Abstract

Infiltration of tumors with effector T cells is positively associated with therapeutic efficacy and patient survival. However, the mechanisms underlying effector T-cell trafficking to the tumor microenvironment remain poorly understood in patients with colon cancer. The polycomb repressive complex 2 (PRC2) is involved in cancer progression, but the regulation of tumor immunity by epigenetic mechanisms has yet to be investigated. In this study, we examined the relationship between the repressive PRC2 machinery and effector T-cell trafficking. We found that PRC2 components and demethylase JMD3-mediated histone H3 lysine 27 trimethylation (H3K27me3) repress the expression and subsequent production of Th1-type chemokines CXCL9 and CXCL10, mediators of effector T-cell trafficking. Moreover, the expression levels of PRC2 components, including EZH2, SUZ12, and EED, were inversely associated with those of CD4, CD8, and Th1-type chemokines in human colon cancer tissue, and this expression pattern was significantly associated with patient survival. Collectively, our findings reveal that PRC2-mediated epigenetic silencing is not only a crucial oncogenic mechanism, but also a key circuit controlling tumor immunosuppression. Therefore, targeting epigenetic programs may have significant implications for improving the efficacy of current cancer immunotherapies relying on effective T-cell-mediated immunity at the tumor site. Cancer Res; 76(2); 275–82. ©2015 AACR.

Introduction

Effector T cells are indispensible for protective tumor immunity and subsequent production of Th1-type chemokines CXCL9 and CXCL10, mediators of effector T-cell trafficking. Moreover, how Th1-type chemokine expression is controlled in the tumor and subsequently how it affects effector T-cell trafficking into the human cancer microenvironment.

Epigenetic regulation, including histone modifications, can mediate gene repression (5). One epigenetic repression machinery involves the polycomb repressive complex 2 (PRC2), which trimethylates histone 3 lysine 27 (H3K27me3; refs. 6, 7). H3K27me3 is a repressive histone mark associated with facultative heterochromatin that functions to recruit regulatory proteins to repress gene transcription (7). PRC2 component, enhancer of zeste homolog 2 (EZH2), is highly expressed in multiple cancers (8, 9). Its role in cancer cell proliferation, invasiveness, and differentiation has been widely studied (8, 9). However, it is not known whether EZH2 and the other PRC2 proteins are involved in the regulation of human T-cell tumor trafficking, and, in turn, tumor immunity. Cancer epigenetic studies suggest that an abnormal evolution of repressive epigenetic marks including histone modification may directly contribute to cancer development and progression (10, 11). However, the nature of cancer epigenetic repression is involved in the control of cancer immunity remains unanswered. Given the relevance of effector T cells and their homing in antitumor immunity (1, 2, 4), we hypothesized that the cancer epigenetic repression machinery mediates Th1-type chemokines and, in turn, alters effector T-cell tumor migration and effective antitumor immunity. The validation of this hypothesis will lead to a notion that cancer epigenetic repression is a novel immune evasive mechanism and the repressive machinery may be a target for novel cancer immunotherapy.
Thus, in the current work, we tested this hypothesis in the context of human colon cancer at the molecular and clinical level.

Materials and Methods

Human subjects and colon cancer tissues
Patients diagnosed with colon carcinomas were recruited in the study. All usage of human subjects in this study was approved by local Institutional Review Boards. Six to 9 fresh colon tissues were collected from patients with colon cancer and ulcerative colitis. Primary colon cancer cells, colon epithelial cells, and all the in vitro functional assays were performed with single cells from fresh colon cancer and colitis tissues as previously described (12). In addition, patients with colorectal carcinoma were evaluated from datasets in Oncomine.org.

Cell culture
Primary colon cancer cell lines (C1) were established from fresh colon cancer tissue. Routine short tandem repeat analysis is done to confirm the uniqueness of the primary line (12). DLD-1 and SW480 cell lines (ATCC) were used in the experiments. Single colon epithelial cells were made from fresh colon tissues from patients with colon cancer and ulcerative colitis. Colon cancer cells were treated with recombinant IFNγ (R&D Systems), DZNep (Sigma), GSK126 (GlaxoSmithKline), and GSK-J4 (GlaxoSmithKline), for different time points and concentrations.

