

## Research Article

**ALDH1A1 Is a Novel EZH2 Target Gene in Epithelial Ovarian Cancer Identified by Genome-Wide Approaches**Hua Li<sup>1</sup>, Benjamin G. Bitler<sup>1</sup>, Vinod Vathipadiekal<sup>4</sup>, Marie E. Maradeo<sup>2</sup>, Michael Slifker<sup>3</sup>, Caretha L. Creasy<sup>5</sup>, Peter J. Tummino<sup>5</sup>, Paul Cairns<sup>2</sup>, Michael J. Birrer<sup>4</sup>, and Rugang Zhang<sup>1</sup>**Abstract**

Epithelial ovarian cancer (EOC) remains the most lethal gynecologic malignancy in the United States. EZH2 silences gene expression through trimethylating lysine 27 on histone H3 (H3K27Me3). EZH2 is often overexpressed in EOC and has been suggested as a target for EOC intervention. However, EZH2 target genes in EOC remain poorly understood. Here, we mapped the genomic loci occupied by EZH2/H3K27Me3 using chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) and globally profiled gene expression in EZH2-knockdown EOC cells. Cross-examination of gene expression and ChIP-seq revealed a list of 60 EZH2 direct target genes whose expression was upregulated more than 1.5-fold upon EZH2 knockdown. For three selected genes (*ALDH1A1*, *SSTR1*, and *DACT3*), we validated their upregulation upon EZH2 knockdown and confirmed the binding of EZH2/H3K27Me3 to their genomic loci. Furthermore, the presence of H3K27Me3 at the genomic loci of these EZH2 target genes was dependent upon EZH2. Interestingly, expression of *ALDH1A1*, a putative marker for EOC stem cells, was significantly downregulated in high-grade serous EOC ( $n = 53$ ) compared with ovarian surface epithelial cells ( $n = 10$ ,  $P < 0.001$ ). Notably, expression of *ALDH1A1* negatively correlated with expression of EZH2 ( $n = 63$ , Spearman  $r = -0.41$ ,  $P < 0.001$ ). Thus, we identified a list of 60 EZH2 target genes and established that *ALDH1A1* is a novel EZH2 target gene in EOC cells. Our results suggest a role for EZH2 in regulating EOC stem cell equilibrium via regulation of *ALDH1A1* expression. *Cancer Prev Res*; 5(3); 484–91. ©2011 AACR.

**Introduction**

Epithelial ovarian cancer (EOC) accounts for more deaths than any other gynecologic malignancy in the United States. EOCs are classified into distinct histologic types including serous, mucinous, endometrioid, and clear cell (1). The most common histology of EOC is serous (~60% of all cancers) and less common histologies include endometrioid, clear cell, and mucinous (1). Recently, an alternative classification has been proposed, in which EOC is broadly divided into 2 types (2). Type I EOC includes mucinous, low-grade serous, low-grade endometrioid, and clear cell carcinomas, and type II EOC includes high-grade serous carcinomas, which is the most lethal histosubtype (2).

Enhancer of zeste homology 2 (EZH2) is a histone methyltransferase that mediates gene silencing by catalyzing the trimethylation on lysine 27 of histone H3 (H3K27Me3; ref. 3). EZH2 is often expressed at higher levels in human EOC cells, and its expression positively correlates with cell proliferation in these cells (4). Further underscoring the importance of EZH2 in EOC, EZH2 knockdown triggers apoptosis and inhibits the invasion of human EOC cells (4). In addition, EZH2 is overexpressed in ovarian tumor-associated endothelial cells, which promotes angiogenesis (5). Finally, there is evidence to suggest that EZH2 is overexpressed in ovarian cancer stem cell-like populations enriched by chemotherapy (6). Accordingly, EZH2 has been suggested as a putative target for developing EOC therapeutics. Thus, it is important to identify EZH2 target genes in EOC to gain insights into the biology of the disease and to facilitate translational EOC research related to EZH2. Although a number of EZH2 target genes have been characterized in a few cancer types, including prostate and breast, using chromatin immunoprecipitation (ChIP)-on-chip analysis (7, 8), studies that aim to globally identify EZH2 target genes in EOC cells have yet to be conducted.

