Experimental Hematology

INVITED REVIEW

Are transplantable stem cells required for adult hematopoiesis?

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Hematopoietic stem cells (HSCs) have been studied intensely for more than half a century. As a result, the properties of HSCs have become a paradigm of adult stem cell biology and function. The "classical" view of hematopoiesis suggests that the HSCs sit at the top of a hierarchy and that differentiation involves sequential production of multipotent and lineage committed progenitors with limited self-renewal capacity. This view of hematopoiesis is certainly valid after transplantation of HSCs, where, with appropriate support, a single HSC can regenerate the entire hematopoietic system of the recipient. However, it is not clear whether HSCs perform the same function during steady-state hematopoiesis. Indeed, studies have shown that the majority of classical HSCs are not required for ongoing steady-state adult hematopoiesis. Several reports suggest that steady-state hematopoiesis relies on highly proliferative cells with more lineage restricted characteristics, a finding that was not anticipated based on results from transplantation experiments. However, other studies indicate a more substantial HSC contribution. Nevertheless, the notion of HSCs as distinct from progenitors appears to be simplistic in view of ample evidence for heterogeneity within the stem cell compartment. In this review we discuss recent results and controversies surrounding HSCs. © 2019 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

The unique ability of hematopoietic stem cells (HSCs) to repopulate the hematopoietic system of a suitable recipient simply through intravenous injection is a property that has enabled their use in clinical applications, for example, treatment of leukemia [1−3]. However, bone marrow transplantation remains a high-risk procedure, preventing its widespread application [4,5]. Because of the clinical importance of HSCs and the potential for HSC research to inform general principles of stem cell biology in other organs, much effort has been devoted to HSC research for more than half a century. Nevertheless, key aspects of HSC biology remain elusive.

We define "classic" HSCs as cells that have the ability to reconstitute both lymphoid and myeloid compartments post-transplantation and sustain long-term production of red blood cells to protect lethally irradiated mice from anemia. Transplantation is a widely used experimental system allowing the functional interrogation of HSCs. Classic HSCs, by definition, must harbor self-renewal capacity to sustain hematopoiesis post-transplantation. Hematopoietic stem cells maintain their capacity for self-renewal over at least three rounds of transplantation into recipients, and at least a subset have the potential to sustain hematopoiesis beyond the normal lifespan of the donor mouse. HSCs are highly enriched in the lineage-negative (Lin−) cKit+Sca1+(LSK) CD34−CD135− [6,7] and LSK CD150+CD48− [8,9] bone marrow populations in wild-type mice. A single human or mouse stem cell prospectively isolated using cell surface markers can reconstitute the hematopoietic system of lethally irradiated mice [10,11], a remarkable testament to the proliferative capacity of stem cells and the resilience of the bone marrow environment after irradiation. Lineage-specific progenitors must have substantial

proliferative capacity to expand sufficiently to replenish the blood, in particular the hematocrit, in the short term after transplantation.

Although the transplantation assay unequivocally demonstrates a truly impressive stem cell capability, mice do not normally exchange bone marrow. Thus, the contribution of HSCs post-transplantation does not necessarily reflect their steady-state properties. Several recent articles challenge the notion that the transplantable, "classically defined" stem cells are required to sustain hematopoiesis in adult animals. In this review, we discuss how the HSC paradigm has shifted in recent years (Figure 1), with a focus on genetic models that examine the impact of HSC depletion and in situ labeling techniques.

Diversity in HSC subsets

Because the majority of mature blood cells have short life spans, very large numbers of cells are produced throughout life [12]. Clonal analysis of HSCs has revealed an underappreciated level of heterogeneity within the stem cell compartment, including lineage-biased stem cells [13−18]. This has led to a clonal diversity model of hematopoiesis, in which the stem cell compartment is diverse in terms of cellular output [19]. Different laboratories have classified lineage-biased stem cells based on cellular output after transplantation, for example, myeloid, balanced, and lymphoid-biased HSCs [13]. In an alternative scheme, α-, β-, γ-, and δ-type stem cells have been defined [16], again

based on their transplantation characteristics and cellular output.

Multipotent progenitor cells are also heterogeneous in terms of self-renewal potential and lineage output [20,21]. At least a subset of cells within the CD150$^-$CD48$^-$LSK population, usually referred to as "multipotent progenitors" (MPPs) or "short-term hematopoietic stem cells" (ST-HSCs), have some capacity for long-term multilineage reconstitution, specifically those negative for CD229 and CD244 expression [9] or expressing *Fgd5* [22], indicating that stem cell potential is not exclusively contained within the phenotypic CD150$^+$CD48$^-$LSK HSC compartment. Interestingly, the repopulating potential of CD150$^-$CD48$^-$LSK cells, as well as canonical HSCs, is greatly enhanced by loss of the tumor suppressor PHF6 [23,24]. Similarly, combined deletion of the tumor suppressor genes *Trp53, p16^{Ink4a},* and *p16^{Arf}* enables long-term replenishment of the hematopoietic system by CD150$^-$CD48$^-$LSK MPP cells [25], assuming the apparent long-term reconstitution is not simply a result of longer-lived mature cells resulting from decreased apoptosis. Furthermore, when drawing conclusions from studies using loss-of-function mouse strains, it is important to consider that stem cell activity outside of the phenotypically defined CD150$^+$CD48$^-$LSK HSC population may indicate altered cell surface marker expression on functional HSCs rather than acquisition of stem cell properties in a progenitor population.

