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The efficiency of murine MLL-ENL–driven leukemia initiation changes with age and peaks during neonatal development

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Key Points

- *MLL-ENL* alters gene expression and initiates leukemogenesis most efficiently in neonatal progenitors.
- *Lin28b* suppresses *MLL-ENL*–driven leukemogenesis, and it potentially protects against leukemia formation during fetal development.

MLL rearrangements are translocation mutations that cause both acute lymphoblastic leukemia and acute myeloid leukemia (AML). These translocations can occur as sole clonal driver mutations in infant leukemias, suggesting that fetal or neonatal hematopoietic progenitors may be exquisitely sensitive to transformation by MLL fusion proteins. To test this possibility, we used transgenic mice to induce one translocation product, *MLL-ENL*, during fetal, neonatal, juvenile and adult stages of life. When *MLL-ENL* was induced in fetal or neonatal mice, almost all died of AML. In contrast, when *MLL-ENL* was induced in adult mice, most survived for >1 year despite sustained transgene expression. AML initiation was most efficient when *MLL-ENL* was induced in neonates, and even transient suppression of *MLL-ENL* in neonates could prevent AML in most mice. MLL-ENL target genes were induced more efficiently in neonatal progenitors than in adult progenitors, consistent with the distinct AML initiation efficiencies. Interestingly, transplantation stress mitigated the developmental barrier to leukemogenesis. Since fetal/neonatal progenitors were highly competent to initiate *MLL-ENL*–driven AML, we tested whether *Lin28b*, a fetal master regulator, could accelerate leukemogenesis. Surprisingly, *Lin28b* suppressed AML initiation rather than accelerating it. This may explain why *MLL* rearrangements often occur before birth in human infant leukemia patients, but transformation usually does not occur until after birth, when *Lin28b* levels decline. Our findings show that the efficiency of *MLL-ENL*–driven AML initiation changes through the course of pre- and postnatal development, and developmental programs can be manipulated to impede transformation.

Introduction

Infant leukemias are clinically and genetically distinct from leukemias that present later in childhood or in adulthood.[1-3] They initiate prior to or shortly after birth, and they can present as either acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML).[4,5] Unlike many other childhood leukemias, infant leukemias are difficult to treat, and most infants die of their disease despite intense chemotherapy.[6-8] Approximately 40% of infants with AML and ~70% of infants with ALL harbor *MLL* (*KMT2A*) rearrangements (eg, *MLL-ENL*, *MLL-AF4*, or *MLL-AF9*).[2,8] These leukemias carry very few additional mutations, and in some cases, the *MLL* rearrangement appears to be the sole clonal driver mutation.[3] *MLL* rearrangements are less common, relative to other mutations, in later childhood and adult leukemias.[2,3,9] In instances when they do occur in adults, the leukemias typically carry additional cooperating mutations,[3] and they often occur in patients who have received prior chemotherapy for

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other malignancies.[10,11] Thus, the genetic changes that cause leukemia in infants are distinct from changes that cause leukemia in adults.

MLL encodes a SET-domain–containing histone 3, lysine 4 (H3K4) methyltransferase that promotes expression of genes associated with self-renewal (eg, *MEIS1* and *HOXA* cluster genes).[12-14] In *MLL*-rearranged leukemias, the C terminus of the protein, including the SET domain, is replaced with one of several potential fusion partners (eg, ENL, AF4, AF9, and AF10).[11,15] MLL fusion proteins recruit the H3K79 methyltransferase DOT1L, and the H3K36 methyltransferase ASH1L, to MLL target loci as part of a multiprotein complex.[16-18] H3K79 and H3K36 dimethylation marks correlate with active gene expression, and both *Dot1l* and *Ash1l* are required for MLL-AF9–driven AML to develop in mice.[17,18] In addition to regulating histone methylation, MLL fusion proteins nucleate a complex, called the superelongation complex, which promotes transcriptional elongation.[19,20] Through these biochemical activities, MLL fusion proteins elicit epigenetic and transcriptional changes that promote self-renewal and distinguish infant leukemias from most other types of childhood and adult AML or ALL.[21-28]

It is not clear why *MLL* rearrangements require so few cooperating mutations to initiate infant leukemias. One possible explanation is that fetal or neonatal hematopoietic progenitors may be inherently more susceptible to transformation by MLL fusion proteins than adult progenitors. Several lines of evidence support this hypothesis. For example, prior studies have shown that human umbilical cord blood progenitors can be immortalized more efficiently by MLL-AF9–expressing lentiviruses than adult bone marrow progenitors.[29] Furthermore, MLL-AF4 has been shown to induce B-cell proliferation, and eventual lymphomagenesis, when it is induced in fetal B-cell progenitors, but it does not cause lymphomas when it is induced after birth.[30] Finally, other leukemogenic mutations have been shown to have developmental context-specific effects on self-renewal and leukemia initiation. *Pten* loss of function and *Flt3*-internal tandem duplication mutations deplete hematopoietic stem cells (HSCs), expand committed progenitor populations, and cause leukemia only in juvenile or adult developmental contexts.[31,32] *MLL* rearrangements may have a similar propensity to cause leukemia in a fetal or neonatal context, particularly when they are expressed at physiologic rather than supraphysiologic levels.

In the current study, we sought to identify the developmental stages that are most permissive for MLL-ENL–driven leukemogenesis. We found that *MLL-ENL* caused AML with the highest penetrance (100%) and the shortest latency when it was induced in neonates as compared with both fetal and adult mice. Even transient suppression of *MLL-ENL* during the neonatal period was sufficient to prevent leukemogenesis in most mice. *MLL-ENL* caused HSC and lineage-committed hematopoietic progenitor cell (HPC) depletion when it was induced in fetal/neonatal mice, but not when it was induced in adult mice. Furthermore, MLL-ENL target genes were activated more efficiently in neonatal progenitors than in adult progenitors. Adult progenitors did give rise to AML with complete penetrance when they were transplanted. These findings show that leukemia initiation efficiency changes as developmental context changes, but the barriers to transformation can be mitigated by transplantation stress.

We tested whether a fetal master regulator, *Lin28b*, could accelerate rates of AML initiation in *MLL-ENL*–expressing mice. *Lin28b* is highly expressed in fetal HSCs and HPCs, but levels decline in neonatal progenitors, both in mice and humans.[32-34] To our surprise, sustained *Lin28b* expression suppressed rather than accelerated AML formation, suggesting that it may afford protection from *MLL* rearrangements during fetal stages before its expression declines in neonates. This may explain why, in humans, *MLL* rearrangements often occur prior to birth but overt leukemias do not usually arise until after birth.[4,5,35] Altogether, the data show that MLL-ENL-mediated changes in gene expression and cell fate are developmental stage-specific, and AML initiation efficiency peaks during the neonatal stage of life. Mechanisms that define this window of peak susceptibility can be manipulated to suppress leukemogenesis.

Materials and methods

Mouse strains and husbandry

The *Col1a1*$^{TetO\_MLL\text{-}ENL}$ line has been previously described.[36] *Vav1-Cre* (008610),[37] *Rosa*$^{LSL\text{-}tTA}$ (011008),[38] *Rosa*$^{LSL\text{-}rtTA\text{-}EGFP}$ (005670),[39] and *Col1a1*$^{TetO\_H2B\text{-}GFP}$ (016836)[40] mice were obtained from The Jackson Laboratory. *Col1a1*$^{TetO\_Lin28b}$ mice were obtained from Hao Zhu (UT-Southwestern) and are now available at The Jackson Laboratory (023911).[41] Doxycycline chow (200 ppm) was purchased from Bioserv. All mouse strains were on a C57BL/6 genetic background. Transplantations were performed as described previously.[32] All animals were housed in a pathogen-free barrier facility, and all procedures were performed according to an Institutional Animal Care and Use Committee–approved protocol.

Flow cytometry

Bone marrow cells were obtained, stained, and analyzed as described previously.[32] Cell counts were measured by hemocytometer. Antibodies are listed in supplemental Materials and methods. Flow cytometry was performed on a BD FACSAria Fusion flow cytometer (BD Biosciences). Bromodeoxyuridine (BrdU) incorporation assays were performed with the APC BrdU Flow Kit (BD Biosciences) according to manufacturer's instructions as described previously.[31] A detailed protocol is provided in supplemental Materials and methods.

