Divisional History and Hematopoietic Stem Cell Function during Homeostasis

Jiajing Qiu,1,3,4 Dmitri Papatsenko,1,3 Xiaohong Niu,1,3 Christoph Schaniel,2,3 and Kateri Moore1,3,*

1Department of Developmental and Regenerative Biology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1496, New York, NY 10029, USA
2Department of Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1496, New York, NY 10029, USA
3Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1496, New York, NY 10029, USA
4The Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1496, New York, NY 10029, USA
*Correspondence: kateri.moore@mssm.edu
http://dx.doi.org/10.1016/j.stemcr.2014.01.016
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SUMMARY

We investigated the homeostatic behavior of hematopoietic stem and progenitor cells (HSPCs) temporally defined according to their divisional histories using an HSPC-specific GFP label-retaining system. We show that homeostatic hematopoietic stem cells (HSCs) lose repopulating potential after limited cell divisions. Once HSCs exit dormancy and accrue divisions, they also progressively lose the ability to return to G0 and functional activities associated with quiescent HSCs. In addition, dormant HSPCs phenotypically defined as multipotent progenitor cells display robust stem cell activity upon transplantation, suggesting that temporal quiescence is a greater indicator of function than cell-surface phenotype. Our studies suggest that once homeostatic HSCs leave dormancy, they are slated for extinction. They self-renew phenotypically, but they lose self-renewal activity. As such, they question self-renewal as a characteristic of homeostatic, non-perturbed HSCs in contrast to self-renewal demonstrated under stress conditions.

INTRODUCTION

Hematopoiesis is a developmental system uniquely suited for studies of regulatory mechanisms governing complex programs of cellular differentiation. The blood consists of at least ten distinct cell types, all with finite life spans that require continuous replenishment throughout life. Hematopoietic stem cells (HSCs) anchor this hierarchical system. These cells can self-renew, die, or commit to programs of differentiation, which give rise to new classes of hematopoietic stem and progenitor cells (HSPCs) distinguished by more restricted self-renewal, proliferative, and differentiation abilities. Clearly, both intrinsic and extrinsic regulatory mechanisms collectively regulate the balance of self-renewal and differentiation in order to ensure life-long, balanced, and multilineage hematopoiesis.

Almost everything we know about HSPC activity has been defined in terms of in vivo transplantation assays. These have been extremely useful in elucidating phenotypically defined compartments of the hematopoietic hierarchy with respect to their long-term (LT) and short-term (ST) repopulating potentials as well as self-renewal abilities in the context of serial transplantation. However, they provide no direct insights into the behavior of HSC populations during normal nonperturbed homeostasis. In actuality, transplantation assays measure a cell’s inherent ability to respond to the extreme stress of the assay itself. Because HSC proliferation and differentiation are inextricably linked, methods to study these cells as they proliferate in situ are necessary. Quiescence has emerged as a hallmark property of HSCs. Primitive HSCs generally reside in the G0 phase of the cell cycle but in broad ranges depending on their phenotype and experimental methodologies (Pietras et al., 2011). However, quiescence measurements provide only a “snapshot” of the immediate status of HSCs. They do not provide information about the duration of quiescence, previous divisional history, the time of entrance into quiescence, and how these aspects correlate with stem cell function. Previous studies have determined the in vivo proliferative status of HSPCs by the incorporation of DNA nucleoside analogs (Cheshier et al., 1999; Kiel et al., 2007). This methodology precludes functional assessment, yielding only correlative information reliant on cell phenotype. More recent studies of HSPC divisional kinetics and subsequent activity employ viable label-retaining cell (LRC) tracking systems. These methods include in vivo biotin labeling (Nygren and Bryder, 2008), in vitro labeling with fluorescent dyes (Takizawa et al., 2011), or dynamic chromosomal labeling with a controllable histone 2B GFP fusion protein (H2BGFP) (Foudi et al., 2009; Schaniel and Moore, 2009; Wilson et al., 2008). These studies revealed HSCs with differential activities and abilities dependent on the context of either homeostasis or stress. Two studies using controllable H2BGFP labeling revealed dormant and activated HSC populations, with the former containing the majority of repopulating stem cell activity (Foudi et al., 2009; Wilson et al., 2008). Dormant HSCs divide very rarely, with less than 1% entering the cell cycle.
Divisional History in Homeostatic HSCs
per day (Foudi et al., 2009; Wilson et al., 2008). In contrast, another study suggested that fast-cycling HSCs contribute to long-term hematopoiesis while slowing down over time (Takizawa et al., 2011). However, this study relied on in vitro labeling followed by transplantation into nonconditioned recipients, a process requiring a range of behaviors not occurring during normal homeostasis. In one study, injury-activated HSCs, defined phenotypically, but not functionally, were shown to go back to dormancy (Wilson et al., 2008). It remains to be seen if homeostatic HSCs that have divided extensively and subsequently returned to quiescence maintain the same functional activities as those that remained dormant.

Our studies employ a transgenic system with H2BGFP expression controlled by an HSPC-specific human (hu) CD34 promoter (Radomska et al., 2002). In this Tet-off system, HSPCs continually incorporate H2BGFP until doxycycline (Dox) is administered (Schaniel and Moore, 2009). We have investigated the properties of HSPCs as they proceed through a divisional cascade defined by progressive label dilution during normal homeostasis. We find that dormancy is a better predictor of stem cell activity than cell-surface phenotypes or snapshot quiescence. Once HSCs leave dormancy and enter the active pool, they progressively lose repopulating and self-renewal activities. Our studies highlight the importance of the active pool in the maintenance of homeostatic hematopoiesis and suggest that, once dormant HSCs are activated, they are slated for extinction. As such, this would provide an important control mechanism for hematological malignancies and other disorders of the hematopoietic system.

RESULTS

Characterization of Stem/Progenitor Cells in huCD34tTA/TetO-H2BGFP Mice

We measured GFP levels in phenotypically defined bone marrow (BM) HSPC subsets in huCD34tTA/TetO-H2BGFP (34/H2B) double transgenic mice prior to a Dox chase. Prospectively identified primitive compartments were analyzed at 6–8 weeks of age to ensure complete labeling and phenotypic stability (Osawa et al., 1996). GFP gating was determined by comparative analysis with single transgenic TetO-H2BGFP mice previously shown to have low levels of background fluorescence (Challen and Goodell, 2008). In 34/H2B mice, high levels of GFP were observed in all HSPC compartments with transplantable activities (Figure 1A). High GFP levels in CD34–CD135– Lineage–Sca-1+ cKit+ (LSK) cells confirm that the huCD34 promoter is active in mouse CD34–HSC. GFP expression is diminished after the CD34+CD135+ LSK multipotent progenitor (MPP) stage. These data demonstrate that the huCD34 promoter directs highest levels of GFP expression in the more-primitive HSPC compartments. We turned off GFP expression during a 12-week Dox chase. Figure 1B displays typical GFP histograms for phenotypically defined repopulating HSCs before and after the chase. High levels of GFP retention are segregated to cells with LT-repopulating HSC phenotypes. A kinetic analysis of label dilution confirmed a previously observed biphasic dilution pattern (Figure S1A available online; Foudi et al., 2009; Wilson et al., 2008). To determine the immediate snapshot cell-cycle status of GFP+ LRC, we stained LSK cells with Pyronin Y and Hoechst 33342 (Py/Ho) (Passegué et al., 2005; Figure 1C). GFP+ cells are distributed throughout all phases of the cell cycle in the no Dox GFP+ fraction, whereas in Dox-treated mice, most GFP+ cells are in G0 (59.2% versus 90.5%, respectively). These data demonstrate that GFP+ LRCs are enriched for quiescent cells that have remained dormant during the prolonged chase period.

In Vitro and In Vivo Activities of GFP Label-Retaining Cells

A distinct advantage of our system is that it allows the use of GFP for viable HSC enrichment without a bias for known phenotypic markers. Because the huCD34tTA

Figure 1. Characterization of Stem/Progenitor Cells in 34/H2B Mice

(A) Percentage of GFP-positive cells in BM compartments of 34/H2B mice. Mean ± SD; n = 3 mice.

(B) GFP histograms of BM compartments in LSK cells before (top) and after (bottom) Dox treatment (12 weeks, chase).

(C) Cell-cycle profiles of LSK cells before and after Dox treatment (12 weeks). All LSK (left), gating for GFP+ LSK (middle), GFP+ LSK (right). Upper panels, before Dox; lower panels, after Dox. G0, Py20+Ho20, G1, Py20+Ho20, S/G2/M, Py30+Ho30 < 2N–4N.

(D) LTC-IC assay of GFP-positive or negative LSK cells. cfus were normalized to the amount derived from 100 cells initiating the LTC. Mean ± SD; n = 3 separate cultures.

(E) LTC-CAFC assay of GFP-positive or negative LSK cells. The frequency of CAFCs was normalized to the amount derived from 100 cells initiating the LTC.

(F) Peripheral blood leukocyte (PBL) chimerism (%CD45.2+ donor-derived cells) 8 months after transplant of GFP-positive and negative LSK cells isolated after 10 weeks chase. 5, 15, 45, 135, or 405 cells were transplanted into SJL mice together with 400,000 SJL BM. Each circle represents an individual mouse (3–5 mice/cell dose). Mice with <1.0% CD45.2+ cells were considered negative. See also Figure S1.
transgene is also expressed in endothelial and some mesenchymal cells, we used LSK cells to exclude nonhematopoietic cells. We observed no significant differences between isolated LSK GFP+ and GFP− cells from mice chased for 12 weeks in primary colony-forming cell (cfu) assays (Figure S1B). We also plated these cells in long-term culture (LTC) (Miller et al., 2008) and in primary limiting-dilution cobblestone-area-forming cell (LD-CAFC) assays (de Haan and Plomacher, 2002). CAFC activity in GFP− cells peaked early and disappeared, whereas CAFC activity in the GFP+ population appeared late and persisted (Figure S1C). After LTC, cells were harvested and plated into cfu assays for LTC-initiating cell (IC) and in secondary LD-CAFC assays to measure primitive progenitors more closely related to repopulating HSCs. Strikingly, no activity was seen when GFP− cells were used to initiate the LTC, whereas high frequencies were present in the GFP+ fraction (Figures 1D and 1E). These data suggest that LSK cells that have divided extensively during the chase period have lost in vitro potential characteristic of primitive HSPCs. We extended our functional studies to in vivo competitive repopulation assays. Peripheral blood from recipients was analyzed for the presence of donor-derived CD45.2+ B, T, and myeloid cells. The repopulating activity of LSK GFP− cells was minimal, whereas LSK GFP+ cells performed extremely well (Figures 1F and S1D). Such repopulation efficiencies are remarkable for LSK cells and suggest that temporally defined quiescence further enriches for HSCs with robust in vivo potential. We also assessed LT self-renewal potential by transplantation into secondary mice. Only BM from primary mice engrafted with GFP+ cells was able to repopulate secondary recipients, demonstrating that LSK GFP− cells had lost their ability to self-renew (data not shown).

