Polycomb Repressor Complex-2 Is a Novel Target for Mesothelioma Therapy

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Abstract

Purpose: Polycomb group (PcG) proteins are critical epigenetic mediators of stem cell pluripotency, which have been implicated in the pathogenesis of human cancers. This study was undertaken to examine the frequency and clinical relevance of PcG protein expression in malignant pleural mesotheliomas (MPM).

Experimental Design: Microarray, quantitative reverse transcriptase PCR (qRT-PCR), immunoblot, and immunohistochemistry techniques were used to examine PcG protein expression in cultured MPM, mesothelioma specimens, and normal mesothelial cells. Lentiviral short hairpin RNA techniques were used to inhibit \( \text{EZH2} \) and \( \text{EED} \) expression in MPM cells. Proliferation, migration, clonogenicity, and tumorigenicity of MPM cells either exhibiting knockdown of \( \text{EZH2} \) or \( \text{EED} \), or exposed to 3-deazaneplanocin A (DZNep), and respective controls were assessed by cell count, scratch and soft agar assays, and murine xenograft experiments. Microarray and qRT-PCR techniques were used to examine gene expression profiles mediated by knockdown of \( \text{EZH2} \) or \( \text{EED} \), or DZNep.

Results: \( \text{EZH2} \) and \( \text{EED} \), which encode components of polycomb repressor complex-2 (PRC-2), were overexpressed in MPM lines relative to normal mesothelial cells. \( \text{EZH2} \) was overexpressed in approximately 85% of MPMs compared with normal pleura, correlating with diminished patient survival. Overexpression of \( \text{EZH2} \) coincided with decreased levels of miR-101 and miR-26a. Knockdown of \( \text{EZH2} \) or \( \text{EED} \), or DZNep treatment, decreased global H3K27Me3 levels, and significantly inhibited proliferation, migration, clonogenicity, and tumorigenicity of MPM cells. Common as well as differential gene expression profiles were observed following knockdown of PRC-2 members or DZNep treatment.

Conclusions: Pharmacologic inhibition of PRC-2 expression/activity is a novel strategy for mesothelioma therapy. Clin Cancer Res; 18(1); 77–90. ©2011 AACR.

Introduction

Malignant pleural mesotheliomas (MPM) are highly lethal neoplasms attributable to asbestos exposure, as well as ill-defined environmental and genetic factors (1). Because of industrialization and long latency associated with these malignancies, the global incidence of MPM continues to increase (2). Currently, results of conventional treatments for MPM are far from optimal; median survivals of patients undergoing aggressive multimodality therapy for MPM range from 14 to 28 months, depending on tumor histology and stage, extent of surgical resection, and response to chemotherapy (3).

During recent years, considerable insight has been achieved regarding the molecular pathogenesis of MPM. These neoplasms exhibit significant, recurrent karyotypic abnormalities (4, 5), perturbed microRNA (miRNA) expression (6, 7), loss of tumor suppressor genes (4), and upregulation of oncogene signaling (5), resulting in cell-cycle dysregulation, resistance to apoptosis (8), and enhanced invasion potential of mesothelioma cells (9). Epigenomic alterations have also been implicated in the pathogenesis of MPM. For example, changes in chromosomal copy number, as well as miRNA expression, correlate with global and site-specific alterations in DNA methylation in these neoplasms (7, 10, 11). In several studies, DNA methylation signatures in MPM seem to coincide with exposure to specific carcinogens (12, 13). In addition, MPM exhibit a global increase in
Polycomb group (PcG) proteins have been implicated in mediating stem cell pluripotency and aggressive phenotype of a variety of human cancers. In this study, we sought to evaluate the frequency and clinical relevance of PcG protein expression in pleural mesotheliomas. Our analysis revealed that EZH2, a core component of polycomb repressor complex-2 (PRC-2), is overexpressed in the majority of pleural mesotheliomas, correlating with decreased patient survival. Inhibition of PRC-2 expression by knockdown or pharmacologic techniques significantly inhibited proliferation, migration, clonogenicity, and tumorigenicity of pleural mesothelioma cells. Collectively, these data show that aberrant PcG protein expression contributes to the pathogenesis of mesotheliomas and suggest that targeting PRC-2 expression/activity may be a novel strategy for the treatment and possible prevention of these neoplasms.

