EZH2 Represses Target Genes through H3K27-Dependent and H3K27-Independent Mechanisms in Hepatocellular Carcinoma

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Abstract
Alterations of polycomb group (PcG) genes directly modulate the trimethylation of histone H3 lysine 27 (H3K27me3) and may thus affect the epigenome of hepatocellular carcinoma (HCC), which is crucial for controlling the HCC cell phenotype. However, the extent of downstream regulation by PcGs in HCC is not well defined. Using cDNA microarray analysis, we found that the target gene network of PcGs contains well-established genes, such as cyclin-dependent kinase inhibitors (CDKN2a), and genes that were previously undescribed for their regulation by PcG, including E2F1, NOTCH2, and TP53. Using chromatin immunoprecipitation assays, we demonstrated that EZH2 occupancy coincides with H3K27me3 at E2F1 and NOTCH2 promoters. Interestingly, PcG repress the expression of the typical tumor suppressor TP53 in human HCC cells, and an increased level of PcG was correlated with the downregulation of TP53 in certain HCC specimens. Unexpectedly, we did not find obvious H3K27me3 modification or an EZH2 binding signal at the TP53 promoters, suggesting that PcG regulates TP53 expression in an H3K27me3-independent manner. Finally, the reduced expression of PcGs effectively blocked the aggressive signature of liver cancer cells in vitro and in vivo.

Implications: Taken together, our results establish the functional and mechanistic significance of certain gene regulatory networks that are regulated by PcGs in HCC.

Visual Overview: http://mcr.aacrjournals.org/content/12/10/1388/F1.large.jpg.

Mol Cancer Res; 12(10); 1388–97. © 2014 AACR.
Materials and Methods

Cell culture

The human HCC cell line HepG2 (wild-type P53) and Hep3B (P53 null) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PLC/PRF5 (human HCC with P53 mutant), SMMC-7721 (human HCC with wild-type P53), and HO-8910 (human ovarian cancer cell) were obtained from the Cell Culture Center of the School of Basic Medicine, Peking Union Medical College (Beijing, China). The cell cultures were performed as previously described (11). These findings provide significant insights into the molecular mechanisms of PcG involvement in hepatocellular carcinogenesis.

Tumor protein p53 (TP53), a typical tumor suppressor, is a major genetic player in the development of various cancers, including HCC (12). The aberrant expression of EZH2 has been associated with P53 alteration and contributed to progression and pool prognosis in human squamous cell carcinoma of the esophagus (13). Activation of TP53 suppresses EZH2 expression through the repression of the EZH2 gene promoter (14). Inversely, the reduction of EZH2 expression by RNA interference increases the expression of TP53 (15). These features point to an interesting negative feedback loop between oncogenic EZH2 and tumor suppressor TP53. Despite the importance of EZH2 negative feedback loop between oncogenic EZH2 and tumor suppressor TP53, a detailed understanding of tumor suppressors by PcG in HCC is not well defined.

In this study, we demonstrate potential tumor-promoting actions of PcG in HCC by the H3K27me3-dependent and H3K27me3-independent regulation of downstream gene expression.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (16). Antibodies are listed in the Supplementary Table S1.

Western blotting and immunofluorescence

Western blotting and immunofluorescence (IF) staining were performed as previously described (16). Antibodies are listed in the Supplementary Table S1.

Cell proliferation assays

Cell growth and viability were evaluated by using either Bromodeoxyuridine (5-bromo-2′-deoxyuridine, BrdUrd) or 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) cell proliferation assays. The cells were plated at 2 × 10^4 cell/mL in 100 μL/well of DMEM media, and the BrdUrd incorporation and detection were performed using a cell proliferation ELISA BrdU kit (Millipore) according to the provided protocol. For the MTT assay, the cells were seeded at a density of 1 × 10^4 cells/well into 96-well plates, and measured as according to the manufacturer’s protocol (Merck). For BrdUrd or MTT detection, the optical density at 450- or 570-nm absorbance was measured by using an ELISA plate reader (Bio-Tek Instruments), respectively. For both assays, the mean absorbance values of at least three independent experiments were used for statistical analysis.

