Enhancer of Zeste Homolog 2 Promotes the Proliferation and Invasion of Epithelial Ovarian Cancer Cells

Hua Li, Qi Cai, Andrew K. Godwin, and Rugang Zhang

Abstract

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb repressive complex 2 (PRC2) that includes noncatalytic subunits suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). When present in PRC2, EZH2 catalyzes trimethylation on lysine 27 residue of histone H3 (H3K27Me3), resulting in epigenetic silencing of gene expression. Here, we investigated the expression and function of EZH2 in epithelial ovarian cancer (EOC). When compared with primary human ovarian surface epithelial (pHOSE) cells, EZH2, SUZ12, and EED were expressed at higher levels in all 8 human EOC cell lines tested. Consistently, H3K27Me3 was also overexpressed in human EOC cell lines compared with pHOSE cells. EZH2 was significantly overexpressed in primary human EOCs (n = 134) when compared with normal ovarian surface epithelium (n = 46; P < 0.001). EZH2 expression positively correlated with expression of Ki67 (P < 0.001; a marker of cell proliferation) and tumor grade (P = 0.034) but not tumor stage (P = 0.908) in EOC. There was no correlation of EZH2 expression with overall (P = 0.3) or disease-free survival (P = 0.2) in high-grade serous histotype EOC patients (n = 98). Knockdown of EZH2 expression reduced the level of H3K27Me3 and suppressed the growth of human EOC cells both in vitro and in vivo in xenograft models. EZH2 knockdown induced apoptosis of human EOC cells. Finally, we showed that EZH2 knockdown suppressed the invasion of human EOC cells. Together, these data demonstrate that EZH2 is frequently overexpressed in human EOC cells and its overexpression promotes the proliferation and invasion of human EOC cells, suggesting that EZH2 is a potential target for developing EOC therapeutics.

Introduction

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of polycomb repressive complex 2 (PRC2; refs. 1–4). In addition to EZH2, PRC2 also contains the noncatalytic subunits embryonic ectoderm development (EED) and suppressor of zeste 12 (SUZ12; ref. 5). PRC2 plays an important role in epigenetic gene silencing via methylation of lysine 27 residue of histone H3 (H3K27) and can add up to 3 methyl groups to the lysine side chain. EZH2 lacks enzyme activity on its own, and has to complex with EED and SUZ12 to attain robust histone methyltransferase activity (5, 6). The trimethylated form of H3K27 (H3K27Me3) is thought to be the main form that confers transcriptional silencing function (7–10).

EZH2 is overexpressed in several types of cancers (11–15) and is correlated with aggressiveness and poor prognosis in breast and prostate cancers (11–13). In breast epithelial cells, EZH2 overexpression causes anchorage-independent growth and increases cell invasiveness in vitro (11). In prostate cancer cells, inhibition of EZH2 blocked the growth of prostate cancer cells (13, 15). In addition, SUZ12 is also upregulated in certain types of cancer, including colon, breast, and liver (16–18).

More than 85% of ovarian cancers are of epithelial origin (19). Epithelial ovarian cancers (EOC) are classified into distinct histologic subtypes including serous, mucinous, endometrioid, and clear cell (19). The most common histology of EOC is serous (50%–60% of all EOCs), approximately, 75% of which is high-grade and 25% is low-grade (20–22). Less common histologies include endometrioid (25%), clear cell (4%), and mucinous (4%; ref. 20, 21). Recently, an alternative classification has gained traction, in which EOC is broadly divided into 2 types (22). Type I EOC includes endometrioid, mucinous, low-grade serous, and clear cell carcinomas, and type II EOC includes high-grade serous carcinomas (22). EOC remains the most
lethal gynecologic malignancy in the Western world (19). Thus, there is an urgent need to identify new targets for developing novel therapeutics for EOC. Although EZH2 is overexpressed in tumor-associated endothelial cells in invasive EOC (23) and regulates tumor angiogenesis in EOC (24), its role in pathogenesis of EOC remains poorly understood. Here, we examined the expression of the subunits of PRC2 and H3K27Me3 in human EOC cell lines. In addition, we determined EZH2 expression in primary human EOCs of different histologic subtypes by immunohistochemistry (IHC). Further, we investigated the effects of EZH2 knockdown by short hairpin RNA (shRNA) on H3K27Me3 expression, cell growth, and invasion of human EOC cells.