Materials and Methods

Cell lines

H28, H2052, and H2452 MPM cell lines were obtained from American Type Culture Collection and cultured in RPMI plus 10% fetal calf serum with Pen/Strep (complete media). LP3 and LP9 normal mesothelial cells were obtained from the Coriell Institute for Medical Research (Camden, NJ) and cultured according to vendor instructions. NCI-SB-NMES1-1 and 2 were isolated from histologically normal mesothelia, cultured and described as such (16). NCI-SB-NMES1-4 were established from primary MPM and cultured in complete RPMI media. These cell lines have been validated by HLA typing and molecular phenotyping relative to the respective primary tumors. All tissues for cell lines or gene expression analysis were procured in accordance with Institutional Review Board (IRB)-approved protocols.

RNA isolation and microarray analysis

Total RNA was isolated from cell lines or snap-frozen surgical specimens using the RNeasy minikit (Qiagen). Normal pleural RNA from individual patients without MPM was obtained from ProteoGenex. Gene expression profiles were analyzed using Gene-Chip Human Genome U133 2.0 plus Arrays (Affymetrix) or Illumina Arrays according to vendor instructions; higher order analysis was carried out using Gene Sprint and Ingenuity Pathway software.

Real-time quantitative RT-PCR

RNA was subjected to reverse transcription using a BioRad RT kit, and gene expression was analyzed using the TaqMan real-time quantitative reverse transcriptase PCR (qRT-PCR) kit and primers/probes listed in Supplementary Table S1.

MicroRNA isolation and quantitation

qRT-PCR analysis was used to determine the relative expression levels of miR-101, miR-26a, miR-26b, and RNU44 in MPM specimens using previously described techniques (17). Briefly, miRNA was isolated using TRIzol reagent (Invitrogen) and the RT2 qPCR-Grade miRNA Isolation Kit (Qiagen). Equal amounts of miRNA were converted into cDNA using miR-101, miR-26a, miR-26b, and RNU44 RT primers (Applied Biosystems), according to the manufacturer’s protocol. Quantitative PCR was done using primers and materials from Applied Biosystems. The C\textsubscript{T} values were used to calculate the relative fold difference in miRNA levels. All experiments were done using biological triplicates. The data were normalized to RNU44 expression levels.

Generation of stable cells expressing shRNA constructs

MPM cell lines were transfected with commercially validated short hairpin RNAs (shRNA) targeting EZH2, or EED, or sham sequences (Sigma) according to the manufacturer’s instructions. Cell lines were selected with puromycin and knockdown confirmed by immunoblot analysis.

Immunohistochemistry of primary tumor samples

Formalin-fixed, paraffin-embedded tissue blocks from primary MPM specimens and unmatched normal pleura from autopsy specimens resected on IRB-approved protocols at the National Cancer Institute (NCI), as well as tissue microarrays (TMA) containing normal mesothelia, MPM, and peritoneal mesotheliomas (PM; US Biomax) were stained with mouse anti-EZH2 (BD Biosciences) at a 1:50 dilution following heat-induced epitope retrieval in a citrate buffer solution. Detection was done using the Ventana Nexus automated immunostainer (Ventana Medical Systems) with a diaminobenzidine chromogen and hematoxylin counterstain. Appropriate tissue controls were included in each run. Hematoxylin and eosin stains were done for morphologic assessment. The immunostains were scored by a board-certified pathologist (HM) in a blinded manner, with extent of staining being graded from 0 to 4 on the basis of percentage of positive cells: 0—no positive cells, 1—less than 25% positive, 2—26% to 50% positive, 3—51% to 75% positive, and 4—76% to 100% positive.

Immunoblotting

Submitted as Supplementary Materials.

