HEMATOPOIESIS

A Critical Role for PU.1 in Homing and Long-Term Engraftment by Hematopoietic Stem Cells in the Bone Marrow

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A LL BLOOD CELLS originate from a common progenitor termed the hematopoietic pluripotent stem cell (HSC). These cells have the capacity to self-renew, provide radioprotection, and long-term repopulating activity.1 Fetal hematopoiesis is a dynamic developmental process dominated by migrating populations of hematopoietic stem cells, progenitors, and precursors.2 The earliest detectable site of hematopoiesis occurs in the yolk sac at day 7. This stage of fetal hematopoiesis, referred to as embryonic or primitive hematopoiesis, is characterized by the generation of nucleated erythrocytes and a limited number of primitive macrophages. The adult/definitive hematopoietic system is thought to be generated from an intraembryonic site. At approximately day 8, hematopoietic activity can be detected in the para-aortic splanchopleura (PAS), which later develops into the aortic-gonad-mesonephros region (AGM).3,4 At day 9, the fetal liver (FL) becomes the active site of fetal hematopoiesis and remains the dominant site until the early neonatal period.5 The shift to the FL involves an initial wave of colonization by primitive erythroid and erythro-myeloid progenitors emanating from the yolk sac, followed by further waves of colonization consisting of circulating committed precursors and uncommitted multipotent progenitors from the PAS/AGM regions.6 Definitive HSC arise intraembryonically in the AGM region at day 10 and subsequently migrate to the yolk sac and FL.7,10 The frequency of HSC peaks in the FL at day 14.5 and then decreases as hematopoiesis gradually shifts to the bone marrow, the principal site of hematopoiesis in the adult.9,11

Regulation of HSC renewal, proliferation, and differentiation is still a poorly understood process. HSC are thought to be subjected to both stochastic and instructive elements within the hematopoietic microenvironment. The microenvironment consists of cellular contacts mediated by a myriad of different adhesion molecules and their cognate ligands together with locally produced cytokines. These factors act together to modulate stem cell differentiation, proliferation, and survival. Changes in the microenvironment that occur as HSC migrate from the FL to bone marrow may be responsible for a number of pronounced phenotypic and functional differences between fetal and adult HSC.1 The importance of the microenvironment has recently been highlighted by the demonstration that the developmental potential of HSC could be reprogrammed by changing their microenvironment.12 Little is known about the molecular mechanisms that enable different hematopoietic microenvironments to alter the functional properties of HSC during different stages of gestation. However, one key regulatory step modulating HSC function is transcription factor-dependent alterations in gene expression. Defining which transcription factors modulate gene expression in HSC is, therefore, fundamental to our understanding of hematopoiesis.

We and others have used gene targeting in mice to probe the function of transcription factors during hematopoiesis.13 The transcription factor PU.1, a member of the ets family of DNA binding proteins, is expressed only in the hematopoietic system.14 Targeted mutagenesis of the PU.1 gene causes a late gestational embryonic lethal phenotype and a profound defect in yolk sac and FL hematopoiesis.15-17 Extensive functional and phenotypic analyses have shown the total absence of lymphoid and myeloid lineages, but normal numbers of both erythroid and megakaryocytic progenitors in the mutants. These results suggest that PU.1 is required for the function of a multipotential lymphoid-myeloid progenitor population in the FL. Furthermore, normal levels of erythropoiesis and megakaryopoiesis in the FL and yolk sac indicate that hematopoiesis was properly initiated at both sites. Additional experimentation, consisting of FL adoptive transfer experiments and the generation of embryonic stem (ES) stem-cell–derived chimeric mice, established that the PU.1 mutation is cell intrinsic and cannot be rescued by a wild-type (WT) microenvironment.17 The inability of PU.1−/− FL cells to offer short-term multilineage reconstitution and radioprotection provided the first indication of a possible dysfunction in the FL HSC compartment. The short duration of...
the radioprotection assay prevented any further conclusions concerning the long-term repopulating potential of PU.1\(^{-/-}\) HSC. In addition, the ability of PU.1\(^{-/-}\) ES cells to contribute to the erythroid lineage in fetal chimeras but not in adult chimeras suggests that the requirement for PU.1 may change during hematopoietic development.\(^{17}\)

