Specification of hematopoietic stem cells in mammalian embryos: a rare or frequent event?

Miguel Ganuza,[1] Wilson Clements,[2] and Shannon McKinney-Freeman[2]

[1]Centre for Haemato-Oncology, Barts Cancer Institute, Queen Mary University of London, London, United Kingdom; and [2]Department of Hematology, St Jude Children's Research Hospital, Memphis, TN

Hematopoietic stem cells (HSCs) are the blood-forming stem cells thought to be responsible for supporting the blood system throughout life. Transplantability has long been the flagship assay used to define and characterize HSCs throughout ontogeny. However, it has recently become clear that many cells emerge during ontogeny that lack transplantability yet nevertheless are fated to ultimately contribute to the adult HSC pool. Here, we explore recent advances in understanding the numbers and kinetics of cells that emerge during development to support lifelong hematopoiesis; these advances are made possible by new technologies allowing interrogation of lifelong blood potential without embryo perturbation or transplantation. Illuminating the dynamics of these cells during normal development informs efforts to better understand the origins of hematologic disease and engineer HSCs from differentiating pluripotent stem cells.

Introduction

Hematopoietic stem cells (HSCs) are the blood-forming stem cells thought to be responsible for maintaining and replenishing blood throughout life and during stress.[1,2] As the first stem cell population identified, HSCs serve as a paradigm for stem cell biology.[1,2] In adult vertebrates, they are found in the bone marrow (mammals) or kidney marrow (fish).[3-5] Classically, they have been most rigorously defined by their functional ability to reconstitute all major blood lineages for long periods of time when transplanted. Transplantability has been exploited as a functional readout to identify their unique molecular and other phenotypic markers. These markers have been used in turn to define their unique transcriptional profiles, interactions with the bone marrow (BM) niche, and functional potential and assess how perturbations to homeostasis (eg, infection, disease, and genetic manipulation) influence numbers and function. Limiting dilution transplantation and imaging of mammalian whole BM have established that transplantable HSCs are extremely rare in adults, although variable with age.[3,6-11] However, if we think of HSCs as the cells that maintain blood for life, a reasonable question is whether transplantability is the best approach to interrogate the size of the entire HSC pool, especially given the many stress hurdles that HSCs must overcome to effectively repopulate and reconstitute the blood of ablated recipients (eg, homing, normoxia, encountering a niche damaged by ablation, and ex vivo manipulation). HSCs capable of durable self-renewal,[12-16] as evidenced by repeated serial transplantation, are experimentally subjected to repeated external stressors and regenerative insult. Indeed, serial transplantation dramatically reduces the clonal complexity of reconstituted marrow.[17] Thus, durably self-renewing HSCs represent rare cells that can survive repeated exposure to intense stress and might not equate perfectly to physiologic HSCs that quietly support blood homeostasis in an unperturbed setting throughout life.

Here, we explore recent advances in understanding the number and dynamics of cells that emerge during development to support lifelong hematopoiesis. Many of these advances are made possible by new technologies and approaches that allow interrogation of lifelong blood potential without embryo perturbation or transplantation. Illuminating the dynamics of these cells during normal development informs efforts to better understand the origins of hematologic disease and engineer HSCs from differentiating pluripotent stem cells.

Historical perspective on transplantability as the gold standard of HSC activity

The history of how HSCs were conceived, identified, and characterized helps to explain the original estimated numbers of clonal specification events and how those numbers expand over the course of ontogeny. At the end of the 1800s, a stem cell foundation for blood had been proposed,[18] and in 1916, Danchakoff[19] articulated a reasonably sophisticated model of stem cells and maturation supporting diverse lineage output and malignancy. Studies in the 1960s by McCulloch and Till and associates[20-24] established assays with quantifiable output, such as splenic colony formation to define clonality and lineage output, which were complemented by in vitro assays pioneered by Bradley and Metcalf[25] and expanded over the years to test hematopoietic potential.[26] In vitro assays, however, were not able to determine self-renewal potential, and ultimately, the gold standard of HSC

potential became the ability to completely reconstitute for a long period of time (months or longer) the blood lineages of an animal lethally irradiated to ablate its hematopoietic system (so-called long-term reconstitution [LTR]) and more stringently to serially reconstitute through multiple subsequent transplantations.[27] Using these assays, multiple groups phenotypically defined the cell populations containing HSCs and other lineage maturation intermediates (eg, size and density, adhesive properties, and cell-surface molecular markers).[28] Refined molecular phenotypes were iteratively developed that highly enriched for both murine (ie, a cocktail of antibodies to eliminate markers of lineage differentiation [Lin−] in combination with Sca-1, c-Kit, CD150, and EPCR and the absence of CD48) and human HSCs (ie, CD34+CD38−CD90+CD45A−CD49f+).[9,10,28-39]

Developmental ontogeny of HSCs

Classical embryology studies implicated endothelial cells as the immediate precursors of the earliest blood cells in vertebrate species, including pig, rabbit, guinea pig, cat, mouse, chick, and human,[18,40-44] and hemogenic endothelium was confirmed in the modern era using techniques including dye-based and genetic in vivo lineage-tracing studies,[45,46] in vitro assays,[47,48] functional genetic studies,[49] and direct imaging in live zebrafish and mouse embryos.[50-52] However, precisely where this hemogenic endothelium was located has been heavily investigated and disputed. The earliest emergence of blood is observed in extraembryonic tissues (the mammalian yolk sac and chick area vasculosa[18,40,43,53-55]), and early studies seemed to provide evidence of diverse lineage potential and LTR by progenitors from that region.[56-59] However, in the 1970s, Dieterlen-Lièvre and associates[60,61] showed, using avian embryos, that all long-term hematopoietic activity is derived intraembryonically from mesodermal tissue containing the dorsal aorta, whereas yolk sac–derived lineages disappeared in adulthood, and this observation was confirmed in amphibian embryos.[62-65] In the 1990s, it was established in mouse that an intraembryonic trunk region termed the aorta-gonado-mesonephros (AGM), containing the primitive dorsal aorta, is a potent source of colony-forming units and HSCs with LTR activity between ∼9 days postcoitum (E9.0) and E10.5.[66-72] These studies raised the possibility that definitive HSC potential observed in the yolk sac might derive from circulating progenitors that originate in the AGM, because circulation initiates at ∼E8.0.[73]

A clear objective was to identify and geographically locate the putative source of intraembryonic HSCs, but it was not clear that the collection of phenotypic markers known to identify adult HSCs would be expressed in developing HSCs. Markers such as c-Kit, Sca-1, CD31, and CDH5[74-76] and genes newly discovered to be functionally important in the specification of HSCs (eg, Runx1[77-79]) were found to label a population in the dorsal aorta of vertebrates with HSC potential. We now know that newly emerging HSCs and hematopoietic progenitors form clusters of c-Kit+ cells in the dorsal aorta (intra-aortic cell clusters) in most vertebrates, often localized near the midcaudal region of the aorta close to the junction with the vitelline artery (VA).[3,66,80,81] Limited numbers of c-Kit+ cells are seen in the aorta at E9.5 (∼0-5 cells).[80] In contrast, large numbers are evident by E10.5 (∼609 ± 84 c-Kit+ cells),[80] with some forming clusters of >10 cells (∼7 large clusters visible at E10.5). c-Kit+ cell numbers begin declining by E11.5 (439 ± 87 c-Kit+ cells; ∼1 large

cluster)[80] as newly specified HSCs and progenitors move on to the fetal liver (FL). By E14.5, only 30 to 40 single c-Kit+ cells are detected in the aortic endothelium.[80] Similarly, the numbers of c-Kit+ clusters and large c-Kit+ clusters localized to the VA and umbilical artery (UA) peak at E10.5 (250-300 c-Kit+ per vessel at E10.5 and 100-200 at E11.5; 5 large clusters at E10.5 and 1 at E11.5 per vessel). Interestingly, the largest c-Kit+ clusters are found in the VA (76 c-Kit+ cells per cluster) and UA (48 c-Kit+ cells per cluster) rather than in the aorta proper (19 c-Kit+ cells per cluster).[80] As investigators sought to better define the phenotype and function of nascent mammalian HSCs, they turned to the functional definition that had been established for adult HSCs: LTR potential in adults or neonates undergoing transplantation.[8,68,82-85]