**Relating Biological Activity to Divisional History**

**Functional Characterization of HSPCs with Varying Levels of GFP Label Retention**

The dramatic differences in biological potency of GFP+ and GFP− cells prompted us to dissect the entire GFP+ compartment to reflect progressive divisional histories. The proliferation index utility in Flowjo software (Treestar) predicts that LSK cells with high levels of GFP need six or seven divisions to completely dilute GFP (Figure S2A). This estimate is only predictive and does not imply that this is a synchronized uniformly dividing cell population. The proliferation index also suggests that only one or two cell divisions separate each successive GFP level. The GFP dilution profile of Dox-treated 34/H2B LSK cells was divided into progressively brighter GFP 0 to GFP 1, GFP 2, GFP 3, and GFP 4 levels (Figure 2A). These fractions were isolated and studied by transplantation and gene-expression profiling. Repopulating activity correlated with GFP retention. Only the GFP3 and GFP4 fractions contained cells that had LT-repopulating abilities (Figures 2B and 2C). Most surprisingly, secondary mouse repopulation potential was restricted to the brightest GFP4 fraction (Figure 2D).

CD48+ cells are devoid of repopulating potential (Kiel et al., 2005). Further enriching the LSK population by excluding CD48+ cells did not show a difference in total cfu (Figure 2E), although there were significant differences in the percentage of more-primitive cfu-mix colonies among the fractions (Figure S2B). LD-CAFC revealed kinetic differences in the appearance and extinction of colonies. Only GFP3 and GFP4 cells displayed CAFC at 4 weeks (Figure 2F). The LTC assays were highly correlated with GFP retention (Figures 2G, 2H, and S2C). Collectively, these in vivo and in vitro assays suggest that, once HSCs initiate a course of cell division, they progressively lose functional

**Figure 2. HSC Activity Correlates with Divisional History**

(A) After 12 weeks chase, the LSK GFP histogram (red line) was divided into five fractions from low- to high-GFP retention. Prior to chase, 90% of LSK cells were in the GFP 3 and GFP 4 fractions (green line). Dotted gray line, H2B GFP control.

(B) Chimerism 5 weeks and 3 and 6.5 months posttransplantation of LSK cells isolated from each GFP level. One hundred cells from each fraction were transplanted into 4–6 SJL-recipient mice together with 200,000 SJL-competitor BM cells. Data are derived from one of two transplant experiments with consistent results. Each circle represents an individual mouse.

(C) Donor reconstitution to each lineage at 3 months after transplant. Mean ± SD; n = 4–6 mice.

(D) Donor reconstitution to each lineage at 3.5 months postsecondary transplantation of pooled BM (2 × 10^6) from primary recipient mice from each GFP group from two transplant experiments. Three to five SJL mice were transplanted per group. Mean ± SD; n = 3–5 mice.

(E) cfu assay of LSKCD48− cells at each GFP level; colonies per 100 cells; mean ± SD; n = 3 separate cultures.

(F) LD-CAFC assay of LSKCD48− cells at each GFP level.

(G) LTC-IC assay of LSKCD48− cells at each GFP level; cfus were normalized to the amount derived from 100 cells initiating the LTC. Mean ± SD; n = 3 separate cultures.

(H) LTC-CAFC assay of LSKCD48− cells at each GFP level; frequency of CAFCs was normalized to the amount derived from 100 cells initiating the LTC.

Significance of donor contribution to total PBL (C and D) and of total colony numbers (G) by Student’s t test; *p < 0.05; **p < 0.01. See also Figure S2.
Figure 3. Phenotypic Analyses of HSPCs within Each GFP Dilution Fraction
Phenotypic analyses of GFP LRCs contained within a specific HSPC phenotype population or as a phenotypic characterization of each GFP level after a 12-week Dox chase period.

(A) The stacked bars are composed of the percentage of each GFP level that contributes to that specific HSPC phenotype as indicated. The percent of GFP4 cells in each phenotype is indicated on top of the bar graph.

(B) The distribution of CD135 and CD34 subfractions of LSK cells in each GFP level.

(C) The distribution of CD48 and CD150 subfractions of LSK cells in each GFP level.

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activities. Most surprisingly, compromised functions are already evident after only a few cell divisions.

**Phenotypic Characterization of BM with Varying Levels of GFP Label Retention**

A potential explanation for the loss of stem cell activity in the dividing fractions would be the loss of cells with an HSC cell-surface phenotype that were initially present within the LSK population. To address this possibility, we performed in-depth analyses of HSPC phenotypes across the divisional cascade. Cells with high levels of label retention, GFP4, are segregated to those with a LT-HSC phenotype (CD135−CD34−LSK 13.4%, CD48−CD150+LSK 17.1%, and CD48−CD150+CD135−CD34−LSK 19.2%), whereas phenotypic ST-HSCs represent 2% to 3% of GFP4 cells and MPPs are almost undetectable in the GFP4 fraction (less than 1%; Figure 3A). The percentages of each population in each GFP compartment from whole BM are presented in Table S1. We next looked at these markers in LSK cells throughout the GFP dilution cascade. Surprisingly, LSKCD34−CD135− and LSKCD34+CD135− cells are present at similar percentages in GFP3 and GFP4 (Figures 3B and S3A). Interestingly, the lower GFP fractions are similar to total LSK cells, although they do not contain repopulating stem cells (Figure 2B). LSKCD48−CD150+ cells are enriched in GFP2, GFP3, and GFP4 fractions, with the GFP3 portion having the highest level (Figure 3C). CD48 appeared in the low-GFP fractions (Figures 3C and S3B). Intrigued with the large percentage of CD34+ and CD150− cells in the high-GFP retaining cells, we asked how CD34 and the MPP marker CD135 were distributed in both LSKCD48−CD150− and LSKCD48−CD150+ populations. CD34−CD135− and CD34−CD135−CD34+ cells were highly enriched in the LSKCD48−CD150− GFP4 population, whereas CD135+ cells appear once these cells exit GFP4 (Figures 3D and S3C). The LSKCD48−CD150+ cells contain large proportions of both CD34+ and CD34− cells in the GFP2, GFP3, and GFP4 compartments with few CD135+ cells (Figures 3E and S3D). Gene-expression profiles of cells used in the transplantation assays shown in Figure 2 support the changing cell-surface markers observed within the divisional cascade (Figure S3E). The persistence of cells defined as ST-HSC (LSKCD34+CD135−) and MPP (LSKCD150−CD48−) in GFP4 cells highlights the heterogeneity of the dormant population. These data also show that the loss of self-renewal potential as LSK cells exit GFP4 cannot be explained by a loss of phenotypically defined LT-HSCs because the GFP3 and GFP4 fractions are almost identical, suggesting that divisional history is a better indicator of stem cell function than phenotype.

**Functional Heterogeneity in the Dormant GFP Label-Retaining Population**

Recent reports have shown CD150 expression heterogeneity in primitive phenotypically defined HSC populations. These studies suggested that CD150−/lo HSCs provide LT-HSC activity in primary transplants but lack self-renewal potential measured in secondary recipients (Moriga et al., 2010; Weksberg et al., 2008). We elected to investigate the functional significance of CD150 expression within the dormant GFP high (Hi) LRC fraction. GFP Hi CD48−LSK cells display three distinct levels of CD150: high (Hi), medium (Med), and low (Low) (Figure 4A). All fractions, including parental CD48−LSK cells, repopulated, but the CD150 Low cells were significantly less robust (Figures 4B and 4C). Recipient BM revealed a gradual decrease in CD45.2+ cells correlated with decreasing donor levels of CD150. Significant differences were seen in all BM HSPC compartments in total CD48−LSK and CD150 Hi recipient groups versus CD150 Low (Figure S4A) groups. The highest secondary reconstitution activity was seen in BM from primary CD150 Hi recipients (Figure 4D). Analyses of secondary recipient BM again revealed robust reconstitution from the CD150 Hi primary donor group (Figure S4B). Although dormant CD150 Low cells can LT-repopulate primary mice, they do not regenerate self-renewing stem cell pools capable of secondary engraftment. The most robust repopulating and self-renewing HSCs were contained within the CD150 Hi portion, extending previous observations to the dormant stem cell pool.

Gene-expression analyses of these same subsets unveiled a surprising degree of specificity that segregates with CD150 levels. Genes are highly differentially expressed in the CD150 Hi, Med, and Low populations (Figures 4E and 4F). Est1, Tgm2, Smacar2, Milt3, Icam1, Hif, and Cdkn1c (p57) emerge as candidate regulators of dormant CD150 Hi cells. These data provide insights into the molecular changes that occur as dormant HSCs downregulate the expression of CD150.

**Correlating Divisional History with Stem Cell Phenotype and Repopulation Potential**

Expression of CD34 has been considered a feature that delineates murine ST/MPP from LT (CD34−) HSPC activities (Osawa et al., 1996; Yang et al., 2005). To our knowledge, additional properties of CD34+ HSPCs have not been

(D) The distribution of CD135 and CD34 subfractions of LSKCD48−CD150− cells in each GFP level.

(E) The distribution of CD135 and CD34 subfractions of LSKCD48−CD150+ cells in each GFP level.

Significance determined by Student’s t test for comparison of the primitive phenotype in each group (green bars): *p < 0.05; **p < 0.01; ***p < 0.001; mean ± SD; n = 3 to 4 mice per analysis. See also Figure S3.
Figure 4. Functional Analysis of GFP-High LRCs with Heterogeneous Expression of CD150

(A) Gating strategy for the isolation of CD150 Hi, Med, and Low subfractions of GFP-high LSKCD48− cells and the parent population. Cells were isolated after 13 weeks chase.