In this report, we have initiated a series of experiments to study the functional behavior of PU.1\(^{-/-}\) fetal HSC and multipotent hematopoietic progenitor cells (HPC) in the adult microenvironment. Competitive repopulation assays (CRA) showed that PU.1\(^{-/-}\) HSC are incapable of establishing long-term repopulation of the erythroid, lymphoid, or myeloid lineages. The lack of contribution suggests that PU.1\(^{-/-}\) fetal HSC are at a competitive disadvantage compared with WT adult HSC in the bone marrow. PU.1\(^{-/-}\) HPC failed to respond synergistically to erythropoietin (Epo) and stem cell factor (SCF), which play an important role during normal definitive erythropoiesis. The expression of c-kit on PU.1\(^{-/-}\) HPC was severely reduced. This may explain the inability of PU.1\(^{-/-}\) HPC to sustain erythropoiesis in the bone marrow. Homing and engraftment studies demonstrated that PU.1\(^{-/-}\) HPC are severely impaired in their ability to migrate to and colonize the bone marrow. PU.1\(^{-/-}\) HPC do not express a number of adhesion molecules, including a-integrins VLA-4/CD49d and VLA-5/CD49e, which have previously been shown to be important for HPC function. The lack of adhesion molecule expression may explain the dysfunctional properties of PU.1\(^{-/-}\) fetal HPC in vivo. Collectively, this study establishes that PU.1 is required for proper migration and engraftment of HPC in the adult bone marrow and suggests a new role for PU.1 in maintaining definitive erythropoiesis.

### MATERIALS AND METHODS

**Mouse strains.** PU.1 WT (PU.1\(^{+/+}\) or PU.1\(^{+/+}\)) and mutant (PU.1\(^{-/-}\)) embryos were generated and genotyped as previously described.\(^{15}\) C57BL/6-Ly 5.1 mice were obtained from Jackson Laboratories (Bar Harbor, ME).

**Antibodies/fluorescence-activated cell sorting (FACS) analysis.** Flow cytometric analysis was undertaken on single-cell suspensions prepared from FL and peripheral blood samples as previously described.\(^{16}\) Cell samples were stained with either fluorescein isothiocyanate (FITC)- or phycocerythrin (PE)-conjugated monoclonal antibodies (MoAbs) as stipulated by the manufacturer (PharMingen, San Diego, CA). PE-conjugated MoAbs included RA3-6B2 (B220), RM4-5 (CD4), and M1/70 (CD11b/Mac-1), FITC-conjugated MoAb included A20 (Ly5.1/CD45.1), 104 (Ly5.2/CD45.2), and 2B8 (c-kit/CD117).

**Erythroid progenitor assay.** E16.5 WT or PU.1\(^{-/-}\) FL single-cell suspensions (2 \(\times 10^5\)) were plated in 1.3 mL of a serum-free methylcellulose medium as described by Ratajczak et al.\(^{18}\) Briefly, a methylcellulose mixture was prepared containing a final concentration of 0.9% methylcellulose, 1% dopedilized bovine serum albumin, 270 µg/mL saturated transferrin, 20 µg/mL insulin, 5.6 µg/mL cholesterol, 2 mmol/L L-glutamine, 0.01% penicillin/streptomycin, and 100 µmol/L monothioglycerol. Methylcellulose cultures were supplemented with Epo (4 U/mL; Amgen, Thousand Oaks, CA) or Epo plus SCF (50 ng/mL; R&D Systems, Minneapolis, MN) to stimulate the generation of erythroid progenitors.

**Hemoglobin (Hbb) assays.** Cystamine-modified hemoglobin samples were analyzed by cellulose acetate electrophoresis as previously described.\(^{17}\) After electrophoresis, cellulose acetate plates were stained in a 1% Ponceau S solution (Sigma Laboratories, St Louis, MO) containing 5% trichloroacetic acid for 5 minutes and rinsed in 5% acetic acid. Quantification was performed by scanning densitometry image analysis to determine relative contributions of donor versus recipient hemoglobin isoforms.

