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The Source and Dynamics of Adult Hematopoiesis: Insights from Lineage Tracing

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Abstract

The generation of all blood cell lineages (hematopoiesis) is sustained throughout the entire life span of adult mammals. Studies using cell transplantation identified the self-renewing, multipotent hematopoietic stem cells (HSCs) as the source of hematopoiesis in adoptive hosts and delineated a hierarchy of HSC-derived progenitors that ultimately yield mature blood cells. However, much less is known about adult hematopoiesis as it occurs in native hosts, i.e., without transplantation. Here we review recent advances in our understanding of native hematopoiesis, focusing in particular on the application of genetic lineage tracing in mice. The emerging evidence has established HSCs as the major source of native hematopoiesis, helped to define the kinetics of HSC differentiation, and begun exploring native hematopoiesis in stress conditions such as aging and inflammation. Major outstanding questions about native hematopoiesis still remain, such as its clonal composition, the nature of lineage commitment, and the dynamics of the process in humans.

Contents

HEMATOPOIESIS: THE ULTIMATE HIERARCHICAL DIFFERENTIATION SYSTEM

The Classical Hierarchical Model of Hematopoiesis

Hematopoiesis is the developmental process occurring in the mammalian bone marrow (BM) that generates all of the cells composing mammalian blood. The hematopoietic system is divided into four major lineages, each serving critical functions for the organism. The first, the erythroid lineage, produces erythrocytes, or red blood cells, that use hemoglobin to carry oxygen to tissues. The second, the megakaryocyte lineage, produces thrombocytes, or platelets, that facilitate blood coagulation (hemostasis) and wound healing. The third, the myeloid lineage, produces granulocytes (neutrophils, eosinophils, basophils, and mast cells), monocytes, and dendritic cells (DCs) that are predominantly involved in innate immunity. Fourth and finally, the lymphoid lineage produces B and T lymphocytes that comprise the adaptive arm of immunity, utilizing somatically rearranged loci encoding antigen receptors to mount specific immune responses and confer long-lasting memory. While these major lineages were defined more than a century ago, progress over the last decades has shown that their ontogenetic and functional differences are not absolute. For example, natural killer (NK) cells and cytokine-producing innate lymphoid cells (ILCs) undergo no somatic rearrangement and participate in innate immune responses via germline-encoded receptors much like myeloid cells, yet they comprise a part of the lymphoid lineage (Geiger & Sun 2016, Vosshenrich & Di Santo 2013). Conversely, plasmacytoid DCs (pDCs)

possess many transcriptomic and morphological features of lymphocytes but are myeloid cells focused on innate cytokine responses to viruses (Reizis 2019). In this sense, hematopoiesis produces a spectrum of cellular output with particularly blurred borders between myeloid and lymphoid lineages.

Mature cell types of these lineages have finite and often very short life spans (e.g., several days for granulocytes and platelets); thus, replenishment of this astounding number and spectrum of cells must occur continuously. The classical model of hematopoietic differentiation is one of a hierarchical tree composed of a series of branching fate decisions that lead to each mature cell type (Orkin & Zon 2008, Shizuru et al. 2005, Weissman & Shizuru 2008). At the very top of the tree is the hematopoietic stem cell (HSC), defined as a cell with the capacity to produce all cell types in the hematopoietic lineage (i.e., multipotency) as well as maintain its own numbers (i.e., self-renewal) (**Figure 1*a***). The gold standard for functional HSCs is the capacity for long-term multilineage reconstitution of myeloablated hosts in primary and serial transplantation (Eaves 2015). Downstream of these long-term reconstituting HSCs (LT-HSCs, hereafter called HSCs for simplicity) are the short-term reconstituting HSCs (ST-HSCs) and multipotent progenitors (MPPs), which are multipotent but show progressively diminishing self-renewal, i.e., they lack long-term reconstitution potential (**Figure 1*a***). These precursors branch off into lineage-committed precursors that lack self-renewal capacity, such as megakaryocyte–erythrocyte progenitors (MEPs), common myeloid progenitors (CMPs), and common lymphoid progenitors (CLPs). Next in the cascade are cell type–specific progenitors such as granulocyte/monocyte progenitors (GMPs) and megakaryocyte progenitors (MkPs), which finally give rise to mature cell types. More recent interpretations of the hierarchical model have therefore viewed it less like a linear scheme and more like a road map with broader distributions of cellular potentials and differentiation pathways (Jacobsen & Nerlov 2019, Laurenti & Gottgens 2018).

The Challenge: From Transplantation to Native Hematopoiesis

The classical hierarchical model of hematopoiesis, which was based almost exclusively on transplantation studies, provided enormous conceptual insights into the process, as well as practical advantages for its study. For example, it facilitates prospective isolation of HSCs and various progenitors based on surface markers, followed by molecular analysis and functional assays. Apart from basic research, HSC isolation and transplantation have enormous clinical significance as critical parts of a lifesaving procedure used to reconstitute hematopoiesis in multiple diseases, including cancer (Copelan et al. 2019). However, the dialectical flip side is that the model describes cellular hierarchy and behavior in transplantation but may or may not fully apply to native hematopoiesis (Hofer et al. 2016). Indeed, transplantation reveals the potential of stem and progenitor cells (i.e., what they can produce when pushed), but not necessarily their actual function (i.e., what they are producing during the steady state). Prospective isolation inevitably focuses on discrete phenotypes rather than spectra of progenitors, and such phenotypes are typically based both on assumptions about their likely nature and on convenient markers that often lack physiological significance. Transplantation introduces a major reset in the form of isolation of cells from their native BM environment and transfer followed by extravasation, homing, and lodging. With respect to HSCs, they do not normally return to the BM after exit in the steady state (McKinney-Freeman & Goodell 2004) and require very large numbers to engraft in unmanipulated BM (Shimoto et al. 2017). That HSCs engraft efficiently upon conditioning such as irradiation (Benveniste et al. 2003) further emphasizes the nonphysiological nature of transplantation into conditioned hosts. Finally, the current transplantation-based hierarchical scheme lacks a real-time scale that describes HSC differentiation into distinct progenitors and mature cell types.

