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INVITED PERSPECTIVE

Lineage commitment of hematopoietic stem cells and progenitors: insights from recent single cell and lineage tracing technologies

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Blood production is essential to maintain human health, and even small perturbations in hematopoiesis can cause disease. Hematopoiesis has therefore been the focus of much research for many years. Experiments determining the lineage potentials of hematopoietic stem and progenitor cells (HSPCs) in vitro and after transplantation revealed a hierarchy of progenitor cell states, where differentiating cells undergo lineage commitment—a series of irreversible changes that progressively restrict their potential. New technologies have recently been developed that allow for a more detailed analysis of the molecular states and fates of differentiating HSPCs. Proteomic and lineage-tracing approaches, alongside single-cell transcriptomic analyses, have recently helped to reveal the biological complexity underlying lineage commitment during hematopoiesis. Recent insights from these new technologies were presented by Dr. Marjorie Brand and Dr. Allon Klein in the Summer 2019 ISEH Webinar, and are discussed in this Perspective.

The hematopoietic system plays a major role in human health and disease, supplying oxygen and nutrients to tissues, supporting healing, and fighting infections. These essential functions are carried out by the various types of mature hematopoietic cells, including red blood cells, platelets, myeloid immune cells (macrophages, neutrophils), and lymphocytes (T cells, B cells, natural killer [NK] cells). Most of the mature cells lack the ability to proliferate and have limited lifespans, so they must be constantly replenished by a process called hematopoiesis [1,2]. Disturbance in the homeostasis of this process results in hematological diseases such as leukemias, lymphomas, anemias, thrombocytopenias, and immunodeficiencies. Hematopoiesis has therefore been the focus of considerable experimental research for many years.

Hematopoiesis is sustained by rare hematopoietic stem cells (HSCs) that have two definitive characteristics [1,2]. HSCs can self-renew, dividing to produce new HSC daughter cells to maintain lifelong hematopoiesis. HSCs are also multipotent; that is, they have the ability to differentiate into any of the adult hematopoietic cell lineages. To produce mature blood cells, the progeny of HSCs undergo lineage commitment, a process of differentiation in which the potential to produce all hematopoietic cell types is progressively lost until they become restricted to forming one type of blood cell. Molecular mechanisms, cellular relationships, and timing of lineage commitment are fundamental to the regulation of blood production in homeostasis and in disease.

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Recently developed technologies including lineage tracing, single-cell transcriptomics, and proteomics have provided important new insights into lineage commitment during hematopoiesis. “Changing Concepts in Hematopoietic Lineage Commitment” was the focus of the Summer 2019 International Society for Experimental Hematology (ISEH) New Investigator Committee Webinar, presented by Dr. Marjorie Brand and Dr. Allon Klein and moderated by Dr. Stephen Loughran. Dr. Brand discussed recent progress in using proteomic approaches to trace lineage commitment while Dr. Klein described how novel lineage tracing methods in combination with single-cell transcriptomics were uncovering new cell fate trajectories. In this Perspective, we provide a brief summary of the classic view of hematopoiesis and a discussion of the topics covered by this recent webinar, which can also be viewed online (https://www.youtube.com/watch?v=RqUsYsXqFfA).

Hierarchical lineage commitment revealed by measuring the potential of isolated HSPCs

The differentiation potential of various hematopoietic stem and progenitor cells (HSPCs) was determined over many years of experimentation, using in vitro colony assays and transplantation of prospectively isolated cells into myelo-ablated mice [1,3]. This allowed HSPCs with varying potentials to be fitted into a cellular hierarchy with HSCs at the apex and mature blood cell types at the base. Hematopoiesis is therefore often depicted as a process of branching transitions between phenotypically identifiable cell states, with lineage commitment occurring during these transitions [4] (Figure 1A).

Studies in the early and late 2000s revealed further complexity, reporting considerable functional and molecular variability between cells with similar cell surface marker phenotypes, and alternative lineage commitment pathways. These included single-cell transplantation, label-retention assays, and molecular analyses, which identified tremendous heterogeneity within the HSC pool, including variability in long-term reconstitution capacity, lineage biases, cell cycle activity, and proliferative history of individual HSCs [5–15]. Moreover, paired-daughter transplant experiments revealed that a subpopulation of HSCs could produce one multipotent daughter cell, and one lineage-committed daughter, indicating that some multipotent stem cells can undergo lineage commitment within a single division, “bypassing” certain differentiation stages [16]. However, it is worth noting that lineage tracing using Flk2-Cre mice suggested that at steady state (non-transplantation settings), the majority of red blood cells and platelets derive from Flk2-expressing multipotent progenitor (MPP) precursors, rather than from the Flk2-negative HSC pool [17].

