REVIEW

Adult murine hematopoietic stem cells and progenitors: an update on their identities, functions, and assays

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The founder of all blood cells are hematopoietic stem cells (HSCs), which are rare stem cells that undergo key cell fate decisions to self-renew to generate more HSCs or to differentiate progressively into a hierarchy of different immature hematopoietic cell types to ultimately produce mature blood cells. These decisions are influenced both intrinsically and extrinsically, the latter by microenvironment cells in the bone marrow (BM). In recent decades, notable progress in our ability to identify, isolate, and study key properties of adult murine HSCs and multipotent progenitor (MPP) cells has challenged our prior understanding of the hierarchy of these primitive hematopoietic cells. These studies have revealed the existence of at least two distinct HSC types in adults: one that generates all hematopoietic cell lineages with almost equal potency and one that is platelet/myeloid-biased and increases with aging. These studies have also revealed distinct MPP cell types that have different functional potential. This review provides an update to these murine HSCs and MPP cells, their key functional properties, and the assays that have been used to assess their potential.

HIGHLIGHTS

- This is a review of murine HSCs and new HSC assays developed in the past 15 years.
- This review summarizes the immunophenotypes and functions of HSCs and MPPs.
- This review provides a summary of studies of platelet/myeloid-biased and lymphoid-biased HSCs.

Our knowledge of hematopoiesis has significantly advanced in recent decades largely because of improved technology. The development of and subsequent improvements in the capacity of flow cytometry–based applications have enabled researchers to identify, isolate, and study populations of hematopoietic cells, including hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs). In 2007, we published an extensive review of methods used to isolate and assay murine HSCs and MPPs [1]. Since then, there have been significant advances in flow cytometry–based methods to purify and study the function of distinct populations of HSCs and MPPs. This review provides an update to our previous one and provides an overview of some of these recent studies. For those who are new to the field, our previous review [1] discusses many different assays used to study HSCs and MPPs, and it is recommended as a companion reading to this review.

In this review, long-term repopulating HSCs (LT-HSCs) refer to multilineage repopulating cells that can generate blood cells for at least 16 weeks after transplantation in recipient mice. Short-term repopulating HSCs (ST-HSCs) are cells that can repopulate mice for at least 12 weeks after transplantation. MPPs are cells that have diminished in vivo repopulating activity compared with both LT-HSCs and ST-HSCs and have varying multipotent potential, depending on the MPP subtype. All studies referenced in this review have performed relevant in vivo repopulating assays to assess the repopulating potential of HSC and MPP populations.

Some of the studies discussed in this review have assessed the in vivo repopulation potential of single cells, which, although technically challenging and requires larger cohorts of recipient mice, is the most stringent test for evaluating HSC/MPP repopulating capacity and enables assessment of the purity of the population identified by fluorescent cell surface markers or reporter mice. However, note that despite these recent advances, the majority, if not all, of the HSC and MPP populations described here rely heavily on immunophenotype. Furthermore, although the function of HSCs has been assessed using single-cell transplants in some studies, the heterogeneous behavior of the HSCs being assessed in these studies suggests that we have not yet achieved 100% pure populations of HSCs (and likely MPPs) using any of the current standard approaches.

HSC POPULATIONS IDENTIFIED USING DIFFERENT COMBINATIONS OF SLAM, CD34, AND CD135 MARKERS

In our previous review, cell surface markers CD34 and CD135 (FLT3) had been used by the Nakauchi and Jacobsen laboratories to identify LT-HSCs (LKS+ CD34− CD135−), ST-HSCs (LKS+ CD34+ CD135−), and a population that consisted of transiently repopulating cells termed MPP cells (LKS+ CD34+ CD135+) [2–4]. The

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Morrison group had also reported a method that isolated HSCs on the basis of the expression of CD150 and CD48 (the SLAM markers), with CD150+ CD48− CD41− cells representing the most primitive cell population [5,6]. Combinations of the SLAM markers (CD150 and CD48), CD34 and/or CD135 have now been used to further purify HSC and MPP populations within lineage-negative, c-KIT+, Sca-1+ (LKS+) cells (also commonly referred to as LSK).

Two separate studies from the Trumpp laboratory identified and explored the functional potential of populations that differentially expressed CD150, CD48, CD135 and CD34 markers within LKS+ cells. In the first study, Wilson et al. [7] showed that differential expressions of the CD150, CD48, CD135, and CD34 markers within LKS+ identified five populations that were termed HSC (LKS + CD150+ CD48− CD34− CD135−), MPP1 (LKS+ CD150+ CD48−CD34+ CD135−), MPP2 (LKS+ CD150+ CD48+ CD34 + CD135−), MPP3 (LKS+ CD150− CD48+ CD34+ CD135−), and MPP4 (LKS+ CD150− CD48+ CD34+ CD135+). A series of elegant studies showed that the HSC population was highly enriched for dormant (very quiescent) HSCs. The HSCs were shown to be activated after hematopoietic stress conditions (treatment of mice with either the chemotherapy agent, 5-fluorouracil, or granulocyte colony-stimulating factor) to self-renew and replenish hematopoiesis and could then return to dormancy [7].

In the second study from the Trumpp group, Cabezas-Wallscheid et al. [8] further explored the functional potential of the HSC and MPP populations identified by Wilson et al. [7]. They also provided a comprehensive proteome, transcriptome, and DNA methylome resource of the HSCs and MPPs [8]. Among the findings of this resource was the enrichment of retinoic acid signaling pathway genes in HSCs. In a subsequent study, they showed that the biologically active vitamin A derivative, all-trans retinoic acid (ATRA), was important in regulating HSC dormancy and self-renewal [9]. These studies independently confirmed the findings of our previous studies that showed that ATRA is a key regulator of murine HSCs and that ATRA treatment increases the serial transplantability of cultured murine HSCs [10−12].

The studies from the Passegue laboratory also used a combination of SLAM and CD135 markers in LKS+ cells to define LT-HSC (LKS + CD150+ CD48− CD135−), ST-HSC (LKS+ CD150− CD48− CD135−), and three MPP types: MPP2 (LKS+ CD150+ CD48+ CD135−), MPP3 (LKS+ CD150− CD48+ CD135−), and MPP4 (LKS+ CD150− CD48+ CD135+) [13]. These studies did not incorporate CD34; hence, the LT-HSC identified by Pietras et al. [13] comprised both the HSC and MPP1 cells separately identified and investigated by the Trumpp laboratory [7,8]. The ST-HSCs were a new population that had not been investigated in the other studies. In contrast, the studies by the Trumpp group revealed that all of the MPP2−MPP4 cells expressed CD34, which was confirmed in the study by Pietras et al. [13]; hence, the properties of these cell types can be directly compared.

Collectively, the studies from the Trumpp and Passegue laboratories concluded that HSCs and ST-HSCs are multipotent HSCs, MPP2 cells are megakaryocyte-biased progenitors, MPP3 cells are myeloid-biased progenitors, and MPP4 cells are lymphoid-biased progenitors [7,8,13]. The functional properties of each of these cell types were also investigated using in vitro and in vivo studies and are summarized in Table 1.