Material and Methods

Cell culture

Primary human ovarian surface epithelial (pHOSE) cells were isolated and cultured as previously described (25). The protocol was approved by Fox Chase Cancer Center (FCCC) institutional review board. Human EOC cell lines A1847, A2780, OVCA3, OVCA5, OVCA10, PEO1, SKOV3, and UPN289 were kindly provided by Drs. Thomas Hamilton and Steve Williams at FCCC and were maintained in 1640 medium, supplemented with 10% FBS, 2 mmol/L of l-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL).

shRNA, lentivirus packaging, and infection

The sense sequences of 2 individual shRNA EZH2 are: 5′-CCAACACAACTCATCCCATTA-3′ and 5′-CGGAAATCTTAAACCAAGAAT-3′, respectively. Lentivirus packaging was performed using virapower system (Invitrogen) according to manufacturer’s instruction. PEO1 and SKOV3 at 40% to 50% confluence were infected with lentivirus expressing shRNA to the human EZH2 gene or vector control. The infected cells were drug-selected with 1 μg/mL (for PEO1) or 3 μg/mL (for SKOV3) of puromycin, respectively.

Human ovarian tissue microarrays

Tissue microarrays, including core samples from 134 primary human EOCs and 46 cases of normal ovary tissues were obtained from FCCC Biosample Repository Core Facility. Use of these human specimens was approved by the Institutional Review Board.

Immunohistochemical staining and scoring

The expression of EZH2 and Ki67 proteins was detected using avidin–biotin–peroxidase methods. Briefly, tissue sections were subjected to antigen retrieval by steaming in 0.01 mol/L of sodium citrate buffer (pH 6.0) for 30 minutes. After quenching endogenous peroxidase activity with 3% hydrogen peroxide and blocking nonspecific protein binding with 1% bovine serum albumin, sections were incubated overnight with primary monoclonal antibody (anti-EZH2: Millipore, 1:100; anti-Ki67: DAKO, 1:100) at 4°C, followed by biotinylated goat anti-mouse IgG (DAKO, 1:400) for 1 hour, detecting the antibody complexes with the labeled streptavidin–biotin system (DAKO), and visualizing them with the chromogen 3,3′-diaminobenzidine. Sections were lightly counterstained with hematoxylin. Tissues in which nuclei were stained for EZH2 or Ki67 protein were considered positive. Two 1-mm cores were examined in each specimen on the tissue microarray and cells were counted in at least 5 high-power fields, with approximately 200 cells analyzed per high-power field.

FACS, immunofluorescence staining, and Western blot analysis

FACS and indirect immunofluorescence (IF) staining were performed as described previously (26–28). The following antibodies were used for IF: rabbit anti-H3K27Me3 (Cell Signaling, 1:1,000), and rabbit anti-H3K9Me3 (Abcam, 1:500). The antibodies used for Western blotting were from indicated suppliers: mouse anti-EZH2 (Millipore; 1:2,500), rabbit anti-H3K27Me3 (Cell signaling, 1:1,000), rabbit anti-H3K9Me3 (Abcam, 1:2,000), mouse anti-histone H3 (Millipore, 1:10,000), mouse anti-GAPDH (Millipore, 1:10,000), rabbit anti-PARP p85 fragment (Promega, 1:1,000), rabbit anti-cleaved caspase 3 (Cell Signaling, 1:1,000), and rabbit anti-cleaved Lamin A (Cell signaling, 1:1,000).

