Spatial and temporal emergence of high proliferative potential hematopoietic precursors during murine embryogenesis

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Edited by Irving L. Weissman, Stanford University School of Medicine, Stanford, CA, and approved February 1, 2001 (received for review January 2, 2001)

During mouse embryogenesis, two waves of hematopoietic progenitors originate in the yolk sac. The first wave consists of primitive erythroid progenitors that arise at embryonic day 7.0 (E7.0), whereas the second wave consists of definitive erythroid progenitors that arise at E8.25. To determine whether these unilineage hematopoietic progenitors arise from multipotential precursors, we investigated the kinetics of high proliferative potential colony-forming cells (HPP-CFC), multipotent precursors that give rise to macroscopic colonies when cultured *in vitro*. No HPP-CFC were found at presomite stages (E6.5–E7.5). Rather, HPP-CFC were detected first at early somite stages (E8.25), exclusively in the yolk sac. HPP-CFC were found subsequently in the bloodstream at higher levels than the remainder of the embryo proper. However, the yolk sac remains the predominant site of HPP-CFC expansion (>100-fold) until the liver begins to serve as the major hematopoietic organ at E11.5. On secondary replating, embryonic HPP-CFC give rise to definitive erythroid and macrophage (but not primitive erythroid) progenitors. Our findings support the hypothesis that definitive but not primitive hematopoietic progenitors originate from yolk sac-derived HPP-CFC during late gastrulation.

In the adult, committed hematopoietic progenitors arise from multipotential precursors that in turn are derived from hematopoietic stem cells (HSCs). 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. It was thought originally that murine progenitors capable of hematopoietic colony formation in adult murine spleens (CFU-S) represented HSCs. However, it was shown subsequently that CFU-S represent multilineage precursors that are incapable of long-term multilineage hematopoietic reconstitution (1).

Several *in vitro* assays define the presence of quiescent bone marrow progenitors that represent cells close to, or actually within, the HSC compartment. "Pre-CFU-S" cells include the long-term culture-initiating cell, the cobblestone area-forming cell, the colony-forming unit blast, the colony-forming unit A, and the high proliferative potential colony-forming cell (HPP-CFC; refs. 2 and 3). HPP-CFC were first described as murine bone marrow cells giving rise to macroscopic colonies (at two weeks of culture) that are greater than 0.5 mm in diameter and consist of >50,000 macrophage-like cells (4). Subsequent studies defined several growth factors, including IL-1α, IL-3, CSF-1, KL (SCF), and G-CSF, that were required for the growth of HPP-CFC and demonstrated that bone marrow HPP-CFC are quiescent cells with relative resistance to 5-fluorouracil treatment (3, 5, 6). Furthermore, HPP-CFC are multipotential cells capable of giving rise to secondary HPP-CFC and other multipotent or unilineage progenitor cells on replating. Although HPP-CFC are enriched in cell populations expressing cell-surface antigens known to enrich for stem cell activity (7, 8), HPP-CFC do not fully comprise the stem cell compartment, because their frequency in the bone marrow is not predictive of HSC frequencies. However, HPP-CFC are the earliest multipotential precursors within the hematopoietic hierarchy that can be cultured *in vitro* without stromal support.

During embryogenesis in the mouse, committed hematopoietic progenitors arise during early gastrulation at embryonic day 7.0 (E7.0; ref. 9). These primitive erythroid progenitors generate large nucleated red cells that sustain embryonic growth until E12.5 when definitive enucleate red cells begin to enter the bloodstream from the liver. The first definitive erythroid progenitors [burst-forming units–erythroid (BFU-E)] originate in the yolk sac at early somite-pair stages at E8.25 (9, 10). It is not known whether these unilineage primitive and definitive erythroid progenitors are derived from multipotential hematopoietic precursors, because multipotential precursors are detected first at E8.5 (11) and CFU-S are not detected until E9.5 (12, 13). Although HPP-CFC have been isolated from the yolk sac and from the embryo proper as early as E9.0 (8), their developmental origin and kinetics have not been investigated systematically. To better understand the origin of the mammalian hematopoietic system, we examined the spatial and temporal distribution of HPP-CFC in staged mouse embryos during gastrulation and early organogenesis. We have determined that HPP-CFC originate in the yolk sac before the onset of circulation where they expand in numbers concomitant with the first definitive hematopoietic progenitors. Subsequently, HPP-CFC are found in increasing numbers within the bloodstream before fetal liver colonization.

