

# Mouse acute leukemia develops independent of self-renewal and differentiation potentials in hematopoietic stem and progenitor cells

Fang Dong,<sup>1-3,\*</sup> Haitao Bai,<sup>1,\*</sup> Xiaofang Wang,<sup>1</sup> Shanshan Zhang,<sup>1</sup> Zhao Wang,<sup>1</sup> Miner Xie,<sup>1</sup> Sen Zhang,<sup>1</sup> Jinhong Wang,<sup>1</sup> Sha Hao,<sup>1</sup> Tao Cheng,<sup>1-4</sup> and Hideo Ema<sup>1-3</sup>

<sup>1</sup>State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China; <sup>2</sup>Center for Stem Cell Medicine, Chinese Academy of Medical Sciences, Tianjin, China; <sup>3</sup>Department of Stem Cell and Regenerative Medicine, Peking Union Medical College, Tianjin, China; and <sup>4</sup>Collaborative Innovation Center for Cancer Medicine, Tianjin, China

## Key Points

- Mouse AML and T-ALL develop from multiple cells of origin: hematopoietic stem and progenitor cells.
- Heterogeneous LSCs are involved in the expansion and maintenance of leukemia.

The cell of origin, defined as the normal cell in which the transformation event first occurs, is poorly identified in leukemia, despite its importance in understanding of leukemogenesis and improving leukemia therapy. Although hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) were used for leukemia models, whether their self-renewal and differentiation potentials influence the initiation and development of leukemia is largely unknown. In this study, the self-renewal and differentiation potentials in 2 distinct types of HSCs (HSC1 [CD150<sup>+</sup>CD41<sup>-</sup>CD34<sup>-</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells] and HSC2 [CD150<sup>-</sup>CD41<sup>-</sup>CD34<sup>-</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells]) and 3 distinct types of HPCs (HPC1 [CD150<sup>+</sup>CD41<sup>+</sup>CD34<sup>-</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells], HPC2 [CD150<sup>+</sup>CD41<sup>+</sup>CD34<sup>+</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells], and HPC3 [CD150<sup>-</sup>CD41<sup>-</sup>CD34<sup>+</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells]) were isolated from adult mouse bone marrow, and examined by competitive repopulation assay. Then, cells from each population were retrovirally transduced to initiate MLL-AF9 acute myelogenous leukemia (AML) and the intracellular domain of NOTCH-1 T-cell acute lymphoblastic leukemia (T-ALL). AML and T-ALL similarly developed from all HSC and HPC populations, suggesting multiple cellular origins of leukemia. New leukemic stem cells (LSCs) were also identified in these AML and T-ALL models. Notably, switching between immunophenotypical immature and mature LSCs was observed, suggesting that heterogeneous LSCs play a role in the expansion and maintenance of leukemia. Based on this mouse model study, we propose that acute leukemia arises from multiple cells of origin independent of the self-renewal and differentiation potentials in hematopoietic stem and progenitor cells and is amplified by LSC switchover.

## Introduction

Leukemia is a clonal malignancy resulting in abnormal hematopoiesis characterized by an accumulation of immature blasts that fail to differentiate into functional blood cells. Leukemia develops through multiple steps in progressive conversion from normal cells to leukemia cells.<sup>1-3</sup>

Clonal evolution in leukemia holds that the genetic and epigenetic changes occur over time in cells derived from a single cell and that, if such changes confer selective advantage, some leukemia clones outcompete others.<sup>4</sup> Clonal evolution can lead to genetic heterogeneity, conferring phenotypic and functional differences among the leukemia cells within a single patient.<sup>5-7</sup> Recent studies have supported complex and branched clonal evolution in the initiation, development, and relapse of

human leukemia.<sup>4,8-10</sup> However, it is unclear at which differentiation stages leukemia clones arise, and how certain clones expand.

The term “cell of origin” is defined as the normal cell in which the first transformation events occur.<sup>11</sup> The cell of origin that received the first oncogenic “hit” would progressively accumulate mutations during the clonal evolution of leukemia. The cell of origin may refer to “leukemia-initiating cells” or “target cells.” It is possible that leukemia cells derived from different cells of origin, such as hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs), may show considerable differences in proliferation potential, differentiation degree, and therapy response.<sup>12-14</sup> The identification of these target cells may allow the earlier detection of malignancy and prevention of disease progression.

Leukemic stem cells (LSCs) are capable of initiating and sustaining leukemia growth after transplantation and are considered biologically distinct cells within leukemia.<sup>15,16</sup> LSCs may refer to “leukemia-propagating cells.”<sup>11</sup> LSCs likely play a role in relapse because the leukemia clone with specific mutations at diagnosis recurrently appears during relapse.<sup>17-19</sup> In this regard, LSCs are an important target in the treatment of leukemia.

The relationship between the cell of origin and LSCs has yet to be elucidated. Considering the long latency in leukemia and technological limitations, it is difficult to clarify the cell of origin in human leukemia. This issue has been addressed by mouse studies using leukemia models. MLL fusion proteins created by chromosomal translocation are frequently associated with the development of acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL).<sup>20</sup> Similar types of leukemia developed with the cellular transduction of fusion oncogenes *MLL-GAS7* and *MLL-ENL*.<sup>21,22</sup> Ectopic expression of the *MLL-AF9* (MA9) fusion gene has been used to induce AML in mice.<sup>23</sup> Analysis of MA9 knock-in mice showed that the transformation efficiency of Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) cells and common lymphoid progenitors (CLPs) was significantly greater than that of granulocyte/macrophage progenitors (GMPs).<sup>14</sup> In most of these studies,<sup>12,23-25</sup> LSK cells were used as the HSC population. However, LSK cells contain different types of HSCs and HPCs.

*NOTCH* gene-activated mutation has been found in >50% of T-cell ALL (T-ALL) patients.<sup>26</sup> Overexpression of the intracellular domain of NOTCH-1 (ICN-1) in mouse HPCs leads to T-ALL.<sup>27,28</sup> In these studies, whether highly purified HSCs or HPCs served as the cell of origin in leukemia has never been examined.

Functional heterogeneity in HSCs and HPCs was recently recognized.<sup>29-35</sup> In this study, based on CD150 and CD41 expression, CD34<sup>-</sup>LSK cells were further divided into HSC1, HSC2, and HPC1 populations. The HSC1 population is enriched in long-term (LT; >6 months) HSCs; the HSC2 population is enriched in short-term (ST; <6 months) HSCs and the HPC1 population is enriched in repopulating common myeloid progenitors (CMPs).<sup>36,37</sup> Similarly, CD34<sup>+</sup>LSK cells were divided into HPC2 and HPC3 populations. The HPC2 population is enriched in colony-forming units in the spleen (Shanshan Zhang, unpublished data), whereas the HPC3 population is enriched in lymphoid-primed multipotent progenitors (LMPPs).<sup>36</sup> The aim of this study was to clarify the effect of different cells of origin on the initiation and development of leukemia. To this end, we examined highly purified HSCs and HPCs as target cells for MA9 AML and ICN-1 T-ALL transformation.

We hypothesized that LT-HSCs, ST-HSCs, and different types of HPCs have varying potentials in the initiation of leukemia. MA9 and ICN-1 were retrovirally overexpressed in highly purified HSC1, HSC2, HPC1, HPC2, and HPC3 cells, followed by transplantation. We then examined whether different HSCs and/or HPCs give rise to different types and degrees of leukemia. Surprisingly, this study showed that the self-renewal and lineage differentiation potentials in HSCs and HPCs did not affect the outcome of leukemogenesis. Moreover, the identification of new LSCs in AML and T-ALL models suggested that heterogeneous LSCs play a role in the development and maintenance of leukemia.

