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Erythro-Myeloid Progenitors: “definitive” hematopoiesis in the conceptus prior to the emergence of hematopoietic stem cells

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Abstract

Erythro-myeloid progenitors (EMP) serve as a major source of hematopoiesis in the developing conceptus prior to the formation of a permanent blood system. In this review, we summarize the current knowledge regarding the emergence, fate, and potential of this hematopoietic stem cell (HSC)-independent wave of hematopoietic progenitors, focusing on the murine embryo as a model system. A better understanding of the temporal and spatial control of hematopoietic emergence in the embryo will ultimately improve our ability to derive hematopoietic stem and progenitor cells from embryonic stem cells and induced pluripotent stem cells to serve therapeutic purposes.

Keywords

Yolk sac; hematopoiesis; erythro-myeloid progenitors; embryogenesis

Introduction to hematopoietic ontogeny

In the adult, both steady state and stress hematopoiesis are dependent on the function of HSCs. While HSCs largely remain quiescent to prevent exhaustion of the hematopoietic system, they must also continuously replenish myeloid- and lymphoid-committed progenitors to supply the need for mature blood cells. The emergence of hematopoietic potential has been most extensively analyzed in the mouse embryo, given the availability both of colony and transplantation assays that functionally define the presence of progenitors and stem cells, respectively, in developmental time and space. The hierarchy of stem and progenitor cells found in the adult is first established during embryonic development, with the emergence of small numbers of HSC beginning at embryonic day (E)10.5 and 5 weeks, in murine and human embryos, respectively [1,2]. However, blood cells are required for embryonic survival prior to the emergence of these first transplantable, adult-repopulating HSCs, and the embryo meets these needs by the transient production of a subset of hematopoietic progenitors in the extraembryonic yolk sac [3–5].

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The first detectable hematopoietic progenitors are found in the yolk sac of the mouse embryo beginning at E7.25. The predominant lineage, in terms of progenitor numbers, is primitive erythropoiesis [4]. The classification of circulating mammalian embryonic erythroblasts as a “primitive” erythroid lineage was initially based on the large size and nucleated status of circulating cells, characteristics associated with the nucleated cells present in fetal and adult non-mammalian species [6]. Extensive analyses of hematopoietic colony-forming activity indicated that the emergence of primitive erythroid progenitors is associated temporally and spatially with macrophage and megakaryocyte lineages [4,7]. These data support the notion that “primitive” hematopoiesis emerges in the yolk sac and is restricted to primitive erythroid, megakaryocyte and macrophage lineages.

Shortly after the onset of primitive hematopoiesis, the first “definitive”, or adult-like, erythroid progenitors, termed burst-forming units erythroid (BFU-E), emerge in the yolk sac of the mouse conceptus beginning at early somite pair stages, i.e. E8.25 [4,8]. Neutrophil, mast cell, and granulocyte-macrophage progenitors display similar temporal kinetics of emergence. Importantly, high proliferative potential colony-forming cell progenitors, that contain multiple myeloid lineage potential and are capable of limited self-renewal expansion, also first emerge in the E8.25 yolk sac just prior to the onset of circulation [9].

Studies in the zebrafish embryo have also provided evidence that distinct primitive and definitive erythroid cells, associated with myelopoiesis, also emerge prior to presumptive HSC [10]. As these HSC-independent definitive progenitors were observed to produce definitive erythroid and myeloid cell types, but not to colonize the zebrafish thymus, this population was termed EMP (erythro-myeloid progenitors). We propose continued use of the term EMP, as it appears to accurately describe this progenitor population in the mouse and zebrafish embryos, as will be discussed. Despite the partial temporal overlap of their emergence within the yolk sac, primitive and EMP-definitive progenitors are distinguishable by their progeny, and should be classified as distinct hematopoietic waves. While the term “definitive” has been used by several authors to refer exclusively to adult-repopulating HSC activity, we propose that “definitive” also accurately describes EMP-derived hematopoiesis, since it contains robust definitive erythroid fate and potential. Describing EMP as “definitive” also recognizes that for over 100 years the terms “primitive” and “definitive” have been used to describe the two distinct types of erythroid cells that sequentially circulate in the embryo [6].

