Inhibition of histone methyltransferase EZH2 depletes leukemia stem cell of mixed lineage leukemia fusion leukemia through upregulation of p16

Koki Ueda,1 Akihide Yoshimi,1,2 Yuki Kagoya,1 Satoshi Nishikawa,1,3 Victor E. Marquez,4 Masahiro Nakagawa1 and Mineo Kurokawa1,2

1Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, Tokyo; 2Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, Tokyo; 3Biologics Research Laboratories, Kyowa Hakko Kirin, Tokyo, Japan; 4Chemical Biology Laboratory Center for Cancer Research, Scientist Emeritus, NCI-Frederick, Frederick, Maryland, USA

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Correspondence
Mineo Kurokawa, Department of Hematology and Oncology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel: +81-3-5800-9092; Fax: +81-3-5840-8667; E-mail: kurokawa-tky@umin.ac.jp

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Leukemia stem cells (LSC) are resistant to conventional chemotherapy and persistent LSC after chemotherapy are supposed to be a major cause of relapse. However, information on genetic or epigenetic regulation of stem cell properties is still limited and LSC-targeted drugs have scarcely been identified. Epigenetic regulators are associated with many cellular processes including maintenance of stem cells. Of note are polycomb group proteins, because they potentially control stemness, and can be pharmacologically targeted by a selective inhibitor (DZNep). Therefore, we investigated the therapeutic potential of EZH2 inhibition in mixed lineage leukemia (MLL) fusion leukemia. Intriguingly, EZH2 inhibition by DZNep or shRNA not only suppressed MLL fusion leukemia proliferation but also reduced leukemia initiating cells (LIC) frequency. Expression analysis suggested that p16 upregulation was responsible for LICs reduction. Knockdown of p16 canceled the survival advantage of mice treated with DZNep. Chromatin immunoprecipitation assays demonstrated that EZH2 was highly enriched around the transcription-start-site of p16, together with H3K27 methylation marks in MLL/ENL and Hoxa9/Meis1 transduced cells but not in E2A/HLF transduced cells. Although high expression of Hoxa9 in MLL fusion leukemia is supposed to be responsible for the recruitment of EZH2, our data also suggest that there may be some other mechanisms independent of Hoxa9 activation to suppress p16 expression, because expression levels of Hoxa9 and p16 were not inversely related between MLL/ENL and Hoxa9/Meis1 transduced cells. In summary, our findings show that EZH2 is a potential therapeutic target of MLL fusion leukemia stem cells.

It has been reported that epigenetic changes including histone modification are broadly linked to leukemogenesis.

1–3) Many drugs modulating histone acetylation have been available for patients with hematological malignancy and they have shown a certain level of anti-tumor effect.4–6) Although aberrant histone methylation or mutations of genes concerning histone methylation have been reported in patients with various types of leukemia,7) drugs targeting histone methylation have not been applied in clinical settings so far. As for aberrant histone methylation, mixed lineage leukemia (MLL) rearranged leukemia is associated with poor prognosis and has been closely investigated over the decades.8,9) For instance, it has been demonstrated that Disruptor of Telomere Silencing 1-like (Dot1L)-mediated di-methylation of Histone H3 lysine 79 (H3K79) is aberrantly methylated in MLL fusion leukemia.10,11) Bernt et al. have shown that Dot1L itself is indispensable for leukemia initiation and maintenance,11) and Dot1L appears to be an ideal target for MLL fusion leukemia. However, pharmacological inhibition of Dot1L resulted in the limited effect against MLL-AF9 leukemia mouse models.12) Furthermore, depletion of Dot1L is considered to suppress normal hematopoiesis.11,13) Thus identification of other therapeutic targets for MLL fusion leukemia is highly anticipated.

Enhancer of Zeste Homolog 2 (EZH2) is the main component of polycomb repressive complex 2 (PRC2) which catalyzes tri-methylation of histone H3 lysine 27 (H3K27) and recruits polycomb repressive complex 1 (PRC1) to the promoter region of target genes.14,15) A growing body of evidence demonstrates that EZH2 and other polycomb group proteins are frequently mutated in patients with hematological malignancy including MLL fusion leukemia.10,16–25) Moreover, inhibitor of EZH2, 3-deazaaneplanocin A (DZNep), was shown to be effective to kill leukemia cells.26,27) However, the effect of EZH2 inhibitor in vivo is not fully investigated.