Proliferation assays

A total of 1,500 cells were plated in triplicate in tissue culture treated 96-well plates and allowed to proliferate for 5 days. Cell proliferation was measured on days 1 to 5 using...
the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). Each experiment was conducted 3 times.

**Cell migration assays**

Cell lines were plated in triplicate to near confluent monolayers in 6-well plates and migration assessed as described (18). For 3-deazaneplanocin A (DZNep) experiments, DZNep at indicated concentrations was added at the time of the scratch and then daily until the conclusion of the experiment. Each experiment was conducted 3 times.

**In vitro DZNep treatment**

DZNep was dissolved in water to a stock concentration of 10 mmol/L and subsequently diluted to experimental concentrations in complete RPMI media. Cells were plated in appropriate tissue culture dishes; the following day, media were replaced with fresh complete media or complete media containing DZNep at the appropriate concentrations. Media were changed daily with fresh DZNep for the 3-day treatment.

**Soft agar clonogenicity**

Submitted as Supplementary Materials.

**Murine xenograft experiments**

Submitted as Supplementary Materials.

**Superarray analyses**

Submitted as Supplementary Materials.

**Gene set enrichment analysis**

Submitted as Supplementary Materials.

**Statistical analysis**

SEM is indicated by bars on all figures and was calculated using Microsoft Office Excel 2007. All experiments were done with at a minimum of triplicate samples, and all $P$ values were calculated with 2-tailed $t$ tests, unless otherwise indicated.

**Results**

**Isolation and characterization of NCI cell lines**

NCI-SB-NMES1 and NCI-SB-NMES2 (NMES1 and 2) were isolated from histologically normal pleura from a 78-year-old female smoker and a 36-year-old female non-smoker, respectively, who underwent thoracotomy for early stage non-small cell lung cancer at the NCI. These lines are capable of growth in tissue culture for approximately 20 to 30 passages before senescence. NCI-SB-MES1-4 (MES1-4) cell lines were established from histologically confirmed MPM resected at the NCI. MES1, 2 and 4 were derived from epithelial tumors, whereas MES3 was established from biphasic MPM. No patient underwent neoadjuvant chemotherapy or radiation therapy prior to resection and isolation of tumor cell lines. Demographic data pertaining to the patients from whom these lines were established, as well as molecular phenotypes of the lines are summarized in Supplementary Table S2. The photomicrographic appearances of NMES1 and 2 and MES1-4 are similar to those corresponding to commercially available normal mesothelial cell cultures, LP3 and LP9, and MPM cell lines H28, H2052, and H2452, respectively (Supplementary Fig. S1A and B).

**Overexpression of EZH2 and EED in MPM cells**

Affymetrix U133 2.0 plus microarrays were used to examine global gene expression profiles, and specifically identify genes encoding PcG proteins in MES1-4, H28, H2052, and H2452 MPM cells relative to LP9 and NMES1. Consistent with previous studies pertaining to gene expression analysis of MPM (11), unsupervised hierarchical cluster analysis of triplicate samples for each cell line showed that gene expression profiles in MPM lines were distinctly different than those observed in NMES1 and LP9 cells (Fig. 1A). Interestingly, MPM lines exhibited overexpression of $EZH2$ (also known as $KMT6$) and, to a lesser extent, $EED$ and $SUZ12$, which encode core components of polycomb repressor complex-2 (PRC-2), recently implicated in the pathogenesis of a variety of human cancers (ref. 15; Fig. 1B). qRT-PCR experiments were undertaken to further examine $EZH2$ and $EED$ expression in MPM cells; primers for $EZH2$ recognized both splice variants. This analysis confirmed that $EZH2$ and $EED$ were overexpressed in MPM lines relative to cultured normal mesothelial cells (Fig. 1C). Subsequent qRT-PCR experiments revealed that both $EZH2$ splice variants were coordinately expressed at comparable levels in MPM cells (data not shown). Immunoblot analysis (Fig. 1D) showed that EZH2 protein levels in cultured MPM cells were higher than those observed in normal mesothelial cells. However, EED levels did not seem to be increased in MPM cells. Additional immunoblot experiments revealed a global increase in the PRC-2–mediated repressive chromatin mark, H3K27Me3 in MPM cells (Fig. 1D).

