The fetal liver is a niche for maturation of primitive erythroid cells

Joan Isern*, Stuart T. Fraser*, ‡Zhiyong He*, and Margaret H. Baron*†§

Departments of *Medicine and ‡Developmental and Regenerative Biology, Oncological Sciences, and Gene and Cell Medicine, and †Black Family Stem Cell Institute, Mount Sinai School of Medicine, New York, NY 10029

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Primitive erythroid cells (EryP) are the earliest differentiated cell type of the mammalian embryo. They appear in the yolk sac by embryonic day 7.5, begin to enter the embryonic circulation 2 days later and continue to mature in a stepwise and synchronous fashion. Like their adult counterparts, EryP enucleate. However, EryP circulate throughout the embryo for several days before the first enucleated forms can be identified in the blood. We have used transgenic mouse lines in which GFP marks EryP to investigate this seemingly long lag and have identified a previously unrecognized developmental niche for EryP maturation. After exiting the yolk sac, EryP begin to express cell adhesion proteins, including α4, α5, and β1 integrins, on their surface and migrate into the fetal liver (FL), where they interact with macrophages within erythroblastic islands. Binding of EryP to FL macrophages in vitro is stage-specific and partly depends on VCAM-1. The ability to tag and track EryP nuclei using a transgenic mouse line expressing an H2B-EGFP fusion allowed us to identify and characterize extruded EryP nuclei and to demonstrate that molecules such as α4, α5, and β1 integrins are redistributed onto the plasma membrane surrounding the extruding nucleus. FL macrophages engulf extruded EryP nuclei in cocultures and in the native FL in vivo. We conclude that EryP home to, complete their maturation, and enucleate within the FL, a tissue that is just developing as EryP begin to circulate. Our observations suggest a simple solution for a puzzling aspect of the development of the primitive erythroid lineage.

enucleation | mouse embryo | primitive erythropoiesis | fetal liver | macrophage

Primrose erythroid cells (EryP) are the first to differentiate in the postimplantation embryo and are detectable within the blood islands of the yolk sac by embryonic day 7.5 (E7.5) (1–3). EryP enter the circulation around E9.5 as a synchronous cohort and continue to mature in a stepwise, developmentally regulated fashion (4). Surprisingly, the terminal event in EryP differentiation, enucleation, is not detected for another 3 days (E12.5) (4, 5); it is largely completed by E15.5 (4).

To tag and track EryP within the mouse embryo, particularly after midgestation, when large numbers of definitive erythrocyte cells (EryD) are present in the blood, we used a transgenic mouse line in which GFP is expressed in EryP (4, 6). We found that circulating EryP expressed a subset of cell adhesion molecules, including integrins, on their surface beginning around E12.5 and then appeared to redistribute some of these proteins, notably α4, α5, and β1 integrins, from the membrane of the nascent EryP reticulocyte to that surrounding the expelled nucleus (4). On the basis of these observations, we proposed that at least a subset of EryP may home to the fetal liver (FL) (4).

The FL is a major site for the development of EryD (7), which mature within erythroblastic islands (EBIs). EBIs, first identified in bone marrow and later in FL and spleen, are morphologically distinct 3D structures comprising a central macrophage surrounded by EryD at various stages of maturation (for a review, see ref. 8). Scanning EM studies have revealed macrophage extensions that surround peripheral erythroblasts, providing intimate membrane contact between these cells. The central macrophages of the EBI are thought to function as nurse cells during erythropoiesis and to provide an essential scavenger function, engulfing nuclei expelled from the maturing erythroblasts (reviewed in refs. 8–10).