Here we show that EZH2 plays a crucial role in maintenance of MLL fusion leukemia and that inhibition of EZH2 can specifically target leukemia initiating cells (LIC) of MLL fusion leukemia.

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Materials and Methods

Leukemia cell lines. Human leukemia cell lines K562, HEL, Kasumi-1, ME-1, MV-4-11 and MOLM13 were cultured in Roswell Park Memorial Institute 1640 (RPMI1640) medium (Wako 189-02025) with 20% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS).

Plasmid construction. The plasmids pMSCV-neo-FLAG-MLL/ENL, pMSCV-IRE-GFP-MLL/AF9, pMXs-neo-E2A/HLF and pMYs-Hoxa9-IRE-Meis1 have been described previously.(31) pMSCV-Tel-PDGF-beta-IRE-AML1/ETO (TPAE) is a gift from Dr. Michael H. Tomasson (Washington University School of Medicine, St. Louis). Mouse p16 DNA was synthesized by PCR using primers (Forward, 5’-GCGAATTCAC-CATGGGTCGCAGGTTCTTGG-3’; Reverse, 5’-GCCCCTGAGCAGCTACTTGTCGTCACTGTTTGTAGTCTTTTGCGCCCGTCGGTCTGG-3’) and cDNA extracted from mouse total bone marrow cells as a template. The product was inserted into pMYs-IRE-GFP at EcoRI and XhoI site.

Short hairpin RNA (shRNA). Specific siRNA oligos targeting murine EZH2 and p16 mRNAs were designed as indicated by Takara Bio (Shiga, Japan) and cloned into pSIREN-RetroQ (harboring puromycin resistant gene) and pSIREN-ZsGreen vectors. Control shRNA is a nonfunctional construct provided from Takara Bio. The target sequences are as follows; EZH2: 5’-ggtggagacgaaactgtt-3’; p16: 5’-caggaaaggaatggcatga-3’.

Retrovirus transduction. Retrovirus transduction was performed to produce immortalized cells, to transplant pre-leukemic cells to mice, and to transduce shRNA into cells. To produce retrovirus, Plat-E packaging cells(39) were transiently transfected with retroviral constructs as described previously.(30) To generate immortalized cells, at least three times of passages were performed in methocult M3434 semisolid medium (Stemcell technolgies, Tokyo, Japan).

Transplantation assay. All transplantation assays were performed using secondary transplantation of leukemic cells. To obtain primary leukemic cells, MLL/ENL, MLL/AF9 or TPAE oncogene was transduced into c-Kit positive bone marrow (BM) cells which were isolated from 8 to 10 week-old C57BL/6 mice (Sankyo Laboratory Service, Tokyo, Japan) with anti-CD117 magnetic beads using the autoMACS apparatus (Miltenyi Biotec, Tokyo, Japan) according to the manufacturer’s instructions. Recipient mice were sublethally irradiated (7.5 Gy) and injected with these pre-leukemic cells. After several months, primary leukemic cells were collected from BM and utilized for transplantation assays.

Flow cytometry. Cell sorting and flow cytometry analysis were performed on FACS AriaII (BD, Tokyo, Japan). Leukemic cells flushed from the tibia, femur, ilium and vertebrae were isolated by density centrifugation using Histopaque-1083 (Sigma-Aldrich Japan, Tokyo, Japan) and prepared for GFP positive cell sorting or leukemic granulocyte macrophage progenitor (L-GMP) analysis. For L-GMP analysis, cells were stained with CD3, CD4, CD8, Gr-1, Ter119 and B220, followed by visualization with streptavidin-APC-Cy5. stained cells were analyzed as described previously. (31)

Quantitative real-time polymerase chain reaction. Real-time PCR was performed using the LightCycler 480 (Roche Diagnostics, Tokyo, Japan) following the manufacturers’ instructions. Results were normalized to GAPDH levels. PCR primers used for quantitative PCR were shown in Table S1.