**Analysis of EZH2 and EED expression in primary mesotheliomas**

qRT-PCR experiments were done to examine expression of $EZH2$ and $EED$ in a panel of primary MPMs resected at the NCI. Interestingly, this analysis revealed upregulation of $EZH2$, but not $EED$, in 5 of 6 primary epithelial MPM relative to normal pleura (Fig. 2A). Immunohistochemistry (IHC) experiments were undertaken to further examine EZH2 expression in these 6 specimens as well as 14 additional MPM resected at the NCI. This analysis revealed no detectable EZH2 expression in normal pleura or stroma; a spectrum of EZH2 expression was detected in mesothelioma cells (Fig. 2B). In general, EZH2 levels tended to coincide with mRNA copy numbers, although some variations were noted, suggesting that posttranscriptional mechanisms also contribute to $EZH2$ overexpression in MPM. Furthermore, EZH2 expression coincided with mRNA copy numbers in MES1-4 cells and correlated with tumors from which the
lines were derived; a gradient of EZH2 expression observed in these primary MPM specimens was maintained when the respective cell lines were passaged in vitro (Supplementary Fig. S2). Overall, 90% of the 20 primary MPM (16 epithelial, 3 biphasic, and one sarcomatoid) specimens expressed EZH2 (Fig. 2C). Additional IHC experiments using TMA revealed that approximately 85% of 45 mesotheliomas (28 MPM and 17 PMs) expressed EZH2; no EZH2 expression was observed in 12 normal pleura or peritoneum specimens or stromal elements (Fig. 2D). The patterns of EZH2 expression in PM were comparable with those observed in MPM (Supplementary Fig. S3).

Additional analysis was done to ascertain whether levels of EZH2 protein expression by IHC coincided with tumor burden (T stage) using 20 NCI samples plus 27 of 28 MPMs from the commercial TMA for which T stage was available. The majority of the MPDs on the TMA were epithelial. EZH2 expression was significantly higher in MPM relative to normal pleura (P < 0.0001). Although EZH2 expression tended to increase with disease burden, this trend was not statistically significant (Z = 0.5; Jonckheere–Terpstra test), possibly because of the small number of samples evaluated (Fig. 3A). Additional analysis was undertaken to ascertain whether intratumoral EZH2 expression detected by Illumina array
Figure 2. Analysis of EZH2 and EED expression in primary mesothelioma specimens and normal mesothelia. A, qRT-PCR analysis of EZH2 and EED expression in 6 primary mesothelioma specimens including 4 from which MES1-4 cell lines were derived, compared with normal pleura samples. EZH2 levels were significantly higher in primary mesotheliomas relative to normal pleura (P < 0.05). This phenomenon was not evident following analysis of EED expression in these specimens. B, representative results of immunohistochemical analysis of EZH2 expression in normal pleura and primary MPM. C, immunohistochemical analysis of 20 primary MPM specimens resected at the NCI, including tumors from which MES1–4 cell lines were derived relative to normal pleura. The majority of mesothelioma specimens expressed EZH2; no EZH2 expression was detectable in normal pleura. D, TMA analysis of EZH2 expression in MPM, PM, normal pleura, or peritoneum.
techniques correlated with survival in 39 patients with locally advanced MPM undergoing potentially curative resections at Brigham and Women’s Hospital (De Rienzo and colleagues, submitted); 24 patients had epithelial mesotheliomas, whereas 7 patients had biphasic and 8 patients had sarcomatoid malignancies. Increased
Figure 4. Effects of stable knockdown of EZH2 and EED in cultured mesothelioma cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001. A, immunoblot analysis of EZH2, EED, and H3K27Me3 levels in MPM lines following transduction with shRNA targeting EZH2, EED, or sham sequences. Knockdown of EZH2 (left) or EED (right) diminishes H3K27Me3 levels in these cells. B, left, effects of knockdown of EZH2 and EED on proliferation of MES1 and H28 cells. The effects of EED knockdown seem to be more pronounced in both of these cells. Similar results were observed for MES2 and H2452 cells. Right, effects of knockdown of EZH2 and EED on migration of MES1 and H28 cells. EZH2 and EED knockdown inhibits migration of MES1 and H28 cells. Similar results were observed for MES2 and H2452 cells. C, effects of knockdown of EZH2 and EED on soft agar clonogenicity of MES1 and H2452 cells. EZH2 and, to a greater extent, EED knockdown significantly inhibits soft agar clonogenicity of MPM cells. D, top, effects of EZH2 knockdown on tumorigenicity of MES1 cells in nude mice. Knockdown of EZH2 mediates a modest increase in time to tumor take, and a significant decrease in average tumor volume and tumor mass of these xenografts. Bottom, effects of knockdown of EED on tumorigenicity of MES1 xenografts in nude mice. Knockdown of EED increases time to tumor take and significantly decreases tumor volume and tumor mass. Results depicted are representative of 2 independent experiments for each condition.
expression of either of the EZH2 splice variants in MPM correlated significantly with shorter patient survival (Fig. 3B). The magnitude of EZH2 overexpression did not seem to correlate with histology. The sample size precluded multivariate analysis to determine whether EZH2 expression was an independent prognosticator of survival in MPM patients.