Sommerkamp et al. [14] recently identified two distinct populations within the ST-HSCs on the basis of their expression of CD34, with CD34+ ST-HSCs being termed MPP5 and CD34− ST-HSCs being termed MPP6 [14]. When 2,000 cells of either type were transplanted together with 2×10^5 competing spleen cells into lethally irradiated recipients, both populations showed multilineage reconstitution, although MPP6 showed increased reconstitution of myeloid cells compared with MPP5 [14]. Repopulation was not assessed beyond 12 weeks, and no other functional data were provided for MPP6; hence, these populations are not included in Table 1 or discussed further in this review.

In order to consolidate the findings of these independent laboratories and avoid terminology confusion, MPP populations were recently summarized and reclassified by Challen et al. [15]. MPP2 has been reclassified as $MPP^{MK/E}$ (megakaryocyte/erythroid-biased), MPP3 has been reclassified as $MPP^{G/M}$ (granulocyte/monocyte-biased), and MPP4 has been reclassified as MPP^{Ly} (lymphocyte-biased). They also suggested renaming ST-HSCs to MPPs (referring to MPPs with unbiased multilineage reconstituting potential) because the authors defined HSCs as cells that have long-term repopulating (and therefore, self-renewal) potential. However, the classical definition of ST-HSCs are cells that show multilineage repopulation in mice for up to 12 weeks after transplantation [1,12]. Interestingly, both the ST-HSC and MPP2 populations currently meet those criteria and are worthy of further investigation to conclusively identify whether they are ST-HSCs or MPPs.

IDENTIFICATION OF PLATELET/MYELOID-BIASED AND LYMPHOID-BIASED HSCS: A NEW CLASSIFICATION OF THE HSC HIERARCHY

It has long been recognized that HSCs do not all behave the same on transplantation into irradiated mice; however, it was unclear as to why this was the case. A seminal transplantation study of single HSCs by the Eaves group [16] used a population of lineage-negative bone marrow (BM) cells further purified on the basis of their intermediate cell surface expression of CD45 and their low intensity of fluorescent dyes rhodamine123 and Hoescht 33342 (the use of each of these dyes to isolate HSCs was reviewed previously by Purton et al [1]). Transplantation of single cells into sublethally irradiated W^{41}/W^{41} mice (which are c-KIT deficient and engraft more readily with very low numbers of HSCs [1,17]) identified that there were four distinct types of murine repopulating cells classified on the basis of their potential to produce myeloid (granulocytes/monocytes), B lymphocytes, and/or T lymphocytes in the mice for up to 24 weeks after transplant. The α-cells were myeloid-biased; β-cells had a balanced production of myeloid cells, B lymphocytes, and T lymphocytes; γ-cells were lymphoid-biased, producing both B and T lymphocytes to similar proportions, with limited myeloid potential; and the δ-cells had a restricted production of T lymphocytes. Note that contribution to platelets could not be assessed in these studies because platelets do not express CD45.1 or CD45.2, which are routinely used to distinguish donor, host, and competing cells in studies that use mice generated on the C57BL/6 background [1].

E-SLAM HSCS

The Eaves group further showed that the endothelial protein C receptor (EPCR) could be used to identify HSCs in both E14.5 fetal liver (FL) and adult BM [18]. Transplantation studies of single cells into

Table 1 Properties of HSC and progenitor cell types isolated based on SLAM, CD34, and CD135 markers

Original name	LT-HSC (Trumpp laboratory) [7,8]	LT-HSC (Passegue laboratory) [13]	MPP1 [7,8]	ST-HSC [13]	MPP2 [7,8,13]	MPP3 [7,8,13]	MPP4 or LMPP [7,8,13]
Revised name [15]	Dormant HSC	LT-HSC	Active HSC	MPP	$MPP^{MK/E}$	$MPP^{G/M}$	MPP^{Ly}
Flow cytometry gating strategy	LKS+ CD150+ CD48− CD34− CD135−	LKS+ CD150+ CD48− CD34+/− CD135−	LKS+ CD150+ CD48− CD34+ CD135−	LKS+ CD150− CD48− CD135−	LKS+ CD150+ CD48+ CD34+ CD135−	LKS+ CD150− CD48+ CD34+ CD135−	LKS+ CD150− CD48+ CD34+ CD135+
Primary transplant Competitive repopulation potential when cotransplanted with 2×10^5 wild-type BM cells (≥0.2% donor cells considered positive) [8].	50 HSC: multilineage, 100% recipients repopulated from 50 HSC at 16 weeks after transplant [8]. 2,000 HSC: donor contribution of approximately 56% (T lymphocytes), 70% (B lymphocytes), 82% (myeloid cells) at 16 weeks after transplant [8].	ND	50 MPP1: 56% of recipients multilineage at 16 weeks after transplant [8].	ND	2,000 MPP2: Rapid myeloid reconstitution, donor contribution of approximately 23% (T lymphocytes), 35% (B lymphocytes), 32% (myeloid cells) at 16 weeks after transplant [8].	2,000 MPP3: Myeloid repopulation between 1 and 3 weeks after transplant, less than 5% multilineage repopulating donor cells at 9−16 weeks after transplant [8].	2,000 MPP4: Transient repopulation of B lymphocytes, less than 1% multilineage donor cell reconstitution at 16 weeks after transplant [8].
Secondary transplant potential (1×10^6 BM cells transplanted from primary recipients of 50 cells and 2×10^5 wild-type BM cells,≥0.2% donor cells considered positive) [8].	8/10 recipients Donor cells detected at 8 weeks after transplant.	ND	0 recipients had detectable donor cells at 8 weeks after transplant.	ND	ND	ND	ND
Competitive repopulation potential when 50 cells were cotransplanted with 3×10^5 Sca-1-depleted BM cells (only the average % reconstitution was provided for each group) [13].	ND	At 4 weeks after transplant: similar donor reconstitution to ST-HSCs, superior myeloid engraftment. At 16 weeks after transplant: approximately 40% multilineage donor cell reconstitution.	ND	At 4 weeks after transplant: similar donor cell reconstitution to LT-HSCs, superior lymphoid engraftment. At 16 weeks after transplant: approximately 5% donor cells, exclusively lymphoid.	ND	ND	ND
Cell cycle [7]	70% in G_0, <2% actively cycling (S +G_2/M)	ND	39% in G_0, 14% actively cycling (S +G_2/M)	ND	<20% in G_0, ~25% actively cycling (S +G_2/M)	<10% in G_0, ~24% actively cycling (S +G_2/M)	<5% in G_0, ~28% actively cycling (S +G_2/M)
Label retaining cells measured at 70 days of chase after 10 −13 days of treatment with BrdU	32.7 ± 4.7% (and approximately 5% after 306 days of chase)	ND	~10%	ND	~7%	~5%	<2%

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Table 1 (*Continued*)

