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MLL5, a histone modifying enzyme, regulates androgen receptor activity in prostate cancer cells by recruiting co-regulators, HCF1 and SET1

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In prostate cancer, the androgen receptor (AR) transcription factor is a major regulator of cell proliferation and metastasis. To identify new AR regulators, we focused on Mixed lineage leukemia 5 (MLL5), a histone-regulating enzyme, because significantly higher MLL5 expression was detected in prostate cancer tissues than in matching normal tissues. When we expressed shRNAs targeting MLL5 gene in prostate cancer cell line, the growth rate and AR activity were reduced compared to those in control cells, and migration ability of the knockdown cells was reduced significantly. To determine the molecular mechanisms of MLL5 on AR activity, we proved that AR physically interacted with MLL5 and other co-factors, including SET-1 and HCF-1, using an immunoprecipitation method. The chromatin immunoprecipitation analysis showed reduced binding of MLL5, co-factors, and AR enzymes to AR target gene promoters in MLL5 shRNA-expressing cells. Histone H3K4 methylation on the AR target gene promoters was reduced, and H3K9 methylation at the same site was increased in MLL5 knockdown cells. Finally, xenograft tumor formation revealed that reduction of MLL5 in prostate cancer cells retarded tumor growth. Our results thus demonstrate the important role of MLL5 as a new epigenetic regulator of AR in prostate cancer. [BMB Reports 2020; 53(12): 634-639]

INTRODUCTION

Prostate cancer (PCa) is the most prevalent cancer in men and the second most cause of cancer-specific mortality in the United States (1). Androgen-deprivation therapy (ADT) is used as principle treatment for locally advanced and metastatic PCas, however, many of them eventually progress to castration-resistant prostate cancer (CRPC) (2). Significant progress has been made during the last decades in the understanding of cancer biology of CRPC related to the androgen receptor (AR) signaling pathway. The first approved AR signaling inhibitor, enzalutamide, has a novel mode of action of targeting AR signaling. The effect of enzalutamide on CRPC patients is rapid and efficient at the first stage, but recurrence of cancer and resistance is a limiting factor for this drug (3). To overcome this clinical burden, investigating AR transcriptional activity and finding alternative modes of action, which could act as targets of additional drugs, is very important. To find novel inhibitors targeting AR activity in CRPC, we focused on epigenetic regulators of AR.

Epigenetic regulation of AR includes DNA methylation, histone acetylation, histone methylation, and alterations in noncoding RNA profiles (4). Among them, we focused on changes in histone methylation. Methylation can activate or repress transcription according to the site of modification (5). For example, methylation of lysine residues 4 and 36 in histone H3 (H3K4, H3K36) usually activates transcription and preserves euchromatic domains, whereas modification of H3K9 and H3K27 represses transcription and forms heterochromatin. Many histone-modifying enzymes have been found to regulate AR activity, including SET9 (6), NSD2 (7), EZH2 (8), LSD1 (9-12), and KDM7A (13). However, their mechanisms of action on AR activity are not yet completely understood. Hence, we have explored another histone-modifying enzyme, mixed lineage leukemia 5 (MLL5), as a novel epigenetic regulator of AR in prostate cancer cells.

MLL5 is a member of the mixed lineage leukemia family of genes, and displays homologies to the trithorax group that plays critical roles in the regulation of homeotic gene (HOX) expression and embryonic development (14). Several reports indicate that MLL5 regulates many important cellular processes including cell cycle progression (15, 16), hematopoiesis (17), and spermatogenesis (18). Human MLL5 gene contains a single plant homeodomain (PHD) zinc finger and a Su(var)3-9 Enhancer-of-zeste and Trithorax (SET) domain. The PHD finger

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domain is involved in chromatin-mediated gene regulation, and the SET domain is frequently found in histone lysine methyl transferases. Although MLL5 was initially categorized under the MLL family together with six other SET methyltransferase domain proteins, it appears to lack intrinsic histone methyltransferase activity towards histones (19). Nevertheless, it is observed that MLL5 binds to gene-rich euchromatin regions via interaction of its PHD finger with the histone mark H3K4-me3 (16), which may be accomplished by recruiting other co-regulators. Particularly, it is reported that MLL5 can interact with Host Cell Factor-1 (HCF-1) in cell cycle progression-related gene promoter regions (20). Moreover, because HCF1 is demonstrated to interact with SET1/Ash2 histone methyltransferase (21), we postulated that MLL5 can recruit SET1/Ash2 through HCF1.

