


## Research Article

# Flt3 ligand-eGFP-reporter expression characterizes functionally distinct subpopulations of CD150<sup>+</sup> long-term repopulating murine hematopoietic stem cells

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The pool of hematopoietic stem cells (HSCs) in the bone marrow is a mixture of resting, proliferating, and differentiating cells. Long-term repopulating HSCs (LT-HSC) are routinely enriched as Lin<sup>-</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells. The Flt3 ligand (Flt3L) and its receptor Flt3 are important regulators of HSC maintenance, expansion and differentiation. Using Flt3L-eGFP reporter mice, we show that endogenous Flt3L-eGFP-reporter RNA expression correlates with eGFP-protein expression. This Flt3L-eGFP-reporter expression distinguishes two LT-HSC populations with differences in gene expressions and reconstituting potential. Thus, Flt3L-eGFP-reporter<sup>low</sup> cells are identified as predominantly resting HSCs with long-term repopulating capacities. In contrast, Flt3L-eGFP-reporter<sup>high</sup> cells are in majority proliferating HSCs with only short-term repopulating capacities. Flt3L-eGFP-reporter<sup>low</sup> cells express hypoxia, autophagy-inducing, and the LT-HSC-associated genes HoxB5 and Fgd5, while Flt3L-eGFP-reporter<sup>high</sup> HSCs upregulate genes involved in HSC differentiation. Flt3L-eGFP-reporter<sup>low</sup> cells develop to Flt3L-eGFP-reporter<sup>high</sup> cells in vitro, although Flt3L-eGFP-reporter<sup>high</sup> cells remain Flt3L-eGFP-reporter<sup>high</sup>. CD150<sup>+</sup>Flt3L-eGFP-reporter<sup>low</sup> cells express either endothelial protein C receptor (EPCR) or CD41, while Flt3L-eGFP-reporter<sup>high</sup> cells do express EPCR but not CD41. Thus, FACS-enrichment of Flt3/ Flt3L-eGFP-reporter negative, Lin<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup>EPCR<sup>+</sup>CD41<sup>+</sup> HSCs allows a further 5-fold enrichment of functional LT-HSCs.

**Keywords:** Bone marrow · Cytokines · Flt3 ligand · Long-term repopulation · Stem cells



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## Introduction

Hematopoietic stem cells (HSCs) in bone marrow maintain the life-long production of all blood cells in mice and humans [1–4].

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The pool of HSCs exists in two cell cycle states, i.e. hypoxic, autophagy-active cells in the G<sub>0</sub> phase of cell cycle, and actively proliferating cells in the G<sub>1</sub>-S-G<sub>2</sub>-M phases. Hypoxic, autophagy-active HSCs can be activated to dividing cells, and dividing cells can become hypoxic, autophagy-active again [5, 6]. HSCs are able to self-renew, or to differentiate into all blood cell types [7]. Moreover, as little as one HSC has the ability to long-term reconstitute a complete hematopoietic system by transplantation [8–10].

Resting HSCs display activated autophagy [11], show reduced metabolism and express hypoxia-related genes [12]. They are thought to localize to hypoxic microenvironments in the bone marrow resulting in maintenance of the HSC pool size [12, 13]. The balance of HSC quiescence, the capacity of self-renewal after activation into proliferation and the decision to differentiate strongly depend on interactions of HSCs with their environments that provide signals through cytokines, e.g. stem cell factor (SCF), fms-like tyrosine kinase-3 ligand (Flt3L), thrombopoietin (TPO), and angiopoietin (Ang1), chemokines, e.g. CXCL12, as well as cell adhesion molecules, e.g. integrins and cadherins, directing HSC functions [12–16].

In adult mouse bone marrow LT-HSCs are identified as Lin<sup>-</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup> (LSK) CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells [17, 18]. Recently, Fgd5 [19] and HoxB5 [20] expression have further narrowed the fraction of true LT-HSCs. Genetically engineered reporter mouse strains for these markers should help in further characterizations and a deeper understanding of their localization and functions within bone marrow. Hence, in potentially heterogeneous HSC populations it remains challenging to further characterize true LT-HSCs.

The cytokine Flt3L has an important role in hematopoiesis [21–23]. It acts on Flt3, its receptor, which is expressed on early hematopoietic progenitors and dendritic cells. Flt3L induces the differentiation of these progenitors, and it also mobilizes HSCs [24–27]. Flt3L-overexpressing transgenic mice have expanded numbers of NK cells, dendritic cells and myeloid cells [28–30]. Flt3L-deficient mice show deficient hematopoiesis, reduced leukocyte cellularity in bone marrow, lymph nodes and spleen, reduced myeloid and B-lymphoid cellularity in bone marrow, but normal cellularity in thymic development and platelets [21]. All of these studies suggest that Flt3L plays an important role in the expansion of early hematopoietic progenitors and the establishment of myeloid and B lymphoid cell lineages.

However, for the maintenance and post-transplantation expansion of HSCs, Flt3L and its receptor are dispensable [31]. Moreover, when HSCs express Flt3 they lose self-renewal capacity, one of the critical capacities of an LT-HSC [32], and gain differentiation capacities [33]. This suggests that Flt3L-signalling via Flt3 terminates LT-HSC status and induce differentiation to short-term (ST<sup>-</sup>) HSCs, multipotent progenitors (MPPs) and more differentiated progenitors.

In a search for environmental cells in bone marrow, which could control dormancy, self-renewal and differentiation of HSCs by Flt3L production, we employed a Flt3L-eGFP reporter mouse strain [34]. To our surprise, we found that a proportion of

bone marrow LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells, themselves, did express Flt3L, detectable by flow cytometry using eGFP as a reporter.

Here, we show that Flt3L-eGFP-reporter expression distinguishes two subtypes of functionally distinct LSK CD34<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells, a Flt3L-eGFP-reporter<sup>low</sup> and a Flt3L-eGFP-reporter<sup>high</sup> population. They differ in their cell cycle status, their pattern of gene expression and in their capacity to long-term repopulate irradiated hosts. The Flt3L-eGFP-reporter<sup>low</sup> population has autophagy- and hypoxia-related genes up-regulated and qualifies as a LT-HSC, while the Flt3L-eGFP-reporter<sup>high</sup> population is cycling, expresses differentiation genes, but does not have LT-HSC qualities. In vitro, Flt3L-eGFP-reporter<sup>low</sup> cells become Flt3L-eGFP-reporter<sup>high</sup>, although Flt3L-eGFP-reporter<sup>high</sup> cells remain Flt3L-eGFP-reporter<sup>high</sup>.

These results demonstrate that the lack of Flt3L mRNA expression in HSCs is yet another marker which further defines the status of LT-HSCs.