In contrast to the relatively rapidly cycling progenitor population, a large proportion of the adult HSC
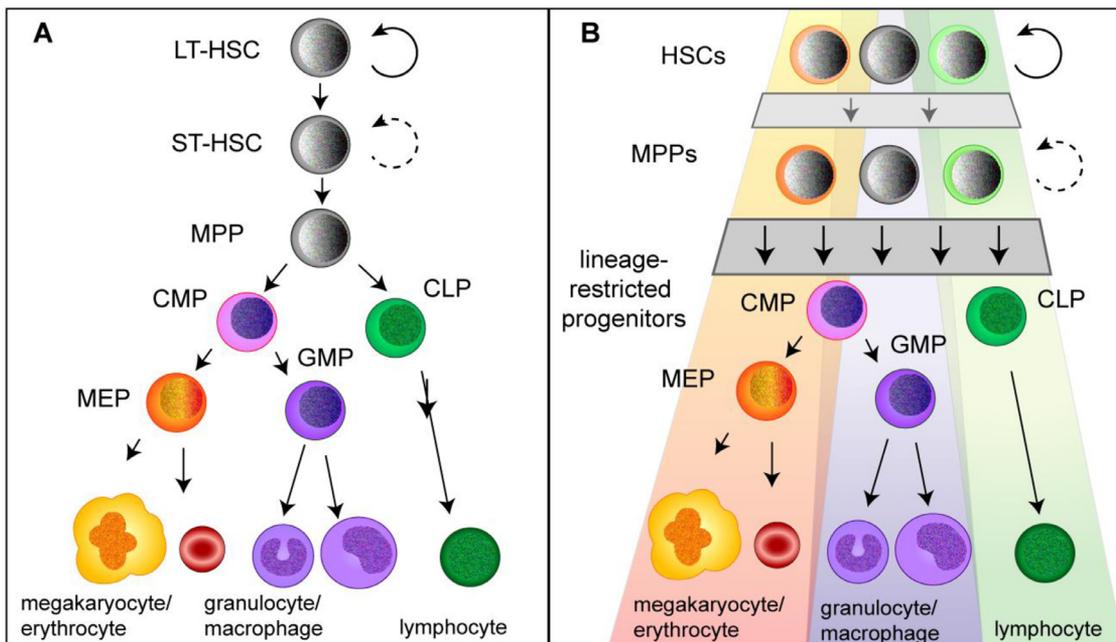
Figure 1. Evolving concepts of hematopoiesis. **(A)** "Classic" model of hematopoiesis. **(B)** Model of hematopoiesis incorporating recent findings, including (1) HSC heterogeneity and lineage-biased output; (2) the continuum of hematopoietic differentiation, as suggested by single-cell RNA sequencing studies; and (3) self-renewal of MPPs based on sustained contribution in situ. LT-HSC=long-term hematopoietic stem cell; ST-HSC=short-term hematopoietic stem cell; MPP=multipotent progenitor; CMP=common myeloid progenitor; CLP=common lymphocyte progenitor; MEP=megakaryocyte/erythrocyte progenitor; GMP=granulocyte/macrophage progenitor.

population is quiescent [26]. Single-cell RNA sequencing has revealed that variation in cycling status is one of the major features of HSC heterogeneity [27,28]. HSCs can be divided into the relatively "fast" cycling cells that divide every 36 days (80%−85% of the population) and more dormant cells (15%−20% of the population) that divide every 145 days, based on models fitted to BrdU and H2B-GFP label retaining data [29,30]. These differences in cell cycle characteristics reflect differences in transplantability, with cells in G_0 having the highest transplantability [31]. Interestingly, HSCs from endosteal niches have greater transplantability compared with HSCs in the more accessible central niche [32], which is reflected in differences in proliferative potential and their location after transplantation [33,34].

Epigenetic regulation of stem and progenitor phenotypes is an important factor in heterogeneity. Myeloid-biased and lymphoid-biased repopulating stem cells retain their characteristics through multiple rounds of transplantation, leading to the suggestion that the bias is "epigenetically" programmed in HSC subtypes [16,35,36]. If this were correct, it would mean that the "epigenetic" program is robustly maintained through a large number of cell cycles and that epigenetic "writers" would be essential for determining HSC phenotype after each cell division. Nonetheless, some stem cells that appear lineage restricted on primary transplantation can regain other lineage potentials in subsequent transplantation rounds [37,38], indicating a degree of plasticity.

Sustained hematopoiesis despite lack of classic HSCs

The lysine (K) acetyltransferase 6A (KAT6A, also called monocytic leukemia zinc finger protein [MOZ]), a MYST family histone acetyltransferase, is an epigenetic "writer" that catalyzes the transfer of an acetyl group to histones. KAT6A (MOZ) was first identified in a recurrent translocation leading to acute myeloid leukemia [39]. *KAT6A*-transformed leukemias typically have a pattern of deregulated *HOX* genes [40,41]. *Hox* genes play a vital role in hematopoiesis [42,43] and in survival of leukemic cells [43,44]. Interestingly, KAT6A is required for normal levels of H3K9 acetylation at *Hox* genes during embryogenesis [45], and mice lacking KAT6A have a homeotic transformation of the axial skeleton [45,46]. In the absence of KAT6A, HSCs fail to develop during embryogenesis [47,48]. However, apart from additional roles in the regulation of pre-B-cell numbers [49] and CD8^{+} memory T cells [50], the formation of embryonic progenitors and mature blood cells is not affected [47]. The histone acetyltransferase activity of KAT6A is required for its role in formation of HSCs [51], suggesting that its epigenetic "writer" function is needed in HSCs.

During adult life, KAT6A is essential for maintenance of classically defined adult HSCs, but not essential for differentiation of most mature blood cells [52].

This suggested to us that studying $Kat6a^{-/-}$ bone marrow might provide insights into the role of HSCs in steady-state hematopoiesis. Conditional knockout of KAT6A in adult mice (using the interferon-inducible *Mx1-Cre* transgene to effect recombination) leads to an acute loss of transplantable stem cells [52]. What happens to adult mice lacking KAT6A function and hence classic HSCs? If HSCs were essential for continual blood production, one might expect that mice lacking HSCs because of loss of KAT6A would become anemic. Surprisingly, mice lacking KAT6A were largely unaffected. There was no change to the hematocrit and no abnormal erythrocytes (in terms of number or morphology), although there were reduced numbers of B cells [52], reflecting a function for KAT6A in this lineage [49]. The CD150^{+}CD48^{−}LSK HSC population did not recover, even after 18 months. Importantly, there was no recruitment of HSCs containing un-recombined $Kat6a^{lox}$ alleles. These results indicate that myeloid and lymphoid cell production was not dependent on a normal population of "classically defined" HSCs.