Here, we report the identification of direct EZH2 target genes in human EOC cells using a combination of genome-wide approaches. Specifically, we identified the genomic loci occupied by EZH2/H3K27Me3 using ChIP followed by next-generation sequencing (ChIP-seq). In addition, we discovered a list of genes whose expression was upregulated

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more than 1.5-fold in EZH2-knockdown EOC cells compared with controls using gene expression microarray analysis. Cross-examination of gene expression profiling and ChIP-seq analysis revealed a list of 60 genes that are direct EZH2/H3K27Me3 target genes, including 56 novel putative EZH2 target genes. For validation, we selected 3 genes that are implicated in regulating stem cells, apoptosis, cell growth, or invasion. We validated their upregulation upon EZH2 knockdown in EOC cells and confirmed the binding of EZH2/H3K27Me3 by ChIP analysis. Interestingly, expression of ALDH1A1, a putative marker for EOC stem cells (9–11), was expressed at significantly lower levels in high-grade serous EOC than in normal human ovarian surface epithelial (HOSE) cells and negatively correlated with expression of EZH2.

## Materials and Methods

### Cell culture, short hairpin RNA, lentivirus packaging and infection

The SKOV3 human EOC cell line was cultured according to the American Type Culture Collection and as previously described (4, 12). SKOV3 cell line identification was further confirmed by DNA Diagnostic Center. The sense sequences of 2 individual short hairpin RNAs (shRNA) to the human *EZH2* genes are as we have previously published (4). Lentivirus packaging was conducted using ViraPower system (Invitrogen) according to the manufacturer's instruction and as previously described (4). Briefly, SKOV3 cells at 40% to 50% confluency were infected with lentivirus expressing shRNA to EZH2 or vector control. The infected cells were drug selected with 3  $\mu\text{g}/\text{mL}$  of puromycin to eliminate noninfected cells.

### Antibodies, Western blot analysis, RNA isolation, and quantitative reverse-transcriptase PCR

The following antibodies were used for Western blot analysis: mouse anti-EZH2 (1:2,500; BD Bioscience), rabbit anti-H3K27Me3 (1:1,000; Cell Signaling), and mouse anti-GAPDH (1:10,000; Millipore). RNA from cultured human SKOV3 EOC cells was isolated using TRIzol (Invitrogen) according to the manufacturer's instruction. For quantitative reverse-transcriptase PCR (qRT-PCR), TRIzol-isolated RNA was further purified using an RNeasy kit (Qiagen) following the manufacturer's instruction. The primers for *ALDH1A1*, *SSTR1*, and *DACT3* genes used for qRT-PCR were purchased from Applied Biosystems. Expression of the housekeeping gene  $\beta$ -2-microglobulin was used to normalize mRNA expression.

### ChIP-seq analysis and ChIP validation for selected EZH2 target genes

Briefly, SKOV3 cells were fixed with 1% formaldehyde for 15 minutes and quenched with 0.125 mol/L glycine. Chromatin was isolated by adding lysis buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.1, 1 mmol/L phenylmethylsulfonyl fluoride) followed by disruption with a Dounce homogenizer. Lysates were sonicated using

a Misonix Sonicator 3000 to shear the DNA to an average length of 300 to 500 bp. Lysates were cleared by centrifugation to collect chromatin suspensions. Prior to their use in the ChIP protocol, protein A agarose beads (Invitrogen) were preblocked using blocking proteins and nucleic acids for 3 hours. For each ChIP reaction, an aliquot of chromatin (20–30  $\mu\text{g}$ ) was precleared with 30  $\mu\text{L}$  preblocked protein A agarose beads for 1 to 2 hours. ChIP reactions were set up using precleared chromatin and antibody (anti-H3K27Me3, Millipore 07-449; anti-EZH2, Millipore 07-689) and incubated overnight at 4°C. Preblocked protein A agarose beads were added and incubation at 4°C was continued for another 3 hours. Agarose beads containing the immune complexes were washed, and the immune complexes eluted from the beads were subjected to RNase treatment at 37°C for 20 minutes and proteinase K treatment at 37°C for 3 hours. Cross-links were reversed, and ChIP DNAs were purified by phenol–chloroform extraction and ethanol precipitation.

