Brief Definitive Report

Clonal expansion capacity defines two consecutive developmental stages of long-term hematopoietic stem cells

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Hematopoietic stem cells (HSCs) replenish millions of mature hematopoietic cell types every second throughout life but also maintain the HSC pool over time. HSC function is assessed by their capacity to repopulate the blood system of lethally irradiated recipient mice in the long term. The most immature HSC pool is functionally heterogeneous, and HSCs vary in their differentiation potential and duration of reconstitution (Copley et al., 2012; Muller-Sieburg et al., 2012). However, the magnitude of repopulation, thus white blood cell output per donor HSC, was only retrospectively associated with specific reconstitution patterns determined by lineage choice (Dykstra et al., 2007). Therefore, it remains unknown whether clonal expansion capacities are predetermined in donor cells or whether the magnitude of repopulation is determined by the microenvironment of the recipient.

Kit expression is widely used for the prospective isolation of HSCs, and the stem cell factor (SCF)–Kit signaling axis is pivotal for normal pool size and function of fetal and adult HSCs (Russell, 1979; Ikuta and Weissman, 1992). Consistently, alterations in Kit signaling profoundly affect adult HSC function (Ogawa et al., 1991; Czechowicz et al., 2007; Waskow et al., 2009; Ding et al., 2012; Deshpande et al., 2013). Furthermore, Kit alleles resulting in hypomorphic expression of the receptor are loss of function alleles (Russell, 1979; Thorén et al., 2008; Waskow et al., 2009), suggesting that reduced densities of Kit expression correlate with loss of “stemness.” In contrast, cells expressing low levels of (Doi et al., 1997; Matsuoka et al., 2011) or lacking (Ortiz et al., 1999) Kit receptor expression were suggested to contain quiescent long-term HSCs (LT-HSCs). However, differences in the clonal expansion capacities of HSCs expressing distinct levels of the Kit receptor were not reported.

Long-term hematopoietic stem cells (HSCs [LT-HSCs]) are well known to display unpredictable differences in their clonal expansion capacities after transplantation. Here, by analyzing the cellular output after transplantation of stem cells differing in surface expression levels of the Kit receptor, we show that LT-HSCs can be systematically subdivided into two subtypes with distinct reconstitution behavior. LT-HSCs expressing intermediate levels of Kit receptor (Kitint) are quiescent in situ but proliferate extensively after transplantation and therefore repopulate large parts of the recipient’s hematopoietic system. In contrast, metabolically active Kithi LT-HSCs display more limited expansion capacities and show reduced but robust levels of repopulation after transfer. Transplantation into secondary and tertiary recipient mice show maintenance of efficient repopulation capacities of Kitint but not of Kithi LT-HSCs. Initiation of differentiation is marked by the transit from Kitint to Kithi HSCs, both of which precede any other known stem cell population.

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To assess whether expansion capacities are predetermined within donor HSCs and whether this function identifies novel cellular subtypes within the most immature HSC pool, we transplanted LT-HSCs that differed in the density of the expression of the Kit receptor. Donor cells repopulated recipient mice to two significantly different magnitudes: HSCs with intermediate levels of Kit receptor expression (Kitint) contained greater expansion capacities compared with HSCs expressing high densities of the Kit receptor (Kithi), suggesting that HSC clonal growth potential is predetermined in a cell-intrinsic fashion. We further provide evidence that these HSC subtypes are two consecutive developmental stem cell stages within the most immature HSC pool and that transit from Kitint to Kithi LT-HSCs marks the onset of differentiation and is associated with significant loss of expansion capacities. Gene expression profiles ex vivo and after SCF trigger suggest that the inherent differences are based on distinct cycling and adhesive activities.