**Association of EZH2 with miR-101 and miR-26 expression in MPM cells and primary MPM specimens**

Recent studies have shown that overexpression of EZH2 in several human cancers coincides with loss of miR-101 or miR-26, which normally target the 3’-untranslated region of EZH2 (19, 20). As such, qPCR experiments were conducted to examine miR-101 and miR-26 expression levels relative to EZH2 expression in primary MPM specimens and normal pleura (Fig. 3C). These experiments showed that miR-101 levels were significantly reduced in MPM cell lines and pleural mesothelioma specimens relative to normal pleura. Furthermore, miR-26a levels were decreased in primary MPM specimens relative to normal pleura. In contrast, levels of miR-26b (which does not target EZH2) in MPM and normal pleura were similar, suggesting that the alterations in miR-101 and miR-26a levels observed in this preliminary analysis were not attributable to a global decrease in miRNA expression (Fig. 3C).

**Effects of EZH2 or EED knockdown in MPM cells**

Additional experiments were carried out to examine whether aberrant PRC-2 activity directly contributes to the malignant phenotype of mesothelioma cells. Briefly, shRNA techniques were used to knockdown EZH2 in cultured MPM cells; the shRNA used for these experiments targeted both splice variants of EZH2. Similar experiments were undertaken to knockdown EED, which although not apparently overexpressed in primary MPM, is critical for maintaining stability of PRC-2 and histone methyltransferase activity of EZH2 (15). Immunoblot analysis confirmed that relative to control sequences, shRNAs depleted EZH2 and EED and decreased global levels of H3K27Me3 in MES1-2, H28, and H2452 (Fig. 4A). Furthermore, knockout of these PRC-2 components resulted in 30% to 50% decreases in proliferation and migration of these 4 MPM lines (Fig. 4B). Interestingly, the effects of EED knockdown on global H3K27Me3 levels, as well as proliferation and migration, seemed to be more pronounced than EZH2 knockdown in MPM cells. Experiments using different shRNAs targeting EZH2 and EED confirmed that knockdown of these PRC-2 components inhibited proliferation of MPM cells, suggesting that the aforementioned results were not due to off-target effects of the shRNAs; the extent of growth inhibition mediated by shRNAs targeting EZH2 or EED seemed to correlate with efficiency of knockdown (Supplementary Fig. S4).

Additional experiments were carried out to ascertain whether knockdown of EZH2 or EED inhibited clonogenicity and tumorigenicity of MPM cells. Knockdown of EZH2 or EED diminished soft agar clonogenicity of MES1 cells by 36% and 66%, respectively, relative to controls. Similarly, knockdown of EZH2 and EED decreased clonogenicity of H2452 cells by 40% and 57%, respectively (Fig. 4C). Subsequent experiments revealed that knockdown of EZH2 or EED modestly delayed tumor take and significantly decreased size and volume of subcutaneous MES1 xenografts in nude mice (Fig. 4D). Consistent with results from immunoblot, proliferation, and migration experiments, the effects of knockdown of EED on clonogenicity and tumorigenicity were more pronounced than those observed following EZH2 knockdown in MPM cells.

