Hematopoietic and Leukemic Stem Cells Have Distinct Dependence on Tcf1 and Lef1 Transcription Factors*

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Hematopoietic and leukemic stem cells (HSCs and LSCs) have self-renewal ability to maintain normal hematopoiesis and leukemia propagation, respectively. Tcf1 and Lef1 transcription factors are expressed in HSCs, and targeting both factors modestly expanded the size of the HSC pool due to diminished HSC quiescence. Functional defects of Tcf1/Lef1-deficient HSCs in multi-lineage blood reconstitution was only evident under competitive conditions or when subjected to repeated regenerative stress. These are mechanistically due to direct positive regulation of Egr and Tcf3 by Tcf1 and Lef1, and significantly, forced expression of Egr1 in Tcf1/Lef1-deficient HSCs restored HSC quiescence. In a preclinical CML model, loss of Tcf1/Lef1 did not show strong impact on leukemia initiation and progression. However, when transplanted into secondary recipients, Tcf1/Lef1-deficient LSCs failed to propagate CML. By induced deletion of Tcf1 and Lef1 in pre-established CML, we further demonstrated an intrinsic requirement for these factors in LSC self-renewal. When combined with imatinib therapy, genetic targeting of Tcf1 and Lef1 potently diminished LSCs and conferred better protection to the CML recipients. LSCs are therefore more sensitive to loss of Tcf1 and Lef1 than HSCs in their self-renewal capacity. The differential requirements in HSCs and LSCs thus identify Tcf1 and Lef1 transcription factors as novel therapeutic targets in treating hematological malignancies, and inhibition of Tcf1/Lef1-regulated transcriptional programs may thus provide a therapeutic window to eliminate LSCs with minimal side effect on normal HSC functions.

Hematopoietic stem cells (HSCs) represent a rare population that self-renew and continuously replenish all blood cells throughout one's lifetime (1, 2). HSCs are regulated by both intrinsic factors and extrinsic signals from the microenvironment to maintain a homeostatic HSC pool at steady state as well as in response to injuries such as radiotherapy or traumatic blood loss (3–5). Leukemic stem cells (LSCs) share similar properties with HSCs, and the self-renewing LSCs are responsible for initiation, maintenance, and propagation of the disease (6, 7). Chronic myelogenous leukemia (CML) is initiated by reciprocal translocation of chromosomes 9 and 22, which generates a constitutively active fusion kinase, BCR-ABL (8). BCR-ABL tyrosine kinase inhibitors (TKIs) including imatinib are effective in inducing remissions and improving survival in CML patients at the chronic phase. Because CML LSCs are not sensitive to imatinib, continuous TKI treatment is necessary to prevent relapse. Molecules and/or pathways that are specifically utilized by the LSCs are of great therapeutic value for eradication of leukemias.

A plethora of data has implicated Wnt signaling pathway in regulation of HSC activities (9, 10). Overexpression of Dkk1 or Wif1, which blocks the interaction between Wnt ligands and their receptors, diminishes HSC quiescence and its repopulation capacity (11, 12). Wnt3a deficiency also greatly impaired HSC activity (13). Because Wnt signaling is involved in proper bone formation (14, 15), blocking Wnt/receptor interaction or germline deletion of Wnt proteins may affect the HSC niche, hence indirectly impacting HSCs. Activation of the canonical Wnt signaling pathway leads to stabilization and nuclear translocation of β-catenin. The role of β-catenin in HSCs has been a highly contentious issue. Depending on the experimental systems utilized, β-catenin activation is reported to have detrimental (16, 17), beneficial (18), or no effect on adult HSCs (19, 20). A recent study reports that the magnitude of β-catenin activation matters, with a narrow window of active β-catenin positively regulating HSC repopulation capacity (21). With regard to necessity of β-catenin, it is essential for definitive hematopoiesis (by embryonic day 10.5) (22), but its requirement in adult HSCs is only evident after serial transplantation (23). Despite the reported discrepancies on its roles in normal HSCs, β-catenin has been consistently demonstrated to be critical for development and maintenance of LSCs in CML (23–25).

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5 The abbreviations used are: HSC, hematopoietic stem cell; BrdU, bromo-2′-deoxyuridine; LSC, leukemic stem cell; CML, chronic myelogenous leukemia; TKI, tyrosine kinase inhibitor; BMT, bone marrow transplantation; PBC, peripheral blood mononuclear cell.
Although β-catenin itself does not have the ability to bind DNA after translocation into the nucleus, it interacts with Tcf/Lef transcription factors to modulate gene expression (9). Tcf/Lef factors contain 4 members, Tcf1, Tcf3, Tcf4, and Lef1, and all have a highly conserved high mobility group (HMG) DNA binding domain. Whereas the requirements for Tcf1 and Lef1 in blood cells, in particular T lymphocytes, have been well documented (9, 26), none of the Tcf/Lef factors has been studied in hematopoietic progenitor cells (HPCs). Although no Tcf/Lef motif is within the Foxo1, 5’-ccacacagttgaagactac and 5’-ctctagttctgctgtcagac; Foxo3, 5’-acacaggctgattagctg and 5’-ctgctacagttgctgacagac; Gfi1, 5’-acacagccagttgctgacagac and 5’-gcacagctgctgacagac; Pten, 5’-gattacagccgtgacctg and 5’-ggtgctaggtgctgacagac; Stat5a, 5’-cttcagttcagttcagctg and 5’-gcacagctgctgacagac; Tall1, 5’-gacctcccatatgagactg and 5’-gatagctgctgctgacagac; Tcf3, 5’-ggttcctcagttgctgacagac to 5’-gtgctcgtgctgctgacagac.

