Yolk-sac hematopoiesis: The first blood cells of mouse and man

James Palis^a and Mervin C. Yoder^b

^aUniversity of Rochester, Department of Pediatrics and Cancer Center, Rochester, NY, USA;

^bIndiana University School of Medicine, Herman B. Wells Center for Pediatric Research, Indianapolis, IN, USA

Objective. To review the process of blood-cell formation in the murine and human yolk sac.

Data Sources. Most articles were selected from the PubMed database.

Data Synthesis. The yolk sac is the first site of blood-cell production during murine and human ontogeny. Primitive erythroid cells originate in the yolk sac and complete their maturation, including enucleation, in the bloodstream. Though species differences exist, the pattern of hematopoietic progenitor cell emergence in the yolk sac is similar in mouse and man. In both species, there is a stage of development where both primitive red blood cells and definitive erythroid progenitors are produced in the yolk sac. An “embryonic” hematopoietic stem cell that engrafts in myeloablated newborn but not adult mice can be detected in the murine yolk sac and embryo. Stem-cell activity in the human yolk sac has not been reported.

Conclusions. The yolk sac is the sole site of embryonic erythropoiesis. However, definitive erythroid, myeloid, and multipotential progenitors also originate in the yolk sac. The relationship between these progenitors and the “embryonic” hematopoietic stem cell has not been elucidated. Yolk sac-derived progenitor cells may seed the developing liver via the circulation and serve as the immediate source of the mature blood cells that are required to meet the metabolic needs of the rapidly growing fetus. © 2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.

In the adult, hematopoiesis is in a steady state as all formed elements of the blood are synthesized from multipotent precursor cells produced by hematopoietic stem cells in the bone marrow. In the fetus, the liver serves as the principal hematopoietic organ. However, the early mammalian embryo requires red cells for growth and survival prior to development of the liver. Large primitive red cells are produced in blood islands of the yolk sac soon after implantation of the embryo. Primitive red cells have marked differences from the definitive red cells later produced in the fetal liver and postnatal bone marrow [1]. A recent review in this journal has discussed the development of the hematopoietic system in the mouse embryo, comparing it to hematopoiesis observed in cultured murine embryonic stem cells [2]. Here we focus on the cell biology of hematopoiesis in the yolk sac, including a more detailed description of primitive erythropoiesis and a discussion of the origins of hematopoi-

etic stem cells. A brief overview of human yolk-sac hematopoiesis is also presented.

Yolk-sac hematopoiesis in the mouse

The first blood and endothelial cells arise in yolk-sac blood islands

The murine yolk sac is a bilayer organ composed of extraembryonic mesoderm cells apposed to visceral endoderm cells. This association of cells derived from mesoderm and endoderm germ layers is termed splanchnopleure. Normal embryonic development is critically dependent on the proper functioning of the yolk sac since its endoderm layer transports and metabolizes maternally-derived macromolecules and synthesizes serum proteins, and its mesoderm layer produces the first blood cells within blood islands (reviewed by [3]). Furthermore, the visceral endoderm is thought to serve as a source of inductive signals important for the formation of blood cells [4,5] and endothelial networks [6] in the yolk sac. GATA-4-null embryonic stem cells form embryoid bodies lacking visceral endoderm and also display markedly reduced blood-island formation, further supporting the con-

Offprint requests to: James Palis, M.D., University of Rochester, Department of Pediatrics, Box 777, Rochester, NY 14642; E-mail: james_palis@urmc.rochester.edu

cept that the visceral endoderm participates in the development and organization of blood islands [7]. Mesoderm/endoderm recombination experiments suggest that these signals act during early gastrulation and are diffusible [5].

Yolk-sac blood islands were first observed in the early 1900's [8]. Detailed light and electron-microscope studies have been performed in several mammalian species, including mouse [9,10] and human (see below). In the mouse, presumptive blood islands, known as mesodermal cell masses or angioblastic cords, arise from proximal mesodermal cells in the visceral yolk sac between E7 and E7.5 (Fig. 1). Blood islands are bordered by visceral endoderm cells facing the yolk-sac cavity and the single cell layer of mesothelium facing the exocoelomic cavity (Fig. 1). Between E8.0 and E9.0, the cells comprising the outer layer of the blood-island cell aggregates assume a spindle shape as they differentiate into endothelial cells. The vast majority of the inner cells progressively lose their intercellular attachments as they differentiate into primitive erythroblasts [9].

