Enucleation of primitive erythroid cells generates a transient population of “pyrenocytes” in the mammalian fetus

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Enucleation is the hallmark of erythropoiesis in mammals. Previously, we determined that yolk sac–derived primitive erythroblasts mature in the bloodstream and enucleate between embryonic day (E)14.5 and E16.5 of mouse gestation. While definitive erythroblasts enucleate by nuclear extrusion, generating reticulocytes and small, nucleated cells with a thin rim of cytoplasm (“pyrenocytes”), it is unclear by what mechanism primitive erythroblasts enucleate. Immunohistochemical examination of fetal blood revealed primitive pyrenocytes that were confirmed by multispectral imaging flow cytometry to constitute a distinct, transient cell population. The frequency of primitive erythroblasts was higher in the liver than the bloodstream, suggesting that they enucleate in the liver, a possibility supported by their proximity to liver macrophages and the isolation of erythroblast islands containing primitive erythroblasts. Furthermore, primitive erythroblasts can reconstitute erythroblast islands in vitro by attaching to fetal liver–derived macrophages, an association mediated in part by α4 integrin. Late-stage primitive erythroblasts fail to enucleate in vitro unless cocultured with macrophage cells. Our studies indicate that primitive erythroblasts enucleate by nuclear extrusion to generate erythrocytes and pyrenocytes and suggest this occurs in the fetal liver in association with macrophages. Continued studies comparing primitive and definitive erythropoiesis will lead to an improved understanding of terminal erythroid maturation. (Blood. 2008;111: 2409-2417)

Introduction

It was recognized in the latter half of the 18th century that enucleation was a unique feature of mammalian erythropoiesis.1 Late-stage definitive erythroblasts in the fetal liver and the postnatal marrow of mammals enucleate by nuclear extrusion. Enucleation begins when vimentin intermediate filaments are lost and the nucleus becomes freely movable within maturing erythroid precursors.2 Soon thereafter, the acentric nucleus is extruded with a thin rim of cytoplasm and an enveloping cell membrane.3-7 The “extruded erythroblast nucleus” then loses phosphatidylinerine asymmetry of its plasma membrane and is rapidly engulfed by macrophage cells.8-10

In contrast to definitive erythropoiesis, where erythrocytes enter the circulation after enucleating, primitive erythroblasts emerge from yolk sac blood islands as immature erythroid precursors and progressively mature in the bloodstream.11,12 The circulation of primitive erythroblasts as nucleated cells has long suggested that they are more similar to the nucleated red cells of birds, fish, and amphibians than the red cells of fetal and adult mammals. However, primitive erythroid precursors in the mouse fetus, unlike avian precursors, lose vimentin intermediate filaments.13 We recognized that primitive erythroblast cells in the murine embryo ultimately enucleate and continue to circulate for several days after birth,12 an observation recently confirmed by others.14 Importantly, we found that primitive erythroblast cells do not decrease in number as they transition from late-stage erythroblasts to erythrocytes between embryonic day (E)12.5 and E16.5, indicating that enucleation is a normal end point of primitive erythropoiesis in the mouse.15 While definitive erythroblasts normally mature and enucleate in association with macrophages in the fetal liver and postnatal bone marrow, it is not clear where and by what mechanism primitive erythroid cells enucleate in the mammalian embryo. Here we show that late-stage primitive erythroblasts in the mouse embryo can physically associate with macrophage cells and that their enucleation leads to a transient population of extruded nuclei (“pyrenocytes”).
were fixed overnight in fresh 4% buffered paraformaldehyde, embedded in paraffin, and sectioned.

**DNA fragmentation assay**

A total of \(2 \times 10^5\) E11.5 fetal blood cells were washed in PBS and lysed in 100 \(\mu\)L lysis buffer (50 mM Tris, 10 mM EDTA, 0.5% SDS, 1 mg/mL protease K, Invitrogen, pH 8.0) at 55°C for 1 hour. DNA was purified by adding an equal volume of water and then extracting twice with 1:1 phenol/chloroform, followed by ethanol precipitation. The DNA was treated with 250 \(\mu\)g/mL RNaseA (Invitrogen) in Tris-EDTA buffer for 1 hour and subjected to electrophoresis on a 1.8% agarose gel. For controls, 2 \(\times 10^6\) murine bone marrow cells and E11.5 fetal blood cells were each resuspended in 1 mL of association media as described below in In vitro reconstitution of erythroblast islands, with the addition of 0.5 \(\mu\)M staurosporine (EMD Biosciences, San Diego, CA) and cultured for 6 or 24 hours at 37°C, 5% CO₂.

