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Journal

Proceedings of the National Academy of Sciences of the United States of America, 115(9)

ISSN

0027-8424

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Publication Date

2018-02-27

DOI

10.1073/pnas.1718446115

Peer reviewed

Respecifying human iPSC-derived blood cells into highly engraftable hematopoietic stem and progenitor cells with a single factor

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Contributed by Yuet Wai Kan, December 24, 2017 (sent for review October 23, 2017; reviewed by Linzhao Cheng and Eric So)

Derivation of human hematopoietic stem cells (HSCs) from induced pluripotent stem cells (iPSCs) offers considerable promise for cell therapy, disease modeling, and drug screening. However, efficient derivation of functional iPSC-derived HSCs with in vivo engraftability and multilineage potential remains challenging. Here, we demonstrate a tractable approach for respecifying iPSC-derived blood cells into highly engraftable hematopoietic stem and progenitor cells (HSPCs) through transient expression of a single transcription factor, *MLL-AF4*. These induced HSPCs (iHSPCs) derived from iPSCs are able to fully reconstitute the human hematopoietic system in the recipient mice without myeloid bias. iHSPCs are long-term engraftable, but they are also prone to leukemic transformation during the long-term engraftment period. On the contrary, primary HSPCs with the same induction sustain the long-term engraftment without leukemic transformation. These findings demonstrate the feasibility of activating the HSC network in human iPSC-derived blood cells through expression of a single factor and suggest iHSPCs are more genomically instable than primary HSPCs, which merits further attention.

human induced pluripotent stem cells | hematopoietic stem cells | MLL-AF4 | engraftability | leukemia

De novo generation of human hematopoietic stem cells (HSCs) from different cell sources remains a high priority for studying and treating blood diseases. Induced pluripotent stem cells (iPSCs) provide alluring probabilities of deriving isogenic HSCs from patients themselves, which would obviate the devastating complication of graft versus host disease (GVHD). However, generating human iPSC-derived functional HSCs has been unexpectedly challenging even when many transcription factors (TFs) are introduced. In particular, when introducing five transcription factors (*ERG*, *HOXA9*, *RORA*, *SOX4*, and *MYB*) into iPSC-derived committed blood cells, those myeloid progenitors can only be reprogrammed into short-term engraftable multipotent progenitors with scant engraftment levels and a strong bias toward myeloid differentiation (1). Not until recently are the long-term engraftable HSCs achievable through transducing seven transcription factors (*ERG*, *HOXA5*, *HOXA9*, *HOXA10*, *LCOR*, *RUNX1*, and *SPI1*) into iPSC-derived hemogenic endothelium (HE) (2). However, the winding road of reprogramming iPSCs through an intermediate state of HE greatly increases the complexity and decreases the efficiency of this method. Therefore, devising a more efficient and simpler strategy is still of great significance for further application.

The myeloid differentiation bias has been a bottleneck for deriving lineage-balanced iHSPCs. Therefore, we reasoned that introducing TFs that not only promote self-renewal but also facilitate lymphopoiesis might correct the myeloid bias of the respecification system. To get more clues for the candidate factors, we evaluated the regulators that have been reported to enhance the multilineage hematopoiesis in the human or mouse reprogramming system (1–4) and found that most mixtures contain regulators such as *HOX*, *MEIS1*, *ERG*, and *GATA3*, that are closely correlated with MLL and/or MLL-fusion proteins (5–8). MLL is a DNA-binding protein that methylates histone H3 lysine 4 (H3K4) and positively regulates the expression of *HOX* genes and *MEIS1*, which are the key regulators of HSCs (9). On the other hand, *MLL* translocations encode truncated MLL with its fusion partners that are able to exert various genetic or epigenetic influences on the targeted cells (10). Moreover, many of the reported HSC reprogramming factors, such as RUNX1, GATA3, and MYB, have direct interactions with MLL (5–7). Given their miscellaneous effects, we reason that leveraging MLL or MLL-fusion proteins might obviate the needs for many extra regulators in the respecification system.

MLL is necessarily required in definitive hematopoiesis (11) and has a critical role in HSC self-renewal (12), but MLL per se does not enhance lymphopoiesis. Among *MLL* translocations, a

Significance

We designed this study to evaluate the feasibility of using only one factor to respecify human induced pluripotent stem cell (iPSC)-derived blood cells into long-term engraftable hematopoietic stem and progenitor cells (HSPCs). By transiently expressing a single transcription factor, MLL-AF4, the iPSC-derived blood cells could gain robust engraftability with multilineage reconstitution potential. Myeloid differentiation bias has been considered a big barrier for deriving functional HSPCs from human iPSCs, and we showed that *MLL-AF4*–induced HSPCs (iHSPCs) were able to reestablish both myelopoiesis and lymphopoiesis without lineage bias in the recipient mice. By parallell comparison of iHSPCs with *MLL-AF4*–induced primary HSPCs, we also found that iHSPCs were more prone to leukemic transformation during the long-term engraftment period, which provided a necessary caveat of using iHSPCs in the actual therapies.

Author contributions: Y.-T.T., L.Y., H.L., S.-J.C., and Y.W.K. designed research; Y.-T.T., L.Y., A.I.B., and J.W. performed research; F.X., M.O.M., and Z.C. contributed new reagents/analytic tools; Y.-T.T. analyzed data; and Y.-T.T., L.Y., H.L., S.-J.C., and Y.W.K. wrote the paper.