Here, we show that MLL5 modulates prostate cancer cell growth and cell migration by altering histone methylation of AR target gene promoters. Considering that the epigenetic regulation of AR by MLL5 is different from existing mechanism of enzalutamide action, MLL5 may be a new target of prostate cancer drug for overcoming enzalutamide resistance.

RESULTS

MLL5 is required for AR activity in prostate cancer cell lines

To uncover novel histone-modifying enzymes that can regulate AR activity, we hypothesized that these enzymes may show changes in prostate cancer tissues compared to normal prostate tissues. We evaluated mRNA expression levels of *MLL5* using the Oncomine database between normal and prostate cancer tissues (www.oncomine.org) and found higher mRNA levels in prostate tissue samples (T) than in normal tissues (N) from two different databases (Supplementary Fig. S1A). Moreover, upon analyzing the data available in the NCBI public database, the *MLL5* mRNA expression level was found to be higher in metastatic prostate tissues than in normal or localized tumor tissues (Supplementary Fig. S1B). To confirm their protein expression in cancer tissues, we extracted proteins from 8 different prostate cancer tissues alone with matching normal tissues. We found that the protein level of MLL5 also showed significant elevation in cancer tissues (Supplementary Fig. S2A). Moreover, immunostaining of MLL5 using a commercial tissue-array slide showed similar results (Supplementary Fig. S2B). When we tested AR-positive and -negative prostate cancer cell lines, the MLL5 protein expression level was found to be higher in AR-negative cell lines (Supplementary Fig. S3A); however, there was no difference observed in its mRNA expression level (Supplementary Fig. S3B). These data encouraged us to examine the function of MLL5 on AR activity. To test the possible role of MLL5 in prostate cancer cells, we generated lentivirus-mediated MLL5 shRNA expressing stable knockdown cell lines in LNCaP and 22Rv1, both of which are known to have active AR activity. The knockdown efficiency was confirmed by quantitative reverse transcriptase-PCR (qRT-PCR) and western blotting (Fig. 1A). To test AR activity, each cell line was activated with dihydrotestosterone (DHT) for 24 h, and the expression of AR target genes was analyzed by western blotting (Fig. 1B) and RT-PCR (Fig. 1C). Protein (Fig. 1B) and mRNA (Fig. 1C) levels of the target genes, including PSA (KLK3), TMPRSS2, and IGF1R, were elevated after DHT treatment in control cells; however, their induction was repressed in MLL5 knockdown cells. AR is known to modulate its own expression at both transcriptional and translational levels (22, 23). The changes in AR protein expression level presented in Fig. 1B might be an outcome of this autoregulatory mechanism, and possibly contribute to the modulation of its activity. These results showed that MLL5 might affect AR activity in prostate cancer cells.

MLL5 is required for prostate cancer cell growth and migration

It is well known that AR plays an important role in prostate cancer cell growth and inhibition of apoptosis (24). To test growth rate differences in MLL5 knockdown cells, we measured the viable cell content among each cell line. Compared to control LNCaP cells, MLL5 knockdown cells showed reduced