Experimental Procedures

Embryo Dissections. Inbred (C57BL6; The Jackson Laboratory) or outbred (ICR; Taconic Farms) mice were mated overnight, and noon of the day of the vaginal plug was defined as E0.5. At specified times, mice were killed and the uteri were removed from the peritoneum and washed with several changes of Iscove's modified Dulbecco's medium (IMDM). Embryos were dissected free of decidual tissues and Reichert's membrane with no. 5 watchmaker's forceps. Presomitic embryos were staged according to established morphological criteria (14), and somite-stage embryos were staged according to somite number. Blood was collected from conceptuses >9 somite pair and was derived principally from large vessels of the embryo proper.

Single-cell suspensions of tissues were obtained by treatment with either 0.25% collagenase (Sigma) with 20% (vol/vol) FCS

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: HPP-CFC, high proliferative potential colony-forming cell(s); HSC, hematopoietic stem cell; CFU-S, colony-forming unit(s)–spleen; BFU-E, burst-forming unit(s)–erythroid; EB, embryoid body; ES, embryonic stem, CSF, colony-stimulating factor; E*n*, embryonic day *n*.

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for 0.5–1.5 h or 0.25% trypsin/EDTA (Worthington) for 3–4 min at 37°C with vigorous pipetting. Cell counts and viability were quantitated after staining with trypan blue. Cell viabilities were routinely greater than 90%.

HPP-CFC Assay. Double-layer agar HPP-CFC cultures were prepared by adding the growth factors IL-1α (1,000 units/ml), IL-3 (200 units/ml), colony-stimulating factor (CSF)-1 (1,000 units/ml), and kit ligand (50 ng/ml) to 1 ml of 0.5% agar (Bacto-agar; Difco) in 35-mm dishes. In some experiments, these growth factors were supplemented with granulocyte/macrophage (GM)-CSF (10 ng/ml), leukemia inhibitory factor (10 ng/ml), vascular endothelial growth factor (50 ng/ml), IL-11 (100 ng/ml), flt3L (10 ng/ml), and thrombopoietin (5 ng/ml). All growth factors were obtained from R & D Systems. Embryonic cells were resuspended in 0.3% Bacto agar and overlayed on the 0.5% agar. After incubation at 37°C in 10% CO_2/85% N_2/5% O_2 (vol/vol) or 5% CO_2/95% room air (vol/vol) for 2 weeks, compact colonies (>0.5 cm) or more diffuse colonies (>1.0 cm) were scored as HPP-CFC by using an inverted microscope.

Replating Experiments: HPP-CFC. Cells from E9.5 and E10.5 yolk sac and embryo tissues were plated into double agar HPP-CFC plates. At 6 to 7 days of culture, individual colonies were plucked, resuspended in warmed PBS to form a single-cell suspension, and replated into double agar HPP-CFC cultures. Secondary HPP-CFC and low proliferative potential-CFC, <0.5 cm in size, were scored after incubation at 37°C in 10% CO_2/85% N/5% O_2 for 2 weeks. The cellular content of replated HPP-CFC was examined microscopically after Wright–Giemsa staining of cytospun colonies (Thermo Bioanalysis, Pittsburgh).

Replating Experiments: Hematopoietic Progenitors. Cells from E8.25–E8.5 embryos (2 to 10-somite pair) were pooled and cultured into six double agar HPP-CFC plates. At 4 days of culture, colonies containing at least 10 cells were plucked from three of the plates, pooled, resuspended in warmed PBS, and replated into 35-mm dishes containing 0.8% methylcellulose (Sigma) supplemented with 10% (vol/vol) plasma-derived serum (Antech, Tyler, TX), 5% (vol/vol) protein-free hybridoma medium (Life Technologies, Rockville, MD), kit ligand (100 ng/ml), erythropoietin (2 units/ml), IL-3 (5 ng/ml), granulocyte/macrophage-CSF (3 ng/ml), G-CSF (30 ng/ml), M-CSF (5 ng/ml), and IL-6 (5 ng/ml). These conditions support the growth of primitive erythroid, definitive erythroid [(BFU-E and colony-forming units–erythroid (CFU-E)], and macrophage progenitors, which can be distinguished by morphology and by gene expression (9, 15). Cultures were maintained at 37°C, 5% CO_2/95% room air for 7 days. CFU-Es were counted at days 2 and 3 and primitive erythroid, BFU-E, and macrophage colonies were counted at days 6 and 7. At 14 days of culture, HPP-CFC were counted from the three control plates and from the three plates that had been plucked at day 4 of primary culture (Fig. 3*A*). The difference in HPP-CFC numbers between control and plucked plates provided an estimate of the number of replated HPP-CFC. HPP-CFC were photographed by using a Polaroid DCM digital camera and the images were processed in PHOTOSHOP (Adobe Systems, Mountain View, CA).