## Results

### Flt3L-eGFP-reporter expression in hematopoietic cell populations of bone marrow

Flt3L is required during the differentiation of early hematopoietic progenitors in the bone marrow [23, 24] and in later steps of hematopoietic cell differentiation, such as NK cells and dendritic cells [35, 36]. Flt3L is produced by a variety of different organs and cell lines [37–41].

In the bacterial artificial chromosome transgenic reporter mouse that expresses eGFP protein under the control of the *flt3l* promoter and cis regulatory elements [34], we detect Flt3L mRNA expression in early hematopoietic cell populations in bone marrow. Thus, eGFP expression signifies *flt3l* transcription. In order to investigate Flt3L expression on single-cell level, we quantified eGFP expression in Flt3L-eGFP reporter mice by flow cytometry.

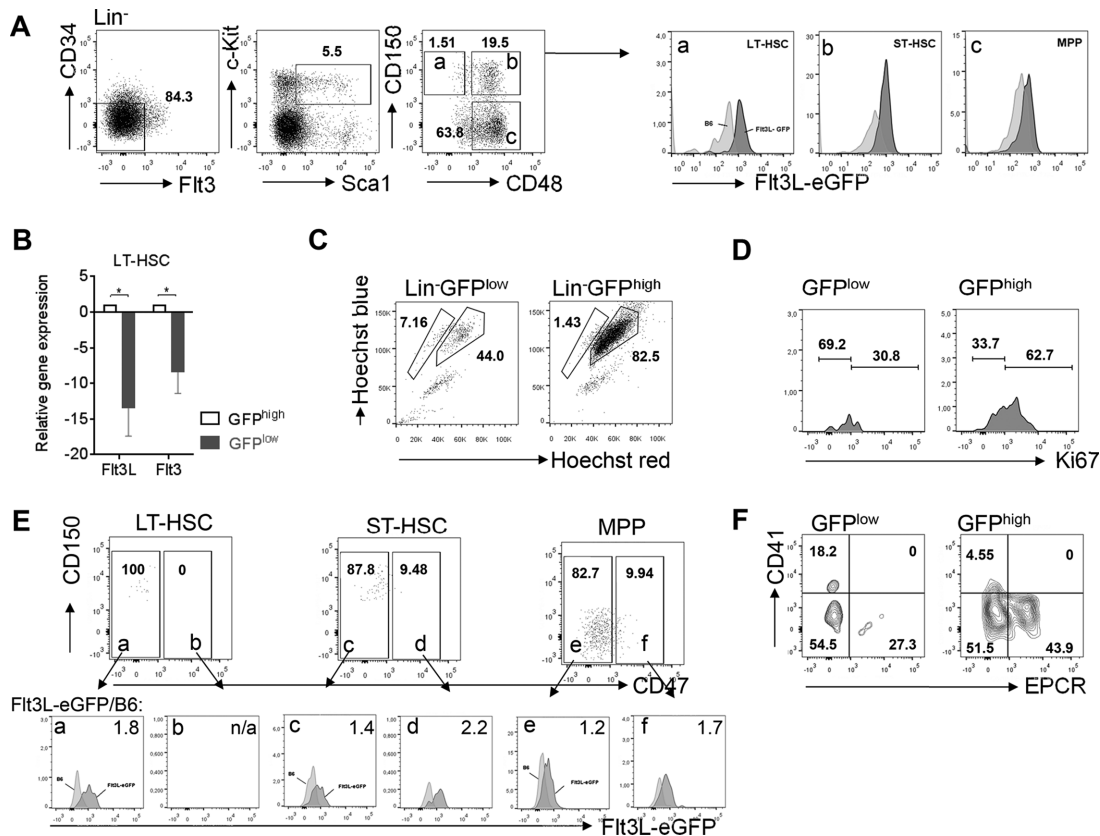
Consistent with earlier findings [34], we found widespread expression of Flt3L-eGFP-reporter in a large variety of hematopoietic cell populations of bone marrow, indicating ubiquitous *flt3l* transcription across different cell types. Nevertheless, there were quantitative differences between cell types. Surprisingly, within the hematopoietic progenitor compartment LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> LT-HSCs showed the highest Flt3L-eGFP level {MFI fold increase (Flt3L-eGFP/wildtype C57BL/6J): ~2}, followed by LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>+</sup> ST-HSCs (MFI fold increase: ~1.4), LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>-</sup>CD48<sup>+</sup> MPPs (MFI fold increase: ~1.2), common-lymphoid progenitors (CLP, Lin<sup>-</sup>CD19<sup>+</sup>c-Kit<sup>+</sup>Sca1<sup>low</sup>IL7R<sup>+</sup>Flt3<sup>+</sup> cells; MFI fold increase: ~1.1), and common-myeloid progenitors (CMP, Lin<sup>-</sup>c-Kit<sup>+</sup>Sca1<sup>-</sup>CD34<sup>+</sup>FcγR<sup>low</sup> cells; MFI fold increase: ~1) (Fig. 1A, Supporting Information Figs. 1 and 2). As described previously [34], the highest Flt3L-eGFP level within differentiated hematopoietic cells was detected in NK cells, followed by T cells,

B cells, dendritic cells and myeloid cells. In CD45<sup>-</sup> non hematopoietic cells such as endothelial cells, Flt3L-eGFP-reporter expression levels were comparable to those of B cells and myeloid cells (data not shown) [34].

LT-HSCs are routinely isolated as LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells. In Fig. 1A and Supporting Information Fig. 2 we show that these LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells

can be subdivided into Flt3L-eGFP-reporter<sup>low</sup> and Flt3L-eGFP-reporter<sup>high</sup> cells.

To confirm Flt3L expression on the molecular level, we separated eGFP<sup>high</sup> from eGFP<sup>low</sup> LT-HSCs and performed qRT-PCR analyses. We detected Flt3L expression in eGFP<sup>high</sup> populations but not in eGFP<sup>low</sup> populations of HSCs (Fig. 1B, Supporting Information Fig. 2). This reconfirm that Flt3L-eGFP reporter mice are a



