDH1A1-negative cells from the A2780cp20 cell line. 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.

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

<b>A2780cp20 cells injected IP</b>	<b>1 mil</b>	<b>250k</b>	<b>100k</b>	<b>25k</b>	<b>5k</b>	<b>1k</b>	<b>Serial transplantation rate</b>
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

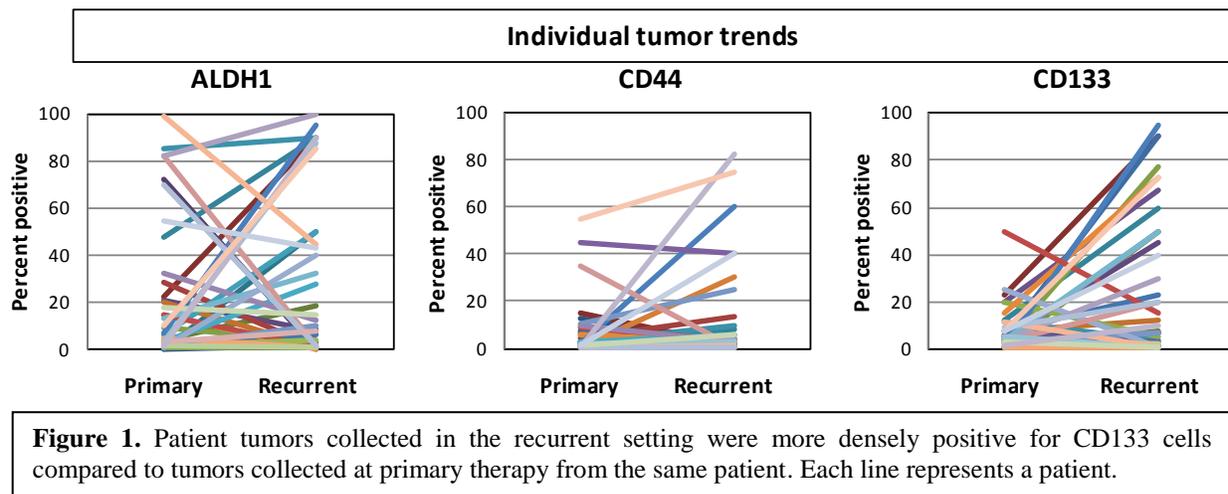
The TD50, or dose of cells required to permit tumor formation in 50% of animals, was 50-fold lower with ALDEFLUOR-positive cells.

An additional important characteristic is demonstration that cancer stem cells have enhanced potential for differentiation. We also demonstrated that tumors formed after injection of ALDEFLUOR-positive cells contained both positive and negative ALDH1A1 populations. However, no ALDEFLUOR-positive cells were found in the tumors that formed after injection of ALDH1A1-negative cells (Figure 4A,B in attached manuscript). This was confirmed with IHC (Figure 4C,D). A similar differentiation capacity was noted *in vitro* (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.

An additional element of this task is to determine whether ALDH1A1-positive cells from patient tumors have enhanced tumorigenicity. Initial attempts to examine this led to few tumors forming from either population, either due to toxicity of the processing procedure required to separate cells to single-cell populations, or because of the inherent low rate of tumor formation from primary xenografts. We have adjusted our initial approach to use tumors growing in mice, established after immediate implantation into mice. These cells will have demonstrated xenograft tumorigenicity, and because they can be collected in a more controlled setting, should require less aggressive and more rapid digestion, enhancing viability. Our protocols for establishing primary xenografts have been optimized, described in more detail under task 2, and will be utilized in the next year.

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*<sup>5</sup>. Many recurrent tumors were indeed composed of a greater number of each of these CSC populations, most significantly in the case of CD133.

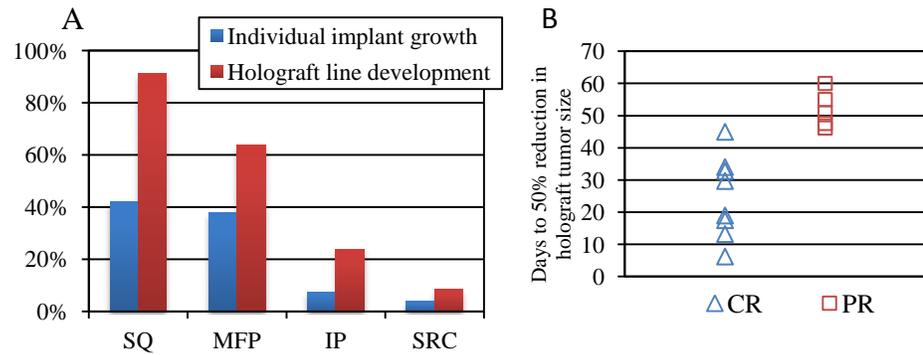


Interestingly, many tumors actually had less of each population in the recurrent tumor, most

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.

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).

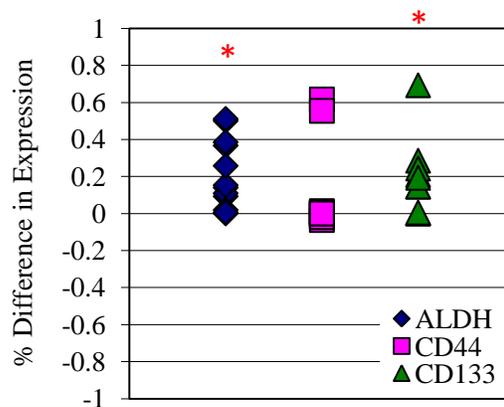


**Figure 2.** “Holografts” are efficiently established after SQ implantation (A), and response to chemo correlates with patient response (B).

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 3). CD44 was only increased in two tumors, and not significant overall. These are consistent findings from patient tumors. However, it is important to note that treated tumors are not composed of ONLY these cells.

Additional studies will explore, on a more high-throughput level, what other populations and pathways are activated in these chemoresistant tumors that allow enhanced survival.

**Figure 3.** Xenografts with a significant response to carboplatin/paclitaxel therapy are enriched in ALDH and CD133-positive cells (\*= $p < 0.05$ )



### Task 3: Target ALDH1 with siRNA *in vivo*

There are no known inhibitors of ALDH1A1 for *in vivo* studies. Therefore, as previously reported, we utilized a method for delivery of siRNA *in vivo* using DOPC nanoparticles. 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  $\mu\text{g}$ , 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 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.

Although the methods used here are being pursued in phase I clinical trials, we are continuing to explore whether other nanoparticle systems might improve delivery of siRNA *in vivo*. We are currently collaborating with a colleague to explore the use of protein cage nanoparticles. These nanoparticles are composed of repeating subunits of peptides, the structure of which can be modified to present ligands for receptor-mediated delivery. If we can enhance delivery to desired cells, such as tumors, doses of siRNA might be increased, and constructs against proteins that would normally be toxic to normal cells might be utilized. Studies in this are

preliminary and ongoing, but we have been able to demonstrate delivery of fluorescent-tagged siRNA to tumor tissues *in vivo*, and siRNA-mediated downregulation of a desired target *in vitro*.

Task 4: Evaluate mechanisms of ALDH1-mediated chemoresistance

We have achieved successful transfection the ALDH1A1-negative A2780ip2 cell line with a construct producing ALDH1A1. The construct was obtained through Addgene (plasmid #11610), produced in the laboratory of Dr. Steven Johnson. However, the ALDH1 protein produced does not appear to be active, as assessed by the Aldefluor assay. Therefore we cannot reasonably expect that biologic effects can be elicited. We are in the process of repeating the transfection, in order to determine the effects of forced overexpression of ALDH1A1 in a null line. In the meantime, microarrays have been completed on ALDH-positive and –negative cells (representative genes presented in Table 1), and confirmation/examination of individual genes is underway. We are also discussing collaboration with a colleague with expertise in metabolism and mitochondrial mechanisms of chemotherapy resistance. Several genes involved in mitochondrial metabolism are overexpressed in the ALDH1-positive cells, using the Illumina microarray data. Confirmation of these genes with qPCR will be performed, as will mitochondrial metabolism experiments that might determine differential regulation of metabolism in ALDH-positive and –negative cells.

**Table 1. Differential expression ALDH-positive and –negative AL2780cp20 cells**

SYMBOL	ALDHneg mean	ALDHpos mean	Ratio Pos:Neg	T-test
<b>OVEREXPRESSED</b>				
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

<b>REDUCED EXPRESSION</b>				
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

### **KEY RESEARCH ACCOMPLISHMENTS:**

- ALDH-positive cells from the A2780cp20 and SKOV3TRip2 cell lines have approximately 50-fold increased tumorigenicity compared to ALDH-negative cells.
- Tumors treated with chemotherapy are enriched in the CD133 CSC population, compared to matched samples collected prior to therapy.
- Tumors collected immediately at the completion of primary therapy are enriched to an even greater degree than tumors collected at first recurrence.
- Efficient establishment of primary xenografts directly from patient tumors is feasible, and mimic patient tumors in histologic make-up, CSC density, and response to chemotherapy.
- Xenograft tumors from mice treated with chemotherapy are similarly enriched in ALDH1 and CD133 CSCs.
- Treatment of tumor-bearing mice with ALDH1A1-targeting siRNA in DOPC sensitized normally-resistant cell lines to cisplatin or paclitaxel.
- Alternatives to DOPC-mediated delivery of siRNA are being explored
- ALDH1-positive and –negative cells have differential expression of multiple genes, and mitochondrial metabolism may contribute to the chemoresistant properties of ALDH-positive cells.

## REPORTABLE OUTCOMES:

- Publications:

**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**. Smoothed Antagonists Reverse Taxane Resistance in Ovarian Cancer. *Mol Cancer Ther*, 11(7): 1587-97, 2012.

- Abstracts presented:

**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.

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 42<sup>nd</sup> 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 42<sup>nd</sup> 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, Newsheer S, Yang SH, Connor MG, Lopez-Berestein G, Sood AK, **Landen CN**. A novel role for the TGF- $\beta$  co-receptor endoglin (CD105) in platinum resistant epithelial ovarian cancer. *43<sup>rd</sup> 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. *43<sup>rd</sup> 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.

- Grants awarded for which data generated by this work contributed preliminary data:
  - Principle 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 (\$60,000 direct) over 1 year.
  - Principle Investigator. *Identifying mediators of chemoresistance in ovarian cancer*. The Norma Livingston Foundation. 50,000, 5/1/2012-4/30/2013.
  