## Materials and methods

### Mice

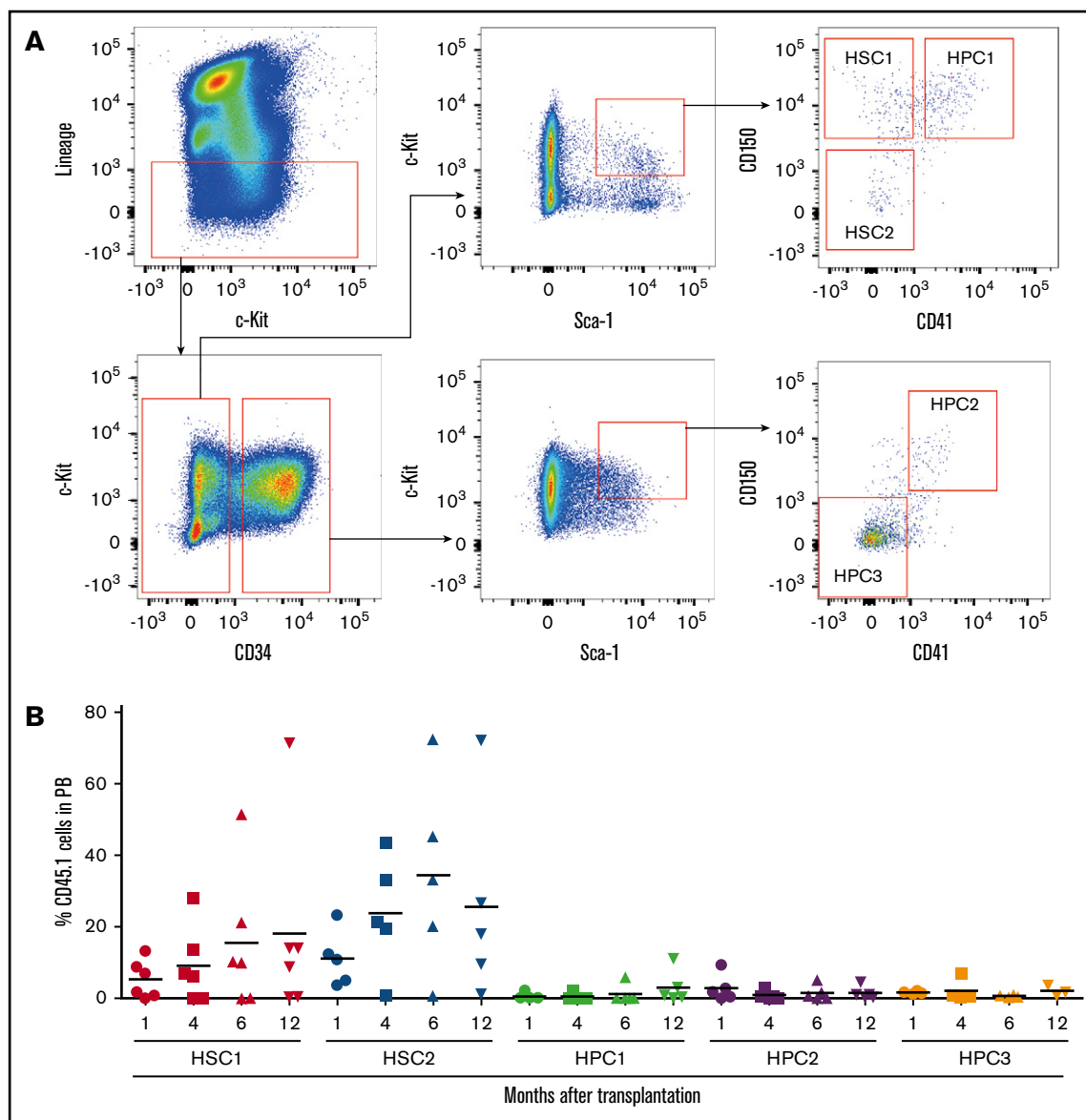
C57/BL6 (B6-Ly5.2) and B6.SJL-Ly5.1 (B6-Ly5.1) mice were maintained at the specific pathogen-free animal facility of the Institute of Hematology and Blood Diseases Hospital. Experimental protocols were approved by the Institute of Hematology Animal Care and Use Committee.

### Retrovirus preparation

MSCV-MLL/AF9-PGK-PURO was generously provided by Chi Wai So (King's College London, London, United Kingdom).<sup>38</sup> The PGK-PURO segment was replaced by internal ribosome entry site (IRES)-green fluorescent protein (GFP) to form the MSCV-MLL/AF9-IRES-GFP construct. The retrovirus vector containing the complementary DNA-encoding ICN-1 was kindly provided by David Scadden (Harvard University, Boston, MA). Retroviral supernatant was generated as previously described.<sup>38</sup> Briefly, HEK293T cells in a 10-cm dish were transfected with 12 μg of pMSCV-IRES-MA9-GFP or 10 μg of pMSCV-IRES-ICN-1-GFP, 6 μg of p-Kat, and 5 μg of VSVG plasmid using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). After 6 to 8 hours, the medium was replaced with fresh growth medium (Dulbecco's modified Eagle medium plus 10% fetal bovine serum; Thermo Fisher Scientific). After 24 to 48 hours, retroviral supernatant was harvested and filtered through a 0.45-μm filter. Filtered virus supernatant was concentrated 50-fold by centrifugation and resuspended by Ham F12 medium (Thermo Fisher Scientific) and then used for the transduction of purified cells.

### HSC, HPC, GMP, and CLP isolation

Bone marrow (BM) cells were collected from femora, tibiae, and iliac crests of female B6-Ly5.1 mice at an age of 7 to 10 weeks. c-Kit<sup>+</sup> cells were enriched by magnetic-activated cell sorting (Miltenyi Biotec) and stained with antibodies purchased from eBioscience and BD Biosciences. Cells were stained with the lineage (Lin) cocktail consisting of biotinylated anti-B220 (RA3-6B2), CD11b (M1/70), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), Ter-119 (TER-119), and Gr-1 (RB6-8C5) antibodies. Biotinylated antibodies were developed by allophycocyanin-Cy7 (APC-Cy7)-conjugated streptavidin. For the sorting of HSCs and HPCs, cells were then stained with APC-conjugated anti-c-Kit (2B8), phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-Sca-1 (E13-161.7), PE-conjugated anti-CD150 (mShad150; BioLegend), fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (RAM34), and eFluor710-conjugated anti-CD41 (MWRReg30) antibodies. For the sorting of GMPs, cells were then stained with APC-conjugated



**Figure 1. Reconstitution potentials of HSC1, HSC2, HPC1, HPC2, and HPC3.** (A) Flow cytometry sorting strategy for HSC and HPC populations. Lin<sup>-</sup> cells were separated into CD34<sup>-</sup> and CD34<sup>+</sup> fractions and then gated on c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells, respectively. According to the expression levels of CD150 and CD41, CD34<sup>-</sup> LSK cells were separated into HSC1, HSC2, and HPC1, and CD34<sup>+</sup> LSK cells were separated into HPC2 and HPC3. (B) The percentage of donor cells in PB. Ten HSC1, 10 HSC2, 10 HPC1, 50 HPC2, and 250 HPC3 cells were mixed with  $5 \times 10^5$  BM cells and injected into lethally irradiated recipient mice. The PB of recipient mice was analyzed at 1, 4, 6, and 12 months after transplantation ( $n = 6$  for each population; however, some mice died after transplantation).

anti-c-Kit, PE-Cy7-conjugated anti-Sca-1, FITC-conjugated anti-CD34, and PE-conjugated anti-FcγRII/III (97) antibodies. For the sorting of CLPs, cells were then stained with APC-conjugated anti-c-Kit, PE-Cy7-conjugated anti-Sca-1, FITC-conjugated anti-CD34, and PE-conjugated anti-CD127 (A7R34) antibodies. Dead cells were excluded by 4',6-diamidino-2-phenylindole staining. HSCs and HPCs were sorted on a FACSAria III with Diva software (BD Biosciences) based on the following surface marker profiles (Figure 1A): HSC1 (CD150<sup>+</sup>CD41<sup>-</sup>CD34<sup>-</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells), HSC2 (CD150<sup>-</sup>CD41<sup>-</sup>CD34<sup>-</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells), HPC1 (CD150<sup>+</sup>CD41<sup>+</sup>CD34<sup>-</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells), HPC2 (CD150<sup>+</sup>CD41<sup>+</sup>CD34<sup>+</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells), HPC3

(CD150<sup>-</sup>CD41<sup>-</sup>CD34<sup>+</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> cells). GMPs and CLPs were gated as CD34<sup>+</sup>FcγRII/III<sup>+</sup>Lineage<sup>-</sup>Sca-1<sup>-</sup>c-Kit<sup>+</sup> cells and IL7-R<sup>+</sup>Lineage<sup>-</sup>Sca-1<sup>low</sup>c-Kit<sup>low</sup> cells, respectively.

### HSC and HPC transplantation

The frequencies of HSC1, HSC2, HPC1, HPC2, and HPC3 cells were about 0.001%, 0.004%, 0.0015%, 0.004%, and 0.04% in BM, respectively. The corresponding numbers of these cells in  $5 \times 10^5$  BM cells as competitor cells were ~5, 20, 7, 20, and 200. In this study, 10 HSC1, 10 HSC2, 10 HPC1, 50 HPC2, and 250 HPC3 cells were mixed with  $5 \times 10^5$  BM cells and injected into mice irradiated at the dose of 9.5 Gy. Twenty microliters of

peripheral blood (PB) from recipient mice was collected via the eye vein after anesthesia at 1, 4, 6, and 12 months after transplantation. ACS solution (Stem Cell Technologies, Vancouver, BC, Canada) was used for red blood cell lysis. The frequencies of Ly5.1<sup>+</sup> donor-derived myeloid cells (Mac-1/Gr-1<sup>+</sup>), B cells (B220<sup>+</sup>), and T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) in PB cells were determined by flow cytometry.

### Detection of transduction efficiencies

Six hundred each of HSC1, HSC2, HPC1, HPC2, and HPC3 were prestimulated overnight with 50 ng/mL murine stem cell factor (mSCF) and 50 ng/mL murine thrombopoietin (mTPO) in SF-O3 medium (Sekisui Medical Co, Ltd, Tokyo, Japan) for 24 hours. MLL-AF9 or ICN-1-GFP-retroviral supernatant (4  $\mu$ L) supplemented with 4 ng/mL polybrene was added to cells. Cells were incubated for 8 to 12 hours and the medium were changed to 10% fetal bovine serum in  $\alpha$ -minimal essential medium with 10 ng/mL mSCF, 10 ng/mL mTPO, 10 ng/mL murine interleukin 3, and 1 IU/mL human erythropoietin. Cells were cultured for an additional 7 days and then collected for the analysis of GFP<sup>+</sup> cells by flow cytometry.

### Retroviral transduction and transplantation

Sorted cells were prestimulated overnight with 50 ng/mL mSCF and 50 ng/mL mTPO in SF-O3 medium for 24 hours. MA9 or ICN-1-GFP-retroviral supernatant (10-15  $\mu$ L) supplemented with 4 ng/mL polybrene was added to cells. Cells were incubated for 24 hours and total cells were injected into B6-Ly5.2 mice irradiated at the dose of 9.5 Gy together with  $5 \times 10^5$  BM cells.