Colonies-forming assays

The colony-forming assays were performed as previously described (16). In brief, 1,000 cells were seeded in 6-well plates for 10 days. Colonies were stained with 0.05% crystal violet, photographed, and counted in triplicate using microscope (Nikon Corporation).

Cell migration assay

Migration assays were performed, as previously described (19), in 24-well transwell plates (Millipore) using uncoated polycarbonate membranes with 8-mm pores. Serum-starved cells were harvested and resuspended at a concentration of 1 × 10^6 cells per 0.2 mL in serum-free medium containing 10% BSA, and added to the upper chamber. At 24 hour after incubation, nonmigrated cells were scraped off the upper side of the filter, and filters were stained with 0.1% crystal violet. The number of migrated cells on the reverse side of the membrane was quantified by average cell counts from six random fields in each well, under a microscope at ×100 magnifications. Each condition was assayed in triplicate wells, and the resulting data were subjected to statistical analysis using the Student t test.

Terminal deoxynucleotidyl transferased UTP nick end labeling assay

Terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL) assay (Roche Applied Science) was used to detect apoptotic cells. Apoptotic cells were determined by...
counting positive-stained cells in five randomly selected fields in each tumor. At least three tumors in each mouse were randomly selected.

**Statistical analysis**

All the statistical analyses were performed using SPSS software version 13.0. Data from cell growth, clonal assays, and cell migration experiments were analyzed by two-tailed Student t tests. The results for parametric variables are expressed as the mean ± SD or the mean ± SEM. In all cases, *P* < 0.05 was considered statistically significant.

**Results**

**A role for EZH2 in hepatic cancer cell growth in vitro and in vivo**

A recent report indicates that knockdown (KD) of EZH2 suppresses HCC tumorigenicity and extrahepatic metastasis in vivo (11); nonetheless, few studies have explored whether other PcG components are involved in the control of the aggressive phenotype of HCC cells. To explore a potential functional implication of PcG components in HCC, we stably transfected HepG2 cells, which are often used to study the behavior of HCC cells (16), with either vectors or constructs expressing one of the 2 to 3 distinct shRNAs that specifically target EZH2, SUZ12, BMI1, or CBX8. Western blotting results indicate that correlated with the reduction level of EZH2, SUZ12, BMI1, and CBX8, the global level of H3K27me3 was remarkably reduced in both PcG KD HepG2 cells (Supplementary Fig. S1A). The CBX8–shRNA3 failed to reduce the expression of CBX8 and was unable to reduce the global H3K27me3 levels (Supplementary Fig. S1A). IF staining results indicate that EZH2 was colocalized with SUZ12 and H3K27me3 at the nucleus (Supplementary Fig. S1B). In addition, the stably ectopic expression of EZH2 substantially increased the levels of SUZ12 and H3K27me3 in HepG2 (Supplementary Fig. S1B). These data indicate that the components of PcG effectively catalyze the global trimethylation of H3K27 in liver cancer cells. Next, the shRNA-mediated KD of EZH2, SUZ12, CBX8, or BMI1 dramatically suppressed the proliferation of HepG2 cells in vitro (Fig. 1A). Control and EZH2 shRNA cells were synchronized in the G0–G1 phase by serum starvation and reentered cell cycle with the restoration of serum culture. Cyclin B1 (CCNB1) protein expression was analyzed by Western blotting, which corresponded to the G2–M checkpoint. We found that both control and EZH2 shRNA HepG2 cells cultured in a serum-free medium had a relatively low level of CCNB1 expression. Following the addition of serum into the