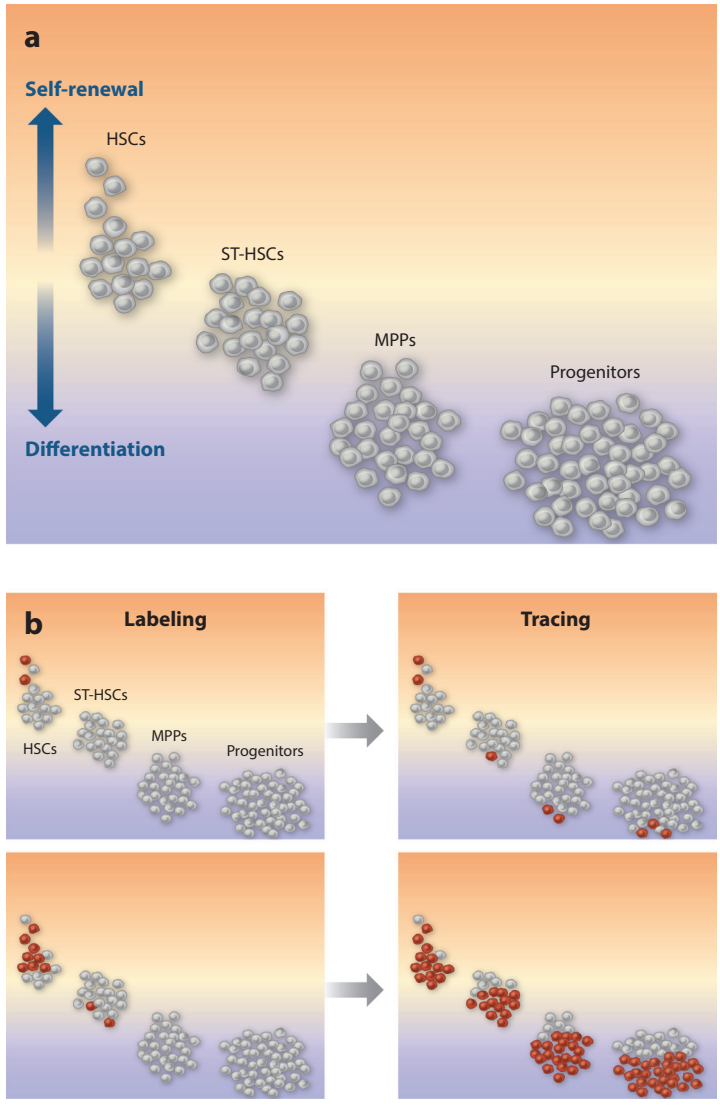

Figure 1

Tracing HSC contribution to native hematopoiesis. (*a*) HSC and progenitor cell populations on a continuum of self-renewal and differentiation. (*b*) Schematic of HSC lineage tracing results, with cells labeled at the start and after tracing highlighted in red. (*Top*) These models have high specificity but low efficiency of HSC labeling (Busch et al. 2015), which may also label rare HSCs with increased self-renewal capacity. (*Bottom*) These models show more efficient HSC labeling (Chapple et al. 2018, Sawai et al. 2016, Säwen et al. 2018), in which the fraction of labeled HSCs increases over time and their progeny become efficiently labeled. Abbreviations: HSC, hematopoietic stem cell; MPP, multipotent progenitor; ST-HSC, short-term reconstituting hematopoietic stem cell.

In this review, we have tried to summarize the current progress in our understanding of native hematopoiesis as it occurs in unmanipulated organisms, i.e., in the absence of cell transplantation. We emphasize the essential relevance of the classical HSC-centric hierarchical model, as well as its adjustment and expansion as applied to the native process. Along the way, we describe outstanding

questions in this emerging field and highlight the power of lineage tracing in answering them. To keep the review focused, we have sought to impose clear limits on its scope. In particular, it covers only mammalian organisms, with emphasis on mouse models; the exciting field of hematopoiesis in nonmammalian species such as invertebrates and fish deserves a separate discussion (Banerjee et al. 2019, de Jong & Zon 2005, Martinez-Agosto et al. 2007). Furthermore, we consider only adult BM-driven hematopoiesis, with embryonic origins and ontogeny of mammalian hematopoiesis being reviewed elsewhere (Dzierzak & Bigas 2018, Palis 2016). Finally, given the central role of HSC in the process and current controversies surrounding it, we primarily focus on HSC differentiation as opposed to the hierarchies within each lineage.

THE TOOLBOX: STUDYING HEMATOPOIESIS BY LINEAGE TRACING

Population-Level Lineage Tracing

To study native hematopoiesis, the goal is to observe and quantify the ongoing process with minimal experimental perturbation. Genetically engineered transgenic (Tg) or knock-in (KI) reporter animal models provide powerful technical approaches based on lineage tracing. Lineage tracing, also known as fate mapping, is a technique whereby the progeny of a cell population of interest can be identified and quantified over time, based on the introduction of an indelible, heritable label. Genetic labeling of the stem and progenitor populations can be achieved using a site-specific recombinase such as Cre recombinase, whose expression is driven by a cell type–specific gene (Jensen & Dymecki 2014). Combined with a reporter allele with a transcriptional stop cassette flanked by Cre substrate LoxP sites, Cre excises the stop cassette and permanently turns on expression of the reporter in the population of interest. This recombined reporter allele is inherited by the progeny of the original Cre-expressing cell, allowing delineation of progenitor–progeny relationships. Moreover, Cre recombinase fused to estrogen receptor (CreER), particularly with its CreERT2 variant that is preferentially activated by the estrogen analog tamoxifen (Feil et al. 1997), enables inducible labeling of the cells of interest by systemic tamoxifen administration. Multiple useful variations of this approach have been developed, e.g., the use of a dual fluorescent switch reporter (Muzumdar et al. 2007) that can serve as an endogenous tracer of proliferation (Upadhaya et al. 2018).

The power of a lineage-tracing system generally relies on several key parameters of cell labeling: specificity, efficiency, and inducibility. Obviously, labeling must be specific to the progenitor population of interest; it is particularly important that the progeny under study do not express the recombinase at any point. However, the process must be efficient enough to label a significant fraction, ideally the majority, of the progenitor population of interest. Lastly, labeling should preferably be inducible, so that legacy labeling from earlier developmental processes would be absent and the time of label initiation and cessation would be known. Of course, Cre recombination is rarely perfect and usually requires a compromise between labeling efficiency and specificity. Importantly, some latitude exists for both parameters; thus, some labeling of downstream and intermediate populations may be tolerated as long as the labeling in the target progenitor population is substantially higher.