### Analysis of recipient mice

PB was sampled from recipient mice every 2 weeks. Donor cells were detected with GFP expression. Mac-1 and Gr-1 were used as myeloid lineage markers. CD3, CD4, and CD8 were used as T-cell makers. B220 was used as B-cell maker. When the frequency of GFP<sup>+</sup> cells was >60% in PB upon flow cytometric analysis (BD FACSCanto II) and the mice showed symptoms of sickness, mice were euthanized under anesthesia and the BM and spleen were analyzed. BM and spleen cells were stained with APC-conjugated anti-c-Kit, PE-Cy7-conjugated anti-Sca-1, PE-conjugated anti-CD150, BV421-conjugated anti-CD34, eFluoro710-conjugated anti-CD41, biotinylated Lin cocktail antibodies. The biotinylated Lin antibodies were developed with APC-Cy7-conjugated streptavidin and analyzed on a BD FACS Aria III. CD11b and CD3/4/8 antibodies were excluded from the lineages cocktail for the analysis of MA9 and ICN-1 mice, respectively. A biotinylated anti-interleukin 7 receptor (IL-7R) antibody was also included in the lineage cocktail for the analysis of MA9 mice. Flow cytometry data were analyzed using FlowJo software (TreeStar).

### Serial transplantation

Cells were sorted from selected populations in the BM of primary leukemic mice and injected into sublethally irradiated (4.75 Gy) mice to observe the development of leukemia. When the secondary recipient mice showed symptoms of illness, the mice were euthanized for the diagnosis of leukemia and the indicated populations were sorted again for tertiary transplantation.

### Statistical analysis

Data are presented as the mean plus or minus standard deviation. GraphPad Prism 5.0 and SPSS 17.0 software was used for all statistical analyses. An unpaired Student *t* test and analysis

of variance were used to generate *P* values for most data sets (\**P* < .05; \*\**P* < .005; \*\*\**P* < .001; NS, not significant).

## Results

### Highly purified HSC and HPC populations showed distinct self-renewal and differentiation potentials

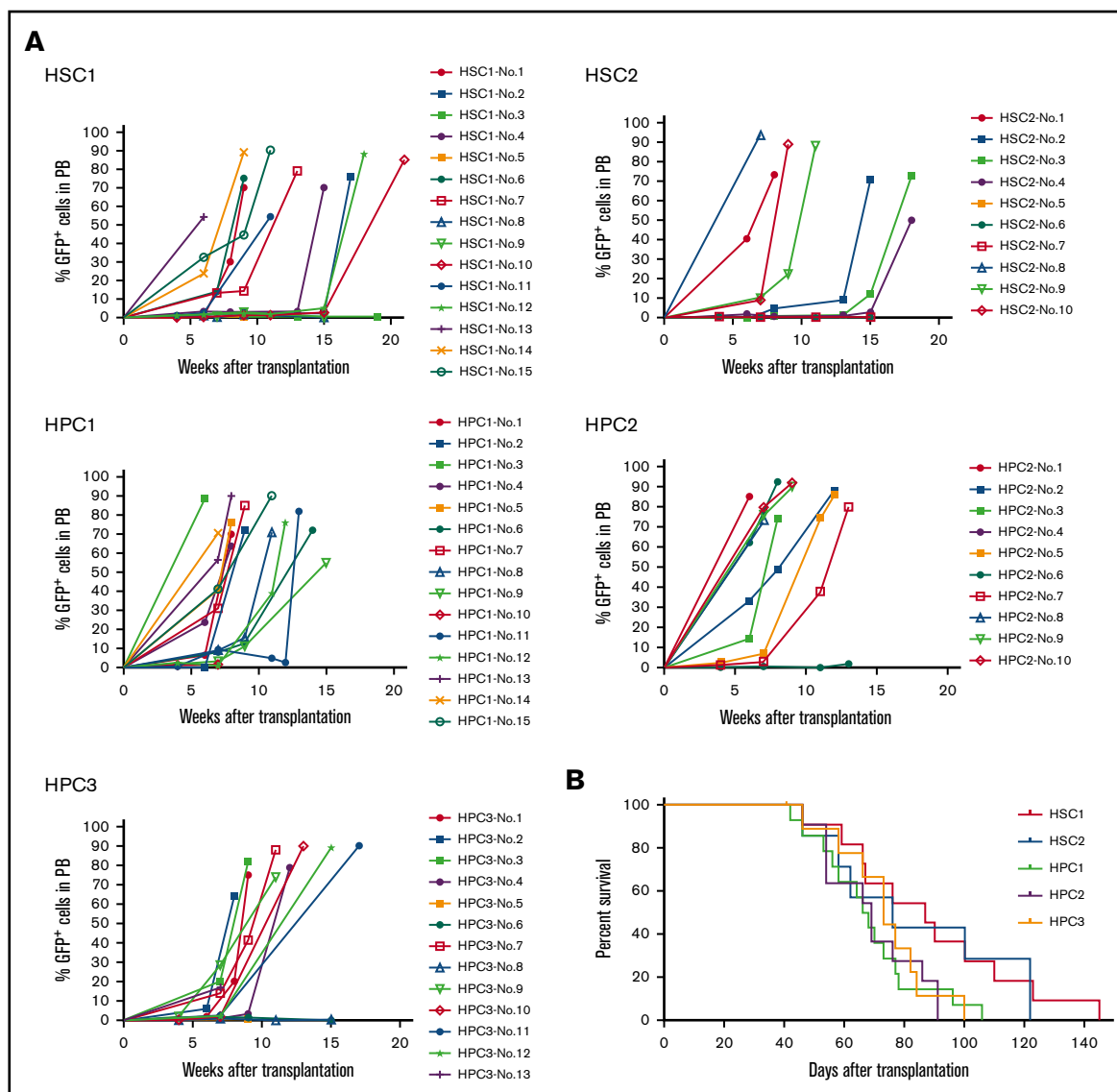
Figure 1A shows the gating strategy of HSC1, HSC2, HPC1, HPC2, and HPC3 cells. Briefly, CD34<sup>-</sup>LSK cells were fractionated into HSC1 (CD150<sup>+</sup>CD41<sup>-</sup>), HSC2 (CD150<sup>-</sup>CD41<sup>-</sup>), and HPC1 (CD150<sup>+</sup>CD41<sup>+</sup>) cells. CD34<sup>+</sup>LSK cells were fractionated into HPC2 (CD150<sup>+</sup>CD41<sup>+</sup>) and HPC3 (CD150<sup>-</sup>CD41<sup>-</sup>) cells. The comparison with the gating strategy of the Trumpp group<sup>39</sup> in supplemental Figure 1A-B showed that our HSC1 and HPC1 overlapped with their HSC. Our HPC2 overlapped with their MPP1 and MPP2. Our HPC3 overlapped with their MPP3 and MPP4. Our HSC2 did not overlap with their populations. To validate their reconstitution potentials in vivo, 10 HSC1, 10 HSC2, 10 HPC1, 50 HPC2, and 250 HPC3 cells were sorted, mixed with  $5 \times 10^5$  BM cells, and transplanted into lethally irradiated mice. The percentages of donor-derived cells and lineage contribution by these 5 populations at 1, 4, 6, and 12 months after transplantation are shown in Figure 1B. HSC1 showed long-term reconstitution, the levels of which continuously increased: the mean percentages of reconstitution levels were 5.2%, 9.1%, 15.4%, and 18.0% at 1, 4, 6, and 12 months, respectively. As shown in supplemental Figure 2, the myeloid lineage was the major component of the reconstitution: the mean percentages of myeloid cells in total donor cells were 59%, 71%, 54%, and 60% at 1, 4, 6, and 12 months, respectively. Similarly, HSC2 cells showed long-term reconstitution: the mean percentages of reconstitution levels were 10.8%, 23.7%, 33.6%, and 26.7% at 1, 4, 6, and 12 months, respectively. In contrast to HSC1 cells, lymphoid-biased reconstitution gradually increased: the mean percentages of lymphoid cells in the total donor cells were 22%, 40%, 83%, and 57% at 1, 4, 6, and 12 months, respectively.

HPC1, HPC2, and HPC3 cells showed very low levels of reconstitution: the mean percentages of reconstitution at 12 months after transplantation were 2.9%, 0.58%, and 0.98%, respectively. Taken together, long-term self-renewal and multiple lineage differentiation potentials of HPC1, HPC2, and HPC3 were significantly lower than HSC1 and HSC2 cells. Myeloid differentiation was preferred by HSC1 cells, whereas lymphoid differentiation was preferred by HSC2 cells.

### HSCs and HPCs with distinct potentials similarly gave rise to AML by overexpression of the MA9 fusion gene

To compare the transduction efficiencies of 5 populations, 600 each of HSC1, HSC2, HPC1, HPC2, and HPC3 cells were transduced by MA9 retrovirus, respectively. The transduction efficiencies of 5 populations were evaluated 7 days after in vitro culture because it was difficult to detect GFP<sup>+</sup> cells in a small number of cells immediately after transduction. The transduction efficiencies of MA9 in each population were <5% and there was no significant difference among the 5 populations (supplemental Figure 3A).