Similar observations have been made using mice in which the phenotypic HSC compartment was experimentally depleted to 4% of normal levels via diphtheria toxin A expression induced by the *Tal1-Cre/ERT* transgene (upon tamoxifen administration) [53]. A substantial loss of repopulating ability post-transplantation was demonstrated, indicating depletion of functional, as well as phenotypic, HSCs. The HSC population remained under 10% for at least 26 weeks, without a compensatory increase in cycling of HSCs. Despite loss of the majority of HSCs, there were normal numbers of mature peripheral blood cell and no signs of hematopoietic failure, even up to 50 weeks post-tamoxifen.

Together, these results suggest that either transplantable HSCs have a much more limited role in steady-state hematopoiesis than previously thought, or there is compensation at the level of primitive progenitors (or residual HSCs) in the absence of normal numbers of classic HSCs.

What contribution do HSCs make to steady-state hematopoiesis?

The experiments described above indicate that the majority of HSCs are not essential for blood formation [52,53]. Therefore, the question arises: To what extent do long-term transplantable HSCs contribute to hematopoiesis under steady-state conditions? In situ labeling studies have confirmed the role of HSCs at the apex of hematopoiesis during embryonic development and in juvenile mice [36,54,55]. However, the contribution of HSCs in adult hematopoietic homeostasis remains a source of controversy, with different conclusions reached using different labeling techniques (Table 1).

Early studies used irradiation-induced chromosomal changes to track HSC contribution without transplantation.

Table 1. Studies investigating contribution/output of HSCs without transplantation in adult mice

Reference	Labeling strategy	Cells labeled at outset	Summary of findings and conclusions	Limitations
Drize et al., 2001 [56]	Sublethal irradiation to induce chromosomal changes	$52.6 \pm 26.4\%$ of colonies Not HSC-specific	Conclude that hematopoiesis is sustained by successive recruitment of clones derived from individual cells	Chromosomal changes and irradiation may affect clonal output
Zavidij et al., 2012 [57]	In vivo labeling of cells with gfp through an in situ lentiviral system	Detected 0.005%−0.5% GFP$^+$ cells in peripheral blood cells for up to 24 mo after intrafemoral injection Not HSC-specific	Conclude that 400 to several thousand clones contribute to steady-state hematopoiesis	Less than 1% of cells labeled Potential for integration site effects
Sun et al., 2014 [58] Rodriguez-Fraticelli et al., 2018 [70]	Dox-inducible hyperactive Sleeping Beauty transposase (ubiquitous *Rosa26* promoter) stimulates mobilization of a specific transposon The integration site of the transposon is used as a molecular tag for that cell and its progeny	30% of HSCs labeled Not HSC-specific: 30% of ST-HSCs and MPPs labeled	90%−98% of granulocyte clones in peripheral blood were detected only at single time points (from 3 to 12 mo), indicating granulopoiesis is driven by successive recruitment of short-lived clones Subsequent study by Roriguez-Faticelli et al. [70] reported that many HSCs and megakaryocyte progenitors shared tags exclusively with no MPP intermediate labeled	Potential for integration site effects Not HSC specific, varying rates of cell turnover of labeled cells Low abundance of HSCs compared with progenitors and mature cells may decrease chance of HSC with tag being present in bone used for analysis Possibility tag may be "lost" from ancestral or transient population because of differentiation or death, which may lead to an underrepresented number of overlapping tags between populations
Busch et al., 2015 [55]	Tamoxifen-inducible *Tie2*^{MCM} (improved Cre fused to MCM-modified estrogen receptor-binding domain) and *Rosa*^{YFP} reporter	~1% HSCs (LSK CD48$^-$ C150$^+$) labeled Likely no ST-HSCs or MPPs labeled	Conclude that at least 30% of HSCs (lower estimate) are productive (i.e., produce YFP$^+$ CD45$^+$ progeny) Labeling of mature cells is not proportional to labeling of HSCs; therefore, the authors suggest that mature cells may be produced from self-renewing progenitors	Only ~1% of HSCs are labeled; therefore, not necessarily representative of the bulk of the HSC pool (there may not be a proportional contribution of labeled to unlabeled HSCs)
Sawai et al., 2016 [60] Upadhaya et al., 2018 [72]	BAC transgenic with a tamoxifen-inducible CreERT2 under the *Pdzk1ip1* promoter, which preferentially labels HSCs	30% of HSCs labeled Progenitors also labeled: ~10% ST-HSCs and ~3% MPPs	Conclude that HSCs are the major source of multilineage hematopoiesis in adult animals, given that more than 60% of myeloid cells are labeled within 32 wk	Approximately 10% of ST-HSCs and MPPs labeled from outset so expansion of this 10% or nonproportional contribution to downstream populations could confound interpretation
Pei et al., 2017 [54]	*Tie2-cre*^{MCM}-driven recombination of a *polylox* allele with unique tag for each clone generated by combination of recombination	Labeling in adults was not restricted to HSCs, also in ST-HSCs and MPPs. Note that this is in contrast to the specificity of the *Tie2-cre*^{MCM} allele in the Busch et al. [55] study, likely because of the increased sensitivity of the *polylox* allele to recombination compared with the *Rosa*^{YFP} reporter construct	Large number of shared molecular tags between HSC and mature cells when recombination was initiated in embryos, but lower overlap of tags between HSCs and mature cells when recombination was initiated at 8 wk of age, i.e., less output from adult compared with embryonic HSCs	Not HSC specific

Table 1 (*Continued*)