The trade-off between efficiency and specificity is particularly relevant for the labeling of HSCs, which present a unique set of challenges. Likely because they are multipotent and not affiliated with any lineage, HSCs appear to lack unique markers that would be both specific and expressed at high levels. Recently, multiple HSC-enriched genes have been used to drive reporter Tg or KI mouse models (**Table 1*a***). Inevitably, the resulting expression was either specific but weak or partial (e.g., *Hoxb5*, *Tie2*) or strong but not completely specific (e.g., *Fgd5*, native *Pdzk1ip1*). Furthermore, some drivers may reflect HSC heterogeneity and preferentially label particular subsets

Table 1 Mouse model for labeling and lineage tracing of HSCs

Gene	Method	Labeling in HSCs	Specificity	Reference(s)
A: Fluorescent reporters				
Ctnnal1	KI: GFP	~50%	Some progenitors, endothelial cells labeled	Acar et al. 2015
Fgd5	KI: mCherry, ZsGreen	~80%	Endothelial cells labeled	Gazit et al. 2014
Gprc5c	BAC Tg: EGFP	~28%	High (dormant HSC subset)	Cabezas-Wallscheid et al. 2017
Hoxb5	KI: tri-mCherry	~22%	High	Chen et al. 2016
Pdzk1ip1	BAC Tg: EGFP	~27%	High	Sawai et al. 2016
Tek (*Tie2*)	Tg: Green Lantern GFP	~5%	Endothelial cells labeled	Ito et al. 2016
Vwf	BAC Tg: EGFP, tdTomato	~60%	MkPs, platelets, endothelial cells labeled	Sanjuan-Pla et al. 2013
B: Lineage-tracing reporters				
Fgd5	KI: CreERT2	~30% (tdTomato)	~10% MPPs labeled	Chapple et al. 2018, Säwen et al. 2018
Krt18	Tg: CreERT2	~2% (YFP)	High	Chapple et al. 2018
Pdzk1ip1	BAC Tg: CreERT2	~20–30% (tdTomato)	~5% of ST-HSCs labeled	Sawai et al. 2016, Upadhaya et al. 2018
Tek (*Tie2*)	KI: Mer-Cre-Mer	~0.5% (YFP)	High	Busch et al. 2015
Vwf	KI: CreERT2-P2A-EGFP	~12% (tdTomato)	Unknown; MkPs, platelets likely labeled	Carrelha et al. 2018

Abbreviations: CreERT2, Cre recombinase fused to estrogen receptor ligand-binding domain with T2 mutation; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; KI, knock-in; MkP, megakaryocyte progenitor; MPP, multipotent progenitor; ST-HSC, short-term reconstituting hematopoietic stem cell; Tg, transgenic; YFP, yellow fluorescent protein.

of HSCs (e.g., *Vwf*, *Gprc5c*). Accordingly, inducible CreER alleles for HSC tracing reflect the expression of their driver genes and may be either relatively inefficient or incompletely specific (**Table 1*b***). Another relevant consideration for the tracing of HSCs is the use of tamoxifen: Although tamoxifen-inducible lineage tracing using CreER is widely used, tamoxifen itself can affect hematopoiesis. In particular, tamoxifen can bind to endogenous estrogen receptors and act as an agonist, causing depletion and impaired function of MPPs at high doses (Sanchez-Aguilera et al. 2014). Therefore, the system must be titrated to the minimal dose of tamoxifen possible, ideally well below the standard cumulative dose of 5 mg/animal.

In addition to Cre-based methods, novel approaches for HSC tracing are being developed such as the modified RCAS (replication-competent avian sarcoma-leukosis retrovirus)/TVA (tumor virus A antigen) system, in which a pseudotyped green fluorescent protein (GFP)-expressing lentivirus infects quiescent TVA-expressing cells. Proof of principle was demonstrated using KI mouse models driving expression of TVA from the HSC-enriched *Krt7* and *Evi1* loci (Tajima et al. 2017). GFP-expressing HSCs were detectable and were subsequently used for lineage tracing, although the low efficiency of labeling remains to be improved in the future.

Clonal Lineage Tracing

In contrast to population-level tracing, several recent approaches facilitate clonal lineage tracing by generating unique DNA and/or protein bar codes within each cell (Kebschull & Zador 2018, Kester & van Oudenaarden 2018). Here we briefly review some of these approaches as applied to hematopoietic cell tracing. From classical (Lemischka et al. 1986) and modern (Zavidij

et al. 2012) studies on retroviral integrations to lentiviral bar code libraries (Naik et al. 2013) to their combination with transcriptomics (Weinreb et al. 2020), retroviral DNA barcoding has been successfully used for clonal tracing in HSC/progenitor transplantation. The study of lentiviral integrations also provided unique insights into human hematopoiesis (Biasco et al. 2016, Scala et al. 2018). Barcoding by integration has been ingeniously applied to native hematopoiesis in the form of induced transposon mobilization, with the resulting transposon random integration sites serving as unique clonal bar codes (Sun et al. 2014). Currently, this approach does not allow cell type–specific transposon mobilization, warranting its further development and combination with Cre-inducible labeling. Similarly, exciting new methods based on CRISPR/Cas9-mediated DNA barcoding (Alemany et al. 2018, Chan et al. 2019, Kalhor et al. 2018) are likely to revolutionize clonal analysis of hematopoiesis; however, challenges of specific cell labeling have precluded their application to adult hematopoiesis so far.

Conversely, cell type–specific Cre-inducible methods of clonal labeling have been developed, such as those based on the original Brainbow or Confetti cassettes of several fluorescent proteins (Livet et al. 2007, Snippert et al. 2010). Recently, the Confetti technology has been directly applied to the study of native hematopoiesis (Ganuza et al. 2017). Further, Tg mice with multiple integrations of such cassettes provide an extended palette of fluorescent labels, which is nevertheless limited by definition (Yu et al. 2016). Another recently developed cell type–specific clonal labeling strategy, termed PolyLox, is based on the consecutive arrangement of multiple LoxP sites with unique intervening sequence elements. This powerful approach has a theoretical capacity for $>10^6$ unique DNA bar codes, although in practice the number of interpretable bar codes has appeared to be significantly fewer, limiting the application to adult hematopoiesis (Pei et al. 2017). These and similar (Weber et al. 2016) emerging methods of cell type–specific clonal labeling will hopefully be developed to expand bar code diversity for a comprehensive analysis of adult native hematopoiesis.

THE ULTIMATE SOURCE: THE ROLE OF HSCs IN NATIVE HEMATOPOIESIS

Challenges to the Central Role of HSCs in Hematopoiesis

The initial analysis of the source of native hematopoiesis produced a striking challenge to the canonical view of the HSC sustaining hematopoiesis throughout adult life. In particular, clonal lineage tracing by transposon mobilization suggested that HSCs provide limited contributions to native hematopoiesis and that the bulk of differentiation arises instead from undefined downstream progenitors (Sun et al. 2014). This conclusion was based on the paucity of shared bar codes among granulocytes collected at different points in time, as well as among granulocytes, lymphocytes, and the HSC compartment. A follow-up study revealed increasing frequency of common bar codes in BM progenitors over time, although long-term tracing was not performed (Rodriguez-Fraticelli et al. 2018). A conceptual caveat to these studies is the lack of cell type specificity in doxycycline-inducible transposon mobilization. Technical caveats include a formal possibility of repeated transposon mobilizations in the same cell over time (e.g., due to sustained leaky expression of the transposase after doxycycline cessation), which would eliminate the original shared bar codes. Furthermore, integration site cloning from a single end of the transposon may be unreliable and remains to be validated.