Original name	LT-HSC (Trumpp laboratory) [7,8]	LT-HSC (Passegue laboratory) [13]	MPP1 [7,8]	ST-HSC [13]	MPP2 [7,8,13]	MPP3 [7,8,13]	MPP4 or LMPP [7,8,13]
BrdU incorporation after 1 hour pulse in vitro [13]	ND	<10%[13]	ND	<10%	>20%	>20%	>20% (slightly less than MPP2 and MPP3)
Proliferative potential during 10 days of liquid suspension culture [13]	ND	High	ND	High	Lower than LT-HSC and ST-HSC, higher than MPP3 and MPP4	Lower than LT-HSC, ST-HSC and MPP2, higher than MPP4	Lowest
CFC potential from single-sorted cells [13]	ND	Highest plating efficiency, high proportion of Meg/E CFCs	ND	Highest plating efficiency	Lower plating efficiency than LT-HSC and ST-HSC, high proportion of Meg/E CFCs	Lower plating efficiency than LT-HSC and ST-HSC	Lowest plating efficiency
Day 12 CFU-S [13]	ND	1/20	ND	1/20	1/80	1/80	1/250
B lymphocyte production in OP9 cocultures [13]	ND	Detectable by day 21 of coculture	ND	ND	1/31	1/40	1/5
Immature T lymphocyte production (DN2= CD44+CD25+, DN3= CD44-CD25+) in OP9-DL1 cocultures during 12 days [13]	ND	Yes, produced some DN2 and DN3 at day 12, very minimal at day 8	ND	ND	Yes, produced DN2 but few DN3, minimal at day 8	Yes, produced more than all other types except MPP4, produces DN2 at day 8, DN2 and DN3 at 12 days	Yes, most robust, produced DN2 and DN3 within 8 days, more DN3 at 12 days
CD41 cell surface expression [13]	ND	Majority of cells + (bright)	ND	Two populations: one with low expression (less than 50% of cells), the other negative expression	Majority of cells + (bright)	Two populations: one with low expression (more than 50% of cells), the other negative expression	Homogenously low/ negative
Vwf-GFP expression [23]	60%	ND	ND	ND	ND	ND	0.22%
ESAM cell surface expression [13]	ND	High	ND	High	Two populations: one high (majority of cells), the other low/ negative	Two populations: one high (majority of cells), the other low/ negative	Two populations: one low/negative (majority of cells), the other intermediate

BM = bone marrow; BrdU = bromodeoxyuridine; CFC = colony-forming cell; CFU-S = colony-forming unit-spleen; DN = double negative; G/M = granulocyte/monocyte-biased; HSC = hematopoietic stem cell; LT-HSC = long-term repopulating hematopoietic stem cell; Ly = lymphocyte-biased; MPP = multipotent progenitor; LMPP = lymphoid-primed multipotent progenitor; MK/E = megakaryocyte/erythroid-biased; ND = not determined; ST-HSC = short-term repopulating hematopoietic stem cell.

sublethally irradiated W^{41}/W^{41} mice revealed that cells that were defined as CD45+, EPCR+, CD48− and CD150+ (E-SLAM HSCs) were the most primitive and were enriched for cells with high self-renewal (SR) capacity (high SR E-SLAM HSCs). From a total of 62 mice that received a single-cell transplantation of CD45+ EPCR+ CD48− CD150+ E-SLAM HSCs, 43% of the single cells had high SR, 13% had low SR, and 2% were short-term repopulating stem cells, with 42% lacking repopulation potential at or beyond 8 weeks after transplantation. By contrast, of the 28 recipients of CD45+ EPCR+CD48− CD150− cells, 7% had high SR, 32% had low SR, 18% were short-term repopulating cells, and 43% lacked repopulation potential. These CD45+ EPCR+CD48− CD150− cells were therefore enriched for low SR potential (low SR E-SLAM HSCs).

Kent et al. [18] performed quantitative polymerase chain reaction (qPCR) studies to compare the expression of transcripts in CD45+ EPCR+ CD48− CD150+ E-SLAM HSCs and CD45+ EPCR+ CD48− CD150− cells. The E-SLAM HSCs were shown to express notably higher levels of transcripts of genes that have previously been shown to regulate HSC SR: *Prnp* [19], *Gata3* [20], and *Bmi1* [21,22]. In a search for new regulators of HSC SR, they constructed LongSAGE libraries generated from E14.5 FL and adult BM HSCs, with gene candidates further narrowed by a comparison with previously published microarrays of these HSC populations. This approach identified 27 candidate genes, nine of which were consistently upregulated in E14.5 FL and BM E-SLAM cells. qPCR studies revealed that transcripts of four of these genes were significantly increased in high SR E-SLAM BM HSCs compared with low SR E-SLAM BM HSCs: *Smarcc2, Rhob, Pld3*, and *Vwf* [18]. Key functional properties of the high SR and low SR E-SLAM HSCs from adult BM are summarized in Table 2.

CD150^{HIGH} MYELOID-BIASED HSCS AND PROGENITORS

The Nakauchi laboratory screened 118 different cell surface markers by flow cytometry to further purify HSCs within LKS+ CD34− HSCs [4]. They showed that the LKS+ CD34− HSCs could be subdivided into three fractions on the basis of their expression of CD150 (high, medium, or negative) and termed them CD150^{high} (which were also shown to express high levels of CD38), CD150^{med}, and CD150^{neg} HSCs [23]. Initial competitive repopulation studies of 10 HSCs competed against 2×10^5 wild-type BM cells revealed that the CD150^{high} HSCs had low peripheral blood chimerism (<25% donor cells) at 2 months after transplantation, however, this increased by 5 months after transplantation. These HSCs were myeloid-biased but did repopulate the lymphoid lineages. The CD150^{med} HSCs showed relatively stable reconstitution at all time points and were lymphoid-biased, but most recipients showed myeloid cell repopulation, and the average donor cell reconstitution was higher than that from both the CD150^{high} and CD150^{neg} HSCs. By contrast, the CD150^{neg} HSCs had the poorest reconstitution potential and were lymphoid-biased with inferior myeloid repopulating potential compared with that of the CD150^{med} HSCs [23].

Competitive transplantation studies of single HSCs confirmed the observations of the competitive transplants of 10 HSCs and revealed that the CD150^{high} HSCs had the highest repopulating capacity in secondary recipients [23]. Furthermore, single CD150^{neg} HSCs had the poorest secondary transplantation potential. Key functional properties of these studies by Morita et al. [23] are summarized in Table 3.

These single HSC studies also suggested the existence of a latent HSC (estimated to be approximately 1/10 CD150^{high} HSCs) [23]. The latent HSC produced virtually undetectable levels of blood cells for approximately 12 weeks after transplantation at or after which they contributed to low levels of myeloid cells. Intriguingly, when BM cells from these primary recipients were transplanted into secondary recipient mice, they robustly reconstituted all cell lineages assessed (myeloid, T lymphoid, and B lymphoid). Furthermore, approximately 1/20 CD150^{high} HSCs showed a low level of myeloid reconstitution and no lymphoid reconstitution in both primary and secondary recipients [23]. A subsequent study revealed that these cells were myeloid-restricted progenitors (MyRPs) that lack SR potential and are formed by asymmetric divisions of HSCs [24].

A limitation of these prior studies was the inability to detect chimerism in the erythroid and platelet lineages. To enable this, they generated Kusabira Orange (KuO) fluorescent reporter mice [25]. Single-cell transplantation studies confirmed that the latent HSCs could repopulate all cell lineages. The effects of aging were also assessed in these studies but are not discussed further in this review, which focuses largely on properties of young HSCs.

VWF-EXPRESSING HSCS

The Jacobsen and Nerlov laboratories generated CD45.2+ *Vwf*-eGFP BAC mice and used them to demonstrate that approximately 60% of adult HSCs defined as LKS+ CD150+ CD48− CD34− cells (which are similar to the HSC population described and characterized by the Trumpp laboratory [7,8]) were *Vwf*-GFP+ [26]. By contrast, the LKS + CD150− CD135+ cells (akin to MPP4/MPP^{Ly}) did not express *Vwf*-GFP. Furthermore, 96% of FL HSCs (identified as being LKS+ CD150+ CD48−) were *Vwf*-GFP+ [26]. These studies confirmed the observations of Kent et al. [18] that *Vwf* may have a role in HSC SR.