Lineage potential and function of EMP

EMP serve as a major source of fetal erythropoiesis

At first glance, the presence of multiple waves of hematopoietic potential in the embryo, including primitive-, EMP-, and HSC-derived hematopoiesis, each with distinct temporal and spatial regulation, seems unnecessarily complicated. However, evidence underscoring the importance of both the primitive and EMP-definitive hematopoietic waves can be drawn from the characterization of embryonic and fetal erythropoiesis. Primitive erythroblasts are distinguished from their definitive counterpart by their large size, expression of embryonic globin genes, and their concurrent maturation and function in the embryonic circulation [11–16]. These differences have enabled us to analyze the relative contribution of each lineage to the fetal circulation over time [12].

The E7.5 murine embryo has less than 20,000 cells [4], but needs to apportion some of these cells as hematopoietic progenitors to provide the first circulating (primitive) erythroid cells, which semi-synchronously mature as they proliferate in the newly established circulatory system (reviewed in [17,18]). By E12.5, they have differentiated into late-stage erythroblasts that are no longer capable of cell division, but rather begin to enucleate to become primitive

erythrocytes [12,16]. However, the continued rapid growth of the mouse embryo requires increasing numbers of erythrocytes to meet tissue oxygen requirements. These requirements are met by the egress of large numbers of enucleated definitive erythrocytes from the fetal liver. Though EMP-derived definitive erythroid progenitors (BFU-E) first arise and expand in numbers in the murine yolk sac between E8.25-E10, by E10.5 over a thousand BFU-E have colonized the newly forming liver [4]. At E11.5, when the first HSC arrives from the aorta-gonad-mesonephros (AGM) [19,20], robust erythropoiesis has already generated all stages of erythroid maturation, culminating in the initial release of the first definitive erythrocytes into the fetal circulation at E11.5 [21]. Taken together, these data support the concept that the first circulating definitive erythrocytes in the mouse embryo ultimately originate from yolk sac-derived EMP.

By E14.5, over 20 million definitive erythrocytes are enumerated in the fetal circulation, far surpassing the numbers of circulating primitive erythrocytes [22]. During this time, HSCs are rapidly increasing in number within the fetal liver [1,19,20]. As EMP- and HSC-derived erythrocytes are morphologically identical, it is currently unclear precisely when the transition from EMP-derived to HSC-derived hematopoiesis occurs. The emergence of transplantable HSC between E10.5 and E12.5 in the aorta, vitelline, and umbilical arteries [1,23,24] commonly results in the notion that HSCs are the sole source of hematopoiesis at this gestational stage. In contrast, detailed analysis of fetal erythropoiesis suggests a model where all three waves of hematopoiesis are present in different phases, with 1) primitive hematopoiesis providing the majority of circulating blood cells, 2) EMP rapidly expanding in numbers and maturing in the fetal liver, and 3) HSC in the initial stages of fetal liver colonization and expansion (Figure 1). Of note, analogous observations have been described in the human embryo from 4 to 6 weeks of gestation [2,25–27].

Recent studies have uncovered distinct capabilities and regulatory mechanisms governing EMP-definitive erythropoiesis. While bone marrow-derived erythroblasts from mouse and human have the ability to proliferate for a limited time *in vitro* when cultured in EPO, SCF, and dexamethasone [28], definitive erythroblasts derived from murine yolk sac and fetal liver have the unique capacity to proliferate for several months when cultured *ex vivo*, all the while maintaining their capacity to differentiate into enucleated erythrocytes [29]. The capability of murine yolk sac and early fetal liver to serve as sources of extensively self-renewing erythroblasts correlates temporally and spatially with the emergence of EMP in the yolk sac and their transition to the fetal liver. Interestingly, primitive erythroblasts lack significant expression of the glucocorticoid receptor and are unable to self-renew *ex vivo* [29].

In addition to the *ex vivo* self-renewal capability of EMP-derived erythroblasts, EMP-derived erythroblasts *in vivo* uniquely regulate the β -globin gene locus. While adult mouse bone marrow-derived definitive erythroblasts only express adult $\beta 1$ - and $\beta 2$ -globins, EMP-derived erythroblasts also express low levels of the embryonic $\beta h 1$ -globin gene [21]. The further study of globin gene regulation during fetal erythropoiesis may provide important insights toward the reactivation of fetal hemoglobin in patients with β -hemoglobinopathies.

