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Are transplantable stem cells required for adult hematopoiesis?

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Abstract

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.

Introduction

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

We will define "classical" HSCs as cells that have the ability to long-term 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. Classical 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, also, the resilience of the bone marrow environment after irradiation. Lineage-specific progenitors must have substantial proliferative capacity in order to expand sufficiently to replenish the blood, in particular the hematocrit, in the short-term after transplantation.

While 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 papers challenge the notion that the transplantable, "classically defined" stem cells are required to sustain hematopoiesis in adult animals. In this review, we will discuss how the HSC paradigm has shifted in recent years (**Fig. 1**), with a focus on genetic models that examine the impact of HSC depletion and *in situ* labeling techniques.

Diversity in HSCs subsets

Since the majority of mature blood cells have short lifespans, very large numbers of cells are produced throughout life [12]. Clonal analysis of HSCs has revealed an under-appreciated 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; e.g. 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 "multi-potent 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 due to 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 population is quiescent [26]. Single-cell RNA-sequencing has shown 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 to 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 classical 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 classical HSCs? If HSCs were essential for continual blood production, one might expect that mice lacking HSCs due to 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 classical HSCs.

What contribution do HSCs have to steady-state hematopoiesis?

The experiments described above show 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. These experiments indicated 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. Due to the small clone size, conclusions could not be drawn about the kinetics of lineage output of long-term labeled cells; however, this work showed 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% 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 only detected at one time point (with 4-5 weeks between sampling), suggesting contribution to granulopoiesis from each tagged clone was short-lived. Lymphocyte clones were generally more stable. While 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 lifespan 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 to LT-HSCs and mature cells could be a result of the relative abundance of MPPs compared to 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 non-specific 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. estimated a lower limit of one in 3 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 lead 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. 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 three days after a single tamoxifen-treatment [60]. With this labeling method, 60-70% 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 and colleagues [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% contribution of HSCs within one 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 and colleagues [60] and the *Fgd5-CreERT2* transgene used by Chapple et al [61] yielded an increased labeling efficiency of HSCs compared to the *Tie2-Cre* transgene used by Busch and colleagues [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 to 0.48% of progenitors were labeled by the *Krt18-CreERT2* transgene [61], which is substantial compared to 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, since 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, more quiescent HSCs 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, since 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 cre-recombinase. Indeed, Sawen and colleagues 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], repeat bleeding [26], or following depletion of progenitor cells by cytotoxic agents such as 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 show 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 while 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 only examine 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 has 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 to 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]. While described as "lineage-restricted" due to their almost exclusive production of platelets *in situ* and upon primary transplantation, these platelet-biased cells are defined as stem cells since they retain multi-lineage 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 shown 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 & considerations

While 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. While HSCs play an ongoing role in adult hematopoiesis, we suggest that progenitor cells may participate in hematopoiesis for 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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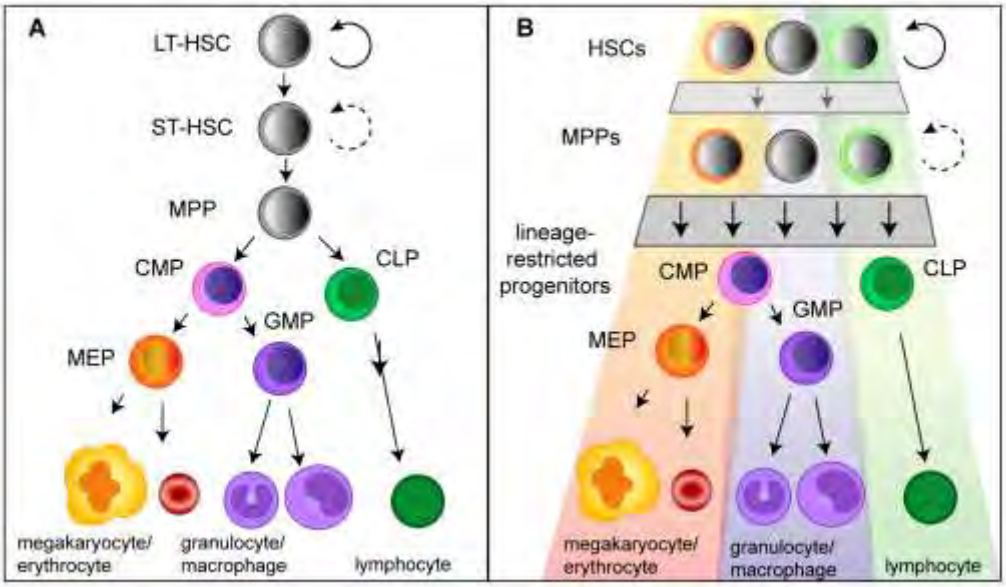

Figure 1: Evolving concepts of hematopoiesis

A) "Classical" 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-seq 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.