The synchronous temporal and intimate spatial appearance of blood cells and endothelial cells in yolk-sac blood islands has led to the concept of a common "hemangioblast" precursor for the blood and endothelial lineages. Recent studies with cells from chick embryos and with embryonic stem (ES) cells have led credence to this concept. In the avian embryo, mesoderm cells expressing the vascular endothelial growth factor (VEGF) receptor-2 (*flk-1*) differentiate in vitro into endothelial cells in the presence of VEGF or into hematopoietic cells in the absence of VEGF [11]. Murine ES cells cultured as embryoid bodies in vitro contain blast colony-forming cells that form both endothelial and hematopoietic cells upon secondary replating [12]. The absence of yolk-sac blood islands in mutant mouse embryos lacking *flk-1* provides further evidence suggesting that endothelial cells and blood cells originate from a common precursor cell [13]. However, no in vivo proof of a clonal rela-

tionship between endothelial cells and hematopoietic cells has yet been presented.

Primitive erythropoiesis originates in the yolk sac

Soon after their initial development in yolk-sac blood islands, primitive erythroblasts enter the newly formed vasculature of the embryo proper. There they continue to divide for several days (Fig. 2), as evidenced by mitotic cells in the bloodstream [14], thymidine incorporation studies [15], and cell-cycle analysis [16]. Primitive erythroblasts differentiate within the bloodstream, gradually accumulating increasing amounts of hemoglobin and becoming progressively less basophilic [10]. Hemoglobin synthesis directed by stable globin transcripts continues until cell replication ceases [17,18]. Primitive murine erythroblasts continue to divide until E13, at which point they reach their steady-state hemoglobin content of 80 pg/cell, nearly four times the amount of hemoglobin found in adult erythrocytes [19].

Initial studies using electrophoretic methods to separate hemoglobins indicated that primitive erythroblasts and adult erythrocytes express different hemoglobins and that adult-type hemoglobin accumulation coincides with the appearance of enucleated fetal liver-derived erythrocytes [20]. Subsequent studies of separated globin chains confirmed the finding that primitive erythroblasts initially synthesize embryonic globins and indicated that they later synthesize adult globins [8,21]. This switch in globin synthesis occurs in all yolk-sac erythroblasts examined by specific antibodies directed against embryonic and adult hemoglobin [21].

Primitive murine erythroblasts attain a mean cell volume of 465–530 fL ([22]; Palis, unpublished data), earning for them the name of megaloblasts. As megaloblasts differentiate in the bloodstream, there is progressive nuclear condensation [20]. Enucleated "megalocytes" having the same size and hemoglobin content as nucleated yolk-sac erythroblasts are first evident at E13 [14]. These cells are thought to be derived from yolk-sac erythroblasts that have undergone enucleation, since they are threefold larger than the fetal liver-

Figure 1. Development of yolk sac blood islands in the mouse embryo between E7.5 and E9.5. Yolk sac blood islands form between the single cell layer of endoderm cells (E) and mesothelial cells (open arrow). Blood islands develop from undifferentiated mesoderm cells (E7.5, closed arrow) that give rise to inner blood cells surrounded by an outer endothelial lining (E9.5, closed arrow).

Figure 2. Cytospin preparation of circulating mouse blood cells at E10.5. The vast majority of cells are differentiating primitive erythroblasts, some still in mitosis (arrow). Rare macrophage cells (m) are also present.

derived “macrocytes” that enter the bloodstream beginning at E12 [19]. While nucleated yolk sac–derived erythroid cells are no longer present in the bloodstream after E16 [23], megalocytes continue to circulate until E18 [14]. Primitive erythroblasts also undergo other changes during differentiation consistent with enucleation, including the loss of the intermediate filament vimentin [16] and loss of histone proteins [24]. These observations indicate that terminal differentiation of primitive erythroid cells results in erythrocytes more similar to the enucleated red cells of mammals rather than the nucleated red cells of birds and fish.

Erythropoiesis in the bone marrow is critically dependent on the growth factor erythropoietin, which is thought to be both a mitogen and survival factor for mature definitive erythroid progenitors [25]. However, the role of erythropoietin in yolk-sac erythropoiesis remains controversial. Early studies indicated that the addition of exogenous erythropoietin did not affect heme synthesis when gastrulating mouse embryos were cultured intact [26]. These results have been widely interpreted to indicate that primitive erythropoiesis is not dependent on erythropoietin. However, in a subsequent study by the same investigator, exogenous erythropoietin caused a significant increase in heme synthesis when added to cultures of disaggregated embryonic cells [27]. Disaggregated E8.5 yolk-sac cells cultured for 18 hours in the presence of erythropoietin contain significantly more hemoglobin⁺ cells and significantly fewer dead cells compared to cultures lacking exogenous erythropoietin [28]. These effects of erythropoietin (increasing erythroid cell numbers and preventing cell death) were not evident when circulating yolk-sac erythroblasts from E9.5 and E10.5 mouse embryos were examined [28]. Other studies have also shown that the addition of exogenous erythropoietin to yolk sacs explanted from E7.5–E8.5 mouse embryos results in increased numbers of primitive erythroid cells and in increased accumulation of embryonic globin transcripts [29,30]. These findings indicate that yolk-sac erythroid cells respond to erythropoietin and suggest that erythropoietin may act as a survival factor for primitive erythroblasts early in their maturation.