- Funding applied for with decision pending:

Synergistic Translational Leverage Award

Role: Initiating PI

Sponsor: CDMRP OCRP

***Epigenetic Dietary Therapeutics in Ovarian Cancer***

\$500,000 in direct costs over 3 years

Major goals of Project: This proposal seeks to determine the efficacy of nutraceuticals EGCG (an active anti-tumor ingredient in green tea) and sulforaphane (concentrated in broccoli sprouts) as a therapeutic against ovarian cancer. These extracts exert epigenetic effects through hypomethylating and deacetylase inhibiting properties, the mechanisms of which will be further delineated. A phase 0 trial is also proposed to determine if changes in tumor gene expression can be induced with short-term use of the nutraceuticals.

Synergistic Translational Leverage Award

Role: Initiating PI

Sponsor: CDMRP OCRP

***Predictors of Response to PARP Inhibitors in Ovarian Cancer***

\$500,000 in direct costs over 3 years

Major goals of Project: To use a primary “holograft” model and a functional XRT-induced Rad51 activity assay to identify tumors with defective homologous recombination and

response to PARP inhibitors. Additionally response to PARP inhibition will be used to develop a PARP-responsive signature.

- Assets:
  - Established reliable method of developing primary ovarian cancer xenografts into SCID mice that may be an important tool for future studies, such as examination of methodologies for testing personalized medicine.
  - Have approximately 25 primary xenografts maintained in mice without having ever been cultured *in vitro*.

## CONCLUSIONS:

These data demonstrate that ALDH1A1-positive cells are more tumorigenic than ALDH1A1-negative cells, contribute to poor patient outcomes, and contribute to chemoresistance. Importantly, these effects can be reversed by downregulating ALDH1A1 expression with nanoparticle-delivered siRNA. Additionally, we have shown that increased tumorigenicity is not only an important *ex vivo* assessment of CSCs, but that they are clinically significant as well, in that chemoresistant tumors have increased density of ALDH and CD133 cells. This suggests that they represent at least part of the chemoresistant population within a heterogeneous tumor. Importantly, they do not seem to explain the entire story, as there are still many CSC-negative cells present at the conclusion of treatment. Additional studies will be performed to determine which other cell types may be present in chemoresistant tumors, and which pathways or mechanisms may be mediating this resistance. In addition, more efficient methods of delivering siRNA will be explored so that when such pathways are identified, methods will be in place to target them without the need for cumbersome and expensive drug development.

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1. Bast RC, Jr., Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer* 2009;9(6):415-28.
2. Dalerba P, Cho RW, Clarke MF. Cancer stem cells: models and concepts. *Annu Rev Med* 2007;58:267-84.
3. Rosen JM, Jordan CT. The increasing complexity of the cancer stem cell paradigm. *Science* 2009;324(5935):1670-3.
4. Landen CN, Jr., Goodman B, Katre AA, *et al*. Targeting aldehyde dehydrogenase cancer stem cells in ovarian cancer. *Mol Cancer Ther* 2010;9(12):3186-99.
5. Steg AD, Bevis KS, Katre AA, *et al*. Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. *Clin Cancer Res* 2012;18(3):869-81.

## APPENDICES:

- Appendix 1: Publication, 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 2: Accepted abstracts
- Appendix 3: Curriculum Vitae, Charles N. Landen, Jr.

## Stem Cell Pathways Contribute to Clinical Chemoresistance in Ovarian Cancer

Adam D. Steg, Kerri S. Bevis, Ashwini A. Katre, et al.

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## Stem Cell Pathways Contribute to Clinical Chemoresistance in Ovarian Cancer

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### 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- $\beta$ , 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

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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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- $\beta$  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  $\mu$ m. 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<sup>2</sup> profiler PCR array

RNA extracted from microdissected samples was converted to cDNA and amplified using the RT<sup>2</sup> FFPE PreAMP cDNA Synthesis Kit (SABiosciences). Quality of cDNA was confirmed with the Human RT<sup>2</sup> 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<sup>2</sup> 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,

TGF- $\beta$ , 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  $\mu$ L of RNase-free water and stored at  $-80^\circ\text{C}$ . 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/ $\mu$ L 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; house-keeping 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'-CUA-CUGAUACUCUGGGAUA-3' or *GLI1\_B* siRNA: 5'-GCAA-AUAGGGCUUCACAU-3'), or one of 2 tested Gli2-targeting constructs (*GLI2\_A* siRNA: 5'-CGAUUGACAUGCGA-CACCA-3' or *GLI2\_B* siRNA: 5'-GUACCAUUACGAGCCU-CAU-3') at a 1:3 siRNA (pmol) to Lipofectamine 2000 ( $\mu$ L) 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/RPMI thereafter. Transfected cells were grown at  $37^\circ\text{C}$  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  $4^\circ\text{C}$ ; or anti- $\beta$ -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  $\beta$ -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  $\mu$ g) for 24 hours in 6-well plates ( $2.5 \times 10^5$  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,  $5 \times 10^5$  cells in a 60-mm dish were transfected with siRNAs and then cultured in RPMI/10% FBS at  $37^\circ\text{C}$  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  $\mu$ g/mL DNase-free RNase A, and 20  $\mu$ g/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  $\text{IC}_{50}$ , cells were first transfected with siRNA (5  $\mu$ g) 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.  $\text{IC}_{50}$  was determined by finding the dose at which the drug had 50% of its effect, calculated by the equation  $[(\text{OD}_{570\text{max}} - \text{OD}_{570\text{min}})/2] + \text{OD}_{570\text{min}}$ .

### 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.

## 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

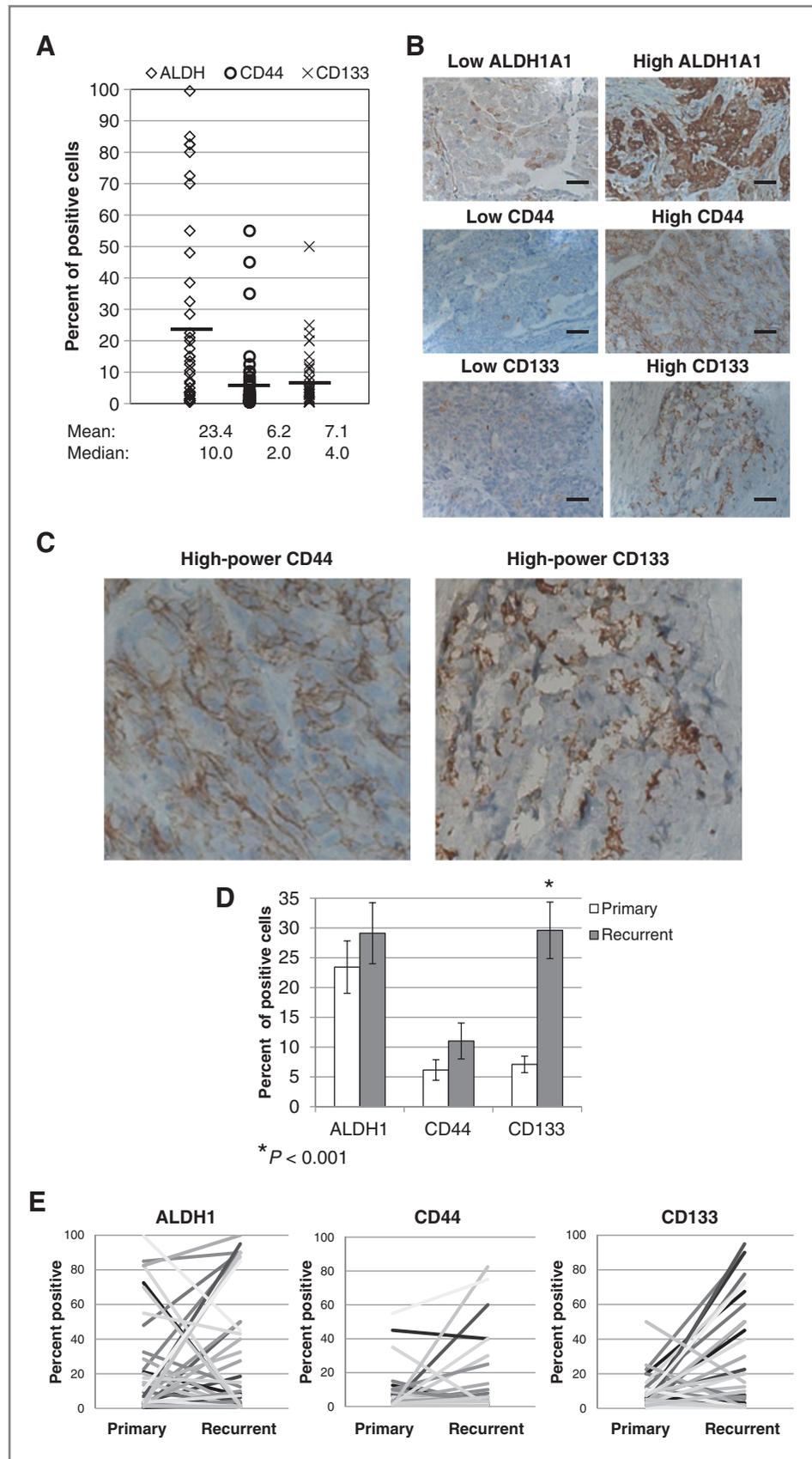
If the CSC hypothesis is clinically significant, then surviving cells would be expected to give rise again to both resistant CSCs and differentiated chemosensitive cells. Clinically this is seen as most patients will again have a response to treatment at first recurrence. Therefore, we examined the pairs 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.