Reviewers: L.C., Johns Hopkins University; and E.S., King's College London.

The authors declare no conflict of interest.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE103420).

See Commentary on page 1964.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718446115/-/DCSupplemental.

Published online January 31, 2018.

major type of rearrangement is MLL-AF4, which is predominantly found in lymphoblastic leukemia. In addition to facilitating lymphopoiesis, *MLL-AF4* also enhances self-renewal of primary HSCs (13), which is possibly related to its function of up-regulating the expression of *CD133* (14). More importantly, transplantation of *MLL-AF4*–transduced HSCs does not result in leukemia, as *MLL-AF4* alone is insufficient to induce leukemogenesis (15–17). Based on these, we thus pinpointed our candidate to *MLL-AF4* and found that *MLL-AF4* alone was sufficient to enable the potent engraftment of iHSPCs with both lymphoid and myeloid reconstitution capability. We also investigated the biological effects of MLL-AF4 exerted on human primary HSPCs so as to examine without bias the cellular properties of iHSPCs under the parallel comparison with bona fide HSPCs.

Results

In Vitro Induction of *MLL-AF4* Can Impart Self-Renewal and Lymphoid Potential to iPSC-Derived Blood Cells. We optimized our previously established reprogramming method (18) to a feeder-free condition and derived iPSCs from peripheral blood (PB)-mobilized HSPCs (CD34-iPSC) and mononuclear cells (MN-iPSC). Pluripotency and normal karyotyping were verified on both iPSCs (Fig. S1 *A*–*E*). For in vitro differentiation of iPSCs toward hematopoietic cells, we also modified our prior strategy (19) to a feeder-free and xeno-free culture condition (Fig. 1*A*). The round-shaped hematopoietic cells could be detected from day 8, when they began to detach from the plate and reached the maximal number at day 12 (Fig. 1*B*). We then collected the detached cells at day 12 and performed colony-forming assay and flow cytometry analysis. Various colony-forming units could be observed by 2 wk of plating (Fig. 1*C*). Flow cytometry analysis showed that CD34-iPSC–derived blood cells had a relatively higher population of $CD45^+CD34^+$ cells (Fig. 1*D* and Fig. S1*F*), which might be ascribed to the epigenetic memory of iPSC related to its somatic cell source (20). Noticeably, the lineage distribution was invariably and predominantly skewed to $CD33^+$ myeloid lineage, corroborating the myeloid-biased property of iPSC-derived blood cells under the regularly cytokine-directed differentiation condition.

Therefore, we constructed an MLL-AF4-GFP plasmid whose expression was doxycycline (Dox) dependent (Fig. S1*G*). MLL-AF4-GFP and reverse tetracycline response transactivator (rtTA) were cotransfected into iPSC-derived blood cells followed by a 3-d Dox induction (Fig. S1*H*). After sorting out GFP^+ and GFP^- cell populations, we extended the induction for another week with cell number counting at each day. In striking contrast with GFP^- population, GFP^+ cells significantly increased until reaching the plateau after 4 d of expansion (Fig. S1*I*). We next examined their self-renewal ability by the serial-replating assay. Within the 7 d of Dox induction after cell sorting, GFP^+ and GFP^- cells were collected at day 1, day 4, and day 7 for the first round plating and continued with the secondary replating after 2 wk of colony forming in the methylcellulose medium. The total number of

