Vector: vector-infected BM cells.

NI: NPMc- and IDH2/R140Q-infected BM cells.

NID: NPMc-, IDH2/R140Q-, and Dnmt3a/R882H-infected BM cells.

NIF: NPMc-, IDH2/R140Q-, and FLT3-ITD–infected BM cells.

NIDF: NPMc-, IDH2/R140Q-, Dnmt3a/R882H-, and FLT3-ITD–infected BM cells.

Data analysis was performed using the GeneSpring GX Version 12.5 software. GSEA (Gene Set Enrichment Analysis) was performed as described (25).

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/ through accession number GSE63638.

Enrichment of 5hmC-containing DNA, deep sequencing, and peak detection

Genomic DNA was purified from BM cells using Nucleospin Tissue (Takara). 5hmC-enriched DNA was obtained using Hydroxymethyl Collector (Active Motif). DNA libraries were generated by following the Illumina protocol for "Preparing Samples for ChIP Sequencing of DNA" (Part# 111257047 Rev. A). In total, 25–40 ng of input genomic DNA or 5hmC-enriched DNA was used. DNA fragments of ~150–300 bp were gel-purified after the adaptor-ligation step. PCR-amplified DNA libraries were quantified on an Agilent 2100 Bioanalyzer and diluted to a concentration of 6–8 pM for cluster generation and sequencing. Thereafter, 100 cycle paired-end sequencing was performed using Illumina HiSeq2000. The acquired data were aligned to the mouse genome, and 5hmC peaks were detected using Avadis software (Strand).

The 5hmc mapping data have been deposited in the NCBI Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/ through accession number GSE63638.

qPCR validation of 5hmC-enriched loci

Input genomic DNA and 5hmC-enriched DNA were used for real-time PCR with Fast Start Universal SYBR Green Master (Roche) and forward and reverse primers. The primers were as follows: *Ebf1*: forward, (TGCGGTTTCCGCGTATTT), and reverse, (CACCAGTGATTTAGGGCTCAGTT); *Spib*: forward, (GGATGCTCTGCGCACACA), and reverse, (CTCGAACAACCCCTGCTGTT).

Results

IDH2/R140Q and NPMc upregulate expression of *Meis1* and *Hoxa9 in vitro*, respectively

To investigate the roles of the aforementioned mutations in AML development, we infected mouse hematopoietic stem/progenitor cells with retroviruses, each encoding *IDH2/R140Q*, *NPMc*, *DNMT3A/R882H*, or *FLT3/ITD*. We employed the most frequently used NPMc mutant containing a duplication of the TCTG tetranucleotide, previously referred to as Mutation A (26). Expression analysis revealed that *Meis1* and *Hoxa9* expression levels were elevated in cells expressing IDH2/R140Q and NPMc, respectively (Figs 1A and 1B). Because the expression of *Hoxa9* immortalizes myeloid progenitor cells (27), we tested whether the NPMc-expressing cells could be maintained *in vitro*. The NPMc-expressing cells formed increased numbers of colonies in methylcellulose media, but could not be maintained for a long time (Fig. 1C). NPMc may inhibit wild-type NPM by localizing it to cytoplasm (28); if so, the levels of wild-type NPM may affect the leukemogenic function of NPMc. To test this idea, we transduced NPMc into $Npm1^{+/-}$ hematopoietic stem/progenitor cells to mimic the situation in an AML patient heterozygous for a mutation in the *NPM1* gene. The $Npm1^{+/-}$ cells expressing NPMc exhibited serial colony-forming activity (Fig. 1C). Moreover, expression levels of Hoxa9 induced by NPMc were higher in the $Npm1^{+/-}$ cells than in the wild-type cells (Fig. 1D). Thus, we used $Npm1^{+/-}$ hematopoietic stem/progenitor cells for further analyses.