In another apparent challenge to the role of HSCs in sustaining hematopoiesis, CreER-mediated inducible diphtheria toxin (DT) expression was used to ablate HSC/progenitor populations in vivo. Progenitors were found to rebound shortly thereafter, whereas HSC frequency remained persistently below 10% of normal numbers, yet steady-state hematopoiesis was largely

unaffected (Schoedel et al. 2016). Similarly, multiple gene targeting studies from the original (Nichogiannopoulou et al. 1999) to the more recent (Sheikh et al. 2016) showed that hematopoiesis may proceed with very few HSCs present. Unlike lineage tracing, which (at least ideally) does not affect cell function, these approaches fundamentally perturb the steady state and likely elicit major compensation mechanisms. As described in its original application to T cell development (Voehringer et al. 2008), DT-mediated ablation potently selects for rare cells that escape it, especially once they exit the Cre-expressing HSC compartment. Given the ability of a single transplanted HSC to repopulate the entire hematopoietic system, the same repopulation may occur due to a very few escaping HSCs in Cre-mediated targeting (Galan-Caridad et al. 2007). In the case of constitutive genetic lesions, compensation by downstream progenitors appears likely, even though it may be inactive in the presence of a functional HSC population.

The Role of HSCs Defined by Lineage Tracing

The definitive application of HSC-specific inducible lineage tracing was based on a novel KI mouse model expressing CreER from the HSC-enriched *Tie2* (*Tek*) locus (Busch et al. 2015). Following tamoxifen administration over 5 days and a subsequent lag period of 1–3 weeks, a very small fraction (~0.5%) of phenotypic HSCs was labeled with an almost absolute specificity. Even though the low frequency precluded continuous monitoring, the endpoint analysis revealed the propagation of label throughout all progenitors as well as mature myeloid and lymphoid cells. The rate of label propagation was estimated to be very low; mathematical modeling of the data suggested that ST-HSCs receive rare input from HSCs and in turn serve as major contributors to hematopoiesis. This landmark study explicitly demonstrated for the first time that HSCs do contribute to steady-state hematopoiesis, whereas the estimated low rate of HSC contribution may reflect specific features of the model. Chief among them is the exceedingly low efficiency of HSC labeling, which may underestimate the differentiation rate in the absence of data on the starting frequency of labeling in each animal. Furthermore, the system may preferentially label a small fraction of relatively dormant HSCs with reduced differentiation (Bernitz et al. 2016, Cabezas-Wallscheid et al. 2017), e.g., due to KI expression specificity and/or the post-tamoxifen lag period (**Figure 1*b***).

Other studies using inducible lineage tracing not only confirmed the contribution of HSCs to steady-state hematopoiesis but also yielded a much higher estimate of its magnitude. Our lab generated a Tg model expressing CreER driven by the HSC-enriched *Pdzk1ip1* (*Map17*) gene. A single low dose of tamoxifen (0.5–1 mg) instantly labeled ~30% of phenotypic HSCs, which manifested undifferentiated phenotypes and transcriptomes, relatively low proliferation rates, and superior reconstitution capacities in transplantation (Sawai et al. 2016, Upadhaya et al. 2018). In contrast to the system used by Busch and colleagues (2015), the fraction of labeled HSCs increased more than twofold over several months to reach ~80%, suggesting that the labeled cells represent top-level HSCs that generate other cells within the HSC population. Subsequent continuous tracing in the blood and BM revealed major contributions to all progenitors and key lineages, yielding greater than two-thirds of HSC-derived platelets and myeloid cells after 6–9 months (**Figure 1*b***). Certain specialized immune cells such as tissue macrophages and innate-like lymphocytes such as B-1a cells are thought to arise during embryogenesis and undergo autonomous maintenance with limited input from the BM (Montecino-Rodriguez & Dorshkind 2012, Varol et al. 2015). These cells became labeled at much lower levels or (in the case of brain and skin macrophages) not at all, proving their partial or complete uncoupling from HSC-driven hematopoiesis. Peripheral cell labeling was always lower than HSC labeling, but only by a small margin that could be eliminated by systemic inflammatory challenge. As an inevitable trade-off with high efficiency,

the specificity of HSC labeling in *Pdzk1ip1*-CreER mice was not absolute, and some downstream progenitors, including ST-HSCs, became labeled at variable low frequency (Sawai et al. 2016, Upadhaya et al. 2018). Notably, this labeling was several times lower than in HSCs at the outset or in mature cells at the endpoint, suggesting that it cannot cause or seriously affect the latter. Collectively, the results suggested that only a small fraction of HSCs is dormant, whereas the majority of the HSC population actively contributes to, and comprises a major lifelong source of, adult hematopoiesis.

Subsequent studies reported a similarly high HSC contribution to native hematopoiesis using different lineage-tracing models. One study utilized *Krt18*-CreER, in which HSCs were labeled specifically at a relatively low rate (~2% at 1 week postinduction), but pan-lineage labeling was observed by 26 weeks and was only slightly lower than in HSCs by 52 weeks (Chapple et al. 2018). This study also reported lineage tracing using *Fgd5*-CreER (Gazit et al. 2014) in which the initial efficiency of labeling was much higher (~30% of HSCs at 1 week postlabeling) and similarly, the labeling of mature cell types was approaching that of HSCs by 26 weeks. As with *Pdzk1ip1*-CreER, the initial labeling of HSCs nearly doubled at the endpoint in both models. These results were further confirmed in yet another study utilizing *Fgd5*-CreER (Säwen et al. 2018). Unlike *Pdzk1ip1*-CreER or *Krt18*-CreER, *Fgd5*-CreER appears to label MPPs detectably; however, this background labeling cannot account for the much higher eventual labeling of mature cells. Collectively, HSC lineage tracing, as well as Confetti-based clonal tracing (Ganuza et al. 2017) and RCAS/TVA-mediated lentiviral delivery to HSCs (Tajima et al. 2017), revealed a long-term, multilineage contribution of the HSC population to steady-state hematopoiesis. These data show that the "HSC-centric" classical scheme is fully applicable to native conditions, with HSCs serving as a major lifelong source of hematopoiesis.

THE TIME DIMENSION: KINETICS OF LINEAGE DIFFERENTIATION IN NATIVE HEMATOPOIESIS

Studies of hematopoietic differentiation have primarily focused on the hierarchy of progenitor emergence and cell fate decisions; an implicit assumption in this approach is that all these events occur on a similar time scale. This assumption was largely consistent with the reality of transplantation, in which all major lineages emerge simultaneously from the transplanted HSCs. However, transplantation by its very nature mobilizes HSCs into overdrive differentiation and fundamentally alters the microenvironment. Indeed, transplantation using specific host conditioning, such as antibody-mediated HSC depletion, produced equally fast myeloid reconstitution but much slower reconstitution of T and B lymphocytes (Czechowicz et al. 2019). These and previous (Schwarz et al. 2007) studies on nonmyeloablative reconstitution suggest major kinetic differences between lymphoid and myeloid differentiation from HSCs. Conversely, in vitro studies suggested megakaryocyte production as the earliest step of HSC differentiation prior to other cell types (Guo et al. 2013).