EMP-derived megakaryopoiesis and myelopoiesis

The primitive and EMP-definitive waves of hematopoiesis share erythroid, megakaryocyte and macrophage potential. Though primitive and definitive erythroid cells have distinct differences in morphology and globin gene expression, little is known about the differences between primitive and definitive megakaryocytes and macrophages. Megakaryocytes derived from the E7.5 yolk sac formed colonies more rapidly than those derived from the adult marrow [30], but it is not known if EMP-derived megakaryocytes mature more rapidly than adult-derived megakaryocytes. Both primitive and definitive megakaryocytes form

proplatelets *in vitro*, and are likely to contribute to the production of embryonic platelets, which first begin to circulate in the mouse embryo beginning at E10.5 [7]. While the function of embryonic platelets remains poorly understood, a role for platelets in the formation of lymphatic vessels in the mouse embryo has recently been established [31].

Primitive and EMP-derived macrophages with similar maturational characteristics have been described in the mouse yolk sac [32]. There is considerable evidence in several model organisms that microglia in the central nervous system are initially derived from the yolk sac [33–36]. Long-lived resident macrophage populations in the central nervous system, liver and skin have recently been shown to arise independently of HSCs [37]. However, it remains unclear if these cells are derived from both primitive and EMP-definitive waves of hematopoiesis. In addition to their well-known role as scavengers of apoptotic cells and debris throughout the embryo, macrophages also constitute a component of the erythroid niche, physically associating with erythroblasts in the fetal liver and the bone marrow in erythroblastic islands [38] (reviewed in [39,40]). While the function of these macrophages during erythroblast maturation remains poorly understood, they certainly serve an important role in the disposal of pyrenocytes generated from the enucleation of maturing primitive and definitive erythroblasts [14,15,41,42].

Despite the emergence of granulocyte progenitors in the E8.5 yolk sac [4], relatively little is known about the ontogeny or embryonic function of the neutrophil lineage. A recent analysis demonstrates circulating murine fetal granulocytes to have limited ability to adhere and roll on endothelium before E15.5 [43]. The developmental origin of these fetal neutrophils remains to be investigated.

Requirement of EMP function for fetal survival

The overlapping temporal emergence of all three hematopoietic waves, along with the shared expression of hematopoietic markers and regulators such as CD41 and kit, has made fate mapping of the hematopoietic lineage contribution and lifespan of each wave [44–46] difficult to determine. However, the targeted disruption of several genes in murine embryos has provided some insights into the critical time windows during which each hematopoietic wave is required. Loss of the transcriptional regulators *Scf/Tal1*, *Gata1*, and *Lmo2* each ablates primitive erythropoiesis, resulting in embryonic lethality by E10.5 [3,47–49]. Conversely, loss of *c-Myb* preserves primitive erythropoiesis [50], but results in the absence of both EMP- and HSC-definitive erythropoiesis, which results in fetal death by E15.5 [51]. Finally, genetic rescue experiments have also provided some insight regarding the contribution of EMP to embryonic blood cell production [5,52,53]. Recent studies have revealed that the preservation of EMP emergence in the absence of transplantable HSCs enables mouse embryos to survive until birth [5]. These embryos demonstrated full myeloid capacity, with a significantly reduced but present lymphoid differentiation *in vivo* [5,53]. These data demonstrate the ability of rescued EMP to provide the mouse embryo with enough blood cells to survive to birth. Interestingly, these findings also highlight the existence of an HSC-independent lymphoid fate, as suggested by others [54–56] (reviewed in [57]). Importantly, this study also demonstrated that loss of EMP, despite the presence of transplantable HSCs, leads to embryonic lethality by E13.5 [5]. These genetic models highlight the requirement for transient fetal erythropoiesis and myelopoiesis to sustain embryonic survival until sufficient functional HSCs can be formed.