Isolation of Lin−c-Kit− BM Cells and Chromatin Immuno-purification (ChIP)—Total BM cells were isolated from WT C57BL/6 mice and depleted of lineage-positive cells. The Lin−BM cells were then incubated with mouse CD117 Microbeads (Miltenyi Biotec) for positive selection of c-Kit+ cells. The Lin−c-Kit− BM cells were cross-linked for 10 min with 1% formaldehyde in medium, then processed using a truChIP Chromatin Shearing Reagent Kit (Covaris) and sonicated for 5 min on Covaris S2 ultrasonicator. The sheared chromatin fragments, in the range of 200–500 bp, were immunoprecipitated with an anti-Lef1 (C12A5, Cell Signaling Technologies) or control rabbit IgG and was washed as described (29). The immunoprecipitated DNA segments were used for quantification by PCR. For calculation of enrichment of Lef1 binding, each anti-Lef1 ChIP sample was first normalized to corresponding IgG ChIP sample, and the signal at a target region was then normalized to that at the Hprt promoter region.

Bone Marrow Transplantation (BMT)—For competitive repopulation assays, the test BM cells were collected from CD45.2+ gene-targeted mice, and the competitor BM cells were from CD45.1+ B6.SJL mice. Both test and competitor BM cells were measured for LSK frequency, and the whole BM cells were mixed at 1:1 LSK ratio (each containing 3,000 LSKs) and then transplanted into lethally irradiated (1,050 rad) CD45.1+CD45.2+ recipients. Sixteen weeks after the transplantation, contribution of test and competitor BM cells to different blood lineages was determined in peripheral nucleated blood cells (PBCs) by flow cytometry.

For serial transplantation assays, total BM cells (2 × 10⁶) from CD45.2+ gene-targeted mice were transplanted into lethally irradiated CD45.1+ primary recipients. Eight weeks later, BM cells from the primary recipients were transplanted into CD45.1+ secondary recipients, and this process was repeated to obtain tertiary recipients. Sixteen weeks after each transplantation, PBCs were analyzed for the contribution from the original donors to different blood lineages.

Tamoxifen Treatment—For induced deletion of Tcfl/H11002 or Lef1/H11002 alleles using CreERT2 in the CML recipients, the mice were treated with tamoxifen at 0.2 mg/g body weight via oral gavage for 4 consecutive days.
CML Model and Treatment with Imatinib—The pMIG-p210BCR-ABL retroviral construct was provided by Dr. Warren Pear (34), and the retrovirus was packaged and used for infection of Lin−/H11002 BM cells as previously described (32). Twenty-four hours after two rounds of spinofection, the infected Lin−/H11002 BM cells containing 3,000–6,000 GFP/H11001 LSK cells along with 2×10^5 protector BM cells were transplanted into irradiated B6.SJL recipients. CML progression in the recipients was monitored through regular evaluation of lethargy, weight loss, splenomegaly, and signs of morbidity or detection of GFP/H11001 Mac1+ leukemic cells in the PBCs. For treatment of CML with TKI, imatinib (Novartis) was obtained from the Pharmacy of University of Iowa Healthcare and Clinics and administered to CML recipients at 100 mg/kg body weight by oral gavage twice a day.

For secondary transplantation of LSCs, more than 5 primary recipients were sacrificed on day 15 post-BMT to harvest BM cells and sorted for GFP/H11001 LSK cells. 10,000–15,000 sorted LSCs were then transplanted into another cohort of CD45.1+ syngeneic mice along with 2×10^5 protector BM cells for CML propagation.

Genetic Complementation with Forced Expression of Egr1—The control and Egr1-pLZRS retroviral vectors were provided by Dr. David Wiest (33), and the retroviruses were packaged to infect Lin−/H11002 BM cells as above. The infected cells were transplanted into irradiated B6.SJL mice at 5×10^5 per recipient without additional protector BM cells. Six weeks later, CD45.2+ GFP/H11001 Lin− BM cells were sorted from the recipients for cell cycle analysis as described above. The sorting isolation of GFP+ cells was used to avoid quenching of GFP fluorescence during intracellular staining for Ki67.