RESULTS AND DISCUSSION
Prospective separation of HSCs with different expansion capacities: Intermediate levels of Kit receptor expression correlate with increased HSC potency
To assess whether distinct levels of Kit cell surface expression mark discrete types of HSCs that differ in their biological properties, we fractionated the HSC compartment into cells expressing high densities of the Kit receptor (Kithi), suggesting that HSC clonal growth potential is predetermined in a cell-intrinsic fashion. We further provide evidence that these HSC subtypes are two consecutive developmental stem cell stages within the most immature HSC pool and that transit from Kitint to Kithi LT-HSCs marks the onset of differentiation and is associated with significant loss of expansion capacities. Gene expression profiles ex vivo and after SCF trigger suggest that the inherent differences are based on distinct cycling and adhesive activities.
already declined in primary recipient mice, suggesting that Kitint HSCs precede Kithi HSCs during differentiation. Disparate repopulation activities after bulk transplantation can be caused by different homing activities or by varying HSC frequencies within the transplanted populations. However, homing activity between Kitint and Kithi cells was indistinguishable (Fig. 1 F), and HSC frequency was also comparable as determined by limited dilution transplantations (Fig. 1 G; Hu and Smyth, 2009), suggesting that differences in repopulation activities are caused by cell-intrinsic differences in the clonal expansion capacities of donor cells. Collectively, these results indicate that clonal expansion capacities are cell-intrinsically

Figure 2. Density of Kit receptor expression defines two functionally distinct populations. (A) BM cells were prepared, and expression of the indicated markers was determined on Kitint and Kithi cells pregated as described above the plots. Isotype control staining on LSK Slam cells is shown in black. Data are representative of two independent experiments each. (B) BrdU was injected into wild-type mice, and after 4 h, BM cells were prepared and BrdU incorporation into LSK Kit+ cells or into LSK Kitint and Kithi cells was measured. (C) Plot shows summary of four mice analyzed in two independent experiments as outlined in B. (D) LSK cells were labeled with BrdU, and 330 d later, Kit expression on LSK CD48−CD150+ BrdU-positive and -negative cells was analyzed. Data are representative of two independent experiments using three mice each. (E) Lineage-depleted BM cells were cultivated with or without SCF for 15 min, and phosphorylation of Akt (top) and Erk (bottom) in LSK Slam Kitint (left) or Kithi (right) HSCs was measured. (F) Plot summarizes data from three independent experiments using cells from 10 (pErk) or 11 (pAkt) mice as shown in E. For controls, four (pErk) or five (pAkt) mice were used. (G) LSK CD135−Kitint or Kithi HSCs were cultivated overnight in the presence or absence of SCF, BrdU was added for 4 h, and its incorporation was subsequently analyzed. Specificity was determined by using the anti-Kit inhibitory antibody ACK2 (Ogawa et al., 1991). The plot summarizes data from two independent experiments using three mice for each group. (H) Single LSK Slam CD135−CD34− Kitint, Kithi, or Kithi HSCs were cultivated in liquid culture in medium supplemented with SCF, Tpo, Il3, and Epo (left). Colony formation was analyzed after 14 d. L, large; M, medium; S, small colonies. Cell type composition of individual colonies is summarized (right). m, macrophage; g, granulocyte; E, erythroblast; M, megakaryocyte. Data shown are pooled from two independent experiments using cells from three mice each. (I) LSK CD135−Kitint or Kithi HSCs were cultivated as described in G, and the frequencies of Kit+ Sca1− expressing cells were determined. Plot summarizes data from three mice. Changes are quantified in the plot on the right. Error bars show SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
predetermined and that different expression densities of the Kit receptor allow for the prospective separation of two subtypes of LT-HSCs differing in that function.

**Level of Kit expression marks two functionally distinct HSC populations**

HSC subtypes with prolonged self-renewal activity can be found in stem cell populations that express low levels of CD49b (Benveniste et al., 2010) or high levels of CD150 (Morita et al., 2010), CD86 (Shimazu et al., 2012), and CD41 (Gekas and Graf, 2013). Furthermore, HSCs that are biased toward the development of either lymphoid or myeloid cells respond differently to stimulation with IFN-α (Essers et al., 2009) or TGF-β (Challen et al., 2010). The latter can modulate Kit cell surface expression (Sansilvestri et al., 1995). However, expression of receptors for TGF-β (*Tgfbr2*) and IFN-α (*Ifnar1*) was comparable on Kit<sup>int</sup> and Kit<sup>hi</sup> HSCs (Fig. 2 A). CD150 expression was inversely correlated with Kit expression, but there was no evidence for differential expression of CD49b, CD41, CD86, or other stem cell markers like Epcr (*Procr*), CD201 (*CD201*), and MPP cells (LSK CD48<sup>+</sup>CD135<sup>−</sup>CD34<sup>−</sup>) were sorted.

