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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 (CSCs) have been identified that have enhanced tumorigenicity and chemoresistance in *ex vivo* models. However, whether these populations are more capable of surviving chemotherapy in *de novo* tumors is unknown.

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

Results—Primary samples were composed of low densities of ALDH1A1, CD44 and CD133. Tumors collected immediately after primary therapy were more densely composed of each marker, while 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 to 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 demonstrating 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.

Keywords

CD133; CD44; aldehyde dehydrogenase; ALDH1A1; endoglin; CD105; gli1; gli2; cancer stem cell; ovarian cancer

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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” (CSCs) or “tumor initiating cells” (TICs) 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 biological processes compared to 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”) (9) and aldehyde dehydrogenase (ALDH1A1)-positive cells (10–13), and are associated with poor clinical 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 in order to achieve durable cures. In the current study, we utilized a unique cohort of matched primary/recurrent ovarian cancer specimens to determine if putative cancer stem cell 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. Additionally, novel genes were revealed to be highly expressed in recurrent samples, specifically endoglin (CD 105) 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 performed using standard techniques (14) on samples collected from matched primary and recurrent tumors taken from 45 patients with ovarian adenocarcinoma, and, with IRB approval, clinical information was collected. Pathology was confirmed and formalin-fixed paraffin-embedded (FFPE) slides were cut at 5 or 10 μm . Antigen retrieval was carried out in citrate buffer (pH 6.0) for 45 mins in an atmospheric-pressure steamer. Slides were then stained using antibodies against ALDH1A1 (Clone 44, BD Biosciences, San Jose, CA), CD44 (Clone 2F10, R&D Systems, Minneapolis, MN) or CD133 (Clone C24B9, Cell Signaling Technology, Danvers, MA) at 1:500 dilution in Cyto-Q reagent (Innovex Biosciences, Richmond, CA) overnight at 4°C. Primary antibody detection was achieved with Mach 4 HRP polymer (Biocare Medical, Concord, CA) for 20 mins at RT, followed by 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 CSC's 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 percent of positive tumor cells across the entire slide. The average number of positive cells for each marker among the 45 primary samples was compared to the average among recurrent samples, with additional subgroup analyses performed as described in the Results section. A subgroup analysis of IHC staining using an antibody against endoglin (Sigma, St. Louis, MO) was also performed.

Laser capture microdissection

Ten micrometer-thick FFPE sections were prepared from 12 matched pairs of ovarian adenocarcinoma patient samples, 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, Mountainview, CA). Care was taken to ensure no stromal cells were collected (see Supplemental Figure 1). RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA) optimized for FFPE samples.

RT² Profiler PCR Array

RNA extracted from microdissected samples was converted to cDNA and amplified using the RT² FFPE PreAMP cDNA Synthesis Kit (SABiosciences, Frederick, MD). Quality of cDNA was confirmed with the Human RT² 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 RT² 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- β and Wnt signaling pathways. PCR amplification was performed 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% fetal bovine serum (Hyclone, Logan, UT). All cell lines were routinely screened for *Mycoplasma* species (GenProbe detection kit; Fisher, Itasca, IL) with experiments performed at 70–80% confluent cultures. Purity of cell lines was confirmed with STR genomic analysis, and only cells less than 20 passages from stocks were used in experiments.

RNA extraction from cell lines

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

Reverse transcription and quantitative PCR

Prior to reverse transcription, all RNA samples were diluted to 20 ng/ μ L using RNase-free water. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA samples were analyzed using quantitative PCR. Primer and probe sets for *ABCG2* (Hs01053790_m1), *ALDH1A1* (Hs00946916_m1), *CD44* (Hs01075861_m1), *CD133* (Hs01009259_m1), *GLI1* (Hs00171790_m1), *GLI2* (Hs00257977_m1) and *RPLP0* (Hs99999902_m1, housekeeping gene) were obtained from Applied Biosystems; primers for *endoglin* (*ENG*; PPH01140F) were obtained from SABiosciences and used according to manufacturer's instructions. PCR amplification was performed 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'-CUACUGAUACUCUGGGGAUA-3' or *GLI1_B* siRNA: 5'-GCAAAUAGGGCUUCACAUA-3'), or one of 2 tested Gli2-targeting constructs (*GLI2_A* siRNA: 5'-CGAUUGACAUGCGACACCA-3' or *GLI2_B* siRNA: 5'-GUACCAUUACGAGCCUCAU-3') at a 1:3 siRNA (pmol) to Lipofectamine 2000 (μ L) ratio. Lipofectamine and siRNA were incubated for 20 min at RT, added to cells in serum-free RPMI to incubate for 6–8 hours, followed by 10% FBS/RMPI thereafter. Transfected cells were grown at 37°C for an additional 48 hours and then harvested for quantitative PCR or Western blot analysis.