Statistical Analysis—Unpaired comparison among different genetic groups was performed using the Student’s t test with a two-tailed distribution assuming equal sample variance. For survival of CML recipients, statistical significance between different genetic and/or treatment conditions was assessed using log-rank test using Prism6 software.

FIGURE 1. Ablating Tcf1 and Left1 causes increase in phenotypic HSCs. A, Tcf1 and Left1 are expressed in HSCs. Indicated cell subsets were sorted from BM cells of WT C57BL/6 mice and analyzed for relative Tcf7 and Left1 expression after normalizing to Hprt. Data are means ± S.D. (n = 3). B and C, analysis of LSKs. BM cells were isolated from mice of indicated genotypes and surface-stained. Representative contour plots are in B, showing the frequency of LSKs. Cumulative data of LSK numbers from 2 tibias, 2 femurs, and 2 humeri are means ± S.D. as summarized in C. D and E, analysis of LT-HSCs. LSKs were fractionated based on CD34 and Flt3 expression to identify CD34+ Flt3+ LT-HSCs, CD34+ Flt3− ST-HSCs, and CD34− Flt3− MPPs. The frequency of each subset is shown in the representative plots (D). The numbers of LT-HSCs per mouse are summarized as means ± S.D. in E. Each dot represents one experimental animal. *, p < 0.05 compared with the control (Ctrl) mice.
Tcf1 and Lef1 Regulate HSC and LSC Self-renewal

Results

Ablation of Tcf1 and Lef1 Causes Moderate Expansion of the HSC Pool—Tcf1 and Lef1 are abundantly expressed in T-lineage cells, and are readily detectable in Flt3− lineage-negative Sca1+c-Kit− cells (Flt3− LSKs), which contain both long-term (LT) and short-term (ST) HSCs (Fig. 1A). Tcf1 expression (encoded by Tcf7) was sharply reduced upon differentiation of HSCs to Flt3+ LSKs, the multipotent progenitors (MPPs). Both factors were further down-regulated in more differentiated Lin− c-Kit+ myeloid progenitors (Fig. 1A). These expression changes suggest a potential requirement for Tcf1 and Lef1 in HSCs.

We used VavCre to excise Lef1-floxed alleles (Lef1FL/FL), which resulted in complete ablation of the Lef1 protein in the BM cells as shown previously (28). We then crossed VavCre-Lef1FL/FL (called Lef1−−) to germline-targeted Tcf7−/− mice to delete both genes. Whereas deleting either factor alone had little effect, Tcf7−/−Lef1−/− mice showed a tendency of increased LSK frequency and numbers in the BM cells (Fig. 1, B and C). We next used the CD34 and Flt3 markers to identify CD34−Flt3− LT-HSCs within the LSK population. No evident changes were detected in Lef1−/− or Tcf7−/− LSKs; however, LT-HSCs exhibited increased frequency in Tcf7−/−Lef1−/− LSKs (Fig. 1D). The absolute numbers of LT-HSCs were elevated in Tcf7−/−Lef1−/− mice accordingly (Fig. 1E). These data demonstrate that loss of Tcf1 and Lef1 resulted in moderate expansion of the pool size of phenotypic HSCs.

Loss of Tcf1 and Lef1 Diminishes HSC Function under Repeated Regenerative Stress and Competitive Condition—HSCs have self-renewal capacity to regenerate themselves under homeostatic conditions or in response to injuries. We used serial transplantation assays, the gold standard for measuring HSC self-renewal. Utilizing disparate CD45 allele markers, we transplanted the same numbers of CD45.2+ BM cells from Lef1−/−, Tcf7−/−, Tcf7−/−Lef1−/− mice or their littermate controls into CD45.1+ recipients. After 16 weeks, their contribution to CD3− non-T cells was measured in PBCs. Representative contour plots (B) and cumulative data (C) from three independent experiments are shown, with 4–12 recipients analyzed. **, p ≤ 0.01; ***, p ≤ 0.001 compared with control (Ctrl) mice.

We then isolated BM cells from the primary recipients followed by transplantation into secondary CD45.1+ recipients, and repeated this process to generate tertiary recipients. Whereas BM cells from all genotypes remained capable of
reconstituting all non-T blood lineages in the secondary recipients (Fig. 2A), in the tertiary recipients, \textit{Lef1}−/− BM cells exhibited moderately reduced contribution, and loss of both Tcf1 and Lef1 significantly impaired blood reconstitution (Fig. 2A). These results demonstrate that Tcf1 and Lef1 contribute to maintaining the regenerative fitness of HSCs.