ChIP DNA was amplified using the Illumina ChIP-Seq DNA Sample Prep Kit. In brief, DNA was resonicated and ends were polished and 5'-phosphorylated using T4 DNA polymerase, Klenow polymerase, and T4 polynucleotide kinase. After addition of 3'-A to the ends using Klenow fragment (3'-5' exo minus), Illumina genomic adapters were ligated and the sample was size-fractionated (300–400 bp) on a 2% agarose gel. After a final PCR amplification step (18 cycles, Phusion polymerase), the resulting DNA libraries were quantified and tested by qPCR at the same specific genomic regions as the original ChIP DNA to assess quality of the amplification reactions. DNA libraries were sequenced on a Genome Analyzer II. Sequences (36-nucleotide reads) were aligned to the human genome (NCBI Build 37.1/hg19) using Eland software (Illumina). Aligned sequences were extended *in silico* at their 3'-ends to a length of 240 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nucleotide bins along the genome. The resulting histograms were stored in BAR (Binary Analysis Results) files. Peak locations were determined using the MACS algorithm.

For validation of binding of EZH2/H3K27Me3 to the genomic loci of the selected EZH2/H3K27Me3 target genes, SKOV3 EOC cells were transduced with lentivirus encoding control or shEZH2. Drug-selected cells were subjected to ChIP analysis as previously described (13, 14). The following antibodies were used to conduct ChIP: anti-EZH2 (C11, BD Biosciences), anti-H3K27Me3 (C36B11, Cell Signaling), and anti-histone H3 (05-928, Millipore). An isotype-matched IgG was used as a negative control. Immunoprecipitated DNA was analyzed with PCR against the genomic regions of *ALDH1A1* (forward: 5'-TGGCACTGGTTATTCAACGTGGTC-3' and reverse: 5'-GAGGGTGAAGCTCTTGTTAGGTTT-3'), *DACT3* (forward: 5'-CACACACACACACAAACAGTGCCT-3' and reverse: 5'-TTCCTCCAAC-TAGGCTGGCAGTTT-3') and *SSTR1* (forward: 5'-TAGCC-TAAGCTGCCTGCTGTGTTA-3' and reverse: 5'-AAAGTG-CATGTGCGGTCTGTTAGC-3'). PCR products were visualized on a 2% agarose gel.

### Gene expression microarray analysis

For gene expression microarray analysis in SKOV3 cells, 500 ng of total RNA was amplified and labeled using Agilent QuickAmp labeling kit following the manufacturer's protocol. A total of 1.65  $\mu$ g of Cy-3-labeled cRNA targets were hybridized onto Agilent 4  $\times$  44 k whole genome arrays for 17 hours at 65°C and washed according to procedure described by Agilent. The hybridized slides were scanned at 5- $\mu$ m resolution on an Agilent scanner (Agilent), and fluorescent intensities of hybridization signals are extracted using Agilent Feature Extraction software.

### Data sets

Gene expression microarray data sets for 53 cases of laser capture and microdissected (LCM) high-grade serous EOC and 10 individual isolations of normal HOSE cells were obtained from Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>; GEO accession number: GSE18521).

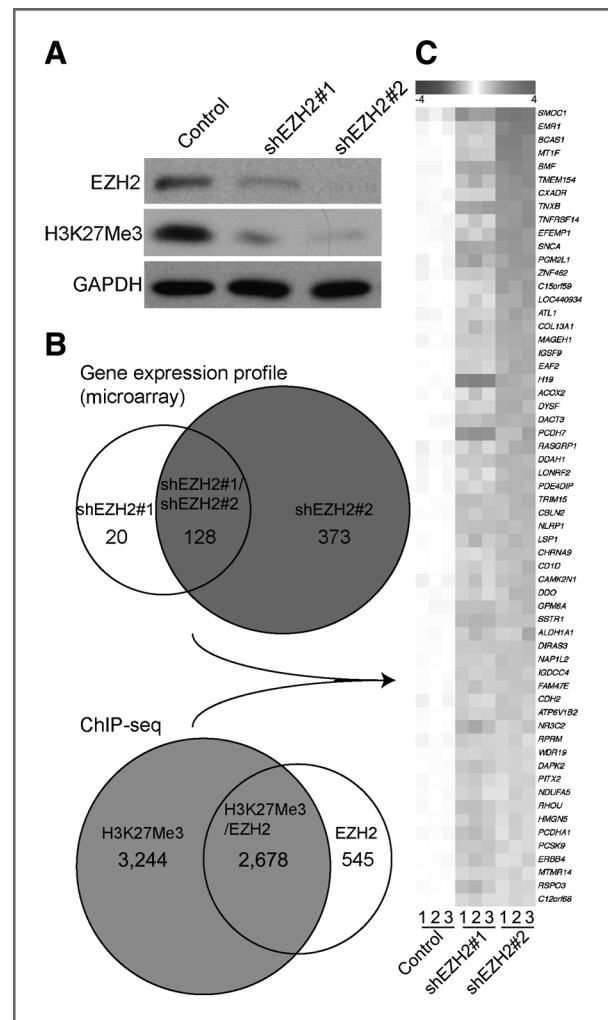
### Statistical analysis

Quantitative data are expressed as mean  $\pm$  SD unless otherwise stated. ANOVA with Fisher least significant difference (LSD) was used to identify significant differences in multiple comparisons. Spearman test was used to measure statistical dependence between EZH2 mRNA levels and ALDH1A1 mRNA levels. For all statistical analyses, the level of significance was set at 0.05.