Establishment of mouse AML model harboring IDH2/R140Q

To establish mouse AML models, we serially infected $Npm1^{+/-}$ hematopoietic stem/progenitor cells with retroviruses encoding *NPMc-ires-EGFP*, *IDH2/R140Q-ires-NGFR*, *DNMT3A/R882H*, and *FLT3/ITD*, and transplanted the infectants into wild-type mice (Fig. 2A). About 60% of the recipient mice died of AML within 140 days after transplantation, and all the mice died within 275 days (Fig. 2B; NIDF). When only three out of the four mutant genes were transduced, the onset of leukemia was delayed in any combinations (Fig. 2B; IDF, NDF, NIF, and NID). These results clearly indicate that all four mutations are necessary for the efficient induction of AML. In our AML model, the expression of IDH2/R140Q and NPMc was monitored by

measuring the expression of NGFR and EGFP, respectively, because these genes were encoded on the same vectors used to express IDH2/R140Q and NPMc (Fig. 2C). We analyzed BM cells derived from AML mice transplanted with NIDF cells, and found that most of the cells expressed both NPMc and IDH2/R140Q (Fig. 2D). We isolated RNA from NIDF-AML cells and confirmed the expression of DNMT3A/R882H and FLT3-ITD by performing PCR with TaqMan probes against human DNMT3A and FLT3 (data not shown). To examine expression of DNMT3A/R882H and FLT3/ITD at the single-cell level, we stained permeabilized AML cells with anti-DNMT3A and anti-FLT3 antibodies. Flow cytometric analysis showed that 30-50% and 20-40% of the AML cells expressed DNMT3A/R882H and FLT3/ITD, respectively (Fig. S1). Morphological analysis revealed that a large population of BM cells derived from NIDF-induced AML mice were blast cells (Fig. 2E). In our analysis of moribund mice transplanted with NID or NIF cells, the percentage of blast cells was around 10%, which did not meet the criteria for AML (Fig. 2E). Most BM cells expressed myeloid markers such as Mac-1 and Gr-1 (Figs 2F and S2A). The percentages of cells expressing B-cell (CD19), T-cell (CD3ε), and erythroid (CD71 and Ter119) lineage markers were extremely low (Fig. S2A). When the NIDF-induced AML BM cells were transplanted into secondary recipient mice, all the mice developed AML (Fig. 2G). The secondary recipients of the AML cells die quicker than the primary recipients. This may be because the number of LSCs in primary recipients was lower than that in secondary recipients. Alternatively, it is also possible that the disease became more aggressive in secondary recipients.

IDH2/R140Q is necessary for the maintenance of AML

To determine whether expression of IDH2/R140Q is required for "maintenance" of AML, we inserted loxP sequences into the 5'- and 3'-regions of IDH2/R140Q within the viral vector (Fig. 3A). *ERT2-Cre*$^{+}$ *Npm1*$^{+/-}$ hematopoietic progenitor cells were infected with the floxed *IDH2/R140Q* and the three other necessary genes [Nf(I)DF], and then transplanted into mice to induce AML. BM cells from the AML mice were subsequently transplanted into secondary recipient mice (Fig. 3B). When the population of the EGFP^{+} leukemic cells within the total leukocyte population in PB of the secondary recipient mice reached 50–80% (7 weeks after transplantation), we commenced treatment with tamoxifen to delete *IDH2/R140Q* (Fig. 3C). The level of

