Polycomb Protein EZH2 Regulates Tumor Invasion via the Transcriptional Repression of the Metastasis Suppressor RKIP in Breast and Prostate Cancer

Gang Ren1, Stavroula Baritaki2, Himangi Marathe1, Jingwei Feng1, Sungdae Park1, Sandy Beach1, Peter S. Bazeley1, Anwar B. Beshir3, Gabriel Fenteany3, Rohit Mehra4, Stephanie Daignault4, Fahd Al-Mulla5, Evan Keller4, Ben Bonavida2, Ivana de la Serna1, and Kam C. Yeung1

Abstract

Epigenetic modifications such as histone methylation play an important role in human cancer metastasis. Enhancer of zeste homolog 2 (EZH2), which encodes the histone methyltransferase component of the polycomb repressive complex 2 (PRC2), is overexpressed widely in breast and prostate cancers and epigenetically silences tumor suppressor genes. Expression levels of the novel tumor and metastasis suppressor Raf-1 kinase inhibitor protein (RKIP) have been shown to correlate negatively with those of EZH2 in breast and prostate cell lines as well as in clinical cancer tissues. Here, we show that the RKIP/EZH2 ratio significantly decreases with the severity of disease and is negatively associated with relapse-free survival in breast cancer. Using a combination of loss- and gain-of-function approaches, we found that EZH2 negatively regulated RKIP transcription through repression-associated histone modifications. Direct recruitment of EZH2 and suppressor of zeste 12 (Suz12) to the proximal E-boxes of the RKIP promoter was accompanied by H3-K27-me3 and H3-K9-me3 modifications. The repressing activity of EZH2 on RKIP expression was dependent on histone deacetylase promoter recruitment and was negatively regulated upstream by miR-101. Together, our findings indicate that EZH2 accelerates cancer cell invasion, in part, via RKIP inhibition. These data also implicate EZH2 in the regulation of RKIP transcription, suggesting a potential mechanism by which EZH2 promotes tumor progression and metastasis.

Introduction

Cancer cells are characterized by an unbalanced and dramatically altered epigenetic state compared with normal counterparts. Histone modifications and DNA methylation are among the most studied epigenetic mechanisms that control the accessibility of target gene promoters to positive or negative transcriptional signals and regulate gene expression. Epigenetic alterations related to transcriptional inactivation of tumor suppressor genes form part of a regulatory mecha-
ectoderm development (eem), and retinoblastoma binding proteins 4 or 7 [RbBP4 (RbAP48) or RbBP7]. These 4 proteins constitute the core components of PRC2. For methylation-dependent gene silencing, PRC2 is thought to be the first complex recruited to DNA through the involvement of various intermediate molecules such as the long noncoding RNA hox transcript antisense RNA (HOTAIR), resulting in EZH2-mediated H3-K27-me3 (9). This mark serves as an anchorage point for the further recruitment of PRC1, DNMT1s, and HDACs, which contribute to chromatin compaction and transcriptional repression (10).

Although polycomb-mediated H3-K27-me3 has been shown to premark genes for de novo DNA methylation in cancer (11), gene silencing by H3-K27 trimethylation independent of promoter DNA methylation is also very frequent in cancer cells (12). This finding supports the significance of expression and activity of H3-K27-me3–inducing EZH2 protein in human malignancies (10). Overexpression of EZH2 was first linked to cancer by microarray studies of breast and prostate cancer (13, 14). Experimental support for the oncogenic action of EZH2 has been provided by induction of anchorage-independent colony growth and promotion of invasion in vitro by overexpression of EZH2 in the breast epithelial cell line H1662 (14). In addition, downregulation of EZH2 expression by siRNA has been shown to decrease the proliferation of prostate cancer cells in vitro (15).

Different mechanisms have been described to explain the overexpression and increased activity of EZH2 in various cancer models. Among them, an acquired missense mutation (V611) within the SET domain of EZH2 associated with enhanced catalytic efficiency for H3-K27 trimethylation has been described in B-cell lymphomas (15), whereas deletions of microRNA-101, a negative upstream regulator of EZH2 expression, have been reported in prostate cancer (16).

The link between EZH2 overexpression and tumor invasiveness/metastasis is supported by the suppressing function of EZH2 on the expression of several miRNAs that regulate the expression and activity of the metastasis suppressor RKIP (22). RKIP has been further identified as a potent inhibitor of Raf-1/MEK/ERK, NF-κB, and G-protein–coupled receptor signaling pathways (23–25). RKIP was initially identified as a potent inhibitor of Raf-1/MEK/ERK, NF-κB, and G-protein–coupled receptor signaling pathways (23–25). RKIP has been further identified as a metastasis suppressor in prostate cancer as its loss of expression in prostate cancer cells conferred a metastatic phenotype, whereas restoration of its expression in a xenograft murine model diminished metastasis (26). Besides prostate cancer, deletion of RKIP expression and activity has now been reported in distant metastases of various cancer types, including breast, gastric, colorectal, and hepatocellular carcinomas, and its expression has been proposed as a prognostic marker for disease-free survival in patients diagnosed with the above cancers. Similar to CDH1, the loss of RKIP expression has been associated with EMT induction, enhanced angiogenesis, and vascular invasion, as well as with protection against drug-, radio- and/or immune-mediated apoptosis during antitumor therapy. Although the critical role of RKIP in tumor progression has been documented, its transcriptional regulation still remains largely unclear. We have previously reported that RKIP expression, similar to CDH1, is directly repressed by the EMT inducer Snail in prostate and breast cancer, however, no other direct or indirect regulators of RKIP transcription have been identified.