![Figure 1. A role for EZH2 in liver cancer cell proliferation and migration. A, the proliferation rates of HepG2 cells that were stably transfected with shEZH2, shSUZ12, shBMI1, or shCBX8 were estimated using an MTT assay. B, the expression of CCNB1 and pCCNB1 was analyzed by Western blotting in shRNA-mediated KD of EZH2 in HepG2 cells at the indicated time points. C and D, the proliferation of HepG2 cells transiently transfected with Luc, EZH2, or SUZ12 siRNA was estimated using an MTT assay. E, HepG2 cells transiently transfected with Luc or EZH2 siRNA were added to the upper-filter, and 1 × 10⁴ cell migration was determined by transwell chamber assay. F, colony-forming assays were performed using HepG2 cells transfected with Luc or EZH2 siRNA.](image-url)
medium, the protein expression of phospho-CCNB1 dramatically increased at 4 to 24 hours after serum stimulation in control HepG2 cells (Fig. 1B, lanes 3–7). However, in shEZH2 HepG2 cells, the protein expression levels started to increase at 6 hours and returned to a level less than baseline after 12 hours (Fig. 1B, lanes 11 and 12). These data indicate that EZH2 promotes HepG2 cell proliferation at least partly by accelerating G2–M progress. Consistent with this hypothesis, the siRNA-mediated KD of either EZH2 or SUZ12 dramatically suppressed the proliferation of HepG2 cells at the indicated time point, as determined by BrdUrd or MTT incorporation, respectively (Supplementary Fig. S1C; Fig. 1C and D). Because cancer metastasis usually involves enhanced cell migration, we sought to determine whether one of the functions of EZH2 is involved in controlling hepatic cancer cell migration. To this end, we performed a modified transwell chamber assay to evaluate the impact of altered EZH2 expression on HCC cell migration. The results indicate that EZH2 siRNA KD significantly reduced the extent of migration (Fig. 2E, \( P < 0.001 \)) and the colony-forming ability of HepG2 cells (Fig. 2F, \( P < 0.001 \)).

We next set out to explore whether PcGs promote the growth of HepG2 cell–derived tumors in nude mice. The size of the solid tumor was measured after various periods following transplantation. The shRNA-mediated KD of EZH2, SUZ12, CBX8, or BMI1 significantly suppressed tumor growth and weight in subcutaneous xenografts (Fig. 2A and B). In addition, the incidence of apoptotic tumor cells, as assessed by the TUNEL assay, was significantly increased in EZH2, SUZ12, and CBX8 shRNA HepG2 xenografts, with no obvious effect observed for BMI1 shRNA (Fig. 2C and D). Collectively, these results strongly argue that both components of PcG play a crucial role in promoting liver cancer in vitro and in vivo.

Identification of downstream pathway of PcG in HCC

Although recent evidence suggests that the abnormal expression of EZH2 and a high level of global H3K27me3 can be attributed to the aggressive nature of HCC (7, 8), the extent of downstream regulation by PcGs in HCC is not well defined. To obtain a broader understanding of the molecular network of PcG in HCC, cDNA microarray analysis was performed on shRNA KDs of EZH2, SUZ12, BMI1, or CBX8 HepG2 cells. Interestingly, the results indicated that PRC1 (CBX8 and BMI1) and PRC2 (EZH2 and SUZ12) display very different characteristics of gene expression regulation in HCC. We found genes that are targeted by PRC2 without PRC1 and vice versa (Fig. 3A and Supplementary Table S3, GSE 54108). The microarray results indicated that EZH2, SUZ12, BMI1, and CBX8 shRNA revealed a significant coregulation of only 61 or 159 genes in HepG2 cells up or down, respectively (Fig. 3B). Figure 3C highlights the major molecular functions (MF) from the list...
Thus, we investigated the regulatory impact that PcG has on the regulation of TP53 expression. To rule out the effect of the TP53 mutation on its own expression, we first performed a mutation screen of the entire TP53 locus in HepG2 cells. We focused our analysis on novel changes that were predicted to affect the protein-coding sequence. We discovered only one variation, P72R, which has been described in a previous report. P72R affects exon 4 of the TP53 gene in HepG2 and is considered a neutral SNP (20). Thus, we investigated the regulatory relationship between PcG and TP53 using HepG2 cells. The reduction of EZH2 levels by shRNA increased the expression of TP53 and its downstream effector P21 in HepG2 cells, as determined by qRT-PCR and Western blotting (Fig. 4A). Similarly, the KD of EZH2 by three distinct siRNA clearly increased TP53 and P21 mRNA expression (Fig. 4B). Next, the shRNA-mediated KD of CBX8 increased the mRNA expression of TP53 and P21 in HepG2 cells (Fig. 4C). The CBX8–shRNA3 failed to reduce CBX8 expression and was unable to increase the level of TP53 or P21 mRNA (Fig. 4C). In time-course detection, the mRNA expression of TP53 was elevated by the substantial KD of EZH2 or CBX8 siRNA at days 2 and 4 in both HepG2 and SMMC-7721 cells (Supplementary Fig. S2A and S2B). Furthermore, the KD of SUZ12 also increased the protein levels of TP53 and P21 in HepG2 cells (Fig. 4D). EZH2 siRNA KD moderately increased the mRNA level of TP53 in another type of human cancer cell line, lung adenocarcinoma A549 (Fig. 4E). However, we did not observe an obvious effect of EZH2 on repressing TP53 mRNA level in other type tumor cell lines, HO-8910, MCF-7, and Wilms (Supplementary Fig. S2C). This result suggests that EZH2 regulates TP53 expression in a tissue-specific manner. Furthermore, inversely correlated with the reduction of PcGs, the mRNA levels of TP53 and CDKN2A were increased in PcG KD HepG2 xenografts (Fig. 4F). Interestingly, we further found that EZH2 did not regulate the TP53 mRNA expression in Hep3B with p53-null background and PLC/PRF/5 with p53-mutant background.
This result suggests that EZH2 represses TP53 expression depending on wild-type TP53 genomic status.