Proof that erythropoietin signaling has a functional role in primitive erythropoiesis comes from the targeted disruption of several murine genes, particularly erythropoietin, the erythropoietin receptor, and the intracellular signaling molecule Jak2 kinase. In each case, there is a fivefold to 20-fold reduction in number of circulating primitive erythroblasts by E11.5 and the mutant embryos die with severe anemia by E13.5 [31–34]. However, the persistence of some hemoglobin-containing primitive erythroblasts in the circulation of mutant embryos is in striking contrast to the complete lack of terminally differentiated definitive erythroid cells in the fetal liver. The relative independence of primitive erythropoiesis from erythropoietin signaling raises the unanswered question of what other cytokine signaling cascades are active in yolk-sac erythroblasts.

Consistent with a role for erythropoietin in the differentiation of primitive red cells, erythropoietin receptor transcripts are present at the earliest stages of yolk-sac blood-island formation in the mouse [30]. Hamster yolk-sac erythroblasts initially express increasing numbers of erythropoietin receptors on their cell surface as they circulate in the bloodstream [35]. Receptor numbers subsequently decrease as terminal differentiation proceeds. While erythropoietin transcripts have been detected in the mouse embryo at E7.5 and in the fetal liver at E14.5, the low levels of erythropoietin expression have hampered the spatial and temporal analysis of its expression during development [36–38]. A small percentage (7%–10%) of a supraphysiologic dose of erythropoietin can cross the mouse placenta just prior to birth (E19) [39]. However, it is not known if physiologic levels of maternal erythropoietin cross the murine placenta, or if maternal erythropoietin can cross the yolk sac, particularly prior to formation of the chorioallantoic placenta, which occurs between E8.5 and E9.5.

The ability to culture hematopoietic progenitors in semisolid medium has contributed significantly to our understanding of the hematopoietic hierarchy in the adult. Early erythroid progenitors in the murine bone marrow are recognized by the generation of large colonies of red cells in methylcellulose (burst-forming unit erythroid; BFU-E) following 7–10 days in culture, while more mature erythroid progenitors generate smaller colonies (colony-forming unit erythroid; CFU-E) within 2–3 days in culture. Wong et al. identified a unique intermediate erythroid progenitor type during gastrulation in the mouse embryo [29,40]. These progenitor colonies, called “day-5 CFU-E,” consisted of approximately 100 red cells expressing both embryonic and adult globins. Day-5 CFU-E progenitors arose early on the eighth gestational day exclusively in the yolk sac and disappeared by E9 [40]. It was subsequently confirmed that day 5 CFU-E are primitive erythroid progenitors (EryP-CFC) that generate colonies in semisolid media with a distinct timing of differentiation, morphology, and pattern of gene expression compared to the definitive BFU-E and CFU-E [41]. EryP-CFC generate colonies comprised of large nucleated primitive erythroid cells that express both embryonic and adult globins. In contrast, BFU-E and CFU-E give rise to colonies comprised of small definitive red cells expressing only adult globins [42].

A more extensive analysis of the development of hematopoietic progenitors in the early postimplantation mouse embryo has recently been carried out [42]. Using carefully staged embryos, it was determined that the first EryP-CFC arise at mid/late primitive streak stages (E7.0), within hours of the start of mesoderm formation (Fig. 3). The spatial distribution of these progenitors within the conceptus and their subsequent expansion in numbers correlated with the formation of extraembryonic mesoderm cells in the visceral yolk sac. EryP-CFC are confined to the yolk sac, where they expand in numbers at E8.25 (early somite pair stages). However, by E9.0 (20–25 somite pair stages) they were no longer

Figure 3. Development of primitive erythroid (EryP-CFC), definitive erythroid (BFU-E), and macrophage (Mac-CFC) progenitors in the mouse yolk sac between E6.5 and E11.5 of gestation. Data derived from Palis et al. [42].

present in the conceptus ([40,42] and Fig. 3). These studies indicate that primitive erythroid progenitor production is confined to the yolk sac and primitive erythropoiesis constitutes the only transient lineage during hematopoietic ontogeny.