Here, we report that the FL provides a previously unrecognized developmental niche for the maturation and enucleation of EryP. As early as E10.5, EryP are found in the FL, within EBIs, and continue to accumulate there through E14.5. One day later, nearly all EryP have enucleated and reentered the circulation. Integrins α4, α5, and β1 are dramatically up-regulated on the surface of EryP that have entered the FL, reflected in greatly enhanced binding to FL macrophages (FLM) in vitro. Blocking of vascular cell adhesion molecule-1 (VCAM-1), a counterreceptor for very late antigen-4 (VLA-4 or α4β1 integrin) expressed on macrophages, abrogates this adhesion. To examine EryP enucleation at higher resolution, we generated a transgenic mouse line in which the nuclei of EryP are marked by a histone H2B-EGFP fusion protein. Extruded EryP nuclei were detected in large numbers in the FL, even at a time when reticulocytes are first detected in the blood. Our findings suggest that EryP enucleation begins earlier than previously believed, and that the resulting reticulocytes may not be released into the circulation immediately. Adhesion molecules (α4, α5, and β1 integrins) were greatly enriched on the surface of the extruded nuclei, demonstrating unequivocally that these proteins are selectively partitioned away from the body of the developing reticulocyte. FLM engulf extruded EryP nuclei in cocultures and in the FL in vivo. These findings suggest a simple explanation for the puzzling lag between the initial formation of EryP in the yolk sac and the appearance in the bloodstream of enucleated EryP and provide important insights into the cellular events involved in the development of this lineage.

Results

Primitive Erythroblasts Accumulate Transiently in the FL. Our previous discovery that cell adhesion molecules are up-regulated on the surface of circulating EryP/GFP(+) cells (4) led us to consider the possibility that EryP home to a fetal tissue such as the liver, where they continue to mature. Indeed, we observed green fluorescence in the FLs of s-globin::KGFP transgenic embryos, in which expression of the GFP reporter is targeted to EryP from a human embryonic (s) globin promoter, (see legend, Fig. 1), from E10.5 through E14.5 (Fig. 1.4 and data not shown). By E15.5, when the majority of EryP (>95%) have enucleated (4), GFP was no longer detectable (Fig. 1A). FACs analysis revealed a peak in the numbers of EryP/GFP(+) cells approximately E13.5–14.5, with a sharp reduction by E15.5 (Fig. 1B).

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§To whom correspondence should be addressed. E-mail: margaret.baron@mssm.edu.

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Immunostaining of FL sections for the erythroid marker Ter119 or the macrophage marker F4/80 revealed that EryP/GFP(+) cells were present within the parenchyma of the FL in close association with macrophages (Fig. 1C). These observations suggest that EryP collect within and possibly home to the FL, a prominent site of definitive hematopoietic differentiation from around midgestation until the time of birth.

To tag, track, and FACS-purify EryP nuclei, we created a “second generation” transgenic mouse line, e-globin::KGFP, which expresses a histone H2B-EGFP fusion protein that labels EryP within this tissue.

EryPs in the FL Up-Regulate Cell Adhesion Molecule Expression and Interact with Macrophages in EBIs. Trafficking of circulating cells to tissues requires up-regulation of surface adhesion molecule expression. We asked whether the progressive up-regulation of cell adhesion molecules detected on EryP in the blood (4) continued after these cells entered the FL. α4, α5, and β1 integrins and CD44 were all dramatically up-regulated on EryP/GFP(+) cells in the FL compared with those in the circulation (Fig. 2A and B). Adhesion protein expression on FL-EryP was not promiscuous, however, because integrins β3 (Fig. 2B) and α2, αV, and β2 (data not shown) were not detected on EryP/GFP(+) in peripheral blood (PB-EryP) or FL. To determine whether EryP/GFP(+) cells are present within EBIs in the FL, native EBIs were isolated on coverslips [supporting information (SI) Fig. S1]. Coverslips were stained with antibodies against Vcam-1 or Mac1 to identify macrophages or for Ter119 to highlight erythroid cells (Fig. 2C). EryP/GFP(+) cells were clearly identified within structures resembling EBIs and containing a central macrophage. EBIs contained both EryP/GFP(+) cells and EryD, which do not express the transgene but stain for Ter119.