To gain insight into the role of the PcG in regulating TP53 expression, we performed qRT-PCR analysis on total RNA extracted from HCC liver samples and their matched non-neoplastic counterparts. First, the whole-exome sequencing of TP53 in the primary HCC tissues of 48 patients excluded the typical TP53 mutation, and we further analyzed the mRNA expression of EZH2, SUZ12, CBX8, BMI1, and TP53. These HCCs included cases with wild-type TP53 (19 cases), TP53 with synonymous mutations (three cases), TP53 with the neutral SNP variation (P72R, 21 cases), and TP53 with four previously unreported SNP variations (a total of five cases; data not shown). Using a large cohort of HCC specimens (n = 48), we confirm here that the expression of EZH2, CBX8, BMI1, and SUZ12 were significantly overexpressed in HCC samples compared with their nonneoplastic counterparts (Fig. 5). In addition, we found that the TP53 mRNA expression levels are decreased in HCC specimens compared with adjacent tissues (Fig. 5). Spearman analysis performed using qRT-PCR expression data indicates that the mRNA expression of TP53 was negatively correlated with the upregulation of EZH2, CBX8, and BMI1, but not SUZ12 (Fig. 5). This observation further suggests a potential role for PcGs in the regulation of TP53 in HCC.

We were interested in whether PcG regulated TP53 transcription through an epigenetic mechanism. Thus, we designed ChIP primers for the regions spanning 25-kb upstream and downstream of the transcriptional start site of the TP53 promoter (Supplementary Fig. S3A) and performed ChIP assays using control and shEZH2 HepG2 cells. IgG and H3-ChIP were used as negative and positive controls, respectively (Supplementary Fig. S3B). Unexpectedly, we did not find any obvious H3K27me3 modification or an EZH2-binding signal at the promoter loci using H3K27me3 and EZH2 antibody ChIP assays, respectively (Supplementary Fig. S3C and S3D), suggesting that EZH2 regulates TP53 expression through an H3K27me3-independent mechanism.