Western blot analysis

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

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 μ g) for 24 hours in 6- well plates (2.5×10^5 cells/well), trypsinized and then re-plated on a 96-well plate at 2,000 cells per well. After 4–5 days, cell viability was assessed by optical density measurements at 570 nm using 0.15% MTT (Sigma) in PBS. For cell cycle analysis, 5×10^5 cells in a 60-mm dish were transfected with siRNAs and then cultured in RPMI/10% FBS at 37°C for an additional 48 hours. Cells were then trypsinized, washed in PBS, and fixed in 100% ethanol overnight. Cells were then centrifuged, washed in PBS, and resuspended in PBS containing 0.1% Triton X-100 (v/v), 200 μ g/mL DNase-free RNase A and 20 μ g/mL propidium iodide (PI). PI fluorescence was assessed by flow cytometry and the percentage of cells in sub-G0, G0/G1, S and G2/M phases were calculated by the cell cycle analysis module for Flow Cytometry Analysis Software (FlowJo v.7.6.1, Ashland, OR). For effects of siRNA-mediated downregulation on cisplatin IC50, cells were first transfected with siRNA (5 μ g) in 6- well plates, trypsinized and then re-

plated on a 96-well plate at 2,000 cells per well, followed by addition of chemotherapy after attachment. IC₅₀ was determined by finding the dose at which the drug had 50% of its effect, calculated by the equation $[(OD570_{MAX} - OD570_{MIN})/2] + OD570_{MIN}$.

Statistical analysis

Comparisons of continuous variables were made using a two-tailed Student's t-test, if assumptions of data normality were met. Those represented by alternate distribution were examined using a nonparametric Mann-Whitney U test. Differences between groups were considered statistically significant at $p < 0.05$. Error bars represent standard deviation unless otherwise stated.

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 Supplemental Table 1, and represent the typical clinical profiles of ovarian cancer patients. 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 demonstrating a putative CSC population in ovarian cancer. The percent of positive ALDH1A1, CD44 and CD133 cells in primary samples averaged 23.4%, 6.2% and 7.1%, respectively (Figure 1A). Representations of high and low-distribution patterns are shown in Figure 1B, and for CD44 and CD133 high power views in Figure 1C. For all three 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 (Figure 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 (Figure 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 compared to primary samples. In order 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 Figure 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 compared to matched primary.

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

If the cancer stem cell hypothesis is clinically significant, then surviving cells would be expected to give rise again to both resistant cancer stem cells 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 based on when their recurrent tumor was collected: 1) in patients who were clinically without evidence of disease, but had other indications for surgery performed within 3 months of completion of primary therapy, termed “persistent tumor,” 2) 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 3) 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%, $p=0.001$) (Figure 2A). By contrast, samples collected at first recurrence before initiating secondary therapy were composed of similar percentages of each marker as their primary tumor (Figure 2B), suggesting 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%, $p=0.027$) (Figure 2C). The percentage of CD133-positive cells increased by more than 2-fold in 50% of treated recurrence samples compared to 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, compared to matched primary. 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 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 to 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 (Supplemental Figure 1). 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 elevated in persistent tumors compared to matched primary, 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 IHC 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 increased CD133 protein expression in recurrent tumors noted by IHC may be due to post-transcriptional or post-translational regulation.

Of the 84 genes examined by the Human Stem Cell Signaling RT² Profiler Array (16), we found that 12 of these genes (14%) were significantly increased in persistent compared to

matched primary tumor. Members of the TGF- β superfamily signaling pathway (*ENG*, *ZEB2*, *LTBP4*, *TGFBR2*, *RGMA*, *ACVR1B* and *SMAD2*) were most commonly significantly increased as well as members of the Hedgehog (*GLI1* and *GLI2*), Notch (*PSEN2*) and Wnt (*FZD9* and *BCL9L*) pathways. Of particular interest, the TGF- β co-receptor 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 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 endoglin positivity than in the matched primary tumor, and that expression was definitively present in tumor cells, not just the vasculature (Figure 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 to 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 Figure 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, two 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 (Figure 3C). ES2 and HeyA8MDR cells transiently transfected with these endoglin-targeting siRNAs demonstrated a significant reduction in viability, as determined by MTT assay (Figure 3D). This effect on viability correlated with the degree of endoglin downregulation, as *ENG_A* siRNA reduced cell viability by 50–84% (in ES2 and HeyA8MDR, respectively, $p<0.001$), while *ENG_B* siRNA had no effect on ES2 and a 64% reduction in HeyA8MDR ($p<0.001$). The variability in effects on the two cell lines may reflect their dependency on endoglin, as HeyA8MDR cells have 3.7-fold higher endoglin expression than ES2 cells. Additionally, 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 performed 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 (Figure 3E). In both ES2 and HeyA8MDR, endoglin knockdown resulted in a significant accumulation of cells in the sub-G0/apoptotic fraction compared to 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 cancer stem cells (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 Figure 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 these hedgehog pathway members may be involved in mediating platinum resistance.