To determine the limiting cell dose for transformation, different numbers of cells (33, 66, 100, 200, 600, and more) from HSC1, HSC2, HPC1, HPC2, and HPC3 populations were transduced with MA9 retrovirus and transplanted along with  $5 \times 10^5$  total BM cells

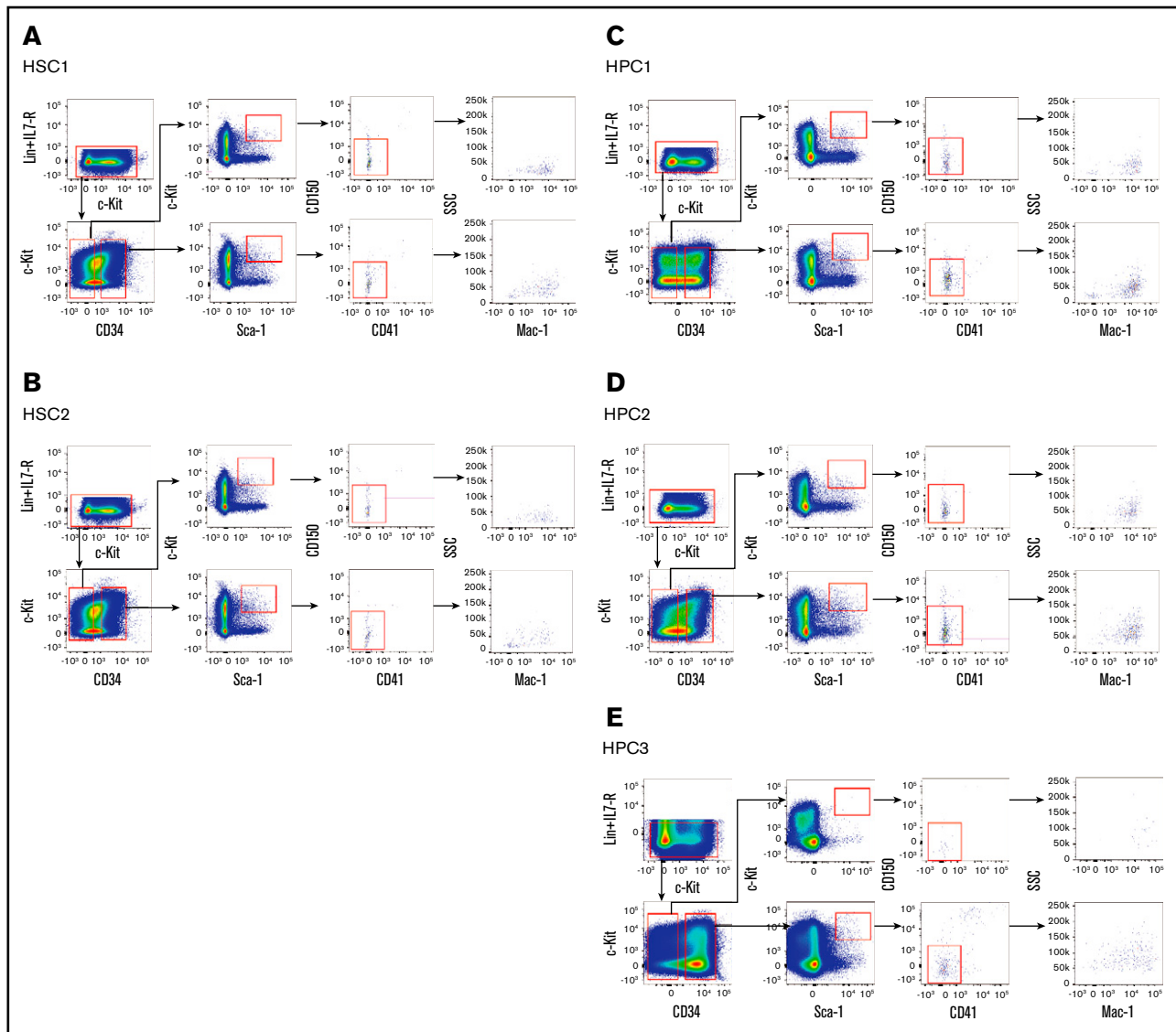


**Figure 2. Transplantation with MA9-transduced HSCs and HPCs.** (A) Percentages of GFP<sup>+</sup> cells in the PB of mice after transplantation (n = 9-15). (B) Survival curves of mice transplanted with each cell population. Leukemic mice from 3 independent experiments were summarized (n = 7-14 per group). The log-rank test was used to compare the percentages of survival.

into lethally irradiated mice. The estimated frequencies of transformation (95% confidence interval) for HSC1, HSC2, HPC1, HPC2, and HPC3 were 1 in 519 (1 in 289-931), 1 in 497 (1 in 252-980), 1 in 306 (1 in 176-533), 1 in 470 (1 in 271-815), and 1 in 510 (1 in 280-928), respectively (supplemental Table 1). The estimated frequencies of the transformation were not significantly different among the 5 populations. Based on these results, the cell dose of 600 cells was chosen for further analysis. To examine more differentiated cells, GMPs and CLPs were transduced by MA9 retrovirus. AML developed from GMPs but not from CLPs (supplemental Table 2).

A total of 600 each of HSC1 and HSC2 and HPC1, HPC2, and HPC3 cells were transduced by MA9 retrovirus and transplanted along with  $5 \times 10^5$  total BM cells into lethally irradiated mice (supplemental Figure 3C). GFP<sup>+</sup> cells were first detected in the PB

of recipients 4 weeks after transplantation. As shown in Figure 2A, the percentages of GFP<sup>+</sup> cells slowly increased in the PB of HSC1, HSC2, HPC1, HPC2, and HPC3 recipients. After the percentage of GFP<sup>+</sup> cells reached 20%, the number dramatically increased, and the mice presented symptoms of illness. The majority of GFP<sup>+</sup> cells in the PB from all 5 groups of mice were positive for Mac-1 but negative for B220 and CD3, indicating that these GFP<sup>+</sup> cells belonged to the myeloid lineage (supplemental Figure 4A). Giemsa staining of PB cells showed morphologically immature cells (supplemental Figure 4B). The spleen weights from all 5 groups of mice were significantly greater than those from normal mice. There was no significant difference in spleen weight among the 5 groups (supplemental Figure 4C). From these data, we gave the diagnosis of AML when the percentage of GFP<sup>+</sup> cells was >20% of the PB cells. The median survival time was 87, 76, 66, 69,



**Figure 3. New LSC detection in MA9 mice.** HSC markers were used to detect leukemic HSC/HPC cells in the BM of MA9 mice. GFP<sup>+</sup> cells were first gated. Lin<sup>-</sup>IL7-R<sup>-</sup>CD34<sup>+/+</sup>c-Kit<sup>-</sup>Sca-1<sup>+</sup>CD150<sup>-</sup>CD41<sup>-</sup>Mac-1<sup>+</sup>(L-CD34<sup>-</sup>/LSK) cells were detected in all MA9 leukemic mice derived from 5 different populations (HSC1 [A], HSC2 [B], HPC1 [C], HPC2 [D], and HPC3 [E]). SSC, side scatter.

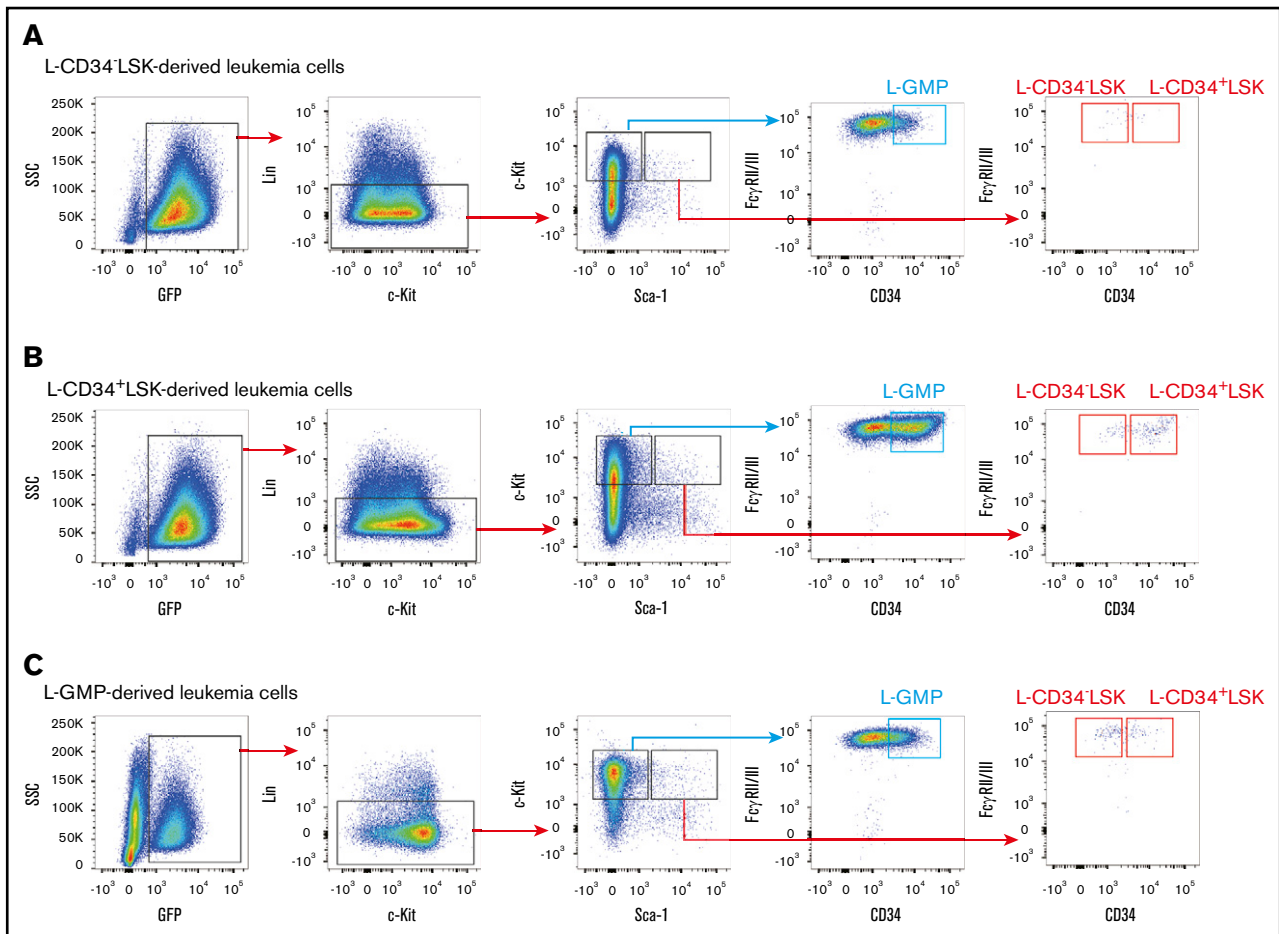
and 66 days, respectively, for mice transplanted with HSC1, HSC2 and HPC1, HPC2, and HPC3 cells. There was no statistically significant difference in survival time (Figure 2B).

