Hematopoietic Stem Cells Are Intrinsically Protected against MLL-ENL-Mediated Transformation

Highlights
A conditional/inducible mouse model of the MLL-ENL fusion oncogene is presented
Leukemia initiation is associated with a window of differentiation
There is an intrinsic block against leukemic transformation at the HSC level

Authors
Amol Ugale, Gudmundur L. Norddahl, ..., Jörg Cammenga, David Bryder

Correspondence
david.bryder@med.lu.se

In Brief
The cellular origin of leukemia driven by MLL fusions has been suggested to underlie heterogeneity in aggressiveness and prognosis. Ugale et al. now use an inducible MLL-ENL mouse model to study leukemia initiation and competence throughout the hematopoietic hierarchy. Although AML development could originate from multiple progenitor subsets, the most primitive stem cells were unexpectedly unable to initiate disease.

Accession Numbers
GSE62734

Ugale et al., 2014, Cell Reports 9, 1246–1255
November 20, 2014 ©2014 The Authors
http://dx.doi.org/10.1016/j.celrep.2014.10.036
Hematopoietic Stem Cells Are Intrinsically Protected against MLL-ENL-Mediated Transformation

Amol Ugale,1,5 Gudmundur L. Norddahl,1,4,5 Martin Wahlestedt,1 Petter Säwen,1 Pekka Jaako,1 Cornelis Jan Pronk,1,3 Shamit Soneji,2,3 Jörg Cammenga,2,3 and David Bryder1,3,*

1Immunology Section, Department of Experimental Medical Science, Biomedical Center D14, Lund University, Klinikgatan 32, 221 84 Lund, Sweden
2Division of Molecular Medicine and Gene Therapy, Biomedical Center A12, Lund University, 221 84 Lund, Sweden
3Lund Stem Cell Center, Biomedical Center B10, Klinikgatan 26, 221 84 Lund, Sweden
4Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC V5Z 1L3, Canada
5Co-first author
*Correspondence: david.bryder@med.lu.se
http://dx.doi.org/10.1016/j.celrep.2014.10.036
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

Studies of developmental pathways of hematopoietic stem cells (HSCs) have defined lineage relationships throughout the blood system. This is relevant to acute myeloid leukemia (AML), where aggressiveness and therapeutic responsiveness can be influenced by the initial stage of transformation. To address this, we generated a mouse model in which the mixed-lineage leukemia/eleven-nineteen-leukemia (MLL-ENL) transcription factor can be conditionally activated in any cell type. We show that AML can originate from multiple hematopoietic progenitor subsets with granulocytic and monocytic potential, and that the normal developmental position of leukemia-initiating cells influences leukemic development. However, disease failed to arise from HSCs. Although it maintained or upregulated the expression of target genes associated with leukemic development, MLL-ENL dysregulated the proliferative and repopulating capacity of HSCs. Therefore, the permissiveness for development of AML may be associated with a narrower window of differentiation than was previously appreciated, and hijacking the self-renewal capacity of HSCs by a potent oncogene is insufficient for leukemic development.

INTRODUCTION

The hematopoietic system is normally under the control of environmentally derived signals and an intricate network of finely tuned transcriptional programs (Orkin and Zon, 2008) that enable rare hematopoietic stem cells (HSCs) to give rise to differentiated progeny in a hierarchical fashion. Therefore, knowledge about the functional and molecular properties of defined hematopoietic progenitor cells (HPCs) at different stages of maturation could provide insights into the etiology of acute myeloid leukemia (AML).

Balanced chromosomal translocations that result in fusion proteins with aberrant transcriptional activities are frequent initiating events in AML. A prototype family of such factors is represented by mixed lineage leukemia-1 (MLL1) gene fusions. In previous studies, retroviral introduction of MLL-ENL or MLL-AF9 fusion genes into committed granulocyte-macrophage progenitors (GMPs) resulted in AML (Cozzio et al., 2003; Krivtsov et al., 2006), demonstrating that MLL-fusion-mediated transformation can involve an early activation of self-renewal. This property is not normally associated with GMPs. However, not all types of HPCs can act as leukemia-initiating cells (LICs). Introduction of MLL-ENL in proliferation-competent B cells did not result in disease (Cano et al., 2008), and AML failed to develop from megakaryocyte/erythroid progenitors and common lymphoid progenitors (CLPs) (Cozzio et al., 2003). In contrast to these data, it was shown that CLPs readily gave rise to AML, but GMPs failed to produce disease, in an endogenous knockin model of MLL-AF9 (Chen et al., 2008).