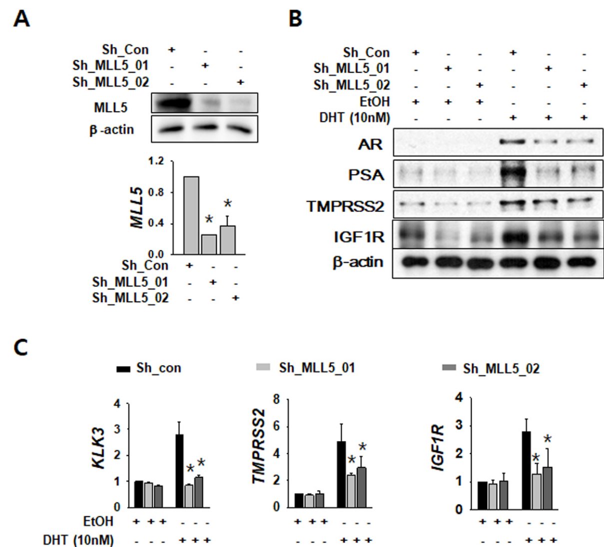

Fig. 1. MLL5 is required for AR activity in prostate cancer cell lines. (A) The efficiency of MLL5 knock-down was measured by comparing protein and mRNA levels of MLL5 in shRNA expressing LNCaP cell line. Whole cell lysates were analyzed with the indicated antibodies (upper figure). Total RNAs from each cell lines were measured by RT-qPCR (lower graph). (B) Protein expression of the AR target was analyzed using western blotting. For inducing AR activity, 10 nM DHT was added after one day of serum deprivation. Whole cell lysates were analyzed with the indicated antibodies. (C) The mRNA levels of AR target genes were analyzed by RT-qPCR in MLL5 knockdown LNCaP cells. Bars represent the means ± SDs of three independent experiments, and * denotes $P < 0.05$ (Student t-test) versus the control shRNA (Sh_con) group.

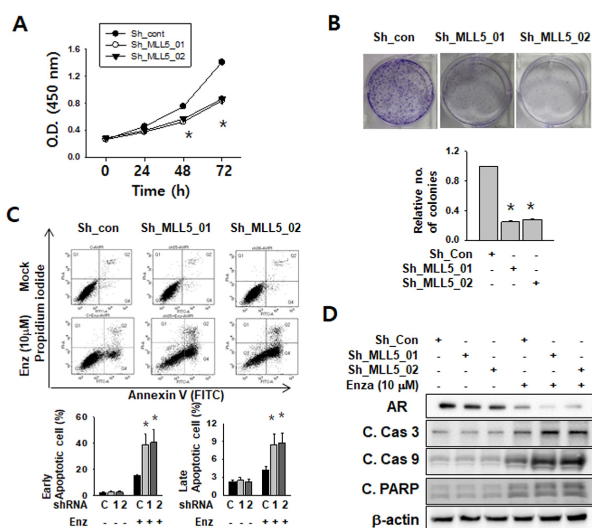

Fig. 2. MLL5 knockdown reduced cell proliferation and induced apoptosis in combination with enzalutamide treatment in LNCaP cells. (A) Time-dependent viability changes of control shRNA- and MLL5 shRNA-expressing LNCaP cells were assessed using EZ-Cytox solution. (B) Crystal violet staining of colonies from a similar number of indicated shRNA-expressing LNCaP cells. The average number of colonies counted is shown in the lower graph. (C) Flow cytometry analysis of annexin-V and propidium iodide (PI) staining of apoptotic cells of each of the indicated stable cells following enzalutamide treatment for 2 days. Graphs of percentages of early apoptosis (EA; PI-negative + annexin-V positive) and late apoptosis (LA; PI positive + annexin-V positive) cells are shown on the bottom. (D) LNCaP cells expressing control or MLL5 shRNAs were treated with enzalutamide (Enz) for 2 days, and whole-cell extracts were analyzed with the indicated antibodies. C. Cas 3 (cleaved caspase 3), C. Cas 9 (cleaved caspase 9), and C. PARP (cleaved PARP). Bars represent the means ± SDs of three independent experiments, and * denotes $P < 0.05$ (Student t-test) versus the control shRNA (Sh_con) group.

growth after 2-3 days (Fig. 2A). Moreover, when we plated a similar number of cells, the number of MLL5 knockdown cell colonies showed a dramatic reduction compared to control cell colonies (Fig. 2B). The AR inhibitor enzalutamide is known to block the binding of androgen to AR. If MLL5 is able to modulate AR activity via an epigenetic mechanism, the mode of action would be different from that of enzalutamide. Therefore, we may expect an additive effect of MLL5 inhibition combined with enzalutamide treatment on AR signaling and cell death effect. To examine the effect of MLL5 in combination with enzalutamide treatment, we tested the reactivity of MLL5 knockdown cell lines with enzalutamide. Apoptotic cells assessed by Annexin V staining showed increased apoptotic cell ratio following enzalutamide treatment, and the number of early and late apoptotic cells was dramatically increased in MLL5 knockdown cells (Fig. 2C). In control cells, enzalutamide treatment alone increased apoptotic signaling molecules, and MLL5 knockdown additionally increased this effect (Fig. 2D). To confirm whether the effect induced through MLL5 knockdown is AR dependent, we generated MLL5 knockdown stable cell lines using AR-negative prostate cancer cells, including PC3 and DU145. However, there was no difference observed in the rate of cell proliferation in these AR-negative cell lines when compared to the control cells (Supplementary Fig. S4). These results showed that MLL5 is required for the growth of prostate cancer cells and may act synergistically with enzalutamide on apoptosis process. The function of AR in prostate cancer cell migration is also well known (25, 26). When we measured the levels of epithelial and mesenchymal protein markers in DHT-treated control cell extract, the epithelial marker E-cadherin level was decreased and those of mesenchymal markers N-cadherin and vimentin increased. These effects were abolished in MLL5 knockdown cell lines (Supplementary Fig. S5A, B). Wound healing assay and trans-well infiltration assay showed that MLL5 knockdown cells migrated less than control cells (Supplementary Fig. S5C, D). These results indicate that MLL5 may regulate the migration ability of prostate cancer cells.