### New LSC populations were found in MA9 AML

Surface markers have been used to isolate LSCs in mouse models and human patients.<sup>40,41</sup> For example, Lin<sup>-</sup>Sca-1<sup>-</sup>c-Kit<sup>+</sup>CD34<sup>+/+</sup>FcγRII/III<sup>+</sup>GFP<sup>+</sup> cells (L-GMP) were shown to be enriched in LSCs in the mouse model and CD123<sup>+</sup>CD34<sup>+</sup>CD38<sup>-</sup> cells were shown to be enriched in LSCs in AML patients.<sup>23,42</sup> Although the surface markers may change after transformation, LSCs seemed phenotypically similar to normal counterparts at least to some extent. As shown in supplemental Figure 5, L-GMP cells were detected in the BM of mice transplanted with MA9-transduced cells from 5 populations. We also attempted to detect LSCs upstream of L-GMP.

The HSC/HPC markers were used to identify new LSCs. As shown in Figure 3, GFP<sup>+</sup> leukemic cells were first gated and then lineage marker-positive cells were excluded. Cells were separated into CD34<sup>-</sup> and CD34<sup>+</sup> cells. LSK cells were detected in both populations. These CD34<sup>-</sup>LSK and CD34<sup>+</sup>LSK cells were negative for CD150 and CD41. Thus, they appeared to be phenotypically similar to normal HSC2 and HPC3 cells. The majority of these CD34<sup>-</sup> and CD34<sup>+</sup> LSK cells expressed Mac-1 as an AML marker. These cells were detected in the BM of mice transplanted with transduced cells from all 5 populations. We named them leukemic CD34<sup>-</sup>LSK (L-CD34<sup>-</sup>LSK) and leukemic CD34<sup>+</sup>LSK (L-CD34<sup>+</sup>LSK) cells.

To determine whether these cells were LSCs, 700 each of L-CD34<sup>-</sup>LSK and L-CD34<sup>+</sup>LSK cells were isolated from mice with AML from all 5 populations (primary AML mice) and transplanted into sublethally irradiated mice. L-GMP cells from primary AML mice were used as a positive control. Supplemental Table 3



**Figure 4. Switchover between L-CD34<sup>-</sup>LSK and L-GMP cells.** L-CD34<sup>-</sup>LSK, L-CD34<sup>+</sup>LSK, and L-GMP cells were isolated from primary AML mice (HSC1:MA9) and transplanted into sublethally irradiated recipient mice individually. When the recipient mice developed leukemia (secondary leukemia), BM cells were analyzed. L-CD34<sup>-</sup>LSK (A), L-CD34<sup>+</sup>LSK (B), and L-GMP (C) cells gave rise to all L-CD34<sup>-</sup>LSK, L-CD34<sup>+</sup>LSK, and L-GMP cells.

shows the results of secondary transplantation. AML developed again at efficiencies ranging from 75% to 100% (secondary AML mice). These results confirmed that L-CD34<sup>-</sup>LSK and L-CD34<sup>+</sup>LSK cells were LSCs, which appeared to be phenotypically heterogeneous.

To examine the relationship among each of L-CD34<sup>-</sup>LSK, L-CD34<sup>+</sup>LSK, and L-GMP cells, serial transplantation was performed. Seven hundred each of L-CD34<sup>-</sup>LSK, L-CD34<sup>+</sup>LSK, and L-GMP cells were sorted from primary AML mice (HSC1:MA9) and transplanted into sublethally irradiated mice. As shown in supplemental Table 4, all recipient mice developed AML again (secondary AML mice). Notably, L-CD34<sup>-</sup>LSK cells generated not only L-CD34<sup>-</sup>LSK cells but also L-CD34<sup>+</sup>LSK and L-GMP cells after secondary transplantation (Figure 4A; supplemental Table 4). Similarly, L-CD34<sup>+</sup>LSK cells generated not only L-CD34<sup>+</sup>LSK cells but also L-CD34<sup>-</sup>LSK and L-GMP cells after secondary transplantation (Figure 4B; supplemental Table 4). Moreover, L-GMP cells generated not only L-GMP cells but also L-CD34<sup>-</sup>LSK and L-CD34<sup>+</sup>LSK cells after secondary transplantation (Figure 4C; supplemental Table 4).

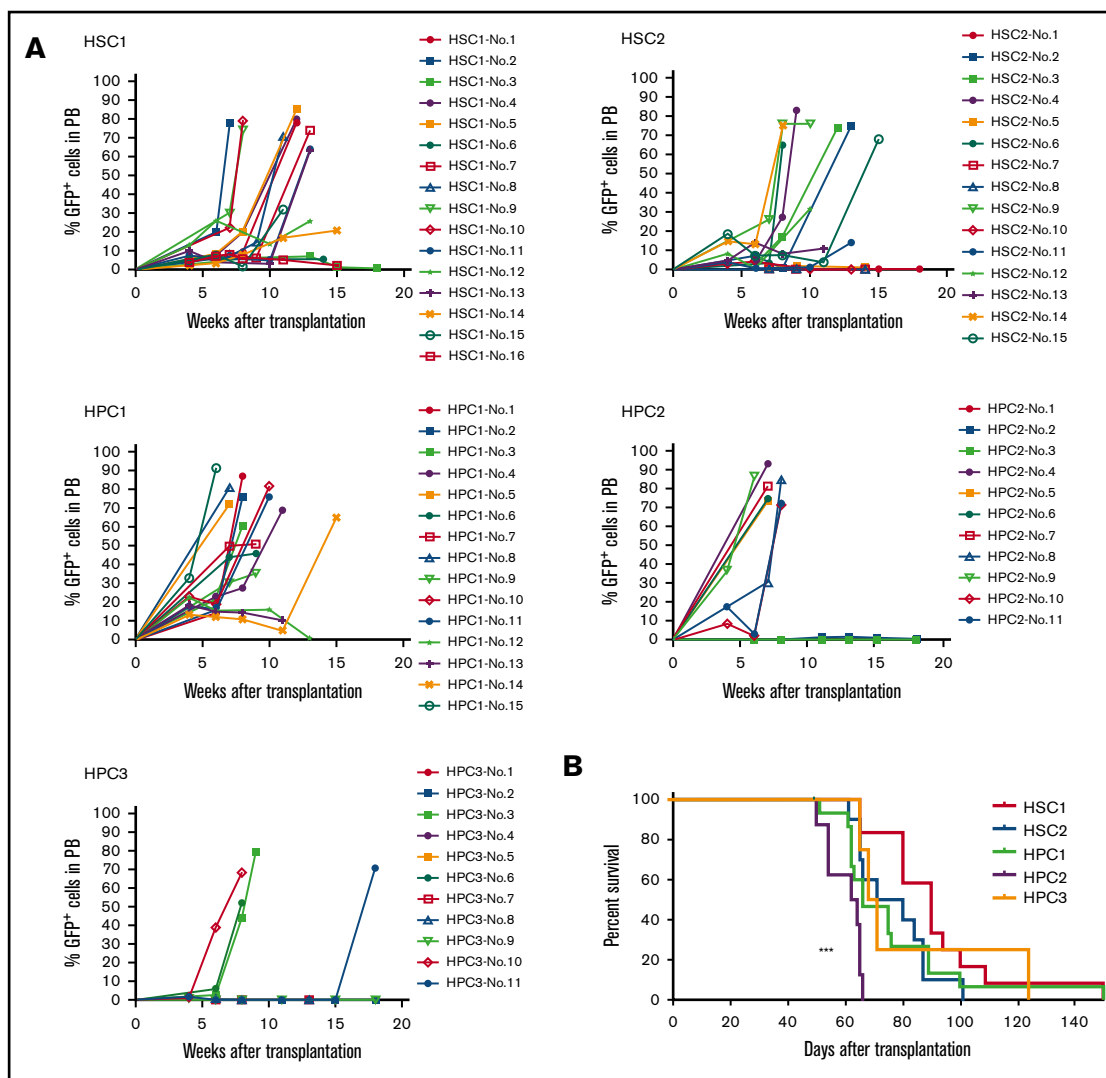
L-CD34<sup>-</sup>LSK, L-CD34<sup>+</sup>LSK, and L-GMP cells from secondary AML mice were transplanted into sublethally irradiated mice. As shown in supplemental Table 4, all recipient mice developed AML again (tertiary AML mice). All of these data suggested the phenotypic

switching among L-CD34<sup>-</sup>LSK, L-CD34<sup>+</sup>LSK, L-GMP cells after transplantation.