Real-time reverse transcriptase PCR
RNA was isolated from the cells by TRIzol (Ambion) and converted to cDNA using reverse transcriptase PCR (cloned AMV reverse transcriptase, Invitrogen). The mRNA was then quantified by real-time RT-PCR using StepOnePlus (Applied Biosystems).

 Lentiviral transduction and transfection
The lentiviral vectors, pGIPZ or pGreen, encoding gene-specific shRNAs were used to transduce colon cancer cells to establish stable cell knockdowns. Lentiviral shRNAs (Supplementary Table S2) were from the Vector Core at the University of Michigan or provided by Arul Chinnaiyan (University of Michigan, Ann Arbor, MI; ref. 8). The lentiviral transduction efficiency was confirmed by GFP, which was coexpressed by the lentiviral vector. The knockdown efficiency was assessed by Western blotting and real-time PCR. For transfections, Eugene HD (Promega) was used to transfect colon cancer cells with PKH3 (empty vector) and pCMV HA JMJD3 (Addgene; #24167) according to the manufacturer’s protocol. The overexpression was confirmed by Western blotting.

ELISA
The protein levels of CXCL9 and CXCL10 were detected by ELISA (R&D Systems) from the supernatants of treated colon cancer cells or single cells from fresh colon cancer and colitis tissue.

Western blot analysis
Western blotting was performed with specific antibodies against Histone H3 (9715, Cell Signaling Technology), β-actin (A5441, Sigma), EZH2 (612667, BD Biosciences), SUZ12 (46264, Santa Cruz Biotechnology), EED (28701, Santa Cruz Biotechnology), and H3K27me3 (07449, Millipore). Signals were detected by ECL reagents (GE Healthcare).

T-cell migration assays
In vitro migration assay was performed in a Transwell system with a polycarbonate membrane of 6.5 mm diameter with a 3 μm pore size as described (13, 14). Activated T cells were treated with anti-human CXCR3 or isotype, and added to the top chamber. Supernatant from the cultured colon cancer cells was added to the bottom chamber. After incubation at 37°C for 12 hours, the phenotype and number of T cells in the top and bottom chambers were determined by FACS (LSRII, BD Biosciences).

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was performed and as previously described (12, 15). Crosslinking was performed with 1% formaldehyde or 1% paraformaldehyde for 10 minutes. Sonication was performed with the Misonix 4000 water bath sonicator unit at 15% amplitude for 10 minutes. Protein/DNA complex was precipitated with specific antibodies against H3K27me3 (6002, Abcam) and IgG control (Millipore). ChIP-enriched chromatin was used for RT-PCR with SYBR Green Master Mix, normalizing to input. Specific primers are listed in supplementary information (Supplementary Table S1; ref. 16).

Statistical analysis
Dependent on data distribution and experimental design, paired or unpaired Student t test and Mann—Whitney U tests were used. Correlation coefficients (Spearman correlation) denoted by r, together with a P value, were computed to measure correlation between different genes. Survival functions were estimated by Kaplan—Meier methods using genes classified as high or low based on mean or median expression values. Data was censored at the last follow-up for patients who were disease-free or alive at the time of analysis. All analyses were done using SAS 9.3 software. P < 0.05 was considered significant.

Results

Inverse correlation exists between Th1-type chemokines and PR2 in colon cancer
Th1-type chemokines CXCL9 and CXCL10 mediate effector CD8+ T-cell tumor trafficking. CD8+ T-cell tumor infiltration is associated with improved cancer patient survival. Th1-type chemokines are correlated with effector T-cell density in some human tumors, including colon cancer, and positively associated with cancer patient survival (17, 18). However, it is poorly understood how Th1-type chemokine expression is controlled in human colon cancer. We found that the levels of CXCL9 and CXCL10 mRNA (Fig. 1A) and protein (Fig. 1B) were higher in colitis colon compared with colon cancer tissue. The chemokines CXCL9 and CXCL10 can be stimulated by IFNγ. When we treated single epithelial cells from the microenvironments of colon cancer, adjacent colon tissue, and colitis with IFNγ, the levels of CXCL9 and CXCL10 were higher in adjacent tissues (Fig. 1C) and colitis tissues (Fig. 1D) than colon cancer tissues. Patients with ulcerative colitis are at increased risk for developing colorectal cancer. The
data suggest that Th1-type chemokine expression in colon cancer may evolve and become repressed when going from inflammatory tissue to cancer.