**Figure 1.** Flt3L-eGFP-reporter expression by hematopoietic progenitor cells in mouse bone marrow. (A) Representative flow cytometry histogram overlays for the expression pattern of Flt3L-eGFP-reporter by hematopoietic progenitor cells in (see also Supporting Information Fig. 2) bone marrow of Flt3L-eGFP reporter mice and the C57BL/6J wild-type mice (B6,  $n = 3$ ). Live cell gates were set for LT-HSCs (LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup>), ST-HSCs (LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>+</sup>), and MPPs (LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>+</sup>). Expression patterns of Flt3L-eGFP-reporter are shown as single-parameter histograms, where the Y-axis display a frequency distribution of the data versus fluorescence intensity. Flow cytometry plots/histograms are from a single experiment representative of four experiments with three mice per experiment. (B) Relative mRNA expression level of Flt3L and Flt3 on sort-purified Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> LT-HSCs (see also Supporting Information Fig. 2). Relative expression of Flt3L and Flt3 mRNA was assessed by quantitative real-time PCR and normalized to GAPDH. qRT-PCR runs were performed on three independent biological samples in technical triplicates. Data are expressed as median  $\pm$  interquartile range ( $n = 3$ ). \* $p < 0.05$  by paired Students  $t$ -test. (C) Representative flow cytometry plots for the percentage of SP and non-SP cells gated on Flt3L-eGFP<sup>high</sup> and Flt3L-eGFP<sup>low</sup> Lin<sup>-</sup> cells ( $n = 3$ ) (see also Supporting Information Fig. 3). Flow cytometry plots/histograms are from a single experiment representative of two experiments with three mice per experiment. (D) Representative flow cytometry histograms for the percentage of Ki67<sup>-</sup> ( $G_0$  cells) gated on Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> LT-HSCs ( $n = 3$ ) (see also Supporting Information Fig. 4). Flow cytometry plots/histograms are from a single experiment representative of three experiments with three mice per experiment. (E) Representative flow cytometry histogram overlays for the expression pattern of Flt3L-eGFP-reporter gated on CD47<sup>-</sup> and CD47<sup>+</sup> hematopoietic progenitor populations in bone marrow of Flt3L-eGFP reporter mice ( $n = 3$ ). The Flt3L-eGFP-reporter MFI fold increase was calculated as the ratio between the eGFP MFI in cells of the Flt3L-eGFP reporter mice and wild-type C57BL/6J mice. A value of one indicates no Flt3L-eGFP-reporter expression. Flow cytometry plots/histograms are from a single experiment representative of two experiments with three mice per experiment. (F) Representative FACS blots for the percentage of EPCR<sup>+/+</sup> and CD41<sup>+/+</sup> cells gated on Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> Lin<sup>-</sup> cells ( $n = 3$ ) (see also Supporting Information Fig. 5). Flow cytometry plots/histograms are from a single experiment representative of three experiments with three mice per experiment. Expression patterns of Flt3L-eGFP-reporter are shown as single-parameter histograms, where the Y-axis display a frequency distribution of the data versus fluorescence intensity. In overlay analyses light grey curves represent C57BL/6J wild-type mice, while dark grey curves represent Flt3L-eGFP-reporter mice. In gene expression analyses open bars represent Flt3L-eGFP-reporter<sup>high</sup> cells, while filled bars represent Flt3L-eGFP-reporter<sup>low</sup> cells. Arrows in Fig. 1A and E indicate direction of gating.

reliable source of Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> HSCs in order to analyze functional differences of these populations [34].

LT-HSCs can also be isolated as hypoxic cells expressing the multidrug transporter proteins MDR1 and ABCG2 as Rho123<sup>low</sup> or Hoechst<sup>low</sup> cells (“side population”, SP cells) [43, 44]. We show that Flt3L-eGFP-reporter<sup>low</sup> Lin<sup>-</sup> cells are enriched for Hoechst<sup>low</sup> cells when compared to Flt3L-eGFP-reporter<sup>high</sup> Lin<sup>-</sup> cells (Fig. 1C, Supporting Information Fig. 3). Moreover, the majority of Flt3L-eGFP-reporter<sup>low</sup> LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells are in the G<sub>0</sub> phase of the cell cycle (~60–80%) as determined by Ki67 staining, while ~40–60% of the Flt3L-eGFP-reporter<sup>high</sup> LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells are actively cycling, Ki67-positive (Fig. 1D, Supporting Information Fig. 4).

CD47 is upregulated on circulating HSCs. Thus, it can be used as a marker for activated HSCs [45]. Confirming these studies, CD47 was only expressed on ST-HSCs and MPPs, but not on LT-HSCs. Moreover, CD47<sup>+</sup> ST-HSCs as well as MPPs showed higher Flt3L-eGFP level, when compared to their CD47<sup>-</sup> counterpart, indicating that in combination with CD47 Flt3L-eGFP-reporter expression marks activated, potentially circulating HSCs (Fig. 1E). Taken together, these data suggest that Flt3L-eGFP-reporter<sup>low</sup>CD47<sup>-</sup> cells characterize a population of long-term resting LT-HSCs that might be located in hypoxic environments of bone marrow in vivo.

Another very well validated HSC marker is EPCR [46]. We show that EPCR surface expression is detected in both, but not all, Flt3L-eGFP-reporter<sup>low</sup> as well as Flt3L-eGFP-reporter<sup>high</sup> HSCs (Fig. 1F, Supporting Information Fig. 5).

Thus, we suggest, that Flt3L-eGFP-reporter expression further narrows the population of LT-HSCs and supports our conclusion of the present work, that Flt3L-eGFP-reporter<sup>low</sup> HSCs are a sub-population of HSCs, which is further enriched for LT-HSCs.

In agreement with previous analyses of Flt3L<sup>-/-</sup> mice [21, 22], these results suggest that Flt3L production and Flt3 expression induces HSC differentiation, and suppression of Flt3L and Flt3 expression conserves the LT-HSC status, and they show that Flt3 and Flt3L are produced by the same cell.

### Flt3L-eGFP-reporter<sup>low</sup> HSCs repopulate transplanted recipients for long periods of time

To test the repopulating capacities of FACS-enriched Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells (CD45.2<sup>+</sup>), 10 of these cells were intravenously co-transplanted with 2 × 10<sup>5</sup> cells of CD45.1 total bone marrow into lethally irradiated C57BL/6 CD45.1 recipient mice and the transplanted host analyzed for engraftment in bone marrow, spleen, thymus, and the peritoneal cavity (PC) 4, 8 and 16 weeks after transplantation.

Four to eight weeks after transplantation, all of the transplanted CD45.2 donor-derived cells generated donor cell chimerisms in ranges between 1 and 20% in the bone marrow, spleen, thymus, and PC (Fig. 2A–F). Thereafter, i.e. 16 weeks

post-transplantation, Flt3L-eGFP-reporter<sup>low</sup> HSC-derived donor cells continued to show engraftment in the different hematopoietic organs and cell lineages, seen in a continued donor cell chimerism (Fig. 2A–F, Supporting Information Fig. 6). By contrast, numbers of Flt3L-eGFP-reporter<sup>high</sup> HSC-derived donor cells decreased significantly between 8 and 16 weeks after transplantation.

These data show that Flt3L-eGFP-reporter<sup>low</sup> as well as Flt3L-eGFP-reporter<sup>high</sup> LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells were competent to repopulate the host, but only Flt3L<sup>low</sup> LSK CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells had long-term repopulating activity.

The Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> donor-derived CD45.2 HSCs showed different kinetics of appearance in bone marrow. Flt3L-eGFP-reporter<sup>high</sup> cells repopulated the bone marrow 4–8 weeks after transplantation, but later declined in numbers. By contrast, Flt3L-eGFP-reporter<sup>low</sup> cells were hardly detectable after 4 weeks, but appeared 8 weeks after transplantation and kept increasing until 16 weeks post-transplantation (Fig. 2A–F). Notably, the majority of the Flt3L-eGFP-reporter<sup>low</sup>-derived donor HSC pool retained a Flt3L-eGFP-reporter<sup>low</sup> phenotype (MFI fold increase: ~1) even 16 weeks post-transplantation and reconstituted all hematopoietic lineages (Fig. 2G, Supporting Information Fig. 6). In contrast, Flt3L-eGFP-reporter<sup>high</sup>-derived donor HSCs showed multi-lineage reconstitution only for 8 weeks upon transplantation, but not thereafter (Supporting Information Fig. 6). These results suggest that HSCs have long-term reconstituting potential as long as they do not express Flt3L, but loose long-term reconstituting capacities once they express Flt3L.

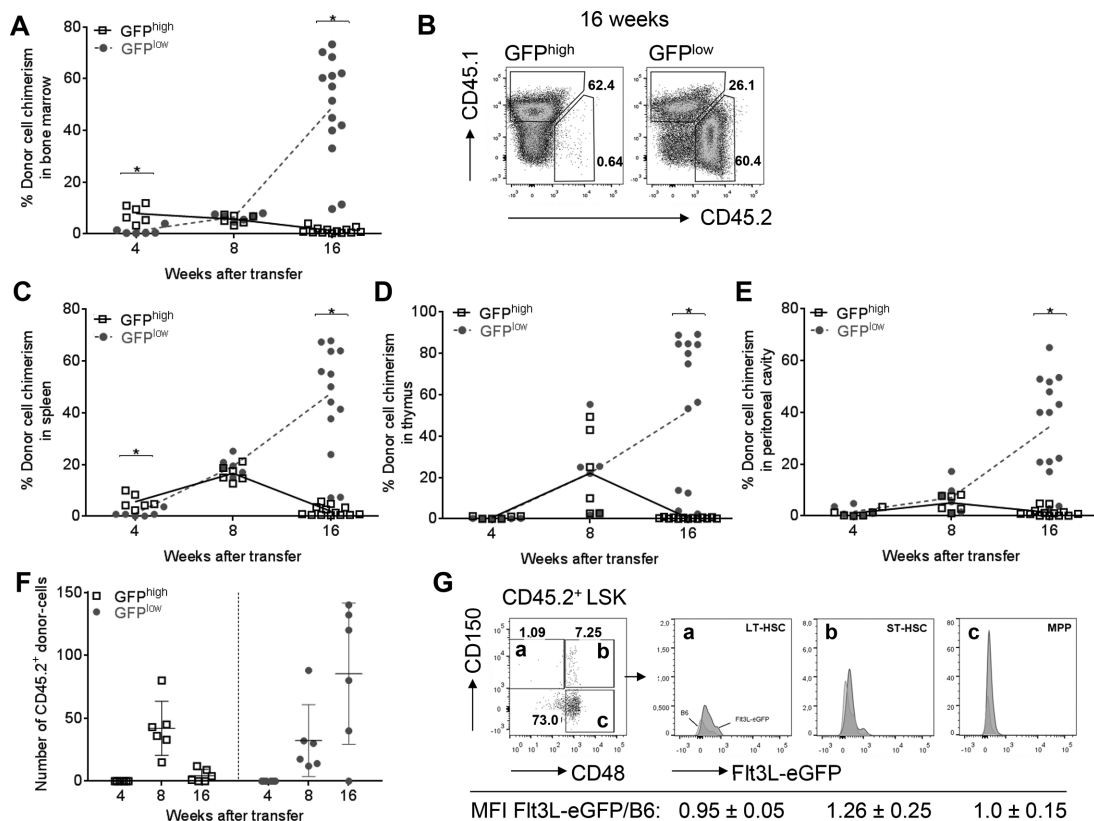
### Flt3L-eGFP-reporter<sup>low</sup> HSCs develop into Flt3L-eGFP-reporter<sup>high</sup> HSCs in vitro

We investigated the relationship of Flt3L-eGFP-reporter<sup>high</sup> with Flt3L-eGFP-reporter<sup>low</sup> LT-HSCs in in vitro cultures. As early as 3 days, and as long as after 10 days in culture, all of the Flt3L-eGFP-reporter<sup>low</sup> HSCs had changed to Flt3L-eGFP-reporter<sup>high</sup> cells, while all of the Flt3L-eGFP-reporter<sup>high</sup> HSCs had remained Flt3L-eGFP-reporter<sup>high</sup> (Fig. 3A–C; Supporting Information Fig. 7). When transplanted after 10 days of culture into irradiated recipients, the originally Flt3L-eGFP-reporter<sup>low</sup>, then Flt3L-eGFP-reporter<sup>high</sup> HSCs had lost their long-term repopulation potential (data not shown).

These results are consistent with the observation by us and others that LT-HSCs spontaneously lose their long-term repopulation potential in tissue culture.

### Differences in gene expression profiles between Flt3L-eGFP<sup>high</sup> and Flt3L-eGFP<sup>low</sup> HSCs

Different HSCs should differ in their gene expression programs, when they change from quiescence and long-term reconstitution potential to self-renewal and to differentiation [47–51].

