Attenuated Acceleration to Leukemia after Ezh2 Loss in Nup98-HoxD13 (NHD13) Myelodysplastic Syndrome

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Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by dysplastic blood cell morphology, ineffective hematopoiesis and a high rate of transformation to acute myeloid leukemia (AML).2 Epigenetic dysregulation underpins the pathogenesis of MDS, with recurrent mutations in epigenetic regulators including TET2 (20%), ASXL1 (14%), DNMT3A (12%), EZH2 (6%) and IDH1/2 (5%).2,3 EZH2, or Enhancer of zeste homolog 2, is a histone methyltransferase and member of the highly conserved polycomb group of proteins, with important roles in regulating gene expression to coordinate self-renewal and differentiation of hematopoietic stem cells (HSCs).4 EZH2 loss-of-function mutations have an adverse effect on prognosis in MDS.2,3 Herein, we describe an in vivo model of attenuated acceleration to leukemia transformation with Ezh2 deletion in a mouse model of MDS.

EZH2, together with other core subunits EED, SUZ12, and RBBP4 form the polycomb repressor complex 2 (PRC2) complex responsible for the repressive tri-methylation modification of lysine 27 on histone 3 (H3K27me3).5 EZH1 and 2 are the only histone methyltransferases responsible for the H3K27 mark in mammals and functional redundancy exists with EZH1.6 EZH2 has important roles in maintaining HSC identity via repression of differentiation genes.5 EZH2 mutations are usually loss-of-function in a broad range of myeloid malignancies including MDS, myeloproliferative neoplasms (MPNs) and AML.2,3,7 In MDS, mutations occur within EZH2 in 6% of cases, however PRC2 is dysregulated in a larger subset of MDS (potentially 25–30% of cases) via gene deletion (del7q36.1) (3–4%),3 ASXL1 mutation (20%), which inhibits PRC2 function,10 or mutations in other PRC2 components (1–2%).11,11 EZH2 mutations occur rarely in de novo AML (~2%),8 but are relatively enriched in AML arising from a precedent MDS (9%).13 EZH2 mutations are a poor prognostic indicator in MDS overall,2 including low-risk MDS, where EZH2 mutation defines a subset with adverse clinical outcomes.14

In mouse models, loss of Ezh2 leads to fatal defects in fetal hematopoiesis, although inductive loss in adult mice leads to a milder phenotype including retained self-renewal of HSCs that are able to engraft in secondary recipients.1 This may reflect increased dependency on Ezh2 in highly proliferative fetal HSCs in the liver, compared to quiescent adult HSCs or may reflect Ezh1 compensation.15 However, the complete loss of PRC2 activity in Eed knockout mice, leads to pancytopenia, defective differentiation and inability to compete with wild-type cells in competitive transplants, demonstrating integral roles of PRC2 signaling in hematopoiesis.16 Inducible Ezh2 knock-out mice develop hematological malignancies with MDS, MDS/MPN17,19 and T-acute lymphoblastic leukaemia (ALL)20 described, but after a long latency suggesting cooperating mutations are required for transformation. Correspondingly, Ezh2 deletion combined with Tet2 deletion17 or Runx1 mutation18 accelerated the MDS disease seen in these respective mouse models.

The Nup98:HoxD13 transgenic (NHD13™) mouse model of MDS and secondary leukemia recapitulates key phases of human disease including a cytopenic phase which progresses at variable latencies to acute leukemia between 6 and 14 months.21 NHD13™ mice express, under the control of the hematopoietic-specific Vav promoter, a fusion oncogene comprising the Nup98 nucleoporin protein and the homeobox protein HoxD13. The Nup98: HoxD13 fusion is found rarely in human MDS or AML, however leukemia arising in NHD13™ is driven by the upregulation of Hox genes, a common mechanism in human disease.22 Additionally, epigenetic dysregulation appears to be an important contributor to Nup98-rearranged leukemia, as evidenced by its frequent fusions with epigenetic regulators.22
Given the driving role of EZH2 dysregulation in MDS and its poor prognostic implications, we sought to study the effects of Ezh2 loss-of-function in the NHD13T mouse model. As Hox gene overexpression is also observed in Ezh2-deleted MDS/AML,23 we hypothesized that the additive upregulation of Hox genes might provide a mechanism of cooperation between Ezh2 deletion and Nup98/HoxD13. In long-term survival studies, we found that Ezh2 deletion minimally accelerated leukemia development and death in NHD13T, demonstrating limited contribution to disease pathogenesis in the context studied.