Leukemia analyses

Mice were euthanized when they became moribund. Spleens and bone marrow were analyzed by flow cytometry with antibodies to CD3, CD11b, Gr-1, and B220 to determine the leukemia type. Spleen sections were stained with hematoxylin and eosin. Bone marrow cells were isolated and spun onto glass slides using a Shandon Cytospin 3. The slides were stained using Protocol Hema 3 Wright-Giemsa stain (Fisher Scientific). CD3 immunohistochemistry was performed with rat anti-CD3 (Bio-Rad, CD3-12) and horseradish peroxidase–conjugated goat anti-rat immunoglobulin G (BioLegend). Sections were developed with the DAB peroxidase substrate kit (Vector Labs) according to manufacturer protocols. Lentivirus transductions and subsequent analyses (supplemental Figure 5) are described in supplemental Materials and methods.

Gene expression analysis by RNA-seq

Ten thousand cells of the indicated phenotypes were double sorted into phosphate-buffered saline with 0.1% bovine serum albumin. Cells were pelleted by centrifugation and resuspended in RLT-plus

RNA lysis buffer (Qiagen). RNA was isolated with RNAeasy micro-plus columns (Qiagen). RNA sequencing (RNA-seq) libraries were generated with Clontech SMRTr kits, and sequencing was performed on a HiSeq3000. Sequences were aligned to the mouse genome (Ensembl release 76 top-level assembly) using STAR version 2.0.4b.[42] The sequence for human *MLL-ENL* was included in the alignment. Linear modeling (limma/voom) was used to compare gene expression across samples. False discovery rates were calculated using the Benjamini and Hochberg method.[43]

Results

MLL-ENL initiates AML more efficiently in fetal/neonatal progenitors than in juvenile/adult progenitors

We used a previously described, tetracycline-regulated *MLL-ENL* transgene (*Col1a1*$^{TetO\_MLL\text{-}ENL}$)[36] to test whether the efficiency of *MLL-ENL* mediated leukemia initiation changes with age. We crossed *Col1a1*$^{TetO\_MLL\text{-}ENL}$ mice with *Vav1-Cre* and *Rosa26-loxP-stop-loxP-tet-transactivator* (*Rosa*$^{LSL\text{-}tTA}$) mice (Figure 1A). The resulting mice (hereafter called tet-OFF-*MLL-ENL*) expressed *MLL-ENL* specifically in hematopoietic cells beginning at embryonic day 10.5 (E10.5) in the absence of doxycycline, concordant with the onset of *Vav1-Cre* expression.[44] To suppress *MLL-ENL* expression until later ages, we fed mothers, and later the pups, doxycycline chow until postnatal day 0 (P0), P21, or 8 weeks after birth. These time points encompassed fetal (E10.5), neonatal (P0), juvenile (P21), and adult (8 weeks old) stages of induction. Analysis of *Col1a1*$^{TetO\_H2B\text{-}GFP}$ reporter mice confirmed that doxycycline could suppress transgene expression from the *Col1a1* locus in nursing neonates, and doxycycline withdrawal at birth led to induction of the transgene within the first 3 weeks of life (supplemental Figure 1A-B)

We observed differences in the penetrance and latency of leukemia formation when we induced *MLL-ENL* expression at different ages (Figure 1B). When *MLL-ENL* was induced at E10.5, a majority of mice developed AML, though the latency varied considerably, and some mice did not develop AML until almost 1 year later. When *MLL-ENL* was induced at P0, all mice died of AML, and almost all deaths occurred between 3 and 4 months after birth. When *MLL-ENL* was induced at P21 or 8 weeks after birth, a majority of mice survived for >1 year without developing signs of illness. All of the moribund mice had AML based on expression of myeloid markers Gr1 and CD11b (Figure 1C), the absence of lymphoid markers (data not shown), and an abundance of blasts in the bone marrow (Figure 1D). Mice that survived for >1 year without illness had only slightly enlarged spleens (supplemental Figure 2A). There was mild hypercellularity within the red pulp, but follicles were evident, and there was no evidence of AML, in contrast to spleens from moribund mice (supplemental Figure 2B-D).

We evaluated expression of the *MLL-ENL* transgene by RNA-seq to confirm that it was expressed in older mice after adult induction. *MLL-ENL* transcript levels were similar to endogenous *Mll1* in both 10-week-old and 14-month-old HPCs and pre–granulocyte-monocyte progenitors (pGMs) after fetal and adult induction, respectively (Figure 1E). However, the transcript was not induced immediately after doxycycline withdrawal in 8-week-old adult mice. At 5 weeks after doxycycline withdrawal (13 weeks old), *MLL-ENL* transcript levels were low in HPCs relative to untreated mice (supplemental Figure 2E). By 4 months after doxycycline withdrawal, *MLL-ENL* transcript levels were similar to levels observed in untreated mice, and they persisted 12 months later (supplemental Figure 2E). Unfortunately, we could not detect MLL-ENL protein levels by western blot, even in AML cells, so we were unable to assess whether protein expression changed with age (supplemental Figure 2F). Nevertheless, AML initiation was most efficient when the *MLL-ENL* transgene was induced in neonatal progenitors.

We next tested whether transient inactivation of *MLL-ENL* in neonatal mice could suppress AML initiation. We administered either normal or doxycycline-containing chow to nursing mothers beginning at P0 (Figure 2A). Doxycycline treatments were discontinued after 4 weeks. As in the initial experiment, sustained *MLL-ENL* expression caused most of the mice to die of AML (Figure 2B). In contrast, a 4-week pulse of doxycycline was sufficient to prevent AML in 15 of 17 mice tested (Figure 2B). The survivors had normal spleen sizes, normal blood counts, and normal bone marrow morphology even at 1 year after birth (Figure 2C-G). Thus, *MLL-ENL*–expressing fetal/neonatal progenitors can lose leukemogenic potential if the mutation is suppressed, even transiently, after birth.

MLL-ENL depletes fetal, but not adult, HSCs and HPCs, and it induces age- and cell-type–specific changes in gene expression

The age-specific effects of *MLL-ENL* on AML initiation raised the question of whether the mutation also has age-specific effects on preleukemic HSCs and HPCs. We measured HSC ($CD150^+CD48^-Lineage^-Sca1^+c\text{-}kit^+$), HPC ($CD150^-CD48^+Lineage^-Sca1^+c\text{-}kit^+$), pGM ($Lineage^-Sca1^-c\text{-}kit^+CD150^-CD105^-CD16/32^-$), and granulocyte-monocyte progenitor (GMPs; $Lineage^-Sca1^-c\text{-}kit^+CD150^-CD105^-CD16/32^+$) numbers after fetal (E10.5) and adult (8 week) *MLL-ENL* induction (supplemental Figure 3A).[45] Fetal *MLL-ENL* induction caused HSC depletion at P0 that persisted at 10 weeks after birth (Figure 3A). HPCs were also depleted in 10-week-old mice after fetal induction (Figure 3B). These phenotypes were consistent with prior observations showing that *MLL-ENL* impairs HSC function,[36] and they were not observed in P0 mice that were exposed to doxycycline via their mothers (supplemental Figure 3B-E). Adult *MLL-ENL* induction did not cause HSC or HPC depletion (Figure 3A-B). Instead, it led to a modest but significant increase in HSC numbers at 4 and 12 months after doxycycline withdrawal (Figure 3A). pGM and GMP numbers were not significantly altered by *MLL-ENL* at either age (data not shown). BrdU incorporation assays revealed a potential explanation for why *MLL-ENL*–expressing HSCs and HPCs were depleted in neonatal mice. *MLL-ENL* impaired both HSC and HPC proliferation in E18.5 fetal mice, consistent with prior observations,[36] but proliferation rates were similar to controls in 10-week-old adult mice (Figure 3C; supplemental Figure 4A-D). Thus, *MLL-ENL*–dependent changes in HSC and HPC fate are age specific.