New insights from lineage-tracing and single-cell approaches

More recently, single-cell and lineage-tracing approaches have provided additional insights into HSC lineage commitment as has been extensively reviewed elsewhere [18–21]. In line with observations by Yamamoto et al. [22], recent single-cell transcriptomic and functional studies have identified a considerable fraction of the murine and human HSC compartments that exclusively adopts a lineage-restricted megakaryocyte fate in vivo [23–26]. It also appears that this fraction expands during aging in mice. Megakaryocyte-primed HSPCs are efficiently driven into maturation by inflammatory signals, resulting in enhanced platelet generation, and it has been suggested that these might serve as an emergency pool for rapid platelet generation in scenarios of high platelet demand, such as upon blood loss or during infection [23,27,28].

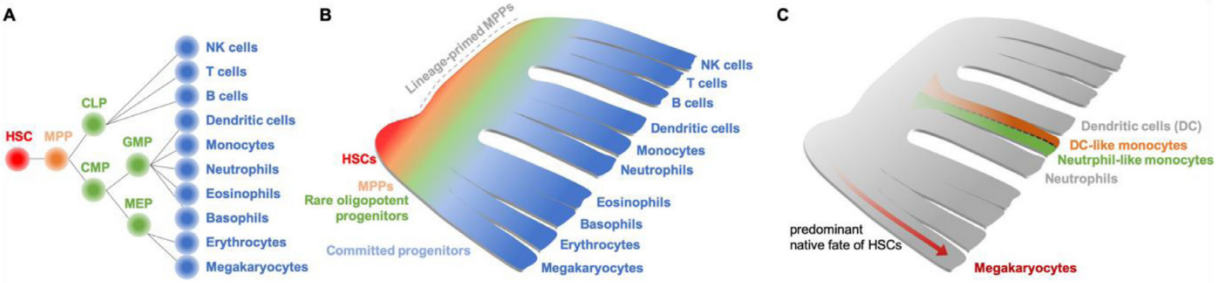

Figure 1. Models of hematopoietic stem cell lineage commitment. **(A)** The classic hematopoietic tree model, which suggested lineage commitment associated with branching transitions between cells with distinct lineage potentials. **(B)** Schematic of hematopoiesis based on recent findings discussed within this Perspective, which suggest that HSC differentiation occurs via a continuum with phenotypically defined progenitor populations representing heterogeneous lineage potential. **(C)** Lineage tracing in combination with single-cell analyses have suggested that (1) the predominant fate of HSCs in native hematopoiesis is megakaryopoiesis, and (2) there may be multiple lineage trajectories that generate equivalent mature hematopoietic cell types. For example, monocytes may be generated via dendritic cell-like or neutrophil-like trajectories.

It is worth noting that a recent study, performing quantitative analyses of the absolute production of mature cells in clonal in vivo assays, revealed a strong erythroid potential of HSCs and MPPs [29]. Additionally, at least two studies have now identified plasticity in the lineage restriction of certain self-renewing stem cell types; stem cells displaying platelet restriction in primary transplantation recipients were capable of additional myeloid and lymphoid fates when differentiated in vitro [30] or, in the case of aged-specific latent HSCs, serial transplantation [22]. Notably, this is different from the previously observed cell-autonomous lineage bias of HSCs and progenitors, by which cells are predisposed toward one lineage, but not fully committed to it [10,31].

Recent fate mapping studies of native hematopoiesis have also identified megakaryocyte-restricted output from HSCs [26]. While early native hematopoiesis fate mapping studies suggested that a large proportion of HSCs were not contributing to steady-state hematopoiesis, these studies did not measure megakaryocyte output [32–34]. In contrast, more recent in vivo lineage-tracing experiments that analyzed megakaryocytic output have found that a large proportion of HSCs produce only megakaryocytic cells, suggesting that this is a predominant native fate of many HSCs that were previously thought to be dormant [26]. One limitation of fate mapping studies is that it is not possible to distinguish a lineage-committed cell from a lineage-primed cell or from a fully multipotent cell that is located in a microenvironment that permits differentiation only into a single lineage.