Lymphoid and long-term hematopoietic potential in the yolk sac

The term EMP implies that these yolk sac-derived, HSC-independent hematopoietic progenitors are devoid of lymphoid potential. This nomenclature was based on a lack of thymus colonization by EMP in the zebrafish embryo [10]. It has been asserted and widely

assumed that the yolk sac only provides erythro-myeloid lineages and that full lympho-myeloid potential is associated with the emergence of HSC [58]. Fully potent HSCs that can engraft adult murine recipients are first detected in the aorta, umbilical and vitelline arterial regions of the embryo beginning at E10.5 [1,23]. However, the finding that certain lymphoid subsets may be uniquely formed during embryogenesis [59,60], and temporally coincide with the emergence of EMP-definitive hematopoietic potential suggests the presence of HSC-independent lymphoid progenitors [54–56]. Moreover, the demonstrated presence of long-term lympho-myeloid potential at E9.0 that is capable of engrafting newborn mouse recipients [61,62], or adult mice after a period of *in vitro* stromal co-culture [63,64], further complicates simplified models of hematopoietic ontogeny. We collectively refer to these cells as “immature HSCs”.

There is some evidence suggesting EMP are distinct from early lymphoid progenitors and immature HSCs. While there are only a handful of lymphoid progenitors and immature HSCs in the yolk sac between E9.0 and E10.5 [55,61], there are hundreds of EMP-derived hematopoietic progenitors in the yolk sac by E9.5 and over a thousand throughout the embryo by E10.5 [4]. Moreover, B-lymphocyte potential prior to 24 somite pairs is restricted to the CD41-negative cell fraction, suggesting that CD41-negative lymphoid progenitors emerge separately from CD41-positive EMP [44,55]. Therefore, it remains to be clarified if EMP constitute a distinct population from immature HSC or lymphoid potential present in the yolk sac. However, the numbers and phenotype of the yolk sac lymphoid potential remains inconsistent with the temporal kinetics and robust erythro-myeloid potential of EMP, suggesting that EMP do not contain lymphoid potential. Taken together, these studies also suggest that the hematopoietic output of hemogenic endothelium is extremely varied.

Developmental origin and regulation of EMP

While zebrafish EMP are observed to both arise and differentiate within the posterior blood island [10], the first EMP in mammals emerge in the extraembryonic yolk sac but differentiate in the fetal liver [25,65]. In the human embryo, BFU-E and granulocyte-macrophage progenitors first emerge in the yolk sac and subsequently decrease in number as they increase in numbers in the fetal liver [25]. A similar kinetic pattern has been observed in the murine conceptus [4], which is consistent with the idea that yolk sac-derived EMP migrate to the fetal liver via the newly established circulatory system [65]. This idea is further supported by the failure of EMP to enter the embryo proper in circulation-deficient *Ncx1*-null embryos or in VE-cadherin-null mouse embryos, which lack an intact vasculature [65,66]. All hematopoietic progenitor activity in these mutant embryos remains confined to the yolk sac. It remains formally possible that the smaller numbers of colony-forming units observed in the embryo proper prior to E10.5 in wild-type embryos [4] are EMP that require shear stress-mediated signals for their robust emergence [67,68]. Indeed, there is some evidence suggesting EMP could originate in other sites of the conceptus beyond the yolk sac. *In vitro* culture of the pre-circulation chorion and allantois has yielded hematopoietic potential, however, at a much lower frequency than the yolk sac [69,70]. Intriguingly, a recent *in vivo* finding indicates that endocardium contributes a small percentage of progenitors with myeloid and rare primitive and definitive erythroid potential to the embryo [71]. The relationship of these progenitors, which depend on the cardiac-specific transcription factors *Nkx2.5* and *Isl1*, to yolk sac-derived EMP that contain robust erythroid potential [4], requires further exploration. However, the low levels of hematopoietic potential reported in these sources, along with the normal numbers of EMP in circulation-deficient embryos, support the conclusion that EMP predominately originate in the yolk sac.

The onset of EMP-definitive hematopoiesis is correlated with the expression of the CD41 integrin, in both mouse and zebrafish [10,44,45]. EMP emerge soon after commitment of CD41^{lo} primitive hematopoietic progenitors from mesoderm [44,72], reviewed in [73]. The first murine EMP capable of generating definitive erythroid colonies in semisolid media are detected at E8.25, exclusively in the yolk sac [4] (see Figure 1). The presence of erythroid and myeloid colony-forming potential in the yolk sac as late as E11.5 suggests, but does not prove, that EMP continue to be formed until midgestation [4,9]. Similarly, Lmo2+Gata1+ EMP numbers in the zebrafish posterior blood islands peak around 30 hours post fertilization (hpf), and decrease by 48 hpf [10], while HSC emergence in the AGM, as presumed by aortic expression of *runx1* and *cmyb*, occurs from 28 hpf to 48 hpf [74–77]. These kinetics suggest that EMP emergence temporally overlaps with HSC emergence both in mouse and zebrafish embryos.