PcG directly controls target gene transcription through H3K27me3

In our previous ChIP-on-chip assay (GSE52300), we screened out the H3K27me3-related target gene network...
of PcG, which contains well-established genes, such as CDKN2A, and other previously unstudied genes in liver cancer cells, including E2F1 and NOTCH2 (21). The functional importance of these genes in controlling liver function has been established (22, 23). This finding suggests a preferential epigenetic action of EZH2 on the regulation of the transcription of E2F1 and NOTCH2 in HCC. To test this hypothesis, we performed ChIP assays using three distinct pairs of ChIP primers at E2F1 promoter loci (Fig. 6A). Using an EZH2-specific antibody, we demonstrated that EZH2 was present at the E2F1 promoter in HepG2 cells (Fig. 6B). As expected, the KD of EZH2 by shRNA decreased the binding of EZH2 to the E2F1 loci (Fig. 6B). EZH2–shRNA also led to a significant decrease in the H3K27me3 level at the promoter of the E2F1 locus (Fig. 6C). In addition, we demonstrated that EZH2 and H3K27me3 were present at the promoter of NOTCH2 and that the KD of EZH2 by shRNA led to a severe decrease in the binding of EZH2 and the H3K27me3 levels at the promoters in HCC (Fig. 6D–F). Therefore, we conclude...
that the transcription of \( E2F1 \) and \( \text{NOTCH2} \) were directly repressed by EZH2-mediated H3K27me3 in HCC cells.

**Discussion**

EZH2 promotes HCC cell motility and metastasis by repressing tumor suppressor miRNAs \((11)\), but the specific gene network and epigenetic characteristics are not well established. The \( CDKN2A \) locus encodes two distinct proteins, \( p16^{\text{INK4A}} \) and \( p14^{\text{ARF}} \), both of which have been implicated in a variety of cancers. In acute myeloid leukemia cells, H3K27me3 and EZH2 seem to be distributed primarily at the promoters of \( CDKN2A \) \((24)\). It has also been demonstrated that H3K27me3 is an early-epigenetic event of \( p16^{\text{INK4A}} \)-silencing in hepatoma cells \((25)\). Our previous results indicated that EZH2 and H3K27me3 cooccupied the promoter of \( CDKN2A \) \((21)\), indicating that H3K27me3-dependent repression is a common feature at \( INK4/ARF \) promoters, including HCC. EZF1, a tumor suppressor, binds to the promoter of \( E2F1 \) and \( EED \) and functionally controls their expression in prostate cancer \((26)\). An interesting negative-feedback loop was found in the present study, in which EZH2 repressed \( E2F1 \) expression by binding to the promoter of \( E2F1 \) in HCC. Our findings highlight H3K27me3 remodeling as an interesting new direction in the elucidation of the \( E2F1 \) transcriptional regulation mechanism.

In agreement with a previous report \((27)\), we identified genes that are targeted by PRC2 without PRC1 and vice versa. Although EZH2 and SUZ12 occupy the promoter regions of thousands of human genes, only 340 genes share H3K27me3 in HepG2 \((21)\). This result suggests that PcG regulates gene expression in both an H3K27me3-dependent and an H3K27me3-independent manner. The mutation-induced inactivation of \( TP53 \), which is one of the important tumor suppressors, has frequently been associated with a variety of human cancers, including HCC. Nevertheless, the transcriptional repression of \( TP53 \) by critical effectors in HCC has not been well defined. In the present study, we found that the expression of \( TP53 \) was dramatically repressed by both PRC1 and PRC2, and a significant negative correlation between the expression of PcGs and \( TP53 \) was found in primary HCC specimens. However, we failed to observe an obvious impact of H3K27me3 on \( TP53 \) expression, suggesting that PcG regulates \( TP53 \) expression in an H3K27me3-independent manner in HCC. This activity is presumably dependent on the direct regulation of \( CDKN2A \) or \( E2F1 \), which are known upstream regulators of the \( TP53 \) pathway \((28, 29)\). The ARF product functions as a stabilizer of the p53 protein, which was responsible for the degradation of p53 \((28)\). \( CDKN2A \) product interacts with and inhibits the oncogenic action of MDM2 by blocking MDM2-induced degradation of p53 and enhancing p53-dependent transactivation and apoptosis \((28)\). Confusingly, we found that both protein and mRNA levels of \( TP53 \) were substantially repressed by PcG in HCC cells. A previous report found a CCGTC-binding factor (CTCF) that binds at the \( P53 \) promoter and protects the \( P53 \) gene promoter against repressive histone marks \((30)\). Interesting findings have shown that EZH2 and SUZ12 were directly bound at \( CTCF \) promoter loci with H3K27me3 modification and that the increased accumulation of H3K27me3 at the \( CTCF \) promoter is associated with the reduction of \( CTCF \) in HCC specimens \((21)\). These results suggest that PcG regulates \( P53 \) expression in HCC at least partly through \( CTCF \) in HCC. Functionally and mechanistically significant work remains to be done to identify the competitive functions between PcG and \( CTCF \) in HCC. \( TP53 \) was frequently mutated in HCC and is a major genetic event for the development of HCC. Our results emphasize the interesting fact that, independent of genetic mutation, the transcriptional silencing of \( TP53 \) by PcG contributes to the aggressive nature of HCC. Recently, Lu and colleagues \((31)\) reported that p53 is not the target of EZH2 in nasopharyngeal carcinoma. Similar to this, we did not observe an obvious regulation effect of EZH2 on repressing \( TP53 \) mRNA level in other type tumor cell lines, including HO-8910, MCF-7, and Wilm. This result suggests an interesting tissue-specific regulation profile of EZH2 on \( TP53 \) expression. Conversely, Tang and colleagues \((14)\) reported that \( P53 \) represses EZH2 transcription through binding to its promoter locus in primary human fibroblasts. These findings suggest a potential feedback loop of tumor suppressor \( P53 \) and tumor promoter gene \( EZH2 \), an important interplay governing tumor activation and suppression in the development of HCC. This process is similar to our previous reports that the interplay between oncogene K-Ras and tumor suppressor menin plays an important role in regulating the development of lung cancer \((17)\). However, it is unclear whether the activated \( P53 \) suppresses the expression of \( EZH2 \) in HCC. The link between \( P53 \) and \( EZH2 \) in controlling aggressive phenotype of HCC needs further investigation.