A2780cp20 (Gli1+/Gli2+) and ES2 (Gli1-/Gli2+) cells were subsequently used for examining the biological 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 (Figure 4B). Importantly, each siRNA construct demonstrated 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 Figure 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 since 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 (Figure 4C). Cisplatin IC₅₀ decreased from 4 μM to 0.8 μM (5.0-fold change) in A2780cp20 cells and from 0.7 μM to 0.15 μM (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 performed 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 Figure 4D, downregulation of Gli1 had little effect on the cell cycle distribution of A2780cp20 cells, with a modest accumulation in the sub-G0 or apoptotic fraction compared to control siRNA (8% to 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% to 32%, $p < 0.001$) in induction of apoptosis compared to 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 cancer stem cell 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 ovarian cancer patients. Additionally, 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 co-receptor endoglin was found to be overexpressed in residual tumor cells, and thus important to the chemoresistant population of tumor cells. 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 vivo*, and now *de novo* models (2, 33). This is clinically evident in the observation that patients often have outstanding initial responses to chemotherapy, suggesting 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 poly (ADP-ribose) polymerase (PARP), could be used to reverse chemoresistance (34). It is reasonable to postulate that cancer stem cells, like normal stem cells, would have enhanced mechanism of DNA repair, allowing for survival with prolonged exposures to DNA-damaging insults. Analysis of RNA from FFPE samples showed 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 performed 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 (TSCs), with a small component of therapy-resistant cells (TRCs). 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 18–24 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 due to 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 in order to achieve increased response rates and survival in ovarian cancer patients.

It is an interesting finding that CD44+ cells were less dense in recurrent tumors than CD133 and ALDH1, despite multiple studies showing that CD44+ cells have CSC properties. Many of these studies have used CD44 in combination with other markers, such as c-kit (4), My88 (5), CD133 (6), and CD24 (35). It is for this reason that we examined CD44 by itself as potentially important, but at the same time may have introduced a limitation by not being able to evaluate dual-positive populations. It is yet to be determined the degree of crossover between individual markers. Likely the combination of markers will identify a more aggressive population than either alone, as previously shown with CD133 and ALDH1 (11), but it is unknown whether such combinations then exclude other aggressive populations. This disparity, however, highlights the limitations in defining the key population by marker status alone, instead relying on clinical behaviors such as resistance to chemotherapy.

Recent studies have shown that developmental pathways (such as Notch, Wnt, Hedgehog and TGF- β) play an important role in the self-renewal and maintenance of CSCs and that inhibiting these pathways may provide useful therapeutic strategies both alone and in combination with traditional chemotherapies (36–37). In our study, genes identified as being significantly overexpressed in persistent tumors included endoglin (a member of the TGF- β superfamily) and the primary mediators of hedgehog transcription, *GLI1* and *GLI2*, among others (Table 2). The most significant and consistent increase in expression from primary to persistent tumor occurred in endoglin (CD105), a TGF- β co-receptor. This molecule interacts with TGF- β receptor II (TGFBR2, which was also significantly increased in persistent tumors (2.76-fold, $p=0.0190$)), both dependently and independently of the TGF- β ligand (38). This interaction subsequently promotes gene transcription mediated by the Smad family of transcription factors (Smad2 and 4). In contrast, a proteolytically cleaved, secreted form of endoglin, known as soluble endoglin (Sol-Eng) appears to inhibit TGF- β signaling by scavenging circulating TGF- β ligands (39). Endoglin is a well-described marker of angiogenesis, whose expression is turned on in growing/sprouting endothelial cells (such as those supplying vasculature to tumors). This characteristic of endoglin has made it a desirable target for anti-angiogenic 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. Based on 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 performed by Li et al., it was demonstrated that endoglin prevents apoptosis in endothelial cells undergoing hypoxic stress, either in the presence or absence of TGF- β ligand (42). It could be speculated that endoglin serves a similar anti-apoptotic function in tumor epithelial cells and thereby promotes ovarian cancer cell survival. Whether this is due to the promotion of TGF- β signaling or through a TGF- β -independent mechanism remains to be determined. Taken together, these data suggest that inhibiting endoglin could be used to target both the tumor and its developing vasculature, thereby having a potentially greater therapeutic benefit. Additional studies will determine the viability of endoglin as a therapeutic target, as antibodies have been developed that disrupt the interaction of endoglin and TGF- β receptor II (43–44).