NHD13T mice were crossed with Ezb20/0 mice24 expressing the polyinosinic-polycytidylic acid (poly I:C)-inducible Mx1-cre recombinase (Mx1) to generate NHD13T with Ezh2 deletion (NHD13T;Mx1;Ezh22/0) and control groups: wild-type (WT) (Ezb20/0 or Mx1), Ezb2-deleted only (Mx1;Ezb22/0) or NHD13T only (NHD13T;Ezb22/0 or NHD13T;Mx1). Poly (I:C) was administered intraperitoneally (6 injections over 2 weeks) to induce Cre recombinase and Ezh2 deletion in 8 to 12-week-old mice. Mice were monitored by monthly peripheral blood analyses and welfare scoring and culled at disease onset (Fig. 1A).

We confirmed absence of Ezb2 RNA and protein in bone marrow cells from Ezb2-deleted mice (Mx1;Ezb22/0 and NHD13T;Mx1;Ezb22/0) (Fig. 1B and C, Supplementary Table 1, Supplemental Digital Content, http://links.lww.com/HS/A40). Consistent with this, H3K27me3 was reduced although not absent (Fig. 1B). Expression of Ezb1 was maintained, although not upregulated, in Mx1;Ezb22/0 and NHD13T;Mx1;Ezb22/0 bone marrow and may explain the persistence of H3K27me3 (Fig. 1C). As H3K27me3 acts to repress gene expression, we confirmed gene de-repression consequences of Ezb2 deletion in our model. In Mx1;Ezb22/0, we observed increased CD109, a gene repressed by Ezb2 with no Ezb1 compensation (Fig. 1D).19 Conversely, there was no significant increase in Sfp1, a gene regulated by both Ezb2 and Ezb1 (Fig. 1D).19 Maintained Nup98:HoxD13 expression was confirmed in NHD13T bone marrow by polymerase chain reaction (PCR) (Supplementary Figure 1, Supplemental Digital Content, http://links.lww.com/HS/A40). Interestingly, bone marrow cells from NHD13T mice had markedly reduced Ezb2 protein expression despite normal mRNA levels, which suggested a post-transcriptional down-regulation of Ezb2. Nevertheless, NHD13T cells had normal levels of the H3K27me3 mark (Fig. 1B). Ezh2 deletion in NHD13T was able to reduce H3K27me3 to levels comparable with Mx1;Ezb22/0 mice and thus still represented a suitable model in which to assess consequences of Ezh2 deletion in a Hox-driven model of MDS.

EZH2 loss-of-function is associated with a poorer prognosis in MDS.2 We therefore sought to examine whether NHD13T;Mx1;Ezb22/0 mice would have a shortened latency to leukemia development compared to the single mutation (Mx1;Ezb22/0 or NHD13T) alone. NHD13T;Mx1;Ezb22/0 mice had similar peripheral blood parameters to NHD13T only mice, with leucopenia and thrombocytopenia by 3 months of age (baseline) and macrocytic anemia by 9 months post poly (I:C). Mx1;Ezb22/0 mice had similar counts to WT at baseline but developed mild leucopenia by 6 months and anemia by 9 months after poly (I:C) (Fig. 1E).