Embryonic Stem (ES) Cell Differentiation. R1 ES cells (passage 15–16) were passaged into IMDM with 2 mM glutamine, 1 mM nonessential amino acids, 10 mM sodium pyruvate, penicillin/streptomycin (100 units/ml and 100 μg/ml), 55 μM 2-mercaptoethanol, 15% (vol/vol) FCS (HyClone), and 1,000 units/ml leukemia inhibitory factor (Life Technologies) 24–48 h before plating into differentiation culture. For the generation of embryoid bodies (EBs), ES cells were plated in bacterial grade Petri dishes at a concentration of 1,000–3,000 cells per ml in 0.9% methylcellulose, 2 mM glutamine, penicillin/streptomycin, 5% (vol/vol) protein-free hybridoma medium (Life Technologies), 200 mg/ml iron-saturated holotransferrin (Sigma), 5 mg/ml ascorbic acid, 450 μM monothioglycerol (Sigma), and 15% (vol/vol) differentiation FCS (StemCell Technologies, Vancouver). Alternatively, EBs were formed by the hanging-drop method with 450 cells per drop (16), and the resulting aggregated EBs were plated into the methylcellulose media for further differentiation. At various days of differentiation, EBs were collected and digested with 0.2% collagenase in 20% (vol/vol) FCS followed by dissociation into a single-cell suspension by passaging through a 20-gauge needle 5 to 10 times. Cell concentrations and viability were measured with trypan blue.

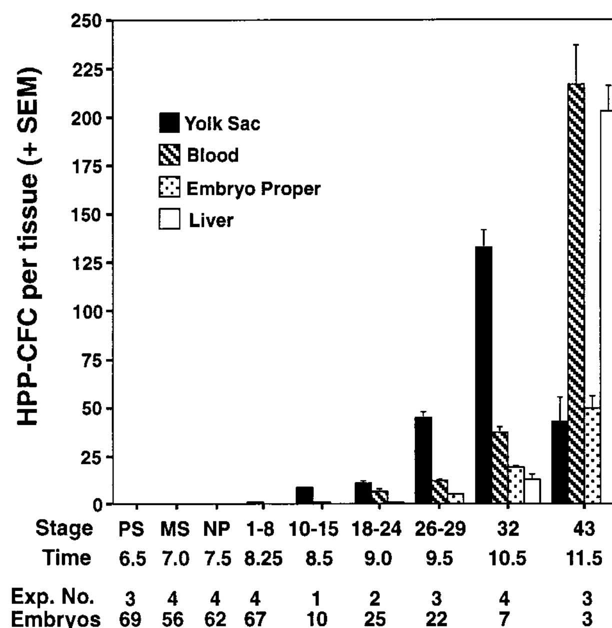


Fig. 1. Distribution of HPP-CFC (mean + SEM) within various compartments of staged mouse conceptuses during gastrulation and early organogenesis (E6.5–E11.5). The liver was assayed separately from the remainder of the embryo proper at 32 and 43 somite pairs. Representative results at each stage are shown; multiple independent experiments (Exp. No.) gave similar results. The total number of embryos (Embryos) investigated at each stage is also listed. PS, prestreak; MS, midprimitive streak; NP, neural plate.

Results

The rapid changes in embryonic growth and the wide intra- and interlitter variation make accurate staging of mouse embryos critically important for the study of developmental events. Therefore, we carefully staged presomite embryos by morphologic criteria (14) and later embryos by somite counts. The specific stages of mouse embryogenesis analyzed in this study with their developmental times and the numbers of embryos examined are summarized in Fig. 1. The quantitation of hematopoietic progenitors present in any given tissue depends on the production of complete single-cell suspensions. Several methods were tested and both collagenase plus serum (10) and trypsin/EDTA (9) produced single-cell suspensions with excellent cell viabilities that generated essentially equivalent numbers of HPP-CFC (data not shown).