Soft agar colony formation assay

A total of 1 × 10⁴ cells per well were inoculated in a 6-well plate in 1.5 mL of RPMI 1640 medium supplemented with 10% FBS and 0.35% agar on a base layer of 1.5 mL of the same medium containing 0.6% agar. Three weeks after plating, the cells were stained with 1% crystal violet (Sigma) in PBS to visualize the colonies. Number of colonies that were larger than 50 μm (approximately 100 cells) in diameter in each well was counted.

Matrigel invasion assay

BD BioCoat Matrigel Invasion Chamber was used to measure cell invasion according to manufacturer’s instruction. Cells (1 × 10⁵ cells per well) suspended in 0.5 mL RPMI 1640 medium were added to the upper compartment of 24-well matrigel-coated or noncoated 8 μm membrane, and RPMI 1640 medium supplemented with 10% FBS was applied to the lower compartment. After incubating 22 hours at 37°C, 5% CO₂, the cells were fixed with 4% formaldehyde and stained with 1% crystal violet in PBS. The number of cells that migrated across control membrane or invaded through Matrigel-coated membrane was determined in 9 fields across the center and the periphery of the membrane.

Annexin V staining for detecting apoptotic cells

Phosphatidylserine externalization was detected using an Annexin V staining kit (Millipore) following manufacturer’s instruction. Annexin V positive cells were detected by
In vivo tumorigenicity assay

A total of \(5 \times 10^6\) cells in PBS (pH 7.3) per mouse were injected subcutaneously into the flank of 6-week-old female nude athymic mice. The mice were sacrificed 4 weeks post-inoculation. Width and length of tumor size were measured and the tumor volume (mm\(^3\)) was calculated using the following formula: tumor volume (in mm\(^3\)) = length \times width \times \frac{1}{2}.

Statistical analysis

Quantitative data were expressed as mean ± SD, unless otherwise stated. Analysis of variance (ANOVA) with Student's \(t\) test was used to identify significant differences in multiple comparisons. The \(\chi^2\) test was used to analyze the relationship between categorical variables. Overall survival was defined as the time elapsed from the date of diagnosis and the date of death from any cause or the date of last follow-up. Disease-free survival was defined as the time elapsed from the date of surgery and the date of the first recurrence. Kaplan–Meier survival plots were generated and comparisons made using the log-rank statistic. For all statistical analyses, the level of significance was set at 0.05.

Results

The catalytic and noncatalytic subunits of PRC2 complex and H3K27Me3 are expressed at higher levels in human EOC cell lines compared with pHOSE cells

Expression of EZH2, EED, and SUZ12 was examined by Western blotting in cultures of pHOSE cells isolated from 5 different individuals and 8 human EOC cell lines. When compared with pHOSE cells, EZH2, EED, and SUZ12 were expressed at higher levels in all human EOC cell lines tested (Fig. 1A). Consistently, the levels of H3K27Me3, the product of EZH2 histone H3 lysine 27 methyltransferase activity, was also increased in human EOC cell lines compared with pHOSE cells (Fig. 1A). On the basis of these results, we conclude that the catalytic and noncatalytic subunits of PRC2 and H3K27Me3 are expressed at higher levels in human EOC cell lines compared with pHOSE cells.

EZH2 is overexpressed in primary human EOCs and its expression positively correlates with expression of Ki67, a cell proliferation marker

We next sought to examine the expression of EZH2, the catalytic subunit of PRC complex, in 134 primary human EOCs and 46 normal ovarian surface epithelium by IHC staining (Table 1; Fig. 1B; Supplementary Fig. S1). The specificity of the EZH2 antibody used for IHC staining was confirmed by the following (Supplementary Fig. S1A and B). First, a single band at right molecule weight (~95KD) was obtained in Western blotting of human EOC cell line SKOV3 using the EZH2 antibody, and this band was absent after expression of shRNA to the human EZH2 gene (shEZH2) that effectively knocked down EZH2 mRNA expression (Supplementary Fig. S1A and data not shown). In addition, EZH2 staining signal was lost when primary anti-EZH2 was replaced with an isotype-matched IgG control (Supplementary Fig. S1B). Importantly, the nuclei of human EOC cells were strongly stained by the anti-EZH2 antibody (Fig. 1B; Supplementary Fig. S1C). By contrast, ovarian surface epithelial cells were negative for EZH2 staining (Fig. 1B).