The Ability of EryP to Bind to FLMs Is Developmentally Regulated and Depends on Vcam-1. To dissect the molecular mechanisms underlying EryP maturation and enucleation, we established an EBI reconstitution assay in which EryP and macrophages are recombined and analyzed for binding. PB-EryP showed stage-dependent adhesion to FLMs (Fig. 3A–C). E12.5–E14.5 PB-EryP bound rapidly (<45 min) to FLMs and formed large rosettes containing up to 20–30 EryP per macrophage (Fig. 3B and data not shown). Few rosettes formed when FLMs were combined with E9.5 EryP which do not express the adhesion molecules we have examined to date (4), Fig. 3D) or E15.5 EryP (nearly all of which have enucleated) (Fig. 3C). The rosettes that did form from E9.5 or E15.5 EryP contained fewer than five EryP per macrophage. Therefore, circulating EryP can interact with FLMs during a distinct developmental window. It is worth

**Fig. 1.** Transient accumulation of EryP within the FL. (A) Brightfield and fluorescent images of whole FLs from E10.5 (Left), E12.5 (left side of Right), and E15.5 (right side of Right) e-globin::KGFP transgenic embryos. The transgene contained a minimal human embryonic e-globin promoter and 3′ splice and polyadenylation signals, a truncated LCR, and a GFP reporter (4, 6). GFP is expressed within the entire EryP cell. (B) Quantitation of GFP(+) cells per FL at different developmental stages. The numbers of embryos and litters examined are indicated. (C) FLs reflect the transient accumulation of EryP in the FL (Giemsa stain, E). Express embryonic in

**GFP(+)** cells (EryD) express βmaj- but not eY-globin (f).
process of enucleating (Fig. 4A Inset) or structures that resembled isolated nuclei (data not shown). To determine whether the GFP(+)/SSC(low) population contains extruded EryP nuclei, we sorted cells from E14.5 e-globin::H2B-EGFP and examined them by fluorescence microscopy. DAPI exclusion of fixed samples confirmed that the GFP(+)/SSC(low) population does not contain dying cells. Fig. 4B shows images of cells from both GFP(+)/SSC(low) and GFP(+)/SSC(high) populations. As we expected, the GFP (+)/SSC(high) population contained nucleated cells with a clearly identifiable cytoplasm and outer membrane, whereas the GFP (+)/SSC(low) population comprised nuclear structures surrounded by only a narrow rim of cytoplasm (Fig. 4B). The GFP(+)/SSC(high) and GFP(+)/SSC(low) populations both stained with DAPI (Fig. 4B) and with DRAQ5 (Fig. 4D), confirming their DNA content.

To test our hypothesis (4) that selected cell adhesion molecules are sorted onto the membrane surrounding the extruding nucleus, we stained blood cells from e-globin::H2B-EGFP embryos with antibodies against α4, α5, and β1 integrins and CD44 and analyzed their expression by FACS. Expression of integrins α4 and β1 (Fig. 4C; α5, data not shown) but not CD44 was much higher on extruded nuclei (red) than on EryP (green), indicating redistribution of these molecules during enucleation. Enucleated EryP and EryD (blue) displayed even lower levels of the integrins. The presence of free nuclei in circulation was consistent with the possibility that EryP enucleate in the blood. To determine whether FL-EryP were the source of the extruded nuclei, we analyzed cells dispersed from FL. A large population of GFP(+)/SSC(low) extruded nuclei was already present in FL at E12.5 and, surprisingly, most (~70%) of the EryP in the FL at this stage had apparently already enucleated (Fig. 4E). By E14.5, very few nucleated EryP remained and the population containing extruded nuclei was also significantly smaller, presumably reflecting active phagocytosis and degradation by macrophages. At E12.5, few GFP(+)SSC(high) expressed α4 or β1 integrin but by E14.5, these molecules were present on more than half of the cells within this population. In contrast, large numbers of the extruded GFP(+)SSC(low) nuclei displayed these adhesion molecules at high levels at both stages.