Definitive erythropoiesis is initiated in the yolk sac

Initial studies of definitive erythroid progenitors in the mouse embryo indicated that BFU-E were first detected at E8.5 and CFU-E at E9.5 [43]. Wong et al. [40] confirmed that BFU-E arose late on the eighth day of gestation and continued to increase in number at E9 and E10. Unexpectedly, these definitive erythroid progenitors were found exclusively in the yolk sac at E8.5, but by E9.0 were also present in the bloodstream and the embryo proper [40,44]. Recently, an analysis of carefully staged mouse embryos revealed that BFU-E originate in the yolk sac of early somite pair embryos at E8.25, just prior to the onset of circulation [42]. Over the subsequent 24 hours, BFU-E increase in numbers within the yolk sac (Fig. 3) and enter the newly formed bloodstream of the embryo proper. CFU-E are subsequently found in the yolk sac, the bloodstream, and the embryo proper, consistent with their differentiation from yolk sac–derived BFU-E. Between E10.5 and E11.5, the number of BFU-E and CFU-E decline in the yolk sac and expand exponentially within the liver rudiment [42].

The fact that definitive erythrocytes are first evident at E12, when they begin their egress from the fetal liver, suggests that the yolk sac–derived definitive erythroid progenitors (BFU-E and CFU-E) cannot fully differentiate within the yolk-sac environment. This conclusion is supported by explant experiments using tissues obtained from 20–25 somite pair mouse embryos [45]. Yolk sacs explanted alone contain a transient wave of erythroid cells expressing embryonic and adult globins, consistent with the presence of primitive erythroid cells. In contrast, yolk-sac tissues cocultured adjacent to liver primordium revealed a subsequent wave of yolk-sac erythroid cells expressing adult globins. These results indicate that definitive erythroid progenitors

are present in the yolk sac and that factors in the embryo proper, but not the yolk sac, support their differentiation. Taken together, these studies indicate that both primitive and definitive erythropoiesis originate in the yolk sac. While the transient wave of primitive erythroid progenitors remains confined to the yolk sac, the second wave of definitive erythroid progenitors enters the circulation and likely seeds the liver primordium beginning at E9.5.

Multiple myeloid lineages originate and expand in the yolk sac

The developmental biology of the macrophage lineage has been recently reviewed in this journal [46] and will be discussed only briefly here. The first macrophage cells identified in the mouse embryo are visible as clusters of free ameboid cells with distinct nucleoli in the yolk sac between E9 and 9.5 [47]. Genes associated with the macrophage lineage, including the macrophage colony-stimulating factor (M-CSF) receptor *c-fms* and F4/80, are first detected in the yolk sac and the head of mouse embryos between E9.0 and E9.5 [48,49]. Unlike tissue macrophages in the adult, which are thought to derive from monocyte cells, embryonic macrophages appear to directly differentiate from macrophage progenitors.

In pioneering work, Moore and Metcalf determined that macrophage progenitors (Mac-CFC) are present in intact presomite embryos and in the yolk sac of early somite pair embryos [50], consistent with the progenitors arising in the yolk sac. A recent detailed analysis identified macrophage progenitors in mid to late primitive streak embryos at E7.0 in the distal regions of the embryo associated with the developing yolk sac [42]. After an initial expansion within the yolk sac (Fig. 3), macrophage progenitors are subsequently found in the bloodstream and throughout the embryo proper.

The yolk sac is also the site of origin of microglial progenitors that form large myeloid colonies in vitro when plated in culture with astroglial monolayers. These microglial precursors were first detected at presomitic stages of development (E7.0) within the yolk sac and subsequently at early stages of somite development (E8.0) in the neural folds [51]. The results of this kinetic analysis suggest, but do not prove, that microglial precursors migrate directly from the yolk sac into the developing central nervous system. These results are consistent with studies performed in the chick, where microglial precursors migrate from the yolk sac to the developing central nervous system (CNS) without circulating in the bloodstream [52]. Upon reaching the central nervous system, the yolk sac–derived microglial precursors proliferate extensively, to eventually comprise the entire population of mature microglial cells in the adult animal. It is not known if these microglial progenitor cells are distinct from the Mac-CFC detected in the yolk sac [42,50].

Mast cell and granulocyte/macrophage (CFU-GM) progenitors are first detected in the yolk sac of the 10–15 somite pair mouse conceptus [42]. These progenitors initially expand in the yolk sac [53], but are subsequently found in higher numbers in the bloodstream and liver of the

fetus, consistent with their migration into the embryo proper from the yolk sac [42]. Thus, like the primitive and definitive erythroid lineages, multiple myeloid lineages also originate and expand in numbers within the yolk sac between E7.0 and 10.5.