In the mouse, EMP are also detected by the cell surface expression of Kit [44,45]. Clusters of Kit^{hi} cells have been visualized to associate with endothelium in the yolk sac vascular plexus as early as 5 somite pairs, i.e. E8.25 [78], suggesting that EMP arise in a spatially distinct pattern when compared to primitive hematopoiesis. However, individual hemangioblasts, which have the *in vitro* potential to generate both primitive erythroid and definitive erythroid progenitors in addition to endothelium and smooth muscle cells [7,79], were detected as blast colony-forming cells in the E7.5 embryo [80], as well as during ESC differentiation [79,81]. These studies support the concept that both primitive and EMP-definitive hematopoiesis originate from cells with endothelial identity [82] (reviewed in [83]). While it is not known if hemangioblasts give rise to all primitive and EMP-definitive hematopoiesis *in vivo*, the investigation of blast colony-forming cell activity *in vitro* has shed light on the sequential steps to hematopoietic commitment from mesodermal derivatives [84,85].

Primitive hematopoiesis appears to emerge directly from mesoderm in yolk sac blood islands [72,86], and can emerge in the absence of the transcription factor Runx1 [87–89], which is required for the endothelial-to-hematopoietic transition [90]. Yokomizo *et al.* [78] observed loss of the Kit^{hi} EMP clusters in Runx1-null yolk sacs, consistent with the idea that EMP, like HSC, emerge from hemogenic endothelium in a Runx1-dependent manner [87–89,91]. Similar observations were made during ESC differentiation, with the dependence of definitive, but not primitive hematopoiesis on Runx1 [82,85]. Moreover, the *in vivo* dependence of EMP on Runx1 in a Tek/Tie2 and VE-Cadherin promoter-dependent manner [90,92], as well as their ability to be rescued by Tek-mediated expression of Runx1 or its requisite binding partner Cbfb [5,93], also supports their endothelial origin.

While HSCs emerge from large embryonic vessels that have already undergone arterial specification, the initial appearance of EMP precedes vascular remodeling. Subsequently, extensive vascular remodeling occurs during the broad timeframe of EMP colony-forming potential. The first EMP emerge at E8.25, when the heart tube begins to contract [94]. Following these first contractions, there is a gradual egress of primitive erythroblasts from the yolk sac primitive vascular plexus [11,94]. As hemodynamic forces increase, the vascular plexus remodels into an arborized network of arteries and veins (reviewed in [95]). This process is mediated in part by shear stress-induced nitric oxide [96], and Notch signaling [97], both of which are required for HSC emergence [98]. It is established that HSC require cell-autonomous expression of Notch1 for activation of Runx1 [99,100] in the aorta. In striking contrast, EMP do not require Notch1 for emergence [99,101], suggesting that Runx1 is activated in an alternate manner to facilitate EMP emergence. In addition, EMP do not require blood flow for specification, as they can form in *Ncx1*^{-/-} embryos [65].

Other signaling pathways have been shown to regulate EMP formation. Retinoic acid is synthesized from vitamin A via *Raldh2* in the visceral endoderm [102]. While retinoic acid dampens primitive erythropoiesis [103], *Raldh2*-null yolk sacs demonstrated a reduced capacity to produce definitive hematopoietic colonies, and only yielded BFU-E in reduced numbers [102]. However, it is clear that retinoic acid signaling affects other aspects of vascular development [104]. Another recent study indicates that Indian Hedgehog can augment EMP colony formation in the murine yolk sac, but not in the para-aortic splanchnopleura, which are the precursor tissues of the AGM region[105].