As shown above, HSCs lacking Tcf1 and/or Lef1 were competent to reconstitute non-T blood lineages in both the primary and secondary recipients in the absence of competitors. We next investigated their competitive repopulation capacity. We obtained BM cells from the CD45.2+ gene-targeted strains (as test HSCs) and those from CD45.1+ WT mice (as competitor

**FIGURE 3. Tcf1 and Lef1 contribute to maintaining HSC quiescence.** A and B, cell cycle analysis. BM cells from mice of indicated genotypes were surface-stained to identify CD34+/Flt3+ LT-HSCs and then intracellularly stained for Ki67 and Hoechst 33342. The percentages of HoechstG0, HoechstG1, and HoechstS/G2/M phase cells in LT-HSCs are shown in representative dot plots (A). Cumulative data on LT-HSCs in G0 and G1 phases are summarized in B. Data are means ± S.D. (n = 5–11 from five experiments). C and D, BrdU incorporation in LT-HSCs. Mice were injected i.p. with 1 mg of BrdU, and 24 h later, BM cells were harvested, surface-stained, and intracellularly stained with anti-BrdU antibody. The percentage of BrdU+ cells in LT-HSCs is shown in representative contour plots (C). Cumulative data from three experiments are summarized in D. E, detection of apoptotic cells. LT-HSCs were identified by surface staining and further stained for AnnexinV and 7-AAD. The percentages of AnnexinV7-AAD+ apoptotic cells in LT-HSCs are summarized (n = 4–8 from 4 experiments). In B, D, and E, the average fold changes of Tcf7−/−Lef1−/− over the control mice are marked in the bars. ns, not statistically significant; **, p < 0.01; ***, p < 0.001 compared with control (Ctrl) mice.
HSCs) and determined the LSK frequency. Because the frequency of LSK cells was elevated in Tcf7-/- Lef1-/- BM cells (Fig. 1 B), for a fair comparison among the test HSCs, we transplanted the same numbers of LSKs into lethally irradiated CD45.1/CD45.2 recipients, instead of using the same numbers of total BM cells. After 16 weeks, we assessed the relative contribution of test HSCs to non-T cell blood lineages in PBCs. The control test BM cells moderately outcompeted the competitor BM cells, constituting 65% of the non-T blood cells (Fig. 2, B and C). Whereas Tcf7-/- BM cells were similarly competent in reconstitution non-T blood cells, the contribution by Lef1-/- BM cells was moderately decreased. On the other hand, the contribution by Tcf7-/- Lef1-/- BM cells was reduced to ~50% of that by control BM cells (Fig. 2, B and C). These observations indicate that Tcf1 and Lef1 have redundant roles in regulating HSC activities, similar to their functions in T lineage cells (26). These analyses collectively identified a requirement for Tcf1 and Lef1 in maintaining functional HSCs, especially under competitive conditions or repetitive regenerative stress.

**Tcf1 and Lef1 Contribute to Maintaining HSC Quiescence**—To elucidate the mechanisms by which Tcf1 and Lef1 regulate the HSC biological activities, we first measured its cell cycle status. By combination of Ki67 and Hoechst33342, which measures cellular proliferation and DNA content, respectively, we found that majority of WT CD34+/Flt3+ LT-HSCs are in HoechstloKi67lo G0 phase, with a small portion in HoechstloKi67lo G1 phase and very few in Hoechstmed-hi Ki67+ S/G2/M phase (Fig. 3 A). Although loss of Tcf1 or Lef1 alone did not have a discernible impact, Tcf7-/- Lef1-/- LT-HSCs showed modest but consistent reduction of the proportion in G0 phase, with corresponding increase of that in G1 phase (Fig. 3 A and B).
Consistent with loss of quiescence, Tcf7−/−Lef1−/− LT-HSCs also exhibited increased incorporation of BrdU into the DNA (Fig. 3, C and D), indicative of more cycling cells. In contrast, AnnexinV+/7-AAD− apoptotic cells in Tcf7−/−Lef1−/− LT-HSCs were not significantly different from those in control LT-HSCs (Fig. 3E). Collectively, Tcf1 and Lef1 contribute to maintaining HSCs at a quiescent state.

Tcf1 and Lef1 Act Upstream of Egr1 and Other HSC Regulators—We next aimed to gain molecular insights into the regulation of HSC quiescence and function by Tcf1 and Lef1. A number of factors have been identified to be responsible for maintaining HSC quiescence, and genetic ablation of these factors leads to moderate expansion of HSCs accompanied by functional defects due to exhaustion (5). These factors include transcription factors such as Egr1 (37), Gfi1 (38), Foxo factors (39, 40), Stat5 (41), E2A/E47 (encoded by Tcf3) (42), and Scl (encoded by Tal1) (43); an ubiquitin ligase Fbw7 (44); a signaling molecule Pten (45); and cyclin-dependent kinase inhibitors such as p27-Kip1 and p57-Kip2 (encoded by Cdkn1b and Cdkn1c, respectively) (46, 47). We sort-purified Flt3+/LSK cells from Tcf7−/−Lef1−/− and control mice and scanned for the expression changes of these known HSC regulators. The expression of Egr1, Tcf3, and Cdkn1c was substantially diminished in Tcf1/Lef1-deficient hematopoietic stem/progenitor cells, whereas other genes were not affected or moderately reduced in expression (Fig. 4A).