Previous studies have implicated hedgehog signaling in multi-drug 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 IC₅₀ 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 anti-apoptotic 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 of the link between Gli2, apoptosis and cisplatin resistance are warranted.

Collectively, the data presented in this study demonstrate 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 in order to achieve durable clinical response in for this disease. In addition, the TGF- β co-receptor endoglin (CD105) and the Hedgehog mediator Gli2 were found to be overexpressed in recurrent ovarian tumors and are promising targets in overcoming chemoresistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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TRANSLATIONAL RELEVANCE

Most ovarian cancer patients will have an excellent response to initial surgical debulking and chemotherapy, but about 75% of patients will later recur and succumb to disease. Based primarily on *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 demonstrated 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 to primary tumor. Further development revealed novel mechanisms of the TGF- β co-receptor 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 demonstrate that therapies targeting these pathways may reverse platinum resistance in ovarian cancer.

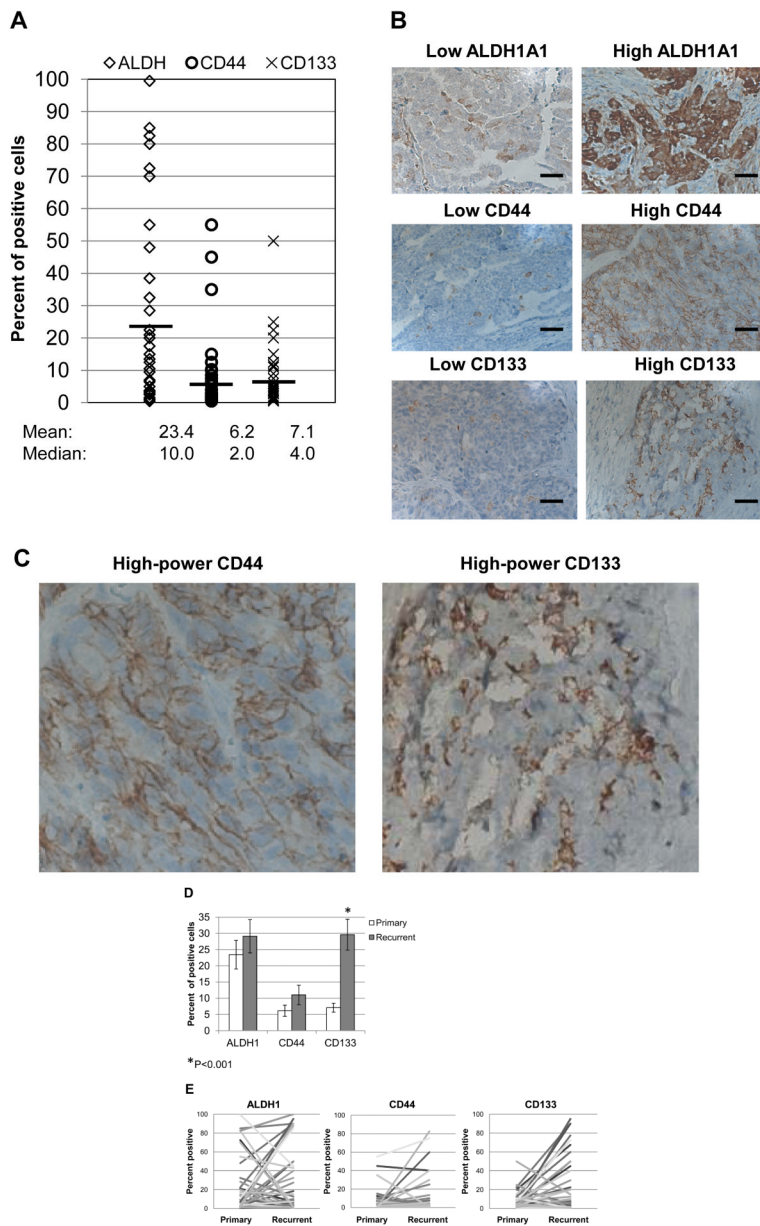


Figure 1. Change in expression of ALDH1A1, CD44 and CD133 from primary to recurrent ovarian cancer
 A) ALDH1A1, CD44 and CD133 expression in 45 high grade ovarian adenocarcinomas was examined using immunohistochemistry. The estimated percentage of positive cells for each sample, with mean (black bars) and median, are shown. B) For all three proteins examined, staining was heterogeneous, rather than diffusely positive. Examples of high and low frequency expression for each are shown (black bar = 100 μ m). C) A higher magnification of CD44 and CD133 expression in primary ovarian cancer specimens, demonstrating cell surface expression. D) The average number of positive cells for ALDH1A1, CD44 and CD133 among the 45 primary samples was compared to the average among matched recurrent samples. Only CD133 was significantly higher in recurrent samples. Error bars represent SEM. *P<0.001. E) In order 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.