When 10 *Vwf*-GFP+ HSCs were transplanted together with 2×10^5 wild-type (WT) CD45.1+ competing BM cells, 15/48 recipients had notable donor cell reconstitution (defined as >1% contribution to at least one of the lineages at both 10 and 16 weeks after transplantation). Of these 15 mice, there was a variable contribution to the different cell lineages at 16 weeks after transplantation, although all recipients had platelet reconstitution. By contrast, 10 of 42 of the *Vwf*-GFP− HSCs showed notable donor cell reconstitution, and these were lymphoid-biased, although they did contribute to the other cell lineages, including platelets.

Single HSCs were transplanted together with 1×10^6 W^{41}/W^{41} CD45.1+ competing BM cells. In these initial studies, only 3 of 17 recipients showed detectable reconstitution, and all recipients had platelet-biased reconstitution, with minimal detection of B and T lymphocytes [26]. Additional transplant studies suggested that the *Vwf*-GFP+ HSCs (termed platelet/biased HSCs) were at the top of the HSC hierarchy and generated *Vwf*-GFP− HSCs, whereas *Vwf*-GFP− HSCs did not robustly generate *Vwf*-GFP+ HSCs [26]. Note that it is currently unclear whether these studies have revealed that *Vwf*-GFP + HSCs show a myeloid/platelet-bias versus myeloid/platelet-restriction. This is an important question in the HSC field, which requires further clarification in future studies.

Table 2 Properties of HSCs identified using EPCR

Original name	E-SLAM HSCs [18]	CD45+ EPCR+ CD48− CD150− cells [18]
Revised name	High SR E-SLAM	Low SR E-SLAM
Flow cytometry gating strategy	CD45+ EPCR+ CD48− CD150+	CD45+ EPCR+ CD48− CD150−
Single-cell transplants assessed at 4 months after transplant into W^{41}/W^{41} mice	43% high SR 13% low SR 2% STRC 42% non-RC 56% of population estimated to be an HSC	7% high SR 32% low SR 18% STRC 43% non-RC 39% of population estimated to be an HSC
Lineage compositions of repopulated mice from single a HSC α = myeloid-biased, β = balanced myeloid and lymphoid, γ = lymphoid-biased (B and T lymphocytes), δ = restricted production of T lymphocytes	~16% α ~61% β ~22% γ 0% δ E-SLAM HSCs repopulated secondary recipients (reported in the text)	0% α ~18% β ~45% γ ~36% δ
Frequency of LTC-IC from single cells	43%	7%

EPCR = endothelial protein C receptor; HSC = hematopoietic stem cell; LTC-IC= long-term culture-initiating cell; RC = repopulating cell; SR = self-renewal; STRC= short-term repopulating cell.

Microarray studies revealed that *Vwf*-GFP+ HSCs had higher expression of megakaryocyte lineage genes compared with *Vwf*-GFP− HSCs, including *Clu, Gpr64, Sdpr, Mpl*, and *Zfpm1*, in addition to genes associated with bipotent megakaryocyte/erythroid progenitors [26]. Multiplex single-cell qPCR of *Vwf*-GFP+ and *Vwf*-GFP− LKS+ CD34− CD150+ CD48− HSCs confirmed expression of these transcripts in most single cells [26].

More recently, the Jacobsen and Nerlov laboratories generated *Vwf*-tdTomato mice, providing a brighter fluorescent reporter mouse to reliably detect the repopulation of single *Vwf*-HSCs [27]. Single HSCs (LKS+ CD34−CD150+ CD48− *Vwf*-tdTomato+) were transplanted together with 2×10^5 CD45.1+ WT BM cells (58 recipients) or 1×10^6 W^{41}/W^{41} CD45.1+ competing BM cells (292 recipients). Comparable results were obtained for both competitive transplant types in terms of the donor cell reconstitution. Approximately 40% of recipients showed 0.1% donor cell contribution to at least one lineage at 16−18 weeks after transplantation, with almost 50% of these recipients showing repopulation in all lineages (platelets, erythrocytes, myeloid, B lymphocytes, and T lymphocytes). Consistent with their previous publication, they observed different proportions of donor cell contribution to the lineages; however, all HSCs contributed to platelet reconstitution [27].

Interestingly, although there was evidence of lineage bias in vivo, when single *Vwf*-tdTomato+ LT-HSCs were isolated from recipient mice that exclusively produced platelets, the HSCs were able to generate other hematopoietic lineages (granulocytes, monocyte/macrophages, and T lymphocytes) in vitro, and gene expression studies of single HSCs indicated that they expressed transcripts associated with those lineages [27]. Collectively, these studies suggested that, irrespective of their in vivo repopulating potential, all *Vwf*-tdTomato+ LT-HSCs retained multipotency. A summary of properties of the platelet-biased and lymphoid-biased HSCs isolated using *Vwf*-reporter mice is in provided in Table 4.

Recognizing that not all laboratories can access *Vwf*-reporter mice, Carrehla et al. [27] searched for flow cytometry markers that could be used instead. The Morrison laboratory had previously reported that LKS+ CD150+ CD48− HSCs lack expression of CD229 [28]. Carrehla et al. [27] performed single-HSC transplants to identify that platelet-biased *Vwf*-tdTomato+ HSCs were LKS+ $CD150+^{high}$ CD48− CD34− $CD229^{low/-}$ and lymphoid-biased *Vwf*-tdTomato+ HSCs were LKS+ $CD150+^{high}$ CD48− CD34− $CD229^{high}$ [27].

Note that *Vwf*-GFP and *Vwf*-tdTomato reporters reliably measure *Vwf* RNA; VWF protein was not confirmed to be expressed by the HSCs likely because it is intracellular and difficult to assess by flow cytometry methods. There is an excellent antibody against human VWF that crossreacts with mouse, and we have used it to detect VWF + cells in paraffin-embedded BM sections obtained from both species [29,30]. It would therefore be of interest to assess VWF expression on cytospins of *Vwf*-GFP/tdTomato+ HSCs by immunohistochemistry-based methods to conclusively determine whether all *Vwf*-GFP or *Vwf*-tdTomato+ HSCs do express VWF protein.

CD41 MYELOID-BIASED HSCS

In complementary studies to those using the *Vwf*-GFP/tdTomato HSCs, Gekas and Graf [31] showed that CD41 is expressed on a population of adult murine HSCs (defined as either LKS+ CD34− CD135− or LKS+ CD150+ CD48−) and that CD41-expressing HSCs increased with aging, marking the majority of HSCs in 16-month-old C57BL/6 mice. CD41 had previously been shown to separate definitive hematopoietic cells (which are CD41+) from endothelial cells (which lack expression of CD41) in the mouse embryo [32,33], and CD41 has been shown to be expressed by yolk sac, fetal, placental, and a small proportion of adult HSCs [32−35]. Interestingly, the studies by Sanjuan-Pla et al. [26] identified that approximately 68% of *Vwf*-GFP+ HSCs coexpressed CD41 protein.