In the present study we question the direct involvement of EZH2 in RKIP transcriptional repression in prostate and breast cancer models as well as whether EZH2 association with increased tumor invasiveness might be attributed to RKIP repression. On the basis of the reported low RKIP levels and the elevated EZH2 expression in invasive prostate and breast carcinomas and metastases, we hypothesized that EZH2 may repress RKIP transcription by inducing histone H3-K27 trimethylation and that the RKIP inhibition is closely related to EZH2-mediated effects on promoting tumor invasion. Our findings support our hypothesis and show that EZH2 accelerates cell invasion, at least in part, via transcriptional repression of the metastasis suppressor RKIP. We further show that the repressive activity of EZH2 on RKIP transcription required the presence of the EMT inducer Snail.

Materials and Methods

Cell lines and reagents

The prostate carcinoma cell lines DU-145, PC-3 (both metastatic bone-derived human androgen-independent prostatic adenocarcinomas), and LNCaP (nonmetastatic bone-derived human androgen-dependent prostatic adenocarcinoma) as well as the breast cancer cell lines T47D, MCF7, and MDAMB231 were obtained from the American Type Culture Collection. The cell lines were cultured in RPMI-1640 (Invitrogen) as described previously (27). The transformed prostate epithelial cell line PrEC-LSHAR expressing SV40 large and small antigens, hTERT and androgen receptor was obtained from Dr. William Hahn (Harvard Medical School, Boston, MA) and was cultured as previously described (28). The HDAC inhibitor SAHA was purchased from Santa Cruz Biotechnology Inc. and diluted in dimethyl sulfoxide. The anti-α tubulin monoclonal antibody (Clone B-5-1-2; T-5168) was obtained from Sigma. The antibodies for E-Cadherin (4065), EZH2 (4905), and Suz12 (3737) were purchased from Cell Signaling Technology. The antibodies for EED (clone H-300; Sc-28701), and RbAp48 (clone K-15; Sc-12434) were obtained from Santa Cruz Biotechnology. The anti-Bmi1 (clone F6) and rabbit anti-RKIP (N-term; 36–0700) antibodies were obtained from Upstate and Invitrogen, respectively. The antibodies used for the chromatin immunoprecipitation (ChIP) assays, were as follows: anti-EZH2 and control IgG (Millipore); rabbit monoclonal anti-SUZ12 (37375; Cell Signaling); polyclonal anti-H3-K9-m3 (39161), anti-H3-K4-m3 (39159), anti-H4-Ac (39179), and anti-H3-K27-m3 (39157; Active Motif). The siRNA against Snail and scrambled siRNA control used for transient cell transfections were obtained from Santa Cruz Biotechnology Inc. For all transient transfections, Lipofectamine 2000 (Invitrogen) was used as a transfection reagent.
Plasmid constructs and retroviral vectors

The wild-type and E-box mutant RKIP promoter (2.2 kb) activities were determined by using the luciferase reporter plasmid constructs RKIP-Luc w/t and RKIP-Luc mut, respectively (29). For the transient ectopic expression of EZH2, EZH2 mutant in SET domain (EZH2 H689A), eed, and Suz12, we used expression plasmids containing the full-length cDNA of the corresponding genes under the control of a cytomegalovirus promoter, as described previously (30). The relevant empty vector was used as a negative control (empty vector control, EVC). The retroviral expression vectors for HOTAIr, miR-101, and miR-145 were kind gifts of Drs. Howard Chang (Stanford University, Stanford, CA; ref. 14) and Yin-Yuan Mo (Southern Illinois University, Carbondale, IL), respectively. The mammalian expression vectors for wild-type and mutant EZH2 as well as Suz12 were kindly provided by Dr. Danny Reinberg (New York University, NY). The retroviral expressing vectors siSUZ12, siEZH2, or siGFP were kindly provided by Drs. Yi Zhang and Yue Xiong (31), respectively (both from University of North Carolina, Chapel Hill, NC). The expression vectors for RKIP were described previously (32).

Cell extracts and Western blot analysis

Cells extracts were prepared and Western blotting was carried out as previously described (27).

Quantitative real-time transcriptase PCR

Total cellular RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed using random hexamer primers (Applied Biosystems). The resulting cDNAs were used for real-time transcriptase (RT)-PCR using SYBR-Green Master PCR mix (QIAGEN) in triplicates. Sample’s running and data collection were carried out on ABI7500 (Applied Biosystems). The retinoic acid receptor (RARE) and hypoxanthine phosphoribosyltransferase (HPRT) were used as an internal standard. The speci mens were changed to regular medium once after selection, viruses were harvested after 24 and 48 hours. Luciferase activity was measured as described earlier. Snail inhibition was confirmed at the protein level using Western blot analysis.

Retroviral infection

To generate retroviruses, all retroviral expression vectors with the exception of pLZBS-hotair were transfected into packaging cell 293-GP2 (Clontech) as previously described. Phoenix amphi packaging cells (Orbigen) were used for retroviral vector pLZBS-hotair. pLZBS-hotair transfected Phoenix cells were selected with puromycin (1.5 μg/mL) for 4 days. Cells were changed to regular medium once after selection, viruses were harvested after 24 and 48 hours.