## Results and Discussion

### Genome-wide mapping of EZH2/H3K27Me3 direct target genes in human EOC cells

To identify genes whose expression was suppressed by EZH2, we conducted gene expression microarray analysis in control and EZH2-knockdown SKOV3 human EOC cells. Two individual shRNAs to the human *EZH2* gene (shEZH2) were used to limit potential off-target effects. Knockdown efficacy was confirmed by immunoblotting analysis (Fig. 1A). EZH2 knockdown notably decreased the levels of H3K27Me3, which is consistent with the idea that EZH2 plays a major role in regulating the levels of H3K27Me3 in human EOC cells (Fig. 1A). A list of 148 genes and 501 genes were upregulated more than 1.5-fold by shEZH2#1 and shEZH2#2, respectively, whereas 128 genes overlapped between the 2 different shEZH2s (Fig. 1B). Further data can be found at GEO database upon publication (GEO accession number: GSE31433). The difference in the number of the genes altered by 2 individual shEZH2s may be due to different degrees of EZH2 knockdown. Consistent with this possibility, shEZH2#2, which decreases EZH2 levels with better efficacy than shEZH2#1, resulted in a greater number of upregulated genes (Fig. 1A and B). Alternatively, the differences in gene upregulation observed with individual EZH2 shRNAs may be due to off-target effects. To avoid this potential issue, we chose to analyze the genes that are upregulated by both shEZH2s. Of note, some of the known EZH2 target genes were approaching the 1.5-fold upregula-



**Figure 1.** Identification of EZH2 target genes in human SKOV3 EOC cells. A, SKOV3 cells were infected with indicated lentivirus encoding shEZH2 or control. Drug-selected cells were examined for expression of EZH2, H3K27Me3, and GAPDH by immunoblotting analysis using indicated antibodies. B, schematic of experimental strategies used to identify EZH2 target genes. Genes whose expression was upregulated more than 1.5-fold upon EZH2 knockdown by 2 individual shEZH2 were identified by global gene expression microarray analysis. Genomic loci occupied by EZH2/H3K27Me3 were profiled by ChIP-seq analysis. C, cross-examination of gene expression profiling and ChIP-seq analysis as illustrated in (B) revealed a list of 60 putative EZH2 target genes in human SKOV3 EOC cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tion cutoff point but were not included in further analysis, including *VASH1* (5) and *E-cadherin* (ref. 15; data not shown). Although the conservative approach we implemented may lead to missing certain EZH2 target genes, we felt these rigorous methods allowed us to minimize false-positive EZH2 target genes in human EOCs.

We next sought to identify genomic loci that are directly bound by EZH2/H3K27Me3. Toward this goal, we conducted ChIP-seq analysis in SKOV3 human EOC cells using antibodies specific to EZH2 or H3K27Me3. EZH2 and H3K27Me3 occupancy was mapped to the genomic loci of

3,223 and 5,922 genes, respectively, and 2,678 genes were associated with both EZH2 and H3K27Me3 (Fig. 1B and Supplementary Table S1). The difference between the number of genes whose locus was occupied by EZH2 and H3K27Me3 may reflect the difference in affinity of the antibodies used for ChIP. Alternatively, for methylated sites not bound by EZH2, it is possible that other epigenetic regulators in addition to EZH2 can also generate H3K27Me3. Consistently, EZH1, a homolog of EZH2 in human cells, is also capable of catalyzing H3K27Me3 epigenetic modifications, albeit at a lower rate than EZH2 (16). Furthermore, genes bound by EZH2, but not H3K27Me3, may reflect an H3K27Me3-independent function for EZH2 as previous reports have suggested (for example, see ref. 17).