**Generation of anti-εγ-globin antibodies**

Antibodies to murine εγ-globin were generated as previously described for anti-βH1-globin antibodies,12 except that a peptide corresponding to amino acids 73 to 86 of εγ-globin (accession number NP_032247) was used as an immunogen.

**Immunohistochemistry and histologic staining**

For βH1- or εγ-globin immunohistochemistry, deparaffinized sections, methanol-fixed cytopsins, or methanol-fixed reconstituted erythroblast islands were pretreated for 15 minutes at 100°C in 0.1 M Tris pH 6.0, incubated with anti-globin antibodies, and visualized using avidin-biotin complex with Vector Red (Vector Laboratories, Burlingham, CA). For double immunohistochemistry, F4/80 antibody (Serotec, Raleigh, NC) staining was performed first, beginning with a 20-minute 3% buffered Triton-X pretreatment. F4/80 antibodies were detected using secondary goat antirabbit antibody conjugated to Alexa Fluor 488 (Invitrogen). For annexin V staining of DNA, photographed using the Hamamatsu Orca digital microscope (4\(\times\), 20\(\times\)) with PE-Ter119 followed by annexin V-Alexa Fluor 488 (Invitrogen) according to manufacturer’s instructions. DNA binding was visualized with a Necchi fluorescence microscope (4\(\times\), 20\(\times\)).

**Vascular morphology**

Smears of E14.5 blood were analyzed for εγ-globin expression and DAPI staining of DNA, photographed using the Hamamatsu Orca digital microscope, and total cellular and nuclear areas of primitive erythroblasts were determined using IPlab software (BD Biosciences, Rockville, MD).

**Amnis ImageStream analysis**

Fixed, permeabilized erythroid cells are very sensitive to lysis and hemoglobin loss; therefore, staining of dissociated fetal liver or peripheral blood was carried out using a method optimized for detection of human fetal hemoglobin containing erythrocytes.16 Briefly, high formaldehyde (4%) fixation was followed by rapid permeabilization in ~20°C acetone and immediate staining with minimal washing. Post-fixation in 1% formaldehyde stabilized staining by fixing antibodies within erythroid cells. Cells were stained with PE-Ter119 (eBioscience, San Diego, CA) and anti–εγ-globin antibodies visualized by binding of a secondary goat antirabbit antibody conjugated to Alexa Fluor 488 (Invitrogen). For annexin V binding studies, fixed fetal blood or adult marrow cells were stained with PE-Ter119 followed by annexin V-Alexa Fluor 488 (Invitrogen) according to manufacturer’s instructions. DNA staining was performed using DAPI according to manufacturer’s instructions. DNA binding was visualized with a Necchi fluorescence microscope (4\(\times\), 20\(\times\)).

**In vitro reconstitution of erythroblast islands with primitive erythroblasts**

**Isolation and stripping of erythroblast islands.** Partially dissociated fetal liver or bone marrow was pelleted gently (500g for 3 minutes) to enrich for islands and resuspended at \(8 \times 10^6\) cells/mL in “bottom media” (10% plasma-derived serum [PDS; Animal Technologies, Tyler, TX], IMDM [Invitrogen], 2 mM glutamine [Invitrogen], 0.15 mM monothialglycerol [MTG; Sigma-Aldrich], and 1 ng/mL M-CFS [Peprotech, Rocky Hill, NJ]); 1 mL of dissociated liver or bone marrow was added to the center of chambers of 2-well Lab-tek slides (Nalge Nunc, Rochester, NY) and allowed to adhere for 4 hours at 37°C, 5% CO₂. Erythroblasts were stripped from macrophage cultures by washing 2 to 3 times in calcium-, magnesium-free PBS allowing the media to sit 30 seconds between washes. Stripping of erythroblasts from attached macrophage cells was confirmed by microscopic observation before proceeding. Occasional unstripped erythroblast islands were observed, particularly near the edge of the chambers. Unstripped definitive erythroblasts were easily distinguished from primitive erythroblasts during the subsequent analysis because of their large nuclear/cyttoplasmic ratio and lack of εγ-globin staining (Figure S1; available on the Blood website; see the Supplemental Materials link at the top of the online article).

**Preparation of fetal blood.** Fetal blood was resuspended at 10⁶ cells/mL in “association media” (bottom media except with 30% PDS, 5% FBS) containing 2 U/mL erythropoietin [Amgen, West Greenwich, RI], 300 μg/mL transferrin [Sigma-Aldrich] and 40 ng/mL IGF-1 [Peprotech]) in plastic dishes. After a 2- to 4-hour incubation at 37°C, 5% CO₂ to allow adherent cells to attach, the supernatant was collected for use in reconstitution assays.