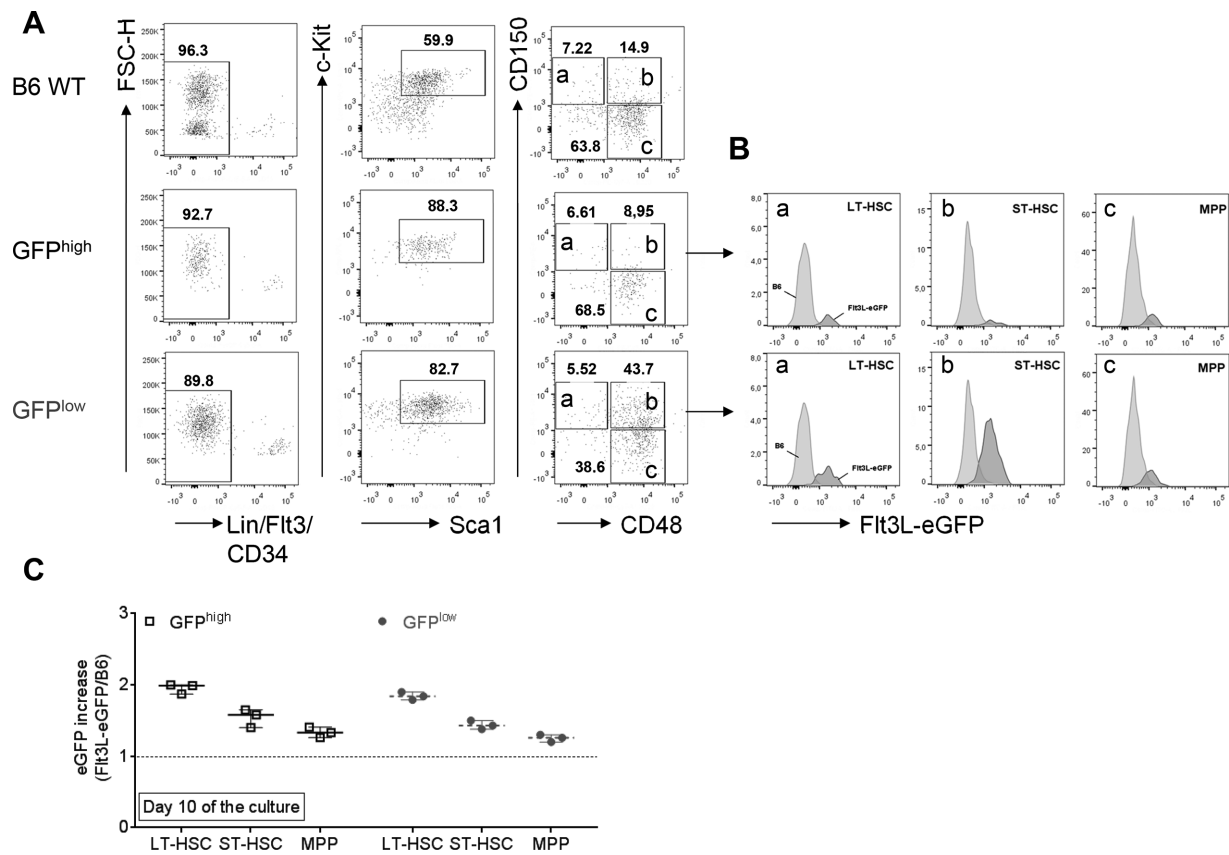


**Figure 2.** Transplantation of Flt3L-eGFP-reporter<sup>low</sup> and Flt3L-eGFP-reporter<sup>high</sup> bone marrow populations into lethally irradiated mice. Ten cells of sort-purified CD45.2 Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> LT-HSCs (see also Supporting Information Fig. 2) were transplanted with CD45.1 total bone marrow cells into lethally irradiated CD45.1 recipients. Mice were analyzed 4, 8, and 16 weeks after transplantation (see also Supporting Information Fig. 6). Recipient mice were sacrificed to analyze bone marrow, spleen, thymus, and the peritoneal cavity for donor-derived CD45.2 or CD45.1 cells by flow cytometry. (A) The percentage of CD45.2 donor cell chimerism in the BM. (B) shows representative flow cytometry plots of the percentage of CD45.1 and CD45.2 donor-derived cells in bone marrow 16 weeks after transplantation. (C) shows the percentage of CD45.2 donor cell chimerism in the spleen. (D) shows the percentage of CD45.2 donor cell chimerism in the thymus. (E) shows the percentage of CD45.2 donor cell chimerism in peritoneal cavity. (F) shows the number of CD45.2 donor-derived hematopoietic progenitor cells in bone marrow. (G) shows representative flow cytometry histogram overlays for the expression pattern of Flt3L-eGFP-reporter by donor-derived hematopoietic progenitor cells in bone marrow of transplanted recipients compared to that of C57BL/6J wild-type mice (B6,  $n = 3$ ). Live cell gates were set for LT-HSCs (LSK CD34<sup>-</sup> Flt3<sup>-</sup> CD150<sup>+</sup> CD48<sup>-</sup>), ST-HSCs (LSK CD34<sup>-</sup> Flt3<sup>-</sup> CD150<sup>+</sup> CD48<sup>+</sup>), and MPPs (LSK CD34<sup>-</sup> Flt3<sup>-</sup> CD150<sup>-</sup> CD48<sup>+</sup>). The Flt3L-eGFP-reporter MFI fold increase was calculated as the ratio between the eGFP MFI in cells of the Flt3L-eGFP reporter mice and wild-type C57BL/6J mice. A value of one indicates no eGFP expression. Black open squares represent Flt3L-eGFP-reporter<sup>high</sup> cells, while grey filled circles represent Flt3L-eGFP-reporter<sup>low</sup> cells. In overlay analyses light grey curves represent C57BL/6J wild-type mice, while dark grey curves represent Flt3L-eGFP-reporter mice. Arrow in Fig. 2G indicates direction of gating. Data in graphs are expressed as median  $\pm$  interquartile range and are pooled from three independent experiments with 6–24 mice per experiment. \* $p < 0.05$  by paired Student's *t*-test.

Finally, we screened Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> HSCs (Supporting Information Fig. 2) for their differential gene expression programs of selected sets of genes connected with niche interaction capacities (e.g. MPL, IL-6R, VCAM1, Ang1, integrins, chemokine receptors) [52, 53], hypoxia (e.g. Grp78, Hif-1 $\alpha$ , ABCG) [43, 44, 53–55], autophagy (e.g. LC3 and APG7) [11], self-renewal by proliferation (e.g. c-Kit, GATA1, p21) [42] and differentiation (e.g. transcription factors, Flk1, CD41, CD45) [56–58].

In our gene expression analyses we detected higher levels of expression for Grp78, Hif-1 $\alpha$ , MPL,  $\beta$ 4-integrin, p21 as well as LC3 and APG7 in Flt3L-eGFP-reporter<sup>low</sup> HSCs when compared to their Flt3L-eGFP-reporter<sup>high</sup> counterparts (Fig. 4A–C). Flt3L-eGFP-reporter<sup>low</sup> HSCs also express Hoxa10, Hoxb5 and Fgd5 transcription factors (Fig. 4D) that have been found

to be expressed in LT-HSCs previously [19, 20]. By contrast, expression levels for genes that are related to hematopoietic differentiation towards myeloid and lymphoid cell lineages, e.g. c-fms, c-Myb, GATA1, and ID2, were decreased in Flt3L-eGFP-reporter<sup>low</sup> HSCs (Fig. 4D). In addition, Flt3L-eGFP-reporter<sup>low</sup> HSCs express increased RNA levels of Flk1 and CD41 [56–58], and express CD41 protein and EPCR [46] on their surface (Fig. 1F; Supporting Information Fig. 5), while expression of EPCR remains, and CD41, CD45 and CD34 are decreased in Flt3L-eGFP-reporter<sup>low</sup> HSCs when compared to in Flt3L-eGFP-reporter<sup>high</sup> HSCs (Fig. 4D). These gene expression analyses support the interpretation that Flt3L-eGFP-reporter<sup>low</sup> HSCs are less mature than Flt3L-eGFP-reporter<sup>high</sup> HSCs and belong to hypoxic, autophagy-active cells. They may be localized in hypoxic stem cell niches. This conclusion is supported by our FACS analyses showing