Macrophage Envelopment of Extruded EryP Nuclei in Vivo and in Vitro. To determine whether macrophages play a role in enucleation, we cultured e-globin::H2B-EGFP PB-EryP alone or with adherent FLMs for 24 h using several different protocols (SI Methods). Nonadherent and loosely adherent cells were collected, stained with DRAQ5 and analyzed by FACS (Fig. S2). Large (FSC(high); SSC(low)) cells were gated and the numbers of enucleated EryP (DRAQ5(intense);GFPe+) measured. Statistically significant enhancement of enucleation in macrophage-containing cultures was not detected under any of the conditions tested, for EryP at E10.5, E12.5, or E14.5. Nevertheless, immunofluorescence analysis of the EBs formed during coculture revealed not only adherent EryP but also EryP reticulocytes (enucleated) and extruded nuclei (Fig. 4F). We have not detected adhesion of enucleated EryP to macrophages in binding assays; therefore, we infer that those reticulocytes bound to FLMs after 24 h must have enucleated during culture. After rinsing the coverslips and stripping off bound EryP, we could identify macrophages containing engulfed nuclei, some in the process of degradation (Fig. 4F). Immunostaining of FL sections (Fig. 4G and Fig. S3B) and sorted F4/80(+);GFPe(+) FLMs (Fig. 4H and Fig. S3C) revealed that FLMs engulf extruded nuclei in vivo and in vitro. We have not observed EryP in association with F4/80(+) macrophages in fetal tissues other than liver.

Discussion

The FL Is a Previously Unrecognized Niche for Primitive Erythroid Development. EryP progenitors form and expand in the yolk sac of the mammalian embryo over a 2-day period (E7.5–E9.5),
enter the newly functional circulation and continue to mature in a synchronous, stepwise developmental progression (4) that terminates in enucleation (4, 5). EryP are detected in the blood throughout gestation and constitute a stable cell population that is present as late as 3 weeks after birth (4). The findings presented here suggest a simple and elegant solution to the puzzling question of why enucleation of EryP is not detected until days after their appearance: terminal maturation, including nuclear extrusion, occurs in the FL, which does not form until midgestation. EryP are not simply nomadic cells but apparently home to the FL.

Function of Adhesion Molecules in Primitive Erythroid Maturation in the FL. After entering the circulation, EryP begin to up-regulate the expression of a variety of adhesion proteins, including α4, α5, and β1 integrins and CD44 (4). Concomitant with their migration into the FL, a further, dramatic increase in adhesion molecule expression occurs. Whether the latter changes are cell autonomous or are triggered by extrinsic signals such as cytokines, interactions with other cells, and/or the hypoxic milieu of the FL is not known. However, they are of functional significance, because the ability of circulating EryP to bind to macrophages is developmentally regulated and maximal around the time of rapid enucleation (4) and EryP within the FL are able to bind to macrophages far more strongly than their circulating counterparts. After enucleation, the ability of circulating EryP to adhere to macrophages is lost and their numbers in the FL decline.

We propose that the alteration in macrophage-binding capacity is mediated, at least in part, by the partitioning of integrins α4, α5, and β1 onto the extruding nucleus. The resulting integrin-poor reticulocytes have now lost their counterreceptor for macrophage VCAM-1 and may more readily reenter the circulation. The redistribution of integrins during enucleation of EryP is likely preceded by modifications in their connections to components of the cytoskeleton (13). Partitioning of cell surface proteins is bidirectional: we have previously shown that Ter119 is present at higher levels on EryP reticulocytes than on nucleated EryP (4). We suggest that the mechanisms underlying enucleation in the primitive and definitive erythroid lineages are at least partly conserved. Selective partitioning of cell surface molecules on definitive erythroblasts (phosphatidylserine, β1 integrin, Ter119, and Emp) has been reported (13–16). Integrins α4 and α5 have also been detected on definitive erythroblasts in the FL (ref. 17 and our unpublished data), and we suspect that they are redistributed during EryD enucleation. Although binding of EryP to FLMs in vitro depended on VCAM-1, little if any decrease in adhesion was observed when blocking antibodies against α4 or β1 integrins were used (data not shown). Similar results were obtained by others using a different α4 integrin blocking antibody, with only a modest inhibition of binding (18). We note that EryP may also interact with other components
of the FL microenvironment (hepatoblasts, endothelial cells, extracellular matrix) during their maturation.