Ferkowicz et al. [33] had previously investigated the function of CD41+ adult HSCs and reported that HSC activity was enriched in

Table 3 Properties of HSCs identified using CD150 expression within the LKS+ CD34− population

Original name	CD150 high [23]	CD150 medium [23]	CD150 negative [23]
Revised name	CD150^{high}	CD150^{med}	CD150^{neg}
Flow cytometry gating strategy	LKS+ CD34− CD150^{high}	LKS+ CD34− CD150^{med}	LKS+ CD34− CD150^{negative}
Primary transplant Competitive repopulation potential 10 HSCs cotransplanted with 2×10^5 wild-type BM cells (≥0.3% donor cells at one or more time point after transplant, monitored up to 5 months after transplant)	19 recipients showed long-term repopulation, myeloid-biased but repopulated all lineages Chimerism was low in 15/19 recipients at early time points and increased during the 5 months after transplant, but remained lower, on average, than CD150^{med} HSCs	19 recipients showed long-term repopulation, lymphoid-biased but repopulated all lineages Chimerism was relatively stable and high (median chimerism ~25%) in 11/19 recipients during the 5 months after transplant	14 recipients showed long-term repopulation, lymphoid-biased, poorer reconstitution of myeloid cells compared with CD150^{med} HSCs Chimerism was relatively stable and low in 11/19 recipients during the 5 months after transplant with the exception of one recipient, which showed increased chimerism after transplant, achieving approximately 75% chimerism
Primary transplant Competitive repopulation potential Single HSCs cotransplanted with 2×10^5 wild-type BM cells (≥0.3% donor cells at one or more time point after transplant, monitored up to 5 months after transplant)	16/40 recipients showed long-term repopulation, myeloid-biased but repopulated all lineages with the exception of 2/20 mice which showed low levels of myeloid cell reconstitution	13/40 recipients showed long-term repopulation, lymphoid-biased but repopulated all lineages	13/40 recipients showed long-term repopulation, lymphoid-biased, poorer reconstitution of myeloid cells compared with CD150^{med} HSCs 1/40 recipients repopulated only T lymphocytes
Secondary transplant 5×10^6 BM cells transplanted from primary recipients of single HSCs competed against 2×10^5 wild-type BM cells (≥0.3% donor cells at one or more time point after transplant, monitored up to 5 months after transplant)	13/13[a] recipients showed long-term repopulation, these were myeloid-biased but most recipients had lymphoid reconstitution 1/10 HSCs were identified to be latent HSCs that showed poor myeloid reconstitution in primary recipients and robust reconstitution in secondary recipients 1/20 cells identified as myeloid-restricted progenitors	4/13[b] recipients showed long-term repopulation. 2 recipients were lymphoid-restricted, two had myeloid-biased multilineage repopulation	2/14[c] recipients showed long-term repopulation, all were lymphoid-biased with minimal myeloid reconstitution 5/12 recipients repopulated only T lymphocytes
Single-cell colony assays	Approximately 42/48 single HSCs formed colonies Colony composition ~60% nmEM ~ 7% nmM ~ 12% nm <1% m Remainder defined as blast-like or "other" colonies	Approximately 45/48 single HSCs formed colonies Colony composition ~30% nmEM ~ 20% nmM ~ 40% nm ~4% m Remainder defined as blast-like or "other" colonies	Approximately 41/48 single HSCs formed colonies Colony composition ~7% nmEM ~ 14% nmM ~ 66% nm ~7% m Remainder defined as blast-like or "other" colonies

BM = bone marrow; HSC = hematopoietic stem cell; m = macrophage colonies; nm = neutrophil/macrophage colonies; nmEM = neutrophil/macrophage/erythroblast/megakaryocyte colonies; nmM = neutrophil/macrophage/megakaryocyte colonies.
a 3 primary recipients died before secondary transplantation.
b 1 primary recipient died before secondary transplantation.
c 2 primary recipients died before secondary transplantation.

the CD41$^{-/lo}$ population [33]. This is consistent with the findings of the Morrison group, who reported that CD41+ cells did not reconstitute hematopoiesis when transplanted into mice [5]. However, Gekas and Graf made the important observation that the CD41 antibody, (clone MWReg30), is a blocking antibody [31]. This meant that it could potentially interfere with the engraftment and, in turn,

Table 4 Properties of HSCs identified using vWF-reporter mice

Original name	*Vwf*-GFP+ platelet-biased HSC [26, 26]	*Vwf*-GFP- lymphoid-biased HSC [26, 27]
Revised name	*Vwf*+ HSC	*Vwf*- HSC
Flow cytometry gating strategy	LKS+ CD150+ CD48− CD34−*Vwf*+ identified using either *Vwf*-GFP or *Vwf*-tdTomato reporter mice	LKS+ CD150+ CD48− CD34−*Vwf*- identified using either *Vwf*-GFP or *Vwf*-tdTomato reporter mice
Primary transplant Competitive repopulation potential 10 HSCs cotransplanted with 2×10^5 wild-type BM cells (≥1% donor cells positive at both 10 and 16 weeks after transplant) Biased reconstitution defined when cells were repopulated >50% higher than the other lineages [26]	15/48 recipients had repopulation in at least one lineage, all recipients repopulated platelets 5/15 were platelet-biased 4/15 were platelet/myeloid-biased 1/15 was myeloid-biased 2/15 were balanced 3/15 were lymphoid-biased	10/42 recipients repopulated mice, all were lymphoid-biased but did contribute to the other lineages
Primary transplant Competitive repopulation potential 1 HSC cotransplanted with 2×10^5 wild-type BM cells (≥0.1% donor cells considered positive at 16−18 weeks after transplant) [27]	25/58 recipients had detectable donor cells in at least one lineage 3/58 recipients were platelet-biased 5/58 recipients were platelet/erythroid/myeloid-biased 6/58 recipients were platelet/erythroid/myeloid/B lymphocyte restricted 11/58 recipients repopulated all lineages	ND
Primary transplant Competitive repopulation potential when single HSCs cotransplanted with 1×10^6 W^{41}/W^{41} BM cells (≥0.1% donor cells considered positive at 16−18 weeks after transplant) [27]	109/292 recipients had detectable donor cells in at least one lineage 12/292 recipients were platelet-biased 3/292 recipients were platelet/erythroid-biased 18/292 recipients were platelet/erythroid/myeloid-biased 54/292 recipients repopulated all lineages	ND
Potential to form other HSC type	Produced high numbers of *Vwf*-GFP- HSCs at 7 and 32 weeks after transplant	Minimal numbers of *Vwf*-GFP+ HSCs produced at 7 and 32 weeks after transplant
CFC potential from single-sorted cells	High cloning efficiency, including large proportions containing megakaryocytes	High cloning efficiency, fewer megakaryocytes

BM = bone marrow; CFC = colony-forming cell; HSC = hematopoietic stem cell.

reconstitution of the CD41-expressing HSCs. To test this, they incubated BM cells obtained from WT mice with either MWReg30 or the control immunoglobulin G (IgG) antibody for 30 min, then mixed the cells 1:1 with untreated competing BM cells and injected them into lethally irradiated congenic mice [31]. There was no difference in the proportions of donor-derived cells in the recipient mice at 1 month after transplantation (a time point that assesses the repopulation of hematopoiesis from MPPs). By contrast, at both 2 and 4 months after transplantation, the donor-derived repopulation was significantly reduced (by approximately 50%) in recipients that received the MWReg30-treated BM compared with those that received IgG control-treated BM. The MWReg30 clone was used in the two previous studies which concluded that adult HSCs were CD41−, thus resolving this discrepancy in the literature [5,33]. It was also used in the studies by Carrelha et al. [27] in their search for cell surface markers that could be used to purify *Vwf*-tdTomato+ platelet-biased and lymphoid-biased HSCs. They reported that there were no differences in the repopulating activity of the CD41+ and CD41− *Vwf*-tdTomato+ HSCs [27]; however, the blocking effects of the CD41 antibody likely contributed to these results. Furthermore, in some single-HSC studies of latent HSCs and MyRPs, Yamamoto et al. [24,25] used CD41 expression to isolate the cells before transplantation, and this may also have influenced the data. It is highly recommended that CD41 not be used to isolate HSCs for transplantation studies.