While it appears clear that cells can lose competence for leukemic transformation with differentiation, the question remains as to whether the most primitive HSCs are imbued with leukemogenic competence. Given the inherent self-renewal and cytotoxic protective properties of HSCs, and assuming that such properties are retained at the apex of the leukemia hierarchy, leukemia initiation at the level of HSCs might be associated with a particularly poor prognosis. Although previous studies have suggested that HSCs are potent LICs (Chen et al., 2008; Cozzio et al., 2003; Krivtsov et al., 2013), and even proposed that HSCs are the predominant targets for AML (Chen et al., 2008; Krivtsov et al., 2013), it was shown that HSCs were not susceptible to transformation in Mn1-induced AML unless Meis1 and Hoxa9/Hoxa10 were also coinjected (Heuser et al., 2011). However, this issue is difficult to address, and in the specific case of Mn1-induced leukemia, is complicated by the fact that the forced expression of Hoxa9/10 and Meis1 in many contexts is leukemic by itself. Other concerns might also apply. For instance, the use of gamma-retroviral models requires induction of proliferation, which, given the quiescent
nature of HSCs, is a potentially significant alteration (Pietras et al., 2011). The purity of isolated HSC subsets can be another confounding factor, as most previous studies utilized only partially purified HSCs intermingled with other, less self-renewing multipotent progenitors (MPPs). Cancer stem cells in human AMLs appear to share common features with such progenitors (Goardon et al., 2011), although information about LICs is difficult to deduce from established leukemia, in which a spectrum of secondary mutations and epigenetic changes has evolved. The issue is further complicated by the fact that the fusion oncogenes themselves can alter the normal rules of differentiation (Drynan et al., 2005).

Here, we developed a transgenic mouse model that allows for the conditional activation of a human MLL-ENL fusion oncogene in otherwise normal cells. Combined with prospective isolations of candidate LICs and adoptive transfers, this approach allowed us to examine leukemia initiation and competence throughout the early hematopoietic hierarchy in detail.

## RESULTS

### Establishment and Functional Validation of a Doxycycline-Inducible MLL-ENL Mouse Model

To create an inducible model for the human MLL-ENL fusion gene, we used the KH2 embryonic stem cell (ESC) system (Beard et al., 2006), in which the M2 reverse tetracycline transactivator (M2-rtTA) is driven constitutively under the Rosa26 promoter. MLL-ENL was targeted to the 3' UTR of the Col1a1 gene under tetracycline-regulated control, allowing MLL-ENL expression upon doxycycline (Dox) supplementation (Figure S1A). Following in vivo induction, we assessed MLL-ENL expression in different hematopoietic stem and progenitor cells (HSPCs; Figure 1A). Although clear expression of MLL-ENL was detected in all of the HSPC subsets evaluated, the levels were in stark contrast to the very high levels observed using retroviral approaches (Chen et al., 2008; Figure 1B).
Leukemic Competence, Disease Latency, and Patterns of Preleukemic Growth Are Hierarchically Structured among Myeloid Progenitors

Using retroviral delivery, Cozzo et al. (2003) showed that both common myeloid progenitors (CMPs) and GMPs could initiate AML. Whereas GMPs represent a rather pure population of lineage-restricted cells (Akashi et al., 2000), the CMP population is more heterogeneous and includes precursors for GMPs (pGMs), bipotential megakaryocyte/erythroid cells (preMeg/Es), monoligal committed megakaryocyte progenitors (MkPs), and some early erythroid (preCFU-E) progenitors (Figure 1A; Pronk et al., 2007). We hypothesized that pGMs could represent candidate LICs, and therefore we first asked whether the related pGM and GMP subsets could exhibit differential responses to MLL-ENL.

We adopted an approach whereby limited numbers of defined HSPCs from Col1a1-tetO-MLL/ENL mice were isolated and transplanted into wild-type (WT) recipient mice under Dox administration (Figure 1C). Starting at 4 weeks after transplantation, the contribution of test cells was evaluated biweekly. We observed a very limited donor myeloid contribution from MLL-ENL-induced GMPs (Figure 1D, upper panel). All mice that received GMPs survived without noticeable abnormalities until week 16. However, one recipient at week 18, two at week 21, and one at week 26 were found to be sick. The remaining seven mice in this group remained disease free (Figure 1E).