**In utero HPC transplants.** PU.1\(^{-/-}\) (Ly5.2) and WT (Ly 5.1) pairs were crossed to generate timed pregnancies. E14.5, AA4.1\(^{+/+}\) WT (Ly 5.1) HPCs were enriched by magnetic bead selection to a cell density of 10\(^5\) µL as described below. Anesthetized pregnant PU.1\(^{-/-}\) females had their uterine horns surgically exposed, and each embryo (Ly 5.2) was injected with 10\(^5\) cells intraperitoneally via 60-µm glass needles through the uterine wall. After replacement of the uterine horns and suturing, the manipulated embryos were carried to term. Pups were genotyped at 3 weeks of age and were subsequently analyzed at 6 weeks to determine HPC contribution.

**CRA.** Recipient animals (C57BL/6-Ly 5.1) were irradiated with a single dose of 960 rads from a \(^{137}\)Cs source. Donor cells (Ly 5.2) were prepared as pooled single-cell suspension (10\(^5\)/mL in phosphate-buffered saline. E14.5, PU.1 WT (+/+ or +/+ or -/-) or mutant (-/-) FLs. Syngeneic competitive bone marrow cells (Ly 5.1) were prepared as a single-cell suspension in PBS. A mixture of 70% (5 \(\times 10^5\)), 80% (1 \(\times 10^5\)), or 90% (2 \(\times 10^5\)) Ly 5.2 FL cells along with a radioprotective dose of syngeneic Ly 5.1 bone marrow cells (2 \(\times 10^5\)) were retro-orbitally injected into anesthetized, lethally irradiated Ly 5.1 animals. After transplantation, the animals were maintained on water containing 20 mL/L of Sulfamethoxazole and Trimethoprim. On a monthly basis, approximately 0.2 mL of peripheral blood was obtained from recipient mice by tail bleeds. The harvested blood was mixed with an equal volume of 2% dextran sulfate, incubated at 37°C to precipitate erythrocytes, and treated with ammonium chloride lysis buffer to lyse any remaining erythrocytes. Mononuclear cells in the resulting supernatant were washed twice with PBS and analyzed by flow cytometry to detect Ly 5.2-derived lymphoid and myeloid cells. Contribution to the erythroid lineage was assessed by analyzing the presence of hemoglobin isoforms as described above.

**Enrichment and fluorescein labeling of FL AA4.1\(^{+/+}\) HPC.** Single-cell suspensions were prepared in Dulbecco’s modified Iscove’s medium (DMIM) from E14.5 FL and pooled as either WT or PU.1\(^{-/-}\) samples as described above. The AA4.1\(^{+/+}\) fraction was isolated from pooled FL cells by magnetic bead positive selection using MidiMACS separation columns and goat anti-rat IgG MicroBeads as recommended by Miltenyi Biotec (Auburn, CA). Briefly, pooled FL cells were incubated at a cell density of 5 \(\times 10^6\) to 1 \(\times 10^7\) cells/mL with saturating amounts of AA4.1 MoAb/MicroBeads for 30 minutes at 4°C. The AA4.1 MoAb/MicroBeads were prepared by mixing an AA4.1 MoAb, obtained from a hybridoma culture supernatant as previously described,\(^{17}\) with goat anti-rat IgG MicroBeads for 15 minutes at 4°C. The samples were loaded onto the MidiMACS separation columns, washed, and eluted as specified by the manufacturer. This procedure routinely resulted in a 100-fold enrichment of AA4.1\(^{+/+}\) cells.

For homing experiments, pooled FL cells were fluorescently labeled with carboxyfluorescein diacetate succinimidyld ester (CFDAse, Molecular Probes, Inc, Eugene, OR), as described.\(^{19}\) Briefly, 1 \(\times 10^7\) cells that have been extensively washed in PBS were incubated with a 10 µmol/L CFDAse solution for 10 minutes at room temperature while slowly agitating. The labeling reaction was quenched by adding an equal volume of newborn calf serum (NCS) and washing extensively with PBS. Alternatively, FL cells were fluorescently labeled using FITC (Isomer I; Molecular Probes) according to Butcher and Weissman.\(^{20}\) Briefly, cells at a density of 3 to 4 \(\times 10^7\) were labeled with a 60 µg/mL solution of FITC for 20 minutes at room temperature in a 1:1 mixture of complete DMIM and 1 \(\times\) PBS (pH 7.4) with a final serum concentration of 5%. After FITC labeling, the cells were washed with complete DMIM and centrifuged through a serum gradient. Labeled cells were
passed through a Nitex membrane before selection with AA4.1 MoAb/MicroBeads.