Lineage tracing has allowed an unbiased analysis of HSC differentiation kinetics, revealing fundamental differences in the emergence of major hematopoietic lineages. Both population-level (Chapple et al. 2018, Säwen et al. 2018, Upadhaya et al. 2018) and clonal (Rodriguez-Fraticelli et al. 2018) tracing revealed the generation of MkPs and/or megakaryocytes or platelets well ahead of other lineages (**Figure 2**). This was confirmed by proliferation tracing, functional analysis, and single-cell RNA sequencing (scRNA-seq), which showed MkPs emerging from labeled HSCs after 2–3 cell divisions within 1 week, followed by erythroid and myeloid progenitors in 2 weeks (Upadhaya et al. 2018). Conversely, lymphoid differentiation of HSCs commenced long after myeloid differentiation (Busch et al. 2015, Chapple et al. 2018, Sawai et al. 2016, Säwen et al.

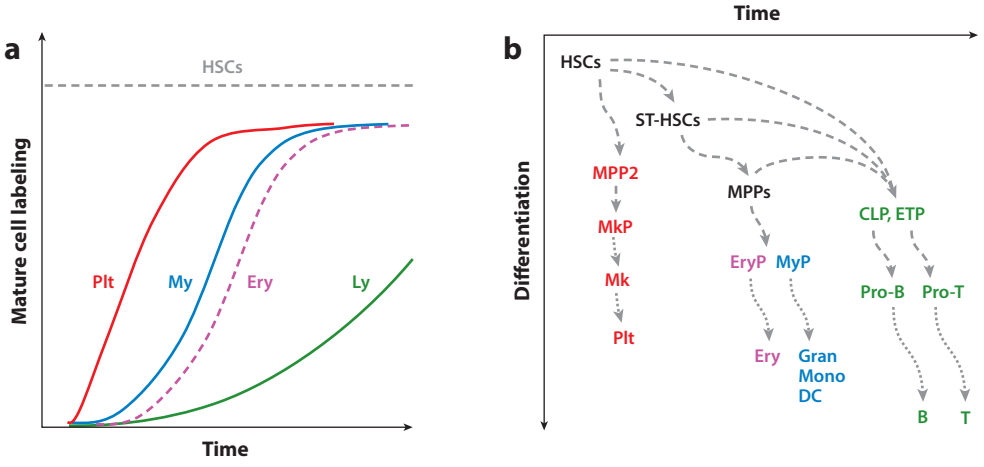

Figure 2

The time dimension of native hematopoiesis. (*a*) Schematic of label accrual in mature platelets, myeloid cells (granulocytes and monocytes) and lymphocytes (T, B, and NK cells) after inducible HSC labeling, based on data from Busch et al. (2015), Chapple et al. (2018), Sawai et al. (2016), Säwen et al. (2018), and Upadhaya et al. (2018). The labeling of erythrocytes was not experimentally measured and is inferred from the labeling of erythroid progenitors. The labeling of peripheral lymphocytes never reached the level of the labeling of platelet/myeloid cells due to the long life spans of lymphocytes. The labeling of HSCs at the endpoint (*gray dashed line*) was only slightly higher than that of peripheral cells (Chapple et al. 2018, Sawai et al. 2016, Säwen et al. 2018). (*b*) A proposed hierarchical scheme of hematopoietic differentiation with the time dimension deduced from the above-mentioned studies. Abbreviations: B, B lymphocyte; CLP, common lymphoid progenitor; DC, dendritic cell; Ery, erythrocyte; EryP, erythrocyte progenitor; ETP, early T lymphocyte progenitor; Gran, granulocyte; HSC, hematopoietic stem cell; Ly, lymphocyte; Mk, megakaryocyte; MkP, megakaryocyte progenitor; Mono, monocyte; MPP, multipotent progenitor; My, myeloid cell; MyP, myeloid cell progenitor; NK, natural killer; Plt, platelet; Pro-B, B lymphocyte progenitor; Pro-T, T lymphocyte progenitor; ST-HSC, short-term hematopoietic stem cell; T, T lymphocyte.

2018) and occurred randomly within 3–6 weeks after HSC labeling (Upadhaya et al. 2018; J.N. Pucella, S. Upadhaya & B. Reizis, unpublished data) (**Figure 2**).

The observed differential production rates of hematopoietic lineages require an explanation, which at this point can be only speculative. The key parameters to consider for each lineage are the evolutionary origin, life span, vital necessity, and likelihood of depletion. By these criteria, platelets are ancient, short lived (~5 days), required for survival on the scale of minutes, and are easy to lose in a massive coagulation due to wounding. Accordingly, HSCs appear primed for platelet production in their expression of megakaryocyte-associated genes (Guo et al. 2013, Kent et al. 2009, Sanjuan-Pla et al. 2013) and markers such as CD150, and in their dependence on shared growth factors such as thrombopoietin (Tpo) (Decker et al. 2018, Qian et al. 2007, Yoshihara et al. 2007). Conversely, T and B lymphocytes are evolutionarily recent, extremely long lived, required for survival in the long term, and hard to lose due to their residence in lymphoid organs and tissues. Myeloid cells and erythrocytes differ in some of these parameters (for example, a life span of 1–2 days versus ~120 days, respectively) but on the whole appear between platelets and lymphocytes in terms of the danger and ease of a sudden loss. Additional reasons for the observed differences may be the mechanisms whereby cell numbers are generated within each lineage. Platelet generation involves limited proliferation (except endoreduplication in megakaryocytes) and thus may require a continuous supply of progenitors. Conversely, committed lymphoid progenitors undergo

massive waves of rapid proliferation during lymphocyte development and selection and therefore may be generated slowly and infrequently from HSCs.