In the last 4 years, large-scale single-cell transcriptomics of the mouse, human, and zebrafish HSPC compartments have provided detailed insights into the transcriptomic landscape of hematopoiesis [35–40]. These studies have suggested that at the mRNA level, hematopoiesis occurs as a continuum rather than by the acquisition of discrete transcriptional states (Figure 1B). With these methods, discrete transcriptional patterns were observed only at the level of mature cell types [35,36,38,39]. In trajectory analyses, early transcriptional lineage priming gradually separates erythroid–megakaryocyte–eosinophil–basophil-primed progenitors from lymphomyeloid-primed progenitors in mouse and human [37–40]. In later stages, lineage-specific gene expression programs are acquired, coinciding with functional lineage commitment [39]. The early separation between the erythroid–megakaryocyte–eosinophil–basophil and lymphomyeloid lineages is also supported by single-cell transcriptomic and single-cell functional assays of downstream progenitor compartments in mouse and human [41–43].

These single cell transcriptomic data sets, however, represent only snapshots of distinct stages of HSC commitment rather than a time-resolved picture of hematopoiesis. To overcome this limitation, new tools combining lineage-tracing and single-cell transcriptomics have recently been developed to address the question of how accurately single-cell RNA sequencing (scRNA-Seq)-inferred hierarchies reflect actual fate choices and to determine transcriptional states upstream of lineage commitment branchpoints [44]. A new method, developed by Klein and colleagues, to overlay lineage relationships from barcoding analysis with single-cell transcriptomics data is providing a powerful approach to interrogate how single HSCs (and their progeny) move through the continuous lineage differentiation models provided by single-cell transcriptomics [26,44]. First, these data reveal that hematopoietic differentiation is not a strict treelike branching process; instead, some cell types appear to reflect more than one possible sequence of molecular events, leading to "loops" as different branches of the tree converge (Figure 1C). This was most apparent for monocytes. Second, sister cell experiments reveal that cells with very similar gene expression profiles can nonetheless be pre-committed to different fates, suggesting that transcriptional circuits alone do not encode the potential of cells toward different fates. Combining CRISPR/Cas9 perturbation with large-scale single readouts [45–47] ex vivo and in vivo is also likely to provide novel insights into the molecular mechanisms driving HSC lineage commitment.

Quantification of lineage-specific transcription factors in single cells during lineage commitment

The studies described above focused on linking cellular differentiation with patterns of mRNA expression. These transcriptional changes are orchestrated by transcription factor proteins that regulate gene expression [48]. The control of myeloid progenitor fate choice by GATA1 and PU.1 (SPI1) is a paradigmatic example of transcription factor levels instructing lineage commitment [49]. GATA1 promotes expression of the erythroid/megakaryocyte program, and PU.1 promotes the granulocyte/monocyte program [50,51]. Furthermore, GATA1 and PU.1 each upregulates its own expression and inhibits the expression of the other transcription factor [52–54]. These properties led to a model in which low levels of GATA1 and PU.1 maintain myeloid progenitors in a metastable multipotential state until a developmental switch occurs when either GATA1 or PU.1 expression dominates, leading to the dominant transcription factor suppressing the expression of the other, while it forms an autoregulatory loop to further activate its own expression and drive lineage commitment [55,56]. This model is supported by the levels of *GATA1* and *PU.1* mRNA detected in populations of cells during differentiation and by studies in

which GATA1 or PU.1 overexpression is able to drive expression of erythroid/megakaryocyte or granulocyte/monocyte programs respectively in cells of the other lineage [57–60]. Other pairs of mutually antagonistic transcription factor switches that similarly control binary lineage commitment decisions during hematopoiesis have been identified, including KLF1 and FLI1, which promote erythroid and megakaryocyte commitment, respectively [49,61–63].

Recent studies measuring dynamic changes in transcription factor abundance at the protein level within single cells have challenged these binary fate models. For example, Hoppe et al. [64] quantified fluorescently tagged GATA1 and PU.1 in a large number of single differentiating HSCs and their progeny for several days. PU.1 was detected in all HSCs and uncommitted progenitors. GATA1 expression was not detected at any time during lineage commitment to the granulocyte/macrophage lineage. In granulocyte/macrophage differentiation events, PU.1 protein levels increased steadily during about half of the events, and in the other half, PU.1 levels dipped transiently before undergoing a similar steady increase. Cells in which GATA1 was detected, even at low levels, invariably continued to express GATA1 and differentiated into $GATA1^{+}PU.1^{-}$ megakaryocytic and/or erythroid cells. During the majority of these megakaryocyte/erythroid differentiation events, downregulation of PU.1 occurred before detection of GATA1. These findings are incompatible with an abrupt GATA1-versus-PU.1 binary switching event driving lineage commitment: uncommitted progenitors contained only PU.1, GATA1 detection was always associated with megakaryocyte/erythroid commitment, and changes in GATA1 and PU.1 protein levels during differentiation occurred gradually.