**Reconstitution and analysis of erythroblast islands.** A total of 1 mL of the nonadherent fetal blood cells was added to the stripped-adherent fetal liver or adult marrow cells and cultured for 24 to 48 hours at 37°C, 5% CO₂. The vast excess of fetal blood cells was then removed by aspiration and the slides briefly rinsed in PB1, fixed in ice-cold methanol for 5 minutes, and processed for immunohistochemistry with anti-εγ-globin and F4/80 antibodies to identify primitive erythroblasts and macrophage cells, respectively. For adherence assays, the number of primitive erythrocytoid cells attached to each of 100 to 400 macrophages was analyzed per experiment. For enucleation assays, the nucleation status of 100 to 1000 primitive erythrocytoid cells attached to macrophage cells was determined after culturing with HO to identify nuclei.

In control experiments, the culture of nonadherent fetal blood cells or of stripped erythroblast islands alone did not result in formation of erythroblast islands containing primitive erythroblasts in either the presence or absence of stripped-adherent liver cells; 8-well Lab-tek slides were also used successfully for reconstitution of erythroblast islands at a ratio of 1:1, 1:10, or 1:100 with HO to identify nuclei.

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erythroblasts using 3 washes with calcium-, magnesium-free PBS, and fixed with 4% formaldehyde.

Reconstitution of erythroblast islands in the presence of α4-integrin blocking antibodies was performed based on Sadahira et al.12 Either α4-integrin blocking antibody (SG31, Santa Cruz Biotechnology, Santa Cruz, CA) or IgG isotype control (BD Biosciences) were added at 10 μg/mL final concentration during the erythroblast island reconstitution with fetal blood cells as described above. After overnight incubation, nonadherent cells were removed by aspiration and fresh association media with 10 μg/mL antibody was added to the chambers for 30 minutes before washing with PB and fixation in ice-cold absolute methanol. After immunohistochemical analysis with anti-εγ-globin and F4/80 antibodies, reconstituted erythroblast islands were analyzed as described above.

Autonomous enucleation assay

Completely dissociated E14.5 liver cells or fetal blood cells were pelleted and resuspended at 4 x 10⁶ cells/mL in media optimized to support erythroid precursor maturation, but not erythroid or myeloid progenitor expansion or maturation (IMDM, 10% serum replacement [Invitrogen], 5% PDS, 10% protein free hybridoma II medium [Invitrogen], 2 mM glutamine, 0.15 mM MTG, 2 U/mL erythropoietin). After a 2-hour preincubation in plastic dishes to allow macrophage adherence, nonadherent cells were transferred to a fresh dish and cultured for 72 hours. Aliquots of cells were removed at 24-hour intervals and assayed for total cell numbers, viability by trypan blue exclusion, and percent nucleation by staining with 1 μg/mL HO.

Results

Primitive erythroblasts do not enucleate by splenic pitting or by karyolysis

It is now recognized that primitive erythroblasts originate in the yolk sac, mature in the bloodstream as a semisynchronous cohort and subsequently enucleate over a relatively narrow temporal window during development.12,14 Because the number of primitive erythroid cells does not decrease as they transition from late-stage erythroblasts to enucleated erythrocytes,12 we estimated the number of primitive erythroid enucleation events per day in outbred mice. Enucleation events were estimated using the percentage of enucleated primitive erythroblasts previously determined for each embryonic day, assuming that there are 6.7 x 10⁶ primitive erythroblasts per fetus and that total primitive erythroblast cell numbers do not decrease between E12.5 and E17.5 of gestation.12 (B) Immunohistochemistry of transverse section of E15.5 mouse embryo with F4/80 (blue) and anti-εγ-globin (red) antibodies reveals the presence of mature macrophage cells and primitive erythroblasts, respectively. At lower power (i), most of the macrophages can be seen in the liver (L) with few in the surrounding body wall and spleen (Sp) or stomach (St). At higher power (ii), infrequent large primitive erythrocytes are seen in vessels in the spleen (arrowhead), but they are not associated with the few splenic macrophages present at this time. Labeled primitive erythroblasts are observed as a subset of blood cells in the aorta (Ao) (iii). Scale bars = 0.1 mm. (C) Analysis of fragmentation of DNA from primitive erythrocyt cells in E15.5 peripheral blood. Lanes 1 and 2 show no fragmentation of E15.5 peripheral blood DNA in either untreated (lane 1) or after 24 hours of treatment with 0.5 μM staurosporine (lane 2). In contrast, bone marrow DNA shows significant laddering after 6 hours (lane 3) or 24 hours (lane 4) of treatment. Vertical lines have been inserted to indicate repositioned gel lanes.