Distribution of HPP-CFC Before the Onset of Circulation. We have determined recently that committed erythroid and macrophage progenitors first arise at mid- to late-primitive-streak stages of embryogenesis at E7.0 (9). Reasoning that multipotential hematopoietic precursors should precede the appearance of com-

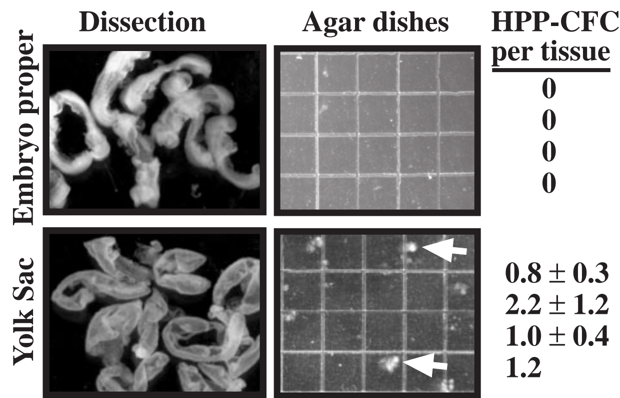


Fig. 2. Dissection of early somite-pair stage embryos (E8.25) into yolk sac and embryo proper tissues, and HPP-CFC (mean ± SEM) derived from those tissues. Grids on the agar dishes represent 2 mm. HPP-CFC are shown by white arrows. The number of HPP-CFC (mean ± SEM) per embryo proper and per yolk sac are shown for four independent experiments. HPP-CFC were plated in triplicate in three of four experiments.

mitted unilineage progenitors, we examined staged embryos before E7.0 for the presence of HPP-CFC. No HPP-CFC were detected during the onset of mesoderm formation at prestreak and early primitive streak stages of development (Fig. 1, E6.5). Furthermore, no HPP-CFC were detected in mid/late primitive-streak (E7.0) embryos, the stage where primitive erythroid progenitors are detected first (9). Nor were HPP-CFC detected in neural plate-stage conceptuses at E7.5, when yolk sac blood islands first become evident morphologically and globin gene expression begins (17, 18). The lack of HPP-CFC during early gastrulation could be caused by the absence of growth factors necessary for the *in vitro* differentiation of an embryonic HPP-CFC. However, the addition of vascular endothelial growth factor, IL-11, thrombopoietin, and flt-3 ligand to the standard assay did not result in the detection of HPP-CFC at presomite stages or in an increase in the numbers of HPP-CFC found at later stages of development (data not shown).

HPP-CFC were detected first in the mouse conceptus at early somite-pair stages (Fig. 1, E8.25). We examined the spatial distribution of these initial HPP-CFC, because at this stage primitive erythroid, macrophage, and the first definitive erythroid progenitors are confined to the yolk sac (9). Separation of the conceptus into yolk sac and embryo proper fractions in four independent experiments revealed that HPP-CFC were found exclusively within the yolk sac at low but reproducible numbers (Fig. 2). Because circulation of blood cells has not yet begun at early somite-pair stages (K. E. McGrath and J.P., unpublished observations), these results indicate that HPP-CFC originate in the yolk sac.

Distribution of HPP-CFC After the Onset of Circulation. HPP-CFC expand >100-fold in number in the yolk sac from E8.5 to E10.5 of mouse embryogenesis (Fig. 1). The yolk sac remains as the site within the conceptus containing the highest number of HPP-CFC during this time period. These results suggest that the yolk sac remains as a hematopoietic organ even after the disappearance of primitive erythroid progenitors at E9.0 (9). Subsequently, the number of HPP-CFC in the yolk sac declines as the liver becomes the predominant hematopoietic organ at midgestation (Fig. 1).

After the onset of circulation at E8.5, HPP-CFC are detected not only in the yolk sac but also in the bloodstream and the embryo proper (Fig. 1). Examination of the bloodstream as early as 10–15 somite pairs revealed low numbers of HPP-CFC (Fig. 1, E8.5), suggesting that HPP-CFC derived from the yolk sac enter the embryo proper with the first primitive erythroblasts. Over the next 48 h, HPP-CFC numbers increase steadily in the bloodstream and in the embryo proper. Between E10.5 and E11.5, there is a dramatic increase in HPP-CFC numbers in the bloodstream and in the fetal liver and a concomitant drop of HPP-CFC numbers in the yolk sac. These kinetics suggest, but do not prove, that yolk sac-derived HPP-CFC seed the fetal liver by way of the bloodstream.