Western blotting. For protein detection, cells were lysed with lysis buffer (10 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 0.1% Aprotinin, 1 mM Na2VO4, 50 mM β-glycerophosphate, 2.5 mM phenytoimethylsulfonylfluoride, and complete protease inhibitor cocktail [Roche Diagnostics]). Lysates were boiled with sample buffer (0.1% Tris-HCl, 4% SDS, 20% Glycerol, 7.5% bromophenol blue) at 100 degrees Celsius for 5 min. For histone detection, 1 x 10^6 cells were lysed with 100 μl of lysis buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.15 M MgCl2, 0.65% NP-40, and complete protease inhibitor cocktail) and centrifuged. The deposit were treated with 100 μl of 0.2 M sulfuric acid and centrifuged for 10 min. The supernatant were soluble in 25 μl of 100% trichloroacetic acid and centrifuged for 10 min. The deposit were washed with acetone and boiled with sample buffer at 100 degrees Celsius for 5 min. The samples were subjected to sodium dodecylsulfate-polyacrylamide gel (10% for protein detection and 15% for histone detection) electrophoresis (SDS-PAGE) and analyzed by western blotting. Antibodies used for Western blotting are shown in Table S2.

Chromatin immunoprecipitation (ChIP). Cells were cross-linked, lysed, sonicated and immunoprecipitated as previously described.(32) Antibodies used for ChIP are shown in Table S2. Bound DNA fragments were eluted and quantified by subsequent quantitative real-time polymerase chain reaction (qRT-PCR). The sequences and locations for mouse p16 gene of primers used for PCR are shown in Table S3.

Results

EZH2 inhibitor is therapeutically active for mixed lineage leukemia fusion mouse models. To clarify whether in vivo administration of EZH2 inhibitor has therapeutically effective against MLL fusion leukemia mice, we employed DZNep, an inhibitor for H3K27 methyltransferase which is feasible for in vivo administration. First, we administered DZNep intraperitoneally to MLL fusion leukemia mouse models and analyzed epigenetic and transcriptional changes in the BM cells. In vivo administration of DZNep prolonged survival of MLL/ENL and MLL/AF9 leukemic mice (Fig. 1a). The BM cells of mice treated with DZNep were globally hypomethylated at H3K27 (Fig. 1b). Next, we investigated the expression of specific genes concerning apoptosis and cellular senescence that are known to be targets of EZH2.(26,27,33) As previously reported,(34) DZNep did not suppress EZH2 transcript level (Fig. S1a) but decreased protein level (Fig. S1b). Most of the genes tested showed a tendency to upregulation by DZNep treatment in vitro (Fig. S1a), among which only p16 was significantly upregulated by in vivo administration of DZNep (Fig. 1c,d). p16 is known to be a tumor suppressor which is typified by association with oncogene-induced senescence (OIS) and is epigenetically regulated by PRC2 and PRC1.(35,37) Loss of epigenetic suppression of p16 is reported to induce depletion of stem cells.(38) Thus, we investigated whether LIC ratio in MLL/AF9 leukemic mice is influenced by administration of DZNep. The LICs in MLL/AF9 leukemic mice are known to be enriched in L-GMP fraction.(31) In FACS analysis, the ratios of L-GMP in mice treated with DZNep were significantly decreased compared with control mice, while administration of typical cytotoxic agent did not reduce them (Fig. 1e, Fig. S2). Moreover, the BM cells from MLL/AF9 leukemic mice treated with DZNep had impaired capacity to

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reconstitute leukemia in vivo and attenuated colony forming capacity in vitro (Fig. 1f,g, Fig. S3). These data suggest that LIC are decreased by DZNep administration in MLL/AF9 leukemic mice.