**Figure 3.** Flt3L-eGFP-reporter<sup>low</sup> HSCs develop into Flt3L-eGFP-reporter<sup>high</sup> HSCs in vitro. LT-HSCs were sort purified from bone marrow of wild-type C57BL/6J mice as well as from bone marrow of Flt3L-eGFP reporter mice (Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> LT-HSCs; see also Supporting Information Fig. 2). Purified bone marrow populations were cultivated for 10 days without serum and stroma cells in serum free medium/ mSCF/ mL-6/ mTPO/ hAng1. (A) Representative FACS stainings of surface markers for hematopoietic progenitors ( $n = 3$ ). (B) Representative histogram overlays for the expression pattern of Flt3L-eGFP-reporter gated on hematopoietic progenitor cells at day 10 of the culture compared to that of C57BL/6J wild-type mice (B6) (see also Supporting Information Fig. 7;  $n = 3$ ). All analyses were performed on live cells. (C) Flt3L-eGFP-reporter MFI fold increase that was calculated as the ratio between the eGFP MFI in cells of the Flt3L-eGFP reporter mice and wild-type C57BL/6J mice ( $n = 3$ ). A value of one indicates no eGFP expression. Expression patterns of Flt3L-eGFP-reporter are shown as single-parameter histograms, where the Y-axis display a frequency distribution of the data versus fluorescence intensity. In overlay analyses light gray curves represent C57BL/6J wild-type mice, while dark gray curves represent Flt3L-eGFP-reporter mice. Black open squares represent Flt3L-eGFP-reporter<sup>high</sup> cells, while gray filled circles represent Flt3L-eGFP-reporter<sup>low</sup> cells. Arrow in Fig. 3B indicates direction of gating. Flow cytometry plots/histograms are from a single experiment representative of three experiments with three samples prepared from pooled mice per experiment.

that Flt3L-eGFP-reporter<sup>low</sup> HSCs are enriched for Hoechst<sup>low</sup> cells (Fig. 1C, Supporting Information Fig. 3), and are Ki67-negative (Fig. 1D, Supporting Information Fig. 4).

In summary, these gene expression analyses allow the conclusion that Flt3L-eGFP-reporter<sup>low</sup> HSCs are enriched for hypoxic, autophagy-active, less differentiated HSCs, while Flt3L-eGFP-reporter<sup>high</sup> HSCs are in majority proliferation-active, more differentiated HSCs.

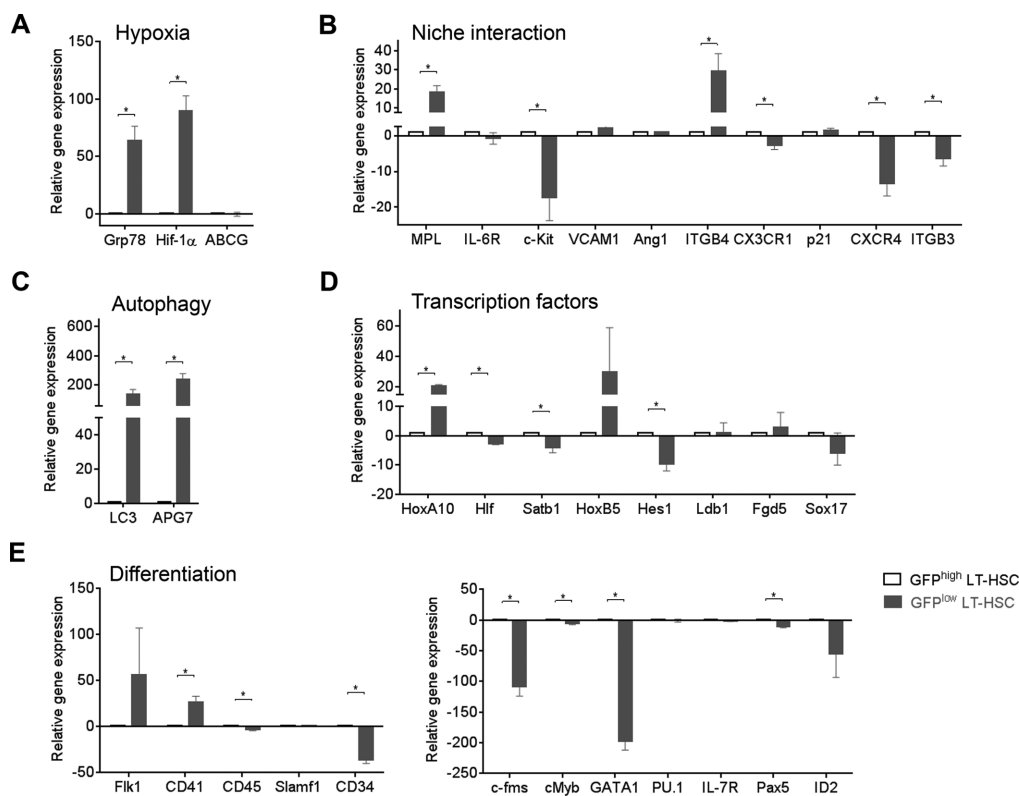
## Discussion

For the phenotypic characterization of the Flt3L-eGFP-reporter high or low expressing HSC subpopulations in the Flt3L-eGFP reporter mice, we make use of high or low mRNA expression of the Flt3L locus, detectable as a consequence of the

Flt3L-gene-associated promoter/enhancer activity which, in turn, drives high or low RNA and protein expression of the inserted reporter eGFP gene. It might be true, but is irrelevant for our distinction of the Flt3L-eGFP-reporter high and low HSCs, that RNA expression-dependent eGFP marker expression might not necessarily correlate with Flt3L-protein expression. Hence, we do not, and do not need to use different levels of Flt3L protein expression for this identification and distinction.

It should also be emphasized that it is not the intention of the present work to identify functions of Flt3L protein in HSC populations.