The PRC2 complex (including EZH2, embryonic ectodermal development (EED), and suppressor of zeste 12 homolog (SUZ12)) represses gene transcription through methylation of H3K27 (6, 19). We hypothesized that the PRC2 complex repressed Th1-type chemokines in colon cancer. To test this hypothesis, we analyzed a colon cancer tissue microarray from Oncomine.org (20) for potential correlations between PRC2 and Th1-type chemokines, CXCL9 and CXCL10. In support of our hypothesis, we found significant negative correlations between the PRC2 complex components and CXCL9 and CXCL10 (Fig. 1E–I). The data suggests that the high levels of PRC2 may control and repress Th1-type chemokine expression in colon cancer.

**PRC2 represses Th1-type chemokine expression in colon cancer**

Given the inverse relationship between PRC2 and Th1-type chemokines (Fig. 1), we investigated whether PRC2 machinery represses Th1-type chemokine expression in colon cancer. We initially examined the potential effect of 3-Deazaneplanocin A (DZNep), a pharmacologic PRC2 inhibitor (19), on Th1-type chemokine expression in human colon cancer cells. In response to IFNγ treatment, we observed that treatment with DZNep led to higher levels of CXCL9 and CXCL10 expression in a primary colon cancer cell line (C1; Fig. 2A and B), DLD-1 (Fig. 2C and D), and SW480 (Fig. 2E and F) colon cancer cells. As expected, DZNep
reduced the expression of EZH2, SUZ12, and EED (Supplementary Fig. S1A). However, the effect of DZNep was specific to chemokines as other IFNγ-associated genes, including IFNγ receptor (IFNGR2; Supplementary Fig. S1B) and HLA-B (Supplementary Fig. S1C) was not affected.

We next genetically knocked down EZH2 expression with lentivirus-based shRNA for EZH2 (shEZH2) in primary colon cancer C1 cells. shEZH2 specifically reduced the expression of EZH2 and SUZ12 (Supplementary Fig. S1D), and resulted in elevated Th1 type chemokine mRNA (Fig. 2G and H) release in response to IFNγ stimulation (Fig. 2G and H). In addition, specific knockdown of EZH2 with shSUZ12 in DLD-1 cells (Supplementary Fig. S1E and S1F) also increased Th1-type chemokine protein expression (Supplementary Fig. S1F). Similar results were observed in shEED-expressing DLD-1 cells (Supplementary Fig. S1G). Thus, the PRC2 complex mediates Th1-type chemokine repression in colon cancer cells.

Th1-type chemokine repression is mediated via H3K27 methylation

The PRC2 complex mediates H3K27 methylation (6, 7). We next examined whether Th1-type chemokine repression mediated by PRC2 depends on H3K27me3 changes at the promotor level of the chemokine genes. To test this possibility, we performed specific ChIP assay to detect H3K27me3 marks, based on the ENCODE database for H3K27me3 in GM12878 cells (Supplementary Fig. S2A; ref. 16). We observed high levels of H3K27me3 marks on the promoter of CXCL9 (Fig. 3A), the intergenic regions in between the CXCL9 and CXCL10 genes (Fig. 3B) and the promoter of CXCL10 (Fig. 3C). Knockdown of SUZ12 with shSUZ12, importantly, removed H3K27me3 on these areas (Fig. 3A–C) and increased Th1-type chemokine expression (Supplementary Fig. S1F). GAPDH and HOXB1 were used as a negative and positive control, respectively (Fig. 3D). Thus, H3K27me3 may be involved in the Th1-type chemokine gene silencing in colon cancer.

GSK126 is a highly selective, potent small-molecule inhibitor of EZH2 methyltransferase activity (21). GSK126 treatment (5 μmol/L) abolished the global level of H3K27me3 without inhibiting EZH2 (Supplementary Fig. S2B). GSK126 treatment led to higher levels of IFNγ-induced CXCL10 (Fig. 3E) expression in primary colon cancer C1 cells. Similar results were observed in DLD-1 cells (Supplementary Fig. S2C and S2D).