Original estimates of the numbers of newly emerging HSCs during mammalian development were based on the transplantation of freshly isolated murine embryonic tissues.[7,8,82,86] When these transplantations are performed at limiting dilution, the number of transplantable HSCs (also known as hematopoietic repopulating units [RUs]) based on Poisson statistics can be estimated.[3,7,8,50,68,70,82,84-89] Via this approach, only 1 to 2 RUs are detected in E10.5 to E11.5 murine embryos.[8,68,70] Thus, nascent HSCs as defined by transplantation are rare at midgestation in the developing mammalian embryo.[90,91] Although these elegant studies shed light on the embryonic origin of HSCs and their migration to successive anatomic locations (AGM, FL, placenta, and BM), transplantation provides only an instantaneous snapshot of the number of RUs present at a particular time and place.[6,92] Moreover, nascent HSCs are not programmed to migrate to or engage with the adult BM niche, as would be required in most transplantation studies. Indeed, one can detect many more RUs when AGM-derived cells are transplanted at limiting dilution into neonates compared with adults.[93] Thus, transplantation-based estimates of nascent HSC numbers overlook cells too immature to repopulate the hematopoietic system of an ablated recipient, but that would ultimately realize their potential to contribute to lifelong hematopoiesis if left developmentally unperturbed. These observations explain why transplantation-based estimates of nascent HSC potential underestimate the actual number of physiologically specified HSCs. The limitations of transplantation-based techniques set the stage for new approaches to estimate clonal specification of HSCs: ex vivo reaggregate culture systems and fate mapping in unmanipulated embryos.

Specification is more frequent than previously thought: new insights from novel approaches

Beginning in the early 2000s, the Medvinsky laboratory[94-97] pioneered a new culture system that allowed for functional detection of immature HSC precursors with LTR potential. Dissected tissues from E8.5 to E11.5 murine embryos were kept intact or dissociated and then reaggregated in the presence or absence of supportive OP9 stromal cells and subsequently cultured as explants at the air-liquid interface in the presence of cytokines and serum.[71,94-98] These studies show that both expansion of existing nascent HSCs and de novo HSC specification are ongoing in cultured embryo explants.[92,97] This system has been used to investigate the hierarchy of HSC precursors between E9.5

and E11.5 by assessing prospectively isolated candidate populations for HSC potential after culture in reaggregated explants.[94,96,97] Combined with subsequent limiting dilution transplants, 1 to 2 E9.5, ~50 E10.5, and ~70 E11.5 immature HSC precursors are detectable in the AGM and major arteries,[95] revealing far greater numbers of cells that ultimately develop transplantable HSC potential than estimated by previous approaches.[7,8,82,86] Moreover, these studies reveal that the number of HSC precursors at E11.5 in the AGM matches the functional HSC number in the E12.5 FL, suggesting that the early FL HSC pool derives from a mixture of maturing and transplantable HSC precursors rather than from extensive division of rare previously specified HSCs.[95] Thus, the E12.5 FL may comprise a niche supportive of HSC maturation, although this model requires further exploration.[6]

Although reaggregated explant cultures have proven extremely useful to uncover and define HSC precursor phenotypes at multiple stages of mammalian ontogeny, they probably do not fully recapitulate the in vivo specification and maturation environment (eg, ex vivo cultures involve supraphysiologic cytokine concentrations). Nonphysiologic RUs may emerge in cultures, and the potential of all legitimate HSC precursors may not be realized.[6,71,94-98] Moreover, transplantation of fresh or cultured embryonic tissues reveals only the functional potential of a given population rather than its actual in vivo fate (ie, contribution to lifelong blood production; Figure 1). Therefore, estimated

numbers of HSCs and HSC precursors that derive from these studies may not reflect in vivo reality. Indeed, the numerous c-Kit[+] intra-aortic cells observed between E10.5 and E11.5 suggest that endothelial-to-hematopoietic transition (EHT) is not a rare event[80] (Figure 1). Determining exactly how many cells in the aorta and UA and VA actually contribute to the adult HSC pool requires noninvasive lineage tracing.

Recent novel noninvasive lineage-tracing approaches allow for tracking and quantification of numbers of hematopoietic precursors with lifelong hematopoietic potential in an unperturbed setting. For example, some fluorescence-labeling methods used to study the clonal origins of blood are based on the *Brainbow* allele, which was initially developed to study neuronal circuitry.[99] The *Brainbow* allele allows inducible random expression of combinations of multiple copies of 4 different fluorescent proteins (CFP, GFP, RFP, and YFP) and thereby produces ~100 detectable colors in neurons.[99] The system was adapted for use in zebrafish as a CRE-inducible *zebrabow* allele (which harbors ~20 transgene insertions and produces 40 distinguishable fluorescent colors).[11] Tamoxifen-inducible *draculin* (*drl:creERT2*; active during early hematopoiesis) in combination with the *zebrabow* allele revealed that zebrafish embryos harbor ~30 HSC precursors (21 pre-HSCs before HSC emergence at 24 hours postfertilization [hpf] and 34 HSCs at 72 hpf).[11] In agreement with this estimate, *hsp70l* LASER-induced labeling of individual HSC precursors in Tg(*cd41:eGFP*; *bactin2:switch*; *hsp70l*;
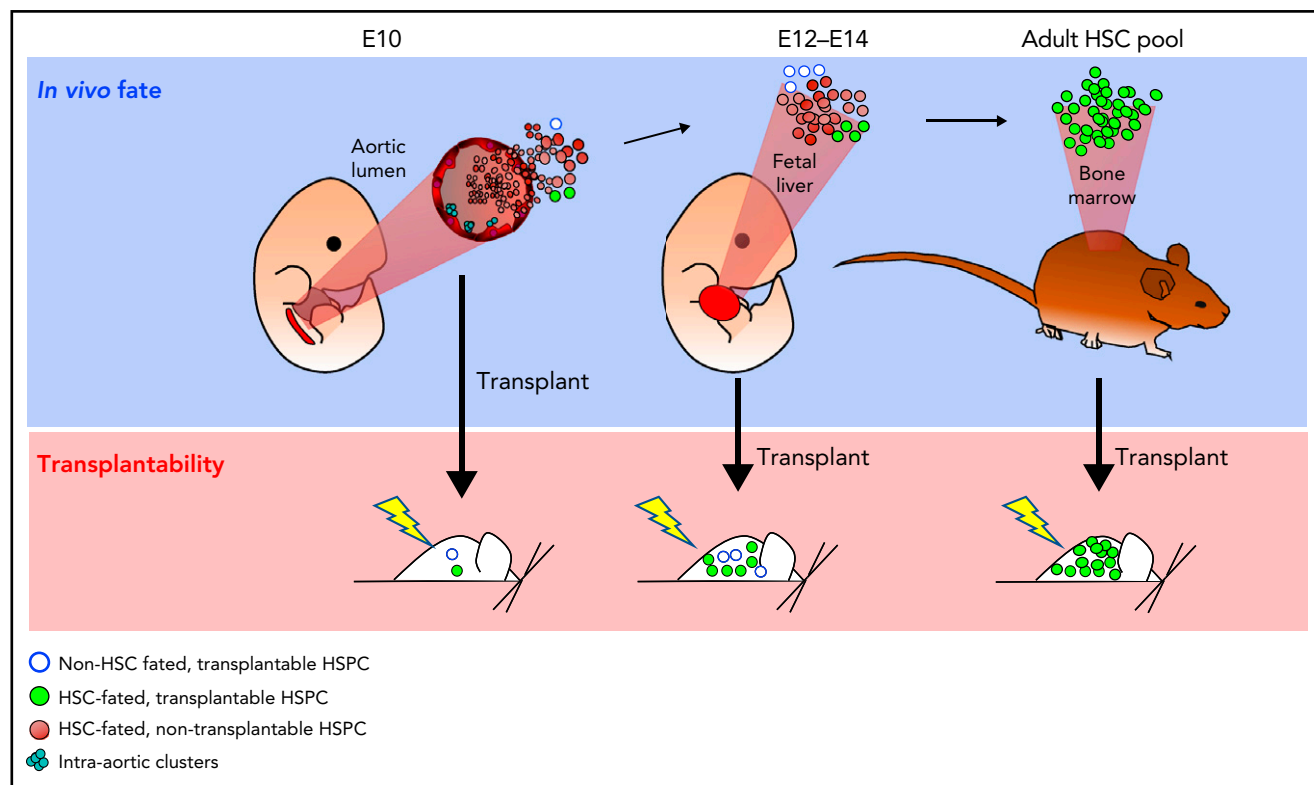
Figure 1. Possible differences in unperturbed in vivo fate and transplantability. At key developmental time points, noninvasive fate mapping studies and phenotypic analyses indicate the presence of far more HSC-fated precursors than are capable of engraftment in transplantation assays. At E10 in the aortic lumen and E12 to E14 in the FL, a few rare HSC-committed hematopoietic stem and progenitor cells (HSPCs) are already sufficiently mature to be transplantable (green), but most HSC-fated HSPCs are not (brown). A small transplantable pool of HSPCs may also be present that would never realize an adult HSC fate without transplantation (blue circles). Differences in unperturbed in vivo fate vs transplantation potential may arise from immaturity of HSPCs at the time of transplantation, stresses associated with ex vivo manipulations, and niche availability.