**Effects of DZNep in MPM cells**

Additional experiments were carried out to ascertain whether pharmacologic agents in early clinical development could recapitulate the effects of EZH2 or EED knockdown in MPM cells. Our analysis focused on DZNep, an S-adenosylhomocysteine hydrolase inhibitor that has been shown to decrease DNA methylation, deplete PRC-2 components, and mediate cell-cycle arrest and apoptosis in cancer cells (21, 22). Preliminary experiments revealed that DZNep inhibited proliferation of MPM cells, with the effects tending to plateau off at high doses, suggestive of cytostasis rather than cell death (Supplementary Fig. S5A); consistent with these findings, Apo-BrdU analysis revealed that the growth inhibitory effects of DZNep (as well as EZH2 or EED knockdowns) were not associated with increased apoptosis (data not shown). Interestingly, cultured normal mesothelial cells (LP9), which exhibit very low level EZH2 expression, seemed relatively resistant to DZNep (Supplementary Fig. S2 and S5A). The growth inhibitory effects of DZNep did not seem to coincide with global DNA demethylation, as evidenced by pyrosequencing of repetitive DNA sequences (representative results pertaining to NBL2 are summarized in Supplementary Fig. S5B). Immunoblot experiments revealed that 72-hour DZNep treatment mediated dose-dependent depletion of EZH2, EED, and H3K27Me3 in MES1-2, H28, and H2452 cells (Fig. 5A; bottom panel); these effects coincided with significantly decreased proliferation and migration of MPM cells (Fig. 5A; top panel, and Fig. 5B). Furthermore, DZNep significantly diminished soft agar clonogenicity of MES1 and H2452 cells (Fig. 5C).

Additional experiments were carried out to examine whether DZNep could inhibit growth of established MPM xenografts. Preliminary experiments showed that the maximum tolerated dose of DZNep administered intraperitoneally in nude mice with tumor xenografts was 2.5 mg/kg twice a day (data not shown). In subsequent experiments, nude mice were injected subcutaneously with MES1 cells sufficient to produce 100% tumor take at 7 days. Commencing on day 7, mice received 3 cycles of DZNep (2.5 mg/kg twice a day qd x3 q7d). This treatment regimen resulted in significant reduction of tumor size after each treatment cycle, with an
approximate 50% reduction in tumor mass at the end of the treatment course (Fig. 5D) and no visible systemic toxicity.

**Inhibition of PRC-2 activity modulates tumor suppressor gene expression**

Because PcG proteins have been implicated in regulating expression of genes modulating cell-cycle progression, differentiation, and stemness (15, 23), focused SuperArray techniques were used to examine whether selective depletion of EZH2 and EED, or DZNep treatment modulated expression of oncogene and tumor suppressors as well as stem cell–related genes in MES1 and H28 cells. This preliminary analysis showed a variety of genes regulating cell-cycle progression and apoptosis, which were modulated by knockdown and/or DZNep treatment, several of which were selected for further analysis (data not shown). Subsequent qRT-PCR experiments confirmed
upregulation of NF1, FOXD3, FHIT, HIC1, p21, and RASSF1A in MES1 and/or H28 cells following knockdown of EZH2 or EED (Supplementary Table S3). In general, the magnitude of induction of these tumor suppressor genes was more pronounced in EED knockdown cells relative to EZH2 knockdowns. DZNep upregulated FHIT, HIC1, p21, and RASSF1A, but not NF1 or FOXD3 in MPM cells, suggesting that DZNep mediates overlapping as well as differential effects relative to EZH2 or EED knockdown in these cells. Immunoblot analysis confirmed results of qRT-PCR experiments (representative data pertaining to p21 and RASSF1A are depicted in Supplementary Fig. S6). Collectively, these data suggest that the growth inhibitory effects of DZNep were not restricted to epigenetic activation of stem cell polycomb targets.