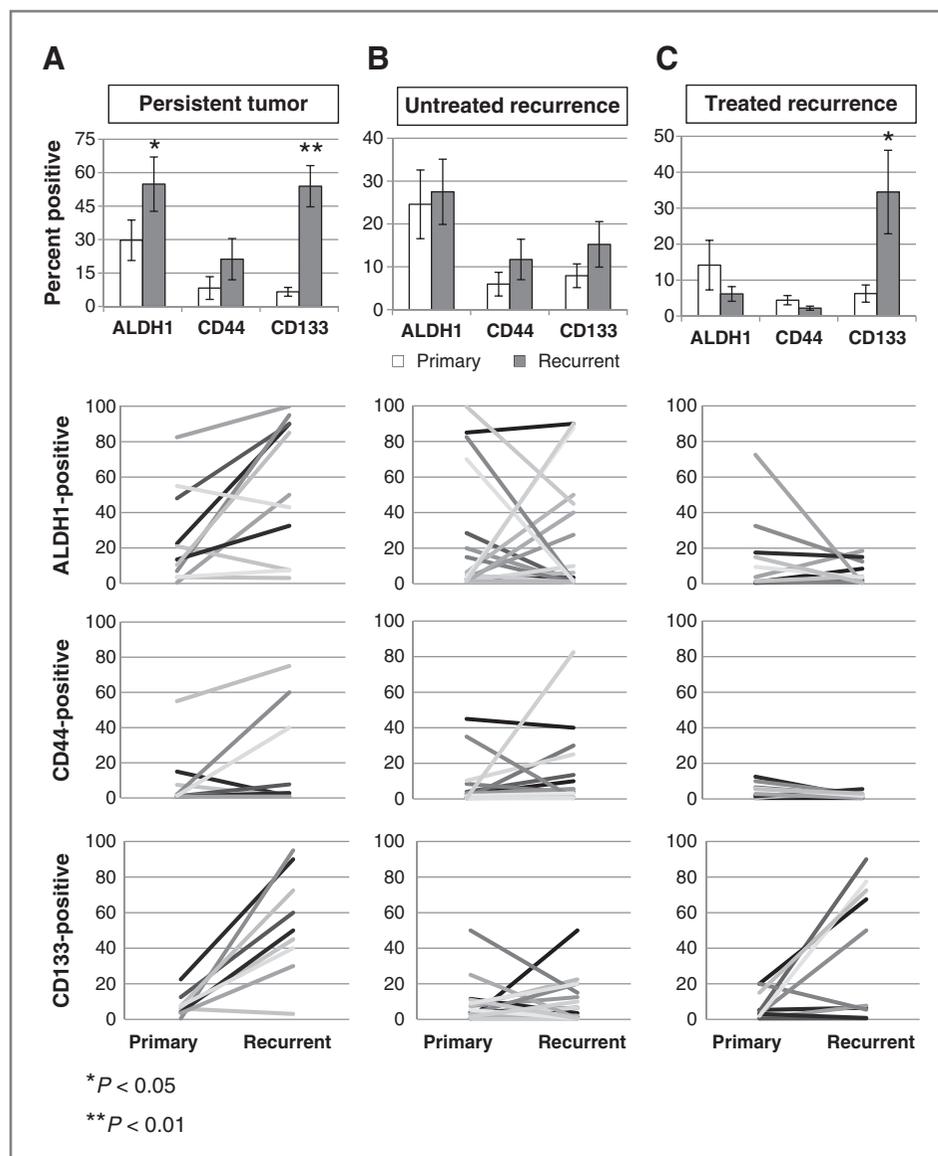
Table 1 illustrates the changes in ALDH1A1, CD44, and CD133 staining from primary to persistent tumor in individual patients. Overall, the percentage of ALDH1A1-, CD44-, and CD133-positive cells increased by more than 2-fold in 64%, 67%, and 89% of persistent tumor specimens, respectively, than in matched 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

**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  $\mu$ m). 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. 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.





**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<sup>2</sup> Profiler Array (16), we found that 12 of these genes (14%) were significantly increased in persistent com-

pared with matched primary tumor. Members of the TGF- $\beta$  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- $\beta$  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

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

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

<sup>a</sup>Persistent compared with primary tumor.

<sup>b</sup>Calculated using paired Student *t* test.

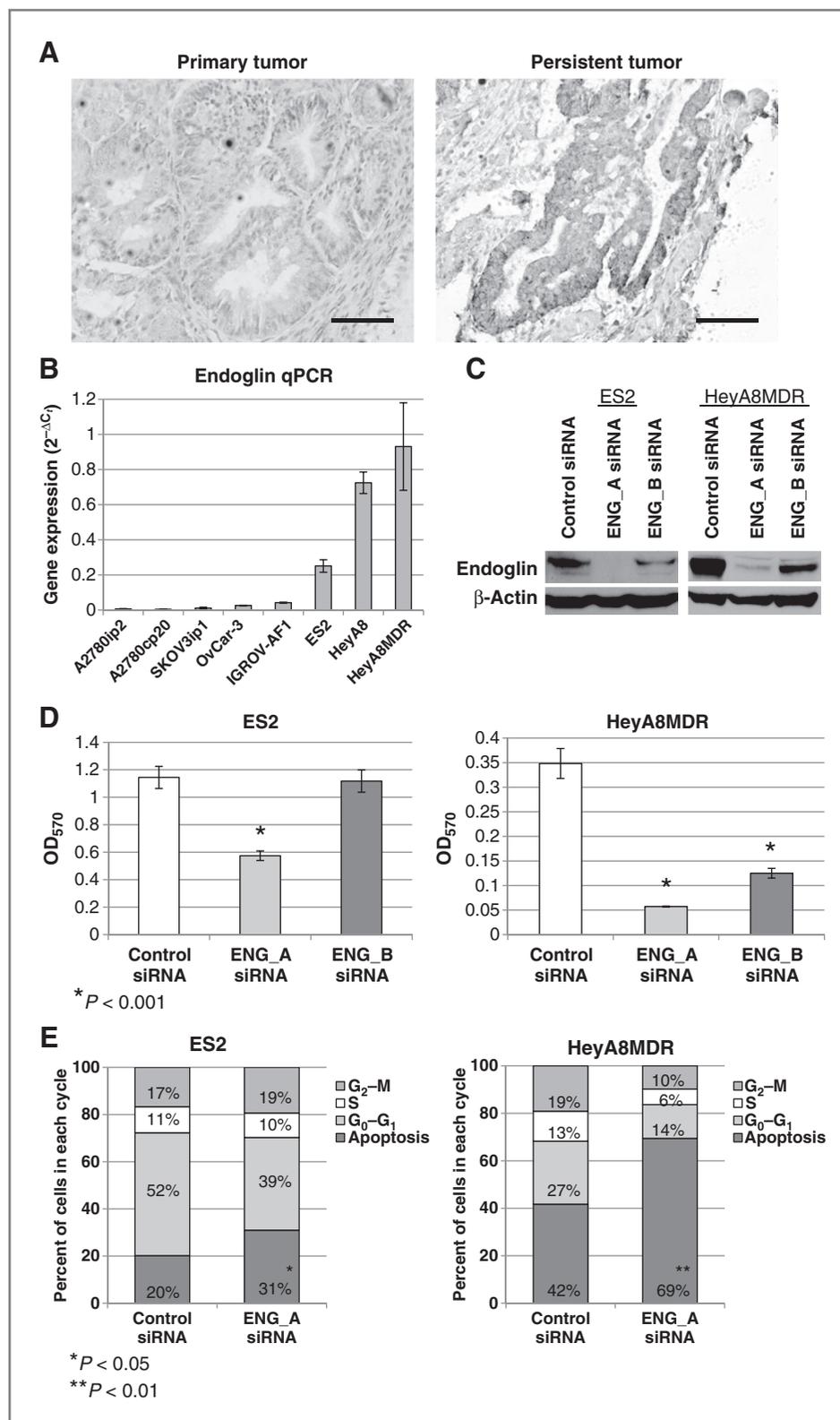


Figure 3. Endoglin is expressed in persistent ovarian tumor and ovarian cancer cell lines, and its downregulation leads to decreased cell viability. A, matched primary/persistent ovarian tumor pairs ( $n = 12$ ) were subjected to IHC analysis of endoglin to evaluate changes in expression. Persistent 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  $\mu$ m). 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  $\Delta C_T$  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.  $\beta$ -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<sub>0</sub> 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

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<sub>0</sub>/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<sup>+</sup>/Gli2<sup>+</sup>) and ES2 (Gli1<sup>-</sup>/Gli2<sup>+</sup>) 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<sub>50</sub> 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

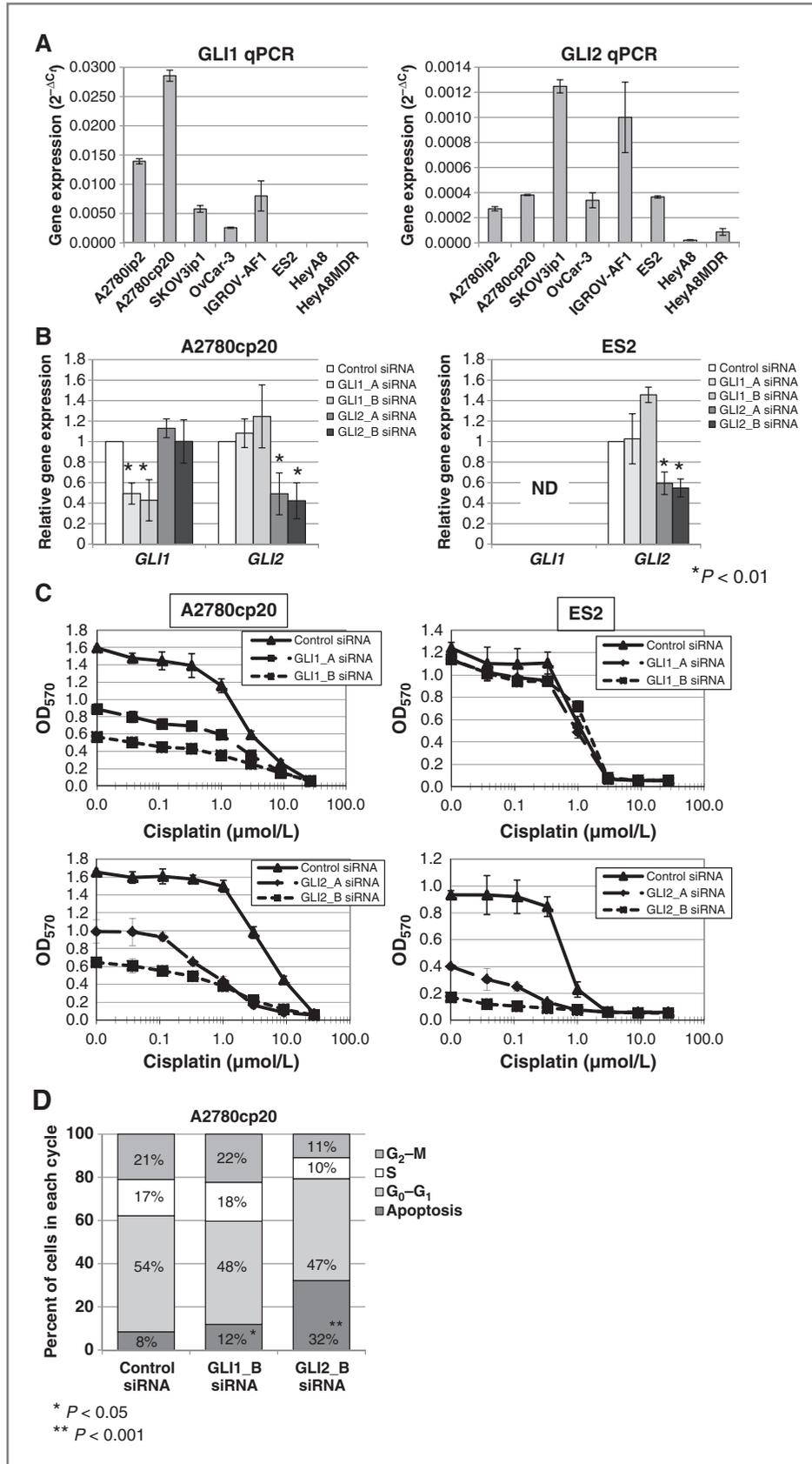
(Table 2), these data make a compelling argument that Gli2 plays a role in platinum resistance, which can be at least partially overcome with Gli2 downregulation. However, Gli1 only appears to contribute to absolute viability, with no platinum-sensitizing effects.

