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Tracking the origin, development, and differentiation of hematopoietic stem cells

Priyanka R Dharampuriya[1,2], Giorgia Scapin[1,2,3], Colline Wong[1,2,5], K John Wagner[1,2,5], Jennifer L Cillis[1,2,3] and Dhvanit I Shah[1,2,3,4]

Purpose of review

The hierarchical nature of the hematopoietic system provides an ideal model system to illustrate the features of lineage tracing. We have outlined the utility of lineage tracing methods in establishing the origin and development of hematopoietic cells.

Recent findings

Methods such as CRISPR/Cas9, Polylox barcoding, and single-cell RNA-sequencing have improved our understanding of hematopoiesis.

Summary

This review chronicles the fate of the hematopoietic cells emerging from the mesoderm that subsequently develops into the adult blood lineages. Specifically, we explain classic techniques utilized in lineage tracing for the hematopoietic system, as well as novel state-of-the-art methods to elucidate clonal hematopoiesis and cell fate mapping at a single-cell level.

Addresses
[1] Division of Hematology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA
[2] Harvard Stem Cell Institute, Cambridge, MA 02138, USA
[3] Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA 02138, USA
[4] Dana-Farber/Harvard Cancer Center, Boston, MA 02115, USA
[5] Boston College, Chestnut Hill, MA 02467, USA

Corresponding author: Shah, Dhvanit I (dshah@research.bwh.harvard.edu, dhvanitshah@gmail.com)

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Introduction

Hematopoiesis, or the process of blood formation, occurs in two waves. The primitive wave creates red blood cells and myeloid cells [1]. The definitive wave begins with the formation of hematopoietic stem cells (HSC), followed by their development and differentiation into four adult blood lineages: erythroid, myeloid, platelet, and lymphoid cells [1,2]. Given the hierarchal nature of hematopoiesis, it is an ideal process for establishing a foundation and then testing state-of-the-art methods in cell fate mapping.

Lineage tracing, a process of marking a single cell and following its progeny [3], establishes the origin, development, and differentiation of a cell by elucidating the identity, number, and location of its descendants [3]. While cell transplantation, time-lapse confocal imaging, parabiosis, and genetic manipulation have established the hierarchical nature of blood formation, the development of quantitative processes and sequencing methods have recently uncovered the clonal nature of hematopoiesis.

Here, we illustrate how past and current advances in lineage tracing have uncovered the hierarchical and clonal nature of hematopoiesis (Table 1).

Lineage tracing in hematopoiesis

Primitive and definitive hematopoiesis

The primitive wave of blood production in the yolk sac produces red blood cells and macrophages [1]. This primitive wave is rapidly replaced by adult-type definitive hematopoiesis in the aorta-gonad-mesonephros (AGM) region [2]. Here, a sheet of lateral mesoderm migrates medially, touches the endoderm, and forms a single aorta tube [4]. Later, the clusters of hematopoietic stem cells emerge in the ventral wall of the dorsal aorta or the AGM region. Next, hematopoietic cells, which are capable of long-term reconstitution, colonize the fetal liver, thymus, spleen, and ultimately the bone marrow [5].

Extraembryonic hematopoiesis

While the first hematopoietic progenitors develop at embryonic day (E)7.0 in the yolk sac [6], the hematopoietic activity also appears in umbilical arteries and the allantois [7], umbilical veins lack hematopoietic potential. This suggests that HSCs arise predominantly during arterial specification. Although HSCs appear in the mouse

placenta [8,9], it is unclear whether placental HSCs arise *de novo* or through colonization upon circulation, or both.

Mesoderm to hemangioblast

In the mouse embryo, the process of gastrulation creates the mesoderm at ~E6.5 [10]. Here, the epiblast cells from the posterior part of the embryo form a transient primitive streak from which the mesoderm emerges. Next, the mesoderm migrates away from the primitive streak, moves laterally and anteriorly, and is patterned into various populations with distinct developmental fates. Early mesodermal cells from the posterior primitive streak are the first source of blood islands in the embryo [11].

Brachyury, a transcription factor within the T-box complex of genes, marks all nascent mesodermal cells. As Brachyury^{+} cells undergo patterning and specification into the skeletal muscle, cardiac muscle, connective tissues, blood, and endothelium, its expression then diminishes [12–14].