Although pGMs and GMPs are believed to be closely related (Pronk et al., 2007), their in vivo behaviors in response to MLL-ENL were strikingly different. MLL-ENL induction led to a substantial pGM-derived myeloid contribution in all mice at 4 weeks (Figure 1D, lower panel). This was associated with subsequent, mostly sharp drops in myeloid contribution up to week 8. Thereafter, we observed rapid rebounds in the levels of peripheral blood (PB) myeloid cells in most (11/13) of the pGM-transplanted mice (Figure 1D, lower panel). Further analysis confirmed a clinical progression to myeloid malignant transformation (Figure S2B; data not shown). Transplantation of bone marrow (BM) cells from diseased mice into secondary hosts led to a rapid propagation of disease (Figure 1E).

**MLL-ENL Is a Potent Inducer of Leukemia in Multiple Progenitor Types but Fails to Induce Leukemia from the Most Primitive Subsets**

We next asked whether and how MLL-ENL might affect other primitive progenitors. We began by assessing CLPs. In agreement with a limited myeloid potential of these cells, five out of seven untreated mice failed to show any evidence of test-cell-derived myeloid reconstitution, and the remaining mice displayed a very low myeloid contribution (0.5% and 0.1%, respectively; Figure 2A). In contrast, MLL-ENL induction of CLPs led to a myeloid contribution in all mice (mean 1.5%, range 0.5%–2.8% at 4 weeks) that persisted at all time points investigated (Figure 2A). At 10 and 16 weeks posttransplantation, two mice that received MLL-ENL-induced CLPs died. Subsequently, the remaining mice exhibited sudden elevations of PB myeloid cells and presented with AML (Figures 2A and 2D). Secondary transplantations of BM cells from diseased CLP-transplanted mice resulted in disease development with a short latency (Figure S3A).

We next tested whether MLL-ENL could also affect preMeg/Es, an early megakaryocytic/erythroid restricted progenitor subset. However, we could not detect any donor-derived reconstitution to the myeloid, B cell, or T cell lineages from such cells, and the mice remained healthy until the experiment’s endpoint (Figure 2D).

In previous studies, it was suggested that HSCs, but not the more downstream GMPs, could initiate AML (Chen et al., 2008). However, in those studies, candidate HSCs were isolated as Lin−Sca1+ckit+ (LSK) cells, which in addition to a minor portion of HSCs contain a larger fraction of various MPP cells (Oguro et al., 2013). Therefore, we investigated this issue more closely in our model by first evaluating LSKCD48−CD150+ cells (referred to as granulocyte-monocyte-lymphoid progenitors [GMLPs]). GMLPs represent the major LSK subset and are functionally distinct from HSCs (Kiel et al., 2005; Oguro et al., 2013; Pronk et al., 2007). Confirming this, the contribution of uninduced GMLPs to the myeloid lineage, a reliable indicator of ongoing HSC activity, was completely absent short term after transplantation (Figure 2A). By contrast, MLL-ENL profoundly affected the in vivo reconstitution activity of GMLPs. Myeloid chimerism from Dox-induced GMLPs was very high at 4 weeks after transplantation (Figure 2A), although this declined somewhat at subsequent time points. At 12 weeks after transplantation, we began to observe an upsurge in myeloid chimerism in these mice. Two induced GMLP recipients spontaneously died following the 14-week analysis, and the remaining mice developed AML within 16 weeks (Figure 2D). Serial transplantation resulted in a rapid disease onset (Figure S3A). With GMLPs, a pronounced effect of MLL-ENL induction extended also to the lymphoid lineages, with high contributions to the peripheral B and T cell lineages (data not shown). However, the rapid rise in chimerism was restricted to myeloid cells.

Next, we evaluated LSKCD48+CD150− cells (hereafter referred to as MPPs). This MPP population has not been extensively characterized. Therefore, we first probed the normal in vivo capacity of these cells (Figure S3B). We then competitively transplanted 500 MPPs per recipient without or with MLL-ENL induction. The contribution to the myeloid lineages was slightly higher in MLL-ENL-exposed MPPs at 4 weeks, but dropped at later time points (Figure 2A). From induced MPPs, one mouse died after the blood analysis conducted 20 weeks posttransplantation. However, somewhat surprisingly, the remaining mice did not show any changes in donor-derived myeloid chimerism or signs of disease development over a period of 50 weeks (Figure 2D).