MLL5 physically interacts with AR and co-factors on the promoters of AR-responsive genes

Although MLL5 does not have methyltransferase activity, histone methylation changes are reported to occur through MLL5 regulation (20). When we measured histone methylation status in MLL5 knockdown prostate cancer cell extract, we found a decrease in H3K4 di-methylation (H3K4-2me) and increase in H3K9 di-methylation (H3K9-2me) (Supplementary Fig. S6A). To further verify histone methylation status on AR target gene promoters, we performed chromatin immunoprecipitation with H3K4-2me and H3K9-2me antibodies. Precipitated DNAs were subjected to PCR with primers of AR target gene promoter sequences. H3K4-2me was decreased and H3K9-2me was increased in MLL5 knockdown cells than in control cells after induction with DHT (Supplementary Fig. S6B). These data showed that MLL5 may modulate histone methylation status of AR target gene promoters. A previous study revealed that MLL5 interacted with HCF1 and functioned as H3K4 methyltransferase on E2F1-responseive promoters (20). We postulated that MLL5 may interact with HCF-1 in prostate cancer cells and methylate H3K4 on the AR responsive element together with SET1, which is known to interact with HCF-1 (21). In DHT-induced LNCaP cells, we immunoprecipitated AR and analyzed the co-immunoprecipitated proteins by western blotting using anti-MLL5, anti-HCF1, and anti-SET1 antibodies. As shown in Fig. 3, AR protein was immunoprecipitated with MLL5, HCF-1, and SET1 proteins (Fig. 3A). When we used an HCF-1 antibody for immunoprecipitation (IP), we also observed co-IP bands of AR, MLL5, and HCF-1 (Fig. 3B). To further analyze protein binding on AR responsive elements (ARE), we performed chromatin immunoprecipitation (ChIP) experiments using anti-AR, MLL5, SET1, and HCF-1 antibodies. Binding of all tested proteins to ARE was increased after DHT treatment in LNCaP cell lines but was decreased in MLL5 knockdown cells