mCherry-T2A-creERT2) fish estimates 25 to 36 HSCs in the ventral dorsal aorta.[11,100] Together, these data suggest a larger number of independently specified clonal HSC precursors than predicted by transplantation approaches in mice (1-2 HSCs at E11.5) and support a model in which HSC specification is more frequent, especially considering that mice are larger than zebrafish.

In mice, Fatima et al[101] sought to label a single E7.5 HSC progenitor via low marking of embryonic blood precursors by exposing *Abcg2-CreERT2*$^{Cre-ERT2/Cre-ERT2}$; *Rosa26Lox-STOP-Lox-YFP*$^{YFP/YFP}$ embryos to a single dose of 4-hydroxytamoxifen at E7.5. ABCG2 is a member of the ATP-binding cassette transporter family expressed by all HSCs that allows for embryonic labeling of some HSC precursors.[102] This strategy resulted in 0.3% to 0.8% labeling of adult myeloid and lymphoid blood lineages, confirming labeling of HSC precursors. Assuming that 0.3% labeling of the blood corresponds to a single labeled HSC, the authors estimated that at least 333 HSC precursors exist in E7.5 to E8.5 murine embryos.[101,102] The inability of this system to determine how many HSC precursors were actually labeled at E7.5 precludes the model from placing an upper bound on the actual number of HSC precursors present at E7.5.

We previously used a CRE-inducible *Confetti* allele,[103] also derived from the *Brainbow* system, to estimate independent clonal HSC specification frequency during embryogenesis at key time points. The *Confetti* allele can be recombined to 1 of 4 colors (GFP, YFP, RFP, or CFP), and expression persists in subsequent cellular progeny.[17,92,103] Although only 4 colors are available, the distribution of colors in the adult HSC-derived peripheral blood can be mathematically correlated to the number of discrete clones present at the time of recombination; if few clones are present at recombination, color distributions show high mouse-to-mouse variance (MtMV), whereas if numerous clones are initially labeled, the variance is small.[17,92] This approach allowed estimates of the number of murine mesodermal blood precursors (*Flk1-Cre*) as ~700 (~E7), endothelial precursors (*VE-cadherin-Cre*) as ~600 (~E8.5), and mature HSC precursors (*Vav1-Cre*) as ~600 (~E11-E14).

The MtMV in the *Confetti* color distribution yields the most accurate estimate of initial labeling events in the range of 50 to 2500 cells; outside this range, estimates are qualitative rather than quantitative (ie, very few vs very large numbers of initially labeled events).[92] Additionally, *Confetti* labeling efficiency needs to be >3%, and >500 cells must be analyzed.[92] Therefore, each of these technical nuances must be considered when evaluating the likely fidelity of estimates of clonal complexity using MtMV of *Confetti* labeling. Furthermore, the system does not allow for the tracking of individual clones. Nonetheless, the evolution of the global clonal complexity of a cellular system as a whole can be followed, allowing one to interrogate the effects of aging and other insults on the global clonal complexity of the blood.[17]

Recently, a number of genetic barcoding technologies have emerged and been applied to adult hematopoiesis. The Sleeping Beauty system, based on a doxycycline-inducible transposase,[104] suggested that native hematopoiesis is maintained by thousands of multipotent progenitors, rather than HSCs, and that HSCs predominantly contribute to megakaryopoiesis.[104,105]

This finding remains controversial, supported by studies where hematopoiesis continued when HSCs were ablated in adults[106-108] but also refuted by lineage-tracing approaches that support a traditional view of HSC contribution to steady-state hematopoiesis.[109-111] Additional innate barcoding systems include Poly-Lox, which generates random barcodes via induced recombination of a cassette containing many *loxP* sites,[112] and CRISPR-based molecular recording or scarring, which revealed skewed contribution of FL HSC precursors and reduced clonal diversity in blood after 5-fluorouracil treatment.[113-115] To date, these technologies have not been reported to estimate frequency of developmental HSC specification, but we are likely to have insights in the near future, including tracking of individual cells and study of lineage output and proliferative behavior. However, if future work establishes definitively that multipotent progenitors contribute substantially to steady-state hematopoiesis, key questions will need to be revisited to clarify our understanding of the establishment of the adult hematopoietic system, such as whether multipotent progenitors are specified independently of classically defined HSCs.[116]

Insights from human studies

Gaining insights into the frequency of HSC specification during human ontogeny has been more challenging, but recent technologic innovations are yielding fresh understanding. Early studies seeking to investigate human clonal dynamics took advantage of X inactivation and the evolution of telomere lengths with age. These and other studies hinted at the highly polyclonal nature of human adult blood and suggested that most HSC expansion takes place during childhood and adolescence and stabilizes in adulthood.[117-120] Observations of shifting patterns of X inactivation with age in women provided early glimpses of age-associated clonal hematopoiesis that have subsequently been laid bare in studies of large cohorts of patients whose blood was subjected to whole-exome sequencing.[119,121-124]

In 2014, Behjati et al[125] reported a proof of principle that somatic mutations acquired naturally over time and detected via whole-genome sequencing (WGS) of many independently expanded clones (murine gut organoids in this case) could be exploited to trace the developmental history of a given tissue. Using estimates of the rate of mutation accrual and the distribution of mutations among independently examined clones, these naturally acquired barcodes can be organized into a hierarchy and placed in ontologic time all the way back to the 2-cell embryo. WGS is now revolutionizing our understanding of the clonal dynamics of human blood development during ontogeny, as well as the clonal evolution of mutations associated with hematologic disease.[121,122,124]

In a groundbreaking study, Lee-Six et al[118] used natural accumulation of somatic mutations in clones of expanded human HSPCs to examine the origins, lineage relationships, and clonal complexity of the human hematopoietic system via WGS. They and others estimated that adult human blood emerges from numerous embryonic clones, with ~50 000 to 200 000 HSCs actively contributing to hematopoiesis in a healthy adult at any given moment and most expansion of adult blood precursors occurring during adolescence.[118,126] This approach has now been applied to expanded HSPC clones isolated from the liver and BM of human fetuses. Here, investigators demonstrated

explicitly that even early in development, HSPCs have acquired dozens of somatic mutations.[127] They also concluded that the cells of a 2-cell embryo do not contribute equally to downstream tissues.[127] Similarly, Ludwig et al[128] recently demonstrated that acquired somatic mutations in the mitochondrial genome, which is almost entirely transcribed, can be exploited in single-cell RNA-sequencing data sets as a useful barcode to examine clonal relationships among sequenced human HSPCs. Most recently, WGS of expanded clones has been applied to illuminate the developmental timing of the acquisition of driver mutations in patients with myeloproliferative neoplasms (MPNs).[129] Here, *JAK2*[V617F] and *DNMT3A* mutations were estimated to arise in early fetal life and childhood, expanding for years (sometimes many decades) before clinical presentation of an MPN. This finding has major implications for thinking about when and how screening should be performed for MPNs. However, because each of these studies depends on the sequencing of clones expanded in vitro, the data are inherently biased for cells capable of in vitro expansion, which may or may not be influenced by disease mutations. Furthermore, it is difficult to exclude mutation acquisition during colony expansion. Finally, the high cost of WGS imposes a limitation on the number of sequenced clones and potentially the ability to effectively extrapolate the data.