Irrespective of its evolutionary origin and significance, the observed differential kinetics of HSC differentiation has major implications for our understanding of the process. It introduces the time dimension into the hierarchical differentiation scheme, emphasizing or rebalancing some of its key parts (**Figure 2*b***). For example, the emergence of MkPs from HSCs appears to precede that of the commonly defined CD150$^-$ MPPs, supporting a separate bypass through the MkP-primed CD150$^+$ MPP2s (Pietras et al. 2015). Furthermore, it generally supports the early split of the megakaryocytic lineage fate from HSCs, as recently proposed (Carrelha et al. 2018, Notta et al. 2016, Rodriguez-Fraticelli et al. 2018), rather than a common differentiation of the megakaryocytic and erythroid lineages, as envisaged in the original differentiation hierarchy. In another example, the early emergence of pDCs simultaneously with myeloid cells (Upadhaya et al. 2018) supports their affiliation with the myeloid lineage (Geissmann et al. 2010) and is inconsistent with the proposed derivation from lymphoid progenitors (Dress et al. 2019, Rodrigues et al. 2018). In general, the dramatic difference in the kinetics of lymphoid and myeloid differentiation poses a major question about its mechanism. One possibility is that a common lymphoid/myeloid progenitor, such as lymphoid-primed MPPs (LMPPs) (Adolfsson et al. 2005, Mansson et al. 2007), predominantly generates myeloid cells and only rarely (and perhaps stochastically) gives rise to lymphoid progenitors. An alternative, however, is that lymphoid and myeloid potentials are determined at the level of HSCs, as discussed in the next section.

THE PROCESS: CLONALITY, PROGENITORS, AND LINEAGE COMMITMENT

Clonal Composition of Adult Hematopoiesis

While the clonal magnitude and spectrum of native hematopoiesis are still poorly understood, recent studies have provided important initial insights. With the exception of the transposon-based analysis (Sun et al. 2014), these studies suggest that the system is highly polyclonal and that individual clones may be stably propagated during steady-state (Zavidij et al. 2012, Ganuza et al. 2019) and interferon-accelerated (Yu et al. 2016) native hematopoiesis. Furthermore, DNA barcoding in embryogenesis revealed clonal divergence between myeloid and lymphoid lineages (Pei et al. 2017), suggesting that HSC populations that give rise to these lineages are distinct and stably maintained in the adult. This observation is consistent with extensive evidence from single-cell HSC transplantations, which revealed HSC subsets that were preferentially contributing (biased) toward certain lineages (Carrelha et al. 2018, Challen et al. 2010, Dykstra et al. 2007, Morita et al. 2010, Muller-Sieburg et al. 2004, Yamamoto et al. 2013). Ultimately, understanding the clonality of hematopoiesis would require inducible HSC labeling with highly diverse clonal bar codes, as well as tracing their dynamics in progenitors and mature cells over time.

The Nature and Stages of Lineage Commitment

The classical scheme of hematopoiesis is based on a hierarchy of progenitors with progressively narrowing differentiation potentials—from multipotent to lineage specific to cell type specific to unipotent immediate precursors of each cell type. An underlying assumption has been that lineage commitment parallels this progression, i.e., most or all cells at each progenitor stage carry the full potential to produce downstream lineages and undergo a cell fate choice depending on cell-intrinsic (e.g., transcription factors) and cell-extrinsic (e.g., cytokines and intercellular signaling pathways) cues (**Figure 3*a***). This assumption was supported by the analysis of cell progeny

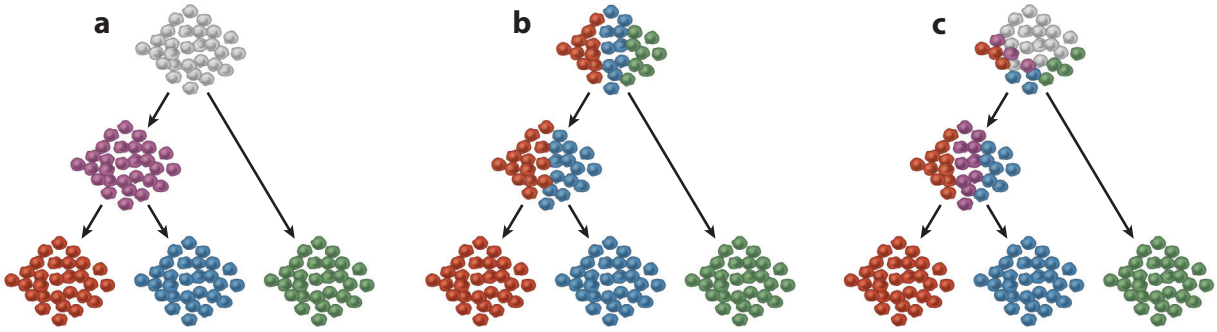

Figure 3

Lineage commitment during native hematopoiesis. Hypothetical differentiation of three mature hematopoietic cell types (*red*, *blue*, and *green*) from upstream progenitors. (*a*) Classical differentiation scenario with progressive lineage commitment in common progenitors (*gray* and *purple*) at each stage. (*b*) A scenario based on several recent studies, envisaging precommitment to each lineage at the earliest progenitor stage. (*c*) A combined scenario that incorporates both precommitted and common progenitors.

in transplantation studies, as well as by functional evidence such as instructive action of lineage-specific cytokines and signaling pathways. Although highly informative, these studies are subject to important caveats. Thus, transplantations may yield variable results depending on the spectrum of resulting cell types examined, as illustrated by studies of macrophage/dendritic cell progenitors (Fogg et al. 2006, Sathe et al. 2014). Similarly, Notch signaling is required to enforce T cell lineage commitment and prevent B cell accumulation in the thymus (Radtke et al. 1999) but does not necessarily block B cell development directly (Feyerabend et al. 2009) and may even precommit earlier progenitors in the BM (Chen et al. 2019). More generally, these experiments reveal lineage potentials in forced conditions, typically at the level of a discrete phenotypically defined population. Not surprisingly, approaches that examined stem/progenitor cells in native conditions and/or at single-cell resolution produced major challenges to the canonical view.

One notable observation from scRNA-seq analysis of myeloid progenitors (Paul et al. 2015) is the near-complete dominance of cells with cell type–specific transcriptomes, suggesting that they represent heterogeneous precommitted rather than common progenitors. Heterogeneity and signs of early commitment have been observed in scRNA-seq of other progenitor populations, including MPPs (Chen et al. 2019). Furthermore, studies using lentiviral barcoding followed by transplantation (Naik et al. 2013, Perie et al. 2015), as well as single-cell cultures of human progenitors (Lee et al. 2017), revealed preferential lineage-specific differentiation of very early progenitors at the level of MPPs or even HSCs. These and other studies brought forward an important conceptual adjustment, namely that lineage commitment does not equal developmental stage and may occur at the highest levels of hematopoietic hierarchy, including HSCs and/or MPPs (**Figure 3*b***). The fact that lineage commitment may "go all the way to the top" is consistent with the above-mentioned notion of lineage-biased HSCs suggested by single-cell transplantations.