Reference	Labeling strategy	Cells labeled at outset	Summary of findings and conclusions	Limitations
Chapple et al., 2018 [61]	*Krt18-CreERT2* and *Rosa26-LSL-YFP* transgenes	$2 \pm 1.2\%$ HSCs labeled Progenitors also labeled: $0.11 \pm 0.21\%$ MPP $0.28 \pm 0.15\%$ HPC-1 $0.48 \pm 0.41\%$ HPC-2	Conclude substantial HSC contribution to adult steady-state hematopoiesis based on labeling of 60% of myeloid cells and 50% of CLPs relative to HSC labeling Platelets reached equilibrium with the percentage of HSCs labeled by 1 y, indicating bias of adult HSCs to contribute to the megakaryocyte/platelet lineage	Labeling of progenitor population from outset may confound interpretation, if the contribution of these labeled progenitors is not proportional/equivalent to the non-labeled population
Chapple et al., 2018 [61]	*Fgd5-ZsGreen-2ACreERT2* knock-in and *Rosa26-LSL-tdTomato* transgene with 5 d of tamoxifen treatment	$32 \pm 10\%$ of HSCs labeled Progenitors also labeled: $\sim$8%−10% MPPs $\sim$<2% of HPC-1 3%−4% HPC-2	Labeling of more than two-thirds of B cells and platelets at 26 wk indicates contribution of HSCs	Low-level labeling of progenitor population from outset may confound interpretation if the contribution of these labeled progenitors is not proportional/equivalent to the non-labeled population
Sawen et al., 2018 [22]	*Fgd5-ZsGreen-2ACreERT2* knock-in and *Rosa26-LSL-tdTomato* transgene with 1-d tamoxifen treatment (in first experiment assessing rate of labeling of progenitors and mature cells)	$5.7 \pm 2.5\%$ of HSCs labeled $0.2 \pm 0.1\%$ of other LSKs labeled	Near equilibrium of CD150−CD48−LSK MPP cells reached by 4 wk Equilibrium of CD150+CD48−LSK MPP-2 cells and megakaryocyte progenitors reached by 32 wk Labeling of mature cells in order of fastest to slowest: platelets, granulocytes, erythrocytes, NK cells, B cells, then T cells; authors conclude that the increase in labeling of mature cells over time indicates continuous input from HSCs	Low level of progenitor population from outset may confound interpretation if the contribution of these labeled progenitors is not proportional/equivalent to the non-labeled population
Carrelha et al., 2018 [38]	*Vwf-CreERT2* line used for in situ studies, 2 mo after tamoxifen pulse	$12 \pm 1.3\%$ of HSCs (LSK CD34− CD48− cells) labeled	Showed that a platelet-biased subset of HSCs contributes to hematopoiesis in situ	Only a specific-subset of HSCs labeled
Yu et al., 2016 [36]	"HUe" transgene consisting of multiple lox sites separating fluorescent reporters enabling unique color based on recombination permutation Used in combination with the poly(I:C)-inducible *Mx1-cre* transgene to mark hematopoietic cells	Not HSC-specific labeling	Based on recombination induced at postnatal day 14 and tracking up to 10 mo, one to four clones make up 80% of the peripheral blood Some clones were stable, some disappeared, some became active at later time points	Although mice were used at a time point when changes in HSC cycling and cell numbers induced by poly (I:C) had returned to normal, it is possible the clonal output of HSCs may have been affected by the poly (I:C) treatment

These experiments indicated that hematopoiesis involved successive recruitment of multiple clones presumed to be derived from a single cell [56]; however, the potential for irradiation and chromosomal changes to influence clonal output is a significant limitation of this technique. Later work drew similar conclusions using an in vivo GFP-lentiviral system to track steady-state hematopoiesis. Because of the small clone size, conclusions could not be drawn about the kinetics of lineage output of long-term labeled cells; however, this work found that some hundreds to thousands of clones contribute to steady-state hematopoiesis, each with a very small contribution [57], based on the assumption that the 0.01%−0.23% of labeled peripheral blood cells were representative of the entire system.

An investigation of hematopoietic clonal dynamics employed a ubiquitously expressed doxycycline-inducible hyperactive Sleeping Beauty transposase to infer hierarchical relationships by using the integration site of the transposon as a tag for each cell and its progeny [58]. Labeling of 20%−30% of hematopoietic stem and progenitor cells with a transposon tag was achieved. Serial peripheral blood analysis every 4−5 weeks over 3−12 months post-doxycycline treatment revealed 65−905 unique tags per mouse in granulocytes at any one time point, demonstrating polyclonal contribution. The majority (90%−98%) of these tags were detected at only one time point (with 4−5 weeks between sampling), suggesting the contribution to granulopoiesis from each tagged clone was short-lived. Lymphocyte clones were generally more stable. Although there was a high concordance of tags within myeloid cell types, few tags were shared initially between lymphoid and myeloid cells. This suggests substantial contribution from lineage-restricted progenitors or potentially may reflect the difference in life span and turnover between lymphoid and myeloid cell types. The number of shared tags between B cells and granulocytes increased with time. Interestingly, LT-HSCs shared few tags with progenitors and mature cells, but there was high overlap between tags in progenitors and downstream progeny, leading the authors to conclude that the majority of differentiated populations were derived from progenitors rather than HSCs and that there was little differentiation of HSCs to progenitors. An important consideration when interpreting these data is whether the increased frequency of shared tags between MPPs and mature cells compared with LT-HSCs and mature cells could be a result of the relative abundance of MPPs compared with LT-HSCs. In this way, there could be a reduced likelihood of capturing an LT-HSC with a shared tag than an MPP with a shared tag. The ancestral HSC may be situated in a bone that was not harvested for experimental analysis or be present at too low a frequency to be detected. Alternatively, the lack of overlap between HSCs and mature cells may reflect a loss of the tag from the HSC population upon differentiation [59].