Dormancy, self-renewal by proliferation and differentiation to more committed hematopoietic progenitors, such as ST-HSCs, MPPs, CLPs and CMPs are properties of HSC pools which control their sizes by interactions of cytokines and chemokines with their receptors, and by integrin- as well as cadherin-mediated



**Figure 4.** Differences in gene expression profiles between Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> LT-HSCs. Panel A-E shows the differential gene expression analyses of (A) hypoxia-related genes, (B) niche-interaction genes, (C) autophagy genes, (D) transcription factors and (E) differentiation-related genes on sort purified Flt3L-eGFP-reporter<sup>high</sup> and Flt3L-eGFP-reporter<sup>low</sup> LT-HSCs (see also Supporting Information Fig. 2). Relative expression of mRNA of selected genes was assessed by quantitative real-time PCR and normalized to murine GAPDH. Data in graphs are expressed as median  $\pm$  interquartile range and are from a single experiment representative of two experiments with three independent biological samples prepared from pooled mice per experiment. qRT-PCR runs were performed in technical triplicates. \* $p < 0.05$  by paired Student's *t*-test. In gene expression analyses open bars represent Flt3L-eGFP-reporter<sup>high</sup> cells, while filled bars represent Flt3L-eGFP-reporter<sup>low</sup> cells.

cell-to-cell contacts in niches of bone marrow [27, 59, 60]. Hypoxic, autophagy-active HSCs divide at best twice a year, while HSCs, activated by inflammation, injury or G-CSF divide as often as once a day. Activation can mobilize activated HSCs to leave their niches and enter blood and peripheral circulation, from where HSCs can return to quiescence by re-entering a hypoxic, autophagy-active HSC state [5, 6, 61–65].

The cytokine Flt3L, in interaction with its receptor Flt3 has been identified as a non-redundant regulator of HSCs [24, 25]. Flt3 is selectively expressed at early stages of hematopoiesis [26, 27, 40]. Mice with deficiencies in either Flt3L or Flt3 show deficient B-, NK-, and dendritic cell development, while myelopoiesis remains unaltered [21, 24]. This suggested that Flt3L/Flt3 interactions are needed for proper lymphoid development, while myeloid cells could develop from HSCs without stimulatory influence of Flt3L and its receptor.

Upregulation of Flt3 expression in HSCs from a LSK Flt3<sup>-</sup> to a LSK Flt3<sup>+</sup> state in bone marrow is accompanied by the loss of long-term reconstituting activity [32], but nothing was known about Flt3L expression. Flt3L has been found to be a stimulator of HSC proliferation and expansion [22, 62–65], and Flt3L-deficient HSCs show reduced *in vivo* reconstitution potential [26].

Since Flt3L-stimulated *in vitro* expanded HSC have been found to lose its long-term reconstituting potential [33]. The number of Flt3L-GFP<sup>low</sup> HSCs ( $5 \times 10^3$ ) is too low for a determination of the expression of Flt3L-protein (assumed to be at best in the range of  $5 \times 10^4$  Flt3L-protein molecules per cell). Thus, we can only hypothesize that, if Flt3L-protein was expressed in activated HSCs, such, autocrine Flt3L-protein expression might influence the generation and maintenance of LT-HSCs *in vivo* in ways, that cannot be stabilized *in vitro*. Alternatively, Flt3L/Flt3 interactions might stimulate differentiation from LT-HSCs to MPPs and CLPs. If so, this would lead to lymphoid deficiencies in Flt3L- and Flt3-deficient mice. In line with this latter activity of Flt3L/Flt3 interactions are the findings that Flt3 and Flt3L are dispensable for maintenance and post-transplantation expansion of LT-HSC pools [31]. It remains to be investigated, what controls the expression of Flt3 and Flt3L, how an autocrine stimulation in HSCs induce hematopoietic differentiation and whether Flt3 overexpression in AML-malignancies already affects this earlier stage of Flt3/Flt3L activity [66–68].

Bone marrow also provides a variety of Flt3L-producing cells, which could be sources of paracrine Flt3/Flt3L stimulation on HSCs. In bone marrow, 95% of all Flt3L is ubiquitously expressed

by hematopoietic cells, such as NK cells, basophils, NK-T cells B cells, granulocytes, monocytes and dendritic cells, while only 5% is produced by non-hematopoietic endothelial and stromal cells [34]. However, when the generation of dendritic cells in spleen was taken as a measure of hematopoietic differentiation, Flt3L production by NK cells, NK-T cells, granulocytes and monocytes was found to redundant and not critical for dendritic cell homeostasis. On the other hand, Flt3L production by T and B cells was found to influence dendritic cell generation. It remains to be investigated, whether HSCs also use Flt3L in an paracrine fashion, e.g. provided by Flt3L-producing environmental cells of their niches.

In conclusion, our expression analyses suggest that, in majority, the Flt3L<sup>-</sup>/Flt3<sup>-</sup> HSCs are hypoxic and autophagy-active cells (Fig. 4). Some of these CD150<sup>+</sup> cells show mRNA expression of Flk1 and CD41, early markers of which appear before CD45 and CD34 expression during differentiation of embryonic stem cells to hematopoietic cells [56–58]. Thus, these Flt3L-eGFP-reporter<sup>low</sup> cells showing high CD41 and low CD45 mRNA expression might define a developmental stage between hemangioblasts and HSCs. When HSCs express Flt3L and Flt3 they upregulate differentiation markers.

Under hypoxia, Hif-1 $\alpha$  regulates the expression of a number of genes that are critical for HSC activity and quiescence, including VEGF, SCF, Ang2, p16, p19, p21, FOXOs, and others, and interacts with other proteins involved in HSC signaling [67]. In fact, *Sironi et al.* [68], described a hypoxia-mediated down-regulation specific for Flt3 that is reversible and proteasome-dependent. Our results suggest that Hif-1 $\alpha$  could also negatively regulate Flt3L expression. This in turn, could then regulate quiescence versus self-renewal and differentiation in hypoxic niches in bone marrow. Whenever HSCs move from the hypoxic, endosteal area to the central non-hypoxic bone marrow Flt3L would become expressed, and HSCs would differentiate. *Chen et al.* [20] have recently shown that HoxB5 expression marks LT-HSCs. Our expression analyses show that Flt3L/Flt3mRNA<sup>-</sup> LT-HSCs differentially express HoxB5. Since we have successfully transplanted as little as 10 of such Flt3L/Flt3mRNA<sup>-</sup> LT-HSCs to obtain long-term repopulation of irradiated hosts our analyses suggest that many of the HoxB5<sup>+</sup> LT-HSCs are Flt3L<sup>-</sup>/Flt3<sup>-</sup>. Future single cell expression analyses should be able to evaluate the precise relationship of HoxB5<sup>+</sup> with Flt3L/Flt3mRNA<sup>-</sup> LT-HSCs.