**Monitoring Primitive Erythroblast Enucleation by Using a Histone H2B-EGFP Transgene Reporter.** The e-globin::H2B-EGFP transgenic mouse line allowed us to identify and isolate newly extruded nuclei from PB and FL from E12.5 to E15.5. Similar structures were first described in the PB of hamster embryos (19) and have recently been reported in mouse embryos (18). The numbers of extruded EryP nuclei in circulation are much lower than in the FL, suggesting that a small fraction briefly escape engulfment by macrophages and enter the bloodstream. (We cannot exclude the possibility that some EryP enucleate while in circulation.) They presumably are phagocytosed later, when they circulate through FL or other tissues. That extruded EryP nuclei cannot be detected in the blood at all strongly suggests that they first become disconnected from EryP reticulocytes and are then engulfed by FLMs. We propose that adhesion molecules such as α4, α5, and β1 integrins create a sticky coating for the “shrink-wrapped” EryP nuclei and facilitate their phagocytosis. A model for the terminal steps in EryP maturation and enucleation is presented in Fig. S4.

Several lines of evidence suggest that macrophages are not essential for enucleation of definitive erythroblasts in vivo (9). In
contrast with others (18), we have not found enhancement of EryP enucleation during coculture on FLMs. We believe our approach is rigorous, because we analyzed all of the cell populations in these experiments. Recently it was reported that proliferation of definitive erythroblasts is stimulated by coculture with macrophages (20). A possible role for macrophages in proliferation and/or later steps in the maturation of EryP maturation remains to be evaluated.

Materials and Methods

Transgenic Mouse Lines. δ-globin::H2B-EGFP transgenic mice were generated by pronuclear injection of a construct analogous to δ-globin::KGFP (4, 6) at the Mount Sinai Mouse Genetics Shared Resource Facility and will be described elsewhere. Transgenic mice were maintained as hemizygotes or homozygotes on an ICR background; transgenic males were crossed with ICR females. All mice were bred at Mount Sinai School of Medicine according to institutional and American Veterinary Medical Association guidelines.

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Supporting Information

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SI Text

Methods

Embryo Collection, Cytological Analyses, Cryosectioning, and Immunohistochemistry. Embryo dissection (day 0.5 of gestation on day of plug), collection of embryonic blood, and Giemsa staining of cytospin preparations were as described (1). Cells were passed through a BD Falcon 40 μm cell strainer (cat. # 352340, Becton-Dickinson Biosciences) to remove clumps. Cryosectioning and immunostaining were performed as described previously (2, 3). All antibodies were used at 1:100 dilution in PBS containing 1% skim milk, 0.1% Tween-20 as described (2). In addition to the antibodies from ref. 2, we also used: PE-conjugated anti-mouse CD11b (Mac1, clone M1/70, cat. # 12-0112-81), eBioscience; Forssman antigen rat monoclonal (clone FOM-1, cat. # T-2113), BMA Biomedicals; rat anti-mouse F4/80 monoclonal (clone BMS8, cat. # ab16911, Abcam). AlexaFluor568-conjugated donkey or goat anti-rat (cat. # A-11057 or A-11077, respectively, Molecular Probes) was used to detect all primary antibodies.

Image Acquisition and Processing. Imaging of embryos and immunostained cells or fetal liver tissue and acquisition and processing of digital images were as described (1–3).

Flow Cytometry. FACS using fluorescently conjugated antibodies [details as described (1, 3)] and biotinylated anti-beta 3 integrin (CD61), clone 2C9.G3 (cat. # 13–0611; eBioscience). Cells were gated by size and granularity. Doublets were excluded by FSC-A/ FSC-W comparison. Dead cells were excluded from the analysis by using DAPI or propidium iodide staining (1). Labeled cells were analyzed using a FACScalibur or LSRII analyzer (Becton Dickinson) in the Mount Sinai Flow Cytometry Shared Research Facility. Data were analyzed using the FlowJo software package (TreeStar). Cell sorting was performed using a FACSVantage (Becton Dickinson) or an Influx Cell sorter (Citopeia) instrument.