Whole transcriptome sequencing was performed on amplified RNA; *n* = 3 for each population. (A) Heat map of the sample to sample Euclidean distance and dendrogram showing sample to sample correlation. All biological replicates cluster well with each other, and all samples of the different populations are clearly separated from each other. (B) Graph shows DESeq-normalized counts for selected genes expressed in Kit<sup>int</sup> or Kit<sup>hi</sup> LT-HSCs. Protein expression from the same genes is depicted in Fig. 2 A. Mean ± SD is shown; *n* = 3; **, *P* < 0.01. (C) Comparison of gene expression profiles between Kit<sup>int</sup> and Kit<sup>hi</sup> LT-HSCs. Red dots represent significant DEGs at a 10% false discovery rate (FDR). (D and E) Plots show biological processes that are enriched in genes down-regulated (D) or up-regulated (E) in LT-HSC Kit<sup>int</sup> cells compared with LT-HSC Kit<sup>hi</sup> cells. Analysis was performed using the GO/BP database of DAVID. Enrichment scores (−log transformation of the DAVID EASE score) were calculated to determine overrepresentation of particular biological processes and are indicated on the *x* axis. (F) Plot shows biological processes that are uniquely enriched in genes differentially expressed in LT-HSC Kit<sup>int</sup> cells after stimulation with SCF compared with LT-HSC Kit<sup>hi</sup> cells after stimulation with SCF. (G) Heat map shows quantitative comparison of the expression level of genes common between DEGs of Kit<sup>int</sup> ± SCF and Kit<sup>hi</sup> ± SCF that are associated with the top five enriched GO biological process terms for these common genes. The number of associated DEGs is indicated in parentheses.

Figure 3. Kit receptor densities are indicative for HSC populations with different molecular expression programs. BM was harvested and Kit<sup>int</sup> or Kit<sup>hi</sup> LT-HSCs (LSK CD48<sup>−</sup>CD41<sup>−</sup>CD150<sup>−</sup>CD135<sup>−</sup>CD34<sup>−</sup>), ST-HSCs (LSK CD48<sup>+</sup>CD135<sup>−</sup>CD34<sup>+</sup>), and MPP cells (LSK CD48<sup>+</sup>CD135<sup>−</sup>CD34<sup>−</sup>) were sorted.
suggesting that high levels of Kit signaling result in differentiation but low levels of Kit signaling result in maintenance of stemness. We conclude that different densities of Kit receptor expression result in altered cell biological consequences after triggering the SCF–Kit signaling axis.

**Kitint and Kithi LT-HSCs are distinct molecular entities**

To test for molecular differences between both HSC populations, we compared the gene expression between Kitint and Kithi LT-HSCs, short-term HSCs (ST-HSCs), and multipotent progenitor (MPP) cells (Fig. 3). Unsupervised clustering revealed great homology between individual samples (Fig. 3 A), and analysis of expression counts of selected transcripts encoding for defined proteins that were previously analyzed by flow cytometry (Fig. 2 C) confirmed the purification strategy of both populations (Fig. 3 B). Comparing gene expression between Kitint and Kithi HSCs revealed 96 up-regulated and 48 down-regulated terms related to cell adhesion pathways for the significantly up-regulated genes, verifying the quiescent state of HSCs that exhibit great expansion capacity after transplantation. Kitint cells inversely correlated with the maintenance of a Kit+ Sca-1+ stem cell surface phenotype after culture (Fig. 2 I), suggesting that high levels of Kit signaling result in differentiation.