ChIP assay

Briefly, the cells were grown to near 70% confluence and cross-linked by adding 3% formaldehyde to a final concentration of 1% and incubated at 37ºC for 15 minutes. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 mol/L. The cells were washed twice with ice-cold PBS and pelleted in PBS-containing protease inhibitors. The nuclei were isolated in a buffer containing 50 mmol/L HEPES-KOH, pH 7.5, 140 mmol/L NaCl, 1 mmol/L EDTA, 10% Glycerol, 0.5% NP-40, 0.25% Triton-X, and protease inhibitors. The nuclei were then subjected to sonication for a total of 4 minutes at 70% amplitude using a Cole Palmer Ultrasonic processor in the following buffer 10 mmol/L Tris-HCl, pH 8.0, 200 mmol/L NaCl, 9 mmol/L EDTA, 0.5 mmol/L EGTA, 0.1% Na-deoxycholate, 0.5% N-laurylsarcosine, and 0.1% Triton X-100. The resulting chromatin was centrifuged for 15 minutes and quantified. Eighty to 100 μg of chromatin was preclaved for 3 hours at 4ºC with 50% slurry of protein A, or G, beads in Tris-EDTA (TE; depending on the isotype of the antibody used) in the presence of 20 μg of salmon sperm DNA and 1 mg of bovine serum albumin per mL. After incubation, the beads were
pellet, and the supernatant was immunoprecipitated with antibodies of interest at 4°C overnight. The immune complexes were collected with Protein A/G agarose beads as prepared for pre-clearing for 4 hours at 4°C. The bead—antibody complex was then washed 5× with RIPA wash buffer (50 mmol/L HEPES, 500 mmol/L LiCl, 0.1 mmol/L EDTA, 1% NP-40, and 7% Nonidet P-40) and once with TE containing 50 mmol/L NaCl. The immune complexes were eluted with 50 mmol/L Tris-Hcl pH8.0, 10 mmol/L EDTA, and 1% SDS. Elution was carried out at 65°C for 30 minutes followed by reversal of cross-links at 55°C for 2 hours followed by phenol–chloroform extraction and ethanol precipitation. The purified DNA was dissolved in 50 μL Tris-EDTA, and 2 μL was used for PCR.

The primers used for ChIP were as follows:

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKIP-F</td>
<td>CCA AAA CCC AAA CAT TTC TCA</td>
<td></td>
</tr>
<tr>
<td>RKIP-R</td>
<td>CCT TGC TTT TCT CCT GCA CT</td>
<td></td>
</tr>
<tr>
<td>Myt1-F</td>
<td>ACA AAG GCA GAT ACC CAA CG</td>
<td></td>
</tr>
<tr>
<td>Myt1-R</td>
<td>GCA GTT TCA AAA AGC CAT CC</td>
<td></td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>TAC TAG CGG TTT TAC GGG CG</td>
<td></td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>TCG AAC AGG AGG AGC AGA GAG CGA</td>
<td></td>
</tr>
</tbody>
</table>

**Invasion assays**

Invasion assays were carried out in 24-well tissue culture plates with Transwell inserts. The polycarbonate membrane (8-μm pore size) of Transwell fluoroblok inserts (Costar) was coated with 90 μL of the diluted (1:25 in serum-free medium) Matrigel (BD Biosciences). Chemoattractive medium (600 μL) was used for PCR.

**Case selection and tissue microarrays**

A prostate cancer progression tissue microarray (TMA) was constructed from cases of clinically localized prostate cancer obtained from a radical prostatectomy series at the University of Michigan (Ann Arbor, MI). Prostate cancer metastases TMA were developed from samples obtained through the Rapid Autopsy Program within the Michigan Prostate SPORE Tissue Core (34). The progression TMA 100 consisted of 309 evaluable cores taken from 99 total patients; 92 cores of nonneoplastic prostate (from 39 cases), 23 cores of benign prostatic hyperplasia (BPH; from 8 cases), 19 cores of PIN (from 12 cases), 142 cores of localized prostate cancer (from 50 cases), and 33 cores of metastatic, hormone-refractory prostate cancer (from 11 cases; ref. 35). Two prostate cancer autopsy arrays 79A and 79B were constructed from soft tissue and bone metastases taken from 30 available autopsies. TMA 79A consists of 303 evaluable cores of primary prostate cancer and soft tissue metastases of the liver, lung, lymph node, adrenal, bladder, dura, and seminal vesicles. The TMA 79B consisted of 129 evaluable cores included 72 cores (from 17 cases) of bone metastases in addition to primary prostate cancer and soft tissue metastases. All tissue procurement and analysis in this study was approved by the Institutional Review Board. Histo-logic processing of all clinical samples was conducted in the University of Michigan Hospital's accredited Pathology Department using a standardized procedure to assure uniform sample preparation for each TMA.

**Immunohistochemistry and evaluation**

TMA slides were deparaffinized, rehydrated to water, and antigen retrieved by pretreatment with citrate buffer, pH 6.0 for 10 minutes with microwaving. After peroxidase blocking, the slide were incubated with 1:400 dilution of RKIP or EZH2 antibodies on a DAKO AutoStainer using the LSAB+ detection kit and counterstained with hematoxylin. Staining intensity was scored by a genitourinary pathologist (to R. Mehra) as negative (1), weak (2), moderate (3), or strong (4) based on the amount of stain detected. The percentage of positively stained cells was determined by counting 100 cells in 2 random fields. Protein levels in each sample were reported as expression index (EI), which is a product of staining intensity and the percentage of positive staining as described previously (36).