(B) Chimerism at 5.5 weeks and 3 and 5 months posttransplantation of GFP-high LSKCD48− cells and the CD150 Hi, Med, and Low subfractions of those cells. One hundred cells per group were transplanted into each of 5–8 SJL mice together with 200,000 BM cells from SJL mice. Each circle represents an individual mouse.

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investigated. Surprisingly, our phenotypic analysis of HSPCs throughout the divisional cascade revealed significant numbers of CD34+CD135− cells in GFP Hi LRCs (Figure 3). Therefore, we divided CD48−CD135−CD150+ LSK cells into GFP Hi and Low portions. These cells were then sorted into GFP Hi CD34+, GFP Low CD34+, GFP Hi CD34−, and GFP Low CD34− compartments (Figure 5A) and functionally analyzed in vitro and in vivo. Both LTC-CAFC and LTC-IC were highly enriched in both CD34− and CD34+ GFP Hi subpopulations, with far fewer in the corresponding GFP Low fractions (Figures 5B and 5C). Both CD34− and CD34+ GFP Hi fractions repopulated robustly (Figures 5D) and throughout the entire posttransplant period (Figure 5A). Analyses of recipient BM revealed additional surprising results. Neither CD34− nor CD34+ GFP Low cells re-established HSPC compartments. On the other hand, there were high levels of HSPC repopulation from both CD34+ and CD34− GFP Hi cells, including a large CD45.2+ LSK CD48−CD150+ compartment (Figure 5F). Perhaps even more surprising is the robust repopulation of secondary mice transplanted with BM from GFP Hi CD34+ primary recipients (Figures 5E and 5B). Analyses of secondary recipient BM finally revealed differences between the initial CD34− and CD34+ GFP Hi subpopulations. CD45.2+ LSK CD48− and LSK CD48−CD150+ fractions were significantly lower in recipients of cells from primary mice engrafted with the CD34+ GFP Hi fraction (Figure 5G). Collectively, these data show that LSK CD48−CD135−CD150+CD34− cells within the dormant population contain robust stem cell activity, whereas cells with an identical phenotype but with a significant history of division have lost this activity. In addition, dormant LSK CD48−CD135−CD150+CD34+ cells also have this capacity but start to lose the ability to re-establish HSPC populations in secondary recipients.

**G0 HSCs with High Divisional History Lose Functional Activity**

We next elected to layer “snapshot” quiescence on temporally defined divisional history to determine the ability of activated HSCs to return to G0. As such, we examined cell-cycle parameters across the divisional cascade and found that, once cells exit dormancy, the proportions of G0 cells decrease in parallel with GFP dilution (Figures 6A and 6B). We then determined the biological activities of the G0 fractions of LSK CD48−CD150+ cells at each GFP level (Figure 6C). We observed dramatic decreases in LTC-CAFC activity in G0 cells within progressive label dilution fractions (Figure 6D). The differences were less pronounced in the LTC-IC assay, but no primitive colonies were seen in the GFP0 to GFP1 fraction (Figure 6E). These data suggest that, once HSCs exit dormancy and start down the divisional cascade, they are less likely to return to G0 and, even if they do, their functional potentials are diminished.

**Relating Gene Expression toDivisional History**

In order to determine global changes in the molecular repertoire within the context of divisional history, we performed gene-expression analyses on the same LSK subpopulations used in transplantation assays (Figure 2). We selected two sets of significant genes based on multiple criteria: an extended set of 5,178 genes and an enriched subset of 871 genes (for details see Supplemental Experimental Procedures). From the latter, we developed a divisional history-related gene-expression signature containing 181 genes (Figures 7A and S6A). The signature genes that are upregulated in progressively more dormant HSCs (highest in GFP4) were further analyzed by functional annotation clustering (Database for Annotation, Visualization and Integrated Discovery [DAVID]). Three overlapping clusters represent 40.7% of these genes: signal transduction, regulation of transcription, and cell-cell/matrix interaction (Figure 7A). The substantial enrichment of cell-cell/matrix-interaction genes highlights the importance of microenvironmental crosstalk in dormant HSCs. Over half of the genes in the regulation of transcription cluster are repressors, including all five chromatin modifiers and two of the five transcription factors. These categories and complete gene lists are in Table S2, and the entire signature is in Table S3. Ingenuity pathway analysis revealed a major network of regulation within the upregulated gene set. Several signaling pathways, including phosphatidylinositol 3-kinase/AKT, mitogen-activated protein kinase, and nuclear factor κB, are linked together in the network. Signature molecules compiled

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(C) Donor reconstitution to each lineage at 3 months post-primary transplantation; mean ± SD; n = 5–8 mice.

(D) Chimerism 1 month after secondary transplantation of pooled BM (2 × 10^6) from each group of primary recipient mice; four or five mice were transplanted per group.

(E) Expression of Ndn, Fstl1, Tgm2, Ltbp3, Fscn, Trib3, and Meg3 by qPCR for CD150 Hi, Med, and Low fractions of GFP-high CD48−LSK cells (n = 3 assays from one sort).

(F) Fluidigm gene-expression qPCR on 200 sorted LSKCD48− cells at varying GFP levels (left) or CD150 Hi, Med, and Low subfractions of LSKCD48− GFP4 cells (right). Results were normalized within each group separately.

Data as mean ± SD; significance of donor contribution to total PBL (C and D) determined by Student’s t test; *p < 0.05; **p < 0.001; ***p < 0.0001. See also Figure S4.
LRCs are predominantly in G0 but appear poised to an opposite expression pattern. Because dormant GFP- were expressed at higher levels in low-GFP cells and pro- encoding cyclins A, B, and E and cyclin-dependent kinases downregulated group, we investigated these further. Genes categories are available in Table S4.

Cyclin-dependent kinase inhibitors were GFP2 sets (Figure S7B). Genes contained within these cate- gories were significantly less enriched in the GFP3 versus GFP3 to GFP2 transitions. Extracellular matrix, cell adhe- sive processes (Figure S6D).

We selected genes with high expression in GFP4 that were rich in cell cycle, DNA replication/repair, and biosyn-thetic processes (Figure S6D).

To interrogate the molecular changes triggered after cell division that may result in the loss of self-renewal capacity, we selected genes with high expression in GFP4 that were specifically downregulated in the GFP4 to GFP3 and the GFP3 to GFP2 transitions. Extracellular matrix, cell adhesion, and cell junction are the top differentially enriched categories in the GFP4 versus GFP3 gene sets. These categories were significantly less enriched in the GFP3 versus GFP2 sets (Figure S7B). Genes contained within these cate- gories are available in Table S4.

Because cell-cycle genes are overrepresented in the downregulated group, we investigated these further. Genes encoding cyclins A, B, and E and cyclin-dependent kinases were expressed at higher levels in low-GFP cells and pro-gressively decreased in cells with higher GFP levels (Figures 7D and S7C). Cyclin-dependent kinase inhibitors were differentially expressed along the divisional gradient. Cdkn1c (p57) was expressed at high levels in GFP3 and GFP4 fractions and decreased with greater divisional histo-ry. In contrast, Cdkn1a (p21) and Cdkn1b (p27) displayed an opposite expression pattern. Because dormant GFP-LRCs are predominantly in G0, but appear poised to respond to microenvironmental cues, we also examined G1 cyclins and their catalytic Cdk partners. Cyclin D3 was specifically upregulated in GFP4 cells that had little to no expression of Cdk4/Cdk6 (Figure 7D). This was con- firmed by single-cell Fluidigm quantitative PCR (qPCR) (Figure 7E). These measurements demonstrated that Cdk6 was more differentially regulated than Cdk4; it was completely absent in GFP4 cells (Figure 7E). These data sug- gest that the lack of cyclin D3 catalytic partners Cdk4/Cdk6 holds G1 progression in check.

Transforming growth factor β (TGF-β) signaling has been implicated in HSC quiescence (Söderberg et al., 2009; Yamazaki et al., 2009; Yamazaki and Nakauchi, 2009) and is overrepresented in the GFP Hi upregulated gene sets. Further investigation revealed that expression of TGF-β upstream modulators, intercellular transducers, and down- stream targets was elevated with increasing GFP label retention levels (Figure 7F). Confirmed genes include: Ltbp3, Fscn1, Pml, Cited2, Fstl1, Hipk2, Crebbp, Ep300, Smad 2, Smad 3, and Smad 7 (Figures S7D and S7E), and Tgm2 (Fig-ure 7C). Interestingly, Ltbp3, Fscn1, Fstl1, and Tgm2 are all involved in extracellular matrix, cytoskeletal, and microenvironmen-tal interactions. Ltbp3 was highly expressed only in GFP4 cells, suggesting involvement in the bioavailability of TGF-β in dormant HSCs.

In summary, the gene-expression landscape changes dramatically as dormant HSCs become activated and traverse through a divisional cascade. In dormant HSCs, the transcriptional and cell-cycle machineries are in repressive but poised states. It appears that dormant HSCs are actively interacting with their environment and that dramatic changes in these interactions are necessary for these cells to become activated.

**DISCUSSION**

Our studies have addressed the functional behavior of HSPCs in the context of their homeostatic divisional...
history. We have determined that, with very few cell divisions, HSCs lose functional activities. We also show that accrued temporal divisional history outweighs both cell-surface phenotype and “snapshot” quiescence in determining HSC potency. Collectively, our results suggest that, during normal homeostasis, HSCs do not undergo self-renewal divisions. They also suggest that, once HSCs exit dormancy and initiate cell division, they will not return to full functional dormancy and are slated for extinction. Although these cells still bear an HSC phenotype, they are not identical functionally. Our studies suggest that we are endowed with a set number of HSCs that in normal situations is sufficient for lifelong hematopoiesis.

In contrast to previous viable LRC studies (Foudi et al., 2009; Wilson et al., 2008), we analyzed five compartments of cells with progressively diluted GFP in homeostatic-labeled and chased HSCs. We analyzed their functional, cellular, and molecular properties over the course of approximately eight cell divisions. To our surprise, initially highly labeled HSCs activated during the chase progressively lose their robust activity over a limited number of cell divisions.