Table 1. The number of replated primary high proliferative potential colony-forming cells (1° HPP-CFC) that give rise to secondary HPP-CFC (2° HPP-CFC) and secondary low proliferative potential colony-forming cells (2° LPP-CFC)

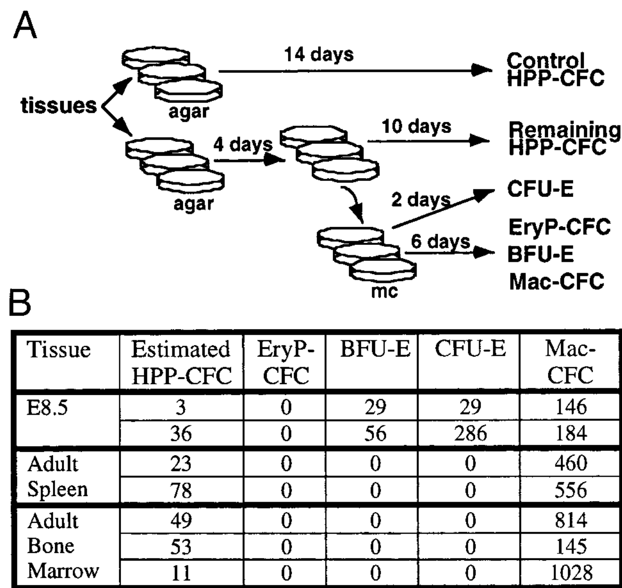
Time	Tissue	Replated 1° HPP-CFC	2° HPP-CFC	2° LPP-CFC
E9.5	Yolk sac	20	3	3
	Embryo proper	20	3	7
E10.5	Yolk sac	20	10	9
	Embryo proper	20	9	7

Twenty primary HPP-CFC from E9.5 and E10.5 yolk sac and embryo proper were replated individually in double agar dishes at days 6 or 7 of primary culture.

Embryonic HPP-CFC Are Multipotential. The multilineage potential of adult bone marrow-derived HPP-CFC has been demonstrated previously by their ability to give rise to secondary HPP-CFC and low proliferative potential (LPP) colonies (LPP-CFC) on replating (8). We therefore examined whether embryonic HPP-CFC derived from E9.5 and E10.5 yolk sac- and embryo-proper tissues can form secondary hematopoietic colonies. Yolk sac-derived and embryo proper-derived primary HPP-CFC gave rise to secondary HPP-CFC and LPP-CFC when individually replated at days 6 and 7 of primary culture (Table 1). The cellular content of 20 individual secondary HPP-CFC derived from primary E9.5 HPP-CFC was examined by staining. All 20 secondary HPP-CFC contained macrophages, neutrophils, and mast cells. As expected, macrophages made up the predominant cell type, constituting 63 ± 12% (mean ± SEM) of the cells, whereas neutrophils and mast cells constituted 24 ± 13% and 13 ± 9%, respectively. These replating results indicate that embryonic HPP-CFC are multipotential and that they have significant proliferative potential consistent with their early position in the hematopoietic hierarchy.

Embryonic HPP-CFC Give Rise to Definitive but Not Primitive Erythroid Progenitors. Adult bone marrow-derived HPP-CFC have been shown also to contain multiple hematopoietic progenitors when replated at day 4 of primary culture (5). To further investigate the multipotentiality of embryonic HPP-CFC, we performed similar replating experiments with E8.25 yolk sac-derived HPP-CFC, compared with adult bone marrow- and spleen-derived HPP-CFC. Cells from each of these tissues were suspended in double agar HPP-CFC cultures and at day 4 of culture, colonies greater than 10 cells were plucked, pooled, and replated in methylcellulose with cytokines that support the growth of erythroid and macrophage progenitors (see Fig. 3*A* for experimental design). HPP-CFC derived from the adult bone marrow and spleen gave rise to secondary macrophage progenitors. In contrast, HPP-CFC derived from E8.25–E8.5 embryos gave rise to macrophage and erythroid progenitors (Fig. 3*B*).

Because the yolk sac at early somite-pair stages (E8.25) contains both primitive and definitive erythroid progenitors, we asked whether yolk sac-derived HPP-CFC can give rise to both types of red cell progenitors. We replated cells from day 4 HPP-CFC in methylcellulose containing growth factors that



Tissue	Estimated HPP-CFC	EryP-CFC	BFU-E	CFU-E	Mac-CFC
E8.5	3	0	29	29	146
	36	0	56	286	184
Adult Spleen	23	0	0	0	460
	78	0	0	0	556
Adult Bone Marrow	49	0	0	0	814
	53	0	0	0	145
	11	0	0	0	1028

Fig. 3. (*A*) Experimental design for replating of day 4 HPP-CFC. Single-cell suspensions of various tissues were plated into six double agar dishes. At day 4, colonies >10 cells were plucked from three dishes, pooled, and replated into methylcellulose (mc) with growth factors that support both primitive erythroid (EryP-CFC), definitive erythroid (BFU-E and CFU-E), and macrophage (Mac-CFC) progenitor differentiation. HPP-CFC and committed hematopoietic progenitors were enumerated at the times shown. (*B*) Hematopoietic progenitors derived from day-4 colonies >10 cells (HPP-CFC) replated from early somite-pair embryos, adult spleen, and adult bone marrow. The number of replated HPP-CFC was estimated by the difference in HPP-CFC between control and remaining HPP-CFC dishes.

support the differentiation of both primitive and definitive erythroid progenitors. Although BFU-E and CFU-E were detected in replated embryonic HPP-CFC, no primitive erythroid progenitors were found. These findings suggest that precirculation HPP-CFC in the yolk sac can give rise to definitive but not primitive erythroid progenitors.