**Meta-analysis of Oncomine database and survival analysis**

The expression RKIP and EZH2 transcripts in prostate cancer tissues were obtained from meta-analysis of our recently established cancer gene microarray meta-analysis public database (37). Heatmaps were generated using the "heatmap.2" function from the "gtools" package in the R statistical program. Neither a column nor row dendrogram was computed to reorder the values (parameters "Rowv" and "Colv" were set to "NULL" and "dendrogram" was set to "none"). No trace line was drawn (parameter "trace" set to "none"), and the bottom and left margins were set to 12 and 9, respectively [parameter "margins" set to "c(12,9)"]. All other parameters used the default settings. The van’t Veer human breast cancer data set consists of 117 breast tumor samples was used for survival analysis as shown in Fig. 1G (38). The association between RKIP/EZH2 expression and relapse-free survival (RFS) within 5 years of diagnosis was analyzed using available outcome data. Samples in the data sets were separated into 4 groups according to RKIP and EZH2 expression. Survival curves were generated using the "survival" package in R. The P value was computed using the "survdiff" function in this package, which compares all 4 groups.

**Statistical analysis**

The Mann–Whitney U and Kruskal–Wallis H tests determined significant differences between values obtained from cells that were treated under different experimental conditions. Analysis of the immunohistochemical staining intensity was also conducted by using the nonparametric Kruskal–Wallis and Wilcoxon rank tests. The Pearson correlation coefficient was used to compare the staining intensities of the 2 markers within the samples and tested against a null
Epigenetic Silencing of RKIP Expression in Cancer Metastasis

Figure 1. Increasing EZH2 followed by decreasing RKIP levels are associated with a progressive cancer disease and are hallmarks for metastasis and poor survival. A, heatmap of RKIP, EZH2, Snail, and E-cad expression profiles obtained by a representative publicly available DNA microarray expression data set. The database consisted of 52 human prostate samples of different histopathology grouped as: NAP, normal adjacent prostate tissue; BPH, benign prostate hyperplasia; PCA, prostate cancer adenocarcinoma; and MPC, metastatic prostate carcinoma. Rows correspond to individual genes and columns represent individual patients. Color density is arranged in order from greatest decrease in expression at left (red) to greatest increase at right (light yellow). EZH2 and Snail mRNA are strongly expressed in MPC, in contrast to RKIP and E-cad. B, box plots presenting the mean ± SEM values of EZH2 and RKIP mRNA expression for each of the indicated groups in the studied DNA microarray database. EZH2 expression is significantly increasing with the severity of the disease (P < 0.05), whereas RKIP is significantly decreasing (P < 0.05). The statistical significance of the expression differences among the various histologic groups was determined using pairwise comparisons. C, EZH2 mRNA expression is inversely correlated with RKIP expression in the studied prostate samples (P = 0.0004). The plot scale represents expression level after logarithmic transformation (log2 value) of the expression ratios for both RKIP and EZH2 (r = 0.67). D, representative staining of EZH2 and RKIP protein expression in PCA and prostate metastatic cancer (PMG) tissue arrays, as assessed by immunohistochemistry. A clear increase in RKIP staining accompanied by decreased RKIP staining is observed in PMC compared with PCA. E, box plots presenting the mean and quartile values of EZH2 and RKIP protein expression in tissue arrays for each of the studied groups. EZH2 protein expression is significantly increasing in metastastic samples (P < 0.001), accompanied by significant decrease in RKIP expression (P < 0.001). The statistical significance of the expression differences among the various histologic groups was determined with Kruskal–Wallis test. F, EZH2 staining density expression is inversely correlated with that of RKIP in the studied samples (Pearson correlation coefficient = -0.42 and P < 0.001). G, high EZH2 and low RKIP mRNA levels are associated with breast cancer metastasis and bad prognosis. Kaplan–Meier curve assessing the disease-free survival of 97 patients with breast cancer based on RKIP and EZH2 mRNA levels obtained by a publicly available DNA microarray expression data set (38). A statistically significant increase in RFS was observed in patients with high RKIP/EZH2 expression ratios (P = 0.0294).

hypothesis that there is a lack of correlation (correlation coefficient = 0).

Probability (P) was set significant at the level of 0.05. All statistical analyses were conducted with the SPSS or SAS 9.2 software.

Results

RKIP expression negatively correlates with EZH2 levels in prostate human cancer samples and the RKIP/EZH2 ratio predicts relapse-free breast cancer survival

As a first step to determine whether EZH2 plays a role in maintaining the repressed state of RKIP expression, we investigated publicly available DNA microarray expression data sets, in search of a correlation between RKIP and EZH2 expression in prostate cancer samples. Ten data sets were identified with normal adjacent prostate tissue (NAP), BPH, localized prostate cancer adenocarcinoma (PCA), and metastasis of prostate cancer (MPC; ref. 33). Only 3 of them included both RKIP and EZH2. As previously reported (13), while the RKIP expression was low, expression of EZH2 was highly upregulated in all 3 data sets as the cancer progressed (P < 0.05 for both EZH2 and RKIP; Fig. 1A and B and Supplementary Fig. S1A). Importantly, we also observed a strong negative correlation between RKIP expression and EZH2; r = -0.67 (P =...
Inverse correlation between RKIP and EZH2 expression levels in prostate and breast cancer cell lines with different metastatic potential

To examine the connection between RKIP and EZH2 in metastasis, we first determined RKIP and EZH2 expression in cancer cell lines of different metastatic potential. Consistent with the expression patterns that were observed in prostate cancer samples, the expression of EZH2 was found to be high in the low RKIP-expressing metastatic prostate cancer cell line DU145, whereas the expression was low in high RKIP-expressing nonmetastatic LNCaP cells (Fig. 2A). Similar results were also observed in metastatic (MDA-MB231) and nonmetastatic (MCF7 and T47D) breast cancer cell lines (Fig. 2B). In addition to EZH2 and RKIP, we also examined the expression levels of other components of the PRCs as well as E-cad, a known target of EZH2 repression in prostate and breast cancers and suggest that EZH2 and RKIP may be in the same regulatory pathway affecting cancer metastasis.