To identify the genes that are directly silenced by EZH2, we cross-examined the gene expression and ChIP-seq data. As a result, we identified a list of 60 EZH2/H3K27Me3 target genes whose expression was upregulated more than 1.5-fold upon EZH2 knockdown in SKOV3 human EOC cells (Fig. 1C). Further confirming our approach, 4 of the 60 identified genes have previously been shown as EZH2/H3K27Me3 target genes, namely, *SNAC* (18), *H19* (19), *DIRAS3* (20), and *DACT3* (21). Notably, Ingenuity networks analysis revealed that the networks enriched by the identified genes included (i) cell death, growth, and proliferation (e.g., *BMF*, *DAPK2*, *NLRP1*, and *DIRAS3*) and (ii) reproductive system development and cancer (e.g., *EAF2*, *ALDH1A1*, *SSTR1*, and *MAGEH1*; data not shown). This is consistent with the proliferation-promoting and apoptosis-suppressing function of EZH2, which we have previously reported in human EOC cells (4).

Interestingly, the number of genes upregulated more than 1.5-fold upon EZH2 knockdown is notably lower than the number of genes whose genomic loci are directly occupied by EZH2/H3K27Me3 (Fig. 1B). This result suggests that additional mechanisms may cooperate with EZH2/H3K27Me3 in silencing or reactivating EZH2 target genes. Consistent with this idea, previous reports have shown that EZH2 target genes are also subject to epigenetic silencing by H3K9Me3 (22) or histone deacetylase (23). This implies that to achieve maximum reactivation of EZH2/H3K27Me3-silenced target genes in human EOC cells, additional epigenetic gene silencing mechanisms may be considered for simultaneous targeting together with EZH2

inhibition. Alternatively, this result may be due to the bivalent modification (i.e., H3K27Me3 and H3K4Me3) at the genomic loci of those upregulated genes, which primes those genes for activation while keeping them silenced (24). Further studies are warranted to differentiate these possibilities.

#### Validation of the selected EZH2 target genes in human EOC cells

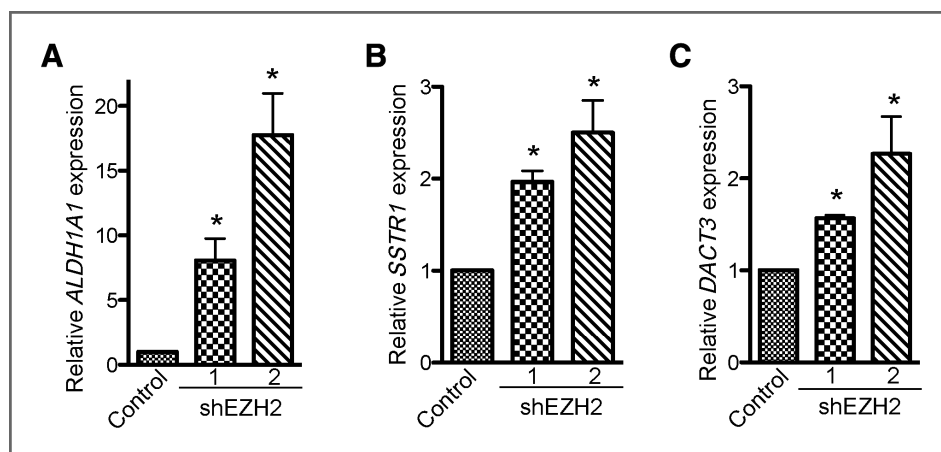
The list of upregulated genes was prioritized for validation by examining their expression in the newly released the Cancer Genomics Atlas (TCGA) ovarian database (25). We first chose those genes whose expression was downregulated more than 2-fold in more than 75% of EOC cases in TCGA ovarian database. In addition, known imprinted genes such as *H19* (19) and *DIRAS3* (20) or poorly annotated genes were excluded. Given that EZH2 promotes proliferation and invasion, suppresses apoptosis, and regulates stem cell-like population in human EOCs (4, 6), we selected 3 identified EZH2/H3K27Me3 target genes with one or more of these roles for validation studies. Those genes are *ALDH1A1* (11), *SSTR1* (26), and *DACT3* (ref. 21; Table 1).

We first validated the upregulation of the selected 3 genes in EZH2-knockdown SKOV3 human EOC cells by qRT-PCR. Indeed, all 3 selected genes were significantly upregulated in shEZH2-expressing SKOV3 cells compared with controls (Fig. 2,  $P < 0.05$  vs. controls.). In addition, all 3 genes were upregulated by both shEZH2s, and there was a correlation between the degree of EZH2 knockdown and the levels of upregulation of these genes (Figs. 1A and 2). We conclude that EZH2 knockdown upregulates the expression of *ALDH1A1*, *SSTR1*, and *DACT3* in SKOV3 human EOC cells.