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

Reference(s)	**Labeling strategy**	**Cells labeled at outset**	**Summary of findings and conclusions**	**Limitations**
Drize et al., 2001 [56]	Sub-lethal irradiation to induce chromosomal changes.	52.6 ± 26.4% of colonies. Not HSC-specific.	Conclude hematopoiesis is sustained by successive recruitment of clones derived from individual cells.	- Chromosomal changes and irradiation may impact clonal output.
Zavidij et al., 2012 [57]	*In vivo* labeling of cells with GFP through an *in situ* lentiviral	Detected 0.005-0.5% GFP^+ cells in peripheral blood cells for up to 24 months after intra-femoral	Conclude 400 to several thousand clones contribute to steady-state hematopoiesis.	- Less than 1% cells labeled. - Potential for integration site effects.

Reference(s)	Labeling strategy	Cells labeled at outset	Summary of findings and conclusions	Limitations
	system.	injection. Not HSC-specific.		
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% HSCs labeled. Not HSC-specific: 30% ST-HSCs and MPPs labeled.	90-98% of granulocyte clones in peripheral blood were detected only at single time-points (from 3-12 months), indicating granulopoiesis is driven by successive recruitment of short-lived clones. Subsequent study by Roriguez-Faticelli et al. 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 to 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 due to differentiation or death, which may lead to an under-represented 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	30% HSCs labeled. Progenitors also labeled: ~10% ST-HSCs and ~3% of MPPs.	Conclude that HSCs are the major source of multi-lineage hematopoiesis in adult animals, given over 60% of myeloid cells are labeled within 32 weeks.	- Approximately 10% of ST-HSCs and MPPs labeled from outset so expansion of this 10% or non-proportional contribution to downstream populations could confound interpretation.

Reference(s)	Labeling strategy	Cells labeled at outset	Summary of findings and conclusions	Limitations
	preferentially labels HSCs.			
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. 2015 study, likely due to the increased sensitivity of the *polylox* allele to recombination compared to the *Rosa*^{YFP} reporter construct.	Large number of shared molecular tags between HSC and mature cells when recombination initiated in embryos, but lower overlap of tags between HSCs and mature cells when recombination was initiated at 8 weeks of age, i.e. less output from adult compared to embryonic HSCs.	- Not HSC specific.
Chapple et al., 2018 [61]	*Krt18-CreERT2* and *Rosa26-LSL-YFP* transgenes.	2 ± 1.2% HSCs labeled. Progenitors also labeled: 0.11 ± 0.21% of MPP 0.28 ± 0.15% of HPC-1 0.48 ± 0.41% of HPC-2.	Conclude substantial HSC contribution to adult steady-state hematopoiesis based on labeling of 60% myeloid cells and 50% of CLP relative to HSC labeling. Platelets reached equilibrium with the percentage of HSCs labeled by 1 year, 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 days tamoxifen treatment.	32±10% of HSCs labeled. Progenitors also labeled: ~8-10%MPPs, ~<2% of HPC-1, 3-4% HPC-2.	Labeling of over two-thirds of B-cells and platelets at 26 weeks 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*	5.7±2.5% of HSCs labeled.	Near equilibrium of CD150–CD48–LSK MPP cells reached by	- Low level of progenitor population from outset may

Reference(s)	Labeling strategy	Cells labeled at outset	Summary of findings and conclusions	Limitations
	knock-in *and Rosa26-LSL-tdTomato* transgene with 1 day tamoxifen treatment (in first experiment assessing rate of labeling of progenitors and mature cells)	0.2±0.1% of other LSKs labeled.	4 weeks. Equilibrium of CD150+CD48-LSK MPP-2 cells and megakaryocyte progenitors reached by 32 weeks. 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.	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 months after tamoxifen-pulse.	12 ± 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 months, one to four clones make up 80% of the peripheral blood. Some clones were stable, some disappeared while some become active at later time points.	- While mice were used at a time point where changes in HSC cycling and cell numbers induced by poly(I:C) have returned to normal, it is possible the clonal output of HSCs may be affected by the poly(I:C) treatment.