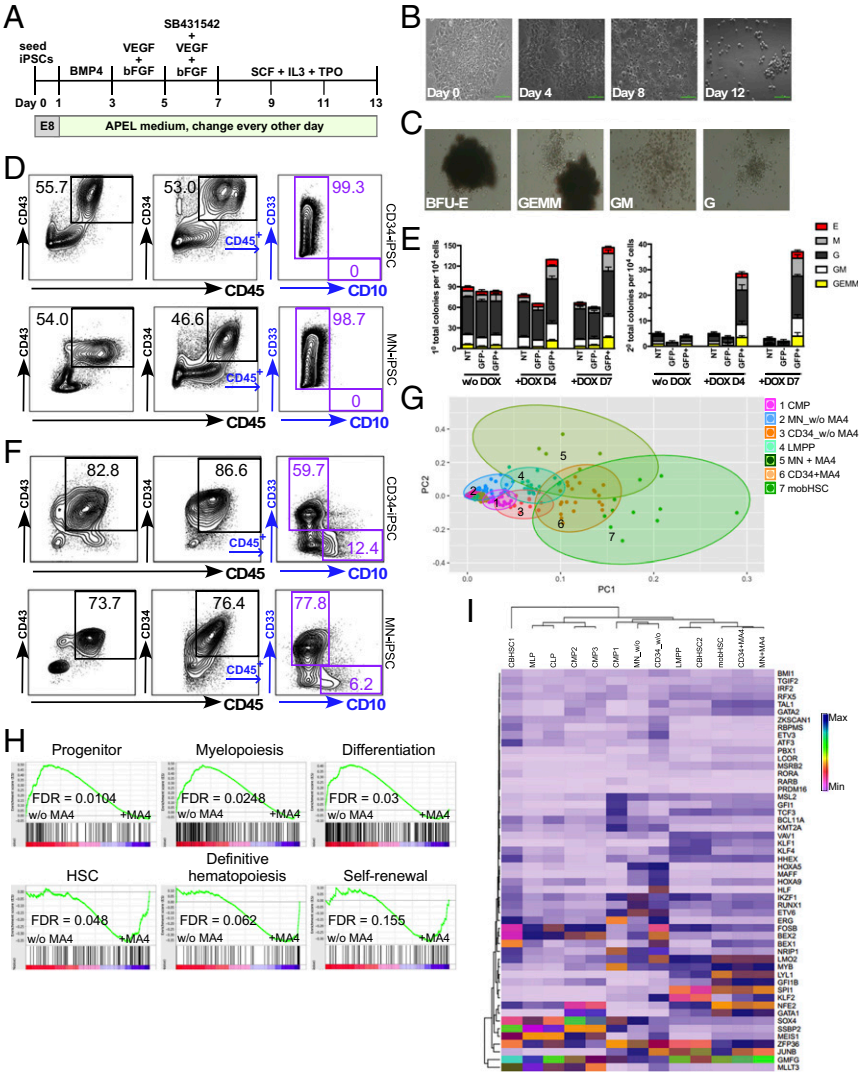

Fig. 1. In vitro induction of *MLL-AF4* in iPSC-derived hematopoietic cells. (*A*) The scheme of in vitro differentiation of iPSCs into hematopoietic cells. (*B*) Morphological change of iPSCs during the in vitro differentiation. (Scale bar, 1 μm.) (*C*) Colony-forming assay of iPSC-derived hematopoietic cells harvested at day 12 of in vitro differentiation. BFU-E, burst-forming unit-erythroid; GEMM, colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte; GM, colony-forming unit-granulocyte, macrophage; G, colony-forming unit-granulocyte. All are shown at 40× magnification. (*D*) Phenotypic analysis of iPSC-derived hematopoietic cells harvested at day 12 of in vitro differentiation. (*E*) The serial replating potential of iPSC-derived hematopoietic cells with or without (w/o) Dox induction. FACS-sorted GFP^+ and GFP^- cells that were immediately seeded on methocult were labeled as "w/o DOX" group. Data are shown as mean ± SD of three independent experiments. (*F*) Flow cytometry analysis of *MLL-AF4*–induced iPSC-derived hematopoietic cell harvested at day 12 of in vitro differentiation. *MLL-AF4* and *rtTA* plasmids were transfected followed by 72-h induction of *MLL-AF4* with the addition of 2 $\mu g \cdot ml^{-1}$ doxycycline. (*G*) Principle component analysis of RNA-Seq data from in vitro-derived HSPCs from iPSC (iPSC-HSPCs, CD34 iPSC, or MN iPSC derived) without *MLL-AF4* transfection (CD34_w/o MA4; MN_w/o MA4) or with *MLL-AF4* transfection (CD34 + MA4; MN + MA4), compared with the peripheral blood mobilized $CD34^+$ HSC (mobHSC), and the publicly available dataset for common myeloid progenitor (CMP) and lymphoid-primed multipotent progenitor (LMPP). (*H*) GSEA signatures of in vitro-derived iPSC-HSPCs without *MLL-AF4* transfection (w/o MA4) or with *MLL-AF4* transfection (+MA4). $P < 0.05$ and false discovery rate (FDR) <0.25 were considered significant conditions. (*I*) Heat map showing relative gene expression of regulatory genes identified as HSC specific in the indicated cell types.

colonies formed by GFP$^+$ cells obviously exceeded GFP$^-$ cells (Fig. 1*E*). Specifically, the later collected GFP$^+$ cells were able to form more colonies at the first round of plating, and these colonies could also give rise to more secondary colonies, demonstrating that the induction of *MLL-AF4* could confer self-renewal potential to the targeted cells. Flow cytometry analysis was performed on GFP$^+$ cells collected at day 4 of Dox induction. The increased CD34$^+$ and CD43$^+$ populations again revealed their enhanced stemness (Fig. 1*F* and Fig. S1*J*). More importantly, the CD10$^+$ lymphoid population was remarkably increased and reached more than 10% among CD45$^+$ cells for both CD34-iPSC and MN-iPSC. These revealed that in vitro induction of *MLL-AF4* could impart self-renewal and lymphoid potential to iPSC-derived blood cells.

To gain more insights into their properties, we analyzed the transcriptomic signatures of *MLL-AF4* transfected blood cells derived from iPSCs after 4-d induction of Dox. We also compared the RNA sequencing (RNA-Seq) data with mobilized HSPCs and the publicly available gene expression data for human cord blood (CB) HSCs and other progenitors (21, 22). Principal component analysis (PCA) placed *MLL-AF4* transfected and nontransfected blood cells derived from iPSCs separately from each other, where the former group was closer to the lymphoid-primed multipotent progenitor and HSPC, while the latter group, to the common myeloid progenitor (Fig. 1*G*). This revealed the phenotypic transition from myeloid-biased progenitor state to multipotent stem and progenitor state after the induction of *MLL-AF4*. In addition, *MLL-AF4* transfected blood cells derived from CD34-iPSC clustered closer to the bona fide HSCs than that of MN-iPSC, implying a more complete conversion of CD34-iPSC–derived HSPCs to the stem cell state. Gene set enrichment analysis (GSEA) indicated that MLL-AF4 could impart self-renewal and definitive hematopoiesis properties to the iPSC-derived blood cells (Fig. 1*H*). Correlation matrix further showed that these *MLL-AF4* transfected blood cells clustered closest to bona fide HSCs than to multilymphoid progenitors (Fig. S1*K*). Genes that represented the key signatures of HSCs (1–4, 23) or directly regulated by MLL-AF4 (24) were significantly up-regulated in transfected blood cells (Fig. 1*I* and Fig. S1*L*), confirming the direct and potent influence exerted by MLL-AF4. These observations thus warranted our attempt to examine the in vivo reconstitution ability of *MLL-AF4* transfected blood cells derived from both iPSCs.