Multipotential hematopoietic

progenitors are first detectable in the yolk sac

In the adult, committed hematopoietic progenitors arise from multipotential precursors. The ordered appearance in the yolk sac of two distinct erythroid and several myeloid progenitor types suggests that the yolk sac contains multipotential hematopoietic precursors. The spleen colony-forming assay (CFU-S) and high proliferative colony-forming assay (HPP-CFC) have been used to determine the presence of multipotential precursors in the early mouse embryo. The former relies on the homing of hematopoietic precursors to the spleen of a lethally irradiated adult recipient, while the latter generates a macroscopic colony of myeloid cells in agar that is dependent on the presence of multiple growth factors [54]. Moore and Metcalf [50] first detected CFU-S8 at E8.5 in the yolk sac but not the embryo proper, suggesting that multipotential precursors arise in the yolk sac. In contrast, Medvinsky et al. [55] did not detect CFU-S8 until E9.5 (26–27 somite pairs), when they were simultaneously identified in the yolk sac and embryo proper. It is not clear what factors are responsible for these discordant findings.

HPP-CFC have been detected as early as E8.0–E8.5 in the mouse conceptus; however, the spatial distribution of these precursors in staged embryos was not examined [56]. We have recently conducted an extensive analysis of the spatial and temporal emergence of HPP-CFC in the mouse embryo [57]. HPP-CFC were not found at presomite stages (E6.5–E7.5), despite examining more than 180 embryos. The first HPP-CFC were detected at early somite stages (E8.25), exclusively in the yolk sac. The yolk sac remains the predominant site of HPP-CFC expansion until E11.5, when the liver supplants the yolk sac as the primary site of hematopoietic progenitor production. These findings indicate that HPP-CFC originate in the yolk sac after the onset of primitive erythropoiesis and suggest that multipotential hematopoietic precursors emerge from the yolk sac concomitant with the definitive erythroid and several myeloid lineages.

The origin of lymphoid precursors remains controversial

Lymphoid cells normally mature during the second half of gestation within the fetal liver and thymus of the embryo proper. The developmental origin of the lymphoid progenitors that seed these organs remains controversial. Several investigators have examined both yolk-sac and embryo-proper tissues for the presence of lymphoid potential. Most of the studies have found the simultaneous appearance of lymphoid progenitors in both yolk sac and embryo proper [58–61]. However, other studies have indicated that lymphoid

potential arises within the embryo proper between E8.5 and E9.5 [62,63] and even earlier if the tissues are first explanted and expanded for several days on a bone marrow stromal cell line [64]. Other investigators have focused exclusively on either the yolk sac or the AGM region as the source of lymphoid potential [65,66]. Ultimately, the source of lymphoid precursors is likely linked to the embryologic origin of hematopoietic stem cells (HSC).

“Embryonic” hematopoietic stem cells

arise before “adult” hematopoietic stem cells

All blood cells are postulated to derive from HSC. These stem cells represent a largely quiescent population that demonstrate self-renewal, replenish circulating blood cells in transplanted recipients, and are resistant to 5-fluorouracil treatment. Studies in the mouse embryo have indicated that HSCs able to engraft adult mice are present in the liver beginning at E11–E12 [67–69]. These fetal liver-derived HSCs express CD34, c-kit, AA4.1, and Sca-1 surface markers, and are thought to migrate to the bone marrow after E15 [70,71]. Since the liver rudiment is colonized by exogenous blood cells [72], HSCs must arise elsewhere. It was initially hypothesized that HSCs originate in the yolk sac, migrate to the liver, and eventually seed the bone marrow [50]. However, the yolk sac does not contain HSCs before E11.5 that are capable of engrafting adult recipients [53,67].

This stem-cell model was further challenged by studies in the avian embryo, where yolk-sac cells do not contribute to long-term adult hematopoiesis (reviewed by [73]). In the avian embryo, foci of hematopoietic cells, found initially in intra-aortic regions, and later in para-aortic clusters, are responsible for long-term erythropoiesis in the adult. Morphologic evidence of intra-aortic cell clusters has also been found in several mammalian species, including the mouse, pig, gerbil, and human [74,75]. Investigations in the mouse embryo indicate that HSCs capable of engrafting adult recipients are associated with the aorta in the AGM region at E10–E10.5, one day before their appearance in the liver and yolk sac [68,76].