We scored expression of EZH2 as low (H-score \(\leq 100\)) or high (H-score > 100) based on the histochemical score (29, 30), which considers both the intensity of staining and the percentage of positively stained cells. EZH2 expression in the surface epithelium of all 46 normal ovaries was scored as low (in fact, negative EZH2 staining). EZH2 was scored as low in 34% (46 of 134) and high in 66% (88 of 134) of primary EOCs tested, respectively. When compared with normal ovarian surface epithelium, EZH2 was expressed at significantly higher levels in primary human EOCs (\(P < 0.001\)). Because EZH2 has been implicated in promoting cell proliferation (12), we stained the same set of primary human EOC specimens with Ki67, a cell proliferation marker, and compared the expression of EZH2 and Ki67 expression in consecutive sections. There
was a significant correlation between EZH2 expression and Ki67 expression ($P < 0.001$; Table 1). Together, we conclude that EZH2 is significantly overexpressed in primary human EOCs compared with normal ovarian surface epithelium and its expression correlates with a high proliferation index revealed by Ki67 staining.

**EZH2 expression is positively correlated with tumor grade but not tumor stage, or overall or disease-free survival**

We next sought to determine the correlation between EZH2 expression and clinical and pathologic features of human EOCs. There was a significantly positive correlation between EZH2 expression and tumor grade ($P = 0.034$; Table 1). However, EZH2 expression was not associated with tumor stage ($P = 0.908$; Table 1). Next, we sought to determine whether EZH2 expression correlates with prognosis of type II high-grade serous histotype EOC patients for which long-term follow-up data were available ($n = 98$). The difference in overall ($P = 0.3$) or disease-free ($P = 0.2$) survival between low EZH2 expression group ($n = 35$) and high EZH2 expression group ($n = 63$) was not significant (Fig. 2).

**EZH2 knockdown inhibits the growth of human EOC cells in vitro and in vivo**

Because EZH2 expression positively correlates with Ki67 expression (Table 1), we sought to determine the effects of EZH2 knockdown on proliferation of human EOC cells. To
knockdown EZH2 expression in SKOV3 cells, we developed 2 individual lentivirus encoded shEZH2. The knockdown efficacy of shEZH2 in SKOV3 cells was confirmed by Western blotting (Fig. 3A). Consistently, the level of H3K27Me3 was significantly reduced by shEZH2 expression in SKOV3 cells as determined by both Western blotting and IF staining (Fig. 3B and C). As a negative control, shEZH2 expression has no effects on the level of trimethylated lysine 9 histone H3 (H3K9Me3) that is generated by histone methyltransferase Suv39H (ref. 31; Fig. 3B and C). Compared with controls, EZH2 knockdown significantly reduced both anchorage-dependent and -independent growth in soft agar in SKOV3 cells (Fig. 3D and E). The degree of growth inhibition by shEZH2 correlated with the level of EZH2 knockdown in SKOV3 cells by 2 individual shEZH2 (Fig. 3), suggesting that the observed growth inhibition was not because of off-target effects. In addition, EZH2 knockdown in PEO1 cells has same effects on the expression of H3K27Me3 and also suppressed both anchorage-dependent and -independent cell growth (Supplementary Fig. S2), suggesting that the observed growth inhibition is not cell line specific.