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<sub>0</sub> 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



**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  $\Delta C_T$  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.

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<sup>+</sup> cells were less dense in recurrent tumors than in CD133 and ALDH1, despite multiple studies showing that CD44<sup>+</sup> 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- $\beta$ ) 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- $\beta$  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- $\beta$  coreceptor. This molecule interacts with TGF- $\beta$  receptor II [TGFBR2, which was also significantly increased in persistent tumors (2.76-fold,  $P = 0.0190$ )], both dependently and independently of the TGF- $\beta$  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- $\beta$  signaling by scavenging circulating TGF- $\beta$  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

prevents apoptosis in endothelial cells undergoing hypoxic stress, either in the presence or absence of TGF- $\beta$  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- $\beta$  signaling or through a TGF- $\beta$ -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- $\beta$  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<sub>50</sub> 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- $\beta$  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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## Targeted therapy against aldehyde dehydrogenase in ovarian cancer

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**OBJECTIVE.** Aldehyde dehydrogenase-1 (ALDH1) expression characterizes a subpopulation of cells with enhanced tumor initiating and differentiating properties in some cancers. We have examined the association of ALDH1 with chemoresistance and whether downregulation of ALDH1 sensitizes cells to chemotherapy in models of ovarian cancer.

**METHODS.** Microarray profiling was performed on SKOV3ip1 and the taxane-resistant SKOV3TRip2 cell lines. Primary ovarian cancer xenografts with and without cisplatin exposure were examined for selection of ALDH1-positive cells. Small interfering RNA (siRNA) was used to downregulate ALDH1 in vitro, and in vivo by incorporation into neutral DOPC liposomes, for evaluation of chemosensitization in an orthotopic model of ovarian cancer.

**RESULTS.** Microarray analysis found 29 genes upregulated and 18 genes downregulated by more than 10-fold when comparing the taxane-resistant SKOV3TRip2 ovarian cancer line compared to its parental SKOV3ip1 line. Included among these was a 92.7-fold higher ALDH1 signature. Increased expression and activity of ALDH1 was confirmed by Western blot and the ALDEFLUOR assay (58% of cells ALDH1-active). In primary ovarian cancer xenografts in NOD-Scid mice, cisplatin treatment resulted in an increase in ALDH1-positive cells, from a baseline of 1% to 38% with therapy ( $p < 0.001$ ). ALDH1-positive cells were not limited to perivascular, hypoxic, or advancing edge regions of the tumor. SiRNA constructs downregulating expression of ALDH1 were identified, and reduced viability of SKOV3TRip2 cells in vitro by 49% ( $p < 0.001$ ). ALDH1 targeting also reduced the docetaxel IC<sub>50</sub> from 178nM to 82nM. In the A2780cp20 cell line (a cisplatin-resistant cell line derived from A2780), ALDH1 siRNA alone reduced growth by

just 16%, but sensitized cells to cisplatin with a reduction in IC50 from 5.1 to 2.0 $\mu$ M. In an in vivo orthotopic model of ovarian cancer, we treated mice with control siRNA, ALDH1-siRNA incorporated into DOPC liposomes, chemotherapy, or combined chemo/ALDH1-siRNA-DOPC. ALDH1 alone or docetaxel alone had minimal effect on SKOV3TRip2 tumor growth, but ALDH1-siRNA-DOPC plus docetaxel reduced growth by 89.8% compared to docetaxel/control siRNA (p=0.003). Similarly, in the A2780cp20 model, ALDH1-siRNA-DOPC alone or cisplatin had a nonsignificant reduction, while ALDH1-siRNA-DOPC plus cisplatin reduced tumor growth by 73.4% compared to cisplatin/control siRNA (p=0.013).

**CONCLUSIONS.** ALDH1 expression is associated with taxane and cisplatin chemoresistance in ovarian cancer cell lines. ALDH1 expression can be induced by cisplatin treatment in vivo, and targeting ALDH1 sensitizes resistant cell lines to taxane and platinum chemotherapy. This enzyme may be important for identification and targeting the chemoresistant population in ovarian cancer.

Targeting hedgehog reverses taxane resistance by Gli-dependent and independent mechanisms in ovarian cancer

American Association of Cancer Research, 2011

**Adam D. Steg, Angela Ziebarth, Ashwini Katre, Charles N. Landen, Jr.**

**Objective:** Recent studies have implicated hedgehog signaling in the formation and continued growth of a variety of malignancies, including ovarian cancer. Several inhibitors of the hedgehog pathway have been identified that block the activity of the Smoothed (Smo) receptor. The goal of this study was to determine the in vitro and in vivo effects of Smo antagonists alone and in combination with chemotherapy in ovarian cancer.

**Methods:** Expression of hedgehog signaling components (Smo, Gli1 and Gli2) was assessed in 3 pairs of parental and chemotherapy-resistant ovarian cancer cell lines (A2780ip2/A2780cp20, SKOV3ip1/SKOV3TRip2, HeyA8/HeyA8MDR) using Western blot and qPCR. Cell lines were exposed to increasing concentrations of two different Smo antagonists (Cyclopamine, LDE225) alone and in combination with carboplatin, paclitaxel, adriamycin, and topotecan. Selective knockdown of Smo, Gli1 and Gli2 was achieved using siRNA constructs. Cell viability was assessed by MTT assay and PARP cleavage was used as an indicator of apoptosis. SKOV3TRip2 orthotopic xenografts were treated with vehicle, LDE225, paclitaxel or combination therapy for 5 weeks. Tumor weight for each treatment group was measured and compared using student's t-test.

**Results:** Expression of Smo and Gli1 was high in A2780ip2/A2780cp20, moderate in SKOV3ip2/SKOV3TRip2 and low/absent in HeyA8/HeyA8MDR. Gli2 was high in SKOV3ip2/SKOV3TRip2, moderate in A2780ip2/A2780cp20 and low in HeyA8/HeyA8MDR. Response to cyclopamine and LDE225 varied among the cell lines examined with IC50s ranging from 7.5 to >20  $\mu$ M. Both agents sensitized chemotherapy-resistant cell lines to paclitaxel (5- to 26-fold, including Smo[low]/Gli1[neg] HeyA8MDR), but not to carboplatin, adriamycin, or topotecan. Selective knockdown of Gli1 and Gli2 resulted in taxane sensitization only in Gli1/2-high A2780cp20 cells (2- to 8-fold). A decrease in acetyl- $\alpha$ -tubulin confirmed microtubule-specific effects of Smo targeting, supporting the taxane specificity of this effect. In vivo, SKOV3TRip2 xenografts treated with LDE225 or paclitaxel alone had slightly less tumor burden than the control group (reduced by 28.1%,  $p=0.42$  and 32.0%,  $p=0.40$ , respectively). Those treated with combined LDE225 and paclitaxel, however, had significantly less tumor burden than those treated with vehicle (70.5% reduction,  $p=0.015$ ).

**Conclusions:** Targeting the hedgehog pathway decreases cell viability and increases taxane sensitivity in taxane-resistant ovarian cancer models. Interestingly, these effects were noted even in cells with little constitutive hedgehog activity. This suggests both Gli-dependent and -independent mechanisms contribute to taxane resistance, expanding the potential use of hedgehog inhibitors to all taxane-resistant tumors.



**Annual Scientific Symposium  
November 3, 2010**

Submitting Investigator:	Charles N. Landen, Jr., MD, MS
Rank, Department:	Assistant Professor, Department of Obstetrics and Gynecology
Abstract Title:	The significance of putative ovarian cancer stem cells to recurrence
Abstract Co-Authors and Affiliation:	Kerri S. Bevis, MD; Adam D. Steg, PhD.; Ashwini Katre, MS; Angela Ziebarth, MD (all Dept of OBGYN); Kui Zhang, PhD (Dept of Biostatistics); Michael G. Conner, MD (Dept of Pathology)

Within heterogeneous tumors, subpopulations labeled cancer stem cells (CSCs) have been identified that have significantly enhanced tumorigenicity and chemoresistance in *ex vivo* models. However, whether these populations are truly more capable of surviving chemotherapy in *de novo* tumors is not known. We hypothesized that CSCs make up a greater portion of chemoresistant recurrent tumors, and therefore may represent the subpopulation within ovarian cancers predominantly contributing to chemoresistance and recurrent disease. We examined 45 matched primary/recurrent tumor pairs of high grade ovarian adenocarcinomas and subjected specimens to immunohistochemistry (IHC) for populations shown to have CSC properties in *ex vivo* studies: CD44, CD133, and ALDH1. The percent of positive CD44, CD133, and ALDH1 cells in primary samples averaged 6.2%, 7.1%, and 23.4%, respectively. When examining recurrent samples, there was a moderate increase CD44-positive cells (to 11.0%,  $p=0.11$ ) or ALDH1-positive cells (to 29.2%,  $p=0.28$ ). However, for CD133, there was a dramatic increase, with 29.6% of cells CD133-positive ( $p=0.0004$ ). Interestingly, when patients were stratified based on the clinical scenario in which the recurrent tumor was sampled, the increases were more pronounced. Of tumors collected immediately after completion of primary therapy, 53.4% of cells were CD133-positive ( $p=0.001$ ), 54.9% were ALDH1-positive ( $p=0.018$ ), and 21.2% were CD44-positive ( $p=0.16$ ). In tumors collected from recurrent platinum resistant patients, 41.6% were CD133-positive ( $p=0.027$ ). Samples collected at first recurrence (before initiating secondary therapy) were composed of similar percentages of each population, suggesting the tumor was repopulated with marker-negative differentiated cells. RNA extracted from laser microdissected tumor cells from a cohort of matched pairs ( $n=12$ ) were subjected to a qPCR array to assess upregulation of stem cell pathways. Of 86 members of the Notch, Hedgehog, Wnt, and TGF- $\beta$  pathways examined, 16% were overexpressed in recurrent specimens. These data indicate that chemoresistant tumor subpopulations are enriched in CD133 and ALDH1 populations, suggesting a contribution of these subpopulations to surviving initial chemotherapy and ultimately recurrent disease. Expression profiling in recurrent samples supports the hypothesis that select subpopulations within a heterogeneous tumor have enhanced chemoresistance due, at least in part, to activation of stem cell pathways.