The *sine qua non* of HSC function is the repopulation of the hematopoietic system of myeloablated recipients following transplantation. For adult recipients, this entails repopulation of the bone marrow. An alternative explanation for the lack of HSC activity prior to mid-gestation is that HSCs do, in fact, exist but are unable to engraft in the adult hematopoietic microenvironment. Evidence that HSCs exist prior to E10 comes from transplant studies using mouse embryos as recipients. E9.0 yolk sac and peripheral blood cells were used to engraft E11–E15 W/W host embryos, which are HSC deficient [77]. While there was tremendous fetal loss caused by the cell injections, 6/163 embryonic recipients showed evidence of long-term engraftment of the erythroid lineage. Unfortunately, donor embryos were not staged, embryo-proper tissues were not studied, and only erythroid engraftment was measured. Weissman et al. [78] injected E8–

E10 yolk-sac cells into E8–E9 donor embryos. Low levels of donor lymphoid cells were identified after birth of the recipients. Again, donor embryos were not staged and embryo-proper cells were not investigated. However, these studies provide evidence suggesting that “embryonic” HSCs exist in the pre-E10 mouse embryo and that these HSCs are capable of homing to fetal hematopoietic organs [79].

More recently, a newborn recipient model of transplantation has been developed [80]. Rationale for these experiments encompassed several principles of developmental hematopoiesis. From E12 until shortly after birth, the murine liver is the predominant site of HSC residence. Though the fetal marrow compartment is seeded with HSC at day E15, the liver retains the majority of HSC activity at birth [81]. Thus, in contrast to the adult recipient in which only bone marrow and spleen are sites for HSC engraftment, the newborn recipient presents the liver, bone marrow, and spleen as potential hematopoietic engraftment sites. However, transplantation of hematopoietic cells into unablated newborn or fetal mice generally fails to result in readily detectable and durable engraftment. Use of mice genetically deficient in HSC activity has been the most successful strategy for achieving donor-cell engraftment in fetal or newborn recipients (see above).

Busulfan is one of several chemotherapeutic agents that is known to cross the placenta and cause untoward effects on fetal development. We [80] determined that certain doses of busulfan could be administered to pregnant mice without fetal wastage. Pregnant mice were treated two days prior to birth with busulfan to reduce endogenous hematopoiesis in the fetuses. Donor cells were injected directly into the livers or intravenously into the facial vein of the newborn pups and donor contribution to peripheral blood cells was monitored for 4–6 months to ensure long-term hematopoietic engraftment. In initial experiments, cells from E10 yolk sacs were able to provide long-term multilineage reconstitution of busulfan-conditioned newborns [82]. Subsequent studies have shown that embryonic HSCs expressing CD34 and c-kit reside both in the yolk sac and in the developing AGM region at E9.0 [83]. A 37-fold preponderance of E9.0 yolk-sac cells expressing CD34 and c-kit suggests, but does not prove, that embryonic HSCs arise in the yolk sac. Tissues derived from earlier developmental stages, particularly prior to the onset of the circulation, will need to be investigated to determine the temporal and spatial origin of embryonic HSCs.

Not only did embryonic HSCs provide long-term engraftment of newborn recipients, but bone marrow cells from these primary recipients at 4–6 months posttransplant engrafted in lethally irradiated secondary adult recipients [84]. These results demonstrate that embryonic HSCs can become “adult” HSCs. At present, little is known of the molecules that effect the change from embryonic to “adult” HSC. The contribution of embryonic HSCs to hematopoiesis in the adult and the relationship of embryonic HSCs to their adult counterparts found later in the AGM region and

fetal liver remain to be elucidated. This information is crucial if one desires to utilize primary embryonic HSC or hematopoietic precursors derived *in vitro* from embryonic stem cells for transplant into adult recipients.

Yolk-sac hematopoiesis in man

The primary sites of hematopoiesis also change in a temporally and spatially ordered fashion during human development. Blood cells first arise in the yolk sac, followed sequentially by the fetal liver and finally the developing long bones [85,86]. The yolk sac serves as the initial site of erythropoiesis from weeks 3–6 of gestation. The liver functions as the primary site of hematopoiesis from weeks 6–22 of gestation, after which the bone marrow becomes the predominant and lifelong site of blood-cell production. Distinct patterns of blood-cell differentiation and proliferation are noted in each specific site of hematopoiesis during ontogeny [87–89]. The ontogeny of blood-cell production in the human embryo has been described using both morphologic approaches and *in vitro* hematopoietic progenitor cell assays. We will briefly review the formation and function of the human yolk sac, the kinetics of blood-cell appearance, and the ontogeny of hematopoietic progenitor cells in the human yolk sac.