### HSCs and HPCs with distinct potentials similarly gave rise to T-ALL by overexpression of the ICN-1 gene

To compare the transduction efficiencies of 5 populations, 600 each of HSC1, HSC2, HPC1, HPC2, and HPC3 cells were transduced by ICN-1 retrovirus, respectively. The transduction efficiencies of 5 populations were detected 7 days after in vitro culture. The transduction efficiency of HPC1 cells was greater than those of HSC2, HPC2, and HPC3 cells ( $P < .05$ ). The transduction efficiency of HPC2 cells was significantly greater than those of HSC2 and HPC3 cells ( $P < .001$  and  $P < .01$ ; supplemental Figure 3B).

To determine the limiting cell dose for transformation, different numbers of cells (33, 66, 100, 200, 600, and more) from HSC1, HSC2, HPC1, HPC2, and HPC3 populations were transduced with ICN-1 retrovirus and then transplanted along with  $5 \times 10^5$  total BM cells into lethally irradiated mice. The estimated frequencies of transformation (95% confidence interval) for HSC1, HSC2, HPC1, HPC2, and HPC3 were 1 in 463 (1 in 268-798), 1 in 562 (1 in 314-1007), 1 in 297 (1 in 174-507), 1 in 370 (1 in 186-735), and 1 in



**Figure 5. Transplantation with ICN-1-transduced HSCs and HPCs.** (A) Percentages of GFP<sup>+</sup> cells in the PB of mice after transplantation (n = 11-16). (B) Survival curves of mice transplanted with each cell population. Leukemic mice from 3 independent experiments were summarized (n = 4-15 per group). The log-rank test was used to compare the percentages of survival (\*\*\**P* < .001).

969 (1 in 437-2146), respectively (supplemental Table 5). No leukemia developed from ICN-1-transduced GMPs or CLPs (supplemental Table 6). To be consistent with the MA9 AML model, the cell dose of 600 cells was chosen for further analysis.

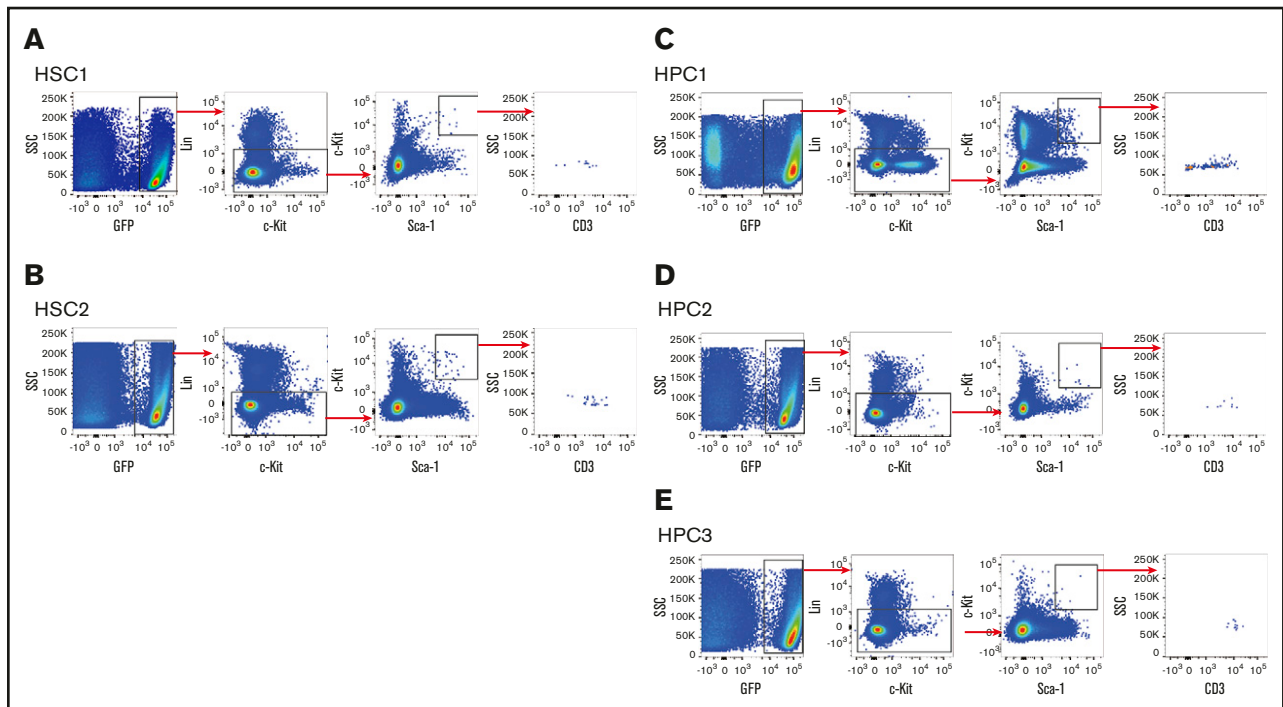
Six hundred each of HSC1, HSC2, HPC1, HPC2, and HPC3 cells were transduced by ICN-1 retrovirus and transplanted along with  $5 \times 10^5$  total BM cells into lethally irradiated mice (supplemental Figure 3C). GFP<sup>+</sup> cells were first detected in the PB of recipients 4 weeks after transplantation. As shown in Figure 5A, the percentages of GFP<sup>+</sup> cells slowly increased in the PB of HSC1 and HSC2 and HPC1, HPC2, and HPC3 recipients. Once reaching 20%, the percentages of GFP<sup>+</sup> cells dramatically increased in the PB from each group and the mice presented symptoms of illness. The majority of GFP<sup>+</sup> cells in the PB from all 5 groups of mice were positive for CD4 and CD8 but negative for B220 and Mac-1/Gr-1, indicating that these GFP<sup>+</sup> cells belonged to the T-cell lineage (supplemental Figure 6A). Giemsa staining of PB cells also showed morphologically immature cells (supplemental

Figure 6B). The spleen weights from all 5 groups of mice were significantly greater than that of normal mice. There was no significant difference in spleen weight among the 5 groups (supplemental Figure 6C). From these data, the diagnosis of T-ALL was given when the percentage of GFP<sup>+</sup> cells was >20% of the PB. The median survival time was 90, 71, 66, 62, and 68 days, respectively, for mice transplanted with HSC1, HSC2 and HPC1, HPC2, and HPC3 cells. HPC2-derived T-ALL showed the shortest survival time compared with the other groups (*P* < .001; Figure 5B).

### New LSC populations were found in ICN-1 T-ALL

The concept and phenotypic markers of LSCs in T-ALL mouse models have been poorly defined.<sup>43</sup> We tried to identify LSCs in the ICN-1 T-ALL model. HSC and HPC markers were used to identify LSCs from T-ALL mice. As shown in Figure 6, GFP<sup>+</sup> leukemia cells were first gated on Lin<sup>-</sup> cells, and then, Sca-1<sup>+</sup> and c-Kit<sup>+</sup> cells were gated (LSK cells). The majority of GFP<sup>+</sup> LSK cells expressed





**Figure 6. New LSK detection in ICN-1 mice.** HSC markers were used to detect leukemic HSC/HPC cells in the BM of ICN-1 mice. GFP<sup>+</sup>Lin<sup>-</sup>IL7-R<sup>-</sup>CD34<sup>+/+</sup>-c-Kit<sup>+</sup>Sca-1<sup>+</sup> (L-LSK) cells were detected in all ICN-1 leukemic mice derived from 5 different populations (HSC1 [A], HSC2 [B], HPC1 [C], HPC2 [D], and HPC3 [E]).

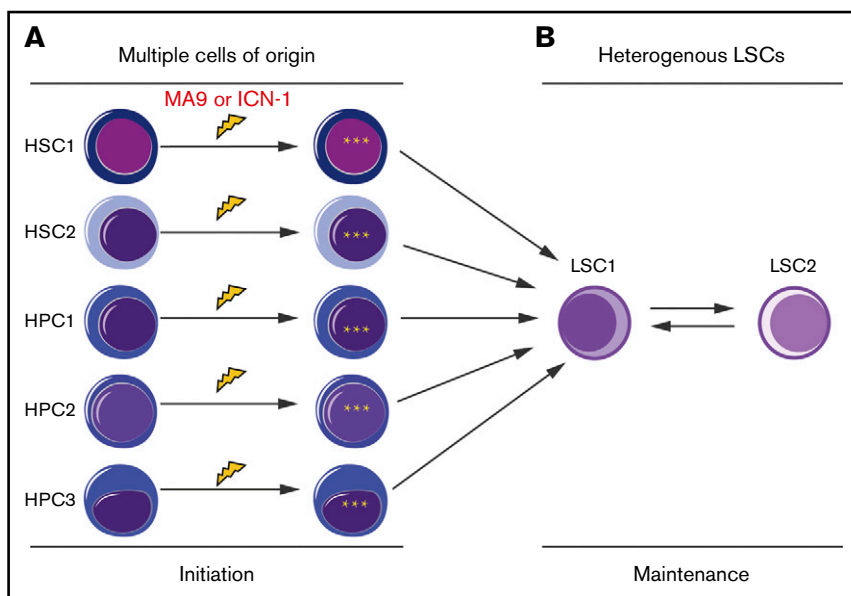
the T-cell lineage marker, CD3. Next, these LSK cells in mice with T-ALL from the 5 populations were sorted and transplanted into sublethally irradiated mice to determine whether they were LSCs. Supplemental Table 7 shows that LSK cells initiated T-ALL again after transplantation. These LSK cells thus appeared to be LSCs in ICN-1 T-ALL. We named them leukemic LSK (L-LSK) cells.