In contrast, large numbers of primitive erythroblasts were identified within large fetal vessels (Figure 1Bii). These findings suggest that the developing splenic rudiment does not play a significant role in the enucleation of primitive erythroblasts cells.

Previous investigators have suggested that primitive erythroblasts enucleate by karyolysis.23,24 If karyolysis occurs as primitive erythroblasts circulate, then we would expect to see morphologic evidence of nuclear fragmentation within primitive orthochromatic erythroblasts. Careful examination of Wright-Giemsa–stained fetal blood smears from E12.5 to E17.5 fetuses failed to reveal any evidence of nuclear fragmentation (data not shown). To more positively identify all primitive erythroblast cells, fetal blood was stained with anti-εγ-globin antibodies and HO. No evidence of karyolysis in circulating primitive erythroblast cells was detected using this staining approach.

To examine whether terminal primitive erythroid maturation is associated with active DNA cleavage as seen in apoptosis, DNA
was isolated from E15.5 peripheral blood cells, where greater than 98% of the nucleated cells are primitive erythroblasts. As shown in Figure 1C, there was no evidence of DNA laddering in primary primitive erythroblasts (lane 1). Even after 24 hours of treatment with the apoptosis inducer staurosporine, there was no evidence of DNA laddering (Figure 1C lane 2). In contrast, DNA isolated from control adult marrow cells treated for 6 or 24 hours with staurosporine showed marked evidence of DNA fragmentation (Figure 1C lanes 3 and 4, respectively).

Appearance of extruded nuclei (“pyrenocytes”) in the bloodstream

Careful examination of the dual anti–ε-globin– and HO-stained E14.5 and E15.5 fetal blood smears revealed the presence of rare cells containing condensed nuclei surrounded by small rims of ε-globin–positive cytoplasm (Figure 2A arrows). Their small size, high nuclear to cytoplasmic ratio, and embryonic hemoglobin content suggested that they were the products of nuclear extrusion from primitive erythroid cells. We reasoned that if this were the case they should be distinctly smaller in overall cell size when compared with late-stage primitive erythroid precursors and yet have a similar nuclear size. To test this prediction, a morphometric analysis of the nuclear and total cell areas of these small ε-globin–positive cells and neighboring ε-globin–positive primitive erythroblasts was conducted. As shown in Figure 2B, there was a significant difference in total cell area, but no difference in nuclear area, suggesting that these small cells constitute a distinct primitive erythroid cell population. Because of their highly condensed nucleus and high nuclear to cytoplasmic ratio, we termed these cells “pyrenocytes.”

To further explore whether pyrenocytes constitute a distinct cell population, while avoiding the bias of their manual identification on blood smears, we took advantage of the multispectral imaging capabilities of the ImageStream flow cytometer (Amnis). This technology captures concurrent brightfield and 4 fluorescent images of single cells that can be analyzed for a combination of morphologic and fluorescent intensity perimeters. Fetal mouse blood was fixed, permeabilized, and stained with anti–ε-globin and Ter119 antibodies and Draq5, to identify nucleated primitive erythroid cells. As shown in Figure 3A (red line), 2 distinct populations of nucleated primitive erythroid cells were identified at E15.5 of gestation. The larger population consisted of primitive orthochromatc erythroblasts (EryP) and the smaller primitive pyrenocytes (rows 3 and 4). (C) Primitive pyrenocytes and primitive orthochromatic erythroblasts (EryP) in the E15.5 circulation have similar nuclear sizes. Error bars represent SD. (D) E15.5 blood stained with annexin V (AnV), Ter119, and the DNA stain Draq5 (shown as merged images), and 7AAD analyzed by the ImageStream. Row 1 is a primitive pyrenocyte that is annexin V–positive and 7AAD-negative. Row 2 is a primitive orthochromatc erythroblast that is negative for annexin V and 7AAD.
population, constituting approximately 2% of the total nucleated primitive erythroid cells, consisted of pyrenocytes, characterized on the ImageStream by their small size and extremely high nuclear to cytoplasmic ratio (Figure 3B rows 3 and 4). The ImageStream software was used to compare the nuclear size of these 2 cell populations. As shown in Figure 3C, both late-stage primitive erythroid precursors (EryP) and primitive pyrenocytes have a similar nuclear size. These findings confirm the morphometric analysis of the blood smears and indicate that primitive pyrenocytes constitute a distinct and previously unrecognized population of cells in the fetal bloodstream. Analysis of E12.5 fetal blood failed to reveal evidence of circulating pyrenocytes (Figure 3A green line). However, a minor population, constituting less than 1% of nucleated primitive erythroid cells, was clearly evident at E14.5 (Figure 3A blue line). These results further support the concept that primitive pyrenocytes are the product of enucleation because their temporal appearance coincides with that of primitive erythroid enucleation events (Figure 1A).