2-HG, which is produced by IDH2/R140Q, was high in both the plasma and PB cells of AML mice, but decreased to the control levels within 7 days after initiation of tamoxifen treatment (Fig. 3D). This confirmed that the floxed *IDH2/R140Q* system was functional. To test the effects of *IDH2/R140Q* deletion on survival, we commenced treatment with tamoxifen when the population of $EGFP^+$ leukemic cells reached 10–15% in PB. While all ten control AML mice died 45–94 days after transplantation, approximately 50% of the AML mice treated with tamoxifen survived for at least 94 days (Fig. 3E). When we began tamoxifen treatment at an earlier stage (with 1–2% $EGFP^+$ cells in PB), five out of nine mice survived for at least 130 days after secondary BMT (Fig. 3F). However, all nine control mice died of AML within 87 days after transplantation (Fig. 3F). The number of $EGFP^+$ leukemic cells did not increase in the PB of mice treated with tamoxifen for 4 weeks, whereas the number of $EGFP^+$ cells in control AML mice increased dramatically over this period (Fig. 3G). To monitor the effects of *IDH2/R140Q* deletion on AML cells, we injected tamoxifen into mice once there was a high population (60–70%) of $EGFP^+$ leukemic cells in PB. The number of $EGFP^+$ cells in PB was drastically reduced after 4 weeks of treatment (Fig. 3H). On the other hand, mice not treated with tamoxifen died within 4 weeks. Using the same set of mice as in Figure 3H, we investigated expression of LSC markers in BM cells of AML mice treated with tamoxifen for 2 weeks. Genotype analysis confirmed that flox-IDH2/R140Q was almost completely deleted after 2 weeks of tamoxifen treatment (Fig. 3I). Flow cytometric analysis revealed that IDH2/R140Q-deleted $EGFP^+$ AML cells were retained, but the populations of these cells that expressed LSC markers (MSCFR, L-GMP, $cKit^+Gr1^-$, and CD34) (29-31) were dramatically reduced in tamoxifen-treated mice (Figs 3I and S2B). We analyzed expression of B-cell (CD19), T-cell (CD3ε), and erythroid (CD71 and Ter119) lineage markers in IDH2/R140Q-deleted AML cells. The differentiated state of the cells was maintained, and most cells were of the myeloid lineage (Fig. S2A). Consistent with decreased populations of cells expressing stem cell markers, the number of $EGFP^+$ AML cells was markedly reduced after an additional 2 weeks of tamoxifen treatment (Fig. 3I). To confirm the decrease in the number of LSCs, we performed a third transplantation using BM cells isolated from mice treated with or without tamoxifen for 2 weeks. When PB cells were analyzed 4 weeks later, the number of AML cells was increased in mice transplanted with untreated BM cells (Fig. 3J). Conversely, AML cells were almost

undetectable in mice transplanted with tamoxifen-treated BM cells (Fig. 3J). All mice transplanted with tamoxifen-untreated BM cells died of AML by 127 days after the third BMT, whereas none of the mice transplanted with tamoxifen-treated BM cells died by 140 days (Fig. 3K). These results strongly indicate that IDH2/R140Q is necessary for the maintenance of the LSC population. To investigate the effect of the IDH2/R140Q deletion on mRNA expression profiles, we performed microarray analysis of BM cells treated with or without tamoxifen for 2 weeks (Table S1). The Meis1 expression level in tamoxifen-treated BM cells was decreased to one third of that of tamoxifen-untreated BM cells (Table S1). This result is consistent with the data showing that IDH2/R140Q upregulated the Meis1 level *in vitro* (Fig. 1B). GSEA (25) using these microarray data showed that gene sets involved in the cell-cycle process (cell replication and nuclear replication) were downregulated in tamoxifen-treated cells compared with tamoxifen-untreated BM cells (Fig. 3L and Table S2). These results suggest that disruption of cell-cycle signaling contributes to the exhaustion of LSCs.