We next tested whether *MLL-ENL* has age-specific effects on gene expression. We performed RNA-seq on HPCs, pGMs, and GMPs that were isolated from control and *MLL-ENL*–expressing mice. After fetal *MLL-ENL* induction, we observed widespread changes in gene expression at P0 (Figure 4A,C). Differentially expressed transcripts included known MLL-ENL targets, such as *Hoxa9*, *Hoxa10*, and *Mecom*, and the HOXA9 target *Igf1* (Figure 4D-G; supplemental Table 1). The expression changes were more extensive in neonatal HPCs than in neonatal pGMs or

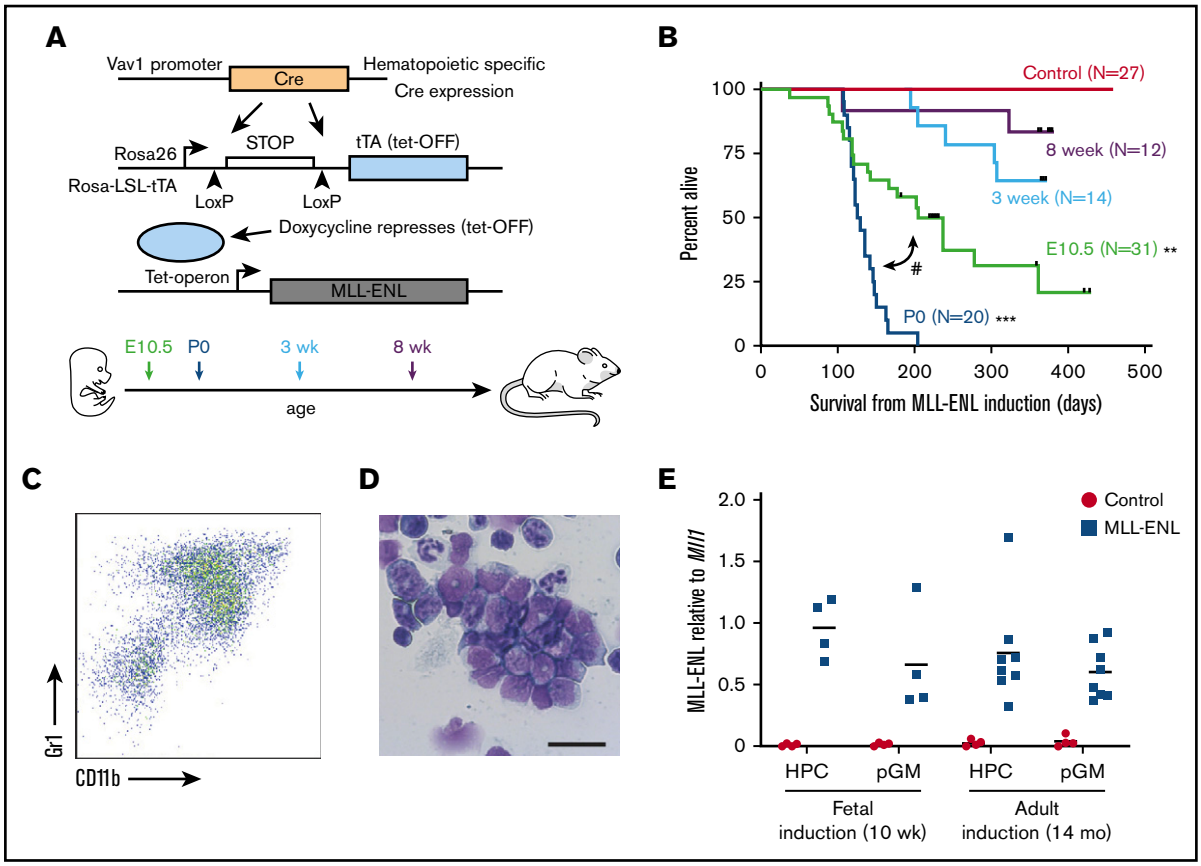

Figure 1. *MLL-ENL* initiates AML most efficiently when it is induced in neonates. (A) Overview of the strategy for inducing *MLL-ENL* at E10.5, P0, and 3 weeks and 8 weeks after birth. (B) Kaplan-Meier survival curves for mice after *MLL-ENL* induction at the indicated ages. $^{**}P < .01$, $^{***}P < .0001$ relative to induction at 8 weeks after birth. $\#P < .0001$ for P0 relative to E10.5 induction. Survival curves were compared with the log-rank test. Group sizes are indicated. (C) Representative flow plot for AML specimens from Tet-OFF-*MLL-ENL* mice. (D) Representative AML cytospin. Scale bar indicates 100 μM. (E) Expression of the *MLL-ENL* transcript by RNA-seq after induction at E10.5. Reads from HPCs and pGMs were mapped to the human *MLL-ENL* sequence for the indicated induction ages and genotypes. Reads per kilobase of transcript per million mapped reads values from each sample were normalized to one allele of endogenous *Mll1*.

GMPs (Figure 4A). Transcriptional changes were also far more extensive in P0 HPCs than in 10-week-old HPCs (Figure 4A,C). After adult *MLL-ENL* induction, the only differentially expressed transcript was *MLL-ENL* itself (Figure 4B). Thus, the transcriptomes of *MLL-ENL*–expressing progenitors change with age.

Our data suggest that there may be a barrier to MLL-ENL target gene expression in adult progenitors that could be overcome simply by increasing transgene expression. To increase *MLL-ENL* transgene expression, we generated mice that were homozygous, rather than heterozygous, for the *Col1a1*$^{TetO\_MLL\text{-}ENL}$ allele. RNA-seq confirmed that, on average, these mice expressed *MLL-ENL* at approximately twice the level observed in heterozygous HPCs, pGMs and GMPs (Figure 5A). Homozygous Tet-OFF-*MLL-ENL* mice had significant reductions in HSC and HPC populations at 10 weeks after birth, though pGM and GMP numbers were not significantly different from littermate *Vav1-Cre*-negative control mice (Figure 5B-E). Gene expression changes were more extensive in homozygous transgenic adult HPCs, pGMs and GMPs (Figure 5F-H; supplemental Table 1), but the number of differentially expressed genes was still small relative to the changes observed in heterozygous neonatal HPCs (Figure 5I-J). Of note, none of these mice received doxycycline, so age, rather than doxycycline clearance rates, accounted for temporal changes in MLL-ENL target gene expression. The data show that neonatal HPCs are far more competent to activate MLL-ENL target genes than adult HPCs, and this effect can be offset, but only partially, by doubling *MLL-ENL* expression levels.

Transplanted adult progenitors are highly competent to initiate MLL-ENL driven AML

Our data show that as hematopoietic progenitors transition from neonatal to adult states, they become less efficient at initiating AML. However, prior work has demonstrated highly penetrant AML formation in recipients of transplanted adult, *MLL-ENL*–expressing HPCs, pGMs, and GMPs.[36] This raised the question of whether transplantation enhances the ability of adult progenitors to initiate AML. To test this possibility, we induced *MLL-ENL* at 8 weeks after birth, as in previous cohorts (Figure 6A). Four months later, we transplanted 300 000 *MLL-ENL*-expressing whole bone marrow cells and 300 000 wild-type CD45.1 competitor cells into lethally irradiated CD45.1 mice. All recipient mice exhibited multilineage