Next, we sought to validate the binding of EZH2/H3K27Me3 to the genomic loci of the selected genes. Toward this goal, we conducted ChIP analysis using antibodies specific to EZH2 or H3K27Me3, respectively. An isotype-matched IgG was used as a negative control, and an antibody to the core histone H3 was used as a positive control for ChIP analysis. Indeed, we observed the binding of both EZH2 and H3K27Me3 to the genomic loci of the selected EZH2 target genes in SKOV3 human EOC cells as determined by ChIP analysis (Fig. 3).

**Table 1.** Three putative EZH2 target genes identified by genome-wide approaches selected for further validation

Gene name	Location	Function	% TCGA cases downregulated >2-fold
NM_000689 ALDH1A1	9q21.13	Cancer stem cell marker	96
NM_001049 SSTR1	14q13	Proliferation and invasion inhibitor, cell signaling	95
NM_145056 DACT3	19q13.32	Apoptosis inducer, Wnt signaling antagonist	76



**Figure 2.** Validation of upregulation of the selected EZH2-silenced target genes in SKOV3 human EOC cells upon EZH2 knockdown by qRT-PCR. SKOV3 cells were infected with lentivirus encoding the indicated shEZH2s or control. After drug selection, mRNA was extracted and examined for expression of (A) *ALDH1A1* mRNA, (B) *SSTR1* mRNA, and (C) *DACT3* mRNA by qRT-PCR. Expression of  $\beta$ -2-microglobulin was used to normalize the expression of *ALDH1A1*, *SSTR1* and *DACT3* mRNA. \*,  $P < 0.05$  compared with controls.

We next sought to determine whether the occupancy of H3K27Me3 on the genomic loci of EZH2 target genes depends upon EZH2. Toward this goal, we conducted the ChIP analysis in EZH2-knockdown SKOV3 EOC cells. Indeed, knockdown of EZH2 severely weakened the association of both EZH2 and H3K27Me3 to the genomic loci of the selected EZH2 target genes (Fig. 3). This result suggests that EZH2 plays a major role in regulating H3K27Me3 modification on the genomic loci of these genes in human EOC cells. This result also implies that the binding of EZH2/H3K27Me3 to the genomic loci of these genes we observed here is specific.

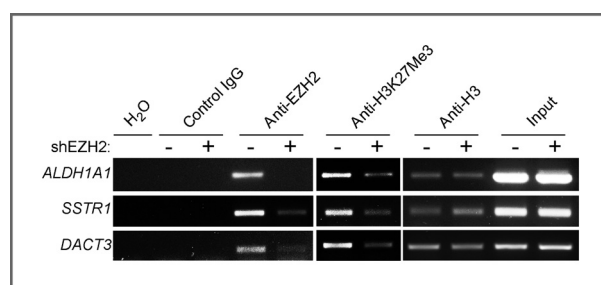
#### Expression of EZH2 inversely correlates with expression of *ALDH1A1*

We next sought to determine whether there is an inverse correlation between expression of EZH2 and expression of the EZH2 target genes that we have identified and validated in this study. In addition to EOC cells, EZH2 is upregulated