NHD13T and Mx1;Ezb22/0 mice had shortened overall survivals compared with WT mice (median 287 days and 273 days, respectively vs undefined, p < 0.0001). Loss of Ezb2 in NHD13T mice conferred additional reduction in median survival (median 241 days, vs NHD13T, p = 0.010 and vs Mx1;Ezb22/0, p = 0.0042), noting however, this was only 32 days shorter than with loss of Ezb2 alone (Fig. 1F). At time of death, Mx1;Ezb22/0, NHD13T and NHD13T;Mx1;Ezb22/0 mice developed a broad range of hematologic malignancies including T-ALL, AML, B-ALL, and MDS (Fig. 1G and Supplementary Tables 2, 3, and 4, Supplemental Digital Content, http://links.lww.com/HS/A40). There was a significant proportion of other acute leukemias that expressed dual-lineage markers akin to mixed phenotype acute leukemias and those that did not express B/T/myeloid markers (acute leukemia [AL]-other). There were no significant differences in proportions of leukemic subtypes as determined by pairwise Fisher exact testing (Supplementary Table 5, Supplemental Digital Content, http://links.lww.com/HS/A40).

Analysis of hematopoietic stem and progenitor cell (HSPC) subpopulations from non-leukemic mice demonstrated an expansion of multipotent progenitor (MPP3) clusters (CD48+, CD150+ fraction of the lineage-negative, Sca1-positive, ckit-positive (LSK) population), which contain the granulocyte/macrophage (GM)-committed and lymphoid-committed MPPs.25 In NHD13T;Mx1;Ezb22/0 and Mx1;Ezb22/0 mice perhaps explaining the preponderance of leukemias with mixed lineage expression in both these genotypes (Supplementary Figure 2, Supplemental Digital Content, http://links.lww.com/HS/A40). Leukemias from Mx1;Ezb22/0 and NHD13T exhibited increased HoxA9 expression compared to WT bone marrow although to a lesser degree in Mx1;Ezb22/0 (mean fold change 1.9 vs 4.3 in NHD13T, p = 0.027). There was no further increase in HoxA9 expression in most leukemias from NHD13T;Mx1;Ezb22/0 mice (Fig. 1H).