Collectively, we identified two discrete subtypes of HSCs that differ in their inherent capacities to expand and to form HSC progeny after transplantation. Kitint HSCs are the most immature HSCs that may be parental to Kithi HSCs. We show that Kit expression levels negatively correlate with differentiation based on transplantation experiments.

**Figure 4. Present and modified model for the initiation of HSC differentiation based on transplantation experiments.** (A) Present model: Kit-positive (plus) LT-HSCs repopulate recipient mice in the long term (each tick on the x axis represents ~16 wk). Kit+ intermediate-term reconstituting cells (ITRCs), ST-HSCs, and MPP cells repopulate recipient mice to lower levels and for much shorter time periods than LT-HSCs. The reconstitution scheme of LT-HSCs, ST-HSCs, and MPP cells is based on the transplantation of 1,500 cells per population (not depicted). The reconstitution scheme of ITRCs was estimated from Benveniste et al. (2010). (B) Modified model: We show that the magnitude and duration of reconstitution differs between HSC subsets and that the most immature LT-HSC compartment can be further subdivided into two novel stem cell subtypes that have inherent differences in their clonal expansion capacities. Kitint HSCs repopulate recipient mice very efficiently (magnitude and duration), whereas Kithi cells can be placed between Kitint LT-HSCs and ITRCs. Both magnitude and duration of reconstitution are reduced compared with Kitint LT-HSCs but increased compared with ITRCs. Q, quiescent LT-HSC.
MATERIALS AND METHODS

Mice. C57BL/6 (B6) and B6.SJL-Ptp∥Pep∥BoyJ (B6.SJL) mice were purchased from the Jackson Laboratory and bred and maintained under specific pathogen-free conditions in the animal facility at the Medical Theoretical Center of the University of Technology Dresden. Experiments were performed in accordance with German animal welfare legislation and were approved by the relevant authorities, the Landesdirektion Dresden.

Transplantation. For competitive transplantation, 50 lineage−Sca−1−Kit− (LSK) CD48−CD41−CD150− (Slam) Kitint or Kithi cells (Fig. 1, B−E [top]) or 1,000 purified LSK Kitint or Kithi cells (Fig. 1, E, bottom) were transplanted together with 5 × 105 nonfractionated BM cells into lethally irradiated (900 cGy) wild-type recipient mice. Test, competitor, and recipient cells carried different CD45 alleles (CD45.1−, CD45.2−, CD45.1−CD45.2+). For serial transplantation, 5 × 105 nonseparated BM cells were injected into secondary lethally irradiated recipients. For limiting dilution analysis, 3, 8, and 20 or 3, 8, and 13 LSK Slam Kitint or Kithi (CD45.1+) cells were injected together with 3 × 103 nonseparated BM cells (CD45.1−CD45.2+) into 10 lethally irradiated wild-type mice (CD45.2−) per donor cell number. 16 wk after transplantation, donor cell chimerism was determined in blood neutrophils, and mice were scored positive when donor contribution was >1%. Frequency of the repopulating cells was calculated using ELDA software. Pairwise differences in active cell frequencies between groups were calculated as described previously (Hu and Smyth, 2009). For the first experiment, donor cells were sorted into one well and separated before transplantation, and for the second experiment, donor cells were sorted into separate wells of a 96-well plate. For homing assays, LSK Kitint (CD45.1+ or CD45.2+) and LSK Kit hi (CD45.2+ or CD45.1+) cells were sorted and mixed. Ratio of mixture was determined by flow cytometry, and a total of 2 × 104 cells were injected into each lethally irradiated recipient mouse (CD45.1−CD45.2+). 16−18 h later, recipient mice were sacrificed, BM cell suspension was prepared, and donor cell ratio in LSK cells was determined.