## Discussion

Our results showed that HSC1, HSC2, HPC1, HPC2, and HPC3 cells, with discrete self-renewal and differentiation potentials, induced similar types of leukemia by transformation with MA9 or ICN-1. Transplantation of HSC1 cells showed that these cells were enriched in myeloid-biased LT-HSCs (Figure 1; supplemental Figure 2), consistent with previous work.<sup>36,44</sup> Transplantation of HSC2 cells showed that these cells were lymphoid-biased LT-HSCs. A previous study suggested that the HSC2 population is enriched in lymphoid-biased ST-HSCs.<sup>36</sup> In this study, the HSC1 and HSC2 gates might have been too close to one another. CD150<sup>med</sup> cells in which some LT-HSCs were present might have been included in the HSC2 population.<sup>31</sup> Nevertheless, HSC1 and HSC2 cells, as well as HPC1, HPC2, and HPC3 cells, all gave rise to AML and T-ALL. In these leukemia models, once transduced with oncogenes, both HSCs and HPCs served as the cell of origin. In this process, the self-renewal and lineage differentiation potentials of HSCs and HPCs seemed to be unessential, supporting data from MA9 knock-in mice.<sup>14</sup> It was reported that CMPs but not GMPs could be transformed by MN1 because the expression of MEIS1/HOX genes is required for MN1 AML.<sup>45</sup> Seemingly, the cell of origin depends on oncogenes. However, we assumed that if CMPs can initiate AML, all cells upstream of CMPs with myeloid differentiation potentials such as HSCs and MPPs should be able to initiate AML.

Retroviral transduction, knock-in mouse, and conditional transgenic mouse strategies have been used for AML models with the MA9 fusion gene.<sup>14,23,46</sup> In general, the expression level of oncogenes is higher in the retroviral system than in the knock-in system because of greater promoter activity and multiple copies of oncogenes in the retroviral system. The transgenic mouse system is perhaps between these 2 systems. A high copy number of oncogenes has been reported in some leukemia patients.<sup>47</sup> The situation in the retroviral system may mimic such leukemia cases. The gene dose has some effects on the transformation efficiency.<sup>14</sup> The knock-in strategy is better than the other 2 systems to compare the transformation of different cell types because the copy number of oncogenes should be equal among different cell types. The onset of oncogene expression is controllable in the retroviral and conditional transgenic systems. The latency is shortest in the retroviral system whereas it is longest in the knock-in system. The transgenic mouse system is perhaps between these 2 systems. It is possible that additional gene mutations are required for a long latency leukemia model. In the retroviral system, the expression of oncogenes is specific to targeted blood cells whereas in the knock-in system, it is not. In addition, the retroviral system is time and money saving, compared with the other 2 systems. Likewise, each system has advantages and technical limitations. Using the retroviral system, we could compare 2 leukemia models. Our focus was whether or not particular types of cells can initiate leukemia. However, the inducible knock-in mouse, if it were available, would be ideal to study the cell of origin with transformation efficiency taken into consideration.

Regarding the transduction and transformation efficiencies for the development of MA9 AML, there was no significant difference among 5 populations (supplemental Figure 3A; supplemental Table 1). These data suggested that the AML development potentials were compared among the 5 populations without bias.



**Figure 7. The cell of origin and LSC-switching model.** (A) Multiple cells, such as HSC1, HSC2, HPC1, HPC2, and HPC3 cells, serve as the cell of origin. After leukemia is initiated, the leukemia pool is expanded and maintained through back-and-forth switching among (B) heterogeneous LSCs (for instance, LSC1 and LSC2). ★★★ Transduced cells by MA9 or ICN-1 virus.

Mice with AML from all 5 populations similarly died (Figure 2B), indicating no significant effect on the progression and aggressiveness of leukemia regardless of the cell of origin. In contrast, in the ICN-1 T-ALL model, the frequency of transformed cells in HPC1 was significantly greater than that in HPC3 (supplemental Table 5). To some extent, this resulted from the higher transduction efficiency of HPC1 than that of HPC3 (supplemental Figure 3C). However, the significantly higher transduction efficiency of HPC1 than that of HSC2 and HPC2, and higher transduction efficiency of HPC2 than that of HSC2 and HPC3 did not contribute to the frequencies of transformed cells among these groups. The transduction efficiency was estimated 7 days after culture whereas the transformation efficiency was estimated several months after transplantation. The effect of oncogenes on the proliferation was involved in both efficiencies. Thus, the transduction efficiencies in vitro may not always influence the transformed efficiencies in vivo. Mice with T-ALL from HPC2 cells died earlier than those from the other populations (Figure 4B). We speculate that the cell of origin may have some effect on the progression and aggressiveness of leukemia. Genes particularly expressed in the HPC2 population may have cooperatively worked with ICN-1 in the proliferation of T-ALL cells. Further studies are required to identify the specific genes expressed in HPC2 and other populations and to address the underlying mechanisms.

LSCs with specific biological and molecular properties play a role in the pathogenesis, resistance, and relapse of leukemia, and LSCs could be a good target for a treatment strategy.<sup>12,46,48</sup> The existence of LSCs in AML has been well documented,<sup>49-51</sup> but their existence in T-ALL is debatable and challenging. In this study, L-CD34<sup>-</sup> LSK and L-CD34<sup>+</sup> LSK cells were identified as LSCs in the MA9 AML model and L-LSK cells in the ICN-1 T-ALL model. L-LSK (L-CD34<sup>-</sup> LSK and L-CD34<sup>+</sup> LSK) cells were more significant in MA9 AML than in ICN-1 T-ALL, presumably because the LSK fraction contains more myeloid progenitor cells than lymphoid progenitor cells in normal BM. LSCs may use their cellular function to expand their pool. L-GMP cells were previously identified as LSCs and have been well characterized in the MA9 AML model.<sup>23</sup> CD3<sup>+</sup>c-kit<sup>+</sup> LSCs in T-ALL from PTEN knockout mice and CD3<sup>low</sup> LSCs in T-ALL from p18 knockout mice have been

reported.<sup>52,53</sup> All of these results suggest that LSCs are heterogeneous in mouse leukemia models, which is consistent with a previous leukemia study in humans.<sup>54</sup>

In the MA9 AML model, myeloid differentiation was suggested to be required for leukemia because L-GMP were not generated in the absence of *Cebpa*, and cytokine-induced myeloid differentiation restored the generation of L-GMP.<sup>55</sup> It is likely that *Cebpa* is highly expressed downstream in all 5 populations and is required for the generation and maintenance of LSCs in MA9 AML. In human T-ALL, the driver event often involves chromosomal translocation of the T-cell receptor gene, suggesting that LSCs in T-ALL are generated from committed T-cell precursors.<sup>56</sup> It is possible that T-cell receptor is highly expressed downstream of all 5 populations and is required for the generation and maintenance of LSCs in ICN-1 T-ALL.

LMPP-like and GMP-like LSCs, in hierarchical order, were detected in human CD34<sup>+</sup> AML,<sup>40</sup> and only LMPP-like LSCs can give rise to GMP-like LSCs but not vice versa. In our model, however, L-CD34<sup>-</sup> LSK, L-CD34<sup>+</sup> LSK, and L-GMP cells switched over among them. In particular, L-GMP cells reverted to generate upstream L-CD34<sup>+/+</sup> LSK cells. Sorted L-GMP were unlikely to have been contaminated with rare cell types like L-CD34<sup>+/+</sup> LSK cells. These data rather suggested the phenotypical plasticity in LSCs. We may not be able to rely on immunophenotypical markers of LSCs when they are targeted for treatment. The analysis of gene expression and chromatin landscapes in different LSCs may provide more useful information on a new therapeutic strategy for leukemia.<sup>13,57</sup>

Clonal hematopoiesis of indeterminate potential (CHIP) was recently described according to sequencing data and has provided new insights into the development of hematopoietic malignancies.<sup>18,58,59</sup> CHIP is considered as the early genetic lesions that take place in preleukemic cells.<sup>60</sup> CHIP can exist for a long time before the onset of malignancy. After multiple steps of mutations and their accumulation, CHIP may finally transform to the cell of origin of leukemia. However, CHIP may not always contribute to the cell of origin of leukemia.

Finally, we propose the cell of origin and LSC-switching model (Figure 7). In this model, acute leukemia has multiple cells of origin

that initiate leukemia. LSCs are phenotypically and functionally heterogeneous. For instance, if there are 2 types of LSCs (LSC1 and LSC2), these LSCs can go back and forth to expand and maintain their pool of LSCs. LSCs have been originally defined as functional cells. We could separate LSCs according to the phenotypic markers, but we should not rely too much on their surface markers for their targeted therapy. To develop new strategies for leukemia therapy, the heterogeneous nature of the cell of origin and LSCs should be taken into account. The identification of multiple cells of origin may facilitate early precision prevention for hematopoietic malignancies.<sup>60</sup> We may also know whether or not and to what extent normal stem and progenitor cells remain in leukemia patients. For instance, if stem cells were not the cell of origin, we may be able to rescue normal stem cells after therapy.