Definitive pyrenocytes, when generated in vitro, rapidly lose phosphatidylserine asymmetry.10 We therefore asked whether circulating pyrenocytes maintain their phosphatidylserine asymmetry. E15.5 blood was stained with annexin V, 7AAD-stained marrow treated overnight with 0.5 µM staurosporine also reveals a large percentage (40%) of annexin V–positive, 7AAD-negative cells. Bone marrow treated with 0.5 µM staurosporine also reveals a large percentage (40%) of annexin V–positive, 7AAD-negative cells.

Table 1. Percentage of analyzed cells stained with annexin V and/or 7AAD

<table>
<thead>
<tr>
<th></th>
<th>7AAD</th>
<th>Annexin V</th>
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<tbody>
<tr>
<td>EryP</td>
<td>93</td>
<td>6</td>
</tr>
<tr>
<td>Pyrenocytes</td>
<td>49</td>
<td>35</td>
</tr>
<tr>
<td>Treated marrow</td>
<td>52</td>
<td>40</td>
</tr>
</tbody>
</table>

Significant numbers of pyrenocytes, but not orthochromatic erythroblasts (EryP), have lost phosphatidylserine asymmetry and therefore are annexin V–positive and 7AAD-negative. Bone marrow treated overnight with 0.5 µM staurosporine also reveals a large percentage (40%) of annexin V–positive, 7AAD-negative cells.

Figure 4. Primitive erythroblasts may associate with fetal liver macrophage cells in vivo. (A) ImageStream images of liver cells viewed in brightfield (BF) or stained with anti–γ-globin (εy) and Ter119 antibodies and Draq5. The right panel is a combination of images of the 3 fluorescent stains. ImageStream analysis software facilitates the identification of different erythroid populations in the liver by quantifying brightfield characteristics (contrast and gradient RMS, ie, irregular surface) as well as fluorescent stain intensities. Primitive orthochromatic erythroblasts (row 1) were contrastlo, gradient RMSlo, εy-globinhi, Ter119lo, Draq5–, Primitive enucleated erythroblasts (row 2) were contrasthi, gradient RMShi, εy-globinlo, Ter119lo, Draq5–. Immature and mature definitive erythroblasts (rows 3 and 4, respectively) were distinguished from primitive erythroid cells by their nuclear size as well as by being contrasthi, gradient RMShi, εy-globinhi, and Ter119+. (B) Cytosinop preparations of E14.5 liver erythroblast islands stained with Wright-Giemsa reveal the presence of small numbers of primitive erythroid cells (*) attached to macrophage cells (m). (C) Immunohistochemistry of E15.5 liver reveals the very common spatial association (arrowheads) of εy-globin–positive primitive erythroid cells (red) with F4/80-positive macrophage cells (blue). Scale bar = 20 µm.

Table 2. Nucleated primitive erythroid cells are enriched in the fetal liver

<table>
<thead>
<tr>
<th>Primate enucleated, %</th>
<th>Blood</th>
<th>Liver</th>
<th>Liver/blood</th>
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<tbody>
<tr>
<td>15</td>
<td>5.6</td>
<td>6.6</td>
<td>1.2</td>
</tr>
<tr>
<td>31</td>
<td>2.2</td>
<td>2.8</td>
<td>1.3</td>
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<tr>
<td>36</td>
<td>1.8</td>
<td>2.4</td>
<td>1.3</td>
</tr>
<tr>
<td>78</td>
<td>0.3</td>
<td>0.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Data from 4 litters of E15.5 fetuses, with progressively higher percentages of nucleated primitive erythroid cells (left column) are shown. For each litter, the ratio of nucleated to enucleated primitive erythroid cells was determined in paired fetal blood and liver samples. As shown in Table 2, this comparison revealed a consistent and significant enrichment (1.2- to 1.7-fold, P ≤ .02) of nucleated cells in the liver compared with the bloodstream, indicating that late-stage primitive erythroblasts are preferentially retained in the liver at E15.5.