Yolk-sac formation differs in man and mouse

The human yolk sac develops in three general phases: 1) a formative period, 2) a functional period, and 3) a period of regression [90]. The first phase can be further subdivided into formation of the primary and secondary yolk sac. The primary yolk sac is formed soon after implantation by proliferation and differentiation of primitive endoderm cells into visceral and parietal endoderm. Some controversy surrounds the identification of the cell lineage from which the first yolk-sac mesoderm cells emerge in the human embryo. In contrast to the mouse embryo, where yolk-sac mesoderm cells are derived from primitive ectoderm (epiblast) during gastrulation, yolk-sac mesoderm cells are thought to arise from primitive endoderm (hypoblast) in the primate embryo (reviewed in [90]). In the rhesus monkey, extraembryonic mesoderm cells appear to delaminate from the parietal endoderm of the hypoblast. Emergence of human and rhesus yolk-sac mesoderm cells occurs prior to formation of the primitive streak, diminishing the likelihood that yolk-sac mesoderm cells arise from the epiblast. Morphologic evidence supporting the endodermal derivation of yolk-sac mesoderm includes the cell-to-cell communication displayed by endoderm and mesoderm cells. Furthermore, some yolk-sac endoderm cells display extensive endoplasmic reticulum and extracellular matrix proteins production that is characteristics of yolk-sac mesoderm [90].

The first stage of yolk-sac formation ends as the primary yolk sac appears to undergo collapse with heterogeneous foci of reexpansion leading to the breaking up of the primary

yolk sac into smaller vesicles (Fig. 4). The secondary yolk sac is subsequently formed at days 12–15 from the remnants of the primary yolk sac (Fig. 4). The secondary yolk sac begins as a small structure with a diameter measuring less than 0.4 mm but steadily increases in size to 2 mm by day 19, 4–5 mm by week 7, and 6–6.5 mm by the end of week 10.

The first yolk-sac blood islands are evident morphologically in the secondary yolk sac beginning at approximately day 18 of gestation [91,92]. The developmental origin of blood cells and endothelial cells that make up the blood islands is also thought to be the endoderm of the yolk sac.

Figure 4. Stages of human yolk sac formation. **(Top)** The primary yolk sac (1°YSC) is formed 7–8 days postconception. Intermediate cells (IC; depicted as white cells) migrate from the endodermal (EL) layer (dark-colored cells) of the primary yolk sac to fill in the space between the endoderm and trophoblastic basal lamina (TBL). **(Middle)** The secondary yolk sac (2°YSC) is formed by the remnants of the primary yolk sac following its collapse and fragmentation. **(Bottom)** By 12 days postconception, the secondary yolk sac has expanded to the edge of the embryonic disk, is lined by endoderm cells (dark cells), and protrudes into the exocoelom (EXO). The mesoderm adjacent to the trophoblastic basal lamina (TBL) has differentiated into mesothelium (MESO). Hematopoietic activity will be present in yolk sac blood islands arising in the secondary yolk sac by week 3 of gestation. Reproduced with permission of F.F. Nogales (ed): *The Human Yolk Sac and Yolk Sac Tumors*, Berlin: Springer-Verlag, 1993, pp. 35–36.

Takashina [93] has provided extensive light and electron microscopic evidence suggesting that blood cells arise in the yolk-sac endoderm and migrate into surrounding yolk-sac mesenchyme. These blood cells become surrounded by other endoderm-derived mesoderm cells that differentiate into endothelial cells and these blood cell–endothelial cell aggregates develop into blood islands. The morphologic studies supporting the origin of human yolk-sac blood islands from the hypoblast are in striking contrast to studies in rodents and birds that indicate blood cells in the yolk sac are derived from the epiblast.

Besides hematopoiesis, the secondary yolk sac is an active site of protein biosynthesis and nutrient transport [90,94]. The endodermal component of the primate secondary yolk sac is a known transporter of proteins and vitamins [95–97]. As the yolk sac matures, an extensive network of superficial capillaries is formed in the mesenchymal layer. The secondary yolk-sac circulation is fed and drained by the vitelline (omphalomesenteric) arteries and veins that connect the embryonic to the extraembryonic circulation. Initially, the vitelline vessels empty into the sinus venosus of the liver, establishing a preportal-type circulation.

Unlike the yolk sac of the mouse that functions throughout gestation, the yolk sac of the human begins regressing around week 10 of gestation. Over the ensuing weeks, there is progressive evidence of tissue degeneration with loss of blood flow through the yolk-sac vascular network [90]. As the yolk sac regresses, new capillaries arising from the developing gut contribute to the vitelline circulation, and as the liver cords proliferate, the portal circulation is established [98].