Illumina array experiments were carried out to examine effects of DZNep on global gene expression in MES1 and H28 cells. Using criteria of more than 1.5-fold change and \( P < 0.05 \) for drug treatment relative to untreated controls, 940 and 513 genes were modulated by DZNep in MES1 and H28, respectively, of which 122 overlapped. Using criteria of more than 1.5-fold change and more stringent adjusted \( P \) values (25), 830 and 435 genes were differentially modulated by DZNep in MES1 and H28, respectively (Fig. 6B); 55% of differentially expressed genes were upregulated by DZNep. Only 11 genes were significantly modulated in both cell lines by DZNep, of which only 3 were simultaneously up- or downregulated (Supplementary Table S4), suggesting significant differences in underlying histology or genotype. Gene set enrichment analysis revealed significant enrichment of EZH2 gene set in MES1, but not H28 cells following DZNep treatment (Table 1). A variety of additional gene sets and ontologies pertaining to proliferation and signal transduction were modulated in these cells (Supplementary Tables S5 and S6).

Discussion

PcG proteins have emerged as critical epigenetic mediators of pluripotency and differentiation of stem cells (26, 27), as well as aberrant gene repression during malignant transformation (15, 28). Two major PRCs,
Each of which contains a variety of core subunits have been identified in mammals. The initiation complex, PRC-2, containing EZH2, SUZ12, PCL, and EED subunits, mediates trimethylation of H3K27. PRC-2 recruits the maintenance complex PRC-1, containing core subunits of PCAF, PHC, RING1, CBX, and SML, as well as SFMBT and L3MBTL proteins that mediate ubiquitination of H2AK119 (15). These histone marks coincide with recruitment of chromatin remodeling complexes, formation of heterochromatin, and repression of gene expression by mechanisms, which to date have not been fully elucidated (29, 30). Of particular relevance with regard to the role of PcG proteins in tumorigenesis are recent observations that PRC-2 inhibits differentiation of normal pluripotent stem cells (31, 32) and that EZH2 is increased and essential for maintenance of cancer stem cells (33, 34).

In this study, we observed overexpression of EZH2 in the majority of cultured MPM lines and primary MPM relative to normal mesothelial cells or pleura. Interestingly, TMA experiments also showed overexpression of EZH2 in PM, suggesting that common epigenetic pathways mediate the pathogenesis of pleural and PMs. Overexpression of EZH2 tended to coincide with tumor burden and correlated with decreased survival of patients with these neoplasms; these findings are consistent with recent studies showing that overexpression of EZH2 in a variety of human malignancies including breast, lung, and esophageal cancers frequently correlates with aggressive tumor phenotypes, advanced stage of disease, and decreased patient survival (35–37).

Knockdown of EZH2 or EED significantly inhibited the malignant phenotype of mesothelioma cells in vitro and in vivo. To the best of our knowledge, these studies are the first to show aberrant expression of PcG proteins in mesotheliomas, and the potential relevance of targeting PRC-2 for the treatment of these malignancies.

Our analysis revealed a spectrum of EZH2 overexpression in pleural mesotheliomas. Variability in EZH2 mRNA levels in MPM could be attributed to stromal elements, which lack EZH2 expression, as these tumors were not microdissected.