We then investigated if Tcf1 and Lef1 directly regulate Tcf1/Lef1-dependent genes. To this end, we searched the proximal regulatory regions, i.e. −1.5 kb to +0.5 kb sequence flanking the transcription start site (TSS) of genes of interest for consensus Tcf/Lef binding motif “CAAAG.” We found three motifs at −1,378, −1,124, and −963 bp upstream of the Egr1 TSS, one at −180 bp upstream of the Tcf3 TSS, and one at −310 bp upstream of the Cdkn1c TSS. We then performed chromatin
immunoprecipitation (ChIP) on primary murine Lin− c-Kit+BMMes progenitor cells using an anti-Lef1 antibody. The progenitor cells were used instead of primary HSCs because HSCs constitute only a rare population in mouse bone marrow but ChIP assay for transcription factor binding usually requires relatively large numbers of purified cells to obtain reliable results. The use of Lef1 antibody is because Lef1 protein was more readily detectable than Tcf1 in Lin− c-Kit+BMMes cells (not shown). Lef1 itself is a direct target gene of the Tcf/Lef factors (28) and used as a positive control. We found that Lef1 exhibited enriched binding at the 5′-regulatory regions of Egr1 and Tcf3 but not that of Cdkn1c (Fig. 4B). These data suggest a direct regulatory role of Lef1, and likely Tcf1 as well, in the expression of key HSC molecules in the hematopoietic progenitor cells.

The phenotypic and functional HSC impairments are strikingly similar between Egr1−/− and Tcf7−/−Lef1−/− mice, including expansion of the HSC pool and loss of HSC quiescence (37). We next investigated if genetic complementation of Tcf7−/−Lef1−/− cells with Egr1 restores HSC quiescence (33). We used retroviral transduction to achieve forced expression of Egr1 in control or Tcf7−/−Lef1−/− Lin−BMMes cells and then transplanted into CD45.1+ hosts to make BM chimeras. The bicistronic pLZRS retroviral vector uses GFP as an expression indicator, and we focused our cell-cycle analysis on sorted CD45.2+GFP−Sca1+LSK cells in the BM chimeras. In the empty pLZRS vector-transduced group, Tcf7−/−Lef1−/− HSCs showed increase in quiescent G0 phase and substantial decrease in S/G2/M phases (Fig. 4, C and D). In steady state, Tcf7−/−Lef1−/− HSCs continued to exhibit reduced portion in G0 phase with a moderate increase in S/G2/M phases (Fig. 4, A and B). Whereas forced expression of Egr1 did not detectably alter the cell cycle status of control HSCs, Egr1-complemented Tcf7−/−Lef1−/− HSCs showed increase in quiescent G0 phase and substantial decrease in S/G2/M phases (Fig. 4, A and B). In steady state, Tcf7−/−Lef1−/− HSCs showed an increase in G1 phase compared with control HSCs (Fig. 3, A and B); however, the difference in G1 phase was not evident between Tcf7−/−Lef1−/− and control HSCs in the BM chimeras, whether transduced with the pLZRS vector or Egr1-expressing retroviruses (Fig. 4, A and B).
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A likely explanation is that when the BM chimeras were analyzed at 6 weeks after HSC transplantation, similar amounts of Tcf7−/−LeF1−/− and control HSCs remained in a cycling-ready state during the process of active reconstitution of hematopoiesis. Collectively, these data support the notion that Tcf1 and LeF1 act, at least in part, through transcriptional activation of Eg1 to maintain HSC quiescence.

Targeting Tcf1 and LeF1 Impairs Self-renewal of LSCs—We next investigated a requirement for Tcf1 and LeF1 in LSCs. Because a moderate impact on HSC functionalities was only evident in the absence of both factors, we focused on Tcf7−/−LeF1−/− cells. We used a preclinical CML model, where we infected Tcf7−/−LeF1−/− or control BM cells with a bicistronic retrovirus expressing the p210BCR-ABL fusion protein along with GFP, followed by transplantation into irradiated CD45.1+ congenic recipients (Fig. 5A) (34). Forced expression of p210BCR-ABL resulted in malignant transformation of hematopoietic stem/progenitor cells and accumulation of GFP+Mac1+ myelogenous leukemic cells. The leukemic cells were readily detectable in the PB cells, and all the recipients succumbed to the disease by day 40 post-BM transplantation (post-BMT) (Fig. 5, B and C). Accumulation of Tcf7−/−LeF1−/− GFP+Mac1+ leukemic cells was not significantly different from that of control leukemic cells during the early stages of disease development (days 10 and 14 post-BMT), albeit it was lower than control on day 18 post-BMT (Fig. 5B). The survival of Tcf7−/−LeF1−/− CML recipients was marginally extended compared with that of control recipients (Fig. 5C). These data suggest that Tcf1/LeF1 deficiency only minimally affected the onset and progression of CML, mirroring their role in HSCs at the steady state.