This study demonstrates a contribution of Ezb2 loss-of-function to NHD13T-driven MDS and leukemia, however, there was only a mild acceleration of disease onset and similar spectrum of blood cancers. The effect of Ezb2 deletion in this model may be abrogated for a number of reasons. First, substantial levels of H3K27me3 were maintained in the absence of Ezb2 expression. From H3K27me3 chromatin immunoprecipitation (ChIP)-sequencing studies, ~79% of Ezb2 target loci had compensatory methylation mediated by Ezb1.19 Functionally, Ezb1 compensation attenuated the hematologic phenotype caused by complete PRC2 dysfunction as has been elegantly demonstrated in Ezb2 knockout vs Eed knockout mice26 and Ezb6/Ezb2 double knockout mice.19 Given that Ezb2 and Ezb1 are the only known methyltransferases capable of H3K27 trimethylation in mammals,6 we presume the residual H3K27me3 demonstrated in the absence of Ezb2 was mediated by Ezb1 with consequent functional compensation for Ezb2 loss in our model, which we believe may have attenuated its phenotypic effects. Nonetheless, Ezh1 mutations are not seen in myeloid malignancy and thus these compensatory mechanisms are also likely to be active in human MDS. Second, Ezb2 protein levels were markedly reduced in NHD13T mice despite RNA expression showing a trendwise increase. Given the maintained H3K27me3 levels, it is likely that Ezb1 compensation was limiting effects of loss of Ezb2. Third, epigenetic mutations, including those in Ezh2, do not occur late in MDS pathogenesis.4 In our study, Ezb2 deletion was induced relatively late in disease pathogenesis after MDS features such as thrombocytopenia and leucopenia were already present in NHD13T. The timing of deletion may have also abrogated the influence of Ezb2 deletion in altering the course of NHD13T disease and is an inherent limitation of the transgenic NHD13 model. Finally, Ezh2 loss has been previously shown to upregulate HOX clusters, including HOXA genes, via reduction of H3K27me3 repression in human MDS.12,23 The potential overlap in mechanisms of transformation with NHD13T and Ezb2 loss through Hox gene dysregulation and lack of further de-
Figure 1. Long-term effects of Ezh2 deletion in NHD13<sup>−/−</sup> leukemia. A) Breeding and experimental schema for long-term survival assessment. PB, peripheral blood. B) Western blot demonstrating Ezh2 and H3K27me3 protein levels in Ezh2<sup>+/−</sup>, NHD13<sup>−/−</sup> and NHD13<sup>+/−</sup>; Ezh2<sup>+/−</sup> leukemias compared to kit-enriched wild-type bone marrow from adult C57BL/6J mice (WT), relative to an Actin loading control. Leukemic bone marrow samples of each genotype were taken from selected mice listed in Supplementary Table 1 (Supplemental Digital Content, http://links.lww.com/HS/A40) and loaded in the listed order. C) Ezh2 and Ezh1 RNA expression levels as measured by qPCR in Ezh2<sup>+/−</sup>, NHD13<sup>−/−</sup> and NHD13<sup>+/−</sup>; Ezh2<sup>+/−</sup> leukemias compared to wild-type whole bone marrow (WT WBM) and WT kit-enriched bone marrow (WT kit). Samples were taken from the same mice as for B). D) Expression of Ezh2 targets as measured by qPCR. E) Peripheral blood counts prior to (baseline) and at 6 months post Poly (I:C). WCC, white cell counts. F) Kaplan-Meier survival curve after Poly (I:C) administration for each genotype. G) Proportions of disease types analyzed at time of cull for each genotype. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; AL, acute leukemia. H) HoxA9 expression by qPCR in Ezh2<sup>+/−</sup>, NHD13<sup>−/−</sup> and NHD13<sup>+/−</sup>; Ezh2<sup>+/−</sup> leukemias compared to WT WBM. Statistics for qPCR experiments show results of unpaired t tests. Statistics for peripheral blood analyses show results of ANOVA testing. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001 and ∗∗∗∗P < 0.0001.
repression of HoxA9 in NHD13T;MxT;Ezh2 Δ/Δ mice may explain the lack of in vivo synergy. 

MxT;Ezh2 Δ/Δ mice in this model exhibited a highly penetrant leukemia with most mice succumbing to acute leukemia during the observation period with a median survival of 273 days. Our findings are most similar to the report by Simon et al where all mice exclusively developed T-ALL after a latency of approximately 10 months.17 In contrast, in other models using a tamoxifen-inducible Cre-ERT, Ezh2-deleted mice developed features of MDS, MPN and MDS/MPN17–19 although in 2 of these reports, had similar overall survival to wild-type controls over a period of 300 days’ observation.17,19 Two studies reported effects of Ezh2 deletion in combination with other mutations seen in myeloid malignancy, Runx1 mutation and Tet2 knockdown and demonstrated greater acceleration of hematological malignancy from Ezh2 deletion compared to the NHD13T background. In combination with Runx1 mutation, Ezh2 loss led to a median overall survival of 262 days compared to ‘not reached’ in either mutation alone over 10 months’ observation.19 Similarly on a Tet2 knockdown background, deletion of Ezh2 accelerated death (median ~180 days) compared with a ~300 day median overall survival in mice with either single mutation.17 Altogether, these studies highlight diverse, context-dependent outcomes of Ezh2 deletion in mouse models.

In conclusion, this study describes a model of EZH2 deletion in MDS, adding to existing literature on the cooperation of Ezh2 with other genetic aberrations in MDS pathogenesis.17,18 Our findings suggest Ezh2 loss may have limited effects in the NHD13T given active Ezb1 compensation and overlapping mechanisms of transformation and highlights caveats in preclinical modeling of disease states.

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