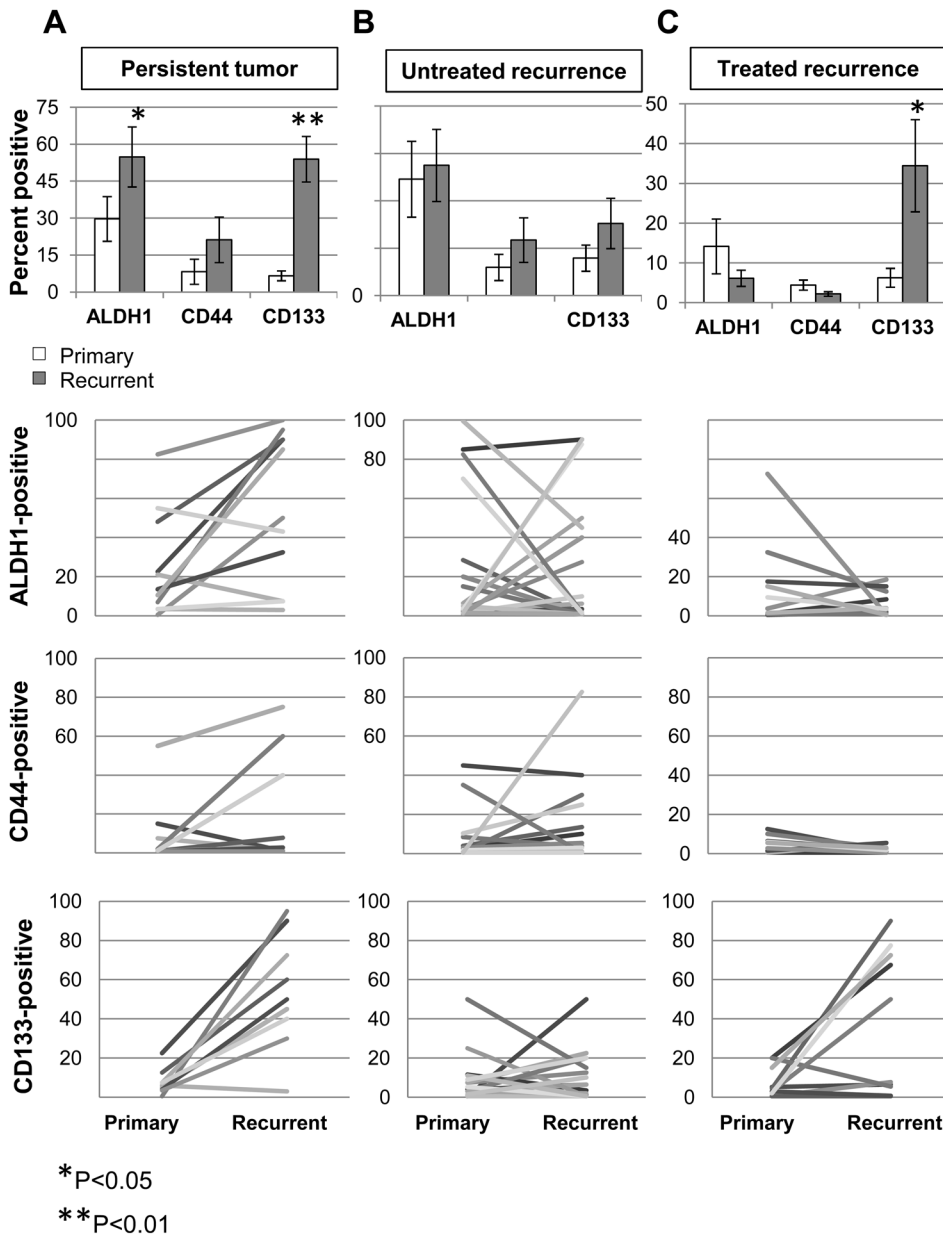


Figure 2. Subgroup analysis of ALDH1A1, CD44 and CD133 based on setting of recurrent tumor collection

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.