***MLL-AF4* Alone Is Sufficient for Enabling the Potent and Multilineage Engraftment of iPSC-Derived Blood Cells.** We next examined the engraftability of iPSC-derived blood cells with or without *MLL-AF4* transfection. Given that *MLL-AF4* was an oncogene, to avoid any potential risk of tumorigenicity, we enforced the transient expression of *MLL-AF4* without integration by using either plasmid or mRNA transfection. We also introduced the TFs (*EARSM*, namely, *ERG*, *HOXA9*, *RORA*, *SOX4*, and *MYB*) that could facilitate the short-term engraftment of iPSC-derived myeloid progenitors (1) to assist *MLL-AF4* and examine whether MLL-AF4 alone was sufficient for imparting engraftability to iPSC-derived blood cells. Newborn NOD-Scid-Il2rg^{null} (NSG) mice were used for xenotransplantation, given that they were more supportive for hematopoietic reconstitution and lymphopoiesis than adult mice (25). To induce the expression of EARSM and/or *MLL-AF4* plasmid, both of which functioned in a Dox-dependent way, their transduced cells were induced by Dox for 48–72 h before transplant, and continued the induction by adding 2 mg/mL Dox to the drinking water of maternal mice for 2 wk so that the transplanted pups could acquire Dox through feeding (Fig. 2*A*).

Eight weeks after transplant, we measured the human cell chimerism in the bone marrow (BM) of recipient mice. Surprisingly, only the *MLL-AF4* plasmid group (6/10) and EARSM plus *MLL-AF4* plasmid group (6/10) showed a significant chimerism (>20%) of donor CD45$^+$ cells (Fig. 2*B*). Nontransfected iPSC-derived blood cells failed to achieve the same engraftment on their own (0.18% of chimerism), even under the assistance of EARSM (0.38% of chimerism) or *MLL-AF4* mRNA (0.22% of chimerism). Also, introducing EARSM did not much influence the effect of *MLL-AF4* plasmid (both around 20% of chimerism), but adding *MLL-AF4* plasmid to EARSM greatly influenced the outcome (from 0.38% to

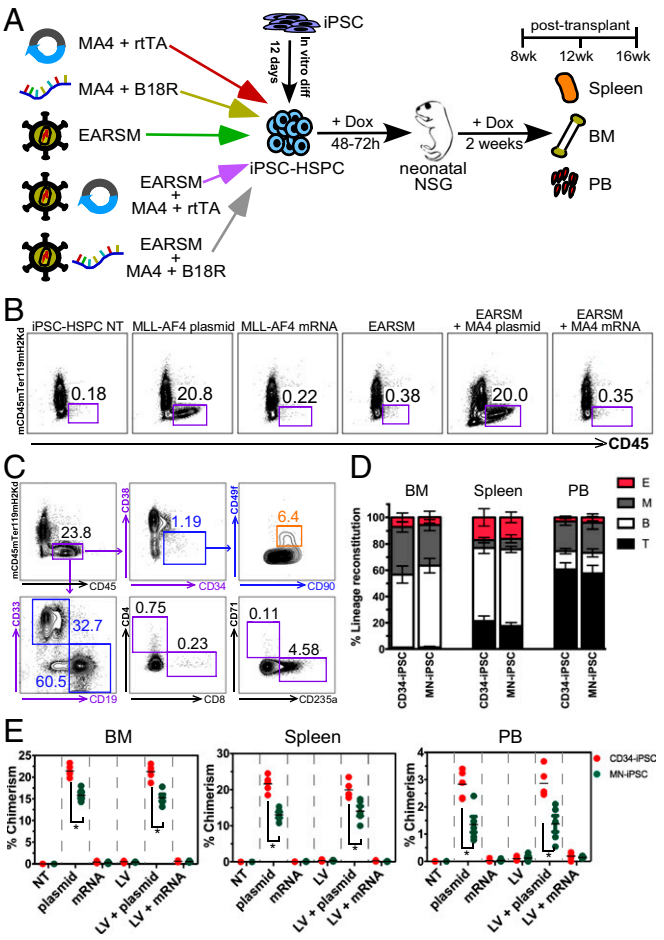

Fig. 2. In vivo induction of *MLL-AF4* in iPSC-derived hematopoietic cells enables potent engraftment and multilineage reconstitution. (*A*) The scheme of transplanting induced iPSC-HSPCs into newborn NSG mice. MA4, MLL-AF4; in vitro diff, in vitro differentiation; BM, bone marrow; PB, peripheral blood. (*B*) The analysis of human cell chimerism in the bone marrow of recipient mice after 8 wk of transplantation. Transplant groups are as described in *A*. NT, nontransfection control. (*C*) Multilineage reconstitution of *MLL-AF4* plasmid-treated iPSC-HSPCs in the BM of recipient mice at 8 wk posttransplant. (*D*) Multilineage reconstitution of human erythroid cells (E; CD235a$^+$ or CD71$^+$), myeloid cells (M; CD33$^+$), B cells (B; CD19$^+$), and T cells (T; CD3$^+$) in the BM, spleen and PB of engrafted mice transplanted with *MLL-AF4* plasmid transfected iPSC-HSPCs at 8 wk posttransplant. (*E*) Total chimerism of human CD45$^+$ cells in the harvested tissues of engrafted mice for each transplant group at 8 wk posttransplant. LV, lentivirus. Data were analyzed for two independent experiments with three mice each time and shown as mean ± SD *$P < 0.05$.