# **A novel role for the TGF-beta co-receptor endoglin (CD105) in platinum resistant epithelial ovarian cancer**

## **Annual Meeting of the Society of gynecologic Oncologists**

Angela Ziebarth<sup>1</sup>, Adam D. Steg<sup>1</sup>, Kerri S. Bevis<sup>1</sup>, Ashwini A. Katre<sup>1</sup>, Somaira Newsheen<sup>2</sup>, Kui Zhang<sup>3</sup>, Hee-Dong Han<sup>4</sup>, Gabriel Lopez-Berestein<sup>4</sup>, Anil K. Sood<sup>4</sup>, Michael Connor<sup>5</sup>, Shih-Hsin Yang<sup>2</sup>, and Charles N. Landen<sup>1\*</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, AL 35294

<sup>2</sup>Department of Radiation Oncology, University of Alabama at Birmingham, Birmingham, AL 35294

<sup>3</sup>Department of Biostatistics, University of Alabama at Birmingham, Birmingham, AL 35294

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**Introduction:** Endoglin (ENG, CD105) is a TGFR co-receptor overexpressed on proliferating endothelial cells but rarely expressed on normal or malignant epithelial cells. Our objective was to evaluate ENG expression in ovarian cancer and examine its potential role in chemoresistance.

**Methods:** Matched primary and persistent-disease ovarian cancer specimens (n=12 pair, recurrent specimens collected within 3 months of completion of primary therapy) were laser microdissected. mRNA was extracted from microdissected cancer cells and subjected to qPCR-array analysis of stem cell family members (n=84). ENG immunohistochemistry was also performed on matched specimens. Western blot and qPCR were used to evaluate ENG expression in multiple ovarian cancer lines. Anti-ENG siRNAs were used to downregulate expression in ES2 and HeyA8MDR cell lines. Effects of ENG-knockdown were evaluated by the MTT assay, cell-cycle analysis, alkaline comet assay, and  $\gamma$ -H2AX foci formation. *In vivo*, an orthotopic mouse model of advanced ovarian cancer was used to determine effects of chitosan-encapsulated anti-ENG siRNA or control siRNA with and without carboplatin.

**Results:** Recurrent ovarian tumors showed upregulation of ENG expression by qPCR when compared to primary specimens (3.77-fold increase, p=0.0023). Tumor-specific expression was confirmed with immunohistochemistry. ENG was noted to be overexpressed in ES2 and

HeyA8MDR cell lines, where siRNA-mediated downregulation decreased cell viability (by 50%,  $p < 0.001$ , and 84%,  $p < 0.001$ , respectively), increased apoptosis (10-27%,  $p < 0.05$ ) and increased cisplatin sensitivity (4.4- and 2- fold , respectively). In an orthotopic mouse model, anti-ENG siRNA/chitosan decreased tumor weight in ES2 and HeyA8MDR models when compared to control (by 41.2%,  $p = 0.001$ ; and 35.6%,  $p = 0.014$ , respectively). ENG inhibition in combination with carboplatin was associated with even greater response when compared to control. Both *in vitro* and *in vivo*, ENG downregulation led to significant DNA damage, as measured by the comet assay and appearance of  $\gamma$ -H2AX foci, suggesting a previously-unrecognized mechanism by which ENG targeting might act synergistically with platinum agents.

Conclusions: ENG appears to be upregulated in chemoresistant ovarian cancer. ENG downregulation promotes apoptosis, induces DNA damage and increases platinum sensitivity both *in vivo* and *in vitro* in ovarian cancer cells. Anti-ENG therapy may allow dual treatment with a direct effect on a subset of tumor cells with ENG-mediated enhanced chemoresistance and potentially on tumor associated endothelial cells.

# **Primary ovarian cancer murine xenografts maintain tumor heterogeneity and biologically correlate with patient response to primary chemotherapy**

Annual Meeting of the Society of Gynecologic Oncologists

Angela Ziebarth<sup>1</sup>, Zachary C. Dobbin<sup>1</sup>, Ashwini A. Katre<sup>1</sup>, Adam D. Steg<sup>1</sup>, Ronald D. Alvarez<sup>1</sup>, Michael G Conner<sup>2</sup>, and Charles N. Landen<sup>1\*</sup>

**Objective:** Preclinical models of ovarian cancer are often limited by their clonality, which lacks the heterogeneity present in patient tumors and limits their ability to predict efficacy of therapeutics. We describe a method for establishing primary xenografts in mice with high efficiency rates, and demonstrate that they retain tumor heterogeneity and demonstrate similar chemoresponsiveness to corresponding patient tumors.

**Methods and Materials:** With IRB and IACUC approval, omental metastatic tumors were collected at the time of primary tumor reductive surgery and implanted into SCID mice in one of four sites: subcutaneous (SQ, 20 sites), mammary fat pad (MFP, 10 sites), subrenal capsule (SRC, 5 sites), and intraperitoneally (IP, 5 mice). Growth in mice was followed with exam and CT scans. Expression of tumor initiating cells (TICs) was evaluated by immunohistochemistry. To determine biologic similarity, a subset of mice were treated with MTD carboplatin and paclitaxel weekly until at least 75% tumor reduction was noted. Descriptive statistics and student's t-test were used for statistical analysis.

## **Results:**

In the first 16 patients collected, tumors ultimately grew from 35% of SQ implants (105/300), 33.6% of MFP (46/137), 4% of SRC (2/50), and 3.1% of IP (2/65) injections ( $p < 0.001$ ). However, samples from 87.5% of patients developed at least one SQ tumor that could be collected and propagated into more mice. The average time to tumor growth was 80.2 days. Murine xenografts were similar to primary human tumors in regards to histology and percentage of TICs (17/20% ALDH1, 2.3/5.5% CD44, and 10.8/3.3% CD133 for mean xenograft /patient TIC, respectively,  $p > 0.05$ ), confirming that xenografts were comprised of a heterogeneous cell set, not just aggressive subpopulations. In 6 xenograft cohorts, mice were treated with MTD dosing and followed for response. Response to treatment in the xenografts correlated to patient clinical response: the mean time to 50% reduction in xenograft volume was 22.56 days in those with a complete response, versus 49.77 days in those with a partial response ( $p = 0.015$ ).

**Conclusions:** Growth of primary xenograft transplants can be achieved with a high success rate after subcutaneous implantation. Xenografts maintain tumor heterogeneity in regards to TIC density and biologic response to chemotherapy. This preclinical model may provide a valuable mechanism by which to study tumor heterogeneity, chemoresistance, and novel therapeutics.

**Targeting the Hedgehog pathway reverses taxane resistance in ovarian cancer**  
**Annual Meeting of the Society of Gynecologic Oncologists, 2011**

Ziebarth, Steg, Bevis, Katre, Alvarez, Landen

**Objectives:** The Hedgehog (Hh) pathway is known to play an important role in stem cell biology and multiple malignancies, but it is not clear what role it may play in chemoresistance. Our objective was to explore the effects of targeting the Hh pathway as a means to reverse taxane resistance in ovarian cancer.

**Methods:** Ovarian cancer cell lines A2780ip2, SKOV3ip2, Hey A8, and their taxol resistant derivatives A2780cp20 (also platinum resistant), SKTRip3, and HeyA8MDR were analyzed for expression of Hh pathway proteins (Smo, Gli 1) by Western blot and qPCR. Cell lines were treated with three different Smo inhibitors: Cyclopamine (Cyp), LDE225 (Novartis), or CUR199691 (CUR, Genentech), alone and combined with paclitaxel. Knockdown of Smo, Gli1, and Gli2 was performed with siRNA. Cell viability was assessed by MTT assay and apoptosis by PARP cleavage. *In vivo*, SKTRip2 orthotopic xenografts were treated with vehicle, LDE225, paclitaxel, or combination therapy for 5 weeks, and IP tumor weights measured and compared using student's t-test.

**Results:** Smo was strongly expressed in the A2780ip2/A2780cp20 and SKOV3ip1/SKTRip3 cell line pairs, but low expression was noted in HeyA8/HeyA8MDR. Gli1 expression was high in A2780ip2/A2780cp20, moderate in SKOV3ip1/SKOV3TRip3, and absent in HeyA8/HeyA8MDR. Cyp, LDE225, and CUR all inhibited growth *in vitro* with IC<sub>50</sub>'s in the range of 7 to 20 $\mu$ M for all cell lines. All agents also significantly sensitized all three taxane-resistant cell lines to paclitaxel, 5 to 45-fold, even in the Smo[low]/Gli1[neg] HeyA8MDR cell line. With specific siRNA-mediated targeting, sensitization to paclitaxel was noted with Smo, Gli1, and Gli2 knockdown only in the in A2780cp20 cell line (3 to 7-fold increased sensitivity, no effect noted on platinum sensitivity). *In vivo*, mice with SKOV3TRip3 xenografts treated with LDE225 or paclitaxel alone had slightly less tumor burden than the control group (reduction in size by 28.1%, p=0.42 and 32.0%, p=0.40, respectively). However, those treated with combined LDE225 and paclitaxel had significantly less tumor burden than those treated with vehicle (70.5% reduction, p=0.015).

**Conclusions:** Inhibitors of the hedgehog pathway demonstrate significant antitumor activity in ovarian cancer, and reverse taxane resistance *in vitro* and *in vivo*. Significantly, these effects were noted even in cell lines with low constitutive Hh pathway activation, expanding its potential use to all taxane-resistant tumors.