Lineage-tracing studies that specifically label HSCs and downstream progeny avoid issues associated with nonspecific tagging. A tamoxifen-inducible Tie2-Cre knock-in used to induce the expression of a fluorescent protein (YFP) in all downstream progeny labeled an average of 1% of HSCs (CD150$^+$CD48$^-$LSK) [55]. By in situ limiting dilution analysis (comparing the percentage of labeled HSCs with the percentage of labeled progeny 6−36 weeks post-labeling), Busch et al. [55] estimated a lower limit of one in three YFP cells contributing to overall hematopoiesis. Importantly, no labeling of ST-HSCs or MPPs was detected until 4 weeks post-treatment, indicating the initial labeling was likely restricted to the HSC population. Although this study concludes that 30% or more of HSCs contribute to steady-state hematopoiesis, equilibrium between the proportion of labeled HSCs and labeled progeny was not achieved within the lifetime of a mouse. The lack of equilibrium between labeled HSCs and labeled progeny led the authors to suggest that mature blood cells must be replenished from a source of cells other than HSCs, indicating that steady-state hematopoiesis might be largely sustained by progenitors with high self-renewal capacity [55].

In contrast, a number of subsequent studies using different HSC-labeling systems concluded that there was substantial ongoing HSC contribution to mature cells during adulthood. Sawai et al. [60] labeled HSCs using a BAC transgene containing a tamoxifen-inducible CreERT2 expressed from a truncated Pdzk1ip1 promoter, achieving genetic tagging of 32.6% of HSCs 3 days after a single tamoxifen treatment. With this labeling method, 60% to 70% of mature cells of most lineages were labeled after 36 weeks, suggesting a considerable contribution of HSCs to hematopoiesis during adulthood. These conclusions were supported by Chapple et al. [61], who used Krt18-CreERT2 transgenic mice with a Rosa26-LSL-YFP reporter to label 2% HSCs. Within 1 year, labeling of B cells was at 50% and myeloid cells was at 60% of the frequency of labeled HSCs. Similarly, labeling of 32% of HSCs using Fgd5-CreERT2 knock-in mice with a Rosa26-LSL-tdTomato reporter yielded comparable results [61]. The Fgd5-CreERT2 system was also employed by Sawen et al. [22], who corroborated findings of Chapple et al. [61], reporting significant contribution of HSCs to progenitors and mature blood cells.

The contribution of HSCs to adult hematopoiesis estimated by Sawai et al., Chapple et al., and Sawen et al. is much larger than the ~30% and 5% contributions of HSCs within 1 year suggested by Busch et al. [55] and Sun et al. [58] respectively. The interpretation of these experiments depends critically on the specificity of the Cre transgenic used for labeling. The Pdzk1ip1 transgene employed by Sawai et al. [60] and the Fgd5-CreERT2 transgene used by Chapple et al [61] yielded an increased labeling efficiency of HSCs compared

with the *Tie2-Cre* transgene used by Busch et al. [55], which should result in a more accurate representation of the overall stem cell compartment. However, these systems also appeared to be more "leaky" as 3% MPPs and "up to 10% of ST-HSCs"' were also labeled from the outset using *Pdzk1ip1* [60]. Between 0.1% and 0.48% of progenitors were labeled by the *Krt18-CreERT2* transgene [61], which is substantial compared with the proportion of HSCs labeled by *Krt18-CreERT2* (2%). Similarly, a subset of progenitor cells was labeled by the *Fgd5-CreERT2* transgene [22,61]. The authors suggest that any tagged populations above these frequencies must be derived from HSCs; however, this is assuming there is a proportional contribution of the labeled ST-HSCs and MPPs to downstream populations, which may not necessarily be the case. This confounds interpretation of HSC contribution and may be one reason for the discrepancy between various studies. Alternatively, because the HSC compartment is known to be heterogeneous, the discrepancy may reflect the labeling of subsets of HSCs with unique properties. Hofer and Rodewald [62] propose that cells labeled by *Tie2-Cre* [55] represent "tip-HSCs," a subset of HSCs at the top of the hierarchy, HSCs more quiescent than those detected by Sawai et al. [60] and Chapple et al [61]. Thus, it is possible that *Tie2*-expressing HSCs contribute less to hematopoiesis than the bulk of the HSCs and that the lack of equilibrium between labeled HSCs and the frequency of labeled progeny could be accounted for by increased contribution of non-*Tie2*-expressing HSCs. Moreover, because the identity of self-renewing cells within the heterogeneous progenitor populations that appear to support steady-state granulopoiesis [55,58] is not precisely determined, it is possible that these include a subset of progenitor cells labeled by activation of a *Pdzk1ip1*-driven, *Fgd5*-driven, and/or *Krt18*-driven crerecombinase. Indeed, Sawen et al. found that *Fgd5*-labeled MPPs could provide long-term multilineage reconstitution post-transplantation, indicating that at least a subset of labeled MPPs have the properties of functional HSCs [22]. Despite the controversies, each of the lineage tracing studies described here demonstrates at least some HSC contribution to adult hematopoiesis. Further work is required to clarify the extent of that HSC contribution and the contribution of progenitor cells.

Contribution of HSCs to stress-response hematopoiesis

Irrespective of the relative contribution of HSCs to steady-state hematopoiesis, deletion studies demonstrate that the majority of HSCs are not strictly required for hematopoiesis [52,53]. Thus, the question remains: Why have these cells evolved? It has been suggested that HSCs represent a "reserve capacity," and it is certainly true that a laboratory mouse is not challenged in the same way as a wild mouse. Supporting this idea, quiescent HSCs enter the cell cycle

under the influence of inflammatory cytokines [63,64] or repeat bleeding [26] or following depletion of progenitor cells by cytotoxic agents such as 5-fluorouracil (5-FU) [65]. Consistently, there appears to be a larger contribution of HSCs to peripheral blood after injection of 5-FU [55] or the interferon response-inducing poly(I:C) [60]. Thus, the activity of HSCs increases in response to stress. Surprisingly, however, mice with depleted HSCs do not exhibit increased sensitivity to stress, including treatment with G-CSF, poly(I:C), or 5-FU [53]. On the other hand, hematopoiesis did fail in mice with more substantial HSC depletion and earlier 5-FU injection [53], indicating that although the bulk of HSCs are not strictly necessary for response to mild hematopoietic stress, they are likely required for response to severe stress.