The hemangioblast, a common precursor for endothelial and hematopoietic cells, is proposed as a site of hematopoiesis based on observations of chick blastoderms cultured *in toto* on coverslips [12] and explant cultures of the caudal region of blastoderms during the gastrulation stage. In both instances, hemangioblasts, which are aggregates of morphologically identical cells, produced endothelial cells and hematopoietic cells [12]. However, fate mapping and chimera studies have failed to provide substantial evidence indicating that there was a common origin for endothelial and hematopoietic cells located in the early mouse yolk sac.

To determine the hematopoietic potential of individual cells in the mouse epiblast, primitive streak, and early yolk sac, Padrón-Barthe *et al.* [13] used an *in vivo* clonal analysis to demonstrate: (I) Early yolk sac blood and endothelial lineages derive from independent epiblast populations, specified before gastrulation. (II) A subpopulation of the yolk sac endothelium has hemogenic activity similar to that found later in the embryonic hemogenic endothelium (HE). (III) HE appears in the yolk sac and produces hematopoietic precursors with markers related to definitive hematopoiesis.

Hemangioblast to hemogenic endothelium

As hemangioblasts and HE develop near each other within the embryo, one hypothesis is that the hemangioblast generates HSCs through the formation of an HE intermediate [14]. This is further supported by observations from single-cell-derived colonies that can produce both hematopoietic and endothelial cells *in vitro*. Utilizing the single-cell-resolution fate maps of the zebrafish late blastula and gastrula, Vogeli and colleagues [15] demonstrated that bipotential progenitors, which can give rise to both hematopoietic and endothelial cells, emerge along the entire lining of the ventral mesoderm. Their results provide *in vivo* evidence to support the existence of the hemangioblast.

Based on ES cell differentiation studies, the hemangioblast to hemogenic endothelium transition occurs in two stages. First, from 36 to 48 hours post-culture, a tight adherent structure arises from the hemangioblast. This is followed by the appearance of non-adherent round cells that proliferate to generate a mature blast colony then. However, it is unknown which mechanisms, if any, regulate or support the emergence of HE from hemangioblasts [14].

A morphological examination demonstrated that the AGM forms HE cells in the ventral wall of the aorta, instead of hemangioblasts that bud off to become HSCs. The program of HE cell development is regulated differently from that of presumptive hemangioblasts, as *Runx1* is critical for HSC formation from the hemogenic endothelium but not HSC formation from yolk sac hemangioblasts [16,17].

Hemogenic endothelium to erythroid/myeloid progenitors (EMP) or hematopoietic stem cells

The AGM is the first site of mammalian intra-embryonic hematopoiesis [18,19]. During E10.5-11.5, hematopoietic cells attached to the aorta bud off from this region [20]. Imaging and lineage tracing studies in zebrafish embryos have established that the first HSCs emerge directly from hemogenic endothelium lining the ventral wall of the dorsal aorta (DA) [21,22••]. These results complement previous studies in the avian, amphibian [21], and mammalian embryo [23,24], which suggests that the cellular mechanisms of HSC generation are conserved across vertebrates.

Zovein *et al.* established that HSCs were generated from Cadherin 5 (Cdh5) precursors, suggesting that HSCs arise from the endothelium, and that AGM-derived endothelial cells contain the majority of HSC potential. The conditional deletion of *Runx1* in Cdh5^{+} cells led to the loss of HSCs, showing that Runx1 is crucial in the transition from endothelium to HSC [25]. However, Anderson *et al.* recently used state-of-the-art parabiosis and mouse chimera studies to establish that Cdh5 is dispensable for the formation, development, and differentiation of HSCs [22••].

Before HSCs appear, committed erythroid/myeloid progenitors (EMPs) emerge from the yolk sac and the HE. The HE is located at sites of EMP and HSC emergence, such as the dorsal aorta, vitelline and umbilical arteries, yolk sac, and placenta. The HE is differentiated from all other endothelial cells by the presence of Runx1 [16]. Runx1 is expressed in hemogenic endothelial cells before the formation of clusters, in the clusters themselves, and in all functional EMPs and HSCs [16,17]. Embryos lacking Runx1 have no EMPs, HSCs, or intra-arterial clusters [16,17,26]. The utility of core binding factor β (CBFβ) for EMP and HSC formation is temporally and spatially distinct, and *Ly6a* explicitly marks the HSC-generating hemogenic endothelium [25].