In this study, we showed that Flt3L-eGFP-reporter expression was one criteria for distinguishment of repopulatable LT-HSCs. It is still unclear whether Flt3L/Flt3 mRNA-expression is reversed, when an activated HSC re-enter a hypoxic, autophagy-active state [6]. Single cell RNA sequencing would be the most informative way to characterize these HSC populations.

If such flexibility of Flt3L/Flt3 mRNA-expression exists, it suggests that the controls of expression of Flt3L/Flt3 could be targets for externally induced down-regulation to stabilize and favor the LT-HSC state for improvements in bone marrow transplantations, and for externally induced up-regulation to terminate a long-term cancer stem cell pool in AML and other early leukemia [69, 70].

## Materials and methods

### Mice

CD45.1 C57BL/6 and Flt3L-eGFP reporter mice (CD45.2 C57BL/6) [34] were bred in our facilities. Mice were maintained under specific pathogen-free conditions, and all animal experiments were approved by the local ethics committee of the German authorities (State Office of Health and Social Affairs Berlin; *Landesamt für Gesundheit und Soziales Berlin*, # G0236-12).

### Antibodies

For analyses by flow cytometry and cell sorting, the following anti-mouse antibodies were used: Mac1 (M1/70), Gr1 (RB6-8C5), Ter119 (TER-119), CD19 (1D3), B220 (RA3-6B2), CD5 (53-7.3), CD3 $\epsilon$  (145-2C11), CD11c (N418), CD4 (GK1.5), CD8 (53-6.7), NK1.1 (PK136), CD41 (MWRReg30), EPCR (RMEPCR1560), CD45.1 (A20), CD45.2 (104), CD47 (miap301), c-Kit (2B8), Sca1 (D7), CD34 (RAM34), Flt3 (A2F10), CD150 (TC15-12F12.2), CD48 (HM48-1), and CD31 (390). All antibodies were obtained from eBioscience (San Diego, CA). Propidium iodide (PI; 5  $\mu$ g/ml, Calbiochem) was added for live–dead cell discrimination.

For intracellular Ki67 staining, BM cells were stained with surface markers, fixed, and permeabilized using a Cytofix/Cytoperm kit according to the manufacturer's instructions (BD) and stained with an anti-mouse Ki67 antibody (SolA15) for 1 h at room temperature. In cell sortings, cells were purified in a purity of >98%. Data of flow cytometry were analyzed by using the FACSDiVa and FlowJoV10 software.

### Hoechst staining

Bone marrow cells were resuspended in PBS supplemented with 2% FCS (Thermo Fisher scientific) and 2mM HEPES buffer (SP-buffer) (Thermo Fisher Scientific), prewarmed to 37°C and incubated with Hoechst 33342 (Molecular Probes, Life Technologies) at 5  $\mu$ g/mL for 2 h at 37°C. All subsequent steps were carried out on ice. Cells were resuspended in ice-cold SP-buffer and stained with antibodies against lineage markers as described above. As negative control, we used bone marrow cells pre-incubated with verapamil (50  $\mu$ M, Sigma Aldrich). Lineage-negative, single, viable and nucleated cells showing a dim staining in the Hoechst blue and Hoechst red channels were identified as HSCs. Cells were analyzed on a LSRII flow cytometer (Aria II, BD Biosciences).

### In vitro culture of bone marrow-derived HSCs

Purified HSCs from bone marrow of 8–10 weeks old male C57BL/6 CD45.1 and C57BL/6 CD45.2 Flt3L-eGFP mice were cultured without any supporting stromal cell layer in serum-free StemPro<sup>®</sup>-34 SFM (Thermo Fisher scientific) supplemented with murine recom-



binant SCF (100 ng/mL; Peprotech), IL-6 (5 ng/mL; Peprotech), TPO (10 ng/mL; Peprotech), and human recombinant angiopoietin 1 (20 ng/mL; Peprotech). Cell suspensions were plated on cell culture flasks and cultivated in a humidified incubator at 37°C, 10% CO<sub>2</sub> for 10 days. Thereafter, cultivated cells were prepared for FACS analyses or transplantation.

### Chimeric transplantation (adoptive transfer)

Before chimeric transplantation, freshly isolated bone marrow cells (male C57BL/6 CD45.2 Flt3L-eGFP or C57BL/6 CD45.1) were washed twice with PBS to remove cell debris and FCS and were passed through a MACS<sup>®</sup> Pre-Separation Filter with 30 μm mesh. Thereafter, 2 × 10<sup>5</sup> cells of C57BL/6 CD45.1 bone marrow together with 10 cells of sorted HSC populations of C57BL/6 CD45.2 Flt3L-eGFP reporter mice were resuspended in a total volume of 100 μL PBS for one C57BL/6 CD45.1 recipient mouse and injected into the lateral tail vein. If not stated otherwise, recipient mice were lethally irradiated with 1010 rad 1 day before the transfer. To prevent the risk of infection after irradiation, recipient mice were fed Baytril (125 mg/l) in the drinking water from one week before the transfer. Mice were analyzed 4, 8, and 16 weeks post-transplantation.

### RNA/qRT-PCR

Cells were homogenized and RNA was isolated via TRIzol total RNA isolation reagent (Thermo Fisher Scientific) using glycogen as a carrier according to the suppliers' recommendation. One hundred ng of total RNA was reverse-transcribed by SuperScript III (Thermo Fisher Scientific) primed with oligo(dT) (Thermo Fisher Scientific).

The cDNA for the specific target assays was then amplified by indicated primers (Supporting information Table 1). RNA abundances of specific genes were normalized to murine GAPDH as endogenous control (Supporting Information Table 1). Each sample was assayed in biological and technical triplicates. RNA from total mouse bone marrow cells was used to construct a standard curve for all inspected genes, proving specificity and reliability of the designed primer. In order to reduce amplification backgrounds with primers, we performed quantitative PCR analyses using a “no template = water control” for every run. The PCR amplification was detected as an increase in fluorescence with the ABI PRISM 7700 instrument. RNA was quantified using the SDS software, version 2.2.2.

### Statistics

For all statistical analyses, PRISM (Version 6, GraphPad, San Diego) software was used. Dispersion is presented as the median ± interquartile range, unless stated otherwise. Statistical analysis

was performed with paired Students *t*-test and *p* values <0.05 were considered significant.

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**Abbreviations:** HSCs: hematopoietic stem cells · LT-HSC: long-term repopulating HSCs · ST-HSC: short-term HSC · Flt3L: fms-like tyrosine kinase-3 ligand · EPCR: endothelial protein C receptor · SCF: stem cell factor · TPO: thrombopoietin · Ang1: angiopoietin · LSK: Lin<sup>-</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup> · MPP: multipotent progenitor · CLP: common-lymphoid progenitor · CMP: common-myeloid progenitors · SP: side population

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