Finally, we investigated how MLL-ENL induction would affect highly purified HSCs. We isolated LSKCD48−CD150+ HSCs from Col1a1-tetO-MLL/ENL mice and competitively transplanted 100 or 1,000 of these cells per mouse with or without MLL-ENL induction. At the lower dose, all uninduced mice were highly reconstituted (Figure 2B, upper left). In contrast, administration of Dox severely compromised the reconstitution potential of Col1a1-tetO-MLL/ENL HSCs (Figure 2B, upper right). Similarly, at the higher cell dose, all control mice were robustly reconstituted (Figure 2B, lower left), whereas two of 13 Dox-treated hosts failed to display HSC-derived reconstitution. The overall myeloid reconstitution in the remaining MLL-ENL-induced mice...
was substantially lower compared with controls and gradually declined (Figure 2B, lower right). Strikingly, we never observed any incidence of myeloproliferation/leukemia from MLL-ENL-induced HSCs. Since AML can develop relatively late (Figure 1E), we extended the monitoring period for HSC-transplanted mice by serial transplantation. Secondary hosts were monitored for an additional 24 weeks (Figure 2C) without showing any signs of disease. The progressive reduction in HSC reconstitution over time led us to speculate that MLL-ENL leads to a block in differentiation. For instance, one could hypothesize that HSCs are unable to differentiate and instead self-renew excessively in response to MLL-ENL. To investigate this, we sacrificed a cohort of mice transplanted with 1,000 HSCs and investigated the contributions of MLL-ENL-induced HSCs to the various HSPC fractions. However, we did not observe a block at any distinct progenitor stage, but rather found an overall reduction in all HSPC subsets assessed (Figure S3C).

**Intrinsic Protection against Transformation at the HSC Level**

The failure of the HSCs and MPPs to transform was a surprising result. Therefore, we decided to further characterize the differential responses by initiating cultures with HSCs, MPPs, GMLPs, and pGMs in the absence or presence of Dox. We also delayed addition of Dox to the cultures. At 10 days after MLL-ENL onset, we evaluated proliferation relative to uninduced cultures. In accordance with their in vivo behavior, GMLPs and pGMs gained an enhanced proliferative capacity immediately following supplementation with Dox (Figure 3A). Of note, Dox addition at days 3 and 4 to pGMs failed to provide an additional proliferative advantage. In striking contrast, continuous MLL-ENL induction in HSCs led to a dramatic reduction in cellular output (Figure 3A). This was not due to a selective toxicity (Figure S4A; data not shown). Proliferation in HSC cultures to which Dox was added at days 1 and 2 was also lower than that observed in noninduced
cultures. However, when Dox was added later, we observed increases in proliferative output (Figure 3A). MPPs responded similarly to HSCs, although they gained a proliferative advantage more rapidly following delayed induction. These findings led us to investigate whether the progeny of transplanted HSCs are leukemia competent. We transplanted 1,000 HSCs with or without MLL-ENL induction. After 4 weeks, noninduced mice were administered Dox. Although no HSC recipients under continuous MLL-ENL expression developed disease, all mice that were administered Dox 4 weeks following transplantation developed AML (Figure 3B).

A potential explanation for the reduced in vivo contribution from MLL-ENL-induced HSCs could be a compromised in vivo homing ability. To investigate this, we isolated HSCs from Col1a1-tetO-MLL/ENL mice provided with Dox food pellets for 14 days, and transplanted 100 HSCs from these mice into mice with or without MLL-ENL induction. Although no significant differences in donor chimerism were observed at 4 weeks, progressive reductions in donor chimerism upon MLL-ENL induction were observed over the period of 14 weeks (Figure 3C; data not shown). Again, no leukemia developed in any mice. An alternative approach generated similar results (Figure S4B), suggesting that compromised homing is unlikely to underlie the functional shortcomings of MLL-ENL-induced HSCs.