### Table 1. EZH2 targets upregulated in MES1 following DZNep treatment

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<td>THBS1</td>
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<td>LAMC2</td>
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<td>TMEM45A</td>
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<td>MGDL</td>
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<td>0</td>
<td>TP53BP2</td>
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Dataset: Ttest_MES1_nm_Dznep_1p5_adjp05
GeneSet: NUYTTEN_EZH2_TARGETS_UP
Enrichment Score (ES): 0.4477096
Normalized Enrichment Score (NES): 2.3391728
Nominal P: 0
FDR Q: 0.001003521
FWER P: 0.007
However, pathologic evaluation confirmed more than 70% tumor cells in specimens submitted for analysis. More importantly, immunostaining showed a range of percent and intensity of EZH2 expression in mesothelioma cells in vivo. Furthermore, patterns of EZH2 expression in primary MPMs were retained in cell lines derived from these tumors, suggesting that differences in EZH2 expression are due to specific genetic/epigenetic factors, which vary among mesotheliomas of similar histologies. Presently, the mechanisms underlying this phenomenon remain unclear. The majority of MPM exhibit disruption of Rb as well as p53-mediated pathways because of allelic loss or epigenetic silencing of p16/p14 (4, 5). A number of PcG genes including EZH2 are potential E2F targets (38). Furthermore, EZH2 expression is negatively regulated by p53 (39), as well as several miRNAs including miR-101 and miR-26a (19, 20). Our preliminary analysis indicated that miR-101 and miR-26a are downregulated in primary MPM relative to normal pleura, suggesting that dysregulation of miRNAs also contributes to overexpression of EZH2 in these neoplasms. Studies are underway to delineate genetic and epigenetic mechanisms regulating EZH2 expression in normal mesothelia and MPM and to further define the prognostic significance of EZH2 overexpression in these malignancies.

Although EED has been extensively studied in stem cells (40, 41), the role of this PRC-2 component in cancer has not been fully defined. EED is necessary for di- and trimethylation of H3K27 and PRC-2–mediated gene silencing in murine embryonic stem cells (42). Direct binding of the WD40 domain of EED is critical for histone methyltransferase activity of the PRC-2 complex and placement of the repressive mark H3K27Me3 in murine embryonic fibroblasts and Drosophila melanogaster embryos (43, 44). EED specifically binds to H3K27Me3 and is necessary for propagation of this repressive mark during cell division. Interestingly, the effects of EED depletion seemed to be more profound than those seen following EZH2 depletion in MPM cells. These results may simply be due to relative knockdown efficiencies; alternatively, they may reflect compensation of EZH2 depletion by EZH1 (45) and magnitude of destabilization of PRC-2 by loss of EED.

Originally developed as an antiviral drug, DZNep has emerged as a novel cancer therapeutic agent (21). This nucleoside analogue induces proteolytic degradation of EZH2 and other PRC-2 components and modest global DNA demethylation (22, 46). Tumor-initiating cells seem exquisitely sensitive to DZNep because of the critical role of PcG proteins in maintenance of cancer stem cells (34). Furthermore, DZNep induces differentiation of cancer stem cells by caspase-dependent degradation of NANOG and OCT4 (47). Our analysis revealed that DZNep depleted EZH2, EED, and H3K27Me3 and upregulated several tumor suppressor genes, including p21 and RASSF1A, which were also activated following knockdown of EZH2 or EED in MPM cells, suggesting that the cytotoxic effects of DZNep are attributable, at least in part, to inhibition of PRC-2 activity. Consistent with this notion, DZNep mediated growth inhibition without apoptosis in MPM cells; similar results were observed following knockdown of EZH2 and EED in these cells. These findings are in accordance with recent observations that knockdown of EZH2 induces cellular senescence in melanoma cells in part by upregulation of p21 (48) and delays G2/M transition without inducing apoptosis in ER-negative breast cancer cells (49).

On the other hand, our current findings do not conclusively implicate depletion of PRC-2 as the major mechanism by which DZNep inhibits the malignant phenotype of pleural mesothelioma cells. Indeed, our highly stringent microarray analysis suggests that DZNep mediates pleiotropic effects in mesotheliomas, which may vary depending on histologic subtype or genotype of these neoplasms. Studies are in progress to further evaluate genomic responses to DZNep in additional cell lines recently established from mesotheliomas of confirmed histologies. Furthermore, experiments are underway to ascertain whether constitutive expression of EZH2 and/or EED abrogates DZNep-mediated cytotoxicity in MPM cells. Whereas the precise mechanisms by which DZNep mediates growth arrest in mesothelioma cells have not as yet been fully elucidated, our findings warrant further analysis of PcG protein expression in mesotheliomas and the development of agents targeting PRC-2 expression/activity for treatment of these malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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