We observed three distinct dormant CD150 populations that correlated with declining repopulation and self-renewal potential. Cells with the highest CD150 levels have the most robust and durable self-renewal potentials. This extends the previously observed functional heterogeneity defined by CD150 expression levels to the dormant HSC pool (Kent et al., 2009; Morita et al., 2010; Weksberg et al., 2008). Specific expression of transcriptional modifiers like Smarca2 and Mllt3 highlight the importance of epigenetic regulation in these cells.

CD34+ LSK cells have been shown to have early, but not sustained, multilineage reconstitution (Osawa et al., 1996). LSKCD34+/CD135− cells were defined as ST-HSCs with limited self-renewal activity (Yang et al., 2005). Wilson et al. (2008) also defined CD48−CD150+CD135−CD34+ cells as part of the MPP population within LSK cells by surface phenotype, but not functional, assays. Our studies revealed a significant amount of CD34+ cells in the dormant fraction that display robust in vitro and in vivo potential, including self-renewal. We showed that, as a percentage of whole BM, the CD34+ fraction was much lower than its CD34− counterpart in the GFP4 population. Therefore, the previous studies might not have captured these rare cells. In addition, functional assessment of CD34+CD150+ cells has not previously been undertaken. One study showed LT repopulation with LSKCD34+Mpl+ cells, although self-renewal was not assessed in secondaries (Yoshihara et al., 2007). This suggests that, with the addition of label-retention, adult CD34+ cells have more potential than previously described. On a cell-for-cell basis, dormant CD34+ cells appear to be as robust as dormant CD34− cells. Ultimately, the initially transplanted GFP Hi CD34− cells outperformed the GFP Hi CD34+ cells in the ability to remake BM HSC populations in secondary mice. Perhaps tertiary transplantation might reveal decreased self-renewal in this cell population. These data also suggest that there is a hierarchical structure within the dormant HSC population with the LSKCD48−CD135−CD34−CD150+ phenotype as the anchor.

Previous studies have shown that cells isolated in different phases of the cell cycle have differential activities. HSCs in G0 have a superior LT reconstitution potential (Passegué et al., 2005), and proliferating HSCs in S/G2/M have an engraftment defect (Bowie et al., 2006). These observations cannot explain our results as we examined G0 cells that differ only in their divisional histories. Although our in vitro assays are highly correlative with in vivo activity (Hackney et al., 2002; Kent et al., 2009), transplant experiments will extend these studies.

It is unclear how divisional history progressively reduces stem cell function. The G1 cell-cycle phase is thought to be the stage when intrinsic and extrinsic cues are processed for self-renewal, differentiation, or apoptosis decisions (Massagué, 2004). It has been proposed that prolonged exposure to stimuli that drive G1 progression facilitates differentiation rather than self-renewal (Orford and Scadden, 2008). During normal homeostasis, cells with increased divisional history accompanied by multiple transitions through G1 might accumulate differentiation signals and lose stem cell potential. However, our studies suggest that this does not necessarily manifest as a change in cell-surface phenotype. Even if cells return to G0 after several rounds of

Figure 6. Cell-Cycle Analysis and Functional Activity of G0 LSKCD48−CD150+ Cells throughout the GFP Dilution Cascade
(A) Cell-cycle profiles of total LSKCD48−CD150+ cells or those at GFP 0, GFP 1, GFP 2, GFP 3, and GFP 4 by DNA content (DAPI) versus Ki67 staining. Cell-cycle phases were determined as G0 Ki67−DAPI−, G1 Ki67+DAPI−, S/G2/M Ki67+DAPI+. (B) Percentage of G0, G1, and S/G2/M cells in total LSKCD48−CD150+ and those at GFP 0, GFP 1, GFP 2, GFP 3, and GFP 4 as determined by Ki67/DAPI staining (mean ± SD; n = 4 mice). Significance of G0 cells determined by Student’s t test; *p < 0.05; **p < 0.01. (C) Isolation of viable G0 LSKCD48−CD150+ cells after 12 weeks chase for in vitro assays. LSKCD48−CD150+ cells were sorted first, followed by Py/Ho staining. G0 cells (Py/G0Ho lower left quadrant) were isolated and separated into GFP 0, GFP 2, GFP 3, and GFP 4 subfractions. (D) LTC-CAFC assay of G0 LSKCD48−CD150+ cells at each GFP level. Significance of total colonies determined by Student’s t test; *p < 0.05; mean ± SD; n = 3 separate cultures.
division, the effect could be irreversible, because the epigenetic landscape and gene-expression programs have changed during previous cell cycling.

In support of this hypothesis, our data reveal unique gene-expression repertoires with each additional division during the initial cell-cycle rounds, as well as genes regulated progressively along the GFP gradient. Genes involved in cell-cycle regulation, energetic, DNA replication/repair, and proliferation were uniquely expressed when cells proceeded through the divisional cascade, whereas genes involved in transcriptional regulation, signal transduction, and cell-to-cell/matrix interaction were upregulated in GFP Hi LRCs. This suggests a continual crosstalk of dormant cells and their microenvironment such that they are poised to rapidly make critical cell-fate decisions. Strikingly, genes involved in niche communication were downregulated immediately when cells exit the dormant pool. This may suggest that the cells leave a niche that maintains quiescence and relocate to a different, more active niche. This is supported by the loss of HSCs returning to G0 after leaving dormancy. It will be necessary to visualize these cells in situ to determine if they are located in different anatomical spaces within the BM. In addition, different sets of epigenetic modifier genes are regulated in either direction along the GFP-dilution cascade, indicating that epigenetic control plays an important role in relating cell division to stem cell fate decisions. Genes involved in apoptosis are enriched in parallel with GFP retention, whereas DNA repair genes are enriched with GFP loss. This suggests that nonproliferating cells undergo apoptosis rather than DNA damage repair to maintain genomic integrity within the population.

Our profiling studies highlighted cell cycle and TGF-β pathway genes. Differentially expressed cell-cycle genes of most interest were Cdkn1c (p57) and Ccnd3 along with its catalytic partners Cdk4 and Cdk6. It has been suggested that quiescent HSCs might access the cell cycle close to the “restriction point” between early and late G1 (Orford and Scadden, 2008). At this point, cells become independent of the mitogenic signaling that induces cyclin D expression (Malumbres and Barbacid, 2001). As such, dormant HSCs would bypass the sensitive early G1 phase and avoid differentiation cues. High expression of p57 specifically in dormant HSCs is interesting as it is the only gene of its class that is induced by TGF-β (Scandura et al., 2004). TGF-β signaling has pleiotropic positive and negative hematopoietic effects, depending on the target cells and their context. Others have observed diverse roles for TGF-β in regulating HSC quiescence. One study suggests that it directly inhibits lipid-raft clustering and upregulates p57 to specifically suppress the activation of cyclin D/Cdk complexes in hibernating HSCs and thus prevents cell-cycle entry (Yamazaki et al., 2009). Our studies support these findings and highlight the lack of Cdk4/Cdk6 in dormant HSC. Another study found differential responses to TGF-β1 in myeloid- and lymphoid-biased HSCs and suggested that this mechanism may explain the differential activation of these subsets (Challen et al., 2010). It is highly likely that the TGF-β pathway plays specific and yet to be understood roles in the regulation of dormant and activated HSCs.

Our studies have elucidated aspects of dormant HSCs. Interestingly, temporally defined dormancy outweighs other indicators of HSC potency. Why HSCs start losing stem cell potential upon cell division during normal...
homeostasis is unclear. HSC self-renewal can only be demonstrated by transplantation studies and transplantation is a high-stress condition. Nevertheless, despite self-renewal under stress conditions, HSCs cannot be serially transplanted indefinitely and aging incurs multiple defects. This suggests that understanding both homeostasis and stress self-renewal processes is necessary to exploit HSCs for therapeutic use. Our studies suggest a radical departure from existing dogma by suggesting that HSCs do not self-renew during normal homeostasis. On the other hand, we do recognize that HSCs can self-renew when under stress. Nevertheless, we still challenge the existing dogma that HSCs self-renew to their initial functional potential once they initiate cell division under any condition. HSCs may not be immortal, nor should they be; in fact, this is the hallmark of leukemia.

**EXPERIMENTAL PROCEDURES**

**Mice**

HuCD34-tTA mice were from Dr. Dan Tenen (Harvard Medical School). Tg (tetO-HIST1H2BJ/GFP) 47Efu/J (TetO-H2BGFP), C57BL/6 (B6), and congenic B6.SJL-Ptgcre-Pepckc/Boj (SJL) mice were purchased from The Jackson Laboratory, bred, and maintained in house. The huCD34-tTA and TetO-H2BGFP strains were backcrossed to B6 mice for at least ten generations. Double-transgenic 34/H2B mice were derived by crossbreeding. Dox (Sigma) was administered to 6–8-week-old 34/H2B mice at 1 mg/ml in drinking water and changed two times per week for 0.5–16 weeks. Animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the Animal Welfare Act.

**Flow Cytometric Analysis, Sorting, and Cell-Cycle Analyses**

**BM analyses:** BM was harvested and stained as previously described (Schaniet al., 2011). Cells were analyzed on an LSR II Flow Cytometer and/or sorted using FACSAriaII, FACSVantage, Influx (all BD Biosciences), or MoFlo (Beckman Coulter Genomics) cell sorters.

**Ki-67 Analysis**

Lineage and CD48-depleted BM was stained for Sca1, cKit, CD150, and Ki-67 as previously described (Schaniet al., 2011) and analyzed on an LSR II.

**Py/Ho Analysis and G0 Cell Assay**

These analyses were performed as previously described (Passegué et al., 2005).

**Hematopoietic Assays**

**In Vitro**

CFU assays were performed in Methocult (M3434; Stem Cell Technologies) according to manufacturer’s instructions. LD-CACF, LTC-CAFC, and LTC-IC assays were done after 4 weeks of LTC on AFT024 cells as previously described (Moore et al., 1997; Schaniel et al., 2011). All values were normalized to 100 initial cell equivalents seeded into LTCs, and limiting dilution frequencies were calculated by Poisson statistics (Taswell, 1981).