Though it is recognized that there are phenotypic differences in EMP- and HSC-producing hemogenic endothelial populations [5], it is currently unknown what intrinsic differences in these precursors result in the production of committed hematopoietic progenitors versus hematopoietic stem cells capable of long-term repopulating activity. Despite the differential requirement for Notch1, several critical master transcriptional regulators downstream of Notch1 in the aorta [77,106,107] appear to be shared both by EMP and by HSC, including *cmyb*, *Runx1*, and *Gata2* [21,87,88,108]. Still other transcriptional regulators are also shared by EMP and HSC, including *Tal1* and *Fli1* [109]. Differential, yet overlapping temporal requirements for *Runx1* activity have recently been observed in EMP and HSC [110], and the dosage of *Runx1* appears to critically regulate emergence of hematopoietic potential [111,112]. Despite the shared emergence of EMP and HSC from hemogenic endothelium, the temporal-spatial emergence and regulation of EMP potential remains poorly understood.

EMP formation during ESC differentiation

Murine and human ESCs are being intensely studied with the goal of generating HSCs *in vitro* to serve therapeutic purposes. To date, the hematopoietic potential derived from differentiating ESCs has revealed a striking similarity with primitive and EMP-definitive hematopoiesis produced in the yolk sac of mammalian embryos [85,113]. The ability of murine ESCs to generate extensively self-renewing erythroblasts *ex vivo* also reflects the appearance of EMP-derived erythropoiesis in differentiating ESCs [29,114]. This essentially faithful recapitulation by ESC/iPSCs of the temporal emergence of the HSC-independent waves of yolk sac hematopoiesis likely explains the difficulty in producing transplantable HSCs *in vitro*. While recent efforts are focusing on the acquisition of lymphoid potential in ESC/iPSCs as a benchmark for identifying the formation of HSC potential, the presence of HSC-independent lymphoid potential in mouse embryos may confound this strategy. A better understanding of the overlap and duration of EMP versus lymphoid emergence and potential during embryogenesis will inform and guide efforts to generate functional blood cell populations in differentiating ESC/iPSCs.

Summary and Final Remarks

The emergence of a transient wave of EMP-definitive hematopoiesis is evolutionarily conserved. In the mouse embryo, yolk sac-derived EMP serve as a critical source of hematopoiesis in the early fetal liver that can support the mouse embryo's need for blood cells from mid-late gestation until birth. This robust wave of multipotent progenitors appears to bridge a gap between primitive hematopoiesis and the ultimate establishment of a functional HSC-derived hematopoietic hierarchy that will persist throughout life. EMP contain a significant erythroid component, with unique regulation of the β -globin locus and the capacity to form extensively self-renewing erythroblasts *ex vivo*. Though their progenitor activity is transient, recent evidence suggests EMP may be a source of specific myeloid lineages, such as microglia, that may persist postnatally. Current evidence supports the concept that EMP lack lymphoid or long-term repopulating potential. However, EMP can be generated during ESC/iPSC differentiation and their ability to rapidly provide robust

erythroid and myeloid potential and to serve as the source of extensively self-renewing erythroblasts makes this cell population of potential clinical interest. The study of EMP fate and potential *in vivo* and *in vitro* will continue to reveal the mechanisms that regulate the emergence of definitive hematopoiesis.

ABBREVIATIONS

EMP	erythro-myeloid progenitors
ESC	embryonic stem cell
iPSC	induced pluripotent stem cell
HSC	hematopoietic stem cell
E	embryonic day
BFU-E	burst-forming units erythroid
AGM	aorta-gonad-mesonephros
Hp	hours post fertilization

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Figure 1. Simplified outline of hematopoietic emergence and differentiation in the mouse embryo

Three major waves of hematopoietic potential emerge sequentially during murine embryogenesis. Primitive hematopoiesis emerges in the yolk sac and primitive erythroid cells subsequently mature in the bloodstream. Erythro-myeloid progenitors (EMP) also emerge in the yolk sac and seed the fetal liver, where they rapidly differentiate, giving rise to the first circulating definitive erythrocytes. These two waves of hematopoiesis also contain megakaryocyte and myeloid potential. Finally, adult-repopulating hematopoietic stem cells (HSC) emerge from major arteries and seed the fetal liver, and eventually the bone marrow. Recent studies suggest that immature HSCs (imHSC), capable of engrafting adult recipients after *in vitro* culture, can give rise to fully functional HSCs. This schema highlights the significant temporal overlap of these distinct waves of hematopoiesis between embryonic days 8.5-12.5.