Contribution of HSCs to erythroid and platelet production

A large proportion of transplantation experiments examine only granulocyte/macrophage/lymphoid reconstitution, because these allow easy separation of donor and recipient via cell surface markers. Preservation of oxygen carrying capacity is of immediate importance, yet erythrocytes are rarely examined. A recent study estimated that 86% of cells produced by HSCs post-transplantation are red blood cells [66], highlighting the substantial contribution of HSCs to the erythroid lineage. Humans with aplastic anemia have a reduced number of phenotypic HSCs as well as reduced HSC function compared with healthy controls, assessed in vitro [67]. Despite substantial loss of HSCs, the number of granulocyte/macrophage progenitor cells is not as severely affected as megakaryocyte/erythroid lineages [68]. Thus, assuming there is not a specific erythroid-lineage defect in these patients, it appears that the erythroid lineage is much more heavily dependent on HSCs for sustained production [68].

Interestingly, recent publications suggest that a subset of HSCs primed for platelet production contribute to long-term hematopoiesis post-transplantation [38,69] and in situ [38]. These cells sit at the top of the HSC hierarchy, at least post-transplantation [69]. Although described as "lineage-restricted" because of their almost exclusive production of platelets in situ and upon primary transplantation, these platelet-biased cells are defined as stem cells because they retain multilineage differentiation in vitro and upon secondary transplantation [38]. Clonal analysis using the Sleeping Beauty transposase indicated that megakaryocyte progenitors might be produced from HSCs independently of other lineages by a differentiation pathway not involving MPPs or megakaryocyte/erythrocyte progenitors, based on shared tags between HSCs and megakaryocyte progenitors, not present in other populations [70]. However, it is possible that failure to detect tags in transient populations may mask a common ancestor. Indeed, the in situ production of both platelets and erythroid

progenitors from FLT3^{+} progenitor cells has previously been demonstrated, albeit not at the clonal level [71]. Notwithstanding the controversy surrounding the in situ differentiation pathway, lineage tracing has consistently revealed that platelets are labeled more rapidly than lymphoid and other myeloid cell populations [22,60,61,72]. Loss of platelets will lead to rapid death by internal bleeding; a lethal dose of the anticoagulant brodifacoum leads to death of mice within 4−9 days [73], whereas the absence of B and T lymphocytes is compatible with life [74], at least in an environment largely protected from pathogens. Possibly, the main function of HSCs in adult hematopoiesis is to support oxygen carrying capacity and blood vessel integrity rather than the innate and adaptive immune system.

Conclusions and considerations

Although HSCs do not appear to be strictly required for in situ hematopoiesis [52,53], labeling studies demonstrate at least some HSC contribution during steady-state hematopoiesis [22,55,58,60,61], confirming the position of HSCs at the top of the adult hematopoietic hierarchy. Recent studies leave little doubt that the hematopoietic stem cell compartment is heterogeneous and that the distinction between "progenitors" and "stem cells" is not as clear as might be expected from transplantation experiments. Although HSCs play an ongoing role in adult hematopoiesis, we suggest that progenitor cells may participate in hematopoiesis longer than predicted by transplantation experiments and, indeed, appear to be able to support hematopoiesis in cases of HSC depletion [52,53]. Single-cell transcriptomic technologies [75−78] indicate that stem and progenitor cells exist as a continuum on a spectrum of differentiation and offer a way forward for characterizing rare heterogeneous cell populations. Recently, inhibitors of epigenetic regulators that control key aspects of HSCs have been identified, for example, KAT6A inhibitors [79], providing new opportunities to study stem cell biology in situ and in a transplant system. Further work delineating the properties of HSC and progenitor subsets labeled by the various existing labeling systems is warranted to advance our understanding of the complexity of HSC and progenitor heterogeneity. The development of novel labeling systems, including technology that labels specific progenitor populations and their progeny in situ, will help clarify the extent of HSC versus progenitor contribution to adult hematopoiesis.

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Conflict of interest disclosure

The authors declare that they have no conflicts of interest, financial or otherwise.

References

1. Thomas ED, Buckner CD, Banaji M, et al. One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood. 1977;49:511–533.
2. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. N Engl J Med. 2010;363:2091–2101.
3. Hamilton BK, Copelan EA. Concise review: The role of hematopoietic stem cell transplantation in the treatment of acute myeloid leukemia. Stem Cells. 2012;30:1581–1586.
4. Copelan EA. Hematopoietic stem-cell transplantation. N Engl J Med. 2006;354:1813–1826.
5. Alter BP. Inherited bone marrow failure syndromes: Considerations pre- and posttransplant. Blood. 2017;130:2257–2264.
6. Adolfsson J, Borge OJ, Bryder D, et al. Upregulation of Flt3 expression within the bone marrow Lin(−)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity. 2001;15:659–669.
7. Yang L, Bryder D, Adolfsson J, et al. Identification of Lin(−)Sca1(+)kit(+)CD34(+)Flt3− short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. Blood. 2005;105:2717–2723.
8. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell. 2005;121:1109–1121.
9. Oguro H, Ding L, Morrison Sean J. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell. 2013;13:102–116.
10. Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science. 2011;333:218–221.
11. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science. 1996;273:242–245.
12. Gordon MY, Lewis JL, Marley SB. Of mice and men ... and elephants. Blood. 2002;100:4679–4680.
13. Muller-Sieburg CE, Cho RH, Karlsson L, Huang JF, Sieburg HB. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. Blood. 2004;103:4111–4118.
14. Challen GA, Boles NC, Chambers SM, Goodell MA. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell. 2010;6:265–278.
15. Benz C, Copley MR, Kent DG, et al. Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. Cell Stem Cell. 2012;10:273–283.
16. Dykstra B, Kent D, Bowie M, et al. Long-term propagation of distinct hematopoietic differentiation programs in vivo. Cell Stem Cell. 2007;1:218–229.
17. Yamamoto R, Morita Y, Ooehara J, et al. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. Cell. 2013;154:1112–1126.