**In Vivo**

SJL (CD45.1) mice were irradiated (10 Gy) and transplanted with freshly isolated 34/H2B (CD45.2) donor cells mixed with BM from SJL mice for competitive repopulation assays. For secondary transplantation, pooled BM from each group was transplanted into secondary SJL mice without competitor cells. Peripheral blood was collected at posttransplant intervals and analyzed for the presence of CD45.2 contribution to T, B, and myeloid cell populations. BM cells from individual primary and secondary mice were analyzed for CD45.2 contribution to whole BM and defined HSPC compartments.

**Microarray and Bioinformatics Analysis**

LSK cells with five different levels of GFP from 0–4 were isolated by fluorescence-activated cell sorting from BM cells taken from multiple cohorts of mice after 12 weeks of Dox treatment. Total RNA was in vitro amplified before hybridization to Affymetrix Mouse 430 2.0 3’ arrays. Microarray data were screened using two statistical criteria to measure the consistencies between biological replicates and probe sets for each gene followed by K-means clustering.

**qPCR and Fluidigm Gene-Expression Assays**

RNA isolated from BM cells was amplified before analysis by qPCR using the SybrGreen (Applied Biosystems) method. Freshly sorted single cells or 200 cell mixtures were preamplified with TaqMan Assay Mix (Applied Biosystems) and then processed on 96-96 Fluidigm Dynamic Array IFCs with a BioMark HD system and then analyzed using BioMark Real-Time PCR Analysis Software (Fluidigm).

See Supplemental Experimental Procedures for details, antibodies, and primers.

**Statistical Analysis**

Statistical significance was determined using unpaired two-tailed Student’s t test with p ≤ 0.05 considered significant.

**ACCESSION NUMBERS**

The microarray expression data were deposited in the Gene Expression Omnibus database, accession number GSE48261.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.01.016.

**ACKNOWLEDGMENTS**

We thank Dr. Dan Tenen (Harvard Medical School) for the huCD34tTA mouse, Dr. Ihor Lemischka and Dr. Saghi Ghaffari for helpful discussions and critical reading of the manuscript, and the Mt. Sinai Flow Cytometry and Mouse Facilities. K.M. was supported by grants from NIH 2RO1HL58739 and the NJ Commission on Science and Technology 06-2042-014-75.
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Supplemental Information

Divisional History and Hematopoietic Stem Cell Function during Homeostasis

Jiajing Qiu, Dmitri Papatsenko, Xiaohong Niu, Christoph Schaniel, and Kateri Moore

Inventory of Supplemental Information

Figure S1, related to Figure 1. Kinetic analysis of GFP dilution for defined populations of HSC phenotypes and functional activities of GFP label-retaining LSK cells

Figure S2, related to Figure 2. Cell divisions required to dilute GFP and CFU-Mix content in relationship to GFP dilution

Figure S3, related to Figure 3. Flow cytometric profiles of GFP label retaining fractions for markers of phenotypic HSPC and their gene expression levels

Figure S4, related to Figure 4. Analyses of BM HSPC populations in primary recipients of GFP Hi CD48-LSK CD150 Hi, Med, and Low cells and in secondary recipients.

Figure S5, related to Figure 5. Donor Contribution to peripheral blood after primary transplantation of phenotypically identical HSCs that were either GFP Hi or Low LRCs and after secondary transplantation

Figure S6 and S7 related to Figure 7. A divisional history gene expression signature, expression profiles and bioinformatic analyses

Table S1, related to Figure 3. The distribution of HSPC compartments at each GFP level in WBM

Table S2, related to Figure 7. Over represented categories of genes in the up regulated expression signature.
Table S3 (excel file), related to Figure 7. 181 Signature gene list

Table S4 (excel file), related to Figure 7. Unique genes at GFP 4 to GFP 3 transition

Supplemental Experimental Procedures

Supplemental References
Figure S1, related to Figure 1. Kinetic analysis of GFP dilution for defined populations of HSC phenotypes and functional activities of GFP label-retaining LSK cells

(A) Percentage of GFP positive cells within each phenotype of huCD34TA X TetO-H2BGFP mice treated with Dox for indicated length of time (n=5 mice per group). Data are presented as mean ± SD.

(B) CFU assay of freshly sorted GFP positive or negative LSK cells after 12 weeks Dox treatment; numbers of each type of colony formed. Data are presented as mean ± SD from 3 separate cultures.

(C) LD-CAFC assay of freshly sorted GFP positive or negative LSK cells. The frequency of CAFC is normalized to 100 LSK cells and determined weekly for 4 weeks.

(D) Donor reconstitution in each lineage 8 months post primary competitive transplantation. Groups of 3-5 mice were transplanted with 405, 135, 45, and 15 LSK GFP- cells, and 135, 45, 15, and 5 LSK GFP + cells respectively together with 400,000 BM cells from CD45.1 mice. Transplanted cells were isolated after 10 weeks of Dox treatment. Each bar represents an individual mouse.
Figure S2, related to Figure 2. Cell divisions required for GFP dilution and CFU-Mix content in relationship to GFP dilution.

(A) The predicted Proliferation Index (FlowJo, Treestar Inc.) using “pulsed” or fully labeled LSK cells. This is a hypothetical prediction based on non-divided cells with a GFP fluorescence intensity of $10^5$, assuming each cell division dilutes GFP signal by one-half. Numbers at the top predict the number of cell divisions needed to dilute GFP signal. 7 cell divisions are required to completely dilute GFP from fully labeled LSK cells.

(B) Percentage of mixed colonies within the total CFU. Mean ± SD, n=3 separate cultures.

(C) Percentage of mixed colonies within the total CFU derives from LTC-ICs. Mean ± SD, n=3 separate cultures.

Significance determined by Student’s T-test, *p<0.05, **p<0.01.
Figure S3, related to Figure 3. Flow cytometric profiles of GFP label retaining fractions for markers of phenotypic HSPC and their gene expression levels.

(A) Flow cytometry profiles of CD135 and CD34 in LSK cells.

(B) Flow cytometry profiles of CD48 and CD150 in LSK cells.

(C) Flow cytometry profiles of CD135 and CD34 in CD48-LSK CD150- cells.

(D) Flow cytometry profiles of CD135 and CD34 in CD48-LSK CD150+ cells.

(E) Microarray gene expression profiles of HSC surface markers along the GFP gradient.
Figure S4

A

CD48-LSK

% CD45.2 BM populations

Total Hi Med Low

Total Hi Med Low

CD48-LSK CD135+CD34+

% CD45.2 BM populations

Total Hi Med Low

Total Hi Med Low

CD48-LSK CD135-CD34+

% CD45.2 BM populations

Total Hi Med Low

Total Hi Med Low

B

WBM

% CD45.2 BM populations

Total Hi Med Low

Total Hi Med Low

Lin-CD48-

Total Hi Med Low

Total Hi Med Low

CD48-LSK

CD48-LSK CD34+

% CD45.2 BM populations

Total Hi Med Low

Total Hi Med Low

CD48-LSK CD34+CD150+

% CD45.2 BM populations

Total Hi Med Low

Total Hi Med Low

CD48-LSK CD34-CD150+

% CD45.2 BM populations

Total Hi Med Low

Total Hi Med Low

CD48-LSK CD150+

% CD45.2 BM populations

Total Hi Med Low

Total Hi Med Low
Figure S4, related to Figure 4. Analyses of BM and HSPC populations in primary recipients of GFP Hi CD48-LSK CD150 Hi, Med, and Low cells and in secondary recipients.

(A) Donor contribution (CD45.2+ cells) to BM and HSPC populations in the BM 5 months after primary transplantation of GFP Hi LRCs with heterogeneous expression of CD150. 5 mice per group initiated the experiment although some died prior to BM analyses.

(B) Donor contribution to HSPC populations in the BM 7 months after secondary transplantation of BM from primary recipients. CD150 Hi GFP Hi LRCs display the most robust ability to reestablish HSPC compartments in secondary mice. 4 mice per group initiated the secondary transplants although some died prior to BM analyses.

Each circle represents an individual mouse. Significance was determined by Students T-test; *p<0.05, **p<0.01, ***P<0.001, ****P<0.0001, *****P<0.000001.
Figure S5, related to Figure 5. Donor Contribution to peripheral blood after primary transplantation of phenotypically identical HSCs that were either GFP Hi or Low LRCs and after secondary transplantation.

(A) CD45.2+ PBLs 1.5, 3 and 5 months after primary transplantation of GFP Hi CD34+, GFP Hi CD34- and GFP Low CD34+, GFP Low CD34- cells that were also CD48-CD135-CD150+ LSK cells. 3-5 SJL (CD45.1) mice were transplanted with 30 cells of each along with 200,000 SJL BM cells.

(B) CD45.2+ PBLs 3 weeks, 3, and 5.5 months after secondary transplantation. 2 X10^6 BM cells pooled from primary recipients in each group were transplanted into 3-4 SJL mice.

Each circle represents an individual mouse.
Figure S6, related to Figure 7. A divisional history gene expression signature, expression profiles and bioinformatic analyses

(A) Clustering analyses to select the gene sets for the divisional history gene expression signatures. K-means clusters showing sets of progressively up or down regulated profiles correlated with GFP levels 0 to 4 are displayed. These were extracted from a pool of 871 significantly enriched genes in the array dataset. See supplemental experimental procedures for details. This resulted in 108 progressively up regulating and 73 down regulating signature genes. The genes within this set are available in Table S3.

(B) Expansion of the significantly changing dataset for functional annotation analyses. Genes were extracted that displayed a progressive profile from an extended pool of 5718 significantly changing genes. See supplemental experimental procedures for details. Selected clusters were combined; this resulted in 958 genes with an up-regulating profile and 892 genes with a down regulating profile from GFP levels 2 to 4. Average profiles are shown for each list with their variance in expression.

(C) A network of interacting genes was developed from the 108 up-regulating genes in the signature list using Ingenuity Pathway Analysis software. Filters were set to consider all molecules and/or relationships, including both direct and indirect interactions. Blue circles with bold names highlight the genes in the network derived from the expression signature. Dashed lines indicate indirect relationships; solid lines indicate direct relationships. Names with all uppercase letters are complexes.