The classical and revisionist views of lineage commitment remain to be reconciled in the context of native hematopoiesis. This awaits the development of a cell type–specific clonal labeling system with a high practical diversity of bar codes. Still, several recent advances are noteworthy. First, scRNA-seq studies of the entire HSC and progenitor compartment (Dahlin et al. 2018, Giladi et al. 2018, Nestorowa et al. 2016, Rodriguez-Fraticelli et al. 2018, Tusi et al. 2018, Weinreb et al. 2020) suggest a gradual accrual of lineage-specific transcriptomes across a spectrum of progenitors downstream of HSCs. Notably, these and other studies (Grover et al. 2016, Kowalczyk et al. 2015, Pei et al. 2020) revealed some heterogeneity within the HSC compartment yet

potential features of lineage-specific priming were either absent or very minor. However, progenitors with mixed transcriptomes consistent with ongoing cell fate choice were detected and supported by genetic and functional analyses. These included progenitors with granulocyte (basophil) and erythroid features (Drissen et al. 2016) or with combined features and specific transcription factors of granulocytes (neutrophils) and macrophages (Olsson et al. 2016). One possible way to capture ongoing differentiation as opposed to the cross-sectional analysis of static progenitor populations is with a combination of scRNA-seq and HSC lineage tracing. This approach revealed the rapid emergence of cells with lineage- and cell type–specific transcriptomes within 2 weeks of HSC labeling. At the same time, progenitors with mixed transcriptomes, including those coexpressing antagonistic transcription factors, were readily detectable (Upadhaya et al. 2018).

Most recently, approaches have emerged to combine scRNA-seq with clonal tracing (Pei et al. 2020, Weinreb et al. 2020). The intersection of clonal bar codes and single-cell transcriptional states from parallel in vitro and transplant experiments has supported the notion of lineage priming in MPPs. Interestingly, progenitor transcriptional state alone was insufficient to fully deduce eventual fate outcomes, likely reflecting as yet unknown epigenetic differences or simply the lack of complete precommitment (Weinreb et al. 2020). Similarly, in situ clonal labeling in early embryogenesis revealed HSCs with full multilineage contributions or stable myelo-erythroid restricted fate bias, whereas HSCs that were biased for the lymphoid lineage or exclusive to only the myeloid or the erythroid lineage were not observed. The labeling of HSC precursors may still predate the full establishment of fate bias in the adult BM HSC pool. Altogether, these studies suggest that lineage commitment may represent a continuum between progressive lineage bifurcations and full precommitment (**Figure 3*c***). While some HSCs and progenitors may be biased toward certain cell fates early on, others may be genuinely multipotent and able to undergo stepwise cell fate choices at each differentiation stage. This scenario may underlie the remarkable plasticity of hematopoietic differentiation and ensure optimal adaptation to both short-term and long-term demands on blood cell production. Critical for the analysis of this and other scenarios is the ability to label and trace specific lineage-biased HSC subsets, a feat that remains to be achieved.

THE PROCESS SUBVERTED: NATIVE HEMATOPOIESIS IN PATHOLOGICAL CONDITIONS

Inflammation and Hematopoiesis

Various stresses, such as injury and infection, lead to tissue and systemic inflammation that promote both defense against invading pathogens and tissue repair (Medzhitov 2008). Often accompanying inflammation are the characteristic mobilization and increased production of myeloid cells (particularly granulocytes) during the so-called emergency hematopoiesis. It is well established that inflammatory cues can be sensed by hematopoietic progenitors, through both direct recognition of microbial products (Nagai et al. 2006) and indirect proinflammatory cytokine signaling (Mirantes et al. 2014, Schuettpelz & Link 2013). HSCs in particular are strongly affected by type I interferon (IFN-α/β) (Essers et al. 2009, Pietras et al. 2014), type II interferon (IFN-γ) (Baldridge et al. 2010), and IL-1 (Pietras et al. 2016). While such acute proinflammatory signals are thought to induce accelerated HSC output by increasing their proliferation, they have also been shown to impair HSC reconstitution potential in the long term (Baldridge et al. 2010, Esplin et al. 2011, Pietras et al. 2016).

With respect to native conditions, the process of inflammation-induced emergency hematopoiesis, as well as the subsequent exhaustion, still remains to be recapitulated and studied. Acute systemic induction of interferon response accelerated the contribution of labeled

endogenous HSCs to all hematopoietic lineages approximately twofold without changing the differentiation pattern (Sawai et al. 2016). Thus, it remains unclear if the increased myelopoiesis in the inflammatory condition originates in the HSC at any time point and whether the preceding inflammation changes the HSC differentiation rate or pattern in the long term. This question is also relevant for the phenomenon of trained immunity, i.e., priming of the immune system by innate stimuli that results in across-the-board enhanced immunity (Netea et al. 2016). Whereas some studies proposed the involvement of HSCs and/or progenitors in this phenomenon (de Laval 2020, Kaufmann et al. 2018, Mitroulis et al. 2018), this remains to be confirmed by lineage tracing in the setting of native hematopoiesis.

Aging and Clonal Abnormalities

During aging, hematopoiesis undergoes profound changes that contribute to and exacerbate the aging process (Montecino-Rodriguez et al. 2013, Snoeck 2013). Hematopoiesis in aging individuals shows a shift toward myeloid differentiation, which contributes to immunodeficiency, as well as predisposes them to myeloproliferative disorders and myeloid leukemia (Adams et al. 2015, Verovskaya et al. 2019). Another abnormality is the emergence of age-related clonal hematopoiesis [ARCH, also referred to as clonal hematopoiesis of indeterminate potential (CHIP)], i.e., the reduction of clonal diversity of the blood system and the emergence of dominant somatic clones (Bowman et al. 2018, Jan et al. 2017, Shlush 2018). ARCH is associated with an increased risk of hematopoietic cancers as well as with overall increased morbidity due to multiple diseases. Age-related defects of hematopoiesis are thought to be caused by impaired function of aging HSCs. Indeed, HSCs from older animals show a reduced capacity to reconstitute irradiated recipients on a per-cell basis, with a particularly impaired lymphoid reconstitution potential consistent with the myeloid shift (de Haan & Lazare 2018, Snoeck 2013). However, the fraction and absolute numbers of HSCs in the BM of aged animals and humans are significantly expanded. Persistent inflammatory stimulation (sometimes called inflamm-aging) is thought to be a major factor facilitating HSC impairment in old age (Kovtonyuk et al. 2016). One scenario to explain HSC abnormalities in aging focuses on the changes in the clonal composition of the HSC population. In particular, HSC aging may be driven by "survival of the laziest," i.e., the expansion of HSC clones with enhanced self-renewal yet impaired differentiation potential, for example, a reduced ability to generate lymphoid lineages (Beerman et al. 2010, Hock 2010, Muller-Sieburg & Sieburg 2008).