Although our data had demonstrated a progressive decline in HSC activity upon MLL-ENL induction, we were perplexed

Figure 3. MLL-ENL Expression Induces Leukemic Activity following HSC Differentiation but Restricts HSC Activity in a Dose-Dependent Manner
(A) Effect of delayed MLL-ENL induction on the proliferation of primitive hematopoietic progenitors. Dox was added at the indicated time points after initiation of cultures with HSCs (red), MPPs (brown), GMLPs (green), and pGMs (blue). Cell numbers were enumerated 10 days after addition of Dox, and proliferation rates are shown relative to uninduced cultures.
(B) Kaplan-Myer curve depicting the survival of mice transplanted with HSCs from Col1a1-tetO-MLL/ENL mice under continuous or delayed MLL-ENL induction.
(C) Myeloid contribution at weeks 4 and 14 in the presence and absence of Dox following transplantation of 100 Col1a1-tetO-MLL/ENL HSCs that were previously induced for 14 days (n = 7 mice/group).
(D) Quantification of MLL-ENL and endogenous Mll1 expression in pGMs isolated from HSC-transplanted mice, GMPs from pGM-transplanted mice, and HSPCs isolated from Col1a1-tetO-MLL/ENL mice induced for 14 days in vivo (the latter expression values also shown in Figure 2B). Shown are the transcript counts per cell of MLL-ENL and endogenous Mll1 ± SD from three biological replicates.
(E) Quantification of MLL-ENL expression in HSCs and pGMs from 3-day induced Col1a1-tetO-MLL/ENL mice. Depicted are copy numbers ± SD.
(F) Mice were competitively transplanted with 650 HSCs and then the donor contribution to the myeloid lineage in the PB of uninduced (closed circles) and mice induced with standard (squares) and high-Dox (triangles) food pellets (n = 7 mice/group) was evaluated. Statistics were determined by Student’s two-tailed t test or log rank test (Mantel-Cox test for survival). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant. Data shown are from one experiment of two with similar results. See also Figure S4.
that leukemia did not develop, given that prominent HSC-derived reconstitution was observed in most recipients. This included the generation of otherwise leukemia-competent progenitors (Figure S3C). To address this issue, we determined the expression levels of MLL-ENL in pGMs developing from HSCs under MLL-ENL induction. These analyses revealed that in comparison with pGMs isolated from Dox-induced Col1a1-tetO-MLL/ENL mice, MLL-ENL expression was significantly lower (3.8-fold) in pGMs from transplanted and induced HSCs. Reduced expression was not observed in GMPs isolated from pGM-transplanted mice (Figure 3D), a scenario in which AML develops. This led us to hypothesize that there might be a selection against HSCs with higher MLL-ENL levels. To address this, we also assessed HSC activity with higher MLL-ENL expression. This revealed that higher MLL-ENL dosages (Figure 3E) severely compromised HSC-derived reconstitution (seven out of seven mice without donor chimera; Figure 3F).

**Transcriptional Signatures upon Leukemia Initiation**

In a previous study, Armstrong et al. (2002) found that MLL-fusion leukemias constitute a distinct entity of AML that is distinguishable by its gene-expression patterns. However, gene-expression studies of established leukemias have provided little information about the initial transcriptional changes that occur in preleukemic cells. Therefore, we decided to investigate the immediate transcriptional responses to MLL-ENL.

We isolated WT and Col1a1-tetO-MLL/ENL pGMs and HSCs that were briefly exposed to Dox (48 hr) in vitro and subjected them to RNA sequencing (RNA-seq). The induction of MLL-ENL resulted in evident transcriptional alterations in both cell types (Figure 4A; Table S1).

Together, our functional data supported a scenario in which inhibition of differentiation is a feature of MLL-ENL-driven leukemic development. To investigate this in detail from a molecular perspective, we used Gene Set Enrichment Analysis (GSEA) (Figure 4B). This revealed that upregulated genes in induced HSCs were strongly associated with the normal HSC state, whereas downregulated genes correlated with the more-differentiated GMLPs (p < 0.001; Figure 4B). Similarly, the upregulated genes in MLL-ENL-induced pGMs were firmly associated with normal pGMs, and the downregulated genes correlated with GMPs (p < 0.001; Figure 4B). Thus, inhibition of differentiation appears to be a key early event in preleukemic cells that applies to both leukemia-competent pGMs and leukemia-incompetent HSCs. To identify additional molecular properties, we searched for overrepresented Gene Ontology (GO) categories among the differentially expressed genes. The GO category “Cell cycle arrest” showed significant enrichment following MLL-ENL induction specifically in HSCs (Figure 4C, left), which was confirmed by direct cell-cycle assessments (Figure 4C, right).