18. Morita Y, Ema H, Nakauchi H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. J Exp Med. 2010;207:1173–1182.

19. Muller-Sieburg CE, Sieburg HB. The GOD of hematopoietic stem cells: A clonal diversity model of the stem cell compartment. Cell Cycle. 2006;5:394–398.

20. Pietras EM, Reynaud D, Kang YA, et al. Functionally distinct subsets of lineage-biased multipotent progenitors control blood production in normal and regenerative Conditions. Cell Stem Cell. 2015;17:35–46.

21. Naik SH, Perié L, Swart E, et al. Diverse and heritable lineage imprinting of early haematopoietic progenitors. Nature. 2013;496:229–232.

22. Sawen P, Eldeeb M, Erlandsson E, et al. Murine HSCs contribute actively to native hematopoiesis but with reduced differentiation capacity upon aging. Elife. 2018:7.

23. McRae HM, Garnham AL, Hu Y, et al. PHF6 regulates hematopoietic stem and progenitor cells and its loss synergizes with expression of TLX3 to cause leukemia. Blood. 2019. Available at: https://doi.org/10.1182/blood-2018-07-860726.

24. Wendorff AA, Quinn SA, Rashkovan M, et al. Phf6 loss enhances HSC self-renewal driving tumor initiation and leukemia stem cell activity in T-ALL. Cancer Discov. 2019;9:436–451.

25. Akala OO, Park I-K, Qian D, Pihalja M, Becker MW, Clarke MF. Long-term haematopoietic reconstitution by Trp53−/−p16Ink4a−/−p19Arf−/− multipotent progenitors. Nature. 2008;453:228–232.

26. Cheshier SH, Morrison SJ, Liao X, Weissman IL. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci USA. 1999;96:3120–3125.

27. Lauridsen FKB, Jensen TL, Rapin N, et al. Differences in cell cycle status underlie transcriptional heterogeneity in the HSC compartment. Cell Rep. 2018;24:766–780.

28. Bernitz JM, Kim HS, MacArthur B, Sieburg H, Moore K. Hematopoietic stem cells count and remember self-renewal divisions. Cell. 2016;167:1296–1309.e1210.

29. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell. 2008;135:1118–1129.

30. Foudi A, Hochedlinger K, Van Buren D, et al. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. Nat Biotechnol. 2009;27:84–90.

31. Passegué E, Wagers AJ, Giuriato S, Anderson WC, Weissman IL. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. J Exp Med. 2005;202:1599–1611.

32. Gong JK. Endosteal marrow: A rich source of hematopoietic stem cells. Science. 1978;199:1443–1445.

33. Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted hemopoietic stem cells: Inferences for the localization of stem cell niches. Blood. 2001;97:2293–2299.

34. Grassinger J, Haylock DN, Williams B, Olsen GH, Nilsson SK. Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential. Blood. 2010;116:3185–3196.

35. Cabezas-Wallscheid N, Klimmeck D, Hansson J, et al. Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. Cell Stem Cell. 2014;15:507–522.

36. Yu VWC, Yusuf RZ, Oki T, et al. Epigenetic memory underlies cell-autonomous heterogeneous behavior of hematopoietic stem cells. Cell. 2016;167:1310–1322.e1317.

37. Yamamoto R, Wilkinson AC, Ooehara J, et al. Large-scale clonal analysis resolves aging of the mouse hematopoietic stem cell compartment. Cell Stem Cell. 2018;22. 600-607 e604.

38. Carrelha J, Meng Y, Kettyle LM, et al. Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. Nature. 2018;554:106–111.

39. Borrow J, Stanton VP Jr, Andresen JM, et al. The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nat Genet. 1996;14:33–41.

40. Camos M, Esteve J, Jares P, et al. Gene expression profiling of acute myeloid leukemia with translocation t(8;16)(p11;p13) and MYST3-CREBBP rearrangement reveals a distinctive signature with a specific pattern of HOX gene expression. Cancer Res. 2006;66:6947–6954.

41. Murati A, Gervais C, Carbuccia N, et al. Genome profiling of acute myelomonocytic leukemia: alteration of the MYB locus in MYST3-linked cases. Leukemia. 2009;23:85–94.

42. Ernst P, Mabon M, Davidson AJ, Zon LI, Korsmeyer SJ. An Mll-dependent Hox program drives hematopoietic progenitor expansion. Curr Biol. 2004;14:2063–2069.

43. Abramovich C, Humphries RK. Hox regulation of normal and leukemic hematopoietic stem cells. Curr Opin Hematol. 2005;12:210–216.

44. Faber J, Krivtsov AV, Stubbs MC, et al. HOXA9 is required for survival in human MLL-rearranged acute leukemias. Blood. 2009;113:2375–2385.

45. Voss AK, Collin C, Dixon MP, Thomas T. Moz and retinoic acid coordinately regulate H3K9 acetylation, Hox gene expression, and segment identity. Dev Cell. 2009;17:674–686.

46. Sheikh BN, Downer NL, Phipson B, et al. MOZ and BMI1 play opposing roles during Hox gene activation in ES cells and in body segment identity specification in vivo. Proc Natl Acad Sci U S A. 2015;112:5437–5442.

47. Thomas T, Corcoran LM, Gugasyan R, et al. Monocytic leukemia zinc finger protein is essential for the development of long-term reconstituting hematopoietic stem cells. Genes Dev. 2006;20:1175–1186.