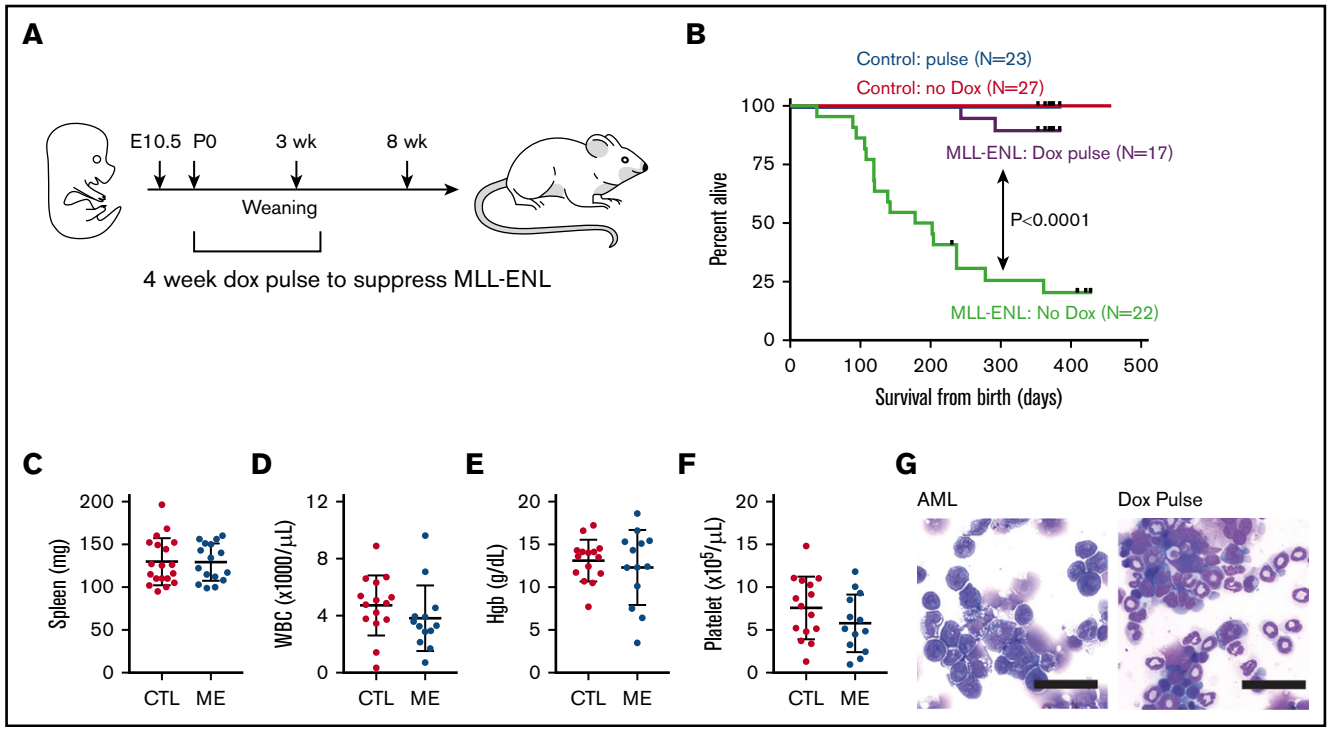

Figure 2. Transient suppression of *MLL-ENL* in neonates prevents AML in most mice. (A) Overview of experimental design. (B) Kaplan-Meier survival curves for Tet-OFF-*MLL-ENL* mice after neonatal suppression of the transgene. The *P* value is indicated and reflects a comparison of mice that received a 4-week doxycycline (Dox) pulse, beginning at P0, to mice that did not receive doxycycline. Group sizes are indicated. Survival curves were compared by the log-rank test. (C-F) Spleen weights, white blood cell (WBC) counts, and hemoglobin (Hgb) and platelet counts for mice that survived for >1 year after transient *MLL-ENL* suppression. All mice were euthanized ~12 months after birth. (G) Representative bone marrow cytospins for mice that developed AML, in the absence of doxycycline, and mice that survived for >1 year after transient *MLL-ENL* suppression. Scale bars indicate 100 μM. For panels C-F, CTL indicates control and ME indicates *Tet-OFF-MLL-ENL* genotypes.

donor engraftment at 4 weeks posttransplant (Figure 6B). CD45.2 peripheral blood chimerism was somewhat higher in recipients of *MLL-ENL*–expressing marrow than in recipients of *Vav1-Cre*–negative control bone marrow. By 3 months posttransplant, the recipient mice began developing AML, and all recipient mice ultimately died (Figure 6C). We repeated the transplantation assays by sorting and transplanting 3000 HPCs or 3000 pGMs so that we could ascertain a cell of origin, as in the prior study by Ugale et al.[36] All recipients developed AML within 120 days after transplantation (Figure 6D). Other stressors, such as single doses of 5-fluorouracil or a 1-week course of polyinosine:polycytosine did not stimulate AML in adult mice (supplemental Figure 5A). Furthermore, adult

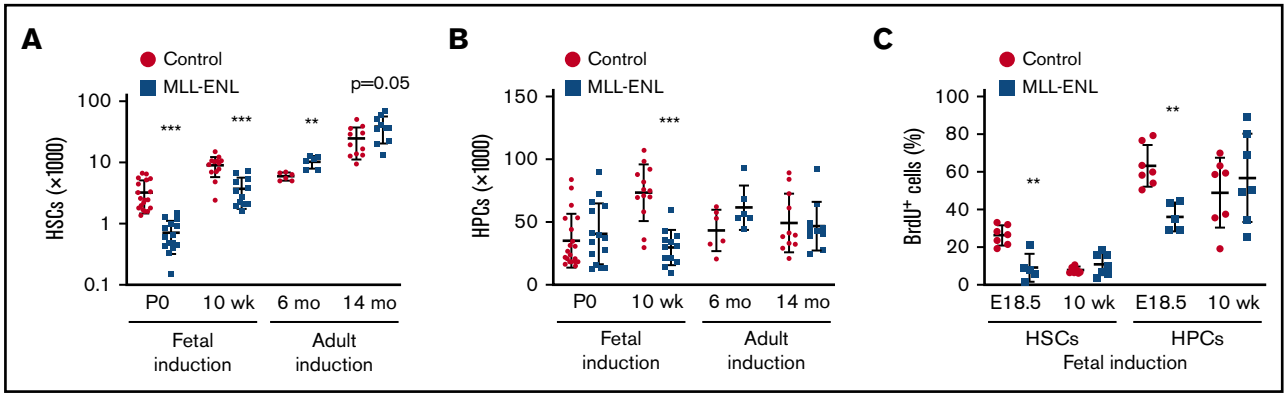

Figure 3. *MLL-ENL* depletes HSC and HPC numbers when it is induced in fetal, but not adult, mice. (A-B) HSC and HPC numbers in P0 liver (n = 15-19) or 10-week-old bone marrow (n = 10-12) after fetal *MLL-ENL* induction or in 6-month-old bone marrow (n = 6 per genotype) or 14-month-old bone marrow (n = 10-11) after adult *MLL-ENL* induction. Bone marrow cell numbers reflect 2 hindlimbs (tibias and femurs). (C) Twenty-four-hour HSC and HPC BrdU incorporation percentages were measured in E18.5 livers (n = 5-7) or 10-week-old bone marrow (n = 7 per genotype) after fetal *MLL-ENL* induction. For all panels, error bars indicate standard deviations. ***P* < .01, ****P* < .001 for Tet-OFF-*MLL-ENL* relative to controls. Groups were compared by the 2-tailed Student *t* test.