Roles of four mutant genes in AML development

Flow cytometric analysis of mice transplanted with NIDF-transduced cells revealed that most NPMc-expressing cells also expressed IDH2/R140Q and that the percentages of NPMc$^+$ IDH2/R140Q$^-$ cells were extremely low in the BM and PB (Fig. 4A). When *Npm1*$^{+/-}$ cells infected with NPMc alone were transplanted into mice, only a small population of NPMc$^+$ cells was detected in the PB of recipients (Fig. 4B). On the other hand, when *Npm1*$^{+/-}$ cells were co-infected with NPMc and IDH2/R140Q (NI), a large population of NPMc$^+$ IDH2/R140Q$^+$ cells was retained in PB (Fig. 4B). The infection efficiencies of the viruses were comparable (NPMc-ires-EGFP: ~40%, and IDH2/R140Q-ires-NGFR: ~50%). We analyzed mice 8 weeks after transplantation to determine the direct effects of each mutant gene. These data indicate that IDH2/R140Q was necessary and sufficient for the engraftment and/or survival of NPMc$^+$ cells *in vivo*. Six months after transplantation of *Npm1*$^{+/-}$ hematopoietic progenitor cells transduced with IDH2/R140Q alone, IDH2/R140Q$^+$ cells were predominant in the BM (Fig. 4C). This indicates that IDH2/R140Q$^+$ cells could be maintained for a long period of time *in vivo*. We isolated RNA from these IDH2/R140Q-expressing cells and performed microarray analysis (Table S3). GSEA using these microarray data showed that the expression of genes upregulated by hypoxia stimuli (32) was increased in

IDH2/R140Q-expressing cells (Fig. 4D and Table S4). These results suggest that the hypoxia pathway may facilitate the engraftment of NPMc$^+$ cells *in vivo*. We analyzed BM cells isolated from mice transplanted with NPMc and IDH2/R140Q transduced cells. Although NPMc$^+$ IDH2/R140Q$^+$ cells were predominant in the BM of recipient mice (Fig. 4E), the proportion of blast cells in the BM was less than 10% at 6 months post-transplantation (Fig. 4F). These data suggest that these mice developed a myeloproliferative neoplasm (MPN)-like disease, not AML. Thus, in addition to IDH2/R140Q and NPMc, the expression of DNMT3A/R882H and FLT3/ITD is also required for efficient development of AML. To investigate the roles of each of these mutants in leukemogenesis, we compared the *in vivo* mRNA expression profiles of control BM cells, NPMc$^+$ and IDH2/R140Q$^+$ (NI) cells, cells expressing all four mutant genes (NPMc, IDH2/R140Q, DNMT3a/R882H, and FLT3/ITD; NIDF), and cells expressing three of the mutant genes (NID or NIF) (Fig. 4G and Table S5). Compared with vector control cells, the expression of *Hoxa9* and *Meis1* was upregulated in NI, NID, NIF, and NIDF cells (Fig. 4H). This is consistent with the finding that NPMc and IDH2/R140Q upregulate the expression of *Hoxa9* and *Meis1*, respectively, *in vitro* (see Fig. 1B). Furthermore, the expression level of *Meis1* was higher in NID and NIDF cells than in NI cells (Fig. 4H), suggesting that DNMT3A/R882H promotes upregulation of *Meis1*. GSEA revealed that, compared with control BM cells, the NI cells expressed significantly higher levels of a set of genes that are upregulated in NPMc$^+$ AML (26), hypoxia (32), and myeloid development (33) (Figs 4I and 4J, Table S6). These results indicate that NPMc and IDH2/R140Q are sufficient to confer some of the properties of NPMc$^+$ AML cells on other cells. Additionally, activation of the hypoxia pathway was consistent with the results obtained with cells expressing IDH2/R140Q only (see Fig. 4D). The myeloid differentiation signature is consistent with the MPN-like phenotype of the NI cells (see Fig. 4F). NID and NIDF cells expressed high levels of a set of genes that are upregulated in hematopoietic early progenitors (34) (compared with NI cells) and hematopoietic stem cells (35) (compared with control BM cells) (Figs 4I and 4J, Table S6). These findings suggest that DNMT3A/R882H may confer stem/progenitor-cell properties on pre-AML cells such as NI cells to induce the development of AML.

