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

Communicated by Elaine Fuchs, The Rockefeller University, New York, NY, February 29, 2008 (received for review December 22, 2007)

Primitive erythroblasts (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 erythroid blast 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.

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 β-globin::KGFP transgenic embryos, in which expression of the GFP reporter is targeted to EryP from a human embryonic (β) globin promoter (see legend, Fig. 1), from E10.5 through E14.5 (Fig. 1A 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 approx- imately E13.5–14.5, with a sharp reduction by E15.5 (Fig. 1B).

Author contributions: J.I. and S.T.F. contributed equally to this work; J.L., S.T.F., Z.H., and M.H.B. designed research; J.L., S.T.F., and Z.H. performed research; J.L., S.T.F., Z.H., and M.H.B. analyzed data; and M.H.B. wrote the paper.

The authors declare no conflict of interest.

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

This article contains supporting information online at www.pnas.org/cgi/content/full/0802032105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA

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

© 2008 by The National Academy of Sciences of the USA
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. 1A). 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::H2B-EGFP, which expresses a histone H2B-EGFP fusion protein that labels chromatin at all phases of the cell cycle (see refs. 11 and 12). The transgene contained the same regulatory elements used for the e-globin::KGFP 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) Right Immunostaining of 20-μm cryosection from E14.5 FL. F4/80 was used as a macrophage marker. sin, sinusoid. (Scale bar, 50 μm.) (Left) High-magnification view of E14.5 FL section. Ter119 was used as a marker for erythroid cells. At this stage, both EryP (green, Ter119(-)) and EryD (red only, Ter119(-)) are seen. (Scale bars, 10 μm.) (D) Sorted Ter119(+)/GFP(+) cells from e-globin::H2B-EGFP FL show the characteristic morphology of EryP (Giemsa stain, E), express e-Y-(but not βmaj-) globin at high levels (F) and are greatly enriched for eY-globin RNA compared with total FL (G). Ter119(+)/GFP(-) cells (EryD) express βmaj-but not eY-globin (F).

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. 3A] 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...
noting that only nucleated EryP were found bound to macrophages; we have previously reported that the surface antigen phenotype of nucleated and enucleated EryP is distinct (4). Consistent with their high-level expression of cell adhesion molecules, the macrophage-binding capacity of FL-GFP(+)/EryP cells was significantly greater than that of PB-EryP (Fig. 3D).

Integrins α4 and β1 (VLA-4) are strongly expressed on EryP within the FL (Fig. 2B). The counterreceptor for VLA-4 is VCAM-1, which we detected on FLMs by immunostaining of native EBs (Fig. 2C) and by FACS analysis of FL cells (data not shown). We found that formation of EryP clusters on FLMs depended partly on VCAM-1 (Fig. 3E). A blocking antibody against VCAM-1 reduced adhesion of cells from total FL (Fig. 3E) or PB-EryP (data not shown) by 75–80%. The experiments of Figs. 1–3 suggest that additional maturation steps, beyond those previously described for circulating EryP (4), occur in the FL.

Distribution of Cell Adhesion Molecules onto Extruding EryP Nuclei. Our previous studies pointed to a selective reorganization of antigens such as α4 integrin from the nascent EryP reticuloctye to the plasma membrane surrounding the expelled nucleus (4). Scatter analysis of PB-EryP/GFP(+) cells from E12.5-E14.5 e-globin::H2B-EGFP embryos revealed the presence of a rare subpopulation of GFP(+) cells (∼0.5–3%) with very low side scatter (SSC; Fig. 4, blue arrow) and forward scatter (FSC; data not shown). These properties are indicative of the low granularity and small size expected for free nuclei. The more prominent GFP(+) SSCneg population contained large (high FSC), granular (high SSC) cells, as expected for EryP. In wet prep of GFP(+) cells from dispersed e-globin::H2B-EGFP transgenic FL, we occasionally identified cells that appeared to be in the process of enucleating (Fig. 4A Inset) or structures that resembled isolated nuclei (data not shown). To determine whether the GFP(+) SSCneg population contains extruded EryP nuclei, we sorted cells from E14.5 e-globin::H2B-EGFP and examined them by fluorescence microscopy. DAPI exclusion of unfixed samples confirmed that the GFP(+) SSCneg population does not contain dying cells. Fig. 4B shows images of cells from both GFP(+) SSCneg and GFP(+) SSCpos populations. As we expected, the GFP(+) SSCpos population contained nucleated cells with a clearly identifiable cytoplasm and outer membrane, whereas the GFP(+) SSCneg population comprised nuclear structures surrounded by only a narrow rim of cytoplasm (Fig. 4B).

The GFP(+) SSCpos and GFP(+) SSCneg 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(+) SSCneg extruded nuclei was already present in FL at E12.5 and, surprisingly, most (∼70%) of the EryP in 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(+) SSCpos 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(+) SSCneg nuclei displayed these adhesion molecules at high levels at both stages.
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 FLMs 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 can 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).
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. ε-globin::*H2B-EGFP transgenic mice were generated by pronuclear injection of a construct analogous to ε-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.

ACKNOWLEDGMENTS. We thank Dr. A.-K. Hadjantonakis (Memorial Sloan Kettering Institute, New York) for an H2B-EGFP fusion construct and for helpful discussions. We are grateful to Dr. M. A. Dyer for comments on the manuscript and to an anonymous reviewer for suggesting that we evaluate engulfment of extruded nuclei by macrophages in vivo. This work was supported by a postdoctoral fellowship from the Cooley’s Anemia Foundation (J.I.) and by the National Institutes of Health (Grants RO1 DK52191, HL62248, and EB02209, to M.H.B.) and the Roche Foundation for Anemia Research (RoFaR, grant to M.H.B.). Transgenic mice were produced by the Mount Sinai Mouse Genetics Shared Research Facility (National Institutes of Health/National Cancer Institute Grant CA88302).