To explore the role of CD41 in adult HSCs, Gekas and Graf used $CD41^{YFP}$ knock-in mice, which they had previously generated by inserting the *Eyfp* gene at the start site of the *GpIIb* (CD41) locus in embryonic stem cells [36]. Homozygous $CD41^{YFP/YFP}$ mice were shown to be akin to CD41 knockout (KO) mice [36] and were subsequently used (and termed CD41-KO) to determine whether CD41 had an important functional role in adult hematopoiesis. At two months of age, the $CD41^{YFP/YFP}$ (CD41-KO) mice exhibited peripheral blood pancytopenia in all lineages (platelets, erythrocytes myeloid cells, B lymphocytes, and T lymphocytes), and this worsened in 9 month- and 10 month-old-mice. The CD41-KO mice had a hypocellular BM at all the time points assessed.

The numbers of HSCs (LKS+ CD34− CD135−) were significantly increased in the CD41-KO mice; however, this was accompanied by notable reductions in ST-HSC (LKS+ CD34+ CD135−) and MPP^{Ly} (LKS+ CD34+ CD135+). The LT-HSC and a mixed population comprising both ST-HSC and MPP^{Ly} (LKS+ CD34+) were shown to have increased apoptosis when compared with the same populations obtained from WT C57BL/6 mice. Furthermore, the CD41-KO LT-HSC, but not the LKS+CD34+ (ST-HSC/MPP^{Ly}) cells, had an increased proliferation rate, assessed at 16 hours after injection of bromodeoxyuridine (BrdU) into mice [31]. Transplantation studies confirmed that the defects observed in CD41-KO mice were intrinsic to the HSC.

To determine whether the CD41-KO HSCs had altered competitive repopulation capacity, 2×10^5 CD41-KO or WT CD45.2+ BM cells were transplanted together with 2×10^5 competing CD45.1+ BM cells into CD45.1/CD45.2 recipients and were assessed for donor cell contribution for up to 6 months after transplantation. The CD41-KO BM cells had an increased donor cell contribution at 1 month after transplantation, but similar donor cell reconstitution at 2 and 4 months after transplantation when compared with WT BM. However, the recipient mice of the CD41-KO BM competitive bone marrow transplant had thrombocytopenia and leukopenia at both 4 and 6 months after transplantation. Additional analyses revealed that the contribution of the WT competing cells to hematopoiesis had been altered when cotransplanted with the CD41-KO BM cells, revealing a feedback mechanism from CD41-KO BM cells that influenced WT hematopoiesis and contributed to the pancytopenia [31]. Although the mechanism was not determined, it is likely that the depletion of megakaryocytes, which also express CD41+ and have been shown to have important roles in regulating HSCs [37–40], contributed to this phenotype.

Microarray studies using WT mice revealed that CD41+ LKS+ CD135− HSCs had increased expression of genes associated with megakaryocyte/platelets (including *Vwf, Selp*, and *Pf4*) and myeloerythroid and megakaryocyte transcription factors, including *Gata1, Zfpm1, Gfi1b* and *Klf1*. Furthermore, the CD41−LKS+ CD135− HSCs were enriched in lymphoid-restricted genes, including *Ftl3, Il7ra*, and members of the *Ikaros* and *Notch* families. The microarray studies also implicated that the CD41+ HSCs were more quiescent than the CD41− HSCs, and this was confirmed by BrdU-labeling studies in WT mice [31].

The numbers of CFCs formed from single-sorted cells were similar for both the CD41− and CD41+ HSCs obtained from WT mice; however, the CD41+ HSCs produced smaller CFCs and had reduced proliferative potential in liquid cultures. Studies performed in vitro indicated that, similar to what was observed for the *Vwf*-GFP + HSCs, the CD41+ HSCs were able to form CD41− HSCs, whereas the CD41− HSCs had limited capacity to produce CD41+ HSCs, although a small, transient population of immunophenotypical CD41+ HSCs were produced from CD41− HSCs at early time points postculture initiation [31].

Finally, using heterozygous $CD41^{YFP/+}$ mice (which were shown to have similar hematopoietic cell content to WT mice), they sorted YFP + (CD41+) and YFP− (CD41−) LT-HSCs (defined as LKS+ CD150 + CD48−) and transplanted 50−100 LT-HSCs together with 2×10^5 competing CD45.1+ BM cells into lethally irradiated recipients. The YFP+ (CD41+) LT-HSCs had notably increased myeloid reconstitution accompanied by notably reduced B lymphocyte repopulation in primary recipients compared with the YFP− (CD41−) LT-HSCs. Although slightly increased in secondary recipients, the myeloid bias was not significantly different to that of the YFP− (CD41−) HSCs [31]. Interestingly, however, most secondary recipients of the BM obtained from primary recipients of the YFP+ HSCs had high donor cell chimerism, whereas the donor cell chimerism observed in YFP− HSCs was variable [31]. Unlike the studies by Sanjuan-Pla et al. [26], the authors did not investigate whether the YFP− (CD41−) LT-HSCs could form YFP+ (CD41+) LT-HSCs and vice versa after transplantation, which would have been of interest. Intriguingly, in validation studies that used BM cells obtained from the $CD41^{YFP/+}$ mice, all c-KIT+ YFP+ cells were shown to express CD41, whereas not all c-KIT+ CD41+ cells expressed YFP [31]. The reasons for this inconsistency in coexpression was unclear. A summary of these studies is provided in Table 5.

EVIDENCE FOR CD41+ AND VWF+ HSCS IN HUMANS

It was previously shown that CD41 was expressed on human cord blood CD34+ cells and, to a lesser extent, on adult G-CSF mobilized CD34+ peripheral blood cells [41]. Comparisons of the properties of CD34+ CD41+ CD42− cells with CD34+ CD41− cells by limiting dilution analyses of long-term culture-initiating cells (LTC-IC [1]) indicated that the CD34+ CD41− cells had a higher frequency of LTC-IC [41]. Both populations obtained from cord blood were capable of multilineage repopulation in NOD/SCID mice, with 9 of 10 mice transplanted with CD34+ CD41− CD42− cells and 6 of 10 mice transplanted with CD34+ CD41+ CD42− cells being positive for human cells [41]. The cord blood CD34+ CD41− CD42− and CD34+ CD41+ CD42− cells had comparable potential to produce T lymphocytes in the in vitro NOD/SCID embryonic thymus hanging drop assay [41]. It is unclear whether, similar to what was observed for mice, the human CD41 antibody used in these studies (clone Tab) is a blocking antibody, which could affect the readout of the in vivo assays.