Adding complexity: transient and tissue-specific populations

Multiple investigations have revealed the existence of transient embryonic HSPCs with restricted lineage potential, generally erythroid and erythromyeloid oligopotency.[130,131] More recently, a suite of studies has defined long-lasting or permanent precursors with highly restricted lineage potential, including tissue-resident macrophage and specific subsets of adult lymphoid cells, which may not derive from HSCs.[66,132-134] Some of these HSPCs may have a role in the emergence of HSCs (eg, proinflammatory AGM-associated macrophages).[135] Here, we briefly highlight additional layers of complexity that derive from these newly discovered transient and restricted lineage HSPCs, as well as how they might confound interpretations of the dynamics and magnitude of lifelong HSC specification during embryogenesis.

During embryonic development, transient hematopoietic waves have been detected that emerge from the yolk sac, contribute with little or no frequency to the adult HSC pool, and harbor erythroid or erythromyeloid potential.[3,71,72,136-139] Primitive erythrocytes arise earliest in blood islands of the yolk sac, and by E8.5 in mouse or 32 hpf in zebrafish, a subsequent oligopotent precursor termed the erythromyeloid progenitor (EMP) is specified.[66,130,136,138] Recently, lineage tracing was performed via activation of *Tg(drl:creERT2;ubi:lox-GFP-lox-mCherry)* reporter fish with TAM at 30 vs 54 hpf, suggesting that definitive HSPCs minimally contribute to embryonic lymphomyelopoiesis in zebrafish.[140] These findings are supported by additional experiments that deplete HSCs with nitroreductase/metronidazole treatments,[140] validating previous observations in mice.[136]

Until recently, it was thought that EMP-derived myeloid cells did not perdure into adulthood, but multiple recent studies have now shown that certain types of adult tissue-resident macrophages, including microglia in the brain, alveolar macrophages of the lung, Kupffer's cells in the liver, and Langerhans cells in the skin, are partly or wholly EMP derived and migrate to the embryo proper, where they differentiate during organogenesis, beginning at ∼E10.5.[132-134] In mammals, these cells are able to self-renew and persist into adulthood, unless faced with unusual challenges, such as organismal irradiation. Interestingly, it seems that in lower vertebrates, such as teleost fish, there are 2 successive waves of microglial specification, where a second HSC-derived population of microglia (development of which relies on colony-stimulating factor 1 receptor b [*csfr1b*]) replaces the earlier EMP-derived wave, so these specification processes may not be completely conserved across vertebrate phyla.[141] Additionally, a transient wave of tissue-resident macrophages derived from the heart endocardium and essential for cardiac valve development has also been discovered via lineage tracing that exploited the endocardial-specific *Nfatc1-Cre* mouse line.[142]

Similarly, it now seems that some embryonic lymphoid populations are also specified independently of HSCs and persist into adulthood.[132,134] Fetal T-lymphoid progenitors have been reported in circulation before the establishment of the thymus.[143-145] Multiple studies have reported development of a restricted lymphoid or T-lineage precursor in the mouse yolk sac or directly from hemogenic endothelium of mouse and zebrafish embryos, and in some cases, these precursors arise earlier than AGM HSCs and in the absence of blood flow (ie, yolk sac in origin), although recent depletion and lineage-tracing studies have revealed an HSC origin for specific innate lymphoid subsets.[146-150] It seems that embryonically derived γδ T cells persist and self-renew in the skin of mice.[151]

A subset of B cells, known as B1a cells, residing in the mucosa and body cavities of adult tissues has also been proposed to have an HSC-independent origin. In vitro lineage potential and in vivo transplantation studies have revealed FL and neonatal HSPCs to be more potent than adult BM HSCs at generating B1a cells.[152] Indeed, adult HSCs do not cell autonomously contain high-level B1a potential (as reviewed by Ghosn et al[153]). Furthermore, B1a potential can be detected in yolk sac and AGM tissues before HSC emergence,[154] and B1a progenitors are detected in *Cbfβ*^{−/−}; *Tek:GFP-Cbfβ* mice, which lack transplantable HSCs.[155] However, B1a progenitors observed in *Cbfβ*^{−/−}; *Tek:GFP-Cbfβ* mice also display maturation defects, raising questions as to whether this model accurately reflects steady-state biology. Furthermore, fate mapping studies have revealed that B1a progenitors are absent in the developing embryo until HSC emergence.[150] Barcoding studies have also revealed the presence of FL HSPCs that give rise to both B2 and B1a progeny when transplanted.[156] These studies were complemented by single-cell transplantations in which phenotypic FL long-term HSCs (LT-HSCs) gave rise to both B2 and B1a cells. In contrast, Ghosn et al[157] failed to observe B1a potential after transplantation of bulk FL LT-HSC. This disparate result might be explained by phenotypic differences in the populations interrogated, because Ghosn et al depleted Mac-1^{+} cells from LT-HSCs; previous work has shown that FL LT-HSCs express Mac-1.[82] In an interesting twist, a fate mapping study in which cells that transit through an Flk2^{+} state are irreversibly switched from TdTomato[+] to GFP[+] showed that an embryonic Flk2^{+} precursor, termed transient HSCs, has multilineage potential with bias toward B1a B and γδ T lymphocytes but does not persist in the adult HSC compartment.[158] It was recently

Table 1. Summary of multiple approaches to study the frequency and dynamics of HSC specification in mice, zebrafish, and humans

Method	Organism	N of HSPC/HSPC precursors specified*	Advantages	Limitations	Equipment/technical requirements	References
IAC cluster cartography	Mouse	609 ± 84 c-Kit$^+$ cells/E10.5 embryo 439 ± 87 c-Kit$^+$ cells/E11.5 embryo	Direct morphologic evaluation; flexibility on use of additional markers	Lack of functional validation; single time point snapshot	Histology equipment; antibodies; confocal microscopy	80
Transplantation of fresh tissues	Mouse	1-2 RUs/embryo at E10.5-E11.5; 60 RUs/ee at E12.5	Well established; statistically robust and simple (Poisson statistics); commercial software available for LDA analysis	Does not score immature HSC precursors	Requires large cohorts of mice as transplant recipients; flow cytometry	7,8,68,82,86
Transplantation of cultured explants	Mouse	60 RUs/ee between E9.5 and E12.5	Allows for quantification of immature HSC precursor potential at early developmental time points (E8.5-E10.5) in murine embryos	Native development perturbed	Requires large cohorts of mice as transplant recipients; flow cytometry; explant culture	94–98
Marking of single blood precursors	Mouse	333 E7.5-E8.5 blood precursors (Abcg2-CreERT2; Rosa26Lox-STOP-Lox-YFP)	Native development is preserved; simple math that assumes observed blood labeling is output of single precursor from which total n of precursors is extrapolated	Difficult to verify single precursor labeling; cannot determine upper bound for n of precursors	*Abcg2-CreERT2; Rosa26Lox-STOP-Lox-YFP* mouse model; single group of mice ($\geq$5 mice); flow cytometry	101
MtMV of *Confetti*-reporter labeling	Mouse	600 blood precursors (E7.5-E12.5)	Native development is preserved; quantitative; simple genetic mouse models; statistically robust and simple; based on Poisson statistics	Individual clones cannot be tracked; requires >3% of *Confetti* labeling and >500 cells examined; quantitative range is limited; commercial software for analyses not available	Cre recombinase mouse models; single group of mice ($\geq$5 mice); flow cytometry with 405, 445, 488, 562, and 640 nm and specific band-pass filter configurations to discriminate *Confetti* colors	17,92
Zebrabow	Zebrafish	20-26 lifelong precursors	Native development is preserved	Reliant on hue and saturation to identify individual clones	Driver and effector transgenic lines; color-clustering computational analysis platform; flow cytometry; confocal and inverted microscope	11
Hsp701 LASER induction	Zebrafish	30 lifelong precursors	Native development is preserved	Reliant on labeling 1 cell, otherwise individual clones cannot be tracked	Micropoint microscope-targeted 440-nm laser; driver and effector transgenic lines; flow cytometry; confocal and inverted microscopes	11

ee, embryo equivalent; IAC, intra-aortic cluster; LDA, limiting dilution analysis.

*N of IAC cells, RUs, or lineage-traced cells.

Table 1. (continued)

Method	Organism	N of HSPC/HSPC precursors specified*	Advantages	Limitations	Equipment/technical requirements	References
Transposase-mediated tagging	Mouse	Not yet employed	Native development is preserved; single clones can be tracked; lineage relationships inferred	Complex genetic mouse model; bioinformatics expertise required to interpret data	Targeted sequencing; bioinformatics expertise	104,105
PolyLox-based barcoding	Mouse	Not yet employed	Native development is preserved; single clones can be tracked; lineage relationships inferred	Complex genetic mouse model; bioinformatics expertise required to interpret data; need to eliminate common rearrangements	Targeted sequencing; bioinformatics expertise	112
CRISPR-based barcoding	Mouse	Not yet employed	Native development is preserved; single clones can be tracked; lineage relationships inferred	Complex genetic mouse model; bioinformatics expertise required to interpret data	Targeted sequencing; bioinformatics expertise	113–115
Somatic mutations	Human		Native development is preserved	Costs limit n of analyzed clones; potential for confounding mutations during cell expansion; biased for sequencing clones able to expand in vitro; complex bioinformatics analyses	WGS platforms; bioinformatics expertise	118

ee, embryo equivalent; IAC, intra-aortic cluster; LDA, limiting dilution analysis.

*N of IAC cells, RUs, or lineage-traced cells.

reported that B2 vs B1a identity can be directed by the nature of the B-cell receptor (BCR) being expressed and that this potential is somewhat plastic (ie, B2 cells can be made to revert to a B1a phenotype simply by switching their BCR to a typical B1a BCR).[159] This raises the question of whether appropriate antigen exposure could be key to the development of B1a cells. Might differential exposure to antigen during in vitro culture assays, transplantation, or steady-state development explain some of the disparate findings described? Further investigation is needed to decipher these puzzles.

Together, these results demonstrate how limited phenotypic markers may be misleading and how functional studies and high-resolution lineage-tracing studies must continue to inform and complement our attempts to understand fate potentials and the clonal establishment of hematopoietic lineage potency. In addition, recent quantitative modeling of normal and malignant HSPC dynamics has highlighted the importance of a unifying mathematic model that could reconcile different observations by considering cell heterogeneity and an extended nonsteady state, in which cells do not evenly contribute to downstream cellular compartments.[160] Early experiments relied on the quantification of transplantable repopulating units as a measurement of HSC numbers and thus only captured a fraction of blood precursors at any given developmental stage. Furthermore, the contribution of each blood precursor to the adult HSC pool may be distinct, hence the importance of unbiased labeling methods to accurately measure blood precursor dynamics and of appropriate mathematic modeling able to integrate cell heterogeneity as defined by murine Cre lines displaying overlapping and nonoverlapping labeling of distinct blood precursors.

Conclusions

Together, lineage-tracing data and genomic mutational studies reveal that HSC specification is not a rare event; 10s of zebrafish HSPCs, 100s of murine HSPCs, and 1000s of human HSPCs contribute to the adult HSC pool[11,92,101,118] (Figure 1). Each of these approaches has its own advantages and limitations (Table 1). Additionally, the presence of transient waves of progeny derived from embryonic HSPCs and long-lived non–HSC-derived cells complicates interpretations of the origins of hematopoietic clonal complexity. Indeed, key for future studies will be to definitively resolve the role of classically transplantable HSCs in steady-state hematopoiesis. This will likely require new tools that allow for clean fate mapping of multipotent progenitors vs HSCs, because all existing published tools show some lack of discrimination in labeling between these two populations.[109-111] Another important question to address is whether multipotent progenitors are specified independently from HSCs during embryogenesis and if they contribute progeny into adult life. Alternatively, these cells might simply be organized into a hierarchy of transplantation and life-long blood potential, consistent with the classic view. The novel approaches described here and others to come should eventually bring some clarity on these outstanding issues.

Authorship

Contribution: W.C., M.G., and S.M.-F. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: M.G., 0000-0002-1070-7961; W.C., 0000-0003-4365-9553.

Correspondence: Shannon McKinney-Freeman, 262 Danny Thomas Place, Memphis, TN 38105; e-mail: shannon.mckinney-freeman@stjude.org.

REFERENCES

1. Laurenti E, Göttgens B. From haematopoietic stem cells to complex differentiation landscapes. Nature. 2018; 553(7689):418-426.

2. Ng AP, Alexander WS. Haematopoietic stem cells: past, present and future. Cell Death Discov. 2017;3:17002.

3. Medvinsky A, Rybtsov S, Taoudi S. Embryonic origin of the adult hematopoietic system: advances and questions. Development. 2011;138(6):1017-1031.

4. Elsaid R, Soares-da-Silva F, Peixoto M, et al. Hematopoiesis: a layered organization across chordate species. Front Cell Dev Biol. 2020;8:606642.

5. Lo Celso C, Fleming HE, Wu JW, et al. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature. 2009;457(7225): 92-96.

6. Ganuza M, Hall T, Obeng EA, McKinney-Freeman S. Clones assemble! The clonal complexity of blood during ontogeny and disease. Exp Hematol. 2020;83:35-47.

7. Gekas C, Dieterlen-Lièvre F, Orkin SH, Mikkola HK. The placenta is a niche for hematopoietic stem cells. Dev Cell. 2005; 8(3):365-375.

8. Kumaravelu P, Hook L, Morrison AM, et al. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. Development. 2002;129(21):4891-4899.

9. Oguro H, Ding L, Morrison SJ. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell. 2013;13(1):102-116.

10. Yilmaz OH, Kiel MJ, Morrison SJ. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood. 2006;107(3):924-930.

11. Henninger J, Santoso B, Hans S, et al. Clonal fate mapping quantifies the number of haematopoietic stem cells that arise during development [published correction

appears in *Nat Cell Biol.* 2017;19(2):142]. *Nat Cell Biol.* 2017;19(1):17-27.

12. Szilvassy SJ, Humphries RK, Lansdorp PM, Eaves AC, Eaves CJ. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. *Proc Natl Acad Sci USA.* 1990; 87(22):8736-8740.

13. Harrison DE, Astle CM, Stone M. Numbers and functions of transplantable primitive immunohematopoietic stem cells. Effects of age. *J Immunol.* 1989;142(11):3833-3840.

14. Benz C, Copley MR, Kent DG, et al. Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell.* 2012;10(3):273-283.

15. Dykstra B, Kent D, Bowie M, et al. Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell.* 2007;1(2):218-229.

16. Kent DG, Copley MR, Benz C, et al. Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. *Blood.* 2009;113(25):6342-6350.

17. Ganuza M, Hall T, Finkelstein D, et al. The global clonal complexity of the murine blood system declines throughout life and after serial transplantation. *Blood.* 2019; 133(18):1927-1942.

18. Maximow A. Untersuchungen über Blut und Bindegewebe. I. Die frühesten Entwicklungsstadien der Blut- unde Bindegewebszellen beim Säugeteireembryo, bis zum Anfang der Blutbildung in der Leber [The earliest developmental stages of blood and connective tissue cells in the mammalian embryo, up to the beginning of blood formation in the fetal liver]. *Arch f mikr Anat u Entwicklungsgesch.* 1909;73: 444-561.

19. Danchakoff V. Origin of the blood cells. Development of the haematopoietic organs and regeneration of the blood cells from the standpoint of the monophyletic school. *Anat Rec.* 1916;10(5):397-413.

20. Becker AJ, McCulloch EA, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature.* 1963;197:452-454.

21. Siminovitch L, McCulloch EA, Till JE. The distribution of colony-forming cells among spleen colonies. *J Cell Comp Physiol.* 1963; 62:327-336.

22. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.* 1961;14:213-222.

23. Wu AM, Till JE, Siminovitch L, McCulloch EA. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. *J Cell Physiol.* 1967; 69(2):177-184.

24. Wu AM, Till JE, Siminovitch L, McCulloch EA. Cytological evidence for a relationship between normal hemotopoietic colony-forming cells and cells of the lymphoid system. *J Exp Med.* 1968;127(3):455-464.

25. Bradley TR, Metcalf D. The growth of mouse bone marrow cells in vitro. *Aust J Exp Biol Med Sci.* 1966;44(3):287-299.

26. Purton LE, Scadden DT. Limiting factors in murine hematopoietic stem cell assays. *Cell Stem Cell.* 2007;1(3):263-270.

27. Topczewska JM, Topczewski J, Solnica-Krezel L, Hogan BL. Sequence and expression of zebrafish foxc1a and foxc1b, encoding conserved forkhead/winged helix transcription factors. *Mech Dev.* 2001;100(2):343-347.

28. Weissman IL, Shizuru JA. The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood.* 2008;112(9):3543-3553.

29. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell.* 2005;121(7): 1109-1121.

30. Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity.* 1994;1(8):661-673.

31. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/ negative hematopoietic stem cell. *Science.* 1996;273(5272):242-245.

32. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science.* 1988; 241(4861):58-62.

33. Balazs AB, Fabian AJ, Esmon CT, Mulligan RC. Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. *Blood.* 2006; 107(6):2317-2321.

34. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci USA.* 1992; 89(7):2804-2808.

35. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci USA.* 1997;94(10):5320-5325.

36. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol.* 1984;133(1): 157-165.

37. Conneally E, Cashman J, Petzer A, Eaves C. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. *Proc Natl Acad Sci USA.* 1997;94(18):9836-9841.

38. Lansdorp PM, Sutherland HJ, Eaves CJ. Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow. *J Exp Med.* 1990;172(1):363-366.

39. Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science.* 2011;333(6039):218-221.

40. Bonnet R. Grundriss der Entwicklungsgeschichte [Outline of Developmental History]. Berlin, Germany: Verlag von Paul Parey; 1891

41. Emmel VE. The cell clusters in the dorsal aorta of mammalian embryos. *Am J Anat.* 1916;19(3):401-421.

42. Jordan HE. Evidence of hemogenic capcity of endothelium. *Anat Rec.* 1916;10(5): 417-420.

43. Sabin FR. Studies on the origin of blood vessels and of red blood corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contrib Embryol.* 1920;9(214):213-262.

44. van der Stricht O. Nouvelles recherches sur la gnese des globules rouges et des globules blancs du sang [New research on the genesis of red and white blood cells]. *Arch Biol (Liege).* 1892;12:199-344.

45. Jaffredo T, Gautier R, Eichmann A, Dieterlen-Lièvre F. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development.* 1998; 125(22):4575-4583.

46. Zovein AC, Hofmann JJ, Lynch M, et al. Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell.* 2008;3(6):625-636.

47. Eilken HM, Nishikawa S, Schroeder T. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature.* 2009;457(7231):896-900.

48. Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V, Lacaud G. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature.* 2009;457(7231):892-895.

49. Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature.* 2009; 457(7231):887-891.

50. Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature.* 2010;464(7285):108-111.

51. Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature.* 2010;464(7285): 116-120.

52. Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature.* 2010; 464(7285):112-115.

53. His W. Untersuchungen über die ester Anlage des Wirblethierleibes [Investigations into the structure of the vertebrate body]. Leipzig, Germany: Vogel, F.C.W.; 1868.

54. Ferkowicz MJ, Yoder MC. Blood island formation: longstanding observations and modern interpretations. *Exp Hematol.* 2005;33(9):1041-1047.

55. Palis J, Robertson S, Kennedy M, Wall C, Keller G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development.* 1999;126(22):5073-5084.

56. Moore MA, Metcalf D. Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br J Haematol.* 1970;18(3):279-296.

57. Weissman I, Pappaoiannou V, Gardner R. Fetal Hematopoietic Origins of the Adult Hemolymphoid System. Differentiation of Normal and Neoplastic Hematopoietic Cells. Cold Spring Harbor, NY: Cold Spring Harbor Lab Press; 1978:33-47.

58. Toles JF, Chui DH, Belbeck LW, Starr E, Barker JE. Hemopoietic stem cells in murine embryonic yolk sac and peripheral blood. *Proc Natl Acad Sci USA.* 1989; 86(19):7456-7459.

59. Liu CP, Auerbach R. In vitro development of murine T cells from prethymic and preliver embryonic yolk sac hematopoietic stem cells. *Development.* 1991;113(4): 1315-1323.

60. Dieterlen-Lievre F. On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol.* 1975;33(3): 607-619.

61. Lassila O, Eskola J, Toivanen P, Martin C, Dieterlen-Lievre F. The origin of lymphoid stem cells studied in chick yold sac-embryo chimaeras. *Nature.* 1978;272(5651):353-354.

62. Maeno M, Todate A, Katagiri C. The localization of precursor cells for larval and adult hemopoietic cells of *Xenopus laevis* in two regions of embryos. *Dev Growth Differ.* 1985;27(2):137-148.

63. Turpen JB, Knudson CM, Hoefen PS. The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: localization of a prospective stem cell compartment. *Dev Biol.* 1981; 85(1):99-112.

64. Ciau-Uitz A, Walmsley M, Patient R. Distinct origins of adult and embryonic blood in Xenopus. *Cell.* 2000;102(6):787-796.

65. Maéno M, Tochinai S, Katagiri C. Differential participation of ventral and dorsolateral mesoderms in the hemopoiesis of Xenopus, as revealed in diploid-triploid or interspecific chimeras. *Dev Biol.* 1985; 110(2):503-508.

66. Cumano A, Godin I. Ontogeny of the hematopoietic system. *Annu Rev Immunol.* 2007;25:745-785.

67. Godin I, Dieterlen-Lièvre F, Cumano A. Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc Natl Acad Sci USA.* 1995;92(3):773-777.

68. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell.* 1996;86(6):897-906.

69. Medvinsky AL, Samoylina NL, Müller AM, Dzierzak EA. An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature.* 1993;364(6432):64-67.

70. Müller AM, Medvinsky A, Strouboulis J, Grosveld F, Dzierzak E. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity.* 1994;1(4): 291-301.

71. Ganuza M, Chabot A, Tang X, et al. Murine hematopoietic stem cell activity is derived from pre-circulation embryos but not yolk sacs. *Nat Commun.* 2018;9(1):5405.

72. Cumano A, Dieterlen-Lievre F, Godin I. Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell.* 1996;86(6):907-916.

73. McGrath KE, Koniski AD, Malik J, Palis J. Circulation is established in a stepwise pattern in the mammalian embryo. *Blood.* 2003;101(5):1669-1676.

74. de Bruijn MF, Ma X, Robin C, Ottersbach K, Sanchez MJ, Dzierzak E. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity.* 2002;16(5):673-683.

75. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development.* 1998; 125(9):1747-1757.

76. Ma X, de Bruijn M, Robin C, et al. Expression of the Ly-6A (Sca-1) lacZ transgene in mouse haematopoietic stem cells and embryos. *Br J Haematol.* 2002;116(2): 401-408.

77. North T, Gu TL, Stacy T, et al. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development.* 1999;126(11):2563-2575.

78. North TE, de Bruijn MF, Stacy T, et al. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity.* 2002;16(5):661-672.

79. North TE, Stacy T, Matheny CJ, Speck NA, de Bruijn MF. Runx1 is expressed in adult mouse hematopoietic stem cells and differentiating myeloid and lymphoid cells, but not in maturing erythroid cells. *Stem Cells.* 2004;22(2):158-168.

80. Yokomizo T, Dzierzak E. Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. *Development.* 2010;137(21):3651-3661.

81. Tavian M, Coulombel L, Luton D, Clemente HS, Dieterlen-Lièvre F, Péault B. Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood.* 1996; 87(1):67-72.

82. Morrison SJ, Hemmati HD, Wandycz AM, Weissman IL. The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci USA.* 1995; 92(22):10302-10306.

83. Mikkola HK, Gekas C, Orkin SH, Dieterlen-Lievre F. Placenta as a site for hematopoietic stem cell development. *Exp Hematol.* 2005;33(9):1048-1054.

84. Yoder MC, Hiatt K, Dutt P, Mukherjee P, Bodine DM, Orlic D. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity.* 1997;7(3):335-344.

85. Yoder MC, Hiatt K, Mukherjee P. In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. *Proc Natl Acad Sci USA.* 1997; 94(13):6776-6780.

86. Ema H, Nakauchi H. Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood.* 2000;95(7):2284-2288.

87. Mikkola HK, Orkin SH. The journey of developing hematopoietic stem cells. *Development.* 2006;133(19):3733-3744.

88. Bowie MB, McKnight KD, Kent DG, McCaffrey L, Hoodless PA, Eaves CJ. Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. *J Clin Invest.* 2006;116(10):2808-2816.

89. Lessard J, Faubert A, Sauvageau G. Genetic programs regulating HSC specification, maintenance and expansion. *Oncogene.* 2004;23(43):7199-7209.

90. Ivanovs A, Rybtsov S, Anderson RA, Turner ML, Medvinsky A. Identification of the niche and phenotype of the first human hematopoietic stem cells. *Stem Cell Reports.* 2014;2(4):449-456.

91. Ivanovs A, Rybtsov S, Welch L, Anderson RA, Turner ML, Medvinsky A. Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. *J Exp Med.* 2011; 208(12):2417-2427.

92. Ganuza M, Hall T, Finkelstein D, Chabot A, Kang G, McKinney-Freeman S. Lifelong haematopoiesis is established by hundreds of precursors throughout mammalian ontogeny. *Nat Cell Biol.* 2017;19(10): 1153-1163.

93. Arora N, Wenzel PL, McKinney-Freeman SL, et al. Effect of developmental stage of HSC and recipient on transplant outcomes. *Dev Cell.* 2014;29(5):621-628.

94. Rybtsov S, Batsivari A, Bilotkach K, et al. Tracing the origin of the HSC hierarchy reveals an SCF-dependent, IL-3-independent CD43(-) embryonic precursor. *Stem Cell Reports.* 2014;3(3):489-501.

95. Rybtsov S, Ivanovs A, Zhao S, Medvinsky A. Concealed expansion of immature precursors underpins acute burst of adult HSC activity in foetal liver. *Development*. 2016;143(8):1284-1289.

96. Rybtsov S, Sobiesiak M, Taoudi S, et al. Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. *J Exp Med*. 2011;208(6):1305-1315.

97. Taoudi S, Gonneau C, Moore K, et al. Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-cadherin+CD45+ pre-definitive HSCs. *Cell Stem Cell*. 2008;3(1):99-108.

98. Gordon-Keylock S, Sobiesiak M, Rybtsov S, Moore K, Medvinsky A. Mouse extraembryonic arterial vessels harbor precursors capable of maturing into definitive HSCs. *Blood*. 2013;122(14):2338-2345.

99. Livet J, Weissman TA, Kang H, et al. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature*. 2007;450(7166):56-62.

100. Xu J, Zhu L, He S, et al. Temporal-spatial resolution fate mapping reveals distinct origins for embryonic and adult microglia in zebrafish. *Dev Cell*. 2015;34(6):632-641.

101. Fatima S, Zhou S, Sorrentino BP. Marking of definitive HSC precursors in E7.5-E8.5 embryos using an Abcg2-CreER lineage-tracing mouse model. *Exp Hematol*. 2018;65:29-33.

102. Fatima S, Zhou S, Sorrentino BP. Abcg2 expression marks tissue-specific stem cells in multiple organs in a mouse progeny tracking model. *Stem Cells*. 2012;30(2):210-221.

103. Snippert HJ, van der Flier LG, Sato T, et al. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell*. 2010;143(1):134-144.

104. Sun J, Ramos A, Chapman B, et al. Clonal dynamics of native haematopoiesis. *Nature*. 2014;514(7522):322-327.

105. Rodriguez-Fraticelli AE, Wolock SL, Weinreb CS, et al. Clonal analysis of lineage fate in native haematopoiesis. *Nature*. 2018;553(7687):212-216.

106. Busch K, Klapproth K, Barile M, et al. Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature*. 2015;518(7540):542-546.

107. Schoedel KB, Morcos MNF, Zerjatke T, et al. The bulk of the hematopoietic stem cell population is dispensable for murine steady-state and stress hematopoiesis. *Blood*. 2016;128(19):2285-2296.

108. Sheikh BN, Yang Y, Schreuder J, et al. MOZ (KAT6A) is essential for the maintenance of classically defined adult hematopoietic stem cells. *Blood*. 2016;128(19):2307-2318.

109. Sawai CM, Babovic S, Upadhaya S, et al. Hematopoietic stem cells are the major source of multilineage hematopoiesis in adult animals. *Immunity*. 2016;45(3):597-609.

110. Chapple RH, Tseng YJ, Hu T, et al. Lineage tracing of murine adult hematopoietic stem cells reveals active contribution to steady-state hematopoiesis. *Blood Adv*. 2018;2(11):1220-1228.

111. Säwen P, Eldeeb M, Erlandsson E, et al. Murine HSCs contribute actively to native hematopoiesis but with reduced differentiation capacity upon aging. *Elife*. 2018;7:e41258.

112. Pei W, Feyerabend TB, Rössler J, et al. Polylox barcoding reveals haematopoietic stem cell fates realized in vivo. *Nature*. 2017;548(7668):456-460.

113. Chan MM, Smith ZD, Grosswendt S, et al. Molecular recording of mammalian embryogenesis. *Nature*. 2019;570(7759):77-82.

114. Spanjaard B, Hu B, Mitic N, et al. Simultaneous lineage tracing and cell-type identification using CRISPR-Cas9-induced genetic scars. *Nat Biotechnol*. 2018;36(5):469-473.

115. Bowling S, Sritharan D, Osorio FG, et al. An engineered CRISPR-Cas9 mouse line for simultaneous readout of lineage histories and gene expression profiles in single cells [published correction appears in *Cell*. 2020;181(7):1693-1694]. *Cell*. 2020;181(7):1693-1694.

116. Dignum T, Varnum-Finney B, Srivatsan SR, et al. Multipotent progenitors and hematopoietic stem cells arise independently from hemogenic endothelium in the mouse embryo. *Cell Rep*. 2021;36(11):109675.

117. Werner B, Beier F, Hummel S, et al. Reconstructing the in vivo dynamics of hematopoietic stem cells from telomere length distributions. *Elife*. 2015;4:e08687.

118. Lee-Six H, Øbro NF, Shepherd MS, et al. Population dynamics of normal human blood inferred from somatic mutations. *Nature*. 2018;561(7724):473-478.

119. Busque L, Mio R, Mattioli J, et al. Nonrandom X-inactivation patterns in normal females: lyonization ratios vary with age. *Blood*. 1996;88(1):59-65.

120. Busque L, Patel JP, Figueroa ME, et al. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nat Genet*. 2012;44(11):1179-1181.

121. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. 2014;371(26):2477-2487.

122. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371(26):2488-2498.

123. Tonon L, Bergamaschi G, Dellavecchia C, et al. Unbalanced X-chromosome inactivation in haemopoietic cells from normal women. *Br J Haematol*. 1998;102(4):996-1003.

124. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med*. 2014;20(12):1472-1478.

125. Behjati S, Huch M, van Boxtel R, et al. Genome sequencing of normal cells reveals developmental lineages and mutational processes. *Nature*. 2014;513(7518):422-425.

126. Osorio FG, Rosendahl Huber A, Oka R, et al. Somatic mutations reveal lineage relationships and age-related mutagenesis in human hematopoiesis. *Cell Rep*. 2018;25(9):2308-2316.e4.

127. Spencer Chapman M, Ranzoni AM, Myers B, et al. Lineage tracing of human development through somatic mutations. *Nature*. 2021;595(7865):85-90.

128. Ludwig LS, Lareau CA, Ulirsch JC, et al. Lineage tracing in humans enabled by mitochondrial mutations and single-cell genomics. *Cell*. 2019;176(6):1325-1339.e22.

129. Williams N, Lee J, Moore L, et al. Phylogenetic reconstruction of myeloproliferative neoplasm reveals very early origins and lifelong evolution. *bioRxiv*; 2020.

130. Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development*. 2007;134(23):4147-4156.

131. Frame JM, McGrath KE, Palis J. Erythro-myeloid progenitors: "definitive" hematopoiesis in the conceptus prior to the emergence of hematopoietic stem cells. *Blood Cells Mol Dis*. 2013;51(4):220-225.

132. Cumano A, Berthault C, Ramond C, et al. New molecular insights into immune cell development. *Annu Rev Immunol*. 2019;37:497-519.

133. Ginhoux F, Guilliams M. Tissue-resident macrophage ontogeny and homeostasis. *Immunity*. 2016;44(3):439-449.

134. Wittamer V, Bertrand JY. Yolk sac hematopoiesis: does it contribute to the adult hematopoietic system? *Cell Mol Life Sci*. 2020;77(20):4081-4091.

135. Mariani SA, Li Z, Rice S, et al. Pro-inflammatory aorta-associated macro-phages are involved in embryonic development of hematopoietic stem cells. *Immunity*. 2019;50(6):1439-1452.e5.

136. Chen MJ, Li Y, De Obaldia ME, et al. Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. *Cell Stem Cell*. 2011;9(6):541-552.

137. He S, Xu J, Qu JY, Wen Z. Lightening the way of hematopoiesis: infrared laser-mediated lineage tracing with high spatial-temporal resolution. *Exp Hematol.* 2020;85: 3-7.

138. Palis J. Hematopoietic stem cell-independent hematopoiesis: emergence of erythroid, megakaryocyte, and myeloid potential in the mammalian embryo. *FEBS Lett.* 2016;590(22):3965-3974.

139. Cumano A, Ferraz JC, Klaine M, Di Santo JP, Godin I. Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multi-lineage reconstitution. *Immunity.* 2001; 15(3):477-485.

140. Ulloa BA, Habbsa SS, Potts KS, et al. Definitive hematopoietic stem cells minimally contribute to embryonic hematopoiesis. *Cell Rep.* 2021;36(11): 109703.

141. Ferrero G, Mahony CB, Dupuis E, et al. Embryonic microglia derive from primitive macrophages and are replaced by cmyb-dependent definitive microglia in zebrafish. *Cell Rep.* 2018;24(1):130-141.

142. Shigeta A, Huang V, Zuo J, et al. Endocardially derived macrophages are essential for valvular remodeling. *Dev Cell.* 2019;48(5):617-630.e3.

143. Douagi I, Colucci F, Di Santo JP, Cumano A. Identification of the earliest prethymic bipotent T/NK progenitor in murine fetal liver. *Blood.* 2002;99(2):463-471.

144. Masuda K, Kubagawa H, Ikawa T, et al. Prethymic T-cell development defined by the expression of paired immunoglobulin-like receptors. *EMBO J.* 2005;24(23):4052-4060.

145. Rodewald HR, Kretzschmar K, Takeda S, Hohl C, Dessing M. Identification of pro-thymocytes in murine fetal blood: T lineage commitment can precede thymus colonization. *EMBO J.* 1994;13(18):4229-4240.

146. Böiers C, Carrelha J, Lutteropp M, et al. Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. *Cell Stem Cell.* 2013;13(5):535-548.

147. Luis TC, Luc S, Mizukami T, et al. Initial seeding of the embryonic thymus by immune-restricted lympho-myeloid progenitors. *Nat Immunol.* 2016;17(12):1424-1435.

148. Tian Y, Xu J, Feng S, et al. The first wave of T lymphopoiesis in zebrafish arises from aorta endothelium independent of hematopoietic stem cells. *J Exp Med.* 2017;214(11):3347-3360.

149. Yoshimoto M, Porayette P, Glosson NL, et al. Autonomous murine T-cell progenitor production in the extra-embryonic yolk sac before HSC emergence. *Blood.* 2012; 119(24):5706-5714.

150. Elsaid R, Meunier S, Burlen-Defranoux O, et al. A wave of bipotent T/ILC-restricted progenitors shapes the embryonic thymus microenvironment in a time-dependent manner. *Blood.* 2021;137(8):1024-1036.

151. Gentek R, Ghigo C, Hoeffel G, et al. Epidermal γδ T cells originate from yolk sac hematopoiesis and clonally self-renew in the adult [published correction appears in *J Exp Med.* 2018;215(12):3213]. *J Exp Med.* 2018;215(12):2994-3005.

152. Beaudin AE, Forsberg EC. To B1a or not to B1a: do hematopoietic stem cells contribute to tissue-resident immune cells? *Blood.* 2016;128(24):2765-2769.

153. Ghosn E, Yoshimoto M, Nakauchi H, Weissman IL, Herzenberg LA. Hematopoietic stem cell-independent hematopoiesis and the origins of innate-like B lymphocytes. *Development.* 2019; 146(15):dev170571.

154. Yoshimoto M, Montecino-Rodriguez E, Ferkowicz MJ, et al. Embryonic day 9 yolk sac and intra-embryonic hemogenic endo-thelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc Natl Acad Sci USA.* 2011; 108(4):1468-1473.

155. Kobayashi M, Shelley WC, Seo W, et al. Functional B-1 progenitor cells are present in the hematopoietic stem cell-deficient embryo and depend on Cbfβ for their development. *Proc Natl Acad Sci USA.* 2014;111(33):12151-12156.

156. Kristiansen TA, Jaensson Gyllenbäck E, Zriwil A, et al. Cellular barcoding links B-1a B cell potential to a fetal hematopoietic stem cell state at the single-cell level. *Immunity.* 2016;45(2):346-357.

157. Ghosn EE, Waters J, Phillips M, et al. Fetal hematopoietic stem cell transplantation fails to fully regenerate the B-lymphocyte compartment. *Stem Cell Reports.* 2016; 6(1):137-149.

158. Beaudin AE, Boyer SW, Perez-Cunningham J, et al. A transient developmental hematopoietic stem cell gives rise to innate-like B and T cells. *Cell Stem Cell.* 2016;19(6):768-783.

159. Graf R, Seagal J, Otipoby KL, et al. BCR-dependent lineage plasticity in mature B cells. *Science.* 2019;363(6428):748-753.

160. Takahashi M, Barile M, Chapple RH, et al. Reconciling flux experiments for quantitative modeling of normal and malignant hematopoietic stem/progenitor dynamics. *Stem Cell Reports.* 2021;16(4): 741-753.