Short-term homing and engraftment of HPC. Fluorescein-labeled AA4.1+ cells (1 to 2 × 10^5) in 0.1 mL of PBS were transferred retro-orbitally into lethally irradiated C57B6 recipients as described above. Recipient animals were killed 5 to 48 hours posttransfer, and single-cell suspensions were prepared from the bone marrow, liver, spleen, and thymus. For the bone marrow, cells were harvested from both the femur and tibia and subsequently pooled. After transfer of the cell suspensions to flat-bottom multiwell tissue plates, the number of fluorescent cells in the entire sample was detected visually using a Nikon (Melville, NY) Diaphot 300 inverted microscope with an external fluorescent light source. Phase contrast visualization was used to confirm the viable nature of the cells counted. Nontransferred fluorescein-labeled cells provided a staining control. After quantification of total cell numbers per sample, results were normalized and reported as fluorescent cells per 10^7 total cells.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of HPC. Total RNA was isolated from E14.5 AA4.1+ pooled FL cells using RNAzol (Tel-Test, Woodlands, TX) according to the manufacturer's specification. Random-primed cDNA was synthesized from 2.0 µg of total RNA using the First Strand Synthesis Kit (Pharmacia Biotech, Upsalla, Sweden) as stipulated by the manufacturer. All PCR reactions were performed using 30 cycles and the following annealing temperature and primer pairs: αδ/VLA-4 (62°C; forward, 5'-CTGCAACGCCACGGTGCAAG-3'; reverse, 5'-GAGATCTGAAAGAAGCTCCAATTCTGTC-3'), αδ/VLA-5 (57°C; forward, 5'-GGCTTTCTCCGTCCTGGTATTAC-3'; reverse, 5'-GATGTTAGGCTGGCCACCTTCC-3'), β2 (62°C; forward, 5'-GTTGACCGTTGAAAGCATGAGGC-3'; reverse, 5'-GGTTGAGATTGAGTGGGAACACTC-3'), β1 (57°C; forward, 5'-CCTCATCTGGCAACATGGTGTCT-3'; reverse, 5'-GGAGGACGGTGAGTACCTGGAAGAG-3'), CD11b/αδ (57°C; forward, 5'-GACCAAGTTACAGCTGTGCTGACT-3'; reverse, 5'-CTTCAGCTGGGCTTTCATCTAAGC-3'), β2/CD18 (62°C; forward, 5'-CTTCTTGGATCTGCTGTC-3'; reverse, 5'-CCAGCTTCTTACGTGTGTTGAGG-3'), hypoxanthine phosphoribosyl transferase (HPRT; 62°C; forward, 5'-CAGCAAGCTAGAAGGATCTGCG-3'; reverse, 5'-GTTGGAGTGGAAAAGGACCTTTC-3'), PCR products were fractionated in 2% agarose and examined further by Southern blot analysis when necessary using cDNA fragments as hybridization probes.

RESULTS

In utero transplantation of WT HSC rescues PU.1+/− animals from a lethal hematopoietic defect. PU.1+/− embryos die by E17.5, apparently due to a severe hematopoietic defect. The mutant embryos are normal morphologically with unimpaired erythocyte and platelet production. Therefore, it is unclear why the embryos die. To determine if the defects found in PU.1+/− embryos are purely hematopoietic in origin, WT (Ly 5.1) hematopoietic progenitors were transplanted in utero. Both donor WT × WT (Ly 5.1) and recipient PU.1+/− × PU.1+/− (Ly 5.2) crosses were performed to generate timed embryos. AA4.1+ HPC were enriched from E14.5 WT (Ly 5.1) pooled FL cells and injected intraperitoneally into recipient embryos (Ly 5.2). AA4.1 is a known surface marker expressed on HSC and multipotent progenitors in the FL. The manipulated embryos were brought to term, genotyped by Southern blot analysis, and analyzed for hematopoietic contributions at 6 weeks of age. PU.1+/− animals were fully rescued by the transplantation of WT HPC and were indistinguishable from PU.1+/+ or PU.1+/− littermates. Flow cytometric analysis of B cells (B220+), monocytes (CD11b+), and T cells (CD4+) demonstrated that only donor-derived progenitors (Ly 5.1) contributed to hematopoiesis in these animals (Fig 1). In contrast, donor cells rarely contributed to hematopoiesis in PU.1+/+ or PU.1+/− recipients. A total of 3 rescued PU.1+/− and 6 transplanted WT animals were examined with equivalent results. The rescue experiments demonstrate that the lethal nature of the PU.1−/− defect is of hematopoietic origin. Furthermore, hematopoiesis can be supported by a PU.1−/− microenvironment.