More recently, single-cell analysis of human fetal and adult BM HSCs revealed that *VWF* was expressed by a subset of these HSCs [42]. The function of the *VWF*-expressing HSC population is yet to be determined and would require the identification of cell surface markers that exclusively identify the *VWF*-expressing HSCs. Furthermore, it is not clear whether, as identified for mice [31], the *VWF*-expressing HSCs coexpress, at least to some extent, CD41.

UPDATES ON ASSAYS USED TO ASSESS HSCS AND MPPS: THEIR BENEFITS AND THEIR LIMITATIONS

In our previous review [1], we also discussed key assays that are used to assess the functions of HSCs and MPPs; this section provides a brief update on such assays.

SUBLETHALLY IRRADIATED RECIPIENTS OF HSCS AND MPPS

One of the in vivo assays from the Passegue laboratory assessed the repopulation potential of the HSC and MPP populations in sublethally irradiated recipients [13]. This assay enables transplantation of HSC and MPP populations without requiring support BM cells to keep the mice alive. These studies used β-actin−GFP mice as donors to enable assessment of the donor cells to platelet reconstitution

Table 5 Properties of HSCs identified using CD41

Original name Revised name	CD41+ HSC [31] Myeloid-biased HSC	CD41− HSC [31] Lymphoid-biased HSC	$CD41^{YFP/+}$ HSC [31] CD41+ HSCs	$CD41^{YFP/-}$HSCs [31] CD41- HSCs
Flow cytometry gating strategy	LKS+ CD41+ CD34− CD135− or LKS+ CD41+ CD150+ CD48−	LKS+ CD41− CD34− CD135− or LKS+ CD41− CD150+ CD48−	$CD41^{YFP}$+ LKS+ CD150+ CD48− LT-HSCs	$CD41^{YFP}$ − LKS+ CD150 +CD48− LT-HSCs
Primary transplant Competitive repopulation potential when 50−100 cells were cotransplanted with 2×10^5 wild-type BM cells (repopulation assessed at 4 months, all mice >0.1% donor cells)	ND (CD41 was identified to be a blocking antibody, all HSC transplants were performed using $CD41^{YFP/+}$ mice)	ND (CD41 was identified to be a blocking antibody, all HSC transplants were performed using $CD41^{YFP/-}$ mice)	Robust repopulation (all 21 recipients had >1% donor cells). A myeloid bias was observed, however, all mice showed donor-derived repopulation in all lineages	11/15 mice had >1% donor cells. A lymphoid-bias was observed, however, all mice showed donor-derived repopulation in all lineages
Secondary transplant BM cells (1/10th of a femur) obtained from primary recipients of $CD41^{YFP/+}$ HSCs	ND	ND	All 10 recipients had >10% donor-derived reconstitution. A myeloid bias was observed, however all lineages were repopulated with the exception of one mouse which lacked donor-derived repopulation in B lymphocytes.	5/10 recipients had >10% donor-derived reconstitution. A lymphoid-bias was observed, however, all lineages were repopulated in 7/10 recipients
Potential to form other HSC type	Yes, assessed in liquid culture only	Transient at early time points after culture initiation only	ND	ND
Cell cycle (assessed at 16 hours after injection of BrdU into mice)	Approximately 2% BrdU+	Approximately 10% BrdU+	ND	ND
CFC potential from single-sorted cells	~85% formed CFCs The majority formed small colonies (<40 cells)	~85% formed CFCs The majority formed large colonies (>2mm in size)	ND	ND

BM = bone marrow; BrdU = bromodeoxyuridine; CFC = colony-forming cell; HSC = hematopoietic stem cell; LT-HSC = long-term repopulating hematopoietic stem cell; ND = not determined.

(platelets do not express CD45.1 or CD45.2, which is the most commonly used method of detecting donor-derived cells in transplant studies in mice [1]). The contribution of the GFP+ donor cells to platelets, nucleated cells, and CD11b+ myeloid cells was assessed in the peripheral blood at regular intervals between 7 and 34 days after transplantation. The populations were transplanted at two different cell doses: 2,000 or 500 cells, the latter reported for the LT-HSC, ST-HSC, and MPP2 populations only.

These studies revealed that 2,000 LT-HSC, ST-HSC, and MPP2 cells robustly reconstituted platelets, nucleated cells, and myeloid cells for more than 1 month after transplantation. By contrast, 2,000 MPP3 and MPP4 cells had limited in vivo repopulating potential, with less than 20% donor contribution to platelets and nucleated cells. The MPP3 showed a transient myeloid bias that notably declined by day 34 after transplantation, whereas MPP4 produced fewer myeloid cells after the first 14 days after transplantation. When 500 cells were transplanted, the LT-HSC had superior platelet repopulating capacity compared with that of ST-HSC and MPP2 cells; however, myeloid repopulation was similar for LT-HSC and ST-HSC.

There were two caveats of this study. First, the individual mice were bled multiple times (every 3−4 days) between 7 and 34 days after transplantation to monitor blood cell repopulation. Other investigators have shown that repeated blood sampling can cause hematopoietic stress and alter responses of HSCs (and MPPs), primarily in response to anemia that can occur in response to regular bleeding [43−45]. Although these prior studies removed high volumes of blood in each bleed (250 μL and above), it has been recommended that mice be bled a maximum of 150 μL/25 g mouse per week and no more than 200 μL/25 g mouse during two weeks [46]. This recommendation has been based on weekly bleeds of larger volumes, not repeated bleeds within the duration of a week; however, it is important to consider the effects of repeated blood sampling on mice, and it has been recommended that the effects of repeated bleeding on erythrocyte parameters (hemoglobin and hematocrit) be monitored during such experiments [46]. Pietras et al. [13] did not report erythroid parameters in their studies.

Second, the repopulation of the mice was assessed for less than 40 days, whereas it is essential to monitor repopulation for a

minimum of 16 weeks after transplantation to assess HSCs [1,16]. It is unclear why they did not monitor the mice beyond 40 days; however, C57BL/6 mice that are irradiated can be susceptible to developing radiation-induced lymphoma, including sublethally irradiated recipients (extensively reviewed by Rivina et al [47] and Purton laboratory, unpublished observations).

THE CHOICE OF COMPETING CELLS CAN INFLUENCE THE READOUT OF COMPETITIVE REPOPULATING IN VIVO ASSAYS

It has previously been recognized that unfractionated BM cells obtained from mice, (which are used as a standard source of competing BM cells in both competitive repopulation assays [48] and limiting dilution assays [49], both of which are explained in our previous review [1]) contain long-lived B and T lymphocytes [50]. However, there are some recent studies that have highlighted additional issues that researchers need to be aware of when using whole BM for such experiments. First, studies from the Bryder laboratory identified that unfractionated BM also contains long-lived progenitor cells, and these can influence the readout of secondary transplantations [50]. This study also showed that the distribution of HSCs in bones obtained from the same mouse can be very different. To overcome this, it is recommended to pool bones from the same mouse before undertaking serial transplant studies.