We next asked whether late-stage primitive erythroblasts are associated, like their definitive counterparts, with fetal liver macrocytes. The discovery of primitive pyrenocytes in the bloodstream suggested that enucleation of primitive erythroblasts may also be occurring within the circulation. However, careful examination of peripheral blood smears failed to reveal evidence of enucleating primitive erythroid forms (data not shown). We therefore investigated whether late-stage primitive erythroblasts, like their definitive counterparts, might be enucleating within the fetal liver. Late-stage primitive erythroblasts (Figure 4A row 1) and primitive erythroblasts (Figure 4A row 2) could be distinguished from definitive erythroid precursors (Figure 4A rows 3 and 4) using ImageStream analysis with a combination of εy-globin and Ter119 expression as well as quantifiable morphologic criteria, including cell size, nuclear size, and the brightfield characteristics of cell contrast and gradient RMS (a measure of surface irregularity). To examine whether primitive orthochromatic erythroblasts are en-
attached liver cells. Immunohistochemical analysis with anti–

slides, endogenous erythroblasts stripped away from the adherent 

Erythroblast islands from E14.5 livers were attached to microscope 

islands at the fetal liver. However, the short preparation time of this experimental approach 

minimizes, but does not exclude, the possibility that these primitive 

erythroid cells became associated with macrophage cells during the 

dissociation step.

To further determine whether primitive erythroblasts associate 

with macrophage cells in vivo, we examined the spatial distribution 

of primitive erythroblasts and macrophage cells. Initial experi-

ments were performed to identify the spatial and temporal distri-

bution of mature macrophage cells in the mid-gestation mouse 

embryo using the F4/80 antibody. At E14.5 to E15.5, when the bulk 

of primitive erythroblasts enucleate (Figure 1A), macrophage cells 

were at highest density in the fetal liver and only scattered 

F4/80-positive macrophage cells were present in other organs, the 

body wall, and the placenta (Figure 1C and data not shown). We 

then examined the distribution of \( \alpha_{\text{EV}} \)-globin–positive primitive 

erythroid cells in relation to F4/80-positive macrophage cells. As 

shown in Figure 4C (arrowheads), many primitive erythroid cells 

were found adjacent to fetal liver macrophage cells at E15.5 of 

gestation.

**Primitive erythroblasts physically interact with fetal liver 

macrophage cells in vitro**

The colocalization of many primitive erythroid cells with macro-

phage cells in the E14.5 to E15.5 liver raised the possibility that 

these cell types are physically interacting. To test this hypothesis, 

we developed an in vitro erythroblast island reconstitution assay. 

Erythroblast islands from E14.5 livers were attached to microscope 

slides, endogenous erythroblasts stripped away from the adherent 

macrophage cells, and E14.5 peripheral blood incubated with the 

attached liver cells. Immunohistochemical analysis with anti–\( \alpha_{\text{EV}} \)-globin and F4/80 antibodies revealed the surprisingly frequent and 
specific association of primitive erythroblasts with macrophage 

cells (Figure 5A). While primitive erythroid cells represented only 

31% of the circulating erythroid cells at E14.5, they constituted 

99% of the cells attached to macrophage cells in vitro.

The binding of primary, late-stage primitive erythroblasts to 

macrophage cells resulted in the reproducible in vitro reconsti-

tution of erythroblast islands. The frequency of macrophage cells 

with 0, 1, 2, 3, or 4 or more primitive erythrocytes is shown in 

Figure 5B (left panel). Reconstituted erythroblast islands, defined 
as 4 or more erythroblasts per macrophage, contained 6.6 plus or 

minus 0.2 (mean ± SEM, \( n = 5 \)) primitive erythrocytes per in 
vitro reconstituted island. Because E14.5 primitive erythroblasts 
can bind to homochronic fetal liver macrophages, we asked 

whether they could also bind to heterochronic and heterotopic 

macrophage cells. Specifically, we found that late-stage primitive 
erythroblasts efficiently bound macrophage cells isolated from 

E11.5 and E17.5 livers and adult marrow (6.1, 6.3, and 5.1 \( \alpha_{\text{EV}} \)-globin–positive cells/reconstituted island, respectively). In addition, 
we found that nucleated primitive erythrocytes bound specifically to F4/80-positive macrophages, but not to adherent 

F4/80-negative cells in the same culture (Figure 5B right panel). 

These results, taken together, indicate that late-stage primitive 
erythrocyte precursors are capable of specifically binding macro-

phage cells.