48. Katsumoto T, Aikawa Y, Iwama A, et al. MOZ is essential for maintenance of hematopoietic stem cells. Genes Dev. 2006;20:1321–1330.

49. Sheikh BN, Lee SCW, El-Saafin F, et al. MOZ regulates B-cell progenitors and, consequently, Moz haploinsufficiency dramatically retards MYC-induced lymphoma development. Blood. 2015;125:1910–1921.

50. Newman DM, Sakaguchi S, Lun A, et al. Acetylation of the Cd8 locus by KAT6A determines memory T cell diversity. Cell Rep. 2016;16:3311–3321.

51. Perez-Campo FM, Borrow J, Kouskoff V, Lacaud G. The histone acetyl transferase activity of monocytic leukemia zinc finger is critical for the proliferation of hematopoietic precursors. Blood. 2009;113:4866–4874.

52. Sheikh BN, Yang YQ, Schreuder J, et al. MOZ (KAT6A) is essential for the maintenance of classically defined adult hematopoietic stem cells. Blood. 2016;128:2307–2318.

53. Schoedel KB, Morcos MNF, Zerjatke T, et al. The bulk of the hematopoietic stem cell population is dispensable for murine steady-state and stress hematopoiesis. Blood. 2016;128:2285–2296.

54. Pei WK, Feyerabend TB, Rossler J, et al. Polylox barcoding reveals haematopoietic stem cell fates realized in vivo. Nature. 2017;548:456–460.

55. Busch K, Klapproth K, Barile M, et al. Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. Nature. 2015;518:542–546.

56. Drize NJ, Olshanskaya YV, Gerasimova LP, et al. Lifelong hematopoiesis in both reconstituted and sublethally irradiated

mice is provided by multiple sequentially recruited stem cells. Exp Hematol. 2001;29:786–794.

57. Zavidij O, Ball CR, Herbst F, et al. Stable long-term blood formation by stem cells in murine steady-state hematopoiesis. Stem Cells. 2012;30:1961–1970.

58. Sun J, Ramos A, Chapman B, et al. Clonal dynamics of native haematopoiesis. Nature. 2014;514:322–327.

59. Lu R. Sleeping beauty wakes up the clonal succession model for homeostatic hematopoiesis. Cell Stem Cell. 2014;15:677–678.

60. Sawai CM, Babovic S, Upadhaya S, et al. Hematopoietic stem cells are the major source of multilineage hematopoiesis in adult animals. Immunity. 2016;45:597–609.

61. Chapple RH, Tseng YJ, Hu T, et al. Lineage tracing of murine adult hematopoietic stem cells reveals active contribution to steady-state hematopoiesis. Blood Adv. 2018;2:1220–1228.

62. Hofer T, Rodewald HR. Differentiation-based model of hematopoietic stem cell functions and lineage pathways. Blood. 2018;132:1106–1113.

63. Essers MAG, Offner S, Blanco-Bose WE, et al. IFN alpha activates dormant haematopoietic stem cells in vivo. Nature. 2009; 458:904–908.

64. Sato T, Onai N, Yoshihara H, Arai F, Suda T, Ohteki T. Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion. Nat Med. 2009;15:696–700.

65. Harrison DE, Lerner CP. Most primitive hematopoietic stem-cells are stimulated to cycle rapidly after treatment with 5-fluorouracil. Blood. 1991;78:1237–1240.

66. Boyer SW, Rajendiran S, Beaudin AE, et al. Clonal and quantitative in vivo assessment of hematopoietic stem cell differentiation reveals strong erythroid potential of multipotent cells. Stem Cell Rep. 2019;12:801–815.

67. Maciejewski JP, Risitano A. Hematopoietic stem cells in aplastic anemia. Arch Med Res. 2003;34:520–527.

68. Notta F, Zandi S, Takayama N, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science. 2016;351:aab2116.

69. Sanjuan-Pla A, Macaulay IC, Jensen CT, et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature. 2013;502:232–236.

70. Rodriguez-Fraticelli AE, Wolock SL, Weinreb CS, et al. Clonal analysis of lineage fate in native haematopoiesis. Nature. 2018; 553:212–216.

71. Boyer SW, Schroeder AV, Smith-Berdan S, Forsberg EC. All hematopoietic cells develop from hematopoietic stem cells through Flk2/Flt3-positive progenitor cells. Cell Stem Cell. 2011;9:64–73.

72. Upadhaya S, Sawai CM, Papalexi E, et al. Kinetics of adult hematopoietic stem cell differentiation in vivo. J Exp Med. 2018;215:2815–2832.

73. Redfern R, Gill JE, Hadler MR. Laboratory evaluation of WBA 8119 as a rodenticide for use against warfarin-resistant and non-resistant rats and mice. J Hyg. 1976;77:419–426.

74. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. Rag-1-Deficient Mice Have No Mature Lymphocytes-B and Lymphocytes-T. Cell. 1992;68:869–877.

75. Paul F, Arkin Y, Giladi A, et al. Transcriptional heterogeneity and lineage commitment in myeloid progenitors. Cell. 2015;163: 1663–1677.

76. Buenrostro JD, Corces MR, Lareau CA, et al. Integrated single-cell analysis maps the continuous regulatory landscape of human hematopoietic differentiation. Cell. 2018;173:1535–1548. e1516.

77. Alberti-Servera L, von Muenchow L, Tsapogas P, et al. Single-cell RNA sequencing reveals developmental heterogeneity among early lymphoid progenitors. EMBO J. 2017;36:3619–3633.

78. Velten L, Haas SF, Raffel S, et al. Human haematopoietic stem cell lineage commitment is a continuous process. Nat Cell Biol. 2017;19:271–281.

79. Baell JB, Leaver DJ, Hermans SJ, et al. Inhibitors of histone acetyltransferases KAT6A/B induce senescence and arrest tumour growth. Nature. 2018;560:253–257.