The association between high EZH2/low RKIP expression and metastasis shown at the mRNA level was further validated at the protein level using prostate TMAs. Human BPH, PCA, and MPC samples were stained for RKIP and EZH2 protein expressions using immunohistochemistry. EZH2 staining density was found higher in MPC samples than in PCA, whereas the opposite results were observed for RKIP (Fig. 1D). For both EZH2 and RKIP the expression differences among BPH, PCA, and MPC samples were statistically significant, thus indicating that among the studied groups the metastatic prostate tumors have the lowest RKIP/EZH2 protein ratios (Fig. 1E). Finally, the staining densities obtained for both RKIP and EZH2 proteins were found inversely correlated with a Pearson correlation coefficient of –0.42 (P = 0.0001; Fig. 1F). On average, the RKIP stain index is 114.9 [95% confidence interval (CI), 98.3–131.4] points higher than the EZH2 stain index in a core (P = 0.0001).

Because low RKIP expression in primary tumors was also a strong positive predictive factor for prostate and breast cancer recurrences (38, 39), we examined the prognostic value of different EZH2/RKIP expression combinations in breast cancers in which the expression of EZH2, RKIP and clinical outcome are available in published microarray expression data set (38). We found that high EZH2 and low RKIP expression were associated more significantly with the development of metastasis within 5 years of primary diagnosis than either high EZH2 or low RKIP expression alone (Fig. 1G). These results implicate EZH2 as another possible repressor of RKIP expression in prostate and breast cancers and suggest that EZH2 and RKIP may be in the same regulatory pathway affecting cancer progression.

EZH2 is directly involved in the suppression of RKIP expression during breast and prostate cancer progression

To investigate the regulatory effects of PcG proteins on RKIP expression, we expressed EZH2 or siRNA for EZH2 or Suz12 by retroviral infection in both prostate and breast cancer cell lines with different metastatic capacities. Ectopic expression of wild-type EZH2 in the nonmetastatic breast and prostate cell lines MCF7 and LNCaP resulted in a significant decrease of baseline RKIP expression when compared with the EVC (Fig. 3A). In contrast, infection of MCF7 cells with a SET domain mutated EZH2 (EZH2 H689A)-expressing retrovirus was incapable of reducing RKIP expression levels (Fig. 3A). Because H689A mutation inactivates EZH2’s methyltransferase activity toward H3K27 our findings suggested that EZH2 enzymatic activity was essential for RKIP repression. This methyltransferase-dependent effect of EZH2 on RKIP expression was also observed in the transformed LSHAR prostate epithelial cell line (Fig. 3A). The inhibitory role of EZH2 on RKIP promoter transcriptional activity was further shown by real-time RT-PCR in LNCaP, MCF7, and LSHAR cells, where the relative RKIP mRNA expression was found significantly reduced only in cells expressing the wild-type (Fig. 3B). To further examine the causal role of EZH2 on RKIP repression, we silenced the EZH2 expression in the metastatic DU145 and MDA-MB231 cell lines using siRNA and monitored the expression of RKIP. siEZH2-treated cells had significantly higher RKIP protein levels than the siGFP control as shown in Fig. 3C. Likewise, the relative RKIP mRNA expression was also significantly increased in siEZH2-expressing DU145 and MDA-MB231 cells (Fig. 3D). In contrast, silencing of Suz12 by siRNA in DU145 did not increase RKIP protein expression (Fig. 3E).

mRNA-101 (miR-101) expression decreases during prostate and breast cancer progression and this abnormal miR-101 downregulation has been proposed as causative factor of EZH2 overexpression in the above tumors (42). Consistent with the notion that EZH2 regulates RKIP expression, ectopic expression of miR-101 in DU145 caused a robust induction of RKIP protein as detected by Western blotting (Fig. 3F). As expected, expression of miR-101 decreased EZH2 protein expression (Fig. 3F). The effect was specific for miR-101 as expression of another miRNA, miR-145, had no effect on the expression levels of EZH2 or RKIP (Fig. 3F). The above findings therefore suggest a direct suppressive role of EZH2 on RKIP expression during breast and prostate cancer initiation and progression.
Epigenetic Silencing of RKIP Expression in Cancer Metastasis

RKIP transcription is repressed via direct recruitment of the EZH2-containing PRC2 complex to the proximal E-boxes of the RKIP promoter

Conceptually, EZH2 could regulate RKIP expression by either one of 2 distinct mechanisms. It may act directly on the RKIP promoter in a cis-binding site-dependent manner. Alternatively, EZH2 can affect the stability of RKIP mRNA. To differentiate between these 2 models we conducted luciferase reporter assays in LNCaP cells using a plasmid carrying a 2.2 kb RKIP reporter and EZH2 expression vectors. Ectopic RKIP expression was served as a loading control. B, protein expression of EZH2, Suz12, eed, RbAP48, Bmi1, RKIP, and E-cad in metastatic prostate cancer cell lines. Tubulin expression was used as an internal control. C, relative mRNA levels of PRC2 subunits EZH2, Suz12, eed, RbAP48 and the metastasis suppressors RKIP and CDH1, as determined by real-time RT-PCR in LNCaP, PC3, and DU145 cell lines. Actin mRNA level was used as internal control. D, relative mRNA expression of EZH2 and RKIP in MDA-MB231, T47D, and MCF7 cell lines. All statistical analyses were conducted using unpaired 2-tailed t test.