Examination of matched primary and recurrent ovarian cancer specimens supports the cancer stem cell hypothesis

Annual Meeting of the Society of Gynecologic oncologists, 2011

Kerri S. Bevis MD, Ashwini A. Katre MS, Adam Steg PhD, Britt K. Erickson MD, Peter J. Frederick MD, Teresa K. Backes BS, Kui Zhang PhD, Michael G. Conner MD, Charles N. Landen, Jr MD

#### Objectives:

Within heterogeneous tumors, subpopulations labeled cancer stem cells (CSCs) have been identified that have significantly enhanced tumorigenicity and chemoresistance in ex vivo models. However, whether these populations are truly more capable of surviving chemotherapy in de novo tumors is not known. We hypothesized that CSCs make up a greater portion of recurrent tumors, and therefore may represent the subpopulation within ovarian cancers predominantly contributing to chemoresistance and recurrent disease.

#### Methods:

45 matched primary/recurrent tumor pairs of high grade papillary serous or endometrioid ovarian adenocarcinomas were subjected to immunohistochemistry (IHC) for populations shown to have CSC properties in ex vivo studies: CD44, CD133, and ALDH1. Additionally, 12 pair in which recurrent tumors were collected immediately after completion of primary therapy were laser microdissected and analyzed with qPCR array for expression of stem cell pathway members.

#### Results:

The percent of positive CD44, CD133, and ALDH1 cells in primary samples averaged 6.2%, 7.1%, and 23.4%, respectively. In recurrent samples, there was a moderate increase in CD44-positive cells (to 11.0%,  $p=0.11$ ) and ALDH1-positive cells (to 29.2%,  $p=0.28$ ). However, for CD133, there was a dramatic increase, with 29.6% of cells CD133-positive ( $p=0.0004$ ). Interestingly, when patients were stratified based on the clinical scenario in which the recurrent tumor was sampled, the increases were more pronounced. Of tumors collected immediately after completion of primary therapy, 53.4% of cells were CD133-positive ( $p=0.001$ ), 54.9% were ALDH1-positive ( $p=0.018$ ), and 21.2% were CD44-positive ( $p=0.16$ ). Samples collected at first recurrence (before initiating secondary therapy) were composed of similar percentages of each population, suggesting the tumor was repopulated with marker-negative differentiated cells. Of 86 members of the Notch, Hedgehog, Wnt, and TGF- $\beta$  pathways examined, 16% were overexpressed in recurrent specimens collected immediately after completion of primary therapy.

#### Conclusions:

These data indicate that chemoresistant tumor subpopulations are enriched in CD133 and ALDH1 populations, suggesting a contribution of these subpopulations to surviving initial chemotherapy and ultimately recurrent disease. Expression profiling in recurrent samples supports the hypothesis that select subpopulations within a heterogeneous tumor have enhanced chemoresistance due, at least in part, to activation of stem cell pathways.

An optimized primary ovarian cancer xenograft model mimics patient tumor biology and heterogeneity.

Annual Meeting of the American Society of Clinical Oncology

Zachary C. Dobbin, Ashwini A. Katre, Angela Ziebarth, Monjri M. Shah, Adam D. Steg, Ronald D. Alvarez, Michael G. Conner, Charles N. Landen.

**Background:** Current xenograft and transgenic models of ovarian cancer are mainly homogeneous and poorly predict response to therapy. Use of patient tumors may represent a better model for tumor biology and offer potential to test personalized medicine approaches, but poor take rates and questions of recapitulation of patient tumors have limited this approach. We have developed a protocol for improved feasibility of such a model and examined its similarity to the patient tumor.

**Methods:** Under IRB and IACUC approval, 23 metastatic ovarian cancer samples were collected at the time of tumor reductive surgery. Samples were implanted either subcutaneously (SQ), intraperitoneally (IP), in the mammary fat pad (MFP), or in the subrenal capsule (SRC) and monitored for tumor growth. Cohorts from 8 xenolines were treated with combined carboplatin and paclitaxel or vehicle, and response to therapy compared between xenografts and patients. Expression of tumor-initiating cell (TIC) markers ALDH1, CD133, and CD44 was assessed by immunohistochemistry in tumors from patients and treated and untreated xenografts.

**Results:** At least one SQ implanted tumor developed in 91.3% of xenografts, significantly higher than in the MFP (63.6%), IP (23.5%), or SRC (8%). Xenografts were similar in expression of putative TIC's compared to patient tumors. The patients and the xenografts also have similar responses to chemotherapy in that xenografts from patients with a partial response responded more slowly than those from patients achieving a complete response (45 vs 21 days,  $p=0.004$ ). Treated xenografts were more densely composed of TICs. ALDH1 increased to 36.1% from 16.2% ( $p=0.002$ ) and CD133 increased to 33.8% from 16.2% ( $p=0.026$ ).

**Conclusions:** Xenoline development can be achieved at a high rate when tumors collected from metastatic sites are implanted SQ. These xenografts are similar to patient tumors with regard to chemotherapy response and TIC expression.. This model may be a more accurate model for *in vivo* pre-clinical studies as compared to current models. Also, as treated xenografts become chemoresistant, this model is well positioned to evaluate targeted therapies aimed at the most aggressive populations in a heterogeneous tumor.

# CURRICULUM VITAE

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

## PRESENT POSITION

*Primary Appointment:* Assistant Professor, Department of Obstetrics and Gynecology,  
Division of Gynecologic Oncology, University of Alabama at Birmingham, Birmingham, AL

*Secondary Appointments:* Assistant Professor, Department of Cell Biology  
Assistant Professor, Department of Pathology  
Graduate Faculty, UAB Graduate School  
Faculty, UAB Medical Scientist Training Program  
Associate Scientist, UAB Comprehensive Cancer Center  
Director of Resident Research, Department of Obstetrics and Gynecology

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## 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	To begin 10/2012

**BOARD CERTIFICATION**

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

**LICENSES**

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

**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-present  
 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-present  
 Reproductive Scientist Development Program Phase II Scholar, 2010-2012  
 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, 1<sup>st</sup> place, UAB CCTS, 2010  
 Listing in Cambridge Who’s Who among Executives and Professions in Research, Medicine, and Healthcare, 2011

**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  
 Gynecologic Oncology Group – Member

The Gynecology and Obstetrics Society, Medical University of South Carolina, 2002-present  
The Felix Rutledge Society, MD Anderson Cancer Center, 2007-present  
UAB Griffin Society, University of Alabama at Birmingham, 2009-present

## **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, Gynecologic Cancer Foundation (GCF), 2010-2013  
Education Committee, Society of Gynecologic Oncologists (SGO), 2011-2013  
Website Development Task Force, Gynecologic Cancer Foundation (GCF), 2011  
Board of Directors, Gynecologic Cancer Foundation (GCF), 2011-2014

### **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  
Endowed chair in Pathology Search Committee, 2011  
Resident Selection Committee, 2011-current  
Clinical Trial Audits, 2011  
Tissue Committee, 2012-current  
Board of Directors, Norma Livingston Foundation, 2012-current

## **GRANT REVIEWER SERVICE**

### **National / International**

Ontario Institute for Cancer Research, Cancer Research Fund Translational Panel, Scientist Reviewer, 2009-11  
External Reviewer, Ovarian Cancer Action Research Centre Quinquennial Review, 2011-12  
CDMRP DOD Ovarian Cancer Research Program, Pathobiology Panel, Scientist Reviewer, 2010-current  
Gynecologic Cancer Foundation Research Grants/Awards Committee, Grant Reviewer, 2010-current  
Ovarian Cancer Research Fund, Ann Schreiber Research Training Program of Excellence, Grant Reviewer, 2012

### **Regional / Institutional**

Ovarian Cancer Pilot Grant, University of Alabama at Birmingham Comprehensive Cancer Center, 2010

## **JOURNAL SERVICE**

### **Editorial Board**

*Gynecologic Oncology Case Report*

### **Ad-hoc Reviewer**

*Journal of Clinical Oncology*

*Cancer Research*

*Clinical Cancer Research*

*Molecular Cancer Therapeutics*

*Oncogene*

*Cancer*

*Genes and Cancer*

*Gynecologic Oncology*

*American Journal of Obstetrics and Gynecology*

*Expert Opinion on Therapeutic Targets*

*Cancer Letters*

*Tumor Biology*

*Neoplasia*

*Journal of Obstetrics and Gynaecology Research*

## **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: Principle Investigator. Sponsor: TRACON Pharmaceuticals, Inc. 9/14/2011-current.  
UAB 0801: Phase I of Intraperitoneal <sup>212</sup>Pb-TCMC-trastuzumab for HER-2 expressing malignancy. Role: Co-Investigator. Sponsor: Areva Med LLC 2/23/2011-current

## 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-present  
Director of Resident Research, Dept of OB/GYN, UAB, 2010-present  
Facilitator, New Student Discussion Group (NSDG), UAB School of Medicine, 2012  
Course Co-Director, Translational Cancer Research, UAB Graduate School, 2012-13

### Postdoctoral fellow mentorship

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

### Graduate Student Advisor Committee Member

Patrick Garcia, PhD candidate. *Development of an animal model of pancreatic cancer for therapeutic intervention.* 2010-present.  
Huaping Chen, PhD candidate. *Epigenetic targeting of ovarian cancer.* 2011-present.  
Matt Schultz, PhD candidate. *The role of STGal-I in the ovarian tumor cell phenotype.* 2012-present.

### Graduate Student Mentorship

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.  
Zachary Dobbins, MD/PhD candidate. *Development and validation of a primary xenograft model in ovarian cancer.* **Primary advisor**, UAB, 2011-present.

### 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, 1<sup>st</sup> 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 predict response to PARP inhibitors.* UAB, 7/2011-6/2014.

### **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. *Combined Hedgehog and Notch targeting in ovarian cancer.* UAB, 2011, 2012. **AWARDS:** 1<sup>st</sup> Place, Oral presentation Short Term Research Category, Medical Student Research Day, 10/25/2011, invited to present at National Student Research Forum 4/2012.

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

### **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) Program of the Medical Scientist Development Program, 2012.

### **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. *Chemoresistance mechanisms of ALDH1 in ovarian cancer.* UAB, 2010-present.

## **RESEARCH FUNDING**

### **Active**

Principle 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.

Principle Investigator, *Characterization and Targeting of the Aldehyde Dehydrogenase Subpopulation in Ovarian Cancer*, OC093443, Department of Defense Ovarian Academy Award, 7/1/2010 – 6/30/2015, \$1,062,125 over 5 years.

Principle 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-current. \$158,078.98 (estimated)

Co-Investigator, U54 pilot project: *BRCA1 Deficiency and Epithelial Ovarian Cancers.* Morehouse School of Medicine/Tuskegee University/University of Alabama Cancer Center Partnership. 9/1/2011-8/30/2012, \$18,000.