\( \alpha_{4} \) integrin is expressed by definitive erythroid precursors and 

mediates some of their physical interactions with macrophage cells 

through VCAM-1, the \( \alpha_{4} \) integrin counter-receptor expressed by 

macrophage cells.\(^{19} \) \( \alpha_{4} \) integrin is expressed on the surface of a 

subpopulation of primitive erythroid cells.\(^{14} \) We found by flow 
cytometry that the majority of F4/80-positive liver cells at E15.5 
express VCAM-1 on their cell surface (data not shown), confirming 
recently published immunohistochemical data that fetal liver 

macrophage cells express VCAM-1.\(^{26} \) Therefore, we tested whether 

\( \alpha_{4} \) integrin mediates adhesion of primitive erythroblasts to macro-

phage cells. The addition of \( \alpha_{4} \) integrin blocking antibody, 

compared with isotype control antibody, resulted in a marked 

reduction in the number of macrophages that form erythroblast 
islands (Figure 5C). In contrast, cultures treated with the isotype 

control antibody had a similar proportion of macrophages in 

erythroblast islands compared with untreated cultures (Figure 5B,C 
left panels). Furthermore, the addition of \( \alpha_{4} \) integrin blocking 

antibody caused a significant reduction in the average number of
primitive erythroid cells per erythroblast island (Figure 5D), while isotype control-treated cultures had no significant effect compared with untreated cultures (6.5 ± 0.3 vs. 6.6 ± 0.2, respectively). These results are consistent with a role for α4 integrin in the adhesive interactions that occur between primitive erythroblasts and macrophage cells.

**Primitive erythroblasts do not autonomously enucleate in vitro but may enucleate when associated with macrophages**

The findings that primitive erythroblasts preferentially localize to the fetal liver in vivo and can physically interact with macrophage cells in vitro raises the question whether primitive erythroid cells, like their definitive counterparts, normally enucleate while associated with macrophages. In control experiments, we first tested whether primitive erythroblasts, like their definitive counterparts, can enucleate without macrophage cells.10,27-29 E14.5 blood and fetal liver cell suspensions, with macrophage cells removed by differential adhesion, were cultured in cytokine conditions optimized to limit the differentiation of contaminating myeloid progenitors. The culture of E14.5 fetal liver cells in vitro led to the progressive increase in both nucleated and enucleated definitive erythroid cells (Figure 6A blue lines). In contrast, culture of fetal blood in the same conditions revealed no change in the number of nucleated primitive erythroblast cells or accumulation of more enucleated cells (Figure 6A red lines).

Because late-stage primitive erythroblasts did not appear capable of autonomous enucleation, we asked whether their coculture with macrophage cells might enhance their enucleation. The in vitro reconstitution assay was used to examine the primitive erythroid cell enucleation in the context of macrophage islands. In control experiments, where fetal blood was cultured without adherent cells, no evidence of autonomous enucleation was found, consistent with the results above (Figure S2). However, after 24 hours of coculture with adherent fetal liver cells, there was a significant increase in enucleation of primitive erythroid cells attached to F4/80-positive macrophages compared with blood cells cultured in parallel without adherent cells (Figure 6B). Furthermore, examination of the macrophage cells after coculture revealed internalized nuclei. The primitive erythroid source of these nuclei was confirmed by prelabeling the nucleated blood cells with cytotracker dyes (Figure 6C). These results, taken together, suggest that primitive erythroblasts enucleate while associated with macrophage cells.

**Discussion**

Enucleation is the hallmark of erythropoiesis in mammals.1 Definitive erythroid precursors in the fetal liver and postnatal bone marrow enucleate by nuclear extrusion. This process results in the production of reticulocytes and small, nucleated cells with a thin rim of cytoplasm and a cytoplasmic membrane that have been referred to as “extruded erythroblast nuclei.”4-7 These “extruded nuclei” undergo rapid loss of cell membrane phosphatidylserine asymmetry and are engulfed by macrophage cells.10,30 Because the cell membrane plays an important role in the biology of these cells, “extruded nucleus” is an inadequate, if not misleading, term. Because they contain an extremely condensed nucleus and have a very high nuclear to cytoplasmic ratio, we propose that these cells be termed “pyrenocytes,” derived from the Greek words “pyren” (πυρην, the pit of a stone fruit) and “cyte” (κυτη, cell).

In contrast to this process of nuclear extrusion seen in definitive erythropoiesis, it has been suggested that primitive erythroid cells in rodents enucleate by karyolysis,33,24 However, we did not find evidence of internal lysis of the nucleus in circulating late-stage primitive erythroblasts or evidence of DNA laddering in these cells even when exposed to staurosporine. In contrast, we identified a transient population of primitive pyrenocytes in the bloodstream.
and fetal liver of mouse embryos that appeared concurrent with the onset of primitive erythroblast enucleation. A similar, transient population of primitive pyrenocytes, described as ‘nuclei with a small amount of cytoplasm,’ has been described in the bloodstream of hamster fetuses. Our studies support the concept that primitive erythroblasts undergo a semisynchronous wave of enucleation by nuclear extrusion to form anucleate primitive erythrocytes and pyrenocytes.