Quantitative Real-Time Reverse Transcription-PCR (QRT-PCR) Analysis. Suspensions of single cells were washed once with PBS containing 10% FBS and collected by centrifugation for 5 min at 200 × g. RNA was purified using a Qiagen RNAeasy mini kit (cat. # 74104). Reverse transcription of total RNA (0.5 to 5.0 μg) was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (cat. # 18080–051, Invitrogen). Gene-specific primers and 5'-FAM (carboxyfluorescein);Black Hole Quencher (FAM.BHQ) probe(s) were designed using Primer Express 3.0 software (Applied Biosystems) and synthesized at the Hartwell Center at St. Jude Children’s Research Hospital (Memphis, TN). Primers and probe sequences for βmajor (βmajor)-globin were:

β-major (forward): GGAGGCCCATGGCAAGAAA
β-major (reverse): GCCCTTGAGGCTGTCCAA

β-major probe: FAM-TGATAACTGGCTTTAAACGTAGGGCTGAATCA-BHQ. Other primers and probe sequences were as reported (4). A series of diluted cDNA samples were used for 40-cycle PCR using the TaqMan system, as described (4). From the C values, the relative transcript concentration was calculated and normalized to that of the internal control according to instructions from Perkin-Elmer-Applied Biosystems, Inc., using an amplicon efficiency of 1.0. Data are represented as the mean of triplicate samples normalized to Gapdh ± SEM; similar results were obtained when one of the other internal controls was used (4). All three control genes (4) were tested in every experiment.

Preparation of Native EBIs. Native EBIs were isolated from fetal livers essentially as described (5, 6). Briefly, murine fetal livers (E12.5–14.5) were dissected and dispersed by either enzymatic digestion with collagenase/DNaseI or mechanically by extensive pipetting. Erythroblastic clusters were enriched by gravity sedimentation and plated on glass coverslips for 2–3 h to allow the EBIs to attach. The coverslips were washed by dipping into PBS to remove nonadherent cells and were then processed for immunofluorescence.

Preparation of Fetal Liver Macrophages and Reconstituted EBIs (Reseeding Assays). For preparation of FLMs, the dispersed fetal liver suspension was filtered through a 40 μm cell strainer, washed twice in PBS containing 5 mM EDTA, and plated onto 12 mm circular glass coverslips in RPMI containing FBS (10%). They were washed 2–3 h later with PBS to remove unattached cells, and residual bound erythroblasts were stripped off using PBS lacking Ca2+ and Mg2+. The remaining adherent fetal liver cells, containing mainly macrophages, were allowed to respread in RPMI plus 10% FBS for up to 6 days in culture. EBIs were reconstituted using a variation on a published protocol (5). EryP/GFP + cells resuspended in binding medium (RPMI containing 2% FBS; 106 in 100 μl) were then added to the cultures, which were incubated for 45 min at 37°C. Coverslips were gently rinsed several times in PBS to remove nonadherent cells and were immediately fixed in 4% paraformaldehyde (PFA) for 15 min at 4°C. After fixation, coverslips were washed in PBS and further processed for staining or scoring. For antibody blocking experiments, E13.5 or E14.5 GFP+/EryP (peripheral blood or fetal liver) were used. FL-EryP were isolated by dispersing and washing (3X) fetal liver cells in PBS containing 5 mM EDTA and resuspending in PBS containing 1% BSA. Blocking antibodies were added to cells in medium at 20 μg/ml. Antibodies used were: rat anti-mouse CD106 (VCAM-1) Low-Endotoxin, Azide-Free purified clone 429 (cat. # 105708, BioLegend); rat anti-mouse CD49d (α4 integrin, cat. # MCA1230EL, Serotec); hamster anti-mouse CD29 (β1 integrin, clone HMb1–1, cat. # 102210, BioLegend). Purified rat IgG2a, kappa chain (cat. # B112192, BioLegend) was used as an isotype control. Binding was quantified in multiple fields using the Automatic Measurement module of Axiovision software (Zeiss). Values measured for blocking antibody-treated cultures were normalized to those from the isotype control, which were set at 100.