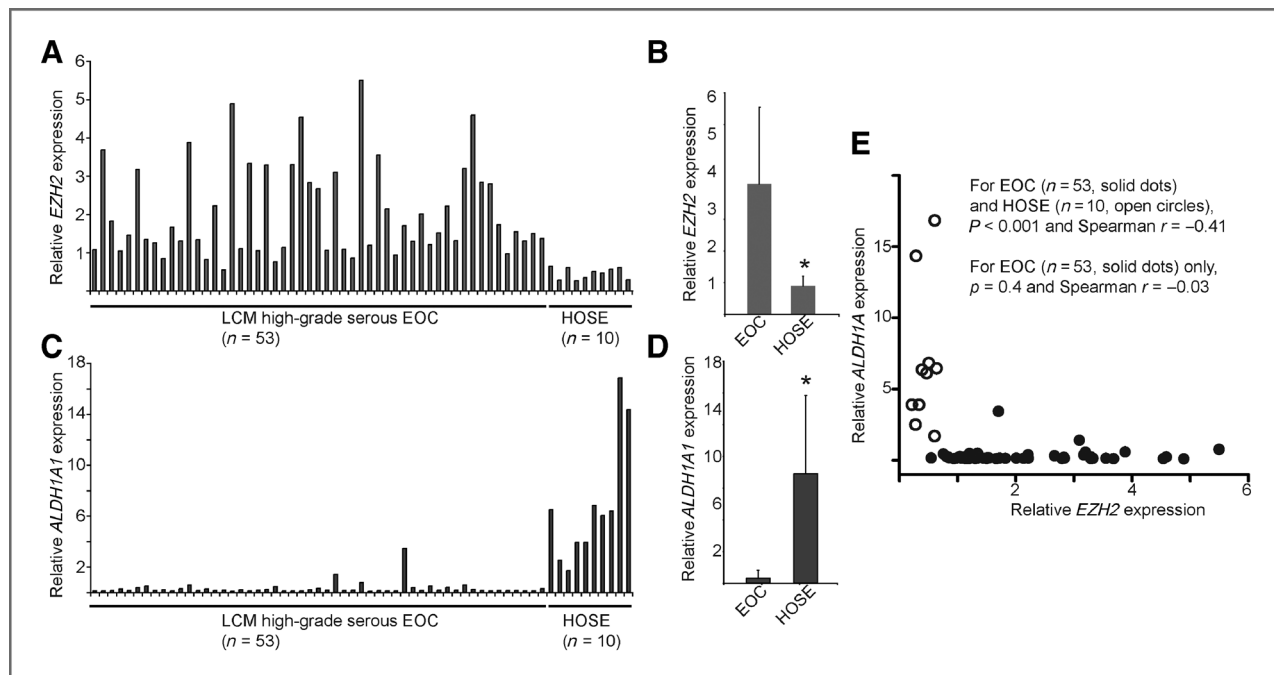
in ovarian tumor-associated endothelial cells (5). To limit the confounding effects of EOC-associated stromal cells (including EOC-associated endothelial cells), we chose to analyze the correlation between expression of EZH2 and its target genes in specimens from LCM high-grade serous subtype EOC, which accounts for the majority of EOC-associated mortalities (27).

EZH2 is expressed at higher levels in human EOC cells than in normal HOSE cells (4). Therefore, we hypothesized that EZH2 target genes that are silenced by EZH2 would be expressed at lower levels in human EOC cells. Toward testing this hypothesis, we examined the expression of EZH2 and the 3 validated EZH2 target genes in a published microarray database, which compares the gene expression profile in 53 cases of LCM high-grade serous EOCs and 10 individual isolations of primary HOSE cells (28). Consistent with our previous report (4), EZH2 was expressed at significantly higher levels in human EOCs than in primary HOSE cells ( $P < 0.001$ ; Fig. 4A and B). Notably, the EZH2 target gene *ALDH1A1* was expressed at significantly lower levels in human EOCs than in normal HOSE cells ( $P < 0.001$ ; Fig. 4C and D). Indeed, there was a negative correlation between expression of EZH2 and its target gene *ALDH1A1* in a Spearman statistical analysis of the cases including both EOC and primary HOSE cells ( $P < 0.001$  and  $r = -0.41$ ; Fig. 4E, including both open circles and solid dots). However, the coefficient Spearman  $r$  is 0.41. This result indicates that other factors may also play a role in the expression relation. Consistently, there is evidence to suggest that Notch signaling also regulates *ALDH1A1* expression (29). In addition, the correlation between expression of EZH2 and *ALDH1A1* is not significant among EOC cases ( $P = 0.81$ ; Fig. 4E, solid dots only). This may be due to the fact the *ALDH1A1* is expressed at very low levels in the vast majority of EOC cases, and thus, the variation in expression may simply be a reflection of experimental variations.

Comparing EOCs with normal HOSE cells, *ALDH1A1* showed a high fold change in expression (>8-fold), whereas *SSTR1* (~3.5-fold) or *DACT3* (<1.5-fold) only showed a moderate to minimal fold change in expression (Fig. 4D and Supplementary Fig. S1). It is possible that EZH2 is the



**Figure 3.** Validation of occupancy of the genomic loci of the selected EZH2 target genes by EZH2 and H3K27Me3 in SKOV3 human EOC cells using ChIP analysis. Control and shEZH2#2-expressing SKOV3 cells were subjected to ChIP analysis using antibodies specific to EZH2 or H3K27Me3, respectively. An isotype-matched IgG was used as a negative control, and an antibody specific to core histone H3 was used as a positive control. After ChIP analysis, the genomic loci of the indicated genes were subjected to PCR amplification using primers detailed in Materials and Methods. Please see Fig. 1A for shEZH2 knockdown efficacy. Shown are representative images of 3 independent experiments. Note that for H3K27Me3 ChIP, a low number of PCR cycles were used compared with EZH2 or histone H3 ChIP to avoid oversaturation of signals.