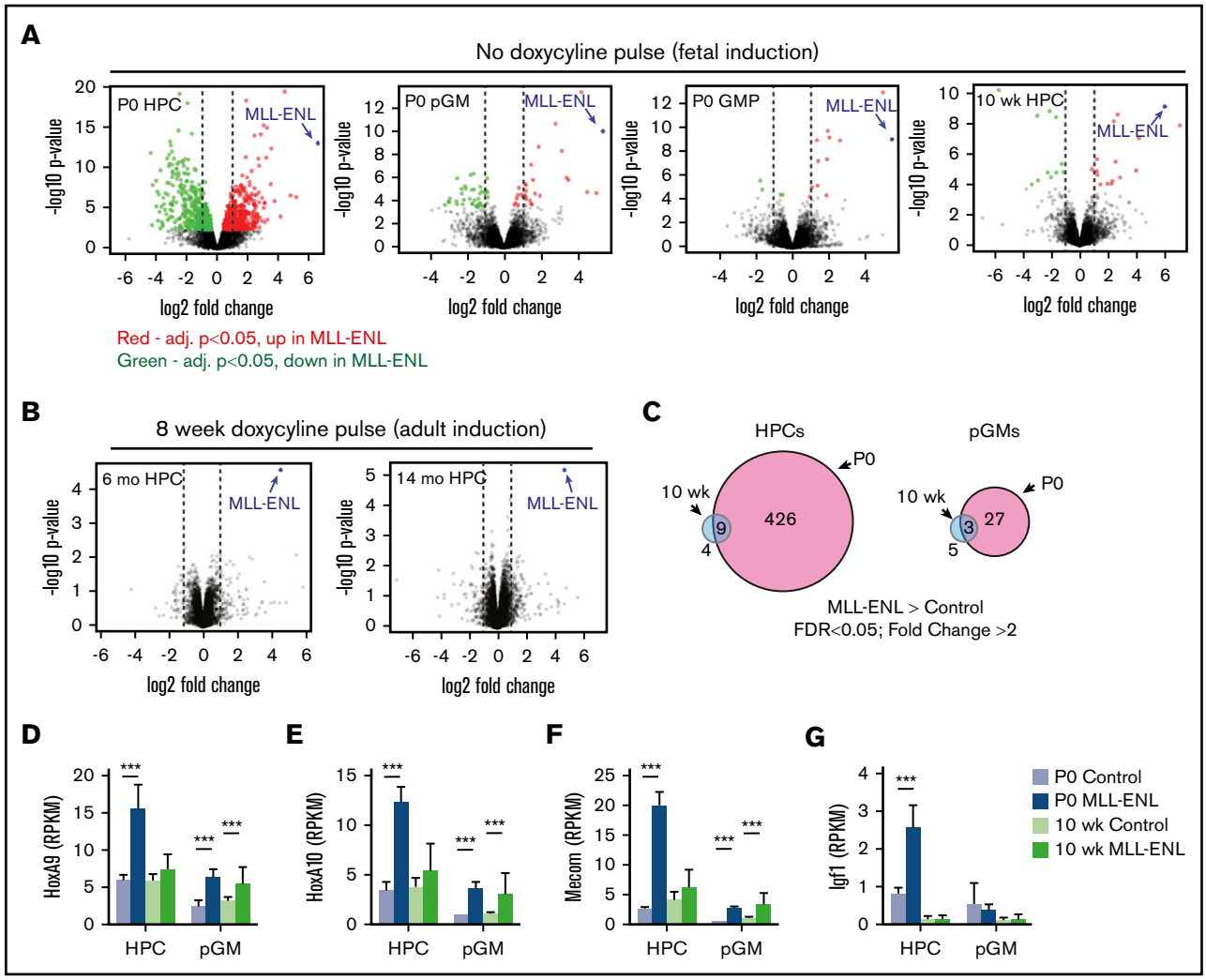

Figure 4. MLL-ENL induces changes in gene expression more efficiently in neonatal HPCs than in older or more committed progenitors. (A) Volcano plots show genes that are significantly induced (red) or significantly repressed (green) in HPCs, pGMs, or GMPs at P0, or in 10-week-old HPCs, after fetal MLL-ENL induction. The *MLL-ENL* transcript was mapped, and expression is indicated as a blue data point. RNA-seq data were analyzed, and adjusted (adj.) *P* values were calculated, as indicated in "Materials and methods." Genes that changed with an adjusted $P < .05$ were considered significantly differentially expressed. (B) Volcano plots showing a lack of differential gene expression after adult *MLL-ENL* induction. The panels indicate gene expression 6-month-old or 14-month-old mice (4 months or 12 months after doxycycline withdrawal). *MLL-ENL* was expressed at these ages as indicated by the blue dots. (C) Venn diagrams comparing the number of genes for the indicated cell types and ages that showed significantly increased expression and a fold change >2. Data reflect fetal *MLL-ENL* induction. FDR, false discovery rate. (D-G) Reads per kilobase of transcript per million mapped reads (RPKM) values for *Hoxa9*, *Hoxa10*, *Mecom*, and *Igf1* at the indicated ages after fetal MLL-ENL induction. Error bars indicate standard deviation. ***Adjusted $P < .001$. For all groups, n = 3 to 4 independent biological replicates.

HPCs expressed lower rather than higher levels of MLL-ENL target genes when they were stressed by ex vivo culture (supplemental Figure 5B). Thus, transplantation stress can mitigate the barrier to AML initiation in adulthood, but other stressors may not, at least when they are transiently applied.

The fetal master regulator, Lin28b, suppresses MLL-ENL–driven AML initiation

The finding that MLL-ENL initiates AML more efficiently in fetal/neonatal progenitors than in adult progenitors raised the question of whether fetal-specific genes could accelerate leukemogenesis. Consistent with this possibility, 3 previously described fetal HSC regulators, *Lin28b*, *Hmga2*, and *Igf2bp2*, were hyperactivated by MLL-ENL in neonatal, but not adult, HPCs (Figure 7A-C). *Lin28b* encodes a fetal master regulator that has been shown to promote self-renewal and fetal lineage priming when it is expressed in adult HSCs.[33,34,46,47] It enhances *Hmga2* and *Igfbp2* expression by inhibiting *let-7* family microRNAs.[33] These genes potentially interact with *MLL-ENL* to promote AML initiation.

To test whether *Lin28b* could accelerate MLL-ENL–driven leukemogenesis, we generated mice to express both *MLL-ENL* and *Lin28b* transgenes from the *Col1a1* allele (*Vav1-Cre; Rosa26*$^{LSL\text{-}tTA}$; *Col1a1*$^{tetO\_MLL\text{-}ENL/tetO\_Lin28b}$). We induced *MLL-ENL* and *Lin28b* expression at P0, since this was the age at which endogenous

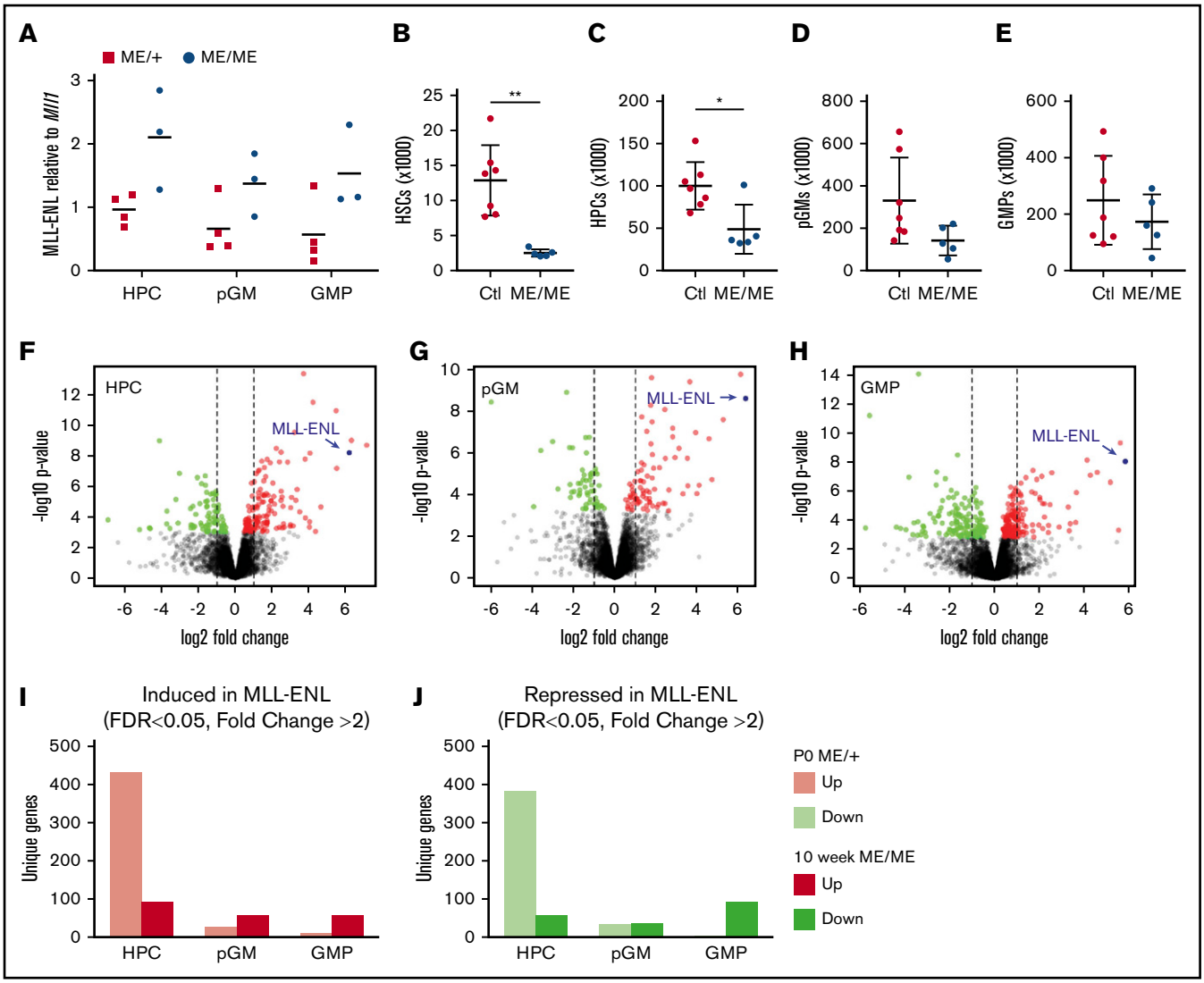

Figure 5. Increased *MLL-ENL* expression partially overcomes the barrier to target gene activation and repression in adult progenitors. (A) *MLL-ENL* transcript levels in *Col1a1*$^{TetO\_MLL\text{-}ENL}$ heterozygous or homozygous mice. Transcript expression was normalized to endogenous *Mll1* in each sample, as in Figure 1E. (B-E) HSC, HPC, pGM, and GMP numbers in 10-week-old, homozygous Tet-OFF-*MLL-ENL* mice after fetal induction. Error bars indicate standard deviations. n = 5 to 8, $*P < .05$, $**P < .01$; groups were compared with the 2-tailed Student *t* test. (F-H) Volcano plots show genes that are significantly induced (red) or significantly repressed (green) in HPCs, pGMs, or GMPs in 10-week-old mice that are homozygous for the *Col1a1*$^{TetO\_MLL\text{-}ENL}$ allele. The *MLL-ENL* transcript was mapped, and expression is indicated as a blue data point. Adjusted *P* values were calculated as indicated in "Materials and methods." (I-J) Numbers of unique genes that were significantly induced or repressed with a fold change >2 in P0 mice with a heterozygous *Col1a1*$^{TetO\_MLL\text{-}ENL}$ allele, as compared with adult mice with a homozygous *Col1a1*$^{TetO\_MLL\text{-}ENL}$ allele. Changes in gene expression were far more extensive in neonatal HPCs than at other time points or in other cell types, despite the presence of only a single *MLL-ENL* transgene. For all groups, n = 4 independent biological replicates. For panels B-E, Ctl indicates control and ME indicates *Tet-OFF-MLL-ENL* genotypes.