Knockdown of EZH2 reveals similar phenotype to DZNep administration in MLL fusion leukemic mice. To confirm whether the effect of DZNep against MLL related leukemia is due to EZH2 inhibition, we transduced shRNAs for EZH2 (shEZH2) into MLL/ENL leukemic cells (Fig. 2a). The transduced cells were FACS sorted and transplanted to sublethally irradiated mice. Knockdown of EZH2 prolonged the survival of MLL/ENL leukemic mice (Fig. 2b). The BM cells transduced with shEZH2 were globally hypomethylated (Fig. 2c), and had diminished colony forming capacity with elevated p16 expression (Fig. 2d,e). The ratios of L-GMP in mice transplanted with shEZH2 transduced cells were markedly reduced (Fig. S4). These data indicate that therapeutic efficacy of DZNep is due to EZH2 inhibition.

Upregulation of p16 as a candidate anti-leukemic effect by EZH2 inhibition. To validate whether upregulation of p16 is responsible for anti-leukemic effect of EZH2 inhibition, we tested shRNA for p16 (shp16) (Fig. 3a). Colony forming capacity of MLL/ENL leukemia cells attenuated by shEZH2 was markedly restored by p16 knockdown (Fig. 3b). Furthermore, similar results were obtained using MLL/ENL leukemia mice treated with shp16 and DZNep (Fig. 3c). In contrast, overexpression of p16 diminished colony number of MLL/ENL leukemia cells (Fig. 3d) and showed decreased leukemia initiating capacity in transplantation assay (Fig. 3e). These data support the idea that p16 is one of the main targets of EZH2 inhibition.

Anti-leukemic effect of EZH2 inhibition may be specific for MLL fusion leukemia. Next, we studied whether anti-leukemic effect for MLL fusion leukemia by EZH2 inhibition can be applied to various kinds of leukemia. We exposed several human leukemia cell lines to DZNep. K562, HEL, Kasumi-1 and ME-1 cells, all of which do not harbor MLL fusion genes were not sensitive to low concentration of DZNep while both of MLL leukemia cell lines Mv4-11 and MOLM13 were sensitive to 50 nM of DZNep (Fig. 4a). Knockdown of EZH2 led to minimal effect on E2A/HLF transduced BM cells or c-Kit positive BM cells as we previously reported, while colony forming capacity of MLL/ENL and Hoxa9/Meis1 transduced cells were strongly suppressed (Fig. 4b). Knockdown of p16 rescued...
colony forming capacity of MLL/ENL or Hoxa9/Meis1 transduced cells by EZH2 inhibition (Fig. 4c). Expression levels of p16 were not influenced by knockdown of EZH2 in the BM cells transduced with leukemia fusion genes other than MLL/ENL (Fig. S5). In addition, survival of mice transplanted with leukemia cells induced by TPAE is not prolonged by DZNep administration (Fig. 4d), although H3K27 were globally hypomethylated (Fig. 4e). These data imply that anti-leukemic effect of EZH2 inhibition through p16 upregulation is specific for MLL fusion leukemia.