Jumonji C (JmjC) domain–containing protein, JMJD3, is an H3K27-specific demethylase (22). As an activator of gene expression, ectopic expression of JMJD3 increased Th1-type chemokine expression (Fig. 3F). GSK-J4, a small molecular catalytic site inhibitor, selectively targets the H3K27-specific JmjC demethylase subfamily (23). GSK-J4 treatment led to reduced CXCL9 and CXCL10 expression in primary colon cancer C1 cells (Fig. 3G and H). Thus, H3K27me3 specific methyltransferase and demethylase regulate Th1-type chemokine repression in colon cancer cells.

PRC2 affects T-cell migration toward colon cancer

Next, we examined whether the PRC2 complex can affect T-cell migration via controlling CXCL9 and CXCL10 expression. We

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**Figure 2.**

PRC2 represses Th1-type chemokine expression in colon cancer. A–F, effects of DZNep on IFNγ-stimulated Th1-type chemokine production. CXCL9 and CXCL10 mRNA levels were detected by real-time PCR in C1 cells (A and B), DLD-1 (C and D), and SW480 (E and F) cells treated with IFNγ (0.5 or 10 ng/mL) and DZNep (0.5, 2, or 5 μmol/L; three independent experiments are shown; *, P < 0.05). G and H, effects of shEZH2 on IFNγ-mediated chemokine production. C1 cells expressing shEZH2 or scr vector were cultured with IFNγ (0.5 ng/mL), and CXCL9 (G) and CXCL10 (H) mRNA was detected by real-time PCR (three independent experiments are shown; *, P < 0.05); fold change shown relative to Scr with IFNγ.
collected supernatants from colon cancer cells transfected with shEZH2 and control lentiviral vectors. We showed that CD4+ (Fig. 4A) and CD8+ (Fig. 4B) T cells more efficiently migrated towards shEZH2 supernatant than control. The migration was blocked by monoclonal antibody (mAb) against CXCR3, the receptor for CXCL9 and CXCL10 on T cells (Fig. 4A and B). Thus, the PRC2 component, EZH2, controls T-cell trafficking toward colon cancer-derived CXCL9 and CXCL10.

Finally, we assessed the clinical relevance of this phenomenon by examining the association of chemokines and the PRC2 complex with patient survival. We first performed survival analysis on chemokines by combining multiple datasets of colorectal carcinoma patients from oncomine.org. The patients were stratified in groups based on the mean expression level of each gene. When we divided patients with high or low expression of both CXCL9 and CXCL10, high CXCL9/CXCL10 associated with better patient overall survival (Supplementary Fig. S3A). As a confirmation, we also observed positive correlation with CD8 and CXCL9 and CXCL10 in different cohorts. It suggests that higher chemokines may lead to higher CD8+ T-cell tumor infiltration (Supplementary Fig. S3B). To examine the association between the 3 immune gene signature (CD8, CXCL9, and CXCL10) and the 3 PRC2 gene components (EZH2, EED, and SUZ12) with patient survival, we divided patients with high or low expression of these genes. We found that high CD8/CXCL9/CXCL10 (Fig. 4C) and low EZH2/SUZ12/EED (Fig. 4D) associated with better patient overall survival. We also analyzed the relationship between PRC2 complex component transcripts and CD4 and CD8 mRNA in patients with colorectal cancer. We found that CD4 and CD8 mRNA expression negatively correlated with the PRC2 complex components, EZH2, SUZ12, and EED mRNA (Fig. 4E-G). Altogether, the data suggests that PRC2 controls T-cell trafficking via repressing Th1-type chemokines and impacts colon cancer pathology.

**Discussion**

Epigenetic changes are biologically vital and often linked to cancer proliferation, progression, and metastasis (8, 9, 24, 25). Our studies have shown that the PRC2 components in colon cancer cells silence the tumor production of Th1-type chemokines, potentially restrain effector T-cell tumor infiltration, and in turn lessen anticancer immunity. Thus, PRC2 and H3K27me3-mediated Th1-type chemokine silencing is a novel immune evasion mechanism in human colon cancer. We propose a unifying mechanistic model of cancer in which epigenetic silencing plays both biologic and immunologic roles in supporting tumor progression.