Snail but not HOTAIR is involved in the EZH2-mediated repression of RKIP promoter

Because none of the PRC2 subunits are sequence-specific DNA-binding proteins, it is not precisely known how EZH2 histone methyltransferase is recruited to target mammalian genes. Among the suggested recruiters is a long noncoding RNA, known as HOTAIR, which was initially implicated in PRC2 recruitment to the human HOXD cluster (43). During breast cancer progression, expression of HOTAIR becomes dysregulated and overexpressed. It has been shown that dysregulated HOTAIR expression induced genome-wide retargeting of PRC2 and promote breast cancer metastasis (44). It is
possible that PRC2 is retargeted to the RKIP promoter leading to its subsequent repression during breast cancer progression. To test this possibility, we ectopically expressed HOTAIR in the breast cancer cell line MDA-MB231 (Fig. 5A) and monitored the expression levels of RKIP (Fig. 5B and C). Overexpression of HOTAIR increased the expression of RKIP (Fig. 5B and C). Relative levels of EZH2/tubulin or RKIP/tubulin are indicated. B, relative RKIP mRNA levels assessed by real-time RT-PCR in LNCap, MCF7 cells, or PrEC expressing the wild-type or H689A mutant EZH2 proteins. Actin mRNA level was used as internal control. C, protein expression of RKIP and EZH2 in the metastatic DU145 and MDA-MB231 cell lines expressing siRNA against EZH2 or siGFP. Relative levels of EZH2/tubulin or RKIP/tubulin are indicated. D, relative RKIP mRNA expression in DU145 or MDA-MB231 cells expressing siEZH2 or siGFP control as assessed by real-time RT-PCR. E, protein expressions of RKIP and EZH2 in DU145 cell expressing silencing Suz12 or GFP control siRNAs as assessed by Western blotting. F, EZH2-mediated RKIP suppression is negatively regulated by miR-101. Downregulation of EZH2 expression by ectopic expression of its suppressor miR-101 upregulates RKIP expression as assessed by Western blotting. miR-145 overexpression was used as negative control.

An alternative explanation for PRC2 recruitment to the RKIP promoter is via transcription factors with direct DNA-binding sites on the target promoter. The only transcription factor that has been reported so far to regulate RKIP transcription is Snail (33). Snail binds directly to E-boxes of RKIP promoter and facilitates its suppression (33). Because EZH2 bound to a region of the RKIP promoter that contains 3 E-boxes, it was possible that Snail is involved in EZH2-mediated RKIP suppression. To test this possibility, we generated an RKIP reporter plasmid with mutated E-boxes that prevent efficient Snail binding (28). Expression of EZH2 in LNCap cells did not have a significant effect on the transfected mutated E-boxes RKIP reporter, indicating that EZH2 suppressive activity on the RKIP promoter depends on Snail binding to the RKIP promoter (Fig. 5D). To further examine the direct involvement of Snail in EZH2-mediated RKIP suppression, we silenced the expression of Snail in LNCap cells by Snail siRNA and monitored the RKIP promoter activity in the presence or absence of EZH2.
expression (Fig. 5E). Snail silencing resulted in reversal of EZH2-mediated inhibition of RKIP promoter activity after ectopic expression of EZH2 (P = 0.061) and significantly reduced the enrichment of RKIP promoter in EZH2 molecules as shown by ChIP analysis on the target promoter (Fig. 5F). The observed differences were not due to differences in levels of Snail expression (Fig. 5G). However, despite repeated attempts we failed to detect the cotargeting of both Snail and EZH2 to the same region in RKIP promoter by sequential ChIP assays (data not shown). Overall, these findings show that Snail is required for EZH2-mediated repression of the RKIP promoter and link the inhibition of RKIP expression with the combined suppressive activities of PRC2 and Snail on the target promoter.

EZH2 accelerates cancer cell invasion by inhibiting RKIP expression

It has been reported that downregulation of EZH2 inhibited cancer cell anchorage-independent growth, proliferation, and invasion (45). Because decreased EZH2 expression also caused an increase in RKIP expression (Fig. 3C), it was possible that gain of RKIP expression is the cause of the observed effects due to the loss of EZH2 expression in cancer cells. To address this possibility we designed an experimental approach whereby we tested whether the decrease of proliferation, anchorage-independent growth, or invasion by silencing of EZH2 in DU145 and MDA-MB231 cell lines could be reversed by ectopic silencing of RKIP expression. While the blockage of RKIP expression did not have observable effect on anchorage-independent growth or proliferation (Supplementary Fig. S1B and S1C), it effectively
Figure 5. Snail and not HOTAIR is involved in EZH2 recruitment to RKIP promoter. A, overexpression of HOTAIR in MDA-MB231 cells infected with HOTAIR-expressing retrovirus or EVC as assessed by real-time PCR. B, qRT-PCR analysis of ABL2 and RKIP mRNA expression in HOTAIR- or EVC-expressing MDA-MB231 cells. The expression of the indicated gene was normalized to the expression level of GAPDH (n ≥ 3). C, Western blot analysis examines expression of EZH2 and RKIP protein in HOTAIR-expressing MDA-MB231 cells. D, RKIP suppression by EZH2 is dependent on Snail binding to proximal E-boxes of RKIP promoter. LNCaP cells were cotransfected with an RKIP-Luc vector carrying mutated E-boxes and EZH2 expression vector or EVC. Twenty-four hours after transfection, cells were harvested for luciferase assay. RLU were determined using a luminometer. E, Snail silencing in LNCaP cells by Snail siRNA causes a marginally significant reversal of EZH2-mediated inhibition of basal RKIP promoter activity. LNCaP cells were initially transfected with Snail siRNA or control siRNA for 48 hours followed by cotransfection with wild-type RKIP-Luc plasmid and EZH2 expression vector or EVC for additional 24 hours. Transfected cells were harvested for luciferase assay as described and the expressions of Snail and EZH2 were assayed by Western blotting (top). F, expression of Snail is required for the binding of EZH2 to the RKIP promoter. ChIPs were carried out in control or snail knockdown DU145 cells using antibodies to RKIP or control IgG. RKIP promoter was amplified with primers that span the putative Snail-binding site and quantified by real-time PCR. GAPDH was used as a negative control. These results are an average of 2 independent experiments carried out in triplicate. G, Western blotting showing protein expressions of Snail and EZH2 in DU145 and LNCaP cell lines expressing siRNA against EZH2 and EZH2 cDNA, respectively. Relative levels of EZH2/tubulin or RKIP/tubulin are indicated.
reversed the decrease in invasiveness due to the loss of EZH2 (Fig. 6A–E). It has been shown that RKIP inhibits cell invasion by repressing the expression of MMP (matrix metalloprotei-