Carrelha et al. [27] also made some very interesting observations about the lineage reconstitution of the single HSCs when competed against either WT or W^{41}/W^{41} BM [27]. Although similar proportions of recipients with distinct lineage biases were observed when either competing BM source was used, the use of WT BM notably increased the detection of lymphoid-biased HSCs and HSCs that repopulated multilineages equivalently. By contrast, the use of W^{41}/W^{41} BM significantly increased the detection of platelet/erythroid-biased HSCs and platelet/erythroid/myeloid-biased HSCs [27]. It is likely that this occurred because c-KIT-expressing hematopoietic cells in W^{41}/W^{41} mice have an impaired response to c-KIT ligand, which is essential in regulating many of the erythroid and myeloid lineages in addition to megakaryocyte progenitors [51].

TIME AFTER TRANSPLANTATION AT WHICH REPOPULATING POTENTIAL IS ASSESSED

A caveat of most studies reviewed here is that although 16 weeks after transplantation is considered sufficient to assess HSCs [1], this may be too early to conclusively differentiate LT-HSCs from ST-HSCs. Transplantation studies by Benenviste et al. [52] suggested that there is an intermediate HSC (superior to ST-HSCs) that can repopulate mice at the clonal level for 6–8 months after transplantation. This study is consistent with a seminal study, in which Jordan and Lemischka [53] used integration site analyses of oncoretrovirally transduced HSCs to show that multiple clones give rise to hematopoiesis between 4 and 6 months after transplantation, after which stable clonal hematopoiesis will occur. The findings of these two independent studies are very important to consider, and I recommend that a minimum of 26 weeks after transplantation should be used to adequately assess HSCs.

RECENT ADVANCES IN TECHNOLOGIES THAT HAVE IMPROVED OUR UNDERSTANDING OF HSCS

It is important to briefly highlight here that technological advances in the past few years have made notable contributions to our ability to identify and study HSCs and MPPs. The first two tools discussed below are innovative methods that have advanced single-cell studies of HSCs. A recent elegant review by Rodriguez-Fraticelli and Camargo [54] thoroughly discussed these two types of single-cell technologies, including caveats of each type of approach, and the pioneering studies are briefly highlighted here.

Cellular barcoding studies have enabled the ability of investigators to track HSCs and their progeny. These studies have included different approaches including gene transduction [55], transposons [56], CRISPR-Cas9 technology [57], and *Polylox* barcoding [58] of HSCs. Seminal contributions from the laboratories who have pioneered the use of cellular barcoding in HSC studies are highlighted here. These are collaborative studies from the de Haan and Bystrykh laboratories, which have used retroviral gene transduction methods of cellular barcoding methods to study the behavior of murine HSCs, including during leukemia development [55,59] and monitoring human HSCs in murine xenotransplantation studies [60–62]. The Camargo laboratory contributed *Sleeping Beauty* transposon [56,63], lentiviral [64], and CRISPR-Cas9 [57] barcoding approaches to study murine HSCs, including in native and stressed conditions. The Rodewald laboratory developed the *Polylox* method, generating a number of useful Cre-*loxP*-driven barcoding approaches including $Rosa26^{Polylox/+}$ C57BL/6 mice [58,65,66]. All these approaches enabled the tracking of HSCs and their progeny, although the sensitivity of identifying HSCs in each of the distinct approaches is different [54].

The development of single-cell RNA-sequencing (scRNA-seq) has advanced the ability to assess gene expression in HSCs at the single-cell level. Notable scRNA-seq studies of murine HSCs that have pioneered studies focused on resolving the heterogeneity of the immunophenotypic definition of healthy murine HSCs are collaborative studies from the Göttgens and Kent laboratories [67,68]. These studies have collectively developed gene sets to identify different types of HSCs and MPPs that are valuable resources for the HSC community.

In addition to the Cre-driven mouse strains that target HSCs and can be crossed to reporter strains for tracing studies (a number previously reviewed by Joseph et al [69]) and the *Vwf*-GFP [26] and *Vwf*-tdTomato [27] reporter mice discussed above, some additional HSC reporter mice have recently been generated to study HSCs. These reporter mice include *Evi1*-IRES-GFP [70], *Fgd5*-ZSGreen [71], *Hoxb5*-tri-mCherry [72], *Pdzk1ip1*-GFP [73], *Krt18* Cre-ER mice crossed to either EYFP or tdTomato reporter strains [74], *Krt7*-GFP [75], *Gprc5c*-EGFP [9], and *Hlf*-tdTomato [76].

Combinations of the aforementioned innovative technologies together with immunophenotypical markers and assays of HSCs can be used to further understand HSCs at the single-cell level, as recently reviewed by Rodriguez-Fraticelli and Camargo [54].

QUESTIONS TO BE RESOLVED IN FUTURE STUDIES

It is an exciting time in the field of HSC biology, with many questions yet to be resolved. First, can additional markers (e.g., combining the cell surface markers used by different investigators) be used to further define the HSC hierarchy? Indeed, in studies from the Pietras

laboratory EPCR was recently combined with LKS+, CD135, CD48, and CD150 to reveal that EPCR can be used to further purify four populations of SLAM HSCs (LKS+, CD135− CD48− CD150+) that were subdivided on the basis of their expression (or lack of expression) of CD34 and/or EPCR [77]. They showed that the CD34-EPCR+ SLAM HSCs had the most robust repopulating potential and were less susceptible to the effects of chronic interleukin 1 (IL-1) infection [77]. These studies also revealed that *Vwf*-GFP-expressing cells were notably reduced in LKS+ CD135− CD48− CD150+ HSCs after chronic IL-1 exposure. By contrast, the expression of *Fgd5*-ZSGreen-positive cells in the SLAM HSCs was unaltered after IL-1 exposure [77]. *Fgd5*-ZSGreen is another reporter mouse that has been used to identify HSCs in mice and, intriguingly, has been shown to be essential for embryonic, but not definitive hematopoiesis [71].

Furthermore, with the exception of the studies from the Eaves laboratory [16,18], all other aforementioned flow cytometry gating strategies used Sca-1 positive cells to isolate HSCs, whereas Sca-1 knockout mice are viable and are generated at normal Mendelian frequencies [78]. Adult Sca-1 knockout mice do, however, have defective hematopoiesis. Intriguingly, these defects are reflective of reduced platelet/myeloid-biased HSCs, with the Sca-1 knockout mice having thrombocytopenia, increased lymphocytes, and reduced myeloid cells, accompanied by reduced numbers of myeloid progenitors and primary and secondary repopulating HSCs [78].

This raises the second question. Have some of the recent HSC studies discussed earlier in this review identified distinct populations of HSCs and, if so, where and when do they arise? Numerous studies have previously reported that embryonic HSCs and adult HSCs are distinct and that different transcription factors are key regulators of such HSCs. For example, *Scl/Tal1* has been shown to be essential for embryonic HSCs but not adult HSCs [79]. Intriguingly, however, embryonic-derived *Scl/Tal1*-targeted HSCs do persist for long term in adult mice [80], and the *hScl*Cre^{ERT} transgenic strain has been used to reliably modify gene expression in adult HSCs by postnatal administration of tamoxifen [81] (and Purton laboratory, unpublished observations). Intriguingly, EPCR, *Vwf*, and CD41 are all highly expressed by embryonic HSCs [18,26,32−35]. Future studies that resolve these questions will not only significantly contribute to our understanding of HSCs during health and disease but also potentially resolve the long-standing controversies on where HSCs reside and are regulated in adult mice [82].

Conflict of Interest Disclosure

The author declares that there is no conflict of interest regarding the publication of this article.

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