Another recent study by Brand and colleagues was the first to quantify the levels of endogenous lineage-specific transcription factors in single cells during hematopoietic differentiation. Mass cytometry time of flight (CyTOF) was used to simultaneously measure 11 cell surface proteins and 16 transcription factors in single human hematopoietic stem and progenitor cells and their progeny at 13 stages of in vitro erythroid development [65]. By use of this technique, KLF1 and FLI1 co-expression was detected in the majority of single megakaryocyte-erythroid progenitors (MEPs), and GATA1 and PU.1 co-expression, in the majority of common myeloid progenitors (CMPs). These data conflict with the lack of co-expression reported by Hoppe et al. [64] and highlight potential limitations in the ability of live imaging of fluorescent labels to detect proteins present at low levels. As MEPs underwent erythroid differentiation, KLF1 levels gradually increased, and FLI1 gradually decreased. Co-expression of KLF1 and FLI1 persisted for 14 days, until the pro-erythroblast stage of differentiation. This demonstrates that lineage commitment is not an abrupt transition from a metastable uncommitted state to one of two distinct lineage-committed states initiated by the rapid switching of a pair of cross-antagonistic, autoregulatory transcription factors. Instead, these findings are consistent with lineage commitment occurring during a continuous process of differentiation in which cells gradually transition along an ordered series of states. Changes in lineage-specific transcription factor levels during lineage commitment were gradual and continuous.

Under the same conditions, artificially increasing FLI1 protein levels in progenitors was sufficient to divert cells from their preferred erythroid trajectory to take on a megakaryocytic fate [65]. This highlights the ability of quantitative changes in transcription factor protein levels to determine cell fate decisions and strongly supports their being the main process that initiates lineage commitment, a finding also compatible with scRNA-Seq data. However, because it is difficult to precisely determine when a cell undergoes lineage commitment, it is difficult to distinguish which changes in transcription factor levels initiate lineage commitment and which are downstream events that reinforce a prior commitment decision. The granulocyte–macrophage versus erythroid–megakaryocyte lineage commitment decision time point, computationally inferred from the genealogy of single cell-tracked differentiating HSCs, occurred much earlier than changes in the level of fluorescently tagged PU.1 [66]. This suggests that PU.1 protein levels do not initiate this lineage commitment decision and indicates that further work is required to determine which transcription factors are responsible.

Conclusions

The development and application of new technologies to study hematopoiesis are creating a more complex but more complete understanding of hematopoietic lineage commitment. This hematopoietic lineage commitment landscape has now been mapped by different technologies in several distinct, but related, ways. One type of map depicts the lineage potential of HSPCs: What lineages can isolated cells differentiate into if placed in appropriate conditions? Another is a map of lineage fates: What lineages do HSPCs produce in situ? The third maps the molecular state of individual HSPCs in particular tissues at snapshots in time. Recent single-cell analyses of hematopoiesis at both the mRNA and protein levels have revealed a continuum of states in hematopoiesis, suggesting that the molecular changes that underlie lineage commitment occur on a time scale comparable to the lifetime of mRNA molecules—hours or days. However, little is known about how rapidly individual cells move

between these molecular states during steady-state hematopoiesis, and it is not yet possible to accurately predict the lineage potential of a single cell from its transcriptome. To better understand the regulation of lineage commitment, links between the three types of map must be discovered, associating the molecular profile of individual cells to their potential and to their most likely fate in the bone marrow. Further characterization of transcription factor protein levels and genomic binding in single cells across different stages of differentiation and new methods combining cell fate tracking and single-cell transcriptomics will provide new information to help address this and allow the development of more accurate models of the molecular regulation of hematopoietic lineage commitment. These findings have important implications for how we interpret the perturbations in hematopoiesis that underlie hematological diseases, as well as our efforts to develop new therapies for these diseases.

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