We next sort-purified GFP+ LSKs from the BM cells of primary recipients as LSCs and transplanted the same numbers of Tcf7−/−LeF1−/− or control LSCs into secondary recipients (Fig. 5D), which is the most stringent test for LSC self-renewal. The control LSCs were able to propagate CML in the secondary hosts, with leukemic cells readily detected in PB cells on day 14 post-BMT; in contrast, Tcf7−/−LeF1−/− LSCs gave rise to substantially lower numbers of leukemic cells (Fig. 5E). Consistent with the difference in leukemia burden, all the secondary recipients of control LSCs succumbed to the disease by day 60 post-BMT, but all those of Tcf7−/−LeF1−/− LSCs survived beyond day 80 post-BMT (Fig. 5F). We demonstrated above that the repopulation capacity of Tcf7−/−LeF1−/− HSCs was only evidently diminished in the tertiary recipients. These data in CML suggest that LSCs are more dependent on Tcf1 and LeF1 for self-renewal than HSCs.

Tcf1 and LeF1 Are Intrinsically Required for LSC Self-renewal—Because Tcf7 was targeted in germline in the Tcf7−/− model, the Tcf7−/−LeF1−/− BM cells may have been exposed to Tcf1-deficient niche environment or have other intrinsic defects, leading to unwanted changes in Tcf7−/−LeF1−/− LSCs. To address these potential caveats, we took advantage of a recently established Tcf7FL/FL strain (29) and generated Tcf7FL/LeF1FL/FL mice. To achieve inducible inactivation of both floxed genes, we used a CreERT2 strain in which the fusion protein of Cre recombinase and estrogen receptor (ER) was driven by the ubiquitously expressed Rosa26 locus (30). In the resulting CreERT2 Tcf7FL/LeF1FL/FL mice, treatment with tamoxifen for 4 consecutive days effectively ablated both Tcf7 and LeF1 genes in hematopoietic cells as determined in splenic T cells (not shown). We used BM cells
from CreERT2 Tcf7\(^{FL/FL}\)/Lef1\(^{FL/FL}\) mice to establish CML so that the LSCs were generated without pre-existing conditions. We left one cohort of primary recipients untreated, and treated another cohort with tamoxifen to induce Tcf7 and Lef1 gene deletion, followed by transplantation of Lin\(^-\) BM cells containing the same numbers of GFP\(^+\) Lin\(^-\) Sca1\(^+\) cells into secondary recipients (Fig. 6A). This approach of using the GFP\(^+\) Lin\(^-\) Sca1\(^+\) phenotype to enumerate LSCs has been shown to have similar capacity as using sorted GFP\(^+\) LSKs to propagate CML in secondary or tertiary recipients (25), with the advantages of obtaining sufficient LSCs from fewer donor mice and avoiding additional stress on LSCs due to cell sorting. In parallel, we used Tcf1/Lef1-sufficient WT BM cells to establish CML coupled with tamoxifen treatment as another control group. LSCs from tamoxifen-treated WT mice and those from untreated CreERT2 Tcf7\(^{FL/FL}\)/Lef1\(^{FL/FL}\) mice had similar capacity to generate leukemic cells and led to death of the secondary recipients by day 50 post-BMT (Fig. 6, B and C). This observation indicates that tamoxifen treatment per se had little impact on CML propagation. In contrast, very few leukemic cells were generated from LSCs derived from tamoxifen-treated CreERT2 Tcf7\(^{FL/FL}\)/Lef1\(^{FL/FL}\) mice, and all the secondary recipients of these LSCs survived beyond day 80 post-BMT. These data demonstrate an intrinsic requirement for Tcf1 and Lef1 in maintaining self-renewal capacity of LSCs.