Lin28b expression naturally declines,[32,33] and we monitored survival. As in the previous analyses, almost all *MLL-ENL*–expressing mice died of AML within 130 days (Figure 7D). However, a majority of compound *MLL-ENL/Lin28b*-expressing mice unexpectedly survived for >300 days (Figure 7D). Most had only slightly enlarged spleens and normal HSC and HPC numbers at 1 year after *MLL-ENL* and *Lin28b* induction, indicating that coexpression of *MLL-ENL* and *Lin28b* was not inherently toxic to hematopoietic progenitors (Figure 7E-G). A few mice in the *MLL-ENL/Lin28b*–expressing cohort did become moribund, and they had markedly enlarged spleens (Figure 7E). However, they did not have AML. Instead, the spleens were infiltrated with malignant CD3$^+$ T cells (Figure 7H-I). T-cell lymphomas have been previously observed in transgenic *Vav1-Lin28b* mice.[48] Thus, the lymphomas that arose in *MLL-ENL/Lin28b*–expressing mice were likely caused by *Lin28b* rather than *MLL-ENL*.

We tested whether other fetal gene products can similarly suppress AML initiation. We transduced E16.5 Tet-OFF-*MLL-ENL* progenitors (Lineage$^-$c-kit$^+$) with lentiviruses to express enhanced green fluorescent protein or 1 of 4 fetal proteins (LIN28B, IGF2BP2,

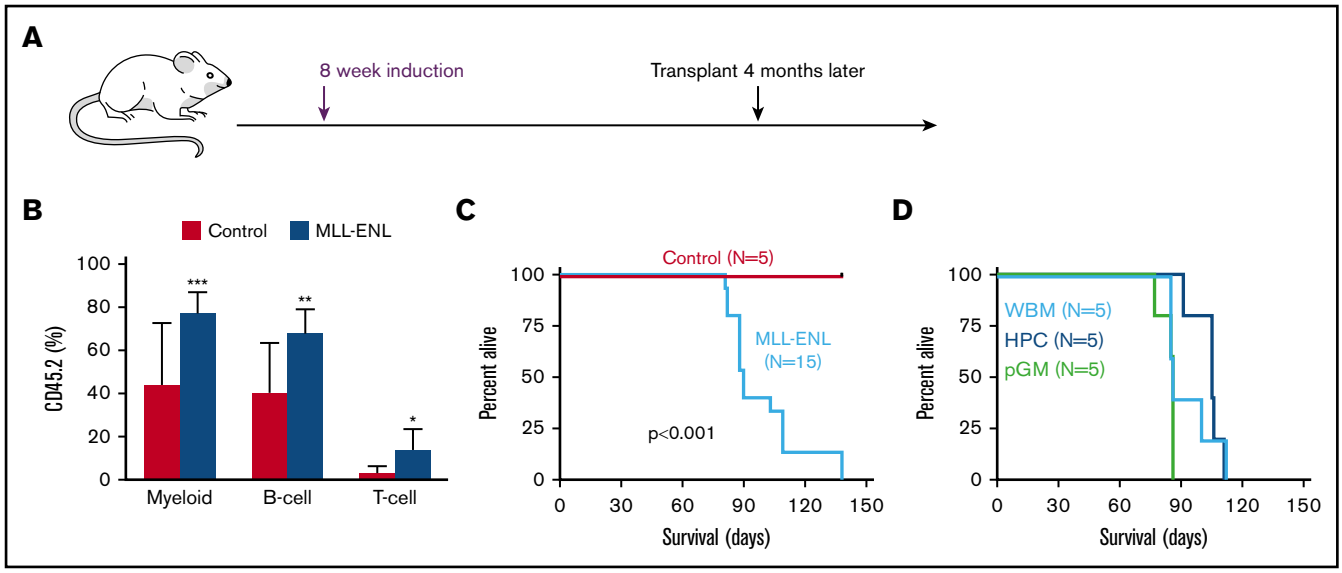

Figure 6. Transplanted HPCs and pGMs give rise to AML after adult *MLL-ENL* induction. (A) Overview of experiment timeline. (B) Percentage of donor (CD45.2) peripheral blood myeloid cells ($CD11b^+Gr1^+$), B cells ($B220^+$), and T cells ($CD3^+$) at 4 weeks after transplantation. $^*P < .05$, $^{**}P < .01$; groups were compared with the 2-tailed Student *t* test. (C) Kaplan-Meier survival curves for mice transplanted with control or *MLL-ENL*–expressing bone marrow. (D) Survival curves for a second cohort of *MLL-ENL*–expressing mice transplanted with 300 000 whole bone marrow cells or 3000 HPCs or pGMs. Group sizes are indicated. Survival curves were compared with the log-rank test.

IGFBP3, and GEM)[32] fused to a 2A-enhanced green fluorescent protein reporter. The transduced cells were transplanted into lethally irradiated recipients (20 000/recipient) along with 300 000 uninfected bone marrow cells. Recipients were monitored until they became moribund. *Lin28b* expression significantly extended survival of recipient mice (supplemental Figure 6A). In contrast, none of the other fetal genes extended survival. Thus, *Lin28b* can suppress AML initiation in a transplantation model, just as it does in Tet-OFF-*MLL-ENL*/*Lin28b* mice, and this phenotype appears to be relatively specific to *Lin28b*.

In contrast to its effect on AML initiation, *Lin28b* did not impair growth of fully transformed AML cells, at least not in vitro. We transduced MOLM14 cells (which express an *MLL* rearrangement) and K562 cells (which do not) with control or *Lin28b*-expressing lentiviruses. Transduced cells had no growth advantage or disadvantage relative to nontransduced cells (supplemental Figure 6B). Altogether, the data suggest that *Lin28b* may help prevent AML initiation in fetal progenitors rather than potentiating transformation. In this model, naturally declining *Lin28b* levels could enhance AML initiation efficiency for a period of time after birth (Figure 7M). This model may explain why AML formation was more penetrant when MLL-ENL was induced at P0 than at E10.5 (Figure 1B).

Discussion

MLL fusion proteins are potent oncoproteins that drive leukemogenesis in infants with very few cooperating mutations.[3] This suggests that fetal or neonatal hematopoietic progenitors may be exquisitely sensitive to MLL fusion proteins. Our data confirm that the efficiency of MLL-ENL-driven AML initiation changes with age, and it peaks shortly after birth. This period correlates with the age at which MLL-ENL most effectively alters gene expression. Indeed, critical effectors of leukemogenesis, such as *Hoxa9*, *Hoxa10*, and *Mecom*, are induced more highly in neonatal progenitors than in adult progenitors. Our data suggest that developmental context plays an important role in shaping the genetic landscapes of *MLL*-rearranged leukemias. Fewer cooperating mutations are observed in infant leukemias because neonatal progenitors are inherently more competent to transform.

It is not clear why adult progenitors respond differently to *MLL-ENL* expression than neonatal progenitors. One possible explanation is that there are epigenetic barriers to target gene expression in adult HPCs, pGMs, and GMPs that do not exist to the same degree in neonatal progenitors. A second possibility is that the MLL-ENL protein may be less stable in adult progenitors than in neonatal progenitors. A third possibility is that yet-to-be identified fetal/neonatal transcription factors collaborate with MLL-ENL to enhance target gene expression and initiate leukemogenesis. These mechanisms are not mutually exclusive, but they all point to a developmental barrier that can be reversed by stress, given the effects of transplantation on leukemogenesis (Figure 6). It should be noted that an inducible MLL-AF9 transgenic mouse has been shown to develop AML after adult transgene induction.[26] This difference could reflect different levels of transgene expression in the different models or important functional differences between MLL-ENL and MLL-AF9. Additional studies are needed to understand how normal temporal changes in HSC, HPC, and pGM epigenomes might alter transcriptional responses to MLL-ENL, how barriers to transformation can be overcome by stress or inflammation, and whether the developmental barriers are relevant to other pediatric AML fusion proteins, such as MLL-AF9.