EZH2 is enriched at the p16 transcription-start-site in MLL fusion leukemia. To clarify the molecular mechanism of p16 regulation by EZH2 in MLL fusion leukemia, we investigated p16 transcription levels and epigenetic status of p16 promoter in MLL/ENL, Hoxa9/Meis1 and E2A/HLF transduced cells. Among several types of immortalized cells and leukemia cells, the transcript level of p16 is remarkably low in MLL/ENL immortalized or leukemia cells (Fig. 5a,b). This suggests existence of specific mechanism of p16 suppression in MLL fusion leukemia and we speculated that difference in epigenetic status between MLL fusion leukemia and other types of leukemia is a possible cause. The promoter of p16 is suggested to locate around the transcription-start-site (TSS), and a previous study using mouse embryonic fibroblasts revealed that H3K27 methylation status around this region is altered by OIS. (37) As expected, our ChIP assay revealed that EZH2 is enriched at the p16 TSS together with Bmi1 and H3K27Me3 in MLL/ENL or Hoxa9/Meis1 transduced cells, while they are not enriched at the p16 TSS of E2A/HLF transduced cells (Fig. 5c, Fig. 5d). In contrast, H3K4Me3 was enriched at the TSS of p16, not only in MLL/ENL and Hoxa9/Meis1 cells, but also in E2A/HLF cells (Fig. S6), which is consistent with the report that H3K4Me3 in the p16 TSS is an initial event after transduction of oncogene leading to p16 upregulation. (37,39) Our data suggest that enriched EZH2 at the p16 TSS maintains H3K27Me3 methylation status against p16 upregulation, leading to suppressed expression of p16 compared with other types of leukemia. Recently, Hoxa9 is reported to be important to recruit EZH2 to the TSS of p16.(40) In fact, Hoxa9/Meis1 transduced cells which mimic downstream signaling of MLL fusion leukemia displayed similar behavior of EZH2 and H3K27Me3 on the p16 gene (Fig. 5c). At this point, our data are compatible to the previous report showing a role of Hoxa9 to recruit EZH2 to the TSS of p16. (40) In fact, Hoxa9/Meis1 transduced cells which mimic downstream signaling of MLL fusion leukemia displayed similar behavior of EZH2 and H3K27Me3 on the p16 gene. However, despite the higher expression of Hoxa9 in Hoxa9/Meis1 transduced cells compared with MLL/ENL transduced cells, the expression of p16 in Hoxa9/Meis1 transduced cells is higher than that of MLL/ENL transduced cells (Fig. 5d). These data suggest that there may be some specific mechanisms other than Hoxa9-mediated recruitment of EZH2 to suppress p16 in MLL/ENL transduced cells.

Discussion
We investigated the impact of EZH2 inhibition in leukemia models. Our data suggest that inhibition of EZH2 in MLL
Fusion leukemia is therapeutically effective by diminishing LIC through upregulation of p16. Recently, genetic deletion of EZH2 is shown to decrease susceptibility to MLL/AF9-induced leukemic transformation. Although these reports suggested the efficacy of EZH2 inhibitors for the treatment of MLL fusion leukemia, in vivo administration of EZH2 inhibitors for MLL rearranged leukemia mouse models had never been verified. We confirmed that treatment with DZNep or knockdown of EZH2 prolonged the survival of MLL fusion leukemic mice in vivo. Previous reports revealed that conditional knock out of EZH2 after transplantation of MLL/AF9 pre-leukemic cells does not prolong survival in the primary transplantation. However, they observed prolonged survival of MLL fusion leukemic mice in the secondary transplantation of EZH2 depleted leukemia cells. In our study, we transplanted the primary MLL fusion leukemia cells after knockdown of EZH2 and found that EZH2 suppression before transplantation reduces LIC and extends survival period. Similarly, we observed that administration of DZNep from day 7 after transplantation also prolonged survival. We presume that the engraftment of MLL fusion leukemia cells is at day 14–18 because white blood cell counts of mice are almost zero within 2 weeks after transplantation. Therefore, transplanted cells are supposed to be exposed by DZNep before engraftment. Tanaka et al. discussed that EZH2 is required for the maintenance of LICs against the stress imposed by transplantation but is dispensable for initiating leukemia. We suppose that their idea also explain our findings, and that depleting EZH2 before engraftment of MLL fusion leukemia cells is important for eradication of LICs. On the other hand, Bmi1, a component of PRC1, was not shown to be a therapeutic target whereas it is required for initiating MLL/AF9 leukemia, which highlights the importance of EZH2 as a therapeutic target among polycomb repressive complexes. At the same time, EZH2 inhibition spares normal hematopoiesis, which suggests that EZH2 is one of the rational therapeutic targets in MLL rearranged leukemia and endorses the future development of EZH2 inhibitors in a clinical setting.

The expression profile of genes targeted by EZH2 is different between in vivo and in vitro models of EZH2 inhibition. Only p16 was significantly upregulated by in vivo administration of DZNep (Fig. 1c), while many genes were upregulated by in vitro exposure of DZNep (Fig. S1a). We suppose that this difference is due to drug concentration and exposure pattern. The BM cells of mice are virtually exposed to DZNep intermittently, because its concentration in mice plasma is lowered to less than 10% within 30 minutes after administration. This fact supports our idea that the effects of DZNep in vitro and in vivo are not the same, and that p16 is a bona fide target of EZH2 in MLL-related leukemia.