HSC development and differentiation

HSCs differentiate into hematopoietic stem-progenitor cells (HSPC), which differentiate into multipotent progenitors (MPP). These multipotent progenitors lose their self-renewal capacity, but can still differentiate into all four adult hematopoietic lineages. The ability for HSCs to differentiate into progenitor cells, while also maintaining an adequate pool of HSCs via balancing self-renewal and differentiation, is essential for maintaining the short lifespan of blood cells [27].

HSCs were purified from mouse bone marrow using cell surface marker Thy-1^{low} Lin (Lineage-markers)$^-$ Sca-1^+ using multi-color fluorescence-activated cell sorting (FACS) and monoclonal antibodies [28,29], with the representation of about 0.05% of C57BL/Ka-Thy-1.1 mouse bone marrow. Then, Morrison *et al.* showed that the Lin$^-$ population of cells included at least three multipotent populations: long-term HSCs, short-term HSCs, and multipotent progenitors [30]. Subsequently, Boyer *et al.* established a lineage tracing mouse model that allowed for direct assessment of HSC differentiation pathways *in vivo* [31].

Using clonogenic B and T cell assays and *in vitro* erythroid potential assays, Lai and colleagues showed that different subsets of MPPs give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). They demonstrated that lymphoid-committed CLPs do not emerge from the same MPP that gives rise to CMPs [32]. Using *in vivo* differentiation, Adolfsson *et al.* have also shown that MPPs lose myeloid lineage differentiation potential during lymphoid lineage differentiation [33]. Injection of lin$^-$CD44$^+$CD25$^-$Sca-1$^+$CD117c-kit$^+$ (LSK), Flt3+ cells into lethally irradiated mice, demonstrated that LSK Flt3$^+$ cells loose MegE differentiation potential before lymphoid lineage commitment, followed by a loss of GM differentiation potential [32].

Common lymphoid progenitors have two CLP subsets: CLP-1 (lin$^-$Sca-1$^+$CD117$^{+/lo}$CD127$^+$CD135$^+$) and CLP-2 (lin$^-$Sca-1$^+$CD117$^-$CD127$^+$CD135$^+$B220$^+$). Clonogenic assays demonstrated that CLP-2 are the most differentiated population with T cell potential before B cell commitment [34]. These CLPs develop into immature early T-lineage progenitors (ETP), characterized as LSK with only low CD127 (IL-7Rα) expression, high T potential, and limited B potential. Using *in vitro* GFP tracing, Pui *et al.* showed that T lymphopoiesis is under the control of Notch signaling. Such ETPs then develop into immature T cells, which migrate to the thymus, where they mature [35].

Using flow cytometry, Loder *et al.* marked each step of B cell development from the CLP by unique gene expression patterns, as well as immunoglobulin H chain and L chain gene loci rearrangements. The rearrangements are in part due to the B cells undergoing V(D)J recombination [36]. Bone marrow-derived immature B cells then migrate to the spleen. Using flow cytometry, Allman *et al.* showed that immature B cells pass through T1 and T2 transitional stages and finally differentiate to a T3 stage [37].

Using single-cell RT-PCR analysis, Hu *et al.* observed that both erythroid and myeloid gene expression programs are initiated by the same progenitor cells (MEPs) before exclusive commitment toward the myeloid or erythroid lineages [38]. Although many of CD34$^+$lin$^-$ primary bone marrow cells shared a specific phenotype, their gene expression varied, as ~50% of the cells expressed mRNA for both β-globin as seen with RBC differentiation and myeloperoxidase as seen with myeloid differentiation.

Recent advances in lineage tracing in hematopoiesis

Here we illustrate state-of-the-art methods in current use for following HSC origin and development (Table 1).