The link between development and leukemia initiation is potentially clinically important. It raises the possibility of identifying patients who are at high risk for developing infant leukemia and temporarily prophylaxing against transformation. There are a number of reasons

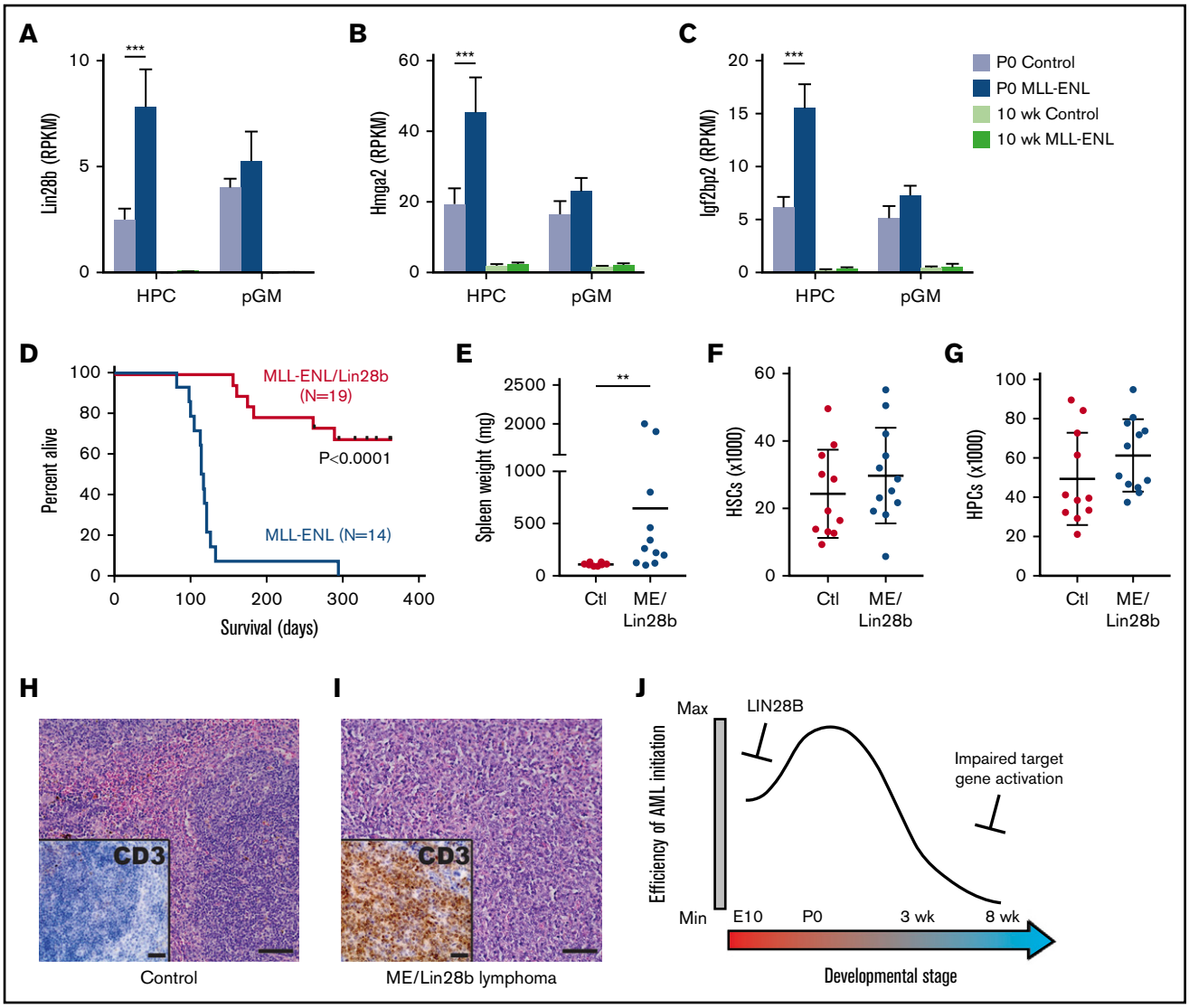

Figure 7. *Lin28b* suppresses *MLL-ENL*–driven AML. (A-C) *Lin28b*, *Hmga2* and *Igf2bp2* were hyper-activated in *MLL-ENL* expressing neonatal, but not adult, HPCs. ***Adjusted $P < .001$. (D) Survival of Tet-OFF-*MLL-ENL* and Tet-OFF-*MLL-ENL/Lin28b* mice after neonatal transgene induction. Curves were compared by the log-rank test. (E-G) Spleen weight and HSC and HPC numbers in surviving Tet-OFF-*MLL-ENL/Lin28b* after they were euthanized at >1 year old. (H-I) Representative spleen histology and CD3 expression in control mice or Tet-OFF-*MLL-ENL/Lin28b* mice that became moribund. Scale bars indicate 100 μM (large panels) or 40 μM (insets). (J) MLL-ENL initiates AML most efficiently when it is induced during the neonatal stage of development. *Lin28b* might protect against AML initiation in utero. At later stages of development, MLL-ENL fails to activate target genes as efficiently as it does in neonates.

that prophylaxis may not be practical: inhibitors of DOT1L and MLL fusion complexes are still in early-phase or preclinical trials,[49,50] the pharmacokinetics of currently available drugs likely precludes prophylactic use,[49] and the sensitivity/specificity of translocations identified in newborn blood remains unclear. Furthermore, additional studies are needed to test whether infection or inflammation can potentiate MLL-ENL–driven leukemogenesis at later stages of development, as this would undermine any protection afforded by age. In principle, if the technical difficulties can be navigated, then transient suppression of the MLL fusion protein could allow children to age out of their window of peak leukemia susceptibility.

In addition to demonstrating a link between developmental context and AML initiation, our studies have shown that *Lin28b* can antagonize rather than facilitate AML initiation. This was surprising, because human *LIN28B* is generally thought to promote pediatric tumor growth (eg, neuroblastoma, hepatoblastoma, and Wilm tumor)[51-53] rather than suppress transformation, and *Lin28b* was upregulated by MLL-ENL in neonates. Nevertheless, the differences between *Lin28b*-expressing and nonexpressing mice were striking, and *Lin28b*-expressing mice did not develop AML (Figure 7G). This finding was consistent with data showing that let-7 microRNAs are elevated in poor prognosis pediatric AML.[2] The human data suggest that LIN28B may function as a tumor suppressor in early childhood AML by suppressing let-7 expression. Since *Lin28b* had no effect on fully transformed AML cells (supplemental Figure 6B), it likely modulates early steps of AML initiation, perhaps by redirecting differentiating HSCs and HPCs toward

fates that do not support efficient transformation, such as megakaryocytic-erythroid progenitors.[36,47,54] Additional studies are needed to test whether *Lin28b* deletion can accelerate leukemogenesis, but our findings raise the possibility of manipulating developmental programs to treat or prevent pediatric malignancies.

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Authorship

Contribution: J.A.M., T.O.-O., and Y.L. conceived, designed, and performed experiments and analyzed data; R.M.P., E.B.C., A.S.C., and S.N.P. performed experiments and analyzed data; W.Y. performed all bioinformatics analyses; D.B. generated the *Col1a1*$^{TetO\_MLL\text{-}ENL}$ mouse line; J.A.M. wrote the manuscript; and all authors reviewed and edited the manuscript.