nase) genes in breast cancer cells (31, 46). Consistently, we observed a good correlation between the MMP13 expression and invasion in MDA-MB231 cells with altered expression of EZH2 or/and RKIP (Fig. 6C, left). Concomitantly, when the prostate epithelial cell line PrEC LSHAR was infected with EZH2-expression retrovirus it acquired increased invasive properties, which were significantly diminished back to the baseline invasion rate after expressing RKIP (Fig. 6F and G).

Altogether, these findings suggest that RKIP inhibition by EZH2 might be part of the molecular mechanism by which EZH2 promotes invasion and metastasis in prostate and breast malignancies.

Discussion

RKIP is a proven metastasis suppressor of prostate and breast cancer. Consistent with its antimetastatic effect, RKIP expression is inversely correlated with tumor aggressiveness and is almost lost in metastatic tumors thus predicting a poor prognosis (22). The molecular mechanisms by which RKIP

![Graphs and images related to the discussion](https://www.aacrjournals.org/cancerres;72(12)/3101)
expression is downregulated during cancer progression and metastasis have not been completely elucidated. Epigenetic silencing by histone methylation has also been shown to play an important role in human cancer metastasis. The histone lysine methyltransferase EZH2 is overexpressed in cancers and is associated with cancer aggressiveness with poor prognosis. In cancer cells, deregulated EZH2 activity represses normal expression of tumor suppressor or metastasis genes. In breast and prostate cancers, among the genes found to be directly targeted and silenced by EZH2 are metastasis suppressor genes CDH1 (19), FOXC1 (47), and DAB2IP (48). In the present study we identify RKIP as an additional transcriptional target of EZH2 and mediator of EZH2 prometastatic effects.

We observed a significant positive association between high EZH2/low RKIP expression ratio and tumor aggressiveness/metastasis in cell lines and human cancer samples. Functionally, we showed that the high EZH2/low RKIP ratio is a strong positive predictive factor for breast cancer recurrence and poor survival. Thus, our results provide for the first time evidence of the involvement of EZH2 in the mechanism that mediates RKIP repression during cancer progression. The recapitulation of EZH2 and RKIP expression levels in cancer cell lines provided us with a genetically tractable system to investigate the causal role of EZH2 in silencing RKIP expression in cancer metastases. Significant inhibition of RKIP expression was observed in low EZH2-expressing cancer cells when expression of EZH2 was restored, whereas EZH2 silencing resulted in recovery of the lost RKIP expression in the high EZH2-expressing metastatic cell lines. Expression of EZH2 in a transformed prostate epithelial cell line with defined genetic alterations was also capable of suppressing RKIP expression, suggesting that abnormally elevated EZH2 levels in normal prostate epithelia may contribute to initiation of tumorigenesis via repression, among other genes, the tumor suppressor RKIP. Suppression of RKIP expression by EZH2 requires its histone lysine methyltransferase catalytic activity implying that the EZH2-mediated regulation of RKIP expression is at the level of transcription initiation.

Three lines of evidence indicate that EZH2 regulates RKIP expression at the level of transcription. First, genetic manipulation of EZH2 expression in cancer cell lines resulted in the change of RKIP mRNA and protein expression levels. Second, the RKIP promoter activity was found significantly diminished after overexpression of the wild-type EZH2 but not the SET mutant EZH2 vectors, indicating that histone modifications related to EZH2-mediated H3-K27-me3 might take place on the RKIP promoter for RKIP suppression. Finally, we showed the physical association of EZH2 and Suz12 with the RKIP promoter in intact cancer cells.

EZH2 is part of a multicomponent protein complex named PRC2. The other components include Suz12, eed, and RbAp48. Consistent with previous findings in human cancer samples (13, 14), only the expression of the EZH2 subunit is elevated in both breast and prostate metastatic cell lines. In normal cells the histone methyltransferase activity of EZH2 requires its binding to Suz12 and eed. Unlike EZH2, silencing of Suz12 by siRNA in high EZH2-expressing DU145 cells did not reveal any significant RKIP induction. On the contrary, in low EZH2-expressing LNCaP cells ectopic expression of Suz12 or eed alone was sufficient to repress RKIP expression. These findings, therefore, suggest that EZH2 catalytic activity may not require the presence of Suz12 and eed when overexpressed in cancer. However, in low EZH2-expressing cancer cells overexpression of other subunits is enough to shore up the activity of the PRC2 complex. In contrast to Suz12, the expression levels of the upstream regulator of EZH2 expression, miR-101, was shown to interfere negatively with EZH2-mediated RKIP suppression. miR-101 has been shown to directly repress EZH2 and abnormal miR-101 downregulation has been described in breast and prostate cancer lines (42), suggesting that it might be the cause of EZH2 overexpression and RKIP inhibition. The reversal of EZH2-2-mediated RKIP suppression by ectopic expression of miR-101 supports the above hypothesis and identifies miR-101 as one of the positive regulators of RKIP expression through EZH2 inhibition.