PU.1−/− HSCs lack long-term repopulating activity in irradiated adult mice. Previous adoptive transfer experiments determined that PU.1−/− fetal HPC are unable to provide radioprotection to lethally irradiated adult recipients. However, the recipient animals did have detectable levels of donor-derived erythrocytes, but no lymphoid or myeloid engraftment, before dying 2 to 3 weeks postirradiation. The early death of the recipient animals receiving PU.1−/− FL cells prevented any further conclusions being drawn concerning HSC and/or HPC function. Therefore, a competitive repopulation assay was used to examine the functional status of PU.1−/− HSC in the adult bone marrow microenvironment. This assay system is designed to test the ability of HSC to offer multilineage reconstitution in vivo in the presence of a competing radioprotective dose of syngeneic bone marrow cells.

E14.5 FL contain the highest level of multilineage repopulating activity. Therefore, CRAs were performed with pooled WT (PU.1+/+ or PU.1+/− littermates) or PU.1−/− E14.5 FL samples.
(Ly 5.2) in competition with a radioprotective dose of syngeneic bone marrow (Ly 5.1). Two groups of 8 recipients received 70% (5 × 10^6) WT or PU.1^-/- FL cells plus 30% (2 × 10^6) syngeneic bone marrow, 2 groups received 80% (1 × 10^6) FL cells with 20% (2 × 10^6) syngeneic marrow, and a final 2 groups received 90% (2 × 10^6) donor to 10% (2 × 10^6) syngeneic. After transplantation, the recipients were bled on a monthly basis and analyzed for donor-derived contribution to the erythroid, lymphoid, and myeloid lineages. Donor-derived erythrocytes were detected by differences in Hbb isoforms. Cellulose acetate electrophoresis was used to distinguish differences between the Hbb^d (Ly 5.2, donor) and the Hbb^s (Ly 5.1, syngeneic) isoforms. Relative contributions of the hemoglobin isoforms were quantified by scanning densitometry. The presence of donor-derived lymphoid and myeloid cells was ascertained by 2-color flow cytometry using lineage- and Ly 5.2-specific MoAbs. The CRA were repeated a total of 3 independent times.

Reconstitution of the erythroid lineage by WT and PU.1^-/- FL cells is shown in Fig 2A. Reconstitution by donor-derived erythrocytes is expressed as the percentage of donor-derived hemoglobin (Hbb^d) in the peripheral blood. At 1 month posttransplant, WT FL cells were contributing to erythrocyte production at nearly the expected ratios based on input percentage. By 2 and 3 months posttransplant, the WT FL cells were contributing at equal to or better than the expected ratios. In contrast, FL cells isolated from PU.1^-/- littermates only produced erythrocytes for approximately 1 month and at greatly reduced levels. Results for reconstitution of the lymphoid and myeloid compartments are shown in Fig 2B. Unlike the contribution to the erythroid lineage by PU.1^-/- HPC, there were no detectable PU.1^-/- (Ly 5.2^-/^-)-derived B cells (B220^+), T cells (CD4^+), or monocytes (CD11b^-) posttransplantation (Fig 2B). Reconstitution was monitored for a total of 6 months with no detectable contribution by PU.1^-/- HSC. For WT FL cells, a substantial portion of mononuclear cells in the peripheral blood expressed Ly 5.2 and lineage markers (B220, CD4, CD11b) for the full course of the experiment. These results demonstrate that PU.1^-/- FL HPC are incapable of contributing to long-term erythropoiesis, lymphopoiesis, or myelopoiesis in the bone marrow.

PU.1^-/- FL erythroid progenitors fail to respond to SCF stimulation. The inability of E14.5 PU.1^-/- FL HPC to support long-term erythropoiesis contrasts with the normal number of FL or yolk sac erythroid progenitors in PU.1^-/- embryos and with the capