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Embryonic Origins of the Hematopoietic System: Hierarchies and Heterogeneity

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ABSTRACT

The hierarchical framework of the adult blood system as we know it from current medical and hematology textbooks, displays a linear branching network of dividing and differentiated cells essential for the growth and maintenance of the healthy organism. This view of the hierarchy has evolved over the last 75 years. An amazing increase in cellular complexity has been realized; however, innovative single-cell technologies continue to uncover essential cell types and functions in animal models and the human blood system. The most potent cell of the hematopoietic hierarchy is the hematopoietic stem cell. Stem cells for adult tissues are the long-lived self-renewing cellular component, which ensure that differentiated tissue-specific cells are maintained and replaced through the entire adult lifespan. Although much blood research is focused on hematopoietic tissue homeostasis, replacement and regeneration during adult life, embryological studies have widened and enriched our understanding of additional developmental hierarchies and interacting cells of this life-sustaining tissue. Here, we review the current state of knowledge of the hierarchical organization and the vast heterogeneity of the hematopoietic system from embryonic to adult stages.

CONCEPTS, FOUNDATIONS, AND HIERARCHIES OF THE BLOOD SYSTEM

Current hematopoietic cell hierarchical models began to take shape following WW2 and the realization that the blood system and particularly the stem cells of the vertebrate bone marrow (BM) are radiosensitive.[1] The discovery of potent BM cells that can regenerate the adult blood system led to the first (allogeneic) BM transplantation[2] and paved the way for the current use of hematopoietic stem cells (HSCs) in treatment of a wide range of (acquired and congenital) blood disorders, malignancies and a growing number of autoimmune diseases.[3] Despite the broad applicability of these transplantations, with often no curative alternatives, the lack of suitable donors and high risk of allogeneic complications/death highlight the need for an unlimited source of engraftable HSCs. Although great progress has been made, many years of research have not yet enabled us to safely engineer customized/patient-specific autologous or "universal" allogeneic long-term, high-level, self-renewing multilineage HSCs, or efficiently expand in vivo-generated HSCs with preservation of all their characteristics.[4–7] The mammalian embryo holds the key to overcome these challenges, given that de novo generation of HSCs from embryonic aortic endothelium occurs only during a brief time in early development. A better understanding of the molecular mechanisms behind the in vivo HSC fate/identity acquisition within the dynamic cell hierarchies in the embryo, is therefore of vital importance and is summarized in this review. Figure 1 shows a timeline summarizing major advances in hematology and hematopoietic development, and Figure 2 shows a simplistic overview of the developmental and adult hierarchies.

ORGANIZATION AND DE NOVO TEMPORAL GENESIS OF DIVERSIFYING CELL LINEAGES DURING EARLY DEVELOPMENT

Transient generation of "primitive" cells

The earliest blood cells appear in the extraembryonic yolk sac (YS) of developing vertebrates and are fleetingly produced. The discovery of blood islands of hemoglobin-containing cells in the chick YS contributed to the initial coining of the term "primitive erythropoiesis."[8] Since then, other "primitive" lineages have been identified in the embryo. These include primitive macrophages and megakaryocytes.[9–12] The primitive erythroid lineage emerges from mesodermal hemangioblastic chords containing polyclonal cells with bipotential and unipotential properties for endothelial and hematopoietic[13] and other mesodermal hemogenic endothelial cells.[14,15]

Initially, it was thought that primitive hematopoietic cells were short-lived and contributed exclusively to the embryonic blood system. Primitive erythrocytes have a limited life span and are known to provide an important role in oxygen transport with production of embryonic hemoglobin that carries oxygen at an affinity more amenable to in utero conditions. As the embryo grows too large to rely on the diffusion of oxygen, the extra- and intraembryonic vascular systems become connected and the circulation efficiently carries primitive cells to distal tissues, concomitant with a developmental molecular switch to fetal/adult type hemoglobin production.

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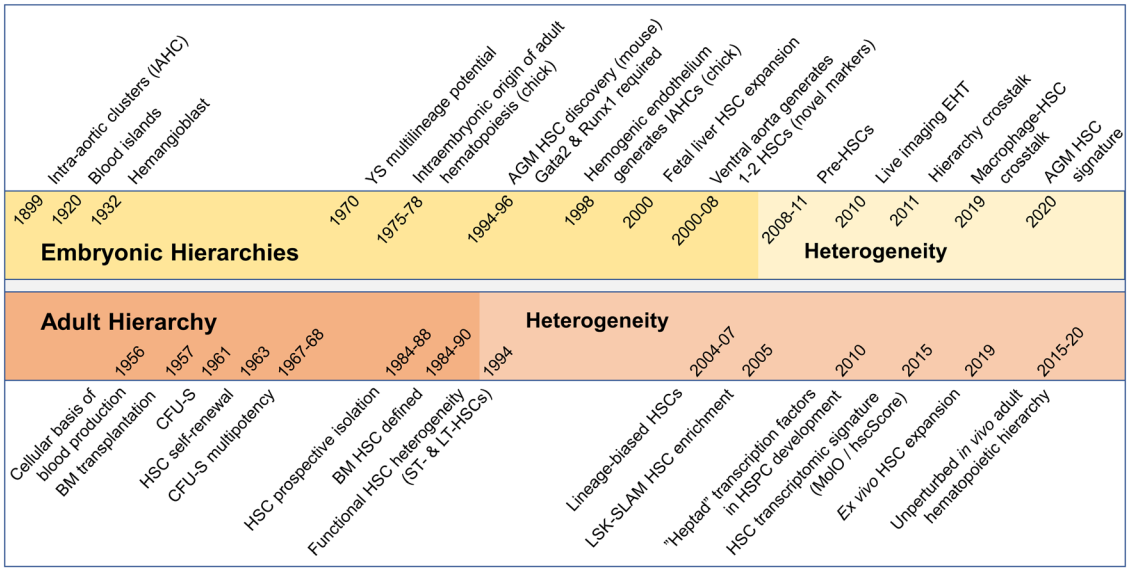

Figure 1. Timeline of major advances in hematology and hematopoietic development. Our understanding of hematopoietic hierarchies is shaped by major breakthroughs in the fields of developmental and adult hematopoiesis. These breakthroughs revealing the generation and organization of the hematopoietic system (from over a century ago to the present time) are summarized for the distinct embryonic hierarchies found in the YS, major vasculature (aorta, umbilical/vitelline arteries), and fetal liver (top panel). Breakthrough findings from the BM are summarized for the adult hematopoietic hierarchy (bottom panel). The foundations for the cellular basis of blood generation and the discovery of the HSC as the founder cell for the adult hierarchy led to the use of these self-renewing, multipotent cells in clinical transplantation therapies for hematopoietic disorders. Clonal transplantation analyses revealed the heterogeneity of HSCs (indicated by lighter-colored panels) in their lineage differentiation bias and longevity. Advancements in cellular (phenotypic, functional) and molecular (transcriptomics, regulatory networks) analyses led to further discoveries. See review for details and references. AGM = aorta-gonad-mesonephros; BM = bone marrow; CFU-S = colony-forming unit-spleen; EHT = endothelial-to-hematopoietic transition; HSC = hematopoietic stem cell; IAHC = intra-aortic hematopoietic clusters; ST-/LT- = short-term/long-term; YS = yolk sac.

In contrast, primitive macrophages are unique in their longevity and are represented by tissue-resident macrophages, such as the microglia that persist through adulthood in the brain.[16] They are produced independently of a monocyte intermediate that is characteristic of later de novo-generated hierarchies. Primitive macrophages play important roles in tissue remodeling and lymphatic development.[5,17] Primitive megakaryocytes have been shown to make platelets but further roles have yet to be determined.[11,18] Although most of these primitive cell types have been examined in mouse embryos, the human YS at 2–3 weeks postconception has been shown to contain similar primitive blood cells.[19]

The "pro-definitive" stage of hematopoiesis

A second transient wave of de novo hematopoietic cell production begins slightly after the initiation of the YS primitive wave. Various hematopoietic progenitor cells (HPCs)—unipotent definitive erythroid and multipotent myeloid (megakaryocyte, mast, granulocyte-macrophage) progenitors—are generated from hemogenic endothelium in the YS.[9–12,14,15,20] Collectively, these cells are known as EMPs (erythroid-myeloid progenitors). The pro-definitive hematopoietic wave begins just prior to the onset of circulation as the vascular system is becoming patent. The expression of CD16/32 (and lack of Sca-1/Ly6a) distinguishes these hematopoietic progenitors from the "primitive" wave cells.[20] Additional transient extra- and intraembryonic mesodermal tissues are developing simultaneously. Cells from the later primitive streak form the chorioallantoic (prospective) placenta, vitelline and umbilical vasculature, and also migrate into the embryo proper to form the aorta.[21,22] These tissues contain EMPs and have a strong myeloid cell potential. Interestingly, the YS-derived EMPs maintain erythropoiesis throughout mouse embryonic development until birth.[23]

After EMP generation in the pro-definitive stage, other more complex progenitors having lymphoid and multilineage (lymphoid-myeloid) potential are also produced in the YS. Lymphoid-primed multipotent progenitors (LMPPs) were discovered and upon differentiation, produce natural killer, T-cells and the B-1a (but not B-2) subset of B-cells.[6,24] Like microglia, B-1a B-cells are self-replenishing and can be considered tissue-resident cells (in the adult peritoneal and pleural cavities) in the sense that they cannot be generated from BM HSCs[25–28] or mouse fetal liver HSCs,[29] although the fetal liver result is still being debated.[30,31] Similarly, the production of T-cell progenitors by the YS is still debatable.[32]

In the 1990s, the intraembryonic mesodermal region (paraaortic splanchnopleura; pSp) was demonstrated to be a hemogenic site.[33–35] Before establishment of circulation in the mouse embryo, the pSp not only produces EMPs but also lymphoid progenitors for the adaptive immune system, as shown by explant cultures. This onset of lymphoid progenitor production in the pSp is earlier than that in the YS. In contrast to the YS, pSp LMPPs (or other precursors) are capable of autonomously producing both B-cell types[25,26,34,36] as well as giving rise to T-cells,[37] thus suggesting that the pSp may be the developmental source of more complex multipotent hematopoietic progenitors.

The pro-definitive wave of de novo hematopoietic generation includes in vivo multipotent colony-forming unit-spleen cells (CFU-S). Originally suggested by Till and McCulloch as the BM "stem cell" source of adult hematopoiesis,[38] CFU-S were found to be present in both the YS and aorta-gonad-mesonephros (AGM; descendent of the pSp) region of the early mouse embryo.[39] Within 8–14 days of transplantation, these progenitors form transient macroscopic erythroid-myeloid cell colonies on the spleens of lethally irradiated adult mice. It was found that AGM-derived CFU-S are more abundant than YS CFU-S and can expand in ex vivo explant cultures,[40,41] again suggesting that the intraembryonic AGM is a more potent source of cells for the adult blood system. Other in vivo multilineage-repopulating progenitors were discovered in the YS and pSp using neonatal and immunodeficient mouse transplantation models.[42–44] Importantly, the early de novo-generated primitive and

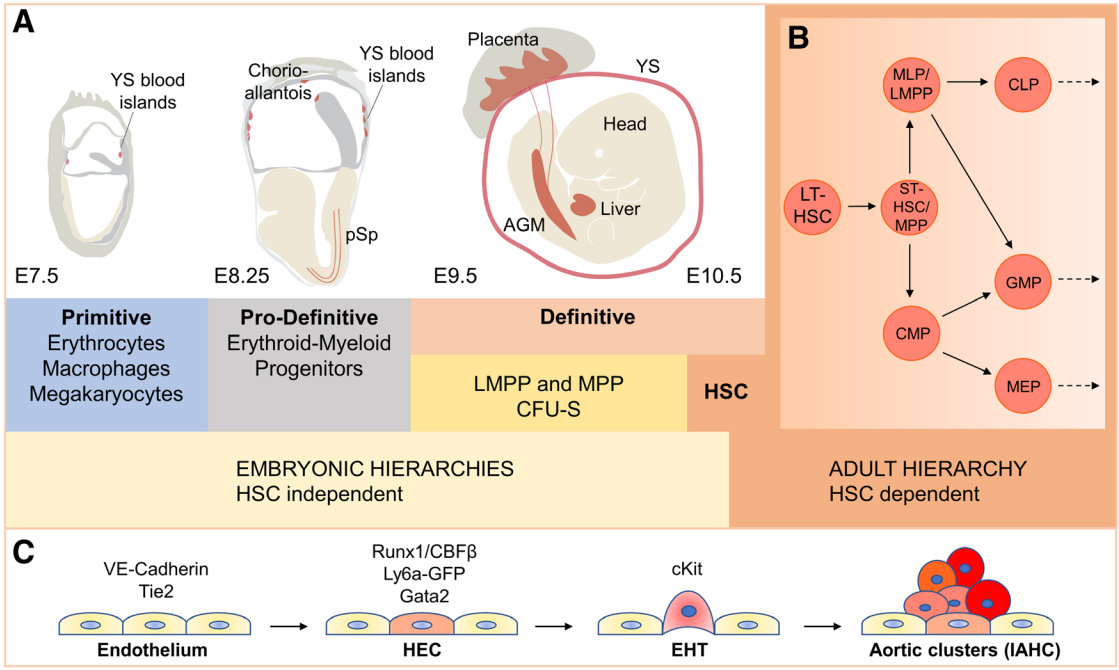

Figure 2. Overview of the developmental and adult hierarchies. (A) Embryonic hematopoiesis occurs in 3 waves. The first (primitive) wave starts at mouse embryonic day (E)7.5 in the YS blood islands with the formation of primitive erythrocytes, megakaryocytes and macrophages. The second (pro-definitive) wave starts just before the onset of circulation at E8.25 with the production of erythroid-myeloid progenitors from the YS. From around E9.0/9.5 these are also found in the blood, pSp, and chorio-allantois/placenta. From around E8.5/9.0, lymphoid potential from LMPPs is detected, slightly earlier in the pSp than YS. At E9.5, a MPP and CFU-S emerge in the YS and AGM region. In the third/adult-definitive wave (E10.5), the first long-term multilineage adult-repopulation HSCs are generated in the AGM. Slightly later, HSCs are found in the umbilical and vitelline arteries, placenta, YS, and head. They will next migrate to and expand in the fetal liver, together with EMPs. Schematic drawings show E7.5, E8.25 and E10.5 embryos with sites of hematopoietic activity colored in red. (B) Adult hematopoietic hierarchy with bifurcating differentiation pathways from the most potent long-term (LT-)HSCs to progenitor intermediates (arrows) and mature cell lineages (not shown; dashed arrows). Advances in single-cell transcriptomics have now led to a hematopoietic tree that depicts differentiation more like a continuum (not shown) rather than a highly-grouped hierarchy with fixed intermediate steps. (C) Representation of morphological and cell-specific marker changes during EHT in the midgestation mouse aorta. Through the expression of pivotal hematopoietic transcription factors (Runx1, Gata2), the HEC fraction (light red) of the endothelium, undergoes EHT to form IAHCs that gain hematopoietic characteristics (dark red) as they advance away from the endothelium. Once required/fully matured, the HSPCs will detach from IAHCs and enter circulation. Onset of expression of specific genes and markers is indicated above the schematic of the different EHT stages. AGM = aorta-gonad-mesonephros; CFU-S = spleen colony-forming unit; CLP = common lymphoid progenitor; CMP = common myeloid progenitor; EHT = endothelial-to-hematopoietic transition; GMP = granulocyte-monocyte progenitor; HSCs = hematopoietic stem cells; IAHCs = intra-aortic hematopoietic clusters; LMPPs = lymphoid-primed multipotent progenitors; MEP = megakaryocyte-erythrocyte progenitor; MLP = multilymphoid progenitor; MPP = multipotent progenitor; pSp = para-aortic splanchnopleura; ST = short-term; YS = yolk sac.

pro-definitive progenitors derived from the YS and pSp are not able to robustly long-term multilineage repopulate the hematopoietic system of adult irradiated recipients. They are considered as HSC-independent progenitors, since their presence in the embryo is prior to the appearance of robust in vivo-repopulating HSCs.[5,45]

The onset of definitive hematopoiesis—generation of adult HSCs and the adult hierarchy

HSCs that are the long-lived foundations of the adult hierarchy are the final hematopoietic cell type to be de novo specified and generated in the embryo. HSC generation occurs only during a short period of developmental time in vertebrate embryos—in midgestation in the mouse embryo, and in the human embryo around 4–5 weeks postconception.[19] In the 1970s exquisite embryo grafting/tracing experiments in chick (and later in amphibian) models of blood development provided the first indication that the life-long adult hematopoietic system originated intraembryonically and not from the extraembryonic YS.[46–49] However, this was controversial in mammalian embryos since in vitro colony-forming unit-culture (CFU-C) assays and in vivo CFU-S assays showed that bi- and multipotential progenitors were present in the YS, earlier than in the embryo body (minus the YS).[50] This dogma on the YS origins of mammalian adult hematopoiesis was finally altered in the 1990s when in vivo transplantations of mouse embryonic hemogenic tissues, including the intraembryonic AGM region, were performed.

The AGM was found to contain HSCs prior to the YS[51] and importantly, that HSCs were autonomously generated in AGM but not in YS explants.[40] These HSCs were found to robustly multilineage repopulate the hematopoietic system of irradiated adults for an entire lifespan and moreover, serial-transplantations demonstrated their stem cell self-renewal properties. HSC de novo generation was localized to intra-aortic hematopoietic clusters (IAHCs) on the ventral wall of the midgestation mouse aorta at the junction with the vitelline artery.[52–55] Out of several hundred IAHCs, which are mainly HPCs, only about 2 are functional HSCs.[56–58] The IAHCs have been considered a "pro-/pre-definitive HSC" compartment comprising of a series of intermediate cell types/states. Pro-HSCs, and Type(T)1 and T2 pre-HSCs are defined by a combination of cell surface markers.[59–61] Although not able to contribute to in vivo-repopulating activity in a direct transplantation scenario, their maturation to HSCs occurs when cultured in a reaggregate system prior to transplantation.

Other hemogenic tissues in the mouse embryo, including the umbilical and vitelline arteries,[62] the embryonic head[63] (demonstrated to generate hematopoietic stem and progenitor cells; HSPCs) and the placenta,[64–66] were also found to contain HSCs, and may be contributing to the cohort of adult HSCs found in the BM. Importantly, there is an exquisite conservation in the temporal and spatial de novo generation of the hematopoietic hierarchies and particularly HSCs, as demonstrated in the human embryo.[35,67]

Altogether, the formation of specialized hematopoietic cells (erythroid, myeloid, and lymphoid) of the primitive and pro-definitive hierarchies occurs, before and in the absence of HSCs, in the early embryo. The most potent and self-renewing stem cells, the HSCs forming the base of the adult HSC-dependent hematopoietic hierarchy, are the final embryonic de novo-specified cell type, and thus the existence of several waves of de novo hematopoietic cell generation has important implications for current approaches attempting the ex vivo production of functional human blood cells and HSCs.

ANCESTRAL RELATIONSHIPS OF DEVELOPMENTAL HIERARCHIES AND HEMATOPOIETIC LINEAGES

How do the primitive, pro-definitive, and definitive developmental hierarchies relate to each other? The advent of molecular manipulations in the mouse model, such as transgenic, knock-in, knock-out, and conditional gene targeting strategies for specific lineages and developmental time points, had a profound influence on understanding the origins, temporal/spatial appearance, heredity, and function of the cells in the distinct embryonic/fetal and adult hierarchies. Cell surface hematopoietic marker phenotyping/sorting of the cells from the embryonic hemogenic sites and multistep culture systems begin to implicate cell-cell interactions between the distinct developmental hierarchies.

Histology: static and vital

The early observations of IAHCs on the lumenal wall of the aorta in a variety of species of vertebrate embryos paved the way for the discovery of hemogenic endothelium.[52,68,69] Notably, IAHCs appear in the human embryo at gestation week 4–5.[70,71] Experiments in the chick embryo, in which aortic endothelium was dye-marked before hematopoietic cluster formation, demonstrated the subsequent appearance of dye-positive hematopoietic cells and firmly established the endothelial cell-to hematopoietic cell-transition (EHT).[69] In mouse, the generation of definitive HPCs and HSCs was found to correspond to the appearance of clusters of hematopoietic cells in the embryonic aorta, and vitelline and umbilical arteries.[56,72] Indeed, most IAHCs (as well as all functional HSCs) were shown to be absent in mouse models in which pivotal hematopoietic transcription factor genes *Runx1* and *Gata2* (amongst others) were deleted in the germ line[5,73,74] or conditionally deleted in the embryonic endothelium.[75–77] Visual time-lapse imaging proof of EHT was obtained from genetically marked zebrafish embryos and thick mouse AGM sections, during the time of hematopoietic cell emergence.[78–80] A transgenic mouse line expressing GFP under the regulatory control of the stem cell marker Sca-1 (Ly6a-GFP;[52,81]) demonstrated the real-time emergence of rare GFP$^+$ hematopoietic cells from GFP$^+$ aortic endothelial cells in the midgestation embryo.[79] Such aortic GFP$^+$ cells, when in vivo transplanted, were found to be fully functional and robust self-renewing HSCs.[52,82] EHT and cluster formation also occurs in the YS in the late pro-definitive wave of hematopoietic cell generation.[83] Recently, EHT has also been observed from BM hemogenic endothelium in the late fetus/young adult chick and mouse.[84] Some de novo generation of HSPCs also appears to occur in the placenta.[66]

Hematopoietic transcription factors: the heptad

The association between transcription factors known to be dysregulated in leukemias and other hematopoietic disorders prompted the examination of their role in normal healthy hematopoietic development. Hematopoietic hierarchies all through development are dependent on a heptad of transcription factors: Gata2, Lmo2, Erg, Fli1, Scl, Runx1, and Lyl1. Together, these heptad factors (in various combinations) activate hundreds of target hematopoietic genes. This was shown in genome-wide screening of consensus transcription factor-DNA binding sites in mouse HPCs.[85] The analogous heptad factor panel was also shown in human HSPCs.[86] These critical hematopoietic heptad factors and others such as Notch and Myb were extensively examined in genetically modified mice (loss/gain of function).[6,17,85,87–89] When deleted in the germ line or in specific lineages, individual factors showed varying effects on hematopoietic cell development and differentiation. Some had profound effects on primitive as well as definitive hematopoiesis, and others such as *Gata2* and *Runx1* were found to be essential in the pro-/definitive stages, whereas primitive hematopoiesis was generally normal.[90,91] Homozygous deletion of these pivotal transcription factors in the germ line resulted in fetal liver anemias and midgestation embryonic lethalities. IAHC formation was disrupted, HPCs were decreased in number or absent, and no functional HSCs were generated. Endothelial-targeted deletion (*VE-Cadherin/Cdh5-Cre*) of *Runx1* abolished HSC formation at the EHT stage,[76] but deletion in *Vav1*-expressing (committed HSC) cells produced no hematopoietic defects[76,92] indicating that the requirement for Runx1 is lost once HSCs are specified. In contrast, similar targeted deletion of *Gata2* shows that it is required not only for EHT and HSC generation but also for the life-long maintenance of HSCs.[77]

Dysregulation of hematopoietic development was also observed in transcription factor haploinsufficient embryos, further underlining the importance of gene dosage in the exquisite control of hematopoietic development or differentiation that likely predisposes for leukemia.[93–95] Inappropriate levels of mouse *Gata2* expression, such as in haploinsufficiency or overexpression lead to disruption of HSC generation, proliferation, and survival.[74,96–100] Haploinsufficiency of *Runx1* has less severe consequences, changing the timing and possibly the sites of HSC generation in the embryo, whereas adult hematopoiesis appears unaffected. Upstream of *Gata2* and *Runx1* transcriptional regulation, several pathways such as Notch,[96] Bmp4,[101,102] or ETS/Erg[103] have been found.

Lineage tracing and clonal marking

A variety of lineage tracing strategies in mouse embryonic, fetal, and adult stages of blood system development have clearly demonstrated that several mature lineages of hematopoietic cells are generated independent of HSCs.[5,45,104] One example is found in the YS-derived primitive cells that become the microglia (tissue-resident macrophages) of the brain. Microglia develop in the absence of *c-myb*, which is required for EMPs, multipotent progenitors, pre-HSCs and HSC formation.[5,45,105] Genetic pulse labeling of *Tie2*-expressing endothelial cells at the primitive YS stage[106] demonstrated that the hematopoietic cells in the fetal brain are derived from the primitive YS-labeled cells. Other experiments using genetic pulse labeling in cells expressing *Runx1* (hemogenic endothelium[16]), *Csf1r* (macrophages[106]) and *cKit* (hematopoietic progenitors[107]) showed that there are different/changing levels of labeled macrophage progeny in the late embryo and the adult mouse, suggesting that tissue-resident macrophages may be replaced with time, especially under conditions of trauma, disease, or inflammation. Presently, the origin of lung and liver tissue-resident macrophages remains controversial.[108,109]

Important insights into the dynamics and longevity of the hematopoietic progeny emerging from the pro-definitive and definitive stages of de novo HSPC generation were obtained from genetic pulse labeling of mouse embryos. Labeling of cells expressing the vascular endothelial cadherin (*Cdh5*) in the mouse embryo[75,76] confirmed that functional HSCs are generated from endothelial cells, and that their progeny contribute to fetal liver, adult BM, thymus, and spleen cells.[75] To determine in what window of developmental time HSCs are generated, temporal pulses of recombination labeling were performed with *Cdh5-CreERT*. Temporal pulsing (with 4-hydroxy-tamoxifen) during the primitive and early pro-definitive stages did not yield

BM HSCs in the adult. However, pulsing slightly before the appearance of the first HSCs in the AGM, did result in labeled progeny in adult BM that included HSCs and cells of the adult hematopoietic differentiation hierarchy. Pulse labeling 1–2 days later similarly resulted in marked progeny in the adult BM, whereas subsequent days of pulse labeling did not. Hence, adult BM HSCs are de novo generated from hemogenic endothelium during a short window of development time (2–3 days) in the midgestation mouse embryo. HSCs generated in the midgestation embryonic aorta do not differentiate into mature blood cells in this niche.[110,111] Instead, these HSCs migrate to the fetal liver, which is a temporary niche for their proliferation and differentiation.[112] Before birth, the BM is established and becomes the permanent niche for HSCs. A recent *Cdh5-CreERT* reporter study uncovered hemogenic endothelial cells in the BM during late fetal and early neonatal stages, some of which de novo-generated HSPCs.[84]

Crosstalk between the hierarchies

A relatively new and important concept has emerged from studies in mouse embryos that have asked whether an adult blood system can be generated in the absence of cells from the earlier hematopoietic waves, or put differently, do the early (primitive) hematopoietic cells provide a niche or inducing function for HSPC generation? This question has particular relevance to our inability to generate robust in vivo-repopulating HSCs from in vitro (embryonic stem cell [ESC]-derived) hematopoietic differentiation cultures.

The evolution of the hematopoietic system began in invertebrates with the simple production of oxygen-carrying cells and innate immune myeloid cells (for growth, survival, tissue remodeling). Later, multipotent and lymphoid progenitors with more complex functions appeared, and finally self-renewing HSCs were generated, and formed the basis of the long-lived robust adult vertebrate hematopoietic system. Mammalian embryos reflect this in the primitive, pro-definitive, and definitive waves of de novo hematopoietic generation (Figure 2). This sequence of blood cell generation is well conserved in all vertebrate embryos thus far examined.

To address crosstalk between hierarchies, complex transgenic mouse models in which the Runx1 transcription factor partner *CBFβ* was deleted, allowed an interesting rescue experiment to be performed. Whereas homozygous deletion of *CBFβ* results in the absence of EMPs and all HSCs in the midgestation embryo, early expression of a *Tie2-CBFβ* transgene in endothelial cells can rescue EMPs but not HSCs. The expression of a *Ly6a-CBFβ* transgene in aortic hemogenic endothelium would be expected to rescue HSCs (see Figure 2A and C). However, functional HSCs in the AGM were not generated. Only when *CBFβ* knockout embryos expressed both the *Tie2-CBFβ* and *Ly6a-CBFβ* transgenes, were AGM HSCs generated.[113] These results suggest that early cohorts of hematopoietic cells (progeny of primitive, pro-definitive, or EMP waves of generation) interact or signal aortic endothelial cells to induce HSC potential or generation.

This "cell-cell interaction" model is supported by experiments in which macrophages from the earliest wave in the YS were ablated,[114] in line with other reports on the essential role of pro-inflammatory signals by (other) primitive cells on HSC emergence.[115–117] Mariani et al.[114] found that Cx3cr1 mediates YS macrophage progenitor recruitment to the AGM niche. Vital time-lapse imaging shows that these macrophages dynamically interact with emerging IAHC cells that are expressing HSC markers. Thus, primitive macrophages and other pro-inflammatory signals are positive regulators of HSC generation and are one of the AGM niche elements required in the definitive wave of HSC generation, and may be part of the niche necessary for the generation of adult HSCs from ESC and induced pluripotent stem cell (iPSC) differentiation cultures.

HETEROGENEITY OF HEMATOPOIETIC CELLS DURING DEVELOPMENT AS REVEALED BY SINGLE-CELL TECHNOLOGIES

Despite the rarity of HSCs as they are generated in the AGM region, their number increases in the fetal liver (through differentiation of pre-HSCs or HSC expansion) to the approximate number found in the adult BM.[59,60,112,118,119] As compared to other hematopoietic cell types in the BM (hematopoietic progenitors, intermediate, and differentiated cells), the number of BM HSCs is rare, comprising about 0.05% to 0.0058% of the adult BM hematopoietic cell compartment.[120,121]

A long-standing challenge has been to isolate HSCs to purity from BM and other tissues such as the fetal liver and AGM region, and to compare their properties such as multilineage potential, lineage bias, metabolic state, self-renewability, and genetic program, at the different developmental stages. Although clonal transplantation analyses demonstrated that single HSCs differ in their relative myeloid and lymphoid outputs (lineage bias),[122] it was postulated that HSCs differ to some degree due to the fact the niche cells that support their generation, expansion and maintenance are quite different.[123,124] Notwithstanding the importance of the extrinsic microenvironment and roles of the various niches throughout development, we focus here on the intrinsic programs of HSCs in the AGM, fetal liver, and adult BM, and summarize studies examining whether the transcriptome of an HSC can be identified and compared between the developmental stages.

Multiplex gene expression before hematopoietic lineage restriction was first examined in single cells of a mouse HPC line and showed the expression of genes related to several differentiated blood lineages.[125] Assumptions were made that stepwise lineage restriction takes place, with lineage potentials segregating in multiple progenitor stages following the HSC stage. In this regard, it is interesting to consider the pivotal hematopoietic transcription factors Runx1 and Gata2. Single-cell expression profiling shows transcripts of both genes in emerging IAHCs/HSCs,[58] fetal liver HSCs,[126] and adult BM HSCs,[127,128] yet gene expression does not always result in an easily measurable cellular function.[129] For example, Runx1 expression or function in the YS primitive stages has been controversial.[130] Splice variants, lineage-specific enhancer elements, varying transcriptional start sites, isoforms, hetero/homodimerization, and pulsatile expression add to the complexity and function of pivotal hematopoietic transcription factors Runx1 and Gata2.[93,130–132] Onset of the hematopoietic transcriptional program occurs with the emergence of the first functional HPCs and IAHCs and is 1 full day before functional HSC detection in the aorta.[133] Hematopoietic fate acquisition, as shown in a mouse ESC model, occurs through *Runx1*-dependent organization of (heptad) transcription factor assemblies at the enhancer/promoter regions[134] and highlights how early combinations of programs could establish the diversity of hematopoietic cells.

If the Waddington epigenetic landscape on the canalization of cells in directional differentiation paths holds true,[135] and there is a distinct and stable program that leads to terminally mature functional blood cells, then it is difficult to explain the layered waves of hemogenesis in the embryo. Many differentiated blood cells are made prior to, and independent of, HSCs and it is possible to convert hematopoietic cells from one lineage to another through the induced expression of specific hematopoietic transcription factors.[136] For example, mouse B-cell transduction with the C/EBPα transcription factor converted them to macrophages through a granulocyte-macrophage progenitor-like transition, thus implicating a level of molecular interconvertibility or transition states in the adult hierarchy.[137] This raises the question—How stable are the transcriptional programs of HSCs or HPCs?

Improved multiparameter immunosorting, clonal functional analyses, and innovative single-cell RNA sequencing (scRNA-seq) methods have together provided insight into the level of intrinsic diversity/similarity of developing hematopoietic cells. Despite the popularity and ease of 10× scRNA-seq, it has shortcomings for the careful study of rare HSCs and low-abundance transcripts.[138] Instead, Illumina (SmartSeq2) scRNA-seq of highly enriched sorted cells generates deep sequence reads that can be used to examine small changes in expression levels, thus allowing classifier gene lists for HSC signatures.[139] Pioneering studies examining highly enriched HSCs and HPCs from the adult BM hematopoietic hierarchy, produced a single-cell resolution map showing that specific characteristics such as metabolic and cell cycle status are not absolute and that early LMPPs and HSCs share common transcriptional networks.[127] Pseudotime ordering of single cells revealing possible dynamics of cell fate regulation[140,141] and diffusion maps[142] resulted in a predictive score for HSC identity, the MolO score and improved hscScore.[128,143,144] The recent establishment of an in vitro HSC expansion culture[145] has led to the identification of single HSC transcriptome signatures and classifiers for human HSCs.[146] Additionally, the unperturbed in vivo adult hematopoietic hierarchy is a focus of profiling studies using unique barcoding systems combined with scRNA-seq.[147–150] Altogether, these single-cell approaches have changed our view on the hierarchical organization of hematopoiesis, switching from discrete compartments containing HSCs and HPCs to a continuum of differentiation with less prominent reservoirs.

ScRNA-seq of surface marker-enriched mouse fetal liver and AGM cells has been performed by several groups.[58,126,141,151–157] The transcriptomes of pre-HSCs (CD45$^-$, T1; and CD45$^+$, T2)[59,60,126] and fetal liver HSCs[126] showed overlap between T1 and T2 pre-HSC clusters by expression dynamics (principal component analysis), but no overlap between pre-HSC and HSC clusters. Many processes and signaling pathways were overrepresented in T1 pre-HSCs, including cytoskeletal-coupled cellular processes, ribosomal subunits, and the mTOR pathway. RICTOR, an mTOR core component was found to be expressed in AGM HSCs but not HPCs.[126] Similar RNA-seq studies also identified the expression of CD27 (*Tnfrsf7*)[58,158] on AGM HSCs. Transcriptomics data have expanded our knowledge and provided important insights into some of the genes that could be involved in the first HSCs, yet heat maps of these enriched single cells still showed heterogeneity.

To more specifically identify the transcriptome of the first functional HSCs emerging in the mouse embryonic aorta, scRNA-seq was performed on highly enriched mouse embryo IAHCs. As the simultaneous analysis of transcriptome and function cannot be performed on the same single cell, iterations of single-cell sorting, sequencing, and functional analyses were performed for high-resolution correlation. Based on the expression of the Gata2 transcription factor and additional surface markers, a convincing profile for the first rare functional HSCs was obtained.[58] From this dataset (of greater than 1000 sorted Gata2-medium expressing [Gata2^{med}] CD31$^+$cKit^{high} IAHC cells), 2 major transcriptomic clusters were found. Functional HSCs were identified in one cluster. Index sorting together with iterative single-cell approaches defined the specific parameters for the high enrichment of HSCs—Gata2^{med}CD31^{high}cKit^{high}CD27^{med}*Ly6a*$^+$. All heptad transcription factors were expressed in the HSCs with some small expression level differences. Surprisingly, the other major cell cluster was *Runx1*$^{-/low}$ and showed no hematopoietic function. This could indicate that these *Runx1*$^{-/low}$ cells are non-hematopoietic by-products of EHT, and possibly arise due to stochastic expression of heptad transcription factors in single cells.[58] These cells may be an essential component of a juxtaposed niche.[58,156,157]

Comparison with other published AGM HSC datasets[126,157] is ongoing and computational analyses are yielding classifier genes that could predict long-term HSC repopulating function from mouse embryonic cells. Further comparisons are underway with single cell datasets of fetal liver and adult BM HSCs to establish the degree of similarity and/or heterogeneity between the HSCs of the developmental stages. Such comparisons will not only allow insights into the specific programs underpinning HSC function but provide clues as to the specific levels, timing and combinations of core HSC genes for the de novo generation of HSCs ex vivo.

SUMMARY

Blood development in vertebrates during embryonic stages is dynamic and is composed of several overlapping layers of hemogenetic events to produce independently generated hematopoietic hierarchies. Mounting evidence implicates a "needs based" physiologic system in which vertebrate embryos generate unique types of primitive and pro-definitive hematopoietic cells at early stages to carry out specific developmental functions. Also, they are likely to prime the developing secondary fetal and adult hematopoietic tissues for reception of the oncoming adult hematopoietic hierarchy of cells. Leaving behind previous hierarchies (which are extinguished or are a long-lived tissue-resident component), an increasingly diverse and heterogeneous hematopoietic system derives from HSCs (the last hematopoietic cell type to emerge in the embryo). This cohort of long-lived multipotent self-renewing HSCs is the basis of adult hematopoiesis and its generation is strictly dependent on the earlier waves.

Molecularly, the layered cellular hierarchies express similar but unique combinations of pivotal intrinsic regulators. Upregulated pivotal transcription factors (heptads and others) during the early onset of hematopoietic fate await full characterization of their downstream targets and importantly, how small expression level changes (such as haploinsufficiency) and combinatorial interactions of transcription factors can lead to diversity in target activation. Single-cell technologies are beginning to reveal the overlaps and differences between the emerging hematopoietic cells and to identify new markers necessary for iterative enrichments to understand the state of transcription factor dynamics and stability. Algorithms have yielded predictions for HSC programs, and further refinement of HSC scores will allow associations of specific genes/groups of genes with specific HSC properties, such as self-renewal, multilineage/lineage bias, proliferation, and longevity, that hold promise for the de novo generation of HSCs for biomedicine. Although closer to an understanding of the programs required to establish the diversity of hematopoietic cells, innovative technical advances with single cells are still needed for simultaneous "omics" analyses (transcriptome, proteome, other) and the testing of in vivo hematopoietic cell function.

AUTHOR CONTRIBUTIONS

CSV and ED wrote the review and made figures. SAM involved in research.

DISCLOSURE

The authors declare no conflicts of interest.

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