As with other hematopoietic phenomena, our understanding of hematopoietic abnormalities in aging is based primarily on transplantation studies. The caveats of such studies have been highlighted in a recent study in which HSC transfer into mildly conditioned recipients (using busulfan instead of irradiation) did not show differences in reconstitution by old versus young HSCs (Montecino-Rodriguez et al. 2019). Furthermore, short-term HSC lineage tracing in older mice has suggested an overall decrease in HSC contribution to all lineages rather than a specific reduction of lymphoid differentiation (Säwen et al. 2018). These observations warrant a comprehensive long-term analysis of native hematopoiesis and HSC function in older animals by lineage tracing. An initial report has suggested that a reduction in the clonal diversity of HSC output, associated with reduced HSC clonal complexity, occurs in aged mice (Ganuza et al. 2019). Still, ARCH and related abnormalities of steady-state hematopoiesis have not been adequately modeled in mice and require a genetic model based on labeling and/or gene targeting specifically in HSCs. Finally, the proposed clonal mechanism of HSC aging can be proven only if specific HSC subpopulations or clones (e.g., the laziest HSCs) could be specifically labeled in vivo and traced during native hematopoiesis.

Feedback from Mature Cells

Given the frequent perturbation of hematopoiesis by inflammation and other insults, mechanisms likely exist that enable sensing of the periphery and return to homeostasis, a process we here refer to as census feedback. The hematopoietic system must maintain certain levels of each cell type for optimal viability and fitness. Factors that may cause sudden fluctuations vary from traumatic injuries to therapeutic side effects and even intended effects, such as with the B cell–depleting antibodies used to treat cancer and autoimmune diseases. The extent of peripheral census feedback to the BM is not well understood, yet it seems to occur at least in some cases. For example, when erythrocyte levels are low, the hormone erythropoietin is secreted by the kidneys and signals to erythrocyte progenitors to augment production of red blood cells (Koury 2005). Similarly, Tpo, which is secreted by the liver and kidneys, is augmented in conditions of low platelet frequency and potently stimulates megakaryocytes through the receptor c-Mpl (Kaushansky 2006). Intriguingly, Tpo can also signal directly to HSCs (Decker et al. 2018, Qian et al. 2007, Yoshihara et al. 2007), suggesting a direct census feedback mechanism. Using antibody-mediated platelet depletion, it was recently shown that HSCs and MPP2s can indirectly sense low levels of platelets and respond with proliferation, whereas longer platelet depletion causes HSC exhaustion (Ramasz et al. 2019). These initial data suggest at least some instances of census feedback from multiple lineages to HSCs and/or progenitors, a process that remains to be proven and analyzed using lineage-tracing models.

THE NEW ERA OF STUDYING NATIVE HEMATOPOIESIS IN HUMANS

The study of human native hematopoiesis is a daunting task, due largely to the inability to artificially impose genetic labels on cells as can be done in animal model systems. Consequently, investigations into human native hematopoiesis have instead cleverly turned to various naturally occurring marks that can be tracked retrospectively to glimpse unperturbed hematopoietic differentiation. Previous attempts to study human native hematopoiesis relied on mathematical modeling based on ratios of maternal to paternal X chromosome inactivation (Catlin et al. 2011) or average telomere length distributions (Werner et al. 2015). These methods were used to infer the population dynamics of the hematopoietic stem cell pool and arrived at the similar conclusion that the pool undergoes expansion from birth until adolescence before leveling off and maintaining a constant size during adulthood.

More recently, lentiviral integration sites created in HSCs and their progenitors during gene therapy were used as stable clonal marks to make inferences about clonal dynamics (Biasco et al. 2016). Although the study is based on transplantation, the authors collected data over several years and reported a phase change from reconstitution to steady-state hematopoiesis after 6–12 months. Once in the steady-state phase, the study described multilineage output from a combination of HSCs and progenitors, providing strong evidence that human HSCs contribute to ongoing hematopoiesis in adulthood. These original observations were further strengthened and expanded in additional patients who underwent autologous transplantation and were followed over 5 years (Scala et al. 2018).

In another ingenious approach, the collection of somatic mutations in a cell's genomic DNA was used as an evolving bar code to delineate relationships among hematopoietic cells (Lee-Six et al. 2018). The modeling of hematopoietic clonal dynamics revealed in vivo multilineage output from adult human stem cell clones, a conclusion in line with the above-mentioned studies using lentiviral barcoding. It was estimated that 50,000–200,000 HSCs actively contribute to producing leukocytes at any given time (Lee-Six et al. 2018). Most recently, a novel technique has been

developed with significant purported advantages in studying clonal dynamics. This method uses the accrual of somatic mutations in mitochondrial DNA (mtDNA) as clonal bar codes (Ludwig et al. 2019). Due to its small size, high copy number, and high rate of mutation, the mitochondrial genome facilitates clonal tracing on a much greater scale than does a nuclear genome and can be combined with scRNA-seq and other techniques that establish cell phenotypes. The method has been demonstrated and characterized on a number of human tissues, including in vitro expanded HSC and progenitor populations and T cells, and awaits its application to human native hematopoiesis. These and related approaches could take advantage of the natural clonal barcoding by genetic and epigenetic events on the timescale of years for the ultimate reconstruction of human hematopoietic hierarchy and homeostasis.

SUMMARY POINTS

1. Lineage tracing is a powerful tool to analyze native hematopoiesis in adult animals, including the function of endogenous hematopoietic stem cells (HSCs).
2. Endogenous HSCs actively contribute to and represent a major sustained source of lifelong hematopoiesis.
3. Adult HSCs continuously generate all blood cell lineages except embryo-derived immune cell types such as tissue macrophages.
4. Initial clonal analysis suggests that normal hematopoiesis is polyclonal and may be derived from lineage-biased clonal pools.
5. HSCs give rise to different blood cell lineages with different kinetics, with particularly fast and slow contributions to platelets and lymphocytes, respectively.
6. The HSC differentiation rate is tunable and can be modulated in conditions such as inflammation and aging.
7. Initial studies suggest that hematopoiesis in human adults is highly polyclonal and driven by HSCs.

FUTURE ISSUES

1. The existence and function of HSC subsets with different lineage potentials should be tested by specific prospective labeling and lineage tracing in native hosts.
2. Cell type–specific, highly diverse clonal labeling systems should be developed and used to dissect the clonal dynamics of endogenous adult HSC populations.
3. Clonal systems should also be applied to elucidate the timing and hierarchy of lineage commitment events during native hematopoiesis.
4. The short-term and long-term effects of perturbations such as inflammation and blood lineage depletion on endogenous HSC differentiation should be examined.
5. The subversion of HSC population dynamics and function during aging and age-associated clonal hematopoiesis should be modeled and studied.
6. Approaches for retracing the clonal dynamics of native hematopoiesis in humans should be developed and applied to both normal and pathological conditions.

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