20% of chimerism). In addition to the potent engraftment, the *MLL-AF4* plasmid group also reestablished the multilineage hematopoiesis in the transplants (Fig. 2*C*). Myeloid bias was not detected as both B-cell (around 60%) and T-cell (around 1%) lineages were successfully reconstituted. Long-term HSC (CD34$^+$CD38$^-$CD90$^+$CD49f$^+$) compartment was also highly detectable within the CD34$^+$CD38$^-$ HSPC population. In addition to the BM, the multilineage hematopoiesis pattern could also be found in the spleen and PB of the recipient mice (Fig. 2*D*). Among recipients with engraftment (>1% of chimerism in the BM was considered engrafted), the CD34-iPSC group showed relatively higher chimerism than the MN-iPSC group (Fig. 2*E*). These observations suggest that only transient induction of *MLL-AF4* without the assistance of other factors was sufficient to enable the potent engraftment of iPSC-derived blood cells. However, this effect was most likely dose dependent in terms of the different performance between the *MLL-AF4* plasmid and mRNA. Since the decaying of in vitro synthesized mRNA in transfected cells was

usually within several hours (26), its short lifetime appears to limit its effectiveness. Thus, we focused on the *MLL-AF4* plasmid group for the following experiments.

iHSPCs but Not Primary HSPCs Are Prone to Leukemic Transformation During the Long-Term Posttransplant Period. We continued to examine the long-term engraftment in the recipients of iHSPCs over 4 mo and observed that $CD45^+$ donor cells dramatically increased in the BM of recipient mice, and the discrepancy of chimerism between CD34-iPSC and MN-iPSC disappeared as the time of posttransplant prolonged (Fig. 3*A* and Fig. S2*A*). By analyzing the long-term engrafted donor cells in the BM of the recipient mice at 16 wk posttransplant, we found a significant decrease on the myeloid lineage, and that the stem/progenitor and B-cell compartments markedly enlarged (Fig. 3*B*). From the shot-term to long-term engraftment, the increase of the B-cell compartment was consistently observable in the BM, spleen, and PB of the recipient mice (Fig. S2*B*), and splenomegaly was a common feature for most of the recipients during the long-term engraftment period (Fig. S2*C*). Moreover, when examining the cellular morphology of the BM cells, hardly any myeloid or erythroid elements could be observed; instead the marrows were overwhelmed with lymphoid cells with an abnormally high nuclear/cytoplasmic ratio (Fig. S2*D*). These abnormal lineage reconstitution patterns suggested a B-cell leukemia transformation might occur during the long-term engraftment period. To further investigate their properties, we also conducted secondary transplantation on newborn mice. Eight weeks after transplant, 3 of 10 recipients of the CD34-iPSC group and 2 of 10 recipients of the MN-iPSC group were dead. Five of 7 and 6 of 8 of the remaining recipients were successfully engrafted. In the BM, all of the engrafted recipients showed the same high chimerism of $CD45^+$ cells (>60%), most of which coexpressed CD10 and CD19 with little or no expression of CD33 or CD3 (Fig. S2*E*). Splenomegaly was a common feature among the recipients, some of which also developed tumors in the intestine (Fig. S2*F*). Wright-Giemsa staining of the harvested BM cells showed a severe shortage of erythrocytes and myeloid cells, but presented uniform-looking lymphocytes with abnormally high nuclear/cytoplasmic ratio, as we observed in the primary recipients (Fig. S2*G*). Together, these observations revealed that the iHSPCs were prone to leukemic transformation during the long-term posttransplant period.

Given that persistent expression of *MLL-AF4* in human CB HSCs was unable to trigger leukemia (15), it was intriguing that our transient induction of *MLL-AF4* in iHSPCs could possibly lead to the leukemia transformation. It should be pointed out that we did not detect the existence of *MLL-AF4* either in the in vivo-derived BM cells as early as 5 wk posttransplant or the in vitro transfected cells after a 2-wk induction (Fig. 2*H*), indicating that *MLL-AF4* was only transiently introduced without being integrated into the genome of the targeted cells.