IDH2/R140Q negatively regulates the 5hmC modification and expression of

differentiation-associated genes

TETs, which convert 5mC into 5hmC, are α-ketoglutarate-dependent enzymes; therefore, the level of 5hmC at TET2 target loci might be decreased in NIDF-AML cells. We isolated 5hmC-enriched DNA from normal BM and NIDF-AML cells and performed high-throughput sequencing of these DNA fragments. We aligned these fragments with the mouse genome and identified gene loci with low levels of 5hmC in AML cells (top 974 genes had a score of >4). Comparison of loci that had a low level of 5hmC with microarray data revealed that one gene was upregulated (>2-fold) and 35 genes were downregulated (<-2-fold) in AML cells compared with normal BM cells (Fig. 5A). Among these 35 downregulated genes, 8 genes were derepressed (>2-fold) after deletion of IDH2/R140Q according to microarray data (Figs 5A and B). The level of the one upregulated gene was not significantly changed after deletion of IDH2/R140Q. Among these 8 genes, we focused on *Ebf1 and Spib*, which are associated with the differentiation of B-cells (36) and macrophages (our unpublished data). Real-time PCR analysis confirmed the downregulation of these genes in Nf(I)DF-AML cells and the derepression of these genes in tamoxifen-treated cells (Fig. 5C). Quantitative PCR analysis indicated that 5hmC modification of these genes was lower in Nf(I)DF-AML cells than in control cells and that they were derepressed to some extent after tamoxifen treatment (Fig. 5D), consistent with the next-generation sequencing data. These data indicate that 5hmC modification and expression of *Ebf1* and *Spib* are reversibly downregulated by IDH2/R140Q.

Discussion

Taken together, our results show that IDH2/R140Q is necessary for the development and maintenance of AML. IDH1/2 mutations have been identified in AML, glioma, and many other cancers (22). Mutant IDHs dysregulate α-KG-dependent dioxygenases, such as TETs, EGLNs, collagen prolyl 4-hydroxylases, and histone demethylases (9-14). Since mutant IDH is an enzyme that acts via a mechanism that is completely different from those of other previously described oncogenes, it is expected to have potential as a therapeutic target. The IDH mutation occurs early in HSCs, and preleukemic cells engendered by the IDH mutation act as a reservoir for the evolution of AML (37,38). IDH-mutated preleukemic cells display chemoresistance, thus making them potential initiators of relapsed disease. These reports point to the importance of

eradicating IDH mutant-expressing cells for complete cure. Recent work demonstrated that selective inhibitors of mutant IDHs impair growth and induce differentiation of IDH mutant-expressing cells *in vitro* (16,17). The results described herein clearly demonstrate that IDH mutations can play critical roles in oncogenesis *in vivo*. The deletion of mutant IDH2 from AML cells leads to the depletion of mutant IDH2-expressing AML stem cells.

To analyze the role of mutant IDH in oncogenesis, we established a mouse model of mutant IDH-mediated AML. As noted previously, IDH1/2 mutations in AML frequently occur simultaneously with mutations in other genes such as *NPM* (about 30% of IDH mutation-positive patients have the NPM mutation), *DNMT3A* (about 40%), and *FLT3* (about 15%) (39). In accordance with these observations, we found that IDH2/R140Q, NPMc, DNMT3A/R882H, and FLT3/ITD cooperatively induced AML in a mouse model (Figs 2B–G). Our present data showed that IDH2/R140Q is necessary for the engraftment or survival of $NPMc^+$ cells *in vivo*. This is consistent with the previous reports showing that *IDH1/2* mutations occur as preleukemic events, followed by NPM1 and FLT3 mutations as late proliferative events (37,38).