(D) Genes involved in cell cycle, DNA replication/repair and oxidative metabolism are enriched in the down regulated gene set. Functional Annotation Clustering (DAVID) was performed on the 958 up regulated and 892 down regulated genes between GFP levels 2 and 4 from the extended pool of 5718 significantly changing genes (see Figure S6 B). Only terms with a p-value (Fisher Exact p-values) less than 0.05 are shown. Annotations chosen for clustering were Gene Ontology categories GO_BP (Biological Process), GO_MF (Molecular Function), GO_CC (Cellular Component), and KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway. Pink bars derive from genes in the up-regulating set and blue bars are from the down regulating set. P value on X axis is shown as negative log10 scale. P value of 0.05 is marked with a black vertical line on each graph.
Figure S7, related to Figure 7. A divisional history gene expression signature, expression profiles and bioinformatic analyses

(A) Genes involved in transcription, signal transduction, and cell-cell matrix interactions are enriched in the up regulating gene set. Functional Annotation Clustering (DAVID) was performed on the 958 up regulated and 892 down regulated genes between GFP levels 2 and 4 from the extended pool of 5718 significantly changing genes (see Figure S6 B). Only terms with a p-value (Fisher Exact p-values) less than 0.05 are shown. Annotations chosen for clustering were Gene Ontology categories, GO_BP (Biological Process), GO_MF (Molecular Function), GO_CC (Cellular Component), and KEGG (Kyoto Encyclopedia of Genes and Genomes)_Pathway. Pink bars derive from genes in the up-regulating set and blue bars are from the down regulating set. P value on X axis is shown as negative log10 scale. P value of 0.05 is marked with a black vertical line on each graph.

(B) Genes were selected from the extended pool of 5718 genes and pair wise comparisons were performed. Genes with high expression in GFP4 that was specifically down regulated in GFP3 and those with high expression in GFP3 specifically down regulated in GFP2 were chosen for functional annotation. The differentially enriched annotations that undergo immediate down regulation upon the transition from GFP4 to GFP3 levels are those involved in cell adhesion, extracellular matrix, cell junction and cadherin binding. The genes within these annotations are available in Table S4.

(C) A heatmap was assembled from microarray data derived from LSK cells isolated at discrete GFP levels from 0 to 4 using the extended pool of 5178 genes. Selected genes encoding cyclins, cdkks and cdk inhibitors that had have either an ANOVA P-value or P2B P-value (*) < 0.05 are listed.

(D) RT-PCR confirmation of TGF-β pathway related genes expressed in LSK cells isolated at each GFP label retaining level.

(E) Additional RT-PCR expression of selected genes in LSK cells with high or low levels of GFP retention after Dox treatment. GFP Hi was gated as GFP4 and GFP Low as GFP0.

Data in (D), (E) are presented as mean ± SD from triplicates. Individual RT-PCRs were repeated at least two times using cells from separate sorts.
Table S1. The distribution of HSPC compartments at each GFP level in whole bone marrow

| Starting Population | %Whole Bone Marrow |
|---------------------|---------------------|
|                     | GFP0    | GFP1    | GFP2    | GFP3    | GFP4    |
| LSK                 | 0.1012% (±0.0215%) | 0.0361% (±0.0065%) | 0.0264% (±0.0041%) | 0.0170% (±0.0056%) | 0.0079% (±0.0049%) |
| LSK CD34+ CD135+    | 0.0508% (±0.0107%) | 0.0095% (±0.0018%) | 0.0026% (±0.0013%) | 0.0006% (±0.0002%) | 0.0003% (±0.0003%) |
| LSK CD34- CD135-    | 0.0438% (±0.0083%) | 0.0219% (±0.0040%) | 0.0159% (±0.0019%) | 0.0068% (±0.0022%) | 0.0028% (±0.0017%) |
| LSK CD150+ CD48-    | 0.0045% (±0.0024%) | 0.0040% (±0.0005%) | 0.0073% (±0.0009%) | 0.0093% (±0.0033%) | 0.0048% (±0.0031%) |
| LSK CD150+ CD48-CD34+CD135- | 0.0023% (±0.0020%) | 0.0071% (±0.0052%) | 0.0013% (±0.0038%) | 0.0130% (±0.0034%) | 0.0032% (±0.0018%) |
| LSK CD150+ CD48-CD34-CD135- | 0.0007% (±0.00007%) | 0.0026% (±0.00019%) | 0.0067% (±0.00008%) | 0.0062% (±0.0003%) | 0.0014% (±0.0002%) |
| LSK CD150+ CD48-CD34-CD135- | 0.0005% (±0.0005%) | 0.0026% (±0.0006%) | 0.0048% (±0.00018%) | 0.0017% (±0.0002%) | 0.0006% (±0.0006%) |

Table S1, related to Figure 3. The distribution of HSPC compartments at each GFP level

Data are presented as the percentage of the designated population in whole bone marrow. The first column indicates the starting population and their percentage. Columns under GFP0, GFP1, GFP2, GFP3, GFP4 indicate each GFP fraction within the starting population on the same row. Data presented as mean ± SD, n=4 mice.
Table S2. Over represented gene categories in the up regulated expression signature

| **Signal Transduction** | **Gene Expression Regulation** |
|-------------------------|--------------------------------|
| **Cell surface receptor** | **Transcription factor** |
| **linked signal**        | **activity** |
| **transduction**         | **(MF.GO:0003700)** |
| **(BP.GO:0007166)**      | **Foxn3** |
|                         | **Runx1t1** |
| **GTPase activity**      | **Chromatin modification** |
| (**MP.GO:0003924**)      | **(BP.GO:0016568)** |
| **Small GTPase**         | **Prdm16** |
| **regulator activity**   | **Cbx6** |
| (**MF.GO:0005083**)      | **Cbx7** |
| **Protein kinase activity** | **Dnmt3a** |
| (**MF.GO:0004672**)      | **Ezh1** |
| **Kinase regulator**     | **Prdm16** |
| **activity**             | **Runx1t1** |
| (**MF.GO:0019207**)      | **Trib3** |

| **Cell-Cell/Matrix Interaction** | **Regulation of transcription, “DNA dependent”** |
|---------------------------------|-----------------------------------------------|
| **Cell junction**               | **(BP.GO:000635)** |
| (**CC.GO:0030054**)             | **(other than above)** |
|                                 | **Methyltransferase** |
|                                 | (**SP.PIR.KEYWORDS**) |
| **Cell adhesion**               | **Dnmt3a** |
| (**BP.GO:0030054**)             | **Ezh1** |
|                                 | **Mettl7a1** |
|                                 | **Prmt2** |
| **Focal adhesion**              | **Ndn** |
| (**KEGG_PATHWAY**)              | **Mettl7a1** |
| **Cd99l2**                      | **Prmt2** |
| **Inadl**                       | **Parp14** |
| **Clnd12**                      | **Zbtb20** |
| **Gabbr1**                      | **Zfp467, 612, 93** |
| **Itns1**                       | **Mettl7a1** |
| **Pank2**                       | **Prmt2** |
| **Pkp2**                        | **Dnmt3a** |
| **Focal adhesion**              | **Ezh1** |
| (**KEGG_PATHWAY**)              | **Mettl7a1** |
| **Itgb5**                       | **Prmt2** |
| **Mylk**                        | **Mettl7a1** |
| **Pik3r1**                      | **Prmt2** |
| **Pdgfd**                       | **Dnmt3a** |
| **Vegfc**                       | **Ezh1** |
| ****                            | **Mettl7a1** |

* repressor, or transcription repression
Table S2, related to Figure 7. Over represented categories of genes in the up regulated expression signature.

List of genes in the 3 main functional annotation clusters derived from the up-regulating genes in the expression signature as diagramed in Figure 7A. The names of 3 clusters shown as are bolded characters. Gene names marked with asterisks are also annotated as repressor or transcription repression.
Supplemental Experimental Procedures

Flow Cytometric Analysis and Sorting
Bone marrow (BM) cells were harvested from long bones (tibias and femurs) by gentle crushing in mortar and pestle in PBS supplemented with 5% NBCS (New Born Calf Serum; Gibco). Bone debris was removed by filtering the cell suspension through 70um strainers (BD). Red blood cells were lysed with ammonium chloride lysis buffer (ACK), and further filtered through 45um cell strainers to obtain a debris free, single-cell suspension. The remaining cells were incubated with a rat anti-mouse lineage cocktail or one conjugated to biotin. When indicated rat anti-mouse CD48 or rat anti-mouse CD135/Flk2/Flt3 was also added into the cocktail. Positive cells were removed by magnetic bead depletion with Sheep-anti Rat IgG Dynabeads® or Dynabeads® Biotin Binder (Life Technologies). Cells were then stained with goat anti rat IgG conjugated to Cy5-PE (or streptavidin conjugated to Cy5-PE; if biotin conjugated lineage cocktail was used) to gate out the remaining positive cells. Cells were subsequently saturated with rat serum and stained with selected fluorochrome conjugated HSPC surface markers as indicated: Sca-1, c-Kit, CD135, CD34, CD150, CD48, IL7R (CD127), FcγR (CD16/32). Cells incubated with Biotin conjugated antibody were further stained with fluorochrome conjugated streptavidin. All antibodies used have been previously determined not to have non-specific staining with isotype controls. Dead cells were excluded by staining with viability dye Propidium Iodide (PI; 1ug/ml; Sigma-Aldrich), or 4′, 6-diamidino-2-phenylindole hydrochloride (DAPI; 1ug/ml; Molecular Probes). LSR II (Becton Dickinson) Flow Cytometer was used for FACS analysis. Cells were sorted in Flow Cytometry Core Laboratory in Icahn School of Medicine at Mount Sinai with FACSARiaII, Influx, FACSVantage all (BD Biosciences) or MoFlo (Beckman Coulter) cell sorters.
## Mouse Hematopoietic Lineage Cocktail Antibodies

| Antibody  | Clone Cocktail of hybridoma supernatant | Cell type                                                                 |
|-----------|------------------------------------------|---------------------------------------------------------------------------|
| CD2       | RM2.2                                    | B cells, T cells, NK & NKT cells                                           |
| CD3       | KT 3-1.1                                  | T cells, NK & NKT cells, Th17 cells, Treg cells                          |
| CD5       | 53.7.3                                    | B cells, T cells                                                          |
| CD5       | 53.6.7                                    | T cells, NK & NKT cells                                                   |
| CD45R(B220) | RA3-6B2                                 | B cells, T cells                                                          |
| Mac-1 (CD11b) | M1/70                                 | Macrophage & Monocyte cells, NK & NKT cells                              |
| Gr-1/Ly6G | RB6-8C5                                   | Myeloid lineage                                                          |
| TER-119   | TER-119                                   | Erythroid cells                                                           |