Coculture of EryP and Macrophages. E14.5 embryonic blood cells (2 × 10^6) were cultured on coverslips with or without adherent FLMs in 0.5 ml EryP medium (7) containing transferrin (30 μg/ml; Roche) for 24 h at 37°C. Nonadherent cells were then removed, loosely adherent cells were collected by gentle washing with PBS, and the two groups of cells were then pooled and analyzed by FACS. Large (FSC<sub>high</sub>SFSc<sub>high</sub>) cells were gated and the numbers of enucleated EryP (DRAQ5<sup>neg</sup>GFP<sup>neg</sup>) measured. In an alternative approach, we focused on the subpopulation of EryP in circulation that can bind to macrophages. We allowed the cells to adhere to macrophages on coverslips for 45 min, then rinsed away all nonadherent cells. Half of the coverslips were then gently stripped of adherent EryP and were cultured for 24 h in the absence of macrophages. The remaining coverslips, con-
taining macrophages with attached EryP, were cultured in parallel for 24 h. This experimental protocol was compared side by side with cultures of total embryonic blood (no preadherence to macrophages; cultured with or without macrophages for 24 h) for embryonic stages E10.5, E12.5, and E14.5. We also performed these studies using FACS-purified E14.5 EryP. For direct visualization of the various cell populations present after culture, cover slips were gently rinsed with PBS, fixed in 4% PFA for 15 min at 4°C, stained with F4/80 and processed for analysis by immunofluorescence. For identification of engulfed nuclei within the macrophages, the bound EryP were stripped by treatment with PBS containing 5 mM EDTA for 15 min at 37°C.

Analysis of F4/80(+)GFP(+) Macrophages from β-globin::H2B-EGFP Fetal Livers. For immunofluorescence analysis, fetal liver cryosections (20 μm) or cytospin preparations from FACS-sorted F4/80(+)GFP(+) cells were stained using unconjugated rat anti-mouse monoclonal F4/80 (Abcam cat. # ab6640–250). For FACS, E14.5 fetal liver cells were stained with F4/80-APC-Cy7 antibody (BioLegend cat. # 122613).


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**Fig. S1.** Protocol for preparation of erythroblastic islands from fetal liver. Flow chart showing steps in preparation of EBIs from fetal livers. Adapted from refs. 1 and 2.
Fig. S2. Summary of EryP-macrophage coculture experiments. (A) Representative data from FACS analysis of EryP from E10.5, E12.5, E13.5, and E14.5 embryos. cH2B-EGFP PB-EryP were cultured alone or with adherent FLMs for 24 h using several different protocols (see SI Methods). Nonadherent and loosely adherent cells were collected, stained with DRAQ5 and analyzed by FACS. Large (FSChighSSChigh) cells were gated and the numbers of enucleated EryP (DRAQ5negGFPneg) measured. We were unable to detect a statistically significant enhancement of enucleation in macrophage-containing cultures under any of the conditions tested, for EryP at E10.5, 12.5, or 14.5 (7 independent experiments, 1–6 coverslips per condition). (B) FACS plots for E14.5 EryP-macrophage coculture data displayed in A (*, E14.5). Populations gated for high FSC and SSC are indicated. The GFPneg (Middle) and DRAQ5negGFPneg (Bottom) populations are indicated.
Fig. S3. Imaging of extruded EryP nuclei and EryP-reticulocytes in EryP-macrophage co-cultures. (A) F from Fig. 4 is shown at high magnification, with DAPI and DIC images included. (B) Additional examples (for Fig. 4G) of merged, rendered confocal images of 20-μm cryosections of E14.5 \( \beta\)-globin::H2B-EGFP FL, immunostained with F4/80 to identify FLMs. (C) Two additional examples of FACS-sorted F4/80(+)GFP(+) macrophages containing H2B-EGFP(+) nuclei (for Fig. 4H). (Scale bars, 10 μm.)
Fig. S4. Model for terminal steps in EryP maturation. EryP in the circulation display little or no α4 and β1 integrin on their surface. Surface expression of specific adhesion molecules (Fig. 2B) is strongly increased on EryP entering the fetal liver, allowing them to adhere to other EryP and to the central macrophages of EBIs. As EryP prepare to enucleate, redistribution of surface antigens (Ter119, white; α4 and β1 integrins, blue) occurs such that the reticulocyte (enucleated EryP) is decorated with Ter119 but displays little if any α4 and β1 integrins. Conversely, the extruded nucleus may be preferentially coated with α4 and β1 integrins, perhaps facilitating engulfment by macrophages. EBI, erythroblastic island.