NPMc and IDH2/R140Q cooperatively activated the Hoxa9/Meis1 pathway, and IDH2/R140Q activated the hypoxia pathway (Figs 1B, 4D, and 4H–I). These two pathways are likely to be important for IDH2/R140Q-mediated engraftment/survival of $NPMc^+$ cells in mice (Figs 4A and 4B). In addition to IDH2/R140Q and NPMc, expression of DNMT3A/R882H and FLT3/ITD is also required for efficient induction of AML. DNMT3A/R882H further upregulated the expression levels of Meis1 (Fig. 4H). Furthermore, DNMT3A/R882H promoted the maintenance of cells in an undifferentiated state (Figs 4I and 4J). Previous studies showed that FLT3/ITD promotes cell growth and survival (40). Taken together, our results suggest that the activation of multiple signaling pathways is required for NIDF cells to induce AML (Fig. 5E).

By deleting floxed IDH2/R140Q from NIDF-induced AML mice, we found that AML cells could not expand *in vivo* (Fig. 3G). Furthermore, the number of AML cells expressing LSC markers in these mice decreased after deletion of IDH2/R140Q; however, the remaining AML cells continued to express myeloid lineage markers (Figs 3I and S2). After deletion of IDH2/R140Q from AML cells in our mouse model, the cells still expressed NPMc. NPMc has been found only in myeloid leukemia patients (26).

These findings suggest that NPMc confers the myeloid phenotype on leukemic cells. It took time for the number of leukemia cells to decrease after deletion of IDH2/R140Q, suggesting that IDH2/R140Q is essential for the survival or inhibition of differentiation of LSCs, rather than for that of more differentiated cells (Fig. 3I). IDH2/R140Q-deleted AML cells showed defects in expansion *in vivo* and failed to induce AML after the third transplantation, indicating that LSCs were exhausted (Figs 3J and K). In accordance with these results, deletion of IDH2/R140Q dramatically prolonged the survival of AML mice (Figs 3E–F). IDH2/R140Q downregulated the 5hmC modification and expression of differentiation-inducing factors (*Ebf1* and *Spib*). These data suggest that IDH2/R140Q-mediated repression of TET reduces 5hmC modification of *Ebf1* and *Spib*. In T-cell development, 5hmC modification reportedly positively correlates with gene expression (41). It is possible that similar transcriptional control mechanisms operate in NIDF-AML cells. Importantly, when LSCs were depleted by IDH2/R140Q deletion, expression of *Ebf1* and *Spib* was derepressed. It is highly probable that re-activation of these differentiation-inducing factors contributes to the differentiation of LSCs.

During the preparation of the manuscript, four IDH mutant-mediated cancer model mice were reported (18-21). One previous report showed that forced expression of Hoxa9 and the IDH1 mutant cooperatively induced MPN-like myeloid leukemia (18). This report is consistent with our results showing that NPMc increases the expression levels of Hoxa9 and cooperates with IDH2/R140Q to induce MPN-like disease. Another report showed that introduction of an IDH2 mutant into FLT3-ITD knock-in hematopoietic progenitor cells induced AML (19). Since FLT3-ITD knock-in mice develop chronic myelomonocytic leukemia (42,43), mutations have accumulated in FLT3-ITD knock-in mice that effectively predispose them to develop AML by introducing the IDH2 mutations. Another report showed that a xenograft model of IDH2 mutant cells was predisposed to develop sarcomas (20). The last report suggested that when the IDH2 mutant is expressed in a well-established surrogate AML model induced by Hoxa9 and Meis1 overexpression, the IDH2 mutant confers IDH mutant-addiction on the AML cells (21). This report suggests that the IDH mutant is still a good therapeutic target for cancer treatment, even if it is acquired during the late evolution of AML. In this report, we show very clearly that the function of IDH2/R140Q is critical for the maintenance of AML. This result strongly suggests that mutant IDHs are promising targets for anticancer therapy.

Acknowledgments

We thank Dr. K. Tamai, Dr. O. Ando (Daiichi Sankyo), and Dr. Y. Kobayashi (National Cancer Center) for discussion. This work was supported in part by Grants-in-Aid from the Ministry of Health, Labor and Welfare; the Ministry of Education, Culture, Sports, Science and Technology; and the National Cancer Center Research and Development Fund.