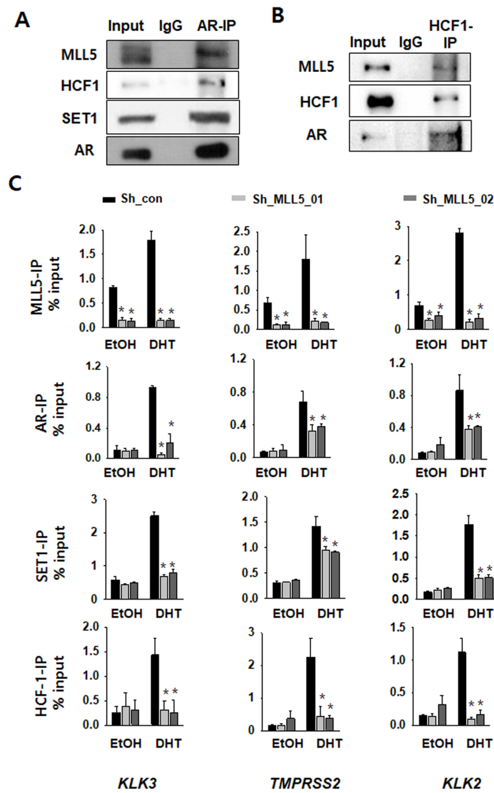

Fig. 3. MLL5 directly interacts with AR with co-factors on AR target gene promoters. (A) LNCaP cells were treated with 10 nM DHT for AR induction, and cell extracts were immuno-precipitated with AR antibody. Input and immune-precipitated (AR-IP) were immunoblotted with the indicated antibodies. (B) The same extract was immunoprecipitated with HCF1 antibody. Input and immune-precipitated (HCF1-IP) were immunoblotted with the indicated antibodies. (C) LNCaP cells stably expressing control or MLL5 shRNAs were treated with 10 nM DHT. Chromatin Immunoprecipitation (ChIP) was performed with sonicated DNAs using the indicated antibodies (left). The primers used for ChIP-qPCR are indicated below. Error bars represent mean ± SD of three independent experiments and * denotes P < 0.05 (Student's t-test) versus the Sh_con group.

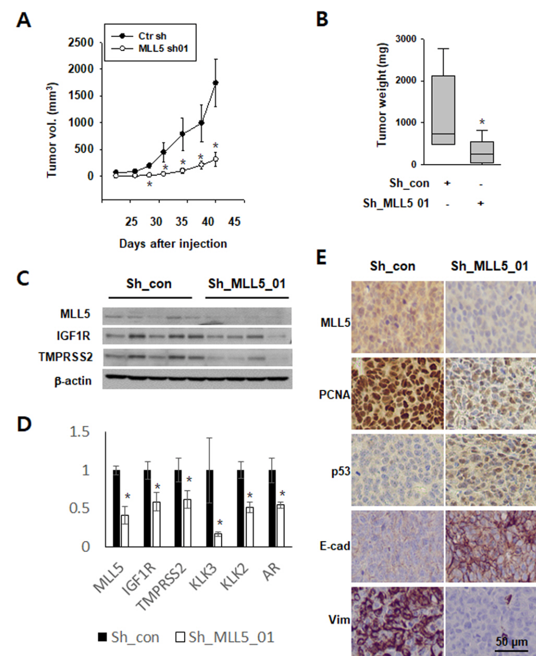

Fig. 4. MLL5 knockdown attenuates tumor growth in human CRPC xenograft model. (A) Tumor growth of control or MLL5 knockdown tumors using 22Rv1 xenograft. (B) Tumor weights of MLL5-suppressed xenograft on the day of sacrifice. Tumors were excised and weighed. A representative graph is shown as vertical box plots and tumor weights are presented as mean (g) ± SEM (n = 5). (C) Total protein was extracted from each xenograft tumors and western blotting was performed with the indicated antibodies. (D) Total RNA was extracted from each xenograft tumor and the indicated mRNA levels were measured. Error bars represent means ± SEM for each group (n = 5). * denotes P < 0.05 (Student's t-test) versus sh-control group. (E) Representative images of immunohistochemical analysis of control (Sh_con) or MLL5 knockdown (Sh_MLL5_01) tumors using the indicated antibodies (left).

(Fig. 3C). These results indicated that MLL5 could directly bind AREs and that MLL5 knockdown decreased AR and co-factor binding at the same site.

MLL5 knockdown reduced tumor growth in a prostate cancer xenograft model

To investigate the role of MLL5 in prostate tumor growth *in vivo*, we injected 22Rv1 cell lines expressing control or MLL5 shRNAs into the flanks of NOD scid gamma (NSG) immunodeficient mice. When tumor growth was monitored for 40 days, the tumors in mice injected with control shRNA grew constantly, while those in MLL5 knockdown cell-injected mice did not (Fig. 4A). On the day of sacrifice, weights of the extracted tumors were measured; the average weight of control tumors was significantly higher than that of MLL5 knockdown tumors (Fig. 4B). Immunoblotting of tumor extracts showed lower protein levels of MLL5 and AR target genes, including IGF1R and TMPRSS2 (Fig. 4C). The mRNA levels of AR target genes in the extracted tumors also showed lower expression in MLL5 knockdown tumors (Fig. 4D). Using immunostaining analysis, the proliferation marker PCNA showed a higher level in control tumor than in MLL5 knockdown tumor. The level of apoptotic marker p53 was higher in MLL5 knockdown tumors. Moreover, staining of EMT markers E-cadherin and vimentin showed that the MLL5 knockdown tumor was more epithelial in nature than control tumor (Fig. 4E). These data indicated that MLL5 is required for growth and migration capability as well as regulation of AR activity of prostate tumors *in vivo*.