Principle Investigator. *Identifying mediators of chemoresistance in ovarian cancer*. The Norma Livingston Foundation. 50,000, 5/1/2012-4/30/2013.

### Prior

Principle 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.

Principle 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.

Principle 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.

Principle 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.

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

Principle 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 over 1 year.

### 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.
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.
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 immunosuppression prolongs the therapeutic effect of adenovirus-mediated factor IX expression. *Hum Gene Ther* 6:1039-1044, 1995.
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.
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* 184(6):1100-1103, 2001.
6. Vermillion ST, **Landen CN**. Prostaglandin inhibitors as tocolytic agents. *Sem Perinatol* 25(4):256-262, 2001.
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.
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.
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.

10. Thaker PH, Deavers M, Celestino J, Thornton A, Fletcher MS, **Landen CN**, Kinch MS, Kiener PA, Sood AK. EphA2 expression is associated with aggressive features in ovarian carcinoma. *Clin Cancer Res* 10(15): 5145-50, 2004.
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. †
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.
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 Research* 65(22): 10562-10568, 2005.
14. **Landen CN**, Kinch MS, Sood AK. EphA2 as a target for ovarian cancer therapy. *Expert Opin Ther Targets* 9(6): 1179-1187, 2005.
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.
16. Sood AK, Bhatti 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
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.
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.
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.
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. †
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.
22. Kamat AA, Bischoff FZ, Dang D, Baldwin MF, Han LY, Lin YG, Merritt WM, **Landen CN**, Lu C, Gershenson DM, Simpson JL, Sood AK.. Circulating cell-free DNA: a novel biomarker for response to therapy in ovarian carcinoma. *Cancer Biol Ther* 5(10): 1369-74, 2006.
23. Merritt WM, Thaker PH, **Landen CN**, Deavers MT, Fletcher MS, Lin YG, Han LY, Kamat AA, Schmandt R, Gershenson DM, Kinch MS, Sood AK.. Analysis of EphA2 expression and mutant p53 in ovarian carcinoma. *Cancer Biol Ther.* 5(10): 1357-60, 2006.
24. **Landen CN**, Lu C, Han LY, Coffman KT, Bruckheimer E, Halder J, Mangala LS, Merritt WM, Lin YG, Gao C, Schmandt R, Kamat AA, Li Y, Thaker P, Gershenson DM, Parikh NU, Gallick GE, Kinch MS, Sood AK. Efficacy and antivasular mechanisms of EphA2 downregulation with an agonistic antibody in ovarian cancer. *JNCI* 98(21): 1558-70, 2006.
25. **Landen CN**, Merritt WM, Mangala LS, Sanguino AM, Bucana C, Lu C, Lin YG, Han LY, Kamat AA, Schmandt R, Coleman RL, Gershenson DM, Lopez-Berestein G, Sood AK. Intraperitoneal delivery of liposomal siRNA for therapy of advanced ovarian cancer. *Cancer Biol Ther* 5(12): 1708-13, 2006. †

26. Lin YG, Han LY, Kamat AA, Merritt WM, **Landen CN**, Deavers MT, Fletcher MS, Urbauer DL, Kinch MS, Sood AK. EphA2 overexpression is associated with angiogenesis in ovarian cancer. *Cancer* 109(2): 332-340, 2006.
27. Kamat AA, Kim TJ, **Landen CN Jr**, Lu C, Han LY, Lin YG, Merritt WM, Thaker PH, Gershenson DM, Bischoff FZ, Heymach JV, Jaffe RB, Coleman RL, Sood AK. Metronomic chemotherapy enhances the efficacy of anti-vascular therapy in ovarian cancer. *Cancer Res* 67(1):281-8, 2007.
28. Lu C, Kamat AA, Lin YG, Merritt WM, **Landen CN**, Kim TJ, Spannuth W, Arumugam T, Han LY, Jennings NB, Logsdon C, Jaffe RB, Coleman RL, Sood AK. Dual targeting of endothelial cells and pericytes in antivascular therapy for ovarian carcinoma. *Clin Cancer Res* 13(14):4209-17, 2007.
29. **Landen CN Jr**, Lin YG, Immaneni A, Deavers MT, Merritt WM, Spannuth WA, Bodurka DC, Gershenson DM, Brinkley WR, Sood AK. Overexpression of the centrosomal protein Aurora-A kinase is associated with poor prognosis in epithelial ovarian cancer patients. *Clin Cancer Res* 13(14):4098-104, 2007.
30. Kim TJ, Ravoori M, **Landen CN**, Kamat AA, Han LY, Lu C, Lin YG, Merritt WM, Jennings N, Spannuth WA, Langley R, Gershenson DM, Coleman RL, Kundra V, Sood AK. Anti-tumor and anti-vascular effects of AVE8062 in ovarian carcinoma. *Cancer Res* 67(19): 9337-45, 2007.
31. Ramirez PT, **Landen CN Jr**, Coleman RL, Milam MR, Levenback C, Johnston TA, Gershenson DM. Phase I trial of the proteasome inhibitor bortezomib in combination with carboplatin in patients with platinum- and taxane-resistant ovarian cancer. *Gynecol Oncol*, 108(1):68-71, 2008
32. **Landen CN Jr**, Lin YG, Armaiz Pena GN, Das PD, Arevalo JM, Kamat AA, Han LY, Jennings NB, Spannuth WA, Thaker PH, Lutgendorf SK, Savary CA, Sanguino AM, Lopez-Berestein G; Cole SW, Sood AK. Neuroendocrine modulation of STAT3 in ovarian cancer. *Cancer Res*, 67(21):10389-96, 2007.
33. Kamat AA, Merritt WM, Coffey D, Lin YG, Patel PR, Broaddus R, Nugent E, Han LY, **Landen CN Jr**, Spannuth WA, Lu C, Coleman RL, Gershenson DM, Sood AK. Clinical and biological significance of vascular endothelial growth factor (VEGF) in endometrial cancer. *Clin Cancer Res* 13(24): 7487-95, 2007.
34. Cole ME, Broaddus R, Thaker P, **Landen C**, Freedman RS; Vanderbilt. Placental-site trophoblastic tumors: a case of resistant pulmonary metastasis. *Nat Clin Pract Oncol* 5(3): 171-5, 2008.
35. **Landen CN Jr**, Birrer MJ, Sood AK. Early genetic events in the pathogenesis of epithelial ovarian cancer. *J Clin Oncol* 26(6): 995-1005, 2008.
36. Merritt WM, Lin YG, Spannuth WA, Fletcher MS, Kamat AA, Han LY, **Landen CN**, Jennings N, Geest KD, Langley RR, Villares G, Sanguino A, Lutgendorf SK, Lopez-Berestein G, Bar-Eli MM, Sood AK. Effects of IL-8 targeted therapy with liposome incorporated siRNA on ovarian cancer growth. *JNCI* 100(5): 359-72, 2008.
37. Lu C, Thaker PH, Lin YG, Spannuth WA, **Landen CN**, Merritt WM, Jennings NB, Langley RR, Gershenson DM, Yancopoulos GD, Ellis LM, Jaffe RB, Coleman RL, Sood AK. Impact of vessel maturation on anti-angiogenic therapy in ovarian cancer. *Am J Obstet Gynecol* 198(4): 477e1-9, 2008.
38. Lu C, Shahzad MMK, Wang H, **Landen CN**, Kim SW, Allen J, Nick AM, Jennings N, Kinch MS, Bar-Eli M, Sood AK. EphA2 Overexpression Promotes Ovarian Cancer Growth. *Can Biol Ther* 7(7): 1098-103, 2008. †
39. Han LY, Fletcher MS, Urbauer DL, Mueller P, **Landen CN**, Kamat AA, Lin YG, Merritt WM, Spannuth WA, Deavers MS, De Geest K, Gershenson DM, Lutgendorf SK, Ferrone S, Sood AK. HLA-I antigen processing machinery component expression and intratumoral T-cell infiltrate as independent prognostic markers in ovarian carcinoma. *Clin Can Res* 14(11): 3372-9, 2008.
40. Lin YG, Immaneni A, Merritt WM, Mangala SL, Armaiz-Pena GN, Kamat AA, Han LY, Spannuth WA, **Landen Jr CN**, Gray MJ, Coleman RL, Bodurka DC, Brinkley WM, Sood AK. Aurora kinase targeting inhibits tumor growth in ovarian cancer. *Clin Can Res* 14(17): 5437-46, 2008.
41. **Landen CN**, Kim TJ, Lin YG, Merritt WM, Kamat AA, Han LY, Spannuth WA, Nick AM, Jennings NB, Kinch MS, Tice M, Sood AK. Tumor-selective response to antibody-mediated targeting of alphavbeta3 integrin in ovarian cancer. *Neoplasia* 10(11): 1259-67, 2008.
42. Shahzad MMK, Lu C, Lee JW, Stone RL, Mitra R, Mangala LS, Lu Y, Baggerly KA, Danes CG, Nick AM, Halder J, Kim HS, Vivas-Mejia P, **Landen CN**, Lopez-Berestein G, Coleman RL and Sood AK. Dual Targeting of EphA2 and FAK in Ovarian Carcinoma. *Can Biol Ther* 8(11): 1027-35, 2009.