If MLL-ENL is capable of restricting both the differentiation and proliferation of HSCs, it is reasonable to assume that transient MLL-ENL expression could be beneficial in terms of preserving HSC activity. To test this, we maintained HSCs from Col1a1-tetO-MLL/ENL mice in the presence or absence of Dox for 10 days and then evaluated their activity. HSCs cultured without MLL-ENL induction proliferated vigorously but failed to provide long-term multilineage reconstitution (Figure 4D). By contrast, cultured and MLL-ENL-induced HSCs displayed robust long-term reconstitution (Figure 4D). Thus, transient MLL-ENL expression preserves at least some degree of functional HSC activity.

The differential effect of MLL-ENL on proliferation led us to speculate that the effect of MLL-ENL was guided strictly by the proliferative status of the target cells. It is well established that fetal liver HSCs are more actively cycling than adult HSCs. Therefore, in a final set of in vivo experiments, we transplanted embryonic day 14.5 (E14.5) fetal liver HSCs from Col1a1-tetO-MLL/ENL mice with or without MLL-ENL induction. However, similar to what was observed for adult HSCs, MLL-ENL induction severely compromised the reconstitution potential of fetal liver HSCs in a dose-dependent manner (Figure 4E).

**DISCUSSION**

Leukemic transformation of primary hematopoietic cells is often regarded as a plastic process wherein various hematopoietic subpopulations can serve as target cells of the disease. At the same time, leukemias present with diverse lineage-associated phenotypes (e.g., myeloid, lymphoid, and, less frequently, erythroid/megakaryocytic forms). This lineage representation is indicative of an interplay between the normal differentiation potential of the target cell and the instructive/permmissive nature of the oncogene. However, a more exact identity of the cell(s) of origin across the spectrum of various subtypes of leukemia remains to be determined.

Using our model, we show that the leukemic potential lies within a relatively narrow window of the hematopoietic hierarchy. Transplantation and induction of prospectively isolated pGMs and GMPs, two closely related cellular states (Pronk et al., 2007), resulted in dramatically different disease development. This observation is in agreement with previous studies on a separate MLL fusion gene (MLL-AF9) and the Meningioma 1 (Mn1) model of leukemia (Chen et al., 2008; Heuser et al., 2011). However, in this work, we were able to pinpoint the developmental switch that limits the leukemic potential. We did this by assaying pGMs rather than the CMP fraction, which represents a more heterogeneous population of hematopoietic progenitors (Pronk et al., 2007). It is of note that Mn1 expression is lost at the GMP stage (Heuser et al., 2011), but the combinatorial overexpression of Mn1, Meis1, and Hoxa9/Hoxa10 can reinforce a susceptibility to transformation of these cells. Presumably, this is also relevant for MLL-fusion-mediated transformation, as both Meis1 and Hox genes are prominent target genes for these fusions, including in our model (data not shown).

Contrary to previous work (Cozzio et al., 2003), we demonstrate that CLPs are highly susceptible to MLL-ENL-mediated transformation. We show that CLPs initially respond to MLL-ENL induction with a continued contribution to the lymphoid lineages and a low, albeit detectable, myeloid output. However, all recipients of induced CLPs succumbed to myeloid leukemia with a germline B cell receptor configuration (data not shown). We believe the discrepancy between our study and previous work on this issue may be due to the different experimental approaches used. An obvious advantage with inducible
**Figure 4. MLL-ENL Induces Both Shared and Cell-Context-Dependent Transcriptional Responses**

(A) Venn diagrams showing the number and overlap of up- and downregulated genes in HSCs and pGMs upon brief exposure to MLL-ENL (based on triplicate RNA-seq samples per group).

(B) The 500 most up- and downregulated genes in both cell types upon MLL-ENL induction were correlated to normal HSCs and GMLPs (n = 4 and 3 microarray samples per group) or normal pGMs and GMPs (n = 3 microarray samples in each group) for MLL-ENL-induced HSCs and pGMs, respectively. Shown are GSEA enrichment plots of these analyses.

(C) Left: heatmap depicting the expression levels (log counts per million [CPM]) of genes belonging to the GO categories “Cell cycle arrest” and “Regulation of cell cycle” in WT and MLL-ENL-induced pGMs and HSCs. Right: cell-cycle analysis of HSCs and pGMs cultured for 96 hr with and without Dox (n = 3, mean ± SD).

(D) Myeloid lineage contribution over time from 100 freshly isolated control HSCs or from 100 cultured HSCs (with or without Dox, n = 5 in each group, mean ± SD).