To further investigate whether our induction and/or transplant conditions were undesirably favorable for leukemogenesis, we conducted the same transplant experiments on primary HSPCs (PB mobilized $CD34^+$ HSPCs). Nontransfected or empty vector transfected primary HSPCs were transplanted as the control group. As previously documented, the engraftment of mobilized HSPCs in immunodeficient mice was less efficient than that of CB or BM HSPCs (27, 28); only ∼5% of chimerism was detected in the BM of the control group (8/10) after 12–16 wk of transplantation, and much less in the PB (around 0.1%) and spleen (around 1%). In contrast, the *MLL-AF4* transfected group (4/5) showed remarkably enhanced chimerism in the BM (33.1%), PB (7.66%), and spleen (14.2%) (Fig. 3*C*). To further interrogate whether *MLL-AF4* transfected primary HSPCs underwent the leukemic transformation, we compared the hematopoietic reconstitution pattern between *MLL-AF4* transfected and control groups. Among the engrafted $CD45^+$ cells, the *MLL-AF4* group had a fourfold increase in $CD34^+$ stem cell compartment, in which the more primitive HSC population of $CD34^+CD38^-$ cells significantly expanded (Fig. 3*D*). However, induction of *MLL-AF4* did not influence the lineage distribution, and the T-cell and myeloid cell compartments were maintained at the same levels as in the control group. Moreover, diverse cell types with normal morphology were detected in the harvested BM cells of *MLL-AF4*–treated group (Fig. S3*A*) with an absence of splenomegaly (Fig. S3*B*). To further confirm their "none-transformed" identity, we also performed secondary transplantation under the same condition as what we did for iHSPC recipients. Despite the HSPC expansion, lineage distribution was normal (Fig. S3*C*) and the harvested BM cells also showed diverse and normal morphology (Fig. S3*D*). Again, no splenomegaly was observed (Fig. S3*E*). Therefore, these results demonstrated that the current scheme of transfecting *MLL-AF4* in vitro and inducing its expression in vivo did not inherently favor the leukemogenesis on the targeted cells.

To better understand the molecular features of the transformed leukemic cells, we harvested BM cells from primary recipients engrafted with *MLL-AF4*–induced iHSPCs at different stages and sorted out $CD45^+$ human cells for RNA sequencing. By measuring the mutations and comparing with in vitro differentiated blood cells transfected with *MLL-AF4* and control groups (normal HSCs and mononuclear cells), we found leukemic mutations that were closely related to B-cell acute lymphoblastic leukemia (B-ALL) were distinctly enriched in in vivo-derived cells but absent from in vitro-derived cells or control groups (Fig. 3*E*). In the period of short-term (9 wk posttransplant) and midterm (12 wk posttransplant) engraftment, canonical B-cell–associated genes such as *PAX5* and *IKZF1* were randomly mutated over the coding region, splicing region, and 3′ UTR with variant frequencies, however, only resulting in single nucleotide variations (SNVs) or some minor indels, which were not considered leukemogenic on their own (29). However, by 16 wk after the transplant, various and randomly occurred fusion transcripts became highly detectable in different recipients (Fig. S4), suggesting a more unstable status of the genome at this time in contrast to the early engraftment period. As consistent with the PCR results (Fig. S2*H*), RNA sequencing did not detect any *MLL-AF4* transcripts in the engrafted BM cells, indicating that the leukemic cells were not likely transformed by *MLL-AF4*.

To understand the entirely different behaviors of *MLL-AF4* transfected primary HSPCs, we also examined their gene expression profiles. PCA analysis showed that wild-type and transfected HSPCs strongly correlated with CB HSCs [from publicly available data (22) as previously described], all of which had very divergent components from those of the iPSC group and B-ALL control (Fig. 3*F*). Also, gene sets involved in stem cell self-renewal, proliferation, and cell-cycle phase transition were highly enriched in *MLL-AF4* transfected HSPCs (Fig. S5*A*). However, despite their facilitated cell-cycle transition, features of G0/G1 and G2/M checkpoints were also enriched while DNA damage was not (Fig. S5*B*), indicating a more stable genetic status of primary HSPCs in contrast to that of iHSPCs. Quantitative PCR also verified the up-regulated expression of self-renewal related marker genes (Fig. S5*C*).

By performing Gene Ontology (GO) analyses on gene signatures representing the top 1,000 most differentially expressed genes in in vivo-derived leukemic cells relative to the in vitro differentiated blood cells from iPSCs, we found that the most enriched molecular features were highly related to cancer, and particularly B-ALL (Fig. 3*G*). Especially, the feature of DNA damage was highly scored, indicating the genomic instability of the engrafted cells, which accounted for the accumulation of genetic lesions ranging from site mutations to fusion transcripts. We also analyzed the most enriched GO features of engrafted primary HSPCs. By comparing with the iHSPC group, we found that those features containing genes that were mostly down-regulated were highly correlated with B-ALL and cancer, such as the B-cell receptor (BCR) signaling pathway, DNA damage, and proliferation (Fig. 3*H*). On the other hand, features containing genes that were mostly up-regulated were strongly related to multilineage hematopoiesis. These molecular discrepancies implied that induction of *MLL-AF4* could incur different biological events in primary HSPCs and iHSPCs, which underlined their inherently distinct cell identity.