43. Lee JW, Han HD, Shahzad MK, Kim SW, Mangala LS, Nick AM, Lu C, Langley RR, Schmandt R, Kim HS, Mao S, Gooya J, Fazenbaker C, Jackson D, Tice D, **Landen CN**, Coleman RL, Sood AK. EphA2 Immunoconjugate as Molecularly Targeted Chemotherapy for Ovarian Carcinoma. *JNCI* 101(17): 1193-205, 2009.
44. Kim T\*, **Landen CN\***, Lin YG, Mangala L, Lu C, Nick AM, Stone RL, Merritt WM, Armaiz-Pena G, Jennings N, Coleman RL, Tice D, Sood AK. Combined targeting of VEGF and alphavbeta3 in ovarian cancer. *Can Biol Ther* 8(23): 2263-73, 2009. †
45. Worley M, **Landen CN**, Slomovitz BM, Malpica A, Palla SL, Ramirez PT. Expression of the Retinoblastoma-Related Gene Rb2/p130 in the Pathogenesis of Serous Carcinoma of the Ovary. *Appl Immunohistochemistry Mol Morph* 18(6): 509-11, 2010.
46. Lu C, Shahzad MMK, Moreno M, Lin YG, Jennings N, Allen JK, **Landen CN**, Mangala LS, Armaiz-Pena GN, Schmandt R, Nick AM, Stone RL, Jaffe RB, Coleman RL, Sood AK. Targeting Pericytes with a PDGF-B Aptamer in Human Ovarian Carcinoma models. *Can Biol Ther* 9(3): 176-82, 2010.
47. Merritt WM, Kamat AA, Hwang JY, Bottsford-Miller J, Lu C, Lin YG, Coffey D, Spannuth W, Nugent E, Han LY, **Landen CN**, Nick AM, Stone RL, Coffman KT, Bruckheimer E, Boaddus R, Gershenson DM, Coleman RL, Sood AK. Clinical and Biological Impact of EphA2 Overexpression and Angiogenesis in Endometrial Cancer. *Can Biol Ther* 10(12): 1306-14, 2010.
48. **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. †
49. Frederick PJ, Ramirez PT, McQuinn L, Milam MR, Weber DM, Coleman RL, Gershenson DM, **Landen Jr CN**. Preoperative Factors Predicting Survival After Secondary Cytoreduction for Recurrent Ovarian Cancer. *Int J Gyn Cancer*, 21(5): 831-6, 2011.
50. 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.
51. Nick AM, Stone RL, Armaiz-Pena G, Ozpolat B, Tekederli I, Graybill WS, **Landen CN**, Villares G, Vivas-Mejia P, Bottsford-Miller J, Kim HS, Lee JS, Kim SM, Baggerly KA, Ram PT, Deavers MT, Coleman RL, Lopez-Berestein G, Sood AK. Silencing of p130cas in Ovarian Carcinoma: A Novel Mechanism for Tumor Cell Death. *J Natl Cancer Inst*, 103(21): 1596-612, 2011.
52. 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.
53. Stone RL, Nick AM, McNeish IA, Balkwill F, Han HD, Bottsford-Miller J, Rupaimoole R, Armaiz-Pena GN, Pecot CV, Coward J, Deavers MT, Vasquez HG, Urbauer D, **Landen CN**, Wei H, Gershenson H, Matsuo K, Shahzad MMK, King ER, Tekederli I, Ozpolat B, Ahn EH, Bond VK, Wang R, Drew AF, Gushiken F, Collins K, DeGeest K, Lutgendorf SK, Chiu W, Lopez-Berestein G, Afshar-Kharghan V, Sood AK. Paraneoplastic Thrombocytosis in Ovarian Cancer. *NEJM*, 366(7): 610-8, 2012.
54. 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.
55. Kim KK, Zsebik GN, Straughn JM, **Landen CN**. Management of Complex Pelvic Masses Using a Multivariate Index Assay: A Decision Analysis. *Gyn Onc*, 126: 364-8, 2012.
56. 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. †

\* Equal contribution

† Selected for Cover Art and/or Issue Highlights

### **INVITED ARTICLES**

1. Tate SN, **Landen CN**. Premalignant Lesions of the endometrium. eMedicine Journal [serial online]. Archive available at: <http://www.emedicine.com/MED/topic3334.htm>. 2005.

2. **Landen CN**, Kinch MS, Sood AK. EphA2 as a target for ovarian cancer therapy. *Expert Opin Ther Targets* 9(6): 1179-1187, 2005.
3. **Landen CN**, Birrer MJ, Sood AK. Early genetic events in the pathogenesis of epithelial ovarian cancer. *J Clin Oncol* 26(6): 995-1005, 2008.

### **ABSTRACT PRESENTATIONS**

1. **Landen C**, Brinkhous K, Monroe M, Liles D, Read M, Roberts H. F.IX Pathophysiology and Gene Therapy of Canine Hemophilia B: Dynamics of F.IX Distribution from Extravascular Sites (Subcutaneous, Intramuscular, and Intraperitoneal), *Proceedings of FASEB*:7:A117, 1992.
2. **Landen CN**, Brinkhous KM, Read MS. Evaluation of Sensitivity of Whole Blood Clotting Time (WBCT), Partial Thromboplastin Time (PTT), and F.IX One-stage Bioassay Tests with Low Plasma F.IX Levels Observed with Transfusion or Gene Therapy in Canine Hemophilia B. *Blood* 82(Suppl. 1):592a, 1994.
3. Eisensmith RC, Fang B, Kay MA, **Landen CN**, Cross RE, Bellinger DA, Read MS, Hu PC, Brinkhous KM, Wood SLC. Gene Therapy for Hemophilia B: Cyclosporin Treatment Increases the Persistence of Adenovirus-Mediated Factor IX Expression in Hemophilia B Dogs. *Blood* 84(Supple. 1):255a, 1994.
4. Liles DK, Monroe DM, Brinkhous KM, Read MS, **Landen CN**, Roberts HR. Extravascular administration of factor IX: Prospects for gene therapy of hemophilia. *Proceedings of The XV Congress of the International Society on Thrombosis and Haemostasis*. Jerusalem, Israel, June 1995.
5. **Landen CN**, Prazma J, Cash A, Shockley WW. The effects of thalidomide and nitric oxide synthase inhibitors on tracheal exudation. *Fax*, February 1996.
6. **Landen CN**, Zhang P, Young RA. Differing mechanisms of inhibition of calcium rises in human uterine myocytes by indomethacin and nimesulide. *Proceedings of District IV of American College of Obstetricians and Gynecologists*, 2000.
7. **Landen CN**, Mathur SP, Richardson MS, Creasman WT. Expression of cyclooxygenase-2 in cervical, endometrial, and ovarian malignancies. *Proceedings of South Carolina/Georgia Section of American College of Obstetricians and Gynecologists*, 2001.
8. **Landen CN**, Collins-Harp BA, Underwood PA. Oncology Patients' Satisfaction with Medical Care. *Proceedings of District IV Meeting of the American College of Obstetricians and Gynecologists*, 2001.
9. **Landen CN**, Das P, Thaker PH, Lutgendorf S, Cole SC, Savary CA, Sood AK. Neuroendocrine modulation of STAT3 in ovarian cancer. *Proceedings of Western Association of Gynecologic Oncologists*, 2004.
10. **Landen CN**, Klingelutz A, Coffin JE, Sorosky JI, Thaker PA, Sood AK. Genomic instability is associated with lack of telomerase activation in ovarian cancer. *Proceedings of the American Association of Cancer Research*, 2004.
11. **Landen CN**, Chavez-Reyes A, Bucana C, Schmandt R, Thaker PH, Kamat AA, Han L, Lopez-Berestein G, Sood AK. Small Interfering RNA (siRNA) in vivo therapy for ovarian carcinoma using a novel nanoparticle. *Proceedings of Society of Gynecologic Oncologists*, 2005.
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83. 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 42<sup>nd</sup> Annual Society of Gynecologic Oncologists Meeting*, 2011.
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90. 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. *43<sup>rd</sup> Annual Society of Gynecologic Oncologists Meeting*, 2012.
91. Yang E, Nowsheen S, Cooper T, **Landen CN**, Bonner J. Poly (ADP-Ribose) Polymerase Inhibition Attenuates Radiation-Induced Non-Homologous End-Joining Repair and Augments Cervical Cancer Response to Radiation. *43<sup>rd</sup> Annual Society of Gynecologic Oncologists Meeting*, 2012.
92. Kim KH, Bevis KS, Walsh-Covarrubias J, Alvarez RD, Straughn JM, **Landen CN**. Optimizing the Research Experience in Gynecologic Oncology Fellowships. *43<sup>rd</sup> Annual Society of Gynecologic Oncologists Meeting*, 2012.
93. 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. §
94. 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. *34<sup>th</sup> Annual Meeting of the Bioelectromagnetics Society*, Brisbane, Australia, 2012.

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§ Selected for Meeting Award

### **BOOK CHAPTERS**

1. **Landen CN** and Holmes MA. Dysmenorrhea, in *Conn's Current Therapy 2002*, R. Rakel and E. Bope, Editors, W.B. Saunders Company, Philadelphia, pp 1076-1078, 2002. ISBN: 072168744X
2. Gernt PR, Norcross ED. *Sleepwell: Surgery and Obstetrics/Gynecology, Vol 2* (Sleepwell Review Series). Blackwell Publishing 2002. ISBN: 0632046643.
3. **Landen CN** and Lopez-Berestein G. Therapeutic Agents and Approaches in the Age of Personalized Cancer Care, in *Targeted Therapy for Solid Tumors and Hematologic Malignancies 2011*. AQ Cardama, DL Gibbons, V Cataldo, Eds. NOVA Science Publishers, New York, NY. ISBN: 978-1-61761-854-3
4. **Landen CN**. Biologic and Targeted Therapies, in *Society of Gynecologic Oncologists Chemotherapy Handbook*, under construction.

### **CONFERENCES AND SYMPOSIA**

#### **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 Gynecologic 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.

Therapeutic silencing of EphA2 by in vivo liposomal siRNA delivery. American Association of Cancer Research Annual Meeting. Anaheim, CA, 4/2005.

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

Overexpression of the centrosomal protein Aurora-A kinase is associated with poor prognosis in epithelial ovarian cancer patients. 37th Annual Society of Gynecologic Oncologists Meeting, Palm Springs, CA, 3/2006.

SiRNA Therapeutics in Ovarian Cancer. 7th Annual International Conference on Ovarian Cancer, Houston, TX, 11/2006.

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  $\alpha\beta 3$  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." 3<sup>rd</sup> 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.” 3<sup>rd</sup> Annual International Conference, Ovarian Cancer: Prevention, Detection and Treatment of the Disease and its Recurrence, Pittsburg, PA, 5/2012.

#### **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.” 42<sup>nd</sup> Annual Meeting of the Society of Gynecologic Oncologists, Orlando, FL, 2011.

#### **Moderator**

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

#### **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.

#### **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.

**Charles N. Landen, Jr., M.D., M.S.**

“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, University of Alabama at Birmingham, 1/2010.

“What’s New in Gynecologic Cancer Research.” Progress in OB/GYN Annual Meeting, University of Alabama at Birmingham, 2/2010.

“Independent targeting of the Notch pathway in tumor cells and tumor stroma.” Cancer Cell Biology seminar series, University of Alabama at Birmingham, 5/2010.

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

“Historical Vignettes in Obstetrics and Gynecology.” Department of Obstetrics and Gynecology Grand Rounds, 7/2010.

“Surgical Management of Gynecologic Malignancies.” Department of Radiology Grand Rounds, 7/2010.

### **Personal Interests**

Family activities

Wife Donna; Kids Sydney, Nicholson, and Jackson

Sports / Crosstraining / Triathlon

Hiking / Camping

Religion / Philosophy / History