ES Cell-Derived EBs Give Rise to HPP-CFC. The differentiation of ES cells into EBs serves as a useful model of early embryonic events. This *in vitro* culture system has been shown to recapitulate embryonic hematopoiesis and has been useful particularly in the functional examination of genes needed for proficient hematopoiesis (19). Furthermore, the appearance of primitive erythroid progenitors proceeds that of definitive erythroid progenitors during EB differentiation, mirroring the kinetics found in normal mouse embryo (9, 19). We therefore examined the temporal relationship of HPP-CFC and primitive erythroid progenitors in EBs from days 3 to 10 of differentiation. Primitive erythroid progenitors were observed as early as day 3, peaked at day 5, and started to decline by day 7 of EB differentiation (Fig. 4), consistent with the observations of others (20). In contrast, HPP-CFC first appeared on day 5 and persisted to day 10 of EB differentiation (Fig. 4). We have observed the presence of HPP-CFC in EBs differentiated as long as 13 days (data not shown). In independent experiments, we have determined that BFU-E first arise no earlier than day 5 of EB differentiation (data not shown). These results indicate that HPP-CFC arise after primitive erythroid progenitors but persist for longer periods during EB differentiation.

We also generated EBs by using the hanging-drop method to quantify the frequency of HPP-CFC per EB. We allowed ES cells (450 cells/drop) to aggregate in hanging drops for 48 h before culture in the methylcellulose-based EB differentiation media.

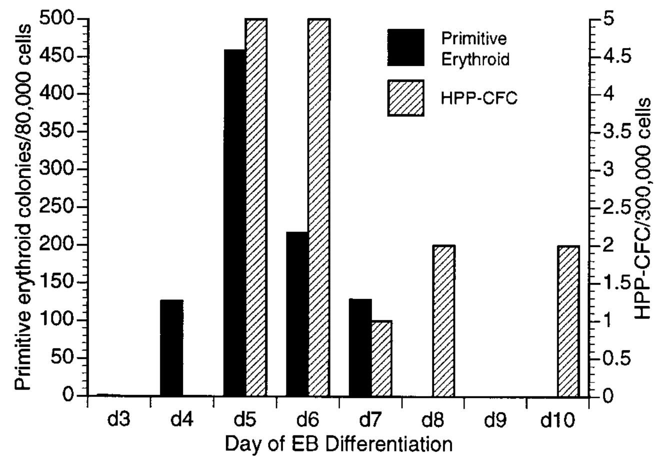


Fig. 4. The temporal appearance of primitive erythroid progenitors and HPP-CFC derived from EBs differentiated for 3 to 10 days. Although the results of a representative experiment are shown, two other independent experiments gave similar results.

To determine the frequency of HPP-CFC per EB, the number of EBs was counted before collection and dissociation. Consistent with our previous results, HPP-CFC were not observed until day 6 of EB differentiation (data not shown). Based on the ratio of cells per EB determined by dividing the total number of cells by the number of EBs harvested, the frequency of HPP-CFC per EB was calculated to be 1 in 33 on day 6 of EB differentiation.

Discussion

The hematopoietic system arises from mesoderm cells exiting the primitive streak during gastrulation. In the mouse embryo, two waves of erythroid progenitors have been detected before the onset of the circulation. The first wave of primitive erythroid progenitors begins at the mid-primitive-streak stage (E7.0) and remains confined to the yolk sac (9). The second wave of definitive erythroid progenitors (BFU-E) begins at early somite-pair stages (E8.25), also within the yolk sac. Unlike the primitive erythroid progenitors, these yolk sac-derived definitive erythroid progenitors are found subsequently in the newly formed bloodstream as well as the embryo proper (9). The developmental origin and lineage relationship of primitive and definitive erythropoiesis is unclear. Differences in developmental timing and cellular morphology of these two distinct erythroid programs have led to the hypothesis that primitive and definitive erythroid progenitors arise from distinct precursors (21). Targeted disruption of the Runx-1 (Cbfa2, AML-1) transcription factor leads to complete disruption of all definitive lineages although leaving primitive erythropoiesis relatively intact, further supporting the concept that primitive erythropoiesis forms as a distinct lineage (22). However, recent investigations of cultured ES cells indicate that primitive and definitive erythroid progenitors arise from an earlier hemangioblast precursor (15, 23). Studies in the *Xenopus* embryo have also supported the concept that primitive and definitive erythroid progenitors arise from a common mesodermal precursor (24).