Table 1

Lineage tracing methods in the study of hematopoiesis

Approach	Utility in hematopoietic development
Mass cytometry (CyTOF)	Allows for simultaneous identification of multiple cell types from heterogeneous sample of blood
Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)	Analyzes protein contents in samples and elucidates glycosylation patterns
Single-cell RNA sequencing	Investigates cell-to-cell variation at transcription level
CRISPR/Cas9-based genome editing	Used to knock out hematopoietic genes and the β-globin gene in human HSCs and allows for multiple genetic modifications
Polylox barcoding	To study origin and clonal composition of HSCs
Multiplexed fluorescent labeling and sequencing	The single-cell transplant data are coupled with single-cell gene expression analysis on different cells to resolve subpopulations with corresponding gene expression and repopulation potential
Tissue engineering & 3D scaffold development	Recreates 3D microenvironment conducive for HSC formation, expansion, development, and differentiation.

CyTOF and MALDI-TOF

While flow cytometry remains vital in HSC research, the technology to identify and quantify cells on a single-cell basis is continually improving.

CyTOF, or mass cytometry, is a quantitative alternative to flow cytometry. Instead of labeling antibodies with fluorochromes, CyTOF uses antibodies labeled with heavy metal ion tags, which are then analyzed by a mass spectroscopic readout [39]. This technique allows for the simultaneous identification of multiple cell types from the same heterogeneous samples of blood.

To examine the interplay between normal human hematopoietic and immunological signaling in the human bone marrow, Bendall *et al.* recently used CyTOF to measure 34 cellular parameters simultaneously at a single-cell level. To analyze intracellular pathways as well as lineage-specific consequences of telomere erosion and the restoration of telomere length in rare HSPC populations, Raval *et al.* simultaneously measured 19 surface markers and 13 intracellular markers [40]. To analyze how growth factors regulate human HSCs, Knapp *et al.* recently measured 43 different surface markers, transcription factors, active signaling molecules, viability, and cell-cycle signals in individual $CD34^+$ cord blood-derived cells [41•].

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) has now been adapted to analyze protein contents in samples [42]. Fuchs *et al.* used matrix-assisted laser desorption and ionization time-of-flight mass spectrometry under standard, nutrient-rich culture conditions, followed by low oxygen and low glucose concentrations to monitor changes in the composition and saturation degree of choline phospholipids of hematopoietic progenitor cells [43].

MALDI-TOF is used to elucidate glycosylation patterns, as well as to determine variations of different proteins. While Reinke *et al.* analyzed N-glycans of membrane proteins of hematopoietic cell lines to assess the various glycosylation patterns [44], Liu *et al.* identified the proteomic changes in myeloid dendritic cells in cases of severe aplastic anemia [45].

Single-cell RNA sequencing

Single-cell RNA sequencing (scRNA-seq) is a robust method to investigate cell-to-cell variation at the transcriptome level in hematopoiesis [46]. Using scRNA-seq, cell purification and functional clonal assays have provided a revised model of hematopoietic development, especially for the T, B, and NK lineages.

Recent single cell studies suggest that oligopotent progenitors contain only a small portion of the hematopoietic hierarchy. Instead, multipotent cells differentiate into unipotent cells of the myeloid (My)-erythroid (Er)-megakaryocyte (Mk) lineages, which suggests two tiers of the human blood hierarchy: one with multipotent cells, and another with cells committed to My, Er, or Mk lineages. However, Mk branching differs in fetal liver and bone marrow. While in the fetal liver, Mk progenitors are enriched, but not restricted, to the stem cell compartment. However, in the bone marrow, Mk fate is coupled to multipotent cells. This result corraborates the two-tier model of adult hematopoiesis, where branching of Mk occurs at the level of HSC/MPPs.

To examine the molecular basis for the two-tier hierarchy, Dick *et al.* have performed low cell-input RNA sequencing, enhanced reduced representation bisulfite sequencing, and ATAC-seq to provide a comprehensive transcriptional and epigenetic roadmap of human HSPCs across development. Additionally, Nestorowa *et al.* used single-cell RNA sequencing to profile more than 1600 single HSPCs to reconstruct differentiation trajectories and dynamic expression changes associated with early lymphoid, erythroid, and granulocyte-macrophage differentiation [47].