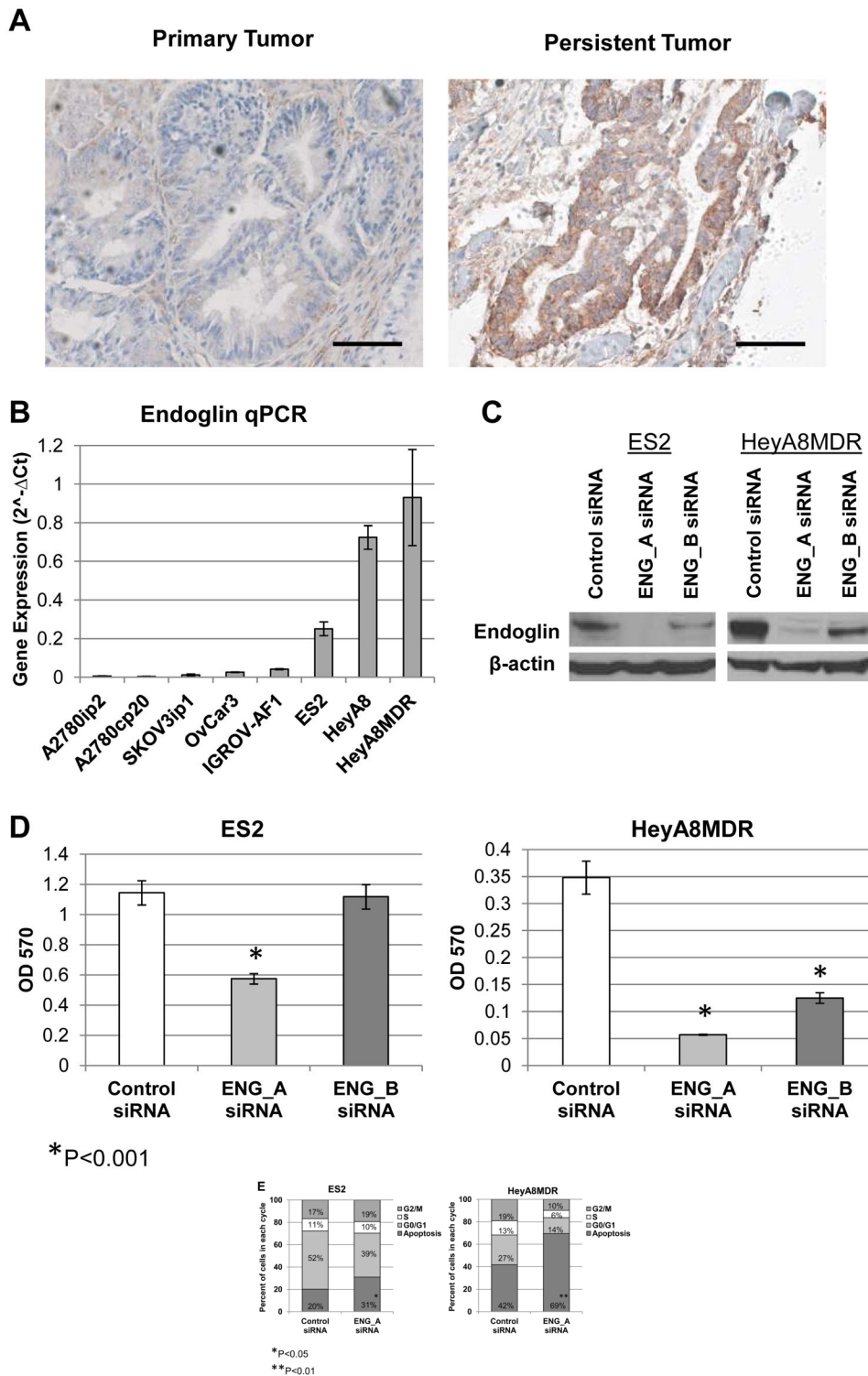
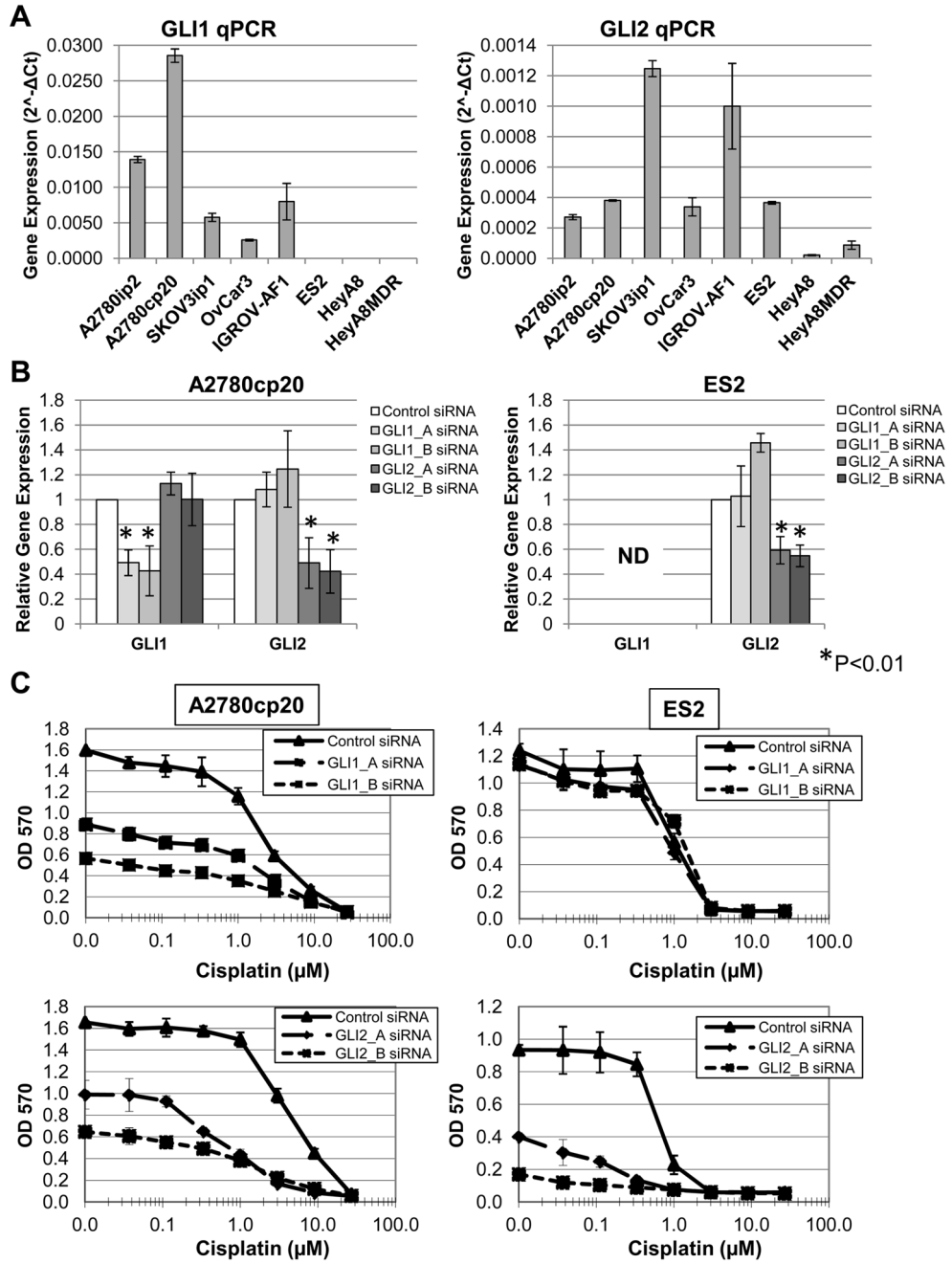