To understand better the embryological origin of the hematopoietic system, we investigated the temporal and spatial distribution and biological characteristics of multipotential hematopoietic progenitors during early postimplantation mouse embryogenesis. The HPP-CFC assay was chosen because HPP-CFC represent the earliest *in vitro* hematopoietic precursors that do not require adult stromal cell support. Because unilineage hematopoietic progenitors were first evident at mid/late primitive-streak stages, we began our analysis at the start of gastru-

lation (E6.5). No HPP-CFC were detected at presomite stages, despite examining more than 180 embryos between days 6.5 and 7.5 of gestation, and despite the addition of several supplemental growth factors to the culture medium. If HPP-CFC are present before somitogenesis, their frequency must be less than ≈1 in 60 embryos at prestreak, mid-streak, and neural-plate stages of mouse development. We first detected HPP-CFC at 1 to 8-somite-pair stages (E8.25) within the yolk sac but not the embryo proper. Cells with myeloid and lymphoid potential have been found slightly later at 10 to 15-somite-pair stages (E8.5) in the yolk sac and embryo proper (11). CFU-S are detected first at the 27-somite pair stage (E9.5) in the yolk sac and the aorta–gonad–mesonephros region of the embryo proper (12). The precirculation embryo proper has been shown to harbor erythroid, myeloid, and lymphoid potential only when cultured on bone marrow stromal cells for 5 days before analysis of progenitor activity (25). Thus, yolk sac-derived HPP-CFC present at 1–8 somite pairs are the earliest directly differentiating multipotential progenitors described during mammalian embryogenesis.

The localization of HPP-CFC to the yolk sac before the onset of circulation indicates that embryonic HPP-CFC originate within the yolk sac. Their distribution correlates temporally and spatially with the first definitive erythroid progenitors (9), suggesting that yolk sac-derived definitive progenitors arise from HPP-CFC precursors. In contrast to these definitive erythroid progenitors, primitive erythroid progenitors are first detected in mid-primitive-streak embryos at E7.0 (9), more than 24 h before the appearance of HPP-CFC. These findings suggest that the primitive erythroid lineage does not arise from HPP-CFC. To further address this question, we replated E8.25-E8.5 HPP-CFC at day 4 of primary culture in conditions that support the differentiation of primitive and definitive erythroid progenitors. Secondary BFU-E and CFU-E, but no primitive erythroid progenitors, were detected. These results support the concept that embryonic HPP-CFC give rise to definitive but not primitive erythropoiesis in the yolk sac.

In contrast to embryonic HPP-CFC, adult bone marrow-derived HPP-CFC do not give rise to erythroid progenitors when replated on day 4 of primary culture (ref. 5; our data). This difference between yolk sac and bone marrow HPP-CFC may be caused by intrinsic biological differences between embryonic and adult HPP-CFC. This hypothesis is supported by our finding that HPP-CFC derived from adult spleen also do not give rise to secondary BFU-E. Our results do not exclude the possibility that embryonic and adult hematopoietic microenvironments effect the development of erythroid progenitors within differentiating HPP-CFC. Finally, it is possible that the yolk sac contains an earlier definitive erythroid precursor that can differentiate in the HPP-CFC culture medium and generate BFU-E when replated on day 4 of primary culture.

The development of techniques that support the differentiation of pluripotent ES cells in culture provides the potential of generating large numbers of hematopoietic stem cells for study. Unfortunately, that potential has yet to be realized. However, our studies demonstrate that HPP-CFC, hematopoietic precursors near the stem cell compartment, do develop during EB differentiation. We observed HPP-CFC beginning from day 5 to at least day 13 of EB differentiation. These observations are consistent with the studies of Hole *et al.* (26), who detected the emergence of multipotential CFU-A beginning between days 4 and 6 of EB differentiation and their persistence as late as day 20 of EB differentiation. Unlike the normal embryo, the frequency of HPP-CFC per EB remains extremely low. By using previously determined cell numbers per EB (19), we estimate the frequency of HPP-CFC per EB increases from 1 per 2,000 EBs on day 5, to 1 HPP-CFC per 100 EBs on day 7. The hanging-drop method of EB formation depends on the aggregation of hundreds of ES cells rather than the clonal growth of one ES cell. By using this alternative method of EB formation, we determined the frequency of HPP-CFC to be 1 in 33 EBs on day 6. This paucity of high proliferative potential hematopoietic progenitors in EBs likely explains, at least in part, the difficulty in reconstituting lethally irradiated hosts with cells derived from *in vitro*-differentiated ES cells.