Our data also imply that EZH2 inhibition exerts the unique anti-leukemic effect against MLL fusion leukemia. The
expression of p16 is remarkably low in MLL/ENL transduced cells. Surprisingly, although basal expression of p16 is suppressed, additional knockdown of p16 increased colony formation in MLL/ENL immortalized cells (Fig. 3b and 4c). Knockdown of p16 also shortened the survival of MLL/ENL leukemic mice (Fig. 3c). Although these results indicate that knockdown of p16 itself exacerbates leukemia, we suppose that down-regulation of p16 also prevent the anti-leukemic effect of EZH2 inhibitor, because the difference of mean survival time between Control/DZNep and shp16/DZNep (9.8 days) is longer than that between Control/DDW and shp16/DDW (7.6 days). Furthermore, overexpression of p16 diminished leukemia initiating capacity of MLL/ENL leukemia cells (Fig. 3d,e). These data support the idea that suppression of p16 is one of the important processes to maintain MLL fusion leukemia, and it is more sensitive to restoration of p16 expression compared with other leukemias. Clinically, chromosomal deletion around p16 is associated with lower survival in MLL rearranged leukemia. The upregulation of p16, which is negatively controlled by EZH2 and Bmi1, is known to be the initial event after transduction of oncogene. We suppose that removal of EZH2 at the p16 promoter restores its expression and induces OIS. Our data suggest that MLL fusion leukemia depends on EZH2 to prevent p16 upregulation while other leukemias do not (Fig. 4, Fig. S5), and it would explain, at least partially, the specificity of EZH2 inhibition for MLL rearranged leukemia. Moreover, a previous report showed that EZH2 is essential for development of fetal hematopoiesis but dispensable for maintenance of adult hematopoiesis. They also revealed that EZH2 is recruited to the TSS of p16 in fetal hematopoiesis, but not in normal adult hematopoiesis. This point is one of the crucial rationales for the clinical use of EZH2 inhibitors because their toxicity potentially spares normal hematopoietic stem cells. It is also important to unveil the factor that recruits EZH2 to the p16 TSS, as it may lead to clarification of other novel targets for the treatment of MLL fusion leukemia.

The recruitment of EZH2 to the p16 TSS is supposed to depend on high expression of Hoxa9 which is upregulated by MLL fusion protein, because the transduction of Hoxa9/Meis1 also recruited EZH2 to the TSS of p16 (Fig. 5c). These data are well consistent with a recent report. Our data also suggest some other mechanisms independent of Hoxa9 activation to suppress p16 in MLL/ENL transduced cells because the expression levels of Hoxa9 and p16 are not inversely related (Fig. 5d).

Collectively, our data indicate that EZH2 plays a crucial role to maintain MLL fusion leukemia through inhibition of p16. This mechanism is specific for MLL fusion leukemia because high expression of Hoxa9 and other possible factors act jointly to recruit EZH2 to the p16 TSS. Removal of EZH2 from the TSS of p16 activates its transcription, and it supposed to be the reason why LIC of MLL fusion leukemia is diminished by EZH2 inhibition. Our findings encourage the development of EZH2 inhibitors that are tolerated in human.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. In vitro effect of DZNep.

Fig. S2. Representative presentation of FACS analysis of MLL/AF9 leukemic mice treated with DDW, DZNep, or AraC.

Fig. S3. Result of limiting dilution transplantation assay of MLL/AF9 secondary leukemia cells.

Fig. S4. Representative presentation of FACS analysis of MLL/AF9 leukemic mice treated with shRNA.

Fig. S5. Relative expression of EZH2 and p16 in immortalized cells.

Fig. S6. Results of ChIP analysis.

Table S1. Primer sequence for expression analysis.

Table S2. Antibodies for Immunoprecipitation, Immunoblotting and ChIP.

Table S3. Primer sequence for ChIP analysis.