We next sought to determine the effects of EZH2 knockdown on the growth of SKOV3 cells in vivo in immunocompromised nude mice. Control and shEZH2 expressing SKOV3 cells were injected subcutaneously into nude mice with 5 × 10⁶ cells per mice and 5 mice in each group. Four weeks after injection, the sizes of xenografted tumors were compared between control and shEZH2 expressing cells (Fig. 4A and B). EZH2 knockdown by shEZH2 in the xenograft tumors was confirmed by IHC staining (Fig. 4C). shEZH2 expression significantly inhibited the growth of xenografted SKOV3 cells (Fig. 4A and B).

**EZH2 knockdown inhibits the invasion of human EOC cells**

EZH2 has been implicated in regulating cell invasion in several types of cancer cells (11, 15, 32, 33). Thus, we sought to determine the effects of EZH2 knockdown on invasion of human EOC cells. Toward this goal, control and shEZH2 expressing SKOV3 cells were tested for their ability to migrate through uncoated control membrane or invade through matrigel-coated membrane. Compared with controls, EZH2 knockdown significantly inhibited the invasion of SKOV3 cells as revealed by a decreased invasion index that is calculated as the ratio between the number of cells invaded through matrigel-coated membrane and the number of cells migrated through control membrane (Fig. 5). Inhibition of invasion was observed by 2 individual shEZH2 in SKOV3 cells (Fig. 5). In addition, the degree of invasion inhibition correlated with the degree of EZH2 knockdown (Figs. 3 and 5), suggesting that this is not because of off-targets effects. On the basis of these results, we conclude that EZH2 knockdown inhibits the invasion of human EOC cells.

**EZH2 knockdown triggers apoptosis in human EOC cells**

We next sought to determine the mechanisms by which EZH2 knockdown inhibits the growth of human EOC cells. DNA content analysis determined by FACS showed that there was no statistical difference in cell-cycle distribution between control and shEZH2 expressing cells (Supplementary Fig. S3). We next examined the markers of apoptosis in control and shEZH2 expressing SKOV3 cells. When compared with controls, markers of apoptosis were significantly induced by shEZH2 expression (Fig. 6). Those apoptotic markers include increased percentage of cells at sub-G1 phase as measured by FACS analysis (Fig. 6A), increased percentage of Annexin V positively stained cells as measured by Guava Nexin assay (Fig. 6B), upregulation of cleaved Lamin A, PARP p85, and caspase 3 (Fig. 6C; ref. 34). Together, we conclude that EZH2 knockdown induces apoptosis of human EOC cells.

**Discussion**

Consistent with our findings, EZH2 mRNA expression was upregulated 2-fold or more in more than 80% of high-grade serous human EOC specimens in the newly released
the Cancer Genomics Atlas (TCGA) serous ovarian cystoadenocarcinoma gene expression database (http://cancergenome.nih.gov/). EZH2 gene is located at chromosome 7q36.1. Gene amplification contributes to EZH2 overexpression in several types of cancer (14, 35). However, TCGA gene copy-number analysis indicates that EZH2 gene amplification occurs only in a very small percentage of EOCs (<10% specimens show >4 copy of EZH2 gene; http://cancergenome.nih.gov/), suggesting that gene amplification is not a major mechanism that leads to EZH2 upregulation in human EOCs. EZH2 is an E2F target gene (35). A very recent study showed that VEGF stimulates EZH2 expression in human EOC cells via E2F family members, E2F1 and E2F3 (24). However, VEGF only stimulates the expression of EZH2 mRNA up to 3-fold (24), which is far below the level of increase in EZH2 mRNA or protein in human EOC cells compared with cultured pHOSE cells (Fig. 1 and data not shown), suggesting additional mechanisms contribute to EZH2 upregulation in human EOC cells. In the future, we will elucidate
additional mechanisms that contribute to EZH2 upregulation in human EOCs.