It has been proposed that self-renewal of stem cells is determined by a balance among three major cellular events, proliferation, survival, and differentiation (48). To further investigate the impact of Tcf1/Lef1 deficiency on self-renewing LSCs, we used Lin\(^-\) BM cells from CreERT2 Tcf7\(^{FL/FL}\)/Lef1\(^{FL/FL}\) or CreERT2 Tcf7\(^{FL/FL}\)+Lef1\(^{FL/FL}\) mice to establish CML, treated the primary recipients with tamoxifen and transplanted enriched LSCs into secondary recipients as in Fig. 6A. Fifteen days post-BMT, we harvested BM cells from the secondary recipients and found that the total BM cells and GFP\(^+\) LSKs from Tcf1/Lef1-deficient LSC recipients were ~29 ± 5.4% and 19.2 ± 3.8% of those from control LSC recipients, respectively. Cell cycle analysis in GFP\(^+\) LSKs in the secondary recipients revealed that both Tcf1/Lef1-sufficient and -deficient LSCs showed similar frequency in the quiescent G0 state or in the actively cycling S/G2/M state (Fig. 7, A and B). On the other hand, Tcf1/Lef1-deficient LSCs in the secondary recipients were severely compromised in survival compared with control LSCs, showing ~3–4-fold increase in Annexin-V\(^+\) Hoechst33342\(^-\) apoptotic population (Fig. 7, C and D). Because of the extreme paucity of Tcf1/Lef1-deficient LSCs in the secondary recipients, it was not feasible to perform colony formation assays which required sorting separation. Nonetheless, the GFP\(^+\) Lin\(^-\) c-Kit\(^+\) transformed myeloid progenitor cells, which are derived from GFP\(^+\) LSKs, exhibited similar frequency in the BM of Tcf1/Lef1-deficient or control LSC recipient mice (Fig. 7E). We infer that loss of Tcf1 and Lef1 did not potently affect LSC differentiation. These data collectively indicate that the impaired self-renewal...
of Tcf1/Lef1-deficient LSCs can be largely attributed to a strong dependence of CML LSCs on Tcf1 and Lef1 for survival.

Targeting Tcf1 and Lef1 Synergizes with TKI in CML Therapy—The TKI therapy is effective in controlling the growth of CML leukemic blasts but lacks strong effect on quiescent LSCs and thus cannot prevent the disease relapse (49). Given the potent impact of targeting Tcf1 and Lef1 on LSC self-renewal, we investigated if the combination of Tcf1/Lef1 deficiency with imatinib could better control CML in the primary recipients (Fig. 8A). After establishing CML using Tcf7L1−/−Lef1−/− or control BM cells, we treated the recipients with imatinib for 3 weeks. By the end of treatment, all the CML recipients were protected from the lethal disease and survived beyond day 50 post-BMT. Both control and Tcf7L1−/−Lef1−/−-derived LSCs engrafted similarly during the early stage of CML onset, as measured by leukemia burdens in the recipient PBGs (Fig. 5B). To determine if imatinib treatment affects LSC persistence, we detected GFP+ LSKs as LSCs in the BM from imatinib-treated recipients and found that Tcf7L1−/−Lef1−/−-derived LSCs were greatly diminished in frequency and numbers (Fig. 8, B and C). Continuous imatinib treatment further prolonged survival of recipients of p210BCR-ABL-infected control BM cells, albeit majority of them eventually succumbed to CML (Fig. 8, A and D). In contrast, recipients of p210BCR-ABL-infected Tcf7L1−/−Lef1−/− BM cells were better protected by imatinib treatment, with more than 80% of the recipients survived at the end of observation period (Fig. 8D). These findings suggest that targeting Tcf1 and Lef1 has synergistic effect with TKI treatment to better control CML progression and relapse.

Discussion

In this study, we identified differential requirements for the Tcf1 and Lef1 transcription factors for the normal HSCs and transformed LSCs. For the HSCs, there is a modest requirement for Tcf1 and Lef1. Loss of both factors resulted in moderately diminished quiescence of HSCs without discernible effect on HSC survival, which leads to an expansion of the HSC pool. Despite the increase in phenotypic HSCs in Tcf1/Lef1-deficient mice, they are functionally inferior, especially under competitive conditions or regenerative stress. HSCs lacking Lef1 alone also showed a moderate reduction in reconstitution of multiple blood lineages under competitive condition. This is line with a recent report that shRNA-mediated knockdown of Lef1 in mobilized BM progenitor cells diminishes spleen-colony formation capacity on day 12 post-BMT (50). Nonetheless, the HSC impairments were more evident in the absence of Tcf1 and Lef1, indicating a functional redundancy. HSCs lacking both Tcf1 and Lef1 were able to successfully repopulate all non-T blood lineages in primary and secondary hosts, but their capacity for blood regeneration was compromised in the tertiary hosts. This functional deficiency remarkably resembles that observed in transgenic expression of Wnt inhibitors such as Dkk1 and Wif1 and in Wnt3a-deficient fetal liver cells (11–13). In those studies where Wnt activity is manipulated, a general concern is that such intervention has a side effect on bone structure and/or stromal cells and thus may have altered HSC niches. Our data on Tcf1 and Lef1, the nuclear effector proteins downstream of the canonical Wnt pathway, provide supporting experimental evidence to the conclusion that Wnt ligands have direct impact on HSCs per se to modulate their regenerative capacity.