Previous reports show that the expression of CXCR3 and CXCL10 correlates with metastasis in certain cancer including colon cancer (26–28). However, whether the expression of CXCR3 in the tumor cells leads to CXCL10 dependent metastasis is not fully elucidated. Nonetheless, our data shows that there is a positive correlation between CD8 and CXCL9 and CXCL10, and...
the expression of CD8, CXCL9, and CXCL10 is positively associated with colon cancer patient survival. In support of this observation, it has been shown that CD8\(^+\) T cells and Th1-type chemokines positively predict colon cancer patient outcome (4, 29–31).

A critical question is what drives the epigenetic changes in the first place. Several reports suggest miRNA involvement of EZH2 regulation (particularly loss of miRNA101) in prostate cancer (8). Further studies will be needed to demonstrate the drivers of this repression in cancer cells versus normal epithelium and whether epigenetic changes are one of the earliest events in cancers, leading to immune evasion. We assume that this mechanism progressively evolves in cancer cells as colon inflammatory tissues express high levels of Th1-type chemokines. In contrast to irreversible genetic mutations, epigenetic alterations can be manipulated, making them a valuable target for therapy (32). To this end, it is crucial to understand the immune-associated epigenetic mechanisms in the human cancer and to dissect how different epigenetic modifiers differentially affect cancer immune signature gene expression. In this regard, we have noticed that GSK126, a compound acting as a direct histone methyltransferase (HMT) inhibitor and DZNep, a compound acting as an indirect HMT inhibitor, have shown certain differences in potencies in promoting cancer Th1-type chemokine expression. Perhaps additional mechanisms of DZNep, such as proteasomal degradation of PCA2 subunits, inhibition of other methylations, and reactivation of thioredoxin-binding protein 2 (TXNIP), which causes disruption of PCA2, could explain this difference (33).

Current cancer immunotherapies (34–36) and classic therapies (1, 3) largely rely on efficient T-cell tumor trafficking and

**Figure 4.**

PRC2 affects CD8\(^+\) T-cell migration toward colon cancer. A and B, effect of shEZH2 on migration of CD4\(^+\) (A) and CD8\(^+\) (B) T cells. shEZH2 and control vector expressing DLD-1 cells were treated with 5 ng/mL IFN\(\gamma\) and the supernatants were collected for T-cell migration assay. Activated T cells were subject to the migration toward the supernatants in the presence of anti-human CXCR3 or control. The migrated T cells were assessed by flow cytometry analysis. One representative of three independent experiments is shown. C, the association between CD8/CXCL9/CXCL10 transcripts and survival in patients with colorectal carcinoma (Smith colorectal, Smith colorectal 2, Jorissen Colorectal 3, and Staub colorectal). The analyses were conducted by combining multiple databases from Oncomine (oncomine.org; \(n = 185, P = 0.0089\)). D, the association between EZH2/SUZ12/EED transcripts and survival in patients with colorectal carcinoma (Smith colorectal, Smith colorectal 2, Jorissen Colorectal 3, and Staub colorectal). The analyses were conducted by combining multiple databases from Oncomine (oncomine.org; \(n = 105, P = 0.037\)). E–G, correlation between PRC2 components and CD4 and CD8A mRNA in patients with colorectal carcinoma. Numbers represent \(r\) values with significance (\(P < 0.05\)). Spearman analysis for correlation was conducted from multiple databases (Khambata-Ford Colon, Staub Colorectal, Smith Colorectal 2, TCGA, and Jorissen Colorectal 3) from Oncomine (oncomine.org). NS, not significant.
T-cell–mediated tumor immunity. Epigenetic reprogramming may unlock Th1-type chemokine repression, transform the tumor from poor T-cell infiltration to rich T-cell infiltration, and, ultimately, improve the effectiveness of any given cancer therapy.

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

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References

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PRC2 Epigenetically Silences Th1-Type Chemokines to Suppress Effector T-Cell Trafficking in Colon Cancer


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