### Cocktail of purified individual antibodies (e-bioscience)

| Antibody  | Clone Cocktail of hybridoma supernatant | Cell type                                                                 |
|-----------|------------------------------------------|---------------------------------------------------------------------------|
| CD3       | 17A2                                      | T cells, NK & NKT cells, Th17 cells, Treg cells                          |
| CD45R(B220) | RA3-6B2                                 | B cells, T cells                                                          |
| CD11b (Mac-1) | M1/70                                 | Macrophage & Monocyte cells, NK & NKT cells                              |
| TER-119   | TER-119                                   | Erythroid cells                                                           |
| Ly-6G (Gr-1) | RB6-8C5                                 | Myeloid lineage                                                          |

### Lineage Biotin Panel (e-bioscience)

| Antibody  | Clone Cocktail of hybridoma supernatant | Cell type                                                                 |
|-----------|------------------------------------------|---------------------------------------------------------------------------|
| CD3e      | 145-2C11                                  | T cells, NK & NKT cells, Th17 cells, Treg cells                          |
| CD45R(B220) | RA3-6B2                                 | B cells, T cells                                                          |
| CD11b (Mac-1) | M1/70                                 | Macrophage & Monocyte cells, NK & NKT cells                              |
| TER-119   | TER-119                                   | Erythroid cells                                                           |
| Ly-6G (Gr-1) | RB6-8C5                                 | Myeloid lineage                                                          |

### Lineage depletion and staining

| Conjugated to | Cat# (Invitrogen) |
|---------------|------------------|
| Dynabeads® Sheep-anti Rat IgG | #11035 |
| Dynabeads® Biotin Binder | #11047 |
| Goat F(ab’)2 anti-rat IgG | Pe-Cy5 #A10691 |

### Other Antibodies Added into the Lineage Cocktail

| Antibody | Clone | Source |
|----------|-------|--------|
| CD48     | OX-78 | Thermo Scientific |
| CD48-biotin | HM48-1 | e-bioscience |
| CD135 (Flk2/Flt3) | A2F10.1 | BioLegend |
Colony-Forming Unit (CFU) Assay

Freshly sort ed cells were suspended in 300 ul α-MEM and vortex mixed with 3ml of cytokine-enriched methylcellulose, Methocult® GF M3434 (Stem Cell Technologies, Vancouver, British Columbia) before plating in triplicate into non-tissue culture treated dish at 1ml per dish. Cells are incubated at 37°C, 5% CO₂ and 100% humidity for 10-12 days. Colonies were then scored as granulocyte/macrophage (GM), high-proliferative-potential (HPP; > 1 mm in diameter) granulocyte/macrophage (HPP-GM), Mix (three or more lineages including erythroid cells) and HPP-Mix. Erythroid cells were stained with freshly prepared erythroid staining solution.

### Antibodies for Individual Stem and Progenitor Populations in BM

| Antibody     | Clone | Conjugate                  | Source              |
|--------------|-------|----------------------------|---------------------|
| Sca-1 (Ly-6A/E) | D7    | PB, APC-Cy7, APC, PE       | Biolegend, eBioscience |
| c-Kit        | 2B8   |                            |                     |
| CD135 (Flk2/Flt3) | A2F10.1 | PE, Biotin, PE, Alexa Fluor 647, 700, Biotin PE-Cy7 | eBioscience, Biolegend |
| CD34         | RAM34 |                            |                     |
| CD48         | TC15-12F12.2 |                     |                     |
| CD150 (SLAM) |       |                            |                     |
| IL7R (CD127) | A7R34 | Biotin PE                  | eBioscience         |
| FcγR (CD16/32) | 2.4G2 |                            | BD Bioscience       |

### Hematopoietic Stem and Progenitor Compartments from BM

| Name       | Cell surface phenotype                                                                 |
|------------|----------------------------------------------------------------------------------------|
| Lin⁻ LSK   | Negative of T, B, NK, myeloid and erythroid lineages                                     |
| LT-HSC     | CD34⁺ LSK (Kiel et al., 2005; Osawa et al., 1996), CD34⁺CD135⁻ LSK (Kiel et al., 2005; Osawa et al., 1996), CD48⁺CD150⁺ LSK (Osawa et al., 1996), CD48⁺CD150⁺CD34⁺CD135 LSK (Wilson et al., 2007) |
| ST-HSC     | CD34⁺CD135⁻ LSK (Yang et al., 2005)                                                    |
| LM-MPP     | CD34⁺CD135⁻ LSK (Kiel et al., 2005), CD48⁺CD150⁻ LSK (Christensen and Weissman, 2001), CD48⁺CD150⁺CD34⁺CD135 LSK (Wilson et al., 2007) |
| CLP        | Lin⁻IL7R⁺cKit⁺Sca1⁺ (Kondo et al., 1997)                                                |
| CMP        | Lin⁻cKit⁺Sca1⁺CD34⁺FcγR⁺ (Akashi et al., 2000)                                          |
| GMP        | Lin⁻cKit⁺Sca1⁺CD34⁺FcγR⁺ (Akashi et al., 2000)                                          |
| MEP        | Lin⁻cKit⁺Sca1⁺CD34⁺FcγR⁺ (Akashi et al., 2000)                                          |
Limiting Dilution – Cobblestone Area Forming Cell (LD-CAFC) Assay

The primitive status of freshly isolated cells was evaluated by LD-CAFC assay. Cells were seeded in limiting dilutions on 20Gy-irradiated monolayers of AFT024 stromal cells (Moore et al., 1997) in 96-well plates and cultured in modified Dexter media at 37°C, 5% CO₂, and 100% humidity. Half volume of the culture medium was changed weekly. Presence of cobblestone areas (CAs) was scored weekly over a 4-week period. Percentage of negative wells for each row was calculated and the frequency of CAs within a cell population determined using Poisson statistics (Taswell, 1981) and presented as the frequency of CA-initiating cells.

Long-Term Culture (LTC) Assays

To determine the long-term potential, purified populations were seeded into LTC on irradiated AFT024 monolayers at 300 cells/well in 6-well plates and cultured in modified Dexter medium with weekly changes of half the medium. At 4 weeks, AFT024/BM cell cultures were harvested and divided into two portions. One portion was re-plated in limiting dilution onto fresh, irradiated AFT024 monolayers in 96-well plates as described above and assayed as described above for LT-CAFC assay. The other portion was plated into 1 ml of Methocult™ GF M3434 in triplicates for evaluation of LTC-initiating cells (LTC-IC), cultured and scored as described above for CFU-assay. All values were normalized to 100 initial cell equivalents that seeded the LTCs.

Competitive Repopulation Transplantation and Analysis

Competitive repopulation transplantations were performed by retro-orbital injection of freshly isolated donor cells (CD45.2) mixed with fresh competitor bone marrow cells from congenic SJL mice (CD45.1) into SJL recipients that had received lethal irradiation (10 Gy split dose of 3 hours apart). Peripheral blood leukocytes (PBL) were collected from the retro-orbital venous plexus at timed interval post transfer. Red blood cells were lysed in ACK buffer and washed. To detect T lymphocyte, B lymphocyte, and myeloid cells, samples were stained with CD4 and CD8a, B220, Mac- and Gr-1. Donor derived cells were detected by CD45.2. The reconstitution capacity was assessed in terms of chimerism (percentage of donor derived cells in the whole peripheral blood or each of the lineages). For secondary transplantation, 2x10⁶ pooled whole bone marrow from each group was transplanted and PBL was analyzed for reconstitution efficiency as above. The BM of primary and secondary mice was analyzed for the presence of CD45.2 cells in HSPCs at the time of sacrifice as described above.
Cell Cycle Analyses with Ki67 Antibody

CD48 and lineage magnetic bead depleted BM cells were stained with anti-Rat PeCy5 (for linCD48-), Sca APC-Cy7, cKit APC, CD150 PeCy7 prior to fixation with 4% paraformaldehyde in 2%NBCS/PBS at 4°C overnight in the dark. Cells were then washed, permeabilized with 0.2% Triton X-100 for 15 mins on ice and stained at room temperature for 60 mins in the dark with Ki-67 PE or isotype control (BD Pharmingen). After wash, cells were stained with 1ug/ml 4', 6-diamidino-2-phenylindole hydrochloride (DAPI, Molecular Probes) for 30 min prior to analysis on an LSRII Flow Cytometer. Doublets were eliminated from the gated populations.

Hoechst/Pyronin Y Cell Cycle Staining of Viable Cells

All stainings were performed in freshly prepared Hoechst staining media (HSM), protected from light. Freshly sorted LSK cells, or CD48- CD150+ LSK cells were incubated for 45 min at 37°C with 20 µg/ml Hoechst 33342 (Invitrogen). Pyronin Y (Sigma-Aldrich) was then added at 1 µg/ml, and the cells were incubated for another 15 min at 37°C, washed, resuspended in 1ug/ml propidium iodide (PI) containing ice cold HSM. Stained cells were immediately analyzed on a FACS analyzer (for LSKs), or further sorted (for CD48- CD150+ LSKs) into GFP 0, 1, 2, 3, 4 sub fractions under Pyronin Y low, Hoechst low gates and plated for in vitro assays. During preparation and FACS analysis/sorting, cells were kept in dark, ice-cold and without dilution by any other fluid.