(E) Fetal liver HSC-derived myeloid contribution in mice that were given a standard dose of Dox pellets at week 16 posttransplantation (left: 200 HSCs/recipient, n = 5 for the no-Dox group and n = 6 for the Dox group) and in the presence of a high concentration of Dox at 4 weeks posttransplantation (right: 100 HSCs/recipient, n = 7 mice/group).
mouse models, in contrast to retroviral delivery, is that in vitro manipulations can be minimized. Our experimental approach is therefore independent of the different sensitivities of different cell types to in vitro manipulation.

Recent studies on both murine cells and primary human samples proposed that gene-expression signatures imposed upon the LICs at transformation share commonalities with normal HSCs (Eppert et al., 2011; Krivtsov et al., 2006; Zhang et al., 2013). Since normal HSCs already harbor an extensive self-renewal capacity, a critical feature of leukemia, HSCs might serve as a potent and susceptible target population for MLL-ENL-induced leukemia. However, contrary to this expectation, we found that HSCs were highly resistant to MLL-ENL-induced transformation. At the same time, our in vitro experiments demonstrated a gain of hyperproliferation to MLL-ENL following only a few differentiation-associated HSC divisions. This is also a highly relevant concern when interpreting results from retroviral approaches, which require induction of proliferation for gene delivery.

Studies on acute lymphoblastic leukemia, myelodysplastic syndromes, chronic myelogenous leukemia, and de novo AML have suggested a role for HSCs as a reservoir for accumulating mutations in the preliminary phases of disease (Hong et al., 2008; Jamieson et al., 2004; Jan et al., 2012; Kico et al., 2014; Nilsson et al., 2002; Shlush et al., 2014). However, because MLL leukemias have a short latency and fewer recurrent mutations than other genetic lesions that result in AML (Cancer Genome Atlas Research Network, 2013), MLL-fusion genes may not require such a cellular reservoir for further mutations. This interpretation would perhaps be in line with the observations made here, since our gene-expression data suggest that although MLL-ENL, regardless of the target cell, represents a potent class II mutation (inhibition of differentiation), inherent class I activity (extensive proliferation) is necessary for disease propagation. However, our data on fetal liver HSCs, a state in which HSCs proliferate more extensively, argue that the proliferative status cannot be the sole factor that explains the HSCs’ lack of LIC competence. Another outstanding question that arises from our work is whether LICs are common for all MLL fusions. For instance, mouse models of the MLL-ENL or reciprocal AF4-MLL fusions (rather than MLL-ENL or MLL-AF9) produce alternative lymphoid or biphenotypic (rather than exclusively myeloid) leukemias (Bursen et al., 2010; Chen et al., 2006). We believe the relatively generic experimental model/approach exploited here could be valuable for addressing this issue.

In conclusion, we have developed a mouse model that allows for a conditional activation of the MLL-ENL oncogene in any normal cell type of choice. Using this model, we observed that overt transformation into AML is confined to a narrower window of differentiation than was previously appreciated, but the aggressiveness of disease can still be predicted from the normal hierarchical position of the target cells. Moreover, short-term in vitro differentiation could abrogate the hyperproliferation mediated by MLL-ENL in otherwise leukemia-competent cells. Together with the observation that HSCs do not seem to be a prominent target of MLL-ENL-mediated transformation, these data argue that differentiation-based therapies are a relevant avenue to pursue for clinical benefit in this aggressive disease.

**EXPERIMENTAL PROCEDURES**

**Generation of Col/α1-tetO-MLL/ENL Mice**

The human MLL-ENL oncogene was subcloned into the EcoRI site of the pBS31 vector and used to target KH2 ESCs (Beard et al., 2006). The engineered ESCs were injected into E3.5 C57BL/6 blastocysts to generate chimeric mice (Lund University Transgenic Animal Facility). MLL-ENL was induced in vivo by administering Dox via standard food pellets (200 mg/kg; Bio-Serv or ssniff Spezialdiäten) or, where indicated, high-Dox food pellets (2g/kg; ssniff Spezialdiäten). Mice were maintained in the animal facilities at the Biomedical Center of Lund University, and all animal experiments were performed with the approval of the local ethics committee.