Primitive erythropoiesis originates in the yolk sac

The most extensive morphologic description of human yolk-sac hematopoiesis was reported by Kelemen et al. [45]. Hematopoietic cells were first observed in the mesenchymal layer of the secondary yolk sac at day 18 postconception. Most blood islands were comprised of hematopoietic cells completely surrounded by endothelial cells; however, some extravascular free aggregates of blood cells were present in the mesenchyme in close proximity to the visceral endoderm. The 3–4-week embryo had more defined vessels than the yolk sac, but many vessels were void of hematopoietic cells. At the sixth week of development, both intraembryonic and extraembryonic blood vessels became macroscopically visible and primitive erythroblasts could be identified in the yolk-sac vessels. An overall decline in hematopoiesis was observed in the human yolk sac after the eighth week of gestation.

Primitive erythroblasts constituted nearly 100% of all nucleated cells in the yolk-sac circulation from the fourth to the fifth week of gestation. By the eighth week, primitive erythroblasts remained the predominant (88%) circulating blood cell but liver-derived definitive red cells were now evident. In the study by Kelemen et al. [85], no primitive erythroblasts were observed in peripheral blood smears at

12 weeks gestation. The only other mature blood cell present in the yolk sac was the macrophage. In contrast to normal fetal liver and bone marrow patterns of differentiation, macrophages in the yolk sac arose without evidence of a monocytic precursor.

Definitive hematopoietic progenitors are produced in the yolk sac

Unlike the mouse, there have been no studies of primitive erythroid progenitors in the human. Huyhn et al. [99] isolated yolk sac, liver, and embryonic tissues from day 25–40 gestation embryos and plated the enzyme disaggregated samples in methylcellulose culture for detection of hematopoietic progenitor cells. While the concentration of BFU-E in the yolk sac and embryo proper were similar, sevenfold to 12-fold higher numbers of CFU-GM were present in the embryo proper compared to the yolk sac. Migliaccio et al. [100] examined the kinetics of definitive hematopoietic progenitors in human yolk sacs from 4.5 to 10 weeks gestation. This stage encompassed the period of yolk sac hematopoietic decline and initiation of liver hematopoiesis. At 4.5 weeks of gestation, BFU-E, CFU-E, and CFU-GM were abundant in the yolk sac, but the concentration of these progenitors subsequently fell precipitously. By 6 weeks of gestation, hematopoietic progenitors were readily detectable in the liver but the yolk sac was devoid of all but a few CFU-E. Over the next three weeks, the concentration of BFU-E, CFU-E, and CFU-GM rose rapidly in the liver and reached a plateau by 8–9 weeks of gestation. In addition, a gradual shift from primarily embryonic to primarily fetal and adult hemoglobin molecules occurs in red blood cells derived from yolk sac and liver BFU-E [101], during the fifth to eighth weeks of gestation. These results suggest that the yolk sac serves as the source of the first definitive erythroid and macrophage progenitors in the human embryo, similar to recent murine studies (above). However, a detailed analysis of embryonic tissues prior to the onset of circulation has not been conducted to confirm this association.

Multipotential hematopoietic progenitors are produced in the yolk sac

HPP-CFC have been localized to both the yolk sac and embryo proper of the day 35–40 gestation embryo cells [99]. CD34⁺ hematopoietic cells recovered from the yolk sac and embryo yielded 45 HPP-CFC and 20 HPP-CFC, respectively, when 10,000 CD34⁺ cells were plated with the growth factors recombinant human stem cell factor (rhuSCF) and interleukin-3 (IL-3). Addition of recombinant human granulocyte (rhuG-CSF) or granulocyte-macrophage (rhuGM-CSF) colony-stimulating factors failed to influence the number or size of the HPP-CFC grown in rhuSCF or rhuIL-3 [99]. All CD34⁺ progenitors and HPP-CFC present in the yolk sac and embryo proper failed to express CD38 or CD33, consistent with the pattern of expression observed for early hematopoietic precursors in the adult marrow.

Summary

We have reviewed the existing data regarding the ontogeny of hematopoiesis in the human yolk sac. As in the mouse, the yolk sac in humans gives rise to differentiating primitive erythroblasts and macrophage cells. At the progenitor level, investigation has been limited to definitive hematopoietic lineages, but the results are most congruent with the yolk sac serving as the first site of BFU-E, CFU-E, and Mac-CFC synthesis. Analysis of the ability of yolk sac cells to engraft in vivo has not been conducted in part because such transplant studies are untenable in human patients. The recent description of human/mouse xenotransplant models for the identification of human SCID-repopulating stem cells may now make these studies feasible. If embryonic HSCs exist in the human embryonic yolk sac, these cells may only be detectable upon transplantation into fetal or newborn rather than adult NOD/SCID mice. The underlying biological behavior of such embryonic HSCs would need to be investigated before the use of yolk-sac cells for human transplantation could be envisioned.

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