Alteration of PcG components was observed in various cancers such as melanoma, lymphoma, and breast and prostate cancer, leading to the hypothesis that PcG has tumor-promoting functions \((32)\). The overexpression of EZH2 promotes the growth and cell invasion of immortalized human mammary epithelial cells \((33)\), and the reduction of EZH2 expression inhibits prostate cancer cell proliferation \textit{in vitro} \((34)\). Further studies demonstrate that the oncogenic effect of EZH2 is linked to the H3K27me3-dependent repression of the tumor suppressor genes, including \( E-cadherin, \) RUNX3, \( INK4/ARF \) (also known as \( CDKN2A \); refs. \(24, 35, 36)\), and others. Here, we found that the alterations of PcG proteins directly modulate H3K27me3 and may thus affect the HCC epigenome, which is crucial for HCC cell growth both \textit{in vitro} and \textit{in vivo}. We also observed that the reduction of EZH2 expression induces G2–M cell-cycle arrest in HCC cells. This finding is consistent with a previous report that \( CDKN2A \) induces G2 arrest in a \( p53 \)-independent manner by preventing the activation of cyclin B1/CDC2 complexes \((37)\). Our findings demonstrate a potential tumor-
promoting action of EZH2 by directly or indirectly regulating the expression of tumor suppressors with critical functions in HCC, at least partly through the CDKN2A–TP53–P21 pathway. These findings also reveal the EZH2 pathway as a potential target for the treatment of EZH2-positive human HCC with EZH2 inhibitors, such as GSK126, which is a specific small molecular inhibitor of the catalytic SET domain of EZH2 (38). Significant work remains to identify the major target of PcGs in HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.B. Gao
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.B. Gao, Q.-L. Zheng, G.-H. Jin
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.B. Gao, K.-L. Li

Acknowledgments
We appreciate the valuable comments from other members of our laboratories.

Grant Support
This work was supported by the grants from the Natural Science Foundation of China (81101763), to S.-B. Gao; 91229111 and 91272719, to G.-H. Jin; 813101754, to B. Xu; and the Natural Science Foundation of Fujian Province (2011J06016 to G.-H. Jin).

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Received January 20, 2014; revised April 30, 2014; accepted May 19, 2014; published OnlineFirst June 10, 2014.

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Molecular Cancer Research

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