## Acknowledgments

This work was supported by grants from the Ministry of Science and Technology of China (2015CB964403 and 2015CB964404), the National Key Research and Development Program of China

Stem Cell and Translational Research (2017YFA0104900 and 2016YFA0100600), Chinese Academy of Medical Sciences Initiative for Innovative Medicine (2016-I2M-1-017 and 2017-I2M-1-015), and the National Natural Science Foundation of China (81470279, 81421002, 81400077, 81670105, 81500085, 81890990, and 81700166).

## Authorship

Contribution: F.D. and H.B. performed all experiments, analyzed data, and wrote the paper; X.W. and Shanshan Zhang assisted with all experiments; Z.W., M.X., and Sen Zhang helped perform flow cytometry analysis; J.W. and S.H. assisted with data analysis; T.C. conducted the research and analyzed data; and H.E. designed the study, interpreted data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Hideo Ema, Institute of Hematology and Blood Diseases Hospital, 288 Nanjing Rd, Tianjin 300020, China; e-mail: hema@ihcams.ac.cn.

## References

1. Jan M, Snyder TM, Corces-Zimmerman MR, et al. Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Sci Transl Med*. 2012;4(149):149ra118.
2. Shlush LI, Zandi S, Mitchell A, et al; HALT Pan-Leukemia Gene Panel Consortium. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia [*Nature*. 2014;508(7496):420]. *Nature*. 2014;506(7488):328-333.
3. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481(7382):506-510.
4. Nowell PC. The clonal evolution of tumor cell populations. *Science*. 1976;194(4260):23-28.
5. Shackleton M, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell*. 2009;138(5):822-829.
6. Ferrando AA, López-Otín C. Clonal evolution in leukemia. *Nat Med*. 2017;23(10):1135-1145.
7. Dick JE. Stem cell concepts renew cancer research. *Blood*. 2008;112(13):4793-4807.
8. Greaves M. Leukaemia 'firsts' in cancer research and treatment. *Nat Rev Cancer*. 2016;16(3):163-172.
9. Landau DA, Carter SL, Getz G, Wu CJ. Clonal evolution in hematological malignancies and therapeutic implications. *Leukemia*. 2014;28(1):34-43.
10. Puente XS, López-Otín C. The evolutionary biography of chronic lymphocytic leukemia. *Nat Genet*. 2013;45(3):229-231.
11. Wang JC, Dick JE. Cancer stem cells: lessons from leukemia. *Trends Cell Biol*. 2005;15(9):494-501.
12. Krivtsov AV, Figueroa ME, Sinha AU, et al. Cell of origin determines clinically relevant subtypes of MLL-rearranged AML. *Leukemia*. 2013;27(4):852-860.
13. George J, Uyar A, Young K, et al. Leukaemia cell of origin identified by chromatin landscape of bulk tumour cells. *Nat Commun*. 2016;7:12166.
14. Chen W, Kumar AR, Hudson WA, et al. Malignant transformation initiated by Mll-AF9: gene dosage and critical target cells. *Cancer Cell*. 2008;13(5):432-440.
15. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*. 1994;367(6464):645-648.
16. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997;3(7):730-737.
17. Shlush LI, Mitchell A, Heisler L, et al. Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature*. 2017;547(7661):104-108.
18. Corces-Zimmerman MR, Hong WJ, Weissman IL, Medeiros BC, Majeti R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proc Natl Acad Sci USA*. 2014;111(7):2548-2553.
19. Garg M, Nagata Y, Kanojia D, et al. Profiling of somatic mutations in acute myeloid leukemia with FLT3-ITD at diagnosis and relapse. *Blood*. 2015;126(22):2491-2501.
20. Meyer C, Hofmann J, Burmeister T, et al. The MLL recombinome of acute leukemias in 2013. *Leukemia*. 2013;27(11):2165-2176.
21. So CW, Karsunky H, Passegué E, Cozzio A, Weissman IL, Cleary ML. MLL-GAS7 transforms multipotent hematopoietic progenitors and induces mixed lineage leukemias in mice. *Cancer Cell*. 2003;3(2):161-171.

22. Cozzio A, Passegué E, Ayton PM, Karsunky H, Cleary ML, Weissman IL. Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes Dev.* 2003;17(24):3029-3035.
23. 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.
24. Somervaille TC, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell.* 2006; 10(4):257-268.
25. Wang Y, Krivtsov AV, Sinha AU, et al. The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. *Science.* 2010; 327(5973):1650-1653.
26. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science.* 2004; 306(5694):269-271.
27. Pear WS, Aster JC, Scott ML, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med.* 1996;183(5):2283-2291.
28. Hu X, Shen H, Tian C, et al. Kinetics of normal hematopoietic stem and progenitor cells in a Notch1-induced leukemia model. *Blood.* 2009; 114(18):3783-3792.
29. Osawa M, Nakamura K, Nishi N, et al. In vivo self-renewal of c-Kit+ Sca-1+ Lin(low/-) hemopoietic stem cells. *J Immunol.* 1996; 156(9):3207-3214.
30. Ema H, Takano H, Sudo K, Nakauchi H. In vitro self-renewal division of hematopoietic stem cells. *J Exp Med.* 2000;192(9):1281-1288.
31. Morita Y, Ema H, Nakauchi H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med.* 2010; 207(6):1173-1182.
32. Benz C, Copley MR, Kent DG, et al. Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell.* 2012;10(3):273-283.
33. Miller PH, Knapp DJ, Eaves CJ. Heterogeneity in hematopoietic stem cell populations: implications for transplantation. *Curr Opin Hematol.* 2013; 20(4):257-264.
34. Babovic S, Eaves CJ. Hierarchical organization of fetal and adult hematopoietic stem cells. *Exp Cell Res.* 2014;329(2):185-191.
35. Kent DG, Dykstra BJ, Eaves CJ. Isolation and assessment of single long-term reconstituting hematopoietic stem cells from adult mouse bone marrow. *Curr Protoc Stem Cell Biol.* 2016;38:2A.4.1-2A.4.24.
36. Yamamoto R, Morita Y, Ooehara J, et al. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell.* 2013;154(5):1112-1126.
37. Ema H, Morita Y, Suda T. Heterogeneity and hierarchy of hematopoietic stem cells. *Exp Hematol.* 2014;42(2):74-82.e72.
38. Liu Y, Cheng H, Gao S, et al. Reprogramming of MLL-AF9 leukemia cells into pluripotent stem cells. *Leukemia.* 2014;28(5):1071-1080.
39. Cabezas-Wallscheid N, Klimmeck D, Hansson J, et al. Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. *Cell Stem Cell.* 2014;15(4):507-522.
40. Goardon N, Marchi E, Atzberger A, et al. Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. *Cancer Cell.* 2011; 19(1):138-152.
41. Jin Y, Zhou J, Xu F, et al. Targeting methyltransferase PRMT5 eliminates leukemia stem cells in chronic myelogenous leukemia. *J Clin Invest.* 2016; 126(10):3961-3980.
42. Jordan CT, Upchurch D, Szilvassy SJ, et al. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia.* 2000;14(10):1777-1784.
43. Tan SH, Bertulfo FC, Sanda T. Leukemia-initiating cells in T-cell acute lymphoblastic leukemia. *Front Oncol.* 2017;7:218.
44. Wang X, Dong F, Zhang S, et al. TGF-β1 negatively regulates the number and function of hematopoietic stem cells. *Stem Cell Reports.* 2018; 11(1):274-287.
45. Heuser M, Yun H, Berg T, et al. Cell of origin in AML: susceptibility to MN1-induced transformation is regulated by the MEIS1/AbdB-like HOX protein complex. *Cancer Cell.* 2011;20(1):39-52.
46. 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.
47. Nibourel O, Guihard S, Roumier C, et al. Copy-number analysis identified new prognostic marker in acute myeloid leukemia. *Leukemia.* 2017; 31(3):555-564.
48. Gentles AJ, Plevritski SK, Majeti R, Alizadeh AA. Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia. *JAMA.* 2010;304(24):2706-2715.
49. Clevers H. The cancer stem cell: premises, promises and challenges. *Nat Med.* 2011;17(3):313-319.
50. Nguyen LV, Vanner R, Dirks P, Eaves CJ. Cancer stem cells: an evolving concept. *Nat Rev Cancer.* 2012;12(2):133-143.
51. Kreso A, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell.* 2014;14(3):275-291.
52. Yuan Y, Yu H, Boyer MJ, et al. Hematopoietic stem cells are not the direct target of spontaneous leukemic transformation in p18(INK4C)-null reconstituted mice. *Cancer Res.* 2006;66(1):343-351.

53. Guo W, Lasky JL, Chang CJ, et al. Multi-genetic events collaboratively contribute to Pten-null leukaemia stem-cell formation. *Nature*. 2008; 453(7194):529-533.
54. Barabé F, Kennedy JA, Hope KJ, Dick JE. Modeling the initiation and progression of human acute leukemia in mice. *Science*. 2007;316(5824):600-604.
55. Ye M, Zhang H, Yang H, et al. Hematopoietic differentiation is required for initiation of acute myeloid leukemia. *Cell Stem Cell*. 2015;17(5):611-623.
56. Look AT. Oncogenic transcription factors in the human acute leukemias. *Science*. 1997;278(5340):1059-1064.
57. 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.
58. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414(6859):105-111.
59. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. *Cell*. 2012;150(2):264-278.
60. Bowman RL, Busque L, Levine RL. Clonal hematopoiesis and evolution to hematopoietic malignancies. *Cell Stem Cell*. 2018;22(2):157-170.