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References

1. Chaudhury SS, Morison JK, Gibson BE, Keeshan K. Insights into cell ontogeny, age, and acute myeloid leukemia. *Exp Hematol*. 2015;43(9):745-755.
2. Bolouri H, Farrar JE, Triche T Jr, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions [published corrections appear in *Nat Med*. 2018;24(4):526 and *Nat Med*. 2019;25(3):530]. *Nat Med*. 2018;24(1):103-112.
3. Andersson AK, Ma J, Wang J, et al; St. Jude Children's Research Hospital–Washington University Pediatric Cancer Genome Project. The landscape of somatic mutations in infant MLL-rearranged acute lymphoblastic leukemias. *Nat Genet*. 2015;47(4):330-337.
4. Gale KB, Ford AM, Repp R, et al. Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. *Proc Natl Acad Sci USA*. 1997;94(25):13950-13954.
5. Ford AM, Ridge SA, Cabrera ME, et al. In utero rearrangements in the trithorax-related oncogene in infant leukaemias. *Nature*. 1993;363(6427):358-360.
6. Salzer WL, Jones TL, Devidas M, et al. Modifications to induction therapy decrease risk of early death in infants with acute lymphoblastic leukemia treated on Children's Oncology Group P9407. *Pediatr Blood Cancer*. 2012;59(5):834-839.
7. Pieters R, Schrappe M, De Lorenzo P, et al. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007;370(9583):240-250.
8. Biondi A, Cimino G, Pieters R, Pui CH. Biological and therapeutic aspects of infant leukemia. *Blood*. 2000;96(1):24-33.
9. Szczepański T, Harrison CJ, van Dongen JJ. Genetic aberrations in paediatric acute leukaemias and implications for management of patients. *Lancet Oncol*. 2010;11(9):880-889.
10. Super HJ, McCabe NR, Thirman MJ, et al. Rearrangements of the MLL gene in therapy-related acute myeloid leukemia in patients previously treated with agents targeting DNA-topoisomerase II. *Blood*. 1993;82(12):3705-3711.
11. Balgobind BV, Zwaan CM, Pieters R, Van den Heuvel-Eibrink MM. The heterogeneity of pediatric MLL-rearranged acute myeloid leukemia. *Leukemia*. 2011;25(8):1239-1248.
12. Jude CD, Climer L, Xu D, Artinger E, Fisher JK, Ernst P. Unique and independent roles for MLL in adult hematopoietic stem cells and progenitors. *Cell Stem Cell*. 2007;1(3):324-337.
13. Hsieh JJ, Cheng EH, Korsmeyer SJ. Taspase1: a threonine aspartase required for cleavage of MLL and proper HOX gene expression. *Cell*. 2003;115(3):293-303.
14. Nakamura T, Mori T, Tada S, et al. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. *Mol Cell*. 2002;10(5):1119-1128.
15. Deshpande AJ, Bradner J, Armstrong SA. Chromatin modifications as therapeutic targets in MLL-rearranged leukemia. *Trends Immunol*. 2012;33(11):563-570.
16. Deshpande AJ, Deshpande A, Sinha AU, et al. AF10 regulates progressive H3K79 methylation and HOX gene expression in diverse AML subtypes. *Cancer Cell*. 2014;26(6):896-908.
17. Bernt KM, Zhu N, Sinha AU, et al. MLL-rearranged leukemia is dependent on aberrant H3K79 methylation by DOT1L. *Cancer Cell*. 2011;20(1):66-78.
18. Zhu L, Li Q, Wong SH, et al. ASH1L links histone H3 lysine 36 dimethylation to MLL leukemia. *Cancer Discov*. 2016;6(7):770-783.
19. Mohan M, Lin C, Guest E, Shilatifard A. Licensed to elongate: a molecular mechanism for MLL-based leukaemogenesis. *Nat Rev Cancer*. 2010;10(10):721-728.
20. Slany RK. The molecular biology of mixed lineage leukemia. *Haematologica*. 2009;94(7):984-993.

21. Krivtsov AV, Twomey D, Feng Z, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature*. 2006;442(7104):818-822.

22. Somervaille TC, Matheny CJ, Spencer GJ, et al. Hierarchical maintenance of MLL myeloid leukemia stem cells employs a transcriptional program shared with embryonic rather than adult stem cells. *Cell Stem Cell*. 2009;4(2):129-140.

23. Mullighan CG, Kennedy A, Zhou X, et al. Pediatric acute myeloid leukemia with NPM1 mutations is characterized by a gene expression profile with dysregulated HOX gene expression distinct from MLL-rearranged leukemias. *Leukemia*. 2007;21(9):2000-2009.

24. Kohlmann A, Schoch C, Dugas M, et al. New insights into MLL gene rearranged acute leukemias using gene expression profiling: shared pathways, lineage commitment, and partner genes. *Leukemia*. 2005;19(6):953-964.

25. Armstrong SA, Staunton JE, Silverman LB, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat Genet*. 2002;30(1):41-47.

26. Stavropoulou V, Kaspar S, Brault L, et al. MLL-AF9 expression in hematopoietic stem cells drives a highly invasive AML expressing EMT-related genes linked to poor outcome. *Cancer Cell*. 2016;30(1):43-58.

27. Garcia-Cuellar MP, Büttner C, Bartenhagen C, Dugas M, Slany RK. Leukemogenic MLL-ENL fusions induce alternative chromatin states to drive a functionally dichotomous group of target genes. *Cell Reports*. 2016;15(2):310-322.

28. Ross ME, Mahfouz R, Onciu M, et al. Gene expression profiling of pediatric acute myelogenous leukemia. *Blood*. 2004;104(12):3679-3687.

29. Horton SJ, Jaques J, Woolthuis C, et al. MLL-AF9-mediated immortalization of human hematopoietic cells along different lineages changes during ontogeny. *Leukemia*. 2013;27(5):1116-1126.

30. Barrett NA, Malouf C, Kapeni C, et al. Mll-AF4 confers enhanced self-renewal and lymphoid potential during a restricted window in development. *Cell Reports*. 2016;16(4):1039-1054.

31. Magee JA, Ikenoue T, Nakada D, Lee JY, Guan KL, Morrison SJ. Temporal changes in PTEN and mTORC2 regulation of hematopoietic stem cell self-renewal and leukemia suppression. *Cell Stem Cell*. 2012;11(3):415-428.

32. Porter SN, Cluster AS, Yang W, et al. Fetal and neonatal hematopoietic progenitors are functionally and transcriptionally resistant to *Flt3*-ITD mutations. *eLife*. 2016;5:e18882.

33. Copley MR, Babovic S, Benz C, et al. The Lin28b-let-7-Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. *Nat Cell Biol*. 2013;15(8):916-925.

34. Yuan J, Nguyen CK, Liu X, Kanellopoulou C, Muljo SA. Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. *Science*. 2012;335(6073):1195-1200.

35. Jones LK, Neat MJ, van Delft FW, et al. Cryptic rearrangement involving MLL and AF10 occurring in utero. *Leukemia*. 2003;17(8):1667-1669.

36. Ugale A, Norddahl GL, Wahlestedt M, et al. Hematopoietic stem cells are intrinsically protected against MLL-ENL-mediated transformation. *Cell Reports*. 2014;9(4):1246-1255.

37. de Boer J, Williams A, Skavdis G, et al. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *Eur J Immunol*. 2003;33(2):314-325.

38. Wang L, Sharma K, Deng HX, et al. Restricted expression of mutant SOD1 in spinal motor neurons and interneurons induces motor neuron pathology. *Neurobiol Dis*. 2008;29(3):400-408.

39. Belteki G, Haigh J, Kabacs N, et al. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Res*. 2005;33(5):e51.

40. Foudi A, Hochedlinger K, Van Buren D, et al. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol*. 2009;27(1):84-90.

41. Zhu H, Shyh-Chang N, Segrè AV, et al; MAGIC Investigators. The Lin28/let-7 axis regulates glucose metabolism. *Cell*. 2011;147(1):81-94.

42. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21.

43. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B*. 1995;57(1):289-300.

44. Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature*. 2009;457(7231):887-891.

45. Pronk CJ, Rossi DJ, Månsson R, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell Stem Cell*. 2007;1(4):428-442.

46. Kristiansen TA, Jaensson Gyllenbäck E, Zriwil A, et al. Cellular barcoding links B-1a B cell potential to a fetal hematopoietic stem cell state at the single-cell level. *Immunity*. 2016;45(2):346-357.

47. Rowe RG, Wang LD, Coma S, et al. Developmental regulation of myeloerythroid progenitor function by the Lin28b-let-7-Hmga2 axis. *J Exp Med*. 2016;213(8):1497-1512.

48. Beachy SH, Onozawa M, Chung YJ, et al. Enforced expression of Lin28b leads to impaired T-cell development, release of inflammatory cytokines, and peripheral T-cell lymphoma. *Blood*. 2012;120(5):1048-1059.

49. Stein EM, Garcia-Manero G, Rizzieri DA, et al. The DOT1L inhibitor pinometostat reduces H3K79 methylation and has modest clinical activity in adult acute leukemia. *Blood*. 2018;131(24):2661-2669.

50. Borkin D, He S, Miao H, et al. Pharmacologic inhibition of the Menin-MLL interaction blocks progression of MLL leukemia in vivo. *Cancer Cell*. 2015; 27(4):589-602.

51. Urbach A, Yermalovich A, Zhang J, et al. Lin28 sustains early renal progenitors and induces Wilms tumor. *Genes Dev*. 2014;28(9):971-982.

52. Nguyen LH, Robinton DA, Seligson MT, et al. Lin28b is sufficient to drive liver cancer and necessary for its maintenance in murine models. *Cancer Cell*. 2014;26(2):248-261.

53. Molenaar JJ, Domingo-Fernández R, Ebus ME, et al. LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression. *Nat Genet*. 2012; 44(11):1199-1206.

54. Wang LD, Rao TN, Rowe RG, et al. The role of Lin28b in myeloid and mast cell differentiation and mast cell malignancy. *Leukemia*. 2015;29(6): 1320-1330.