DISCUSSION

In metastatic castration-resistant prostate cancer (CRPC) patients,

second-generation antiandrogens such as enzalutamide extend the overall survival by a median of 4-5 months. However, acquired resistance to enzalutamide is common with recurrence and progression of cancer. Many approaches have been developed, including co-treatment with existing cancer drugs, to overcome this resistance (27). Finding new AR regulating mechanisms different from that of enzalutamide might be a way to overcome enzalutamide resistance. Towards the aim of finding new regulatory mechanisms of AR, we explored the role of MLL5, a histone modifying enzyme in prostate cancer. We showed that AR activity changed through reduced binding and H3K4 methylation on target promoters in MLL5-repressed prostate cancer cells (Fig. 3). Synergistic effects of enzalutamide and MLL5 knockdown on prostate cancer cell growth and induction of apoptosis further demonstrated the possibility of the MLL5 inhibitor as a cancer drug overcoming enzalutamide resistance (Fig. 2C, D).

This study revealed a new epigenetic regulator, MLL5, as a co-factor for AR activity. In a previous study, MLL5 was reported to interact with HCF-1 in the E2F1 responsive element (20). Because E2F1 is known to collaborate and physically interacts with AR in many AR-regulated promoters (28), it further strengthens our result that MLL5 interacts with AR through HCF-1. Notably, Retinoblastoma protein (pRb), which interacts with E2F1, is also known to regulate AR-responsive genes including PSA (29). The interaction of HCF-1 with SET1 is well established in many studies (21, 30). Because purified MLL5 protein does not have intrinsic methyl-transferase activity (19), methylation changes in H3K4 in MLL5 knockdown cells may have occurred due to the recruited SET1 enzyme. By showing physical interaction of MLL5 and SET1 through AR immunoprecipitation, we confirmed this speculation (Fig. 3A). Additionally, we demonstrated that physical interaction of HCF1 and SET1 with ARE were decreased in MLL5 knockdown cells using a chromatin precipitation method (Fig. 3C).

In prostate cancer tissues, we observed higher expression of MLL5 compared to normal tissues. Based on our data, patients with higher MLL5 expression are likely to exhibit higher AR activity. Because it is well known that prostate cancer progression is tightly correlated with AR activity (31), measuring MLL5 expression in prostate cancer can be used as a prognostic marker for its progression or predicting therapeutic response to AR-targeting treatments.

In conclusion, we confirmed that a new epigenetic regulator MLL5 physically interacted with AR and co-regulators and presented molecular mechanisms of epigenetic regulation. Our findings further suggest that MLL5 could be a possible therapeutic target for CRPC, especially with enzalutamide resistance.

MATERIALS AND METHODS

Detailed information is provided in Supplementary information.

Materials

RPMI-1640, DMEM, trypsin, anti-biotics, Trizol and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum and culture media were obtained from Hyclone Laboratories Inc. (South Logan, UT). Anti-MLL5 antibody was purchased from Abcam (Cambridge, England). Antibodies against PSA, TMPRSS2, AR, IGF1R and beta-actin were purchased from SantaCruz Biotechnology (Santa Cruz, CA), and antibodies against total histone, H3K4, H3K9, H3K27, cleaved PARP, cleaved Caspase 3 and cleaved Caspase 9 were from Cell Signaling (Danvers, MA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines, plasmids, virus production and infection

The LNCaP, 22Rv1, and 293T cell lines were purchased from the American Type Culture Collection (Rockville, MD). LNCaP and 22Rv1 cells were cultured in RPMI-1640 medium at $37^{o}C$ in 5% CO_2, which was supplemented with 10% fetal bovine serum. For gene silencing, the pLKO.1-puro lentiviral vector was purchased from Sigma-Aldrich, and oligonucleotides for control shRNA or *MLL5* shRNA were inserted into the vector using the AgeI and EcoRI restriction enzymes. For lenti-virus packaging, the 293T cell line was transfected with pLKO.1 vectors and helper DNAs using Lipofectamine 2000 (Invitrogen). The media was changed after 16 hours, and the virus stocks were harvested after 48 hours. Cells were infected overnight with lentivirus with 6 μg/ml polybrene. 48 hours after transduction, cells were selected with 2 μg/ml puromycin until the knock-down was confirmed by RT-PCR or western blotting.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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