EZH2 has been demonstrated as a prognostic marker for breast and prostate cancers and positively correlates with disease-free survival and overall survival in those patient populations (11–13). In addition, EZH2 overexpression correlates with more advanced disease stages of breast and prostate cancers (11, 13). However, EZH2 expression was not a prognostic marker in other types of cancers including renal clear cell carcinoma and hepatocellular carcinomas (33, 36). We showed that there was no significant correlation between EZH2 expression and disease-free or overall survival in the tested high-grade serous histotype EOC patients (Fig. 2). Consistent with this, although EZH2 correlates with Ki67 expression (Table 1), EZH2 expression was not a prognostic indicator for either overall or disease-free survival in the tested high-grade serous histotype EOC patients (Fig. 2).

Interestingly, when compared with normal ovarian surface epithelium, EZH2 expression is significantly upregulated (up to 23-fold) in ovarian epithelial inclusion cysts (44), which are thought to be the precursor lesion of a subset of EOC (45). This suggests that EZH2 overexpression is an early event during EOC development. Although ovarian surface epithelium is thought to be the cell origin of EOC (46), there are still several histopathology-based theories that differ in their explanations about the origins of EOC (46–48). Notably, recent evidence suggests that a proportion of high-grade serous EOC may arise from distal fallopian tube

**Figure 4.** EZH2 knockdown suppresses the growth of SKOV3 cells in vivo in immunocompromised mice. A, 5 × 10⁶ control or shEZH2 expressing SKOV3 cells were injected subcutaneously into immunocompromised nude mice (n = 5). Four weeks postinjection, tumors were removed from mice, and the size of tumors was measured. Mean of tumor sizes with SEM. B, xenografted tumors formed by control or shEZH2 expressing SKOV3 cells were sectioned and stained for EZH2 expression. Bar = 50 μm.

**Figure 5.** EZH2 knockdown suppresses the invasion of SKOV3 cells. A, equal number of control and shEZH2 expressing SKOV3 cells were assayed for migration through uncoated control membrane or invasion through matrigel-coated membrane. The cells migrated through control membrane or invaded through matrigel-coated membrane were stained with 1% crystal violet in PBS. B, quantitation of A. Relative percentage of shEZH2 expressing cells migrated through control membrane or invaded through matrigel-coated membrane compared with controls was indicated. Mean of 3 independent experiments with SD. *, P < 0.05. C, invasion index of shEZH2 expressing SKOV3 cells compared with controls. Invasion index is the ratio between cells invaded through matrigel-coated membrane and cells migrated through control membrane. Mean of 3 independent experiments with SD. *, P < 0.05.
Therefore, it will be interesting to examine EZH2 expression in normal fallopian tube epithelium. Multiple genes have been implicated in EZH2 inhibition–induced apoptosis. For example, FBXO32 contributes to EZH2 inhibition induced–apoptosis in breast cancer cells (45), and Bim expression has been demonstrated to mediate EZH2 inhibition–induced apoptosis in prostate cancer cells (49). Likewise, E-cadherin, DAB2IP, and SLIT2 have all been implicated in mediating increased invasiveness conferred by high levels of EZH2 expression (32, 50–52).

Further studies are warranted to delineate the molecular mechanisms by which EZH2 overexpression promotes proliferation and invasion of human EOC cells. In summary, the data reported here show that EZH2 is overexpressed in approximately 66% of primary human EOCs and its overexpression correlates with a high proliferation index and tumor grade in EOCs. Knockdown of EZH2 inhibits the growth of human EOC cells in vitro and in vivo. EZH2 knockdown induces apoptosis of human EOC cells. In addition, EZH2 knockdown suppresses the invasion of human EOC cells. Further, inhibition of the growth and invasion of human EOC cells induced by EZH2 knockdown correlates with a decrease in the levels of H3K27Me3, suggesting that EZH2 histone methyltransferase activity is critical for its function in human EOC cells. Together, our data imply that EZH2 is a potential target for developing epigenetic modifying therapeutics for EOC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were declared.

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