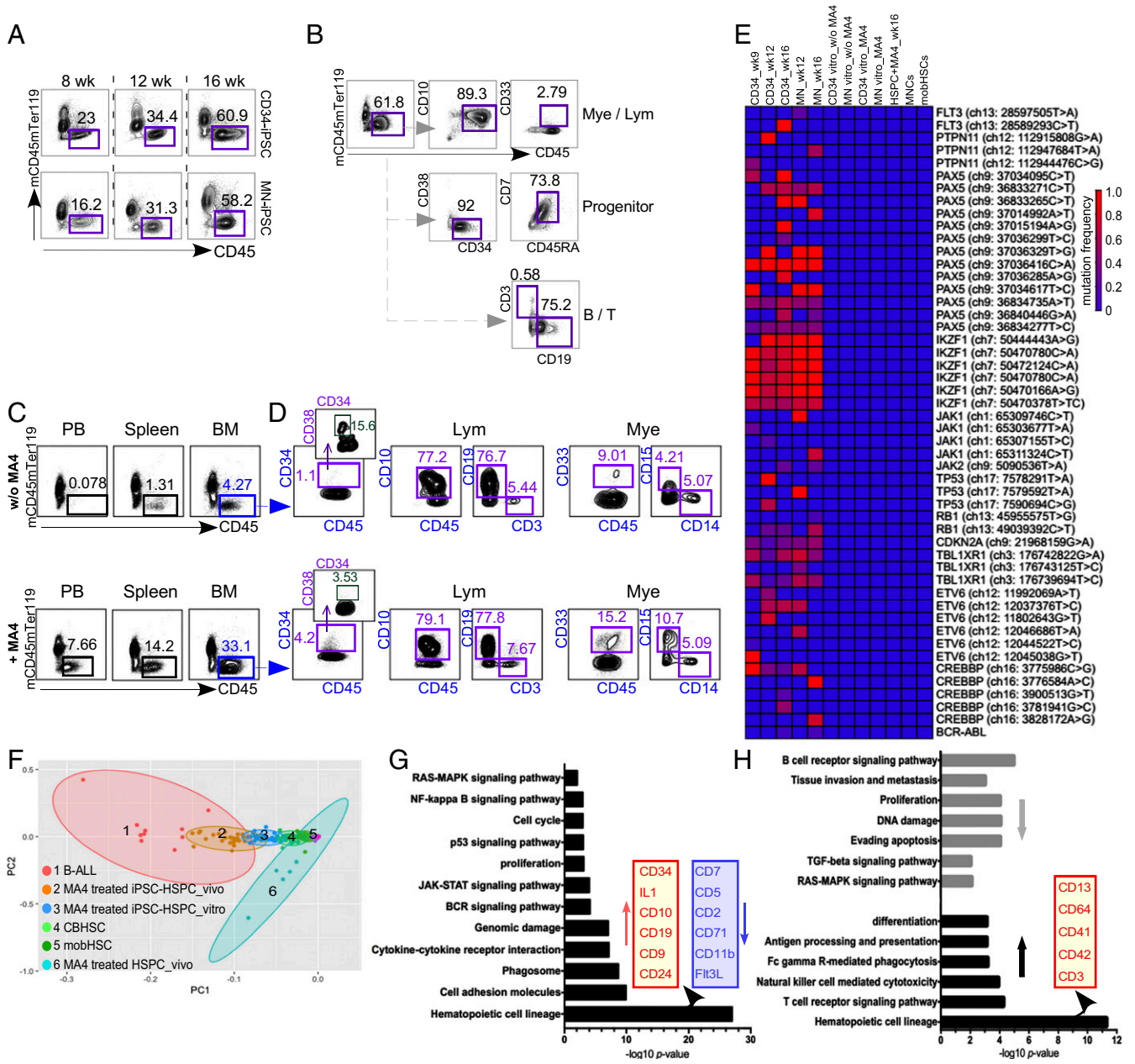

Fig. 3. iHSPCs but not primary HSPCs undergo the leukemic transformation during the long-term engraftment period. (*A*) Chimerism of human cells in the bone marrow of recipient mice over 16 wk posttransplant. (*B*) Phenotypic analysis of engrafted human cells in the bone marrow (BM) of recipient mice with long-term engraftment. Gated human CD45$^+$ cells were analyzed for myeloid (CD33) and lymphoid (CD10) (Mye/Lym) lineage distribution, percentage of progenitor (CD34$^+$CD38$^-$, CD7$^+$CD45RA$^+$), and B cell (CD19) and T cell (CD3) compartments (B/T). (*C*) Engraftment of primary HSPCs with (+MA4) or without (w/o MA4) transfection of *MLL-AF4* in the peripheral blood (PB), spleen, and BM of recipient mice after 12–16 wk of transplantation. (*D*) Phenotypic comparison of engrafted human CD45$^+$ cells in the BM of *MLL-AF4* transfected transplants or nontransfected transplants. (*E*) Profiling of B cell leukemia-associated mutations in the in vitro-derived iPSC-HSPCs without *MLL-AF4* transfection (CD34 vitro_w/o MA4; MN vitro_w/o MA4) or with *MLL-AF4* transfection (CD34 vitro_MA4; MN vitro_MA4), and in vivo-derived human CD45$^+$ cells engrafted in the BM of primary recipients over 9–16 wk posttransplant with *MLL-AF4* transfection. CD34_wk9, CD34-iPSC derived HSPCs transfected with *MLL-AF4* were transplanted and harvested for human CD45$^+$ cells at 9 wk posttransplant; the same meaning for MN-iPSC group. HSPC + MA4_wk16, BM cells harvested from engrafted recipients transplanted with primary HSPCs with *MLL-AF4* induction at 16 wk posttransplant; MNCs, peripheral blood-derived mononuclear cells; mobHSC, mobilized HSCs. (*F*) PCA analysis of RNA-Seq data from in vivo-derived human CD45$^+$CD34$^+$ cells in the bone marrow of primary recipient mice transplanted with *MLL-AF4* transfected primary HSPCs at 16 wk posttransplant (MA4 treated HSPC_vivo), compared with the *MLL-AF4* transfected iPSC-HSPCs (CD34-iPSC derived) before transplantation (MA4-treated iPSC-HSPC_vitro) or 16 wk posttransplant (MA4-treated iPSC-HSPC_vivo), and mobilized HSC (mobHSC). (*G*) GO analysis of in vivo-derived human CD45$^+$ cells from engrafted iHSPCs (16 wk posttransplant) compared with the in vitro-derived iPSC-HSPCs. Significantly deregulated genes enriched in hematopoietic cell lineage signature were indicated. (*H*) GO analysis of enriched features of *MLL-AF4* transfected primary HSPCs compared with *MLL-AF4* transfected iPSC-HSPCs. In vivo-derived cells of both groups were harvested at 16 wk posttransplant. Gray bars indicate the enriched features containing genes that are mostly down-regulated, and the opposite for the black bars. All RNA-Seq data were generated from three independent biological replicates and analyzed by the average FPKM (fragments per kilobase of transcript per million mapped reads) value.