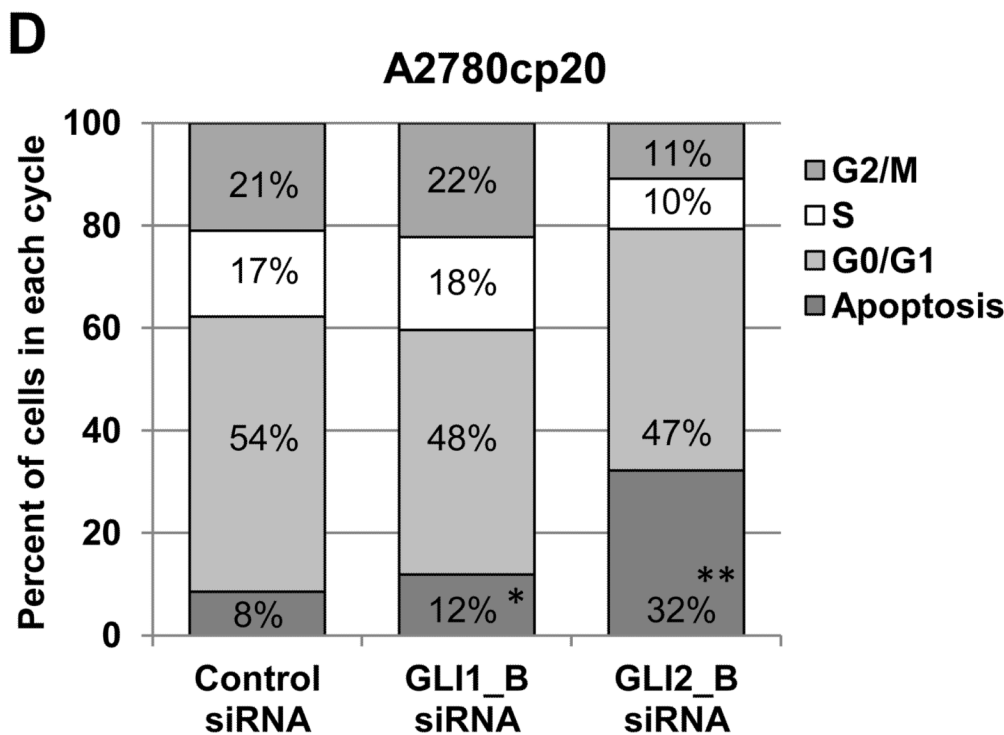