**Figure 4.** EZH2 targets ALDH1A1 in high-grade serous subtype EOC. A, relative expression of *EZH2* mRNA in 53 cases of LCM high-grade serous EOC and 10 individual isolations of normal HOSE cells. B, quantitation of (A). \*,  $P < 0.001$  compared with high-grade serous EOC. C, same as (A), but for relative expression of *ALDH1A1* mRNA. D, quantitation of (C). \*,  $P < 0.001$  compared with high-grade serous EOC. E, correlation between expression of *ALDH1A1* and *EZH2* as determined by Spearman statistical analysis using GraphPad Prism version 5.0 software.

major epigenetic regulator of ALDH1A1, whereas additional epigenetic silencing mechanisms may contribute to suppression of the other 2 validated EZH2 target genes. In support of this possibility, EZH2 knockdown induced much greater levels of upregulation for ALDH1A1 (up to 22-fold) than the other 2 EZH2 target genes (Fig. 2).

Next, we sought to examine the correlation between expression of EZH2 or ALDH1A1 and survival of patients with high-grade serous EOC. Consistent with our previous report (4), the difference in overall survival between high and low EZH2 expression in patients with high-grade serous EOC was not significant ( $P = 0.1684$ ; Supplementary Fig. S2). In addition, the difference in survival between low ALDH1A1 expression group and high ALDH1A1 group was not significant ( $P = 0.7789$ ; Supplementary Fig. S2). In contrast to our results, high ALDH1A has previously been reported to be associated with poor prognosis in patients with EOC (10, 30). The basis for this discrepancy remains to be determined. However, the discrepancy could be due to different methods that were used in this study (microarray) versus the other 2 studies (immunohistochemical staining).

ALDH1A1 has been reported as a marker of cancer stem cells in certain types of cancers including breast and ovarian (10, 29, 31–33). Likewise, EZH2 plays an important role in stem cell biology (34). Our data suggest that EZH2 directly regulates the levels of ALDH1A1 in EOC cells, implying that EZH2 may regulate the EOC stem cell population by controlling the levels of ALDH1A1 expression. Similarly, it has been shown that EZH2 directly regulates the epigenetic status of Nanog, an important factor for both embryonic

stem cell and induced pluripotent stem cells, to balance the equilibrium between self-renewal and differentiation of stem cells (35). Similarly, other putative markers of cancer stem cells [such as CD133 (ref. 36) and TACSTD2 (ref. 37)] have been also reported to be hypermethylated in cancerous cells. While the expression in stem cells will be masked by the vastly more abundant non-stem cell population in our or any similar analysis, it is nevertheless intriguing that differential expression of ALDH1A1 has been described as a marker of stemness in cancer, including in EOC (10, 33). Together, our data suggest that EZH2 may regulate the EOC stem cell population by controlling the levels of ALDH1A1 expression. Our future work will test this hypothesis.

In the present study, using a combination of global gene expression profiling and genome-wide ChIP-seq analysis, we identified a list of 60 EZH2 direct target genes, whose expression was upregulated more than 1.5-fold upon EZH2 knockdown in human EOC cells. These genes include 56 novel putative EZH2 target genes and 4 known EZH2 target genes. We validated 3 selected EZH2 target genes that are implicated in regulating cancer stem cells, cell proliferation, apoptosis, and cell invasion. We showed that ALDH1A1, a putative marker of EOC stem cells (11), was expressed at lower levels in high-grade serous EOCs than in normal HOSE cells, and there was a negative correlation between expression of EZH2 and expression of ALDH1A1. Further studies are warranted to mine the data presented here as well as functional characterization of the identified EZH2 target genes. These studies should provide important insights

into the biology of EOC development and the identification of potential candidate targets for prevention and intervention of EOC.

#### Disclosure of Potential Conflicts of Interest

C.L. Creasy and P.J. Tummino are employees and stockholders of GlaxoSmithKline Pharmaceuticals. No potential conflicts of interests were disclosed by other authors.

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