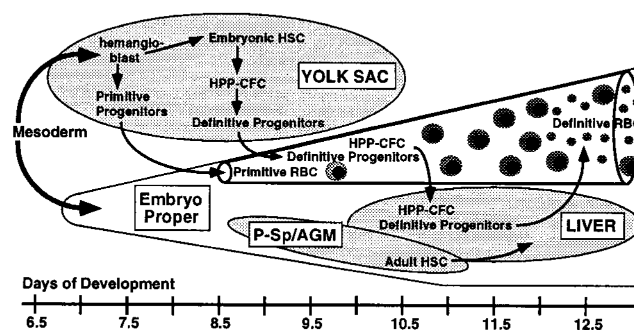


Fig. 5. Model of early hematopoietic ontogeny in the mouse embryo. P-Sp/AGM, para-aortic splanchnopleura/aorta-gonad-mesonephros region; RBC, red blood cells.

The temporal appearance of HPP-CFC was compared with that of primitive erythroid progenitors during EB differentiation. In multiple independent experiments, we observed the appearance of primitive erythroid progenitors at day 3, whereas HPP-CFC were not detected before day 5 of EB differentiation. These results are consistent with our findings in gastrulating mouse embryos and provide further evidence that multipotential definitive precursors arise well after the appearance of the primitive erythroid lineage. Furthermore, our findings strengthen the contention that *in vitro* EB differentiation recapitulates the initial stages of embryonic hematopoiesis.

Taken together with the work of others (23), our findings support the model of early hematopoietic ontogeny shown in Fig. 5. During early gastrulation, a subset of mesoderm cells destined for extraembryonic sites differentiates into hemangioblasts that rapidly give rise to the first endothelial cells and to primitive erythroid cells within yolk sac blood islands (23). With continued differentiation, these hemangioblasts give rise to definitive hematopoietic lineages, as evidenced by the presence of HPP-CFC (this study) and definitive erythroid progenitors (9) within the yolk sac of 1 to 8-somite-pair embryos. We hypothesize that these definitive precursors arise from "embryonic" HSCs that can repopulate fetal and newborn but not adult recipients. Yolk sac cells from E8.5–E9.5 conceptuses have been shown to engraft the erythroid and lymphoid compartments of fetal recipients (27, 28). Embryonic cells as early as E9.0 expressing high levels of CD34 contain embryonic HSCs capable of engrafting newborn recipients (8, 29). This transient population of CD34++ cells, present predominantly within the yolk sac, are enriched in HPP-CFC and committed definitive hematopoietic progenitors (8).

The yolk sac remains the predominant site of HPP-CFC expansion through E9.5. Subsequently, HPP-CFC numbers decrease in the yolk sac and increase dramatically in the bloodstream and fetal liver. These findings support the concept that two distinct waves of hematopoiesis originate in the yolk sac. The first is a primitive erythroid wave that is responsible for the synthesis of all circulating red cells in the mouse embryo until E12. The second is a definitive hematopoietic wave, consisting of HPP-CFC and multiple definitive lineages, that is responsible for the initial colonization of the fetal liver. It was hypothesized initially that HSCs arise in the

yolk sac where they give rise to primitive erythropoiesis before migrating to the fetal liver to initiate definitive hematopoiesis (30). HSCs capable of engrafting adult recipients are not present in the mouse embryo before E10.5-E11 in the aorta–gonad–mesonephros region (31). These "adult" HSCs are found subsequently in the yolk sac and liver beginning at E11.5 (31, 32). These findings have led to the hypothesis that HSCs capable of definitive erythropoiesis arise intraembryonically at midgestation (33). However, embryonic HSCs, capable of engrafting newborn recipients and providing long-term engraftment of secondary adult recipients, have been found in the yolk sac and the aorta–gonad–mesonephros region as early as E9.0 (29). We postulate that these embryonic HSCs arise in the precirculation yolk sac and give rise to the initial definitive wave of hematopoiesis. Proof that embryonic HSCs arise during gastrulation awaits future studies.

We thank Gordon Keller for critically reading the manuscript and for many helpful discussions. This work was supported by National Institutes of Health Grants RO1 HL59484 (to J.P.) and RO1 HL63169 (to M.C.Y.), and an Individual Research Service Award Grant CA84677 (to R.J.C.).

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