Figure 3. Endoglin is expressed in persistent ovarian tumor and ovarian cancer cell lines and its downregulation leads to decreased cell viability.<

A) Matched primary/persistent ovarian tumor pairs (n = 12) were subjected to immunohistochemical analysis of endoglin to evaluate changes in expression. Persistent tumors were found to have a higher density of endoglin staining compared to primary

specimens. Representative histologic sections are shown for a matched pair (black bar = 100 μm). B) mRNA expression of endoglin was quantified in 8 different ovarian cancer cell lines using quantitative PCR. Gene expression is shown as \log_2 transformed ΔC_T values (difference between the C_T value of the gene of interest (endoglin) and that of the housekeeping gene (RPLP0)). C) Downregulation of endoglin in ES2 and HeyA8MDR cells using 2 different siRNA constructs was determined by Western blot analysis. β actin was used as a loading control. D) ES2 and HeyA8MDR cells transiently transfected with anti-endoglin siRNAs had decreased viability as determined by MTT assay. E) Cell cycle analysis (PI staining) revealed that downregulation of endoglin led to an accumulation of both ES2 and HeyA8MDR cells in the sub-G0 or apoptotic fraction. Data are representative of 3 independent experiments. * $P < 0.001$.





*P<0.05

**P<0.001

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. Gene expression is shown as log₂ transformed ΔC_T values. B) Downregulation of Gli1/2 in A2780cp20 and ES2 cells using 2 different siRNA constructs was determined by quantitative PCR. Each siRNA construct demonstrated 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-G0 or apoptotic fraction. Data are representative of 3 independent experiments.

Table 1

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

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

* An increase or decrease more than 2-fold designated by arrow NC - Density of cells did not change by more than 2-fold NM - Not measured due to insufficient tumor

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 Fold Change*	p-value [†]	# decreased >50%	# increased >2-fold
Putative CSC Markers					
Aldehyde dehydrogenase 1A1 (ALDH1A1)		2.46	0.2343	3	6
CD44 molecule (CD44)		4.08	0.0023	2	9
Prominin 1 (PROM1/CD133)		1.11	0.8877	4	5
ATP-binding cassette, sub-family G, member 2 (ABCG2/BCRP)		7.65	0.0163	1	5
Human Stem Cell Signaling RT² Profiler PCR Array					
Endoglin (ENG)	TGFβ	3.77	0.0023	0	9
Zinc-finger E-box binding homeobox 2 (ZEB2)	TGFβ	3.66	0.0062	1	9
Presenilin 2 (PSEN2)	Notch	3.30	0.0071	0	7
GLI family zinc finger 1 (GLI1)	Hedgehog	10.21	0.0076	1	10
GLI family zinc finger 2 (GLI2)	Hedgehog	7.61	0.0111	2	9
Latent transforming growth factor beta binding protein 4 (LTBP4)	TGFβ	4.69	0.0146	1	9
Transforming growth factor, beta receptor II (TGFBR2)	TGFβ	2.76	0.0190	0	8
RGM domain family, member A (RGMA)	TGFβ	7.84	0.0204	2	9
Activin A receptor, type IB (ACVR1B)	TGFβ	2.20	0.0275	0	4
Frizzled homolog 9 (FZD9)	Wnt	10.43	0.0393	2	8
SMAD family member 2 (SMAD2)	TGFβ	1.79	0.0435	1	6
B-cell CLL/lymphoma 9-like (BCL9L)	Wnt	2.06	0.0463	1	6

* Persistent compared to primary tumor

[†] Calculated using paired Student's t-test