Consistent with the detection of bound EZH2 in the RKIP promoter, we found the RKIP promoter marked with H3-K27 trimethylation (me3). In addition to H3-K27-me3, we also detected the presence of H3-K9-me3 repressing marks on the RKIP promoter in DU145 cells, which indicates that in addition to the EZH2 methyltransferase activity other histone methyltransferases also play a role in RKIP suppression. Unexpectedly, activation-related histone modifications H3-K4-me2 and H4-Ac were also detected on the RKIP promoter. Colocalization of activating and repressing marks has been detected in a vast number of gene promoters in the human genome and the gene expression levels was shown to be correlated with both the absolute and relative levels of the activating H3-K4-me3/H4-Ac and the repressive H3-K27-me3/H3-K9-me3 modifications (9). The expression of RKIP is low in cancer cells. However, its expression is not completely shut-off and can be reactivated. Our data, therefore, suggest that these histone modifications together may define the chromatin dynamics important for RKIP repression and derepression in response to different stimuli.

Another important component for EZH2-mediated RKIP suppression was shown to be the presence of HDAC, as cell treatment with the HDAC inhibitor SAHA abolished the suppressive activity of EZH2 on the RKIP promoter. This finding suggested that either SAHA relieves EZH2 catalytic activity on the RKIP promoter, or EZH2-mediated H3K27me3 of RKIP promoter might not be sufficient for promoter silencing and other repressing enzymes including HDACs are necessary to be recruited and interact with the initial PRC2 complex for further chromatin compaction and transcriptional repression of RKIP. This is consistent with the fact that H3K27me3 mark usually serves as an anchorage point for the further recruitment of additional repressive elements on the target promoters such as PRC1, DNMTs, and HDACs (49). Accordingly, we believe that SAHA more likely obstructs the preservation of the suppressive mark on the RKIP promoter rather than the initial EZH2-mediated H3-K27 trimethylation of the RKIP promoter.

To delineate how EZH2 is recruited to the RKIP promoter we examined the involvement of the known PRC2 recruiter HOTAIR. The absence of changes in RKIP expression after ectopic expression of HOTAIR argues that HOTAIR might not
participate in the recruitment process. Contrarily, silencing of Snail, a transcription factor with high expression levels in metastatic breast and prostate tumors and a direct repression on the RIKIP promoter (33), resulted in inhibition of RIKIP promoter enrichment in EZH2 molecules and reversal of EZH2-mediated repression of RIKIP promoter activity. EZH2 recruitment was dependent on the efficient binding of Snail on the E-boxes present on the RIKIP promoter, as mutations in E-boxes abolished the suppressive effect of EZH2 on the RIKIP promoter activity. Thus, our work identifies Snail as one of the possible recruiters of EZH2 to RIKIP promoter and links the reduction of RIKIP expression in metastatic prostate and breast malignancies with the combined suppressive activities of PRC2, HDAC, and Snail on the target promoter. However, these findings motivate further studies to test whether our previously shown Snail-mediated RIKIP suppression requires the presence of EZH2-triggered histone modifications. Furthermore, at present it is not clear whether Snail plays a direct role in tethering EZH2 to the RIKIP promoter.

RIKIP inhibits cancer metastasis at least via inhibition of EMT-associated cancer cell invasion and the corresponding mesenchymal cell phenotype (50). On the other hand, abnormally elevated EZH2 levels promote cell invasion leading to cancer metastasis. We showed that by genetically manipulating the expression levels of RIKIP we were able to reverse the EZH2-mediated cancer cell invasive phenotype. This observation shows one potential mechanism by which EZH2 accelerates cancer cell invasion and metastasis through the inhibition of RIKIP expression. Additional studies in in vivo metastatic models are needed to validate the dependence of EZH2 prometastatic activity on RIKIP suppression.

In conclusion, our findings provide evidence that histone modifications, regulated by PcG proteins, and HDACs, are involved in the transcriptional repression of the metastasis suppressor gene RIKIP, thereby permitting tumor cell expansion. Collectively, these studies confer new data on the molecular mechanism by which EZH2 promotes cancer progression and aggressiveness in breast and prostate malignancies and identifies novel targets for therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Drs. Howard Chang, Yin-Yuan Mo, Danny Reinberg, Yi Zhang, and Var Xiong for reagents and Eric Tsung and Vinod Saladi for technical assistance.

Grant Support

This work was supported by NIH grant (RO1CA133479) to K.C. Yeung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 28, 2011; revised March 7, 2012; accepted March 23, 2012; published OnlineFirst April 13, 2012.

References

Polycomb Protein EZH2 Regulates Tumor Invasion via the Transcriptional Repression of the Metastasis Suppressor RKIP in Breast and Prostate Cancer

Gang Ren, Stavroula Baritaki, Himangi Marathe, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-3546

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/04/13/0008-5472.CAN-11-3546.DC1

Cited articles
This article cites 48 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/12/3091.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/72/12/3091.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.