On the mechanistic side, the expression of Egr1, Tcf3 and Cdkn1c depended on Tcf1 and Lef1 in hematopoietic stem/progenitor cells. In addition, the 5’-regulatory regions of Egr1 and Tcf3 were bound by Lef1 in BM progenitor cells, albeit we cannot exclude the possibility that Cdkn1c may be regulated by Tcf1/Lef1 factors through distal elements. Egr proteins are the immediate early response transcription factors and are involved in stress responses in many tissues. The molecular connection between Tcf1/Lef1 and Egr proteins has been observed in immature thymocytes as well (51). By genetic complementation, we showed that forced expression of Egr1 was sufficient to rectify the diminished quiescence in Tcf1/Lef1-deficient HSCs. These data suggest that Egr1 is one of the key target genes regulated by Tcf1 and Lef1 in preventing excessive HSC cycling. Cell cycle regulators are essential for maintaining HSC quiescence (52). Among the Cip/Kip family of cyclin-dependent kinase inhibitors, only genetic targeting of Cdkn1c (the p57-Kip2) caused evident loss of HSC quiescence, albeit it does have cooperative roles with other family members, p21-Cip (encoded by Cdkn1a) and p27-Kip1, in the maintenance of HSCs (46, 47). The role of Tcf1 and Lef1 in HSC quiescence may be also mediated by regulation of the p57-Kip2, suggesting a multifaceted regulatory circuit under the control of Tcf1/Lef factors. Future studies should address a possible redundancy of Tcf1/Lef1 with Tcf3 and Tcf4 factors in the same family, which will further elucidate their downstream genes that contribute to regulation of HSC homeostasis.

β-Catenin stabilization and nuclear translocation are key events upon activation of the canonical Wnt pathway. The role of β-catenin in HSCs has been a highly contentious issue. Induced deletion of β-catenin alone or in combination with its homologue γ-catenin did not have a detectable impact on HSCs and blood lineages under homeostatic conditions (19, 20). Under competitive conditions, the β-catenin-deficient BM cells showed diminished contribution to all blood lineages, and their contribution was further reduced when transplanted into secondary recipients (23). Deletion of both Tcf1 and Lef1 showed similar effect, with no obvious impact on HSCs at the steady state but diminishing their competitive repopulation capacity. Our new observations on Tcf1/Lef1 deficiency reiterate the intrinsic requirements of Wnt-β-catenin pathway in HSCs. Our data are also in line with a recent finding that β-catenin is required for HSC regeneration and BM recovery after radiation or chemotherapy (53).

Unlike the moderate impact on normal HSCs, Tcf1 and Lef1 deficiency more potently affected LSCs in the preclinical CML model. Imatinib treatment prolonged the survival of primary CML recipients and hence facilitated detection of LSCs at a later time point after BM transplantation. In this context, targeting Tcf1 and Lef1 greatly diminished numbers of LSCs in the BM. Furthermore, Tcf1/Lef1-deficient LSCs failed to propagate the disease after transplantation into the secondary recipients. Mechanistically, the self-renewing CML LSCs critically depend on Tcf1 and Lef1 for survival, whereas their proliferation and
differentiation were not significantly altered due to Tcf1/Lef1 deficiency. Therefore, Tcf1 and Lef1 are essential for maintaining the pool size and self-renewal capacity of LSCs. In support of this notion, it was previously reported that forced expression of Lef1 in BM progenitors causes B lymphoblastic and acute myeloid leukemia, which are likely propagated by LSCs with lymphoid characteristics (54). An important goal in chemotherapy is to eliminate malignant cells and minimize damage to normal cells. Our studies show that HSCs and LSCs have differential requirements for Tcf1/Lef1 in their self-renewal capacity. Tcf1 and Lef1 can therefore be exploited as novel therapeutic targets in eradicating LSCs in hematological malignancies with less adverse effect on normal hematopoiesis.

Aberrant activation of β-catenin is a hallmark event in tumor initiation, growth and metastasis, and has been a well sought-after drug target in cancer therapy (55). It has been demonstrated that genetic ablation or pharmacological inhibition of β-catenin expression synergizes with imatinib in improving survival of CML mice; however, this effect is only observed after the LSCs were serially transplanted into tertiary recipients (25). In contrast, the synergy of Tcf1/Lef1 deletion and imatinib was evident in the primary recipients where the CML was first established. These observations imply that the role of Tcf1 and Lef1 in LSCs may not be mediated exclusively by β-catenin. In fact, our recent studies showed that Tcf1 and Lef1 directly interact with Runx3 transcription factor and this interaction contributes to Cd4 gene silencing in CD8+ T cells (29). Another study mapped genome-wide binding locations of Tcf1 and Runx1 transcription factors in a mouse EML (erythroid, myeloid, and lymphocytic) multipotent hematopoietic precursor cells (56). Significantly, the Tcf1 and Runx1 binding peaks overlapped at more than 40% of all their binding locations. The differential use of cofactors (β-catenin versus Runx proteins) by Tcf1 and Lef1 merits further investigation. It is currently recognized that transcriptional programs can be druggable targets (57). Detailed delineation of Tcf1/Lef1-dependent transcriptional program in HSCs and LSCs will facilitate identification of small molecules to antagonize Tcf1/Lef1 activities in LSCs and further in vivo assessment of their therapeutic potentials in treating leukemia.

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