It was observed 50 years ago that definitive erythroblasts mature while attached to a central macrophage cell within erythroblast islands. Subsequently, it was found that erythroblast islands could be isolated in vitro and that erythroblast cells could be stripped from, and subsequently added back to, the macrophage cells to reconstitute islands. Using a similar experimental approach, we found that circulating late-stage primitive erythroid precursors can reconstitute erythroblast islands by attaching to fetal liver- or adult marrow-derived macrophage cells. This surprising finding indicates that circulating primitive erythroblasts have the potential to physically interact with macrophage cells. Studies of erythroblast islands in the bone marrow indicate that several adhesion molecules mediate the physical interactions between definitive erythroblasts and macrophage cells, including α4 integrin, emp, and ICAM-4. Recent studies indicate that primitive erythroblasts also express emp and ICAM4 (data not shown). It will be of interest to determine which other adhesion molecules are expressed by primitive erythrocytes and whether these molecules contribute to the physical interactions that occur with macrophage cells.

Several findings support the concept that primitive erythroblast-macrophage interactions may occur in vivo. First, the ratio of nucleated to enucleated primitive erythroblasts is higher in the fetal liver than in the bloodstream, indicating that nucleated primitive erythroblasts are enriched in the liver. Second, immunohistochemical studies indicate that primitive erythroid cells and macrophage cells are in close physical proximity in the fetal liver but not the fetal spleen or placenta. Finally, primitive erythroblasts were occasionally found to constitute components of erythroblast islands isolated from the fetal liver. Macrophage cells in the fetal liver and bone marrow engulf definitive pyrenocytes that have lost phosphatidylserine asymmetry. We found that a significant proportion of primitive pyrenocytes in the fetal bloodstream are annexin V−positive and that nuclei derived from primitive erythroblasts are present within macrophage cells after in vitro coculture. It is likely that the enucleation of primitive erythroblasts in close physical proximity to macrophage cells facilitates the engulfment of newly formed pyrenocytes and that some primitive pyrenocytes may temporarily escape this fate in vivo leading to their transient appearance in the bloodstream.

A fundamental difference between primitive and definitive erythropoiesis is the maturation of primitive erythrocytoid cells in the circulation while definitive erythrocytoid cells mature extravascularly in erythroblast islands. It has been proposed that macrophage cells serve several roles to facilitate the maturation of definitive erythroid precursors including the provision of cytokines and iron, the enhancement of erythroblast proliferation, and the bringing of erythrocytoid cells in close proximity for regulatory purposes as, for example, fas–FasL interactions. In contrast, primitive erythrocytoid cells mature in the bloodstream and appear to have circumvented macrophage interactions during early steps of erythroblast maturation. A specific role for macrophages in the process of definitive erythrocytoid cell enucleation remains unclear, particularly because large percentages of definitive erythroblasts can enucleate in vitro in the absence of macrophage or stromal cells and in vitro culture of maturing definitive erythroblasts does not enhance their enucleation. In striking contrast, primitive erythrocytoid cells are unable to enucleate autonomously in vitro; however, coculture of late-stage primitive erythroblasts with macrophages leads to a small, but significant, increase in enucleation compared with blood cells cultured in parallel without macrophages. It is currently not known whether circulating primitive erythroblasts contain inhibitory mechanisms preventing enucleation or whether they lack intrinsic machinery necessary for autonomous nuclear extrusion. The continued study of the similarities and differences between primitive and definitive erythropoiesis will lead to an improved understanding of erythroblast maturation and the terminal steps of erythroid differentiation.

Acknowledgments

The authors thank Rachael Emerson and Sarah Mack for assistance with immunohistochemistry.

This work was supported by National Institutes of Health and Dean’s Research Award (University of Rochester).

Authorship

Contribution: K.E.M. designed and performed experiments, analyzed data, and wrote the manuscript; P.D.K. performed experiments and generated antibody, fetal dissections, and morphometry; A.D.K. performed experiments and animal husbandry; R.L.P. performed experiments; T.P.B. assisted with flow cytometry; J.P. analyzed data, and wrote the manuscript.

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

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References

Enucleation of primitive erythroid cells generates a transient population of "pyrenocytes" in the mammalian fetus

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