Discussion

Although the de novo generation of human HSCs has now been attainable (2), devising a more efficient and simpler strategy is still of great significance for further application. Here, by using a single factor of *MLL-AF4*, we were able to derive highly engraftable iHSPCs that can reconstitute both lymphoid and myeloid lineages in recipient mice. This system is highly operable and efficient. First, the targeted cells for *MLL-AF4* respecification are iPSC-derived blood cells, which can be efficiently derived in vitro. Second, the ectopic expression of *MLL-AF4* is mediated by the plasmid in a transient and nonintegrative way. Third, the engraftment levels of iHSPCs are as high as primary HSPCs. Last but not the least, multilineage hematopoiesis, including lymphopoiesis, can be detected after long-term engraftment. We thus demonstrate that the de novo derivation of long-term engraftable HSPCs from human iPSCs can be achieved by using a single factor.

By comparing in parallel the *MLL-AF4*–induced iHSPCs with primary HSPCs, we also find that iHSPCs are more prone to leukemic mutations after transplantation. iPSC bears high oncogenic risks resulting from the genetic and epigenetic aberrations due to reprogramming and culture adaptation, which may dramatically increase the chances of DNA damage in its derivatives when undergoing lineage differentiation (30, 31). For instance, the tumorigenicity of iPSC derivatives has been evidenced in iPSC-derived neural stem and progenitor cells, which form tumors after a long period of transplantation (32).

In our study, the genetic aberrations are only found in engrafted cells instead of in vitro-derived iPSC-HSPCs. This precludes the possibility that leukemic mutations are directly passed from the original iPSCs; instead, the posttransplant period plays a vital role in the induction of leukemogenesis. In our model, it is also possible that the newborn NSG mice provide a permissive microenvironment, enabling the transformation of iHSPCs.

Another consideration is the dosage effect. Ectopically expressed TFs usually exert distinct functions on targeted cells upon different expression levels (33). Hence, it will be interesting to subtly titrate the in vitro transfection dosage and in vivo induction time of MLL-AF4 so that the transfected iHSPCs might behave normally when currently "overdosed" MLL-AF4 is lowered to an underthreshold level. However, the same overdosed MLL-AF4 was also introduced into primary HSPCs, resulting in only enhanced engraftment without the development of leukemia. This striking comparison thus evidences the inherently different potential for tumorigenesis between iHSPCs and bona fide HSPCs.

Although further investigations are needed to secure a comprehensive scrutiny of the cellular identity of iHSPCs, our study has shed light on the feasibility of achieving engraftable human HSC-like cells by respecifying iPSC-derived blood cells with a single factor, and has also highlighted the need for consideration of the discrepancy between iPSC-derived HSPCs and primary HSPCs, which is critical to the translation of this technology into actual therapies.

Materials and Methods

Frozen human PB mobilized CD34$^+$ cells and PB mononuclear cells were purchased from AllCells (www.allcells.com). The protocols involving human iPSCs were approved by Human Research Committee and Human Gamete, Embryo and Stem Cells Research Committee (GESCR) in University of California, San Francisco (UCSF). The studies involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of UCSF, and all of the experiments were performed in accordance with approved protocols. All of the related methods including but not limited to the iPSC generation, hematopoietic differentiation of iPSCs, in vitro transduction of hematopoietic cells, mouse transplantation, flow cytometry, RNA-Seq, and data analysis are described in detail in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank J.-W. Yu and Z.-X. Qi (Cytogenetics Laboratory, UCSF) for performing karyotyping of iPSC; J. Levy and F. Teque for helping with the lentivirus titration; F. Chehab, G. Dieter, and A. Bárcena for their spirited discussions; the Beijing Genomics Institute (BGI) for RNA-Seq and data computing; and the UCSF Flow Cytometry Core Facility in the Diabetes Research Center (DRC) supported by NIH Grant P30 DK063720. This work was supported by NIH Grant P01DK088760 (to Y.W.K.), a Program for Breakthrough Biomedical Research award (to Y.W.K.), which is partially funded by the Sandler Foundation, the Chinese National Key Basic Research Project 2013CB966800 (to S.-J.C. and H.L.), the Shu Guang Project 14SG15 (to H.L.), and the Samuel Waxman Cancer Research Foundation (to S.-J.C. and H.L.). Y.-T.T. was awarded a scholarship under the State Scholarship Fund from the China Scholarship Council.

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