**Cell Isolation and Adoptive Transfers**

HSPCs were isolated as described previously (Figure S2A; Norddahl et al., 2011; Pronk et al., 2007) from CD45.2+ Col/α1-tetO-MLL/ENL mice and competitively transplanted into lethally irradiated (800 rad) young CD45.1 mice 3–4 hr after irradiation. For serial transplantation experiments, 5 × 10^6 unfractionated BM cells from primary diseased mice were transplanted into sublethally irradiated (500 rad) CD45.1+ recipients (n = 3 recipients per primary leukemia). Fetal liver HSCs were isolated from E14.5 embryos using a LinSca1+c-c-kit+CD48+CD150+ phenotype. Recipients were put on a Dox-containing diet 5 days prior to transplantation. PB blood analyses were conducted as described previously (Norddahl et al., 2011). Diseased mice were subjected to Sysmex and necropsy analyses. Survival rates were plotted using the Kaplan-Meier method. Sudden elevations in donor myeloid chimerism were considered events for plotting event-free survival. The antibody clones and suppliers are provided in Supplemental Experimental Procedures. Cells were sorted on a FACS Aria II or III cell sorter (Becton Dickinson). All flow-cytometry and FACS data were analyzed using FlowJo software (Treestar).

**In Vitro Culture Assays**

HSPCs were cultured in standard culture conditions (37°C, 95% humidity, and 5% CO2) in OptiMEM (Invitrogen) supplemented with 10% fetal calf serum in the presence of stem cell factor (10–50 ng/ml), interleukin-3 (5 ng/ml), and granulocyte-colony stimulating factor (5 ng/ml) (all from PeproTech). Detailed procedures for all in vitro experiments are described in the Supplemental Experimental Procedures.

**Quantitative RT-PCR**

Quantitative RT-PCR was performed as described previously (Norddahl et al., 2011). The procedure used for absolute quantification of mRNA and the primers used for quantitative RT-PCR are listed in the Supplemental Experimental Procedures.

**Genome-Wide Expression Profiling**

A total of 2,000 HSCs and pGMs (triplicates) from WT and Col/α1-tetO-MLL/ENL mice were cultured in the presence of Dox for 48 hr. Cells were harvested and total RNA was extracted using the RNeasy Micro Kit (Qiagen) for mRNA purification. For details, see the Supplemental Experimental Procedures.

**Statistical Analysis**

Data were analyzed using Microsoft Excel (Microsoft, http://www.microsoft.com) and GraphPad Prism (GraphPad Software). Significance values were calculated by Student’s two-tailed t test or the log rank test (Mantel-Cox test) for Kaplan-Meier curves.

**ACCESSION NUMBERS**

The GEO accession number for the RNA sequence data reported in this paper is GSE62734.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cellrep.2014.10.036.

AUTHOR CONTRIBUTIONS

A.U. and G.L.N. designed and performed experiments, analyzed data, wrote the paper, and contributed equally to the work. M.W. and S.S. analyzed data. P.S., P.J., and C.J.P. performed experiments. J.C. provided essential intellectual support and edited the paper. D.B. conceived and supervised the study and wrote the paper.

ACKNOWLEDGMENTS

We thank Dr. Robert Slany for providing the MLL-ENL cDNA; Drs. R. Jaenisch and C. Beard for providing input on the KHZ recombination system, and Drs. Naoki Hosen, Derrick Rossi, and Cord Brakebusch for their help in the initial stages of the ESC work. We also thank Keith Humphries for a critical reading of the manuscript, Gerd Sten and Eva Erlandsson for expert technical support, and the staff of the Lund Transgenic Mouse Facility for blastocyst injections. This work was supported by grants from the Swedish Cancer Society (a project grant and a Young Investigator Award position grant), the Swedish Medical Research Council, ERC Consolidator grant 615068, the Swedish Pediatric Leukaemia Foundation, the Crafoord Foundation, and the Gunnar Nilsson Cancer Foundation to D.B. The Genome Technology Access Center, Department of Genetics, Washington University School of Medicine, assisted with genomic analysis and is partially supported by NCI Cancer Center Support Grant P30 CA91842 to the Siteman Cancer Center, ICTS/CTSA Grant UL1TR000448 from the National Center for Research Resources (NCRR, a component of the NIH), and the NIH Roadmap for Medical Research. This publication is solely the responsibility of the authors and does not necessarily represent the official view of the NCRR or NIH.

Received: December 2, 2013
Revised: September 18, 2014
Accepted: October 13, 2014
Published: November 13, 2014

REFERENCES