Flow cytometry. BM cell suspensions were prepared, stained, and analyzed as described previously (Arndt et al., 2013). Antibodies (clones in parentheses) used are as follows: CD3 (2C11; 17A2), CD11b (M1/70), CD19 (1D3), CD34 (RAM34), CD45.1 (A20), CD45.2 (104), CD45R (RA3-6B2), CD86 (GL1), CD117 (2B8), CD135 (A2F10), Gr-1 (RB6-8C5), Nk1.1 (PK136), Sca1 (D7), Ter119 (Ter119), CD41 (MWReg30), Epcr (PK136), CD49b (DX5), and IfnR1 (MAR-1-A3; all eBioscience); CD48 (HM48-1); CD34 (ST-HSC) Kitint or Kit hi cells, LSK CD48+CD135− (ST-HSC), and LSK CD48−CD135+ (MPP) cells were sorted (FACSAria II; BD) and immediately lysed in μMACS mRNA isolation lysis buffer (Miltenyi Biotec). For SCF trigger experiments, 7,500 sort-purified LSK CD135−Kit−Kithi cells were incubated in StemSpan medium (STEMCELL Technologies) supplemented with 50 ng/ml rmSCF for 11 h and subsequently lysed. Lysates were cleaned using LysateClear Columns (Miltenyi Biotec), and mRNA was directly isolated from the lysis buffer using SeraMag oligo(dT)14 beads (Thermo Fisher Scientific). The mRNA was eluted in a volume of 5 μl of 10 mM Tris-HCL and directly subjected to subexponential RNA amplification using the WT-Ovation System (Nugen Technologies). Samples were prepared according to the manufacturer’s instructions, but stopped before final Post-SPIA Modification. After bead-based purification (XP beads; Agencourt), randomly primed second strand synthesis was performed using second Strand Synthesis Module from New England Biolabs, Inc. After DNA shearing by ultrasonication (Covaris S2) and treatment with S1 nuclease (New England Biolabs, Inc.), samples were subjected to standard Illumina fragment library preparation using indexed adaptors. Resulting libraries were pooled in equimolar quantities for 75-bp single-read sequencing on Illumina HiSeq 2000 and distributed on several lanes, resulting in ~30–90 million reads per sample.

Bioinformatic analysis. Alignment of the short reads to the mm9 transcriptome was performed with pBWA software, and a table of read counts per gene was created based on the overlap of the uniquely mapped reads with the Ensembl Genes annotation version 61 for mm9, using BEDTools (version 2.21; Quinlan and Hall, 2010). The raw read counts were then normalized with the DESeq R package (version 1.8.1; Anders and Huber, 2010), and the sample to sample Euclidean distance was computed based on the normalized counts to explore sample to sample correlation. After normalization, testing for differential expression was performed with DESeq, and accepting a maximum of 10% false discoveries (10% FDR), resulted in 96 up-regulated and 48 down-regulated genes in Kitint HSCs. For SCF trigger experiments, DEGs between Kitint versus Kitint + SCF and Kitint + SCF versus Kithi + SCF were compared, and DEGs unique to Kitint + SCF versus Kitint + SCF or DEGs common to both gene lists were identified. To identify enrichment for particular biological processes associated with the DEGs, the DAVID GO_BP_FAT database (Huang et al., 2009) was used. Enrichment scores were calculated (−log transformation of the DAVID EASE score) to determine overrepresentation of particular biological processes. To quantify gene expression levels, an expression score defined as the median of all normalized counts of the DEGs associated with that particular GO term was calculated. Subsequently, expression scores for the top five GO biological process terms across the four experimental conditions were depicted in a heat map.

We thank S. Piontek and S. Böhme for expert technical assistance and V. Grinenko and B. Wielockx for discussion.
C. Waskow is supported by the Center for Regenerative Therapies Dresden, by the Deutsche Forschungsgemeinschaft (DFG) Sonderforschungsbereich (SFB) 655 (B9), SFB 127 (A3), and FOR2033 (A3), and by a grant from the European Union (FP7, CELL-PID). A. Dahl is supported by DFG SFB 655 (Deep Sequencing Group).

The authors declare no competing financial interests.

Submitted: 29 May 2013
Accepted: 6 December 2013

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