Media and Buffers:

Ammonium Chloride Red Cell Lysis Buffer (ACK):
0.15 M Ammonium Chloride, 10 mM Potassium Bicarbonate, 0.1 mM EDTA, pH 7.2-7.4

Hoechst Staining Media (HSM):
In HBSS containing 10% FCS, 20 mM Hepes, 1 g/liter glucose, and 50 µg/ml Verapamil
Modified Dexter Media:
DMEM, 10% FBS, 10% horse serum, 5x10^{-6} M beta-mercaptoethanol, and 1x10^{-7} M hydrocortisone

Erythroid Staining Solution:
2ml 1M Tris-HCl, pH 7.5, 100ul 30% H_{2}O_{2}, 100ul 10 mg /ml 2,7-diaminofluorene in 90% glacial acetic acid, 8ml H_{2}O
**Microarray Amplification and Hybridization**

Total RNA extracted from seven to sixty thousand sorted BM LSKs at GFP level 0, 1, 2, 3, 4 in triplicates (except GFP0 in duplicates, GFP 4 in 4 replicates) was purified with RNeasy Micro Kit (Qiagen). In vitro RNA amplification was performed with SuperScript™ RNA Amplification System (Invitrogen). Biotin-labeled cRNA was synthesized with Enzo BioArray High Yield RNA transcript labeling kit T7 (Enzo Life Science) and then fragmented following the manufacturer’s protocol for Affymetrix GeneChip Target Preparation. Hybridization to Affymetrix Mouse 430 2.0 3’ gene expression array was performed by Microarray Resource Facility, Mount Sinai School of Medicine. Both raw image (.dat) and probe cell intensity data (cel) were generated with the Affymetrix Gene Chip Operating Software (GCOS). Probe level expression values were extracted by Robust Multi-array Averaging (RMA) normalization.

**Microarray data screening and gene lists**

Two major criteria were used to determine whether gene expression was significantly changed among GFP data series. First, standard ANOVA test (ANOVA $P$ - value) (Ayroles and Gibson, 2006) determined the consistency between different biological replicas for each probe. Second, P2B $P$ - value was calculated from Pearson correlation $r$ between the profiles for the best two ANOVA scored probes $X_{i,1}$ and $X_{i,2}$, which reflected the agreement between independent probes of the same genes:

$$P_{i}^{P2B} = P\left\{ r \mid X_{i,1}, X_{i,2} \right\};$$

The multiple probes for a gene were merged using the following rule: if ANOVA $P$-values were similar for the 2 best ANOVA scored probes, an average score was calculated for each gene $i$, each experiment $j$: if the ANOVA $P$-values were strikingly different (>3x), only the best probe was taken into account for each gene $i$, each experiment $j$:

$$x_{i,j} = x_{i,1}^j \quad \text{if } P_{i,1} < \frac{P_{i,2}}{3}$$

$$x_{i,j} = \text{average}(x_{i,1}^j; x_{i,2}^j) \quad \text{if } P_{i,1} \geq \frac{P_{i,2}}{3}$$

Based on the above two statistical scores - ANOVA $P$-values and P2B $P$-values, two pools of genes were established according to different criteria. “Enriched pool” contains 871 genes, passing both ANOVA cutoff $P = 0.05$ and P2B cutoff $P = 0.05$. “Extended pool” contains 5178 genes, passing either ANOVA cutoff $P=0.05$ or P2B cutoff $P=0.05$. “Enriched pool” contained fewer false-positives (FP), but was missing many genes that have changing expression profile.
(more false-negatives, FN); the "Extended pool" recovered more genes (lower FN rate) at the expense of higher FP rate.

To characterize major gene functions related to divisional history (FP tolerating, FN non-tolerating), the “Extended pool” of 5178 genes was subjected to K-mean clustering (K=10). Gene clusters of progressively up or down profiles from GFP2 to 4 were combined to extract 958 up or 892 down regulating genes.

To generate a divisional history related Gene Signature (FN tolerating, FP non-tolerating), the “Enriched pool” of 871 genes was subjected to 20 nodes K-mean clustering. Gene clusters with progressively up or down profiles from GFP0 or GFP1 towards GFP4 were combined and extracted. To assure that every gene in the list has progressive profile, occasional genes of profile outliers from K mean clustering were excluded so that the relative value (range from -2 to 2) of GFP2 is no lower than GFP1 by 0.05, GFP3 no lower than GFP2 by 0.05, GFP4 no lower than GFP3 by 0.1, regardless of the changes between GFP0 and GFP1; vise versa for down-regulating genes. By this means, we made our Signature Gene List of progressively regulated genes, 108 genes up, 73 genes down.

Functional Annotation Clustering was performed with DAVID Bioinformatics Resource 6.7 (The Database for Annotation, Visualization and Integrated Discovery) (Huang da et al., 2009). Software was available at http://david.abcc.ncifcrf.gov. Interactive Pathway Analysis (IPA) was performed through the use of IPA (Ingenuity® Systems, www.ingenuity.com). Gene expression data was analyzed with Cluster 3.0 software available at http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm (de Hoon et al., 2004) and heatmaps were generated with Java Treeview available at http://jtreeview.sourceforge.net (Saldanha, 2004).

Quantitative RT-PCR
Total RNA isolated from BM LSK cells at GFP level 0, 1, 2, 3, 4 or CD48-LSKGFP Hi cells, and GFP Hi LSKCD48-CD150 hi/med/low cells were trizol-extracted (Invitrogen), column-purified with RNeasy Micro Kit (Qiagen) and amplified with SuperScript™ RNA Amplification System (Invitrogen). Amplified aRNA was reverse transcribed utilizing High Capacity cDNA Reverse Transcription Kit (ABI). Primers were obtained from Integrated DNA Technologies (See supplemental material). Real-Time PCR reactions were performed in triplicates with the
SybrGreen method (Applied Biosystem) on Step One Plus Real-Time PCR System (Applied Biosystems). Expression values were normalized to Gapdh amplification.

**Fluidigm Q-PCR**

Single cells, either LSK or CD48-LSK at GFP level 0, 2, 3, 4, or a bulk of 200 cells of CD48-GFP4 LSK cells, total or CD150 Hi/Med/Low were sorted into RT-STA (specific target amplification) mix, which combined the following: 2X Reaction Mix of Cells Direct™ One-Step qRT PCR Kits (Invitrogen), 0.2x TaqMan Assay Mix (Applied Biosystems), and SuperScript™ III RT/Platinum® Taq Mix. A 15’ reverse transcription (RT) at 50°C followed by pre-amplification of 22 cycles was performed according to manufacturer’s protocol. Pre-amplified cDNAs were 1:5 diluted, mixed with TaqMan Gene Expression Assays, Taqman Universal Master Mix (Applied Biosystems) and GE Sample and Assay Loading Reagents (Fluidigm) for Q-RT PCR using BioMark 96.96 Fluidigm array. Raw data were analyzed using BioMark™ Real-Time PCR Analysis Software v2.0 (Fluidigm). For single cell Fluidigm a total of 96 single cells with all primer pairs were amplified on the same array and normalized against Gapdh. All 96 cells express both Gapdh and Actb with the exception of 3-5 cells that were excluded from the analyses. All cells with detectable signal of the specified gene were analyzed and presented. Each rectangle represented one single cell. Heatmaps were centralized to the median value among all cells and hierarchically clustered by centered correlation.

| Taqman probe ID for Fluidigm Q-PCR | Id       |
|-----------------------------------|----------|
| Smarca2                           | Mm00508992_m1 |
| Prdm16                            | Mm00712556_m1 |
| Mllt3                             | Mm00550927_m1 |
| Icam1                             | Mm00516027_g1 |
| Hlf                               | Mm00723157_m1 |
| Cdkn1c                            | Mm01272135_g1 |
| Cd34                              | Mm00519283_m1 |
| Cdkn1a                            | Mm00432448_m1 |
| Cdkn1b                            | Mm00438168_m1 |
| Cndd3                             | Mm01612362_m1 |
| Ccnb2                             | Mm01171453_m1 |
| Ccne2                             | Mm00438077_m1 |
| Ccna2                             | Mm00438064_m1 |
| Cdk1                              | Mm00772472_m1 |
| Cdk2                              | Mm00443947_m1 |
| Cdk6                              | Mm01311342_m1 |
| Cdk4                              | Mm00726334_s1 |
### Primers for Regular RT-PCR

| Gene  | Primer 5’            | Primer 3’          |
|-------|----------------------|-------------------|
| Ndn-L | CCAGGGGCACACTGATAGTT |                   |
| Ndn-R | GCACGAAAGCACAAAGTGA |                   |
| Fst1-L| CTGAGTACCAGAGGGCAAGC|                   |
| Fst1-R| TCAACCCAAGTCTCCCAAC|                   |
| Tgm2-L| GCCAGAGAACTGGAGGTCAG|                   |
| Tgm2-R| GGCTACAGGTCTGGTGCACT|                   |
| Ltbp3-L| TGTCTGCTTTTATCCCCAAGG|                   |
| Ltbp3-R| CAGCAGTGTCAGGCACATCTA|                   |
| Fscn1-L| AACCCTTGGCCTTTCAAACT|                   |
| Fscn1-R| CATGGAAAGAAGGGGACAGA|                   |
| Trib3-L| ACTTGCTGTCGGAATTCAG|                   |
| Trib3-R| GACTGTGGGCCTGGGTACTA|                   |
| Meg3-L| TGGTAATGCGTAGCTCTTG  |                   |
| Meg3-R| TTCGCTGTGTCCTGTCTCCTC|                   |
| Vegfc-L| AGCCAACAGGGATAATGGATG|                   |
| Vegfc-R| CACAGCGGCATACCTCTCA  |                   |
| Smad3-L| GGGCAACAAGTCAACAAGT|                   |
| Smad3-R| CTGGCTGCTAAAGGAGTGA  |                   |
| Cited2-L| ACTGCCATCTCGCTCTTCT|                   |
| Cited2-R| CACTGACGACATCCACACC|                   |
| Pml-L | TGAGAGCGAGGAAGCAGTGA |                   |
| Pml-R | AAATGGGACACACAGCAAT  |                   |

### Primers for Genotyping

| Primer  | Sequence                  |
|---------|---------------------------|
| hCd34-5’| AGA AGA GAT GAG GTG TGA GGA T |
| hCd34-3’| GGA TCC ACA AGA ATG AGC ATG TA |
| GFP-5’  | AGC TGA CCC TGA AGT TCA TCT G |
| GFP-3’  | GTC GGC CAT GAT ATA GAC GTT G |
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