CRISPR/Cas9-based genome editing

The CRISPR/Cas9-based, genome editing system, targets specific sequences in the genome for the generation of mouse lines with point mutations, deletions, conditional deletions, or reporter expression [48]. It has extensively been used to knockout hematopoietic genes and the β-globin gene in human HSCs [49••]. Since the CRISPR/Cas9 system can be multiplexed for manipulations of multiple genes at once [50], it allows for multiple genetic modifications in a single mouse line for the investigation of multiple hematopoietic genes within the same organism [51].

Polylox barcoding

Polylox barcoding is a recombinase-based approach that enables fate mapping in the hematopoietic system under physiological conditions [52••]. The Polylox locus consists of an array of unique DNA sequences interspersed with loxP sites. Barcodes get created *in vivo* through Cre-dependent recombination [52••].

The Polylox system shares some features with the CRISPR/Cas9-based lineage-tracing approaches, allowing the barcode generation *in vivo* [52••]. However, in the Polylox system, each of the individual DNA blocks is about 170 base pairs long, and it is necessary to sequence across the whole locus to obtain the full barcode information [52••]. Therefore, CRISPR–Cas9-based systems where barcodes can differ by just single nucleotides may be more prone to PCR and sequencing artifacts than the Polylox system.

To study the origin and clonal composition of HSCs in the adult bone marrow after embryonic barcode induction,

Pei *et al.* recently treated embryos containing the Polylox locus at E9.5 by administering the mother a single dose of tamoxifen. They then could determine the barcodes of sorted single HSCs [52••]. Such high-resolution fate mapping by Polylox barcoding of embryonic HSC progenitors and adult HSCs supports a bifurcating tree model of hematopoiesis, which was proposed in the 1980s [53] but has not yet been tested under physiological conditions.

Multiplexed fluorescent labeling and sequencing

Heterogeneity among cells within tissues is recognized in both normal and malignant blood development [46,54–56]. The hematopoietic system contains populations of cells with divergent properties and distinctive behaviors, such as cell production and lineage bias [57,58]. HSCs exhibit a bias toward myeloid, lymphoid, or megakaryocytic lineage upon transplantation of single cells [57,59,60] with *ex vivo* barcoding, transplantation of populations of cells [55,61–65,66••,67], or by retrotransposon tagging of endogenous cells [68].

The single-cell transplant data are coupled with single-cell gene expression analysis on different cells to resolve subpopulations with corresponding gene expression and repopulation potential [69•]. Overlaying *in vivo* functional behavior of endogenous HSC clones with their gene expression and epigenetic characteristics has coupled the function of gene expression and chromatin state at the clonal resolution and established the cell-autonomous epigenetic constraints bound the HSC function.

Tissue engineering and 3D scaffold development

The fate and development of HSCs is dependent on their tissue microenvironment during fetal and adult development, such as AGM, fetal liver, and bone marrow regulates HSC formation, expansion, and maintenance. Therefore, it is prudent to recreate a 3D microenvironment using biomaterials-based 3D bioprinted scaffold.

Wagner *et al.* demonstrated that the self-renewal capacity of HSCs was higher in 2D co-culture with bone-marrow-derived adherent cells [70]. Then, Taqvi *et al.* and Ferreira *et al.* used biocompatible materials to culture umbilical cord blood-derived $CD34^+$ HSPCs on constructs with a variety of pore size and topology [71,72]. Recently, microfluidics-based organ-on-a-chip approach has been utilized to investigate the roles on wall shear stress of hematopoietic development [73].

To accurately mimic tissue architecture and components, we are analyzing the composition of scaffolding proteins, matrix-bound soluble factors, 3D structure, topography, nanoroughness, stiffness, and biophysical properties of hematopoietic tissue microenvironment. Given the recent advances in tissue engineering and 3D bioprinting, we are hopeful that 3D bioprinted AGM, fetal liver, and bone marrow will be developed for HSC formation, expansion, and development.

Conclusion

Lineage tracing has established a family tree of hematopoiesis from the mesoderm to adult hematopoietic lineages. While recent advances in quantitative approaches to cell fate mapping have found clonality of HSCs, it is unclear when and where HSCs become lineage-biased during their development. We have illustrated how classical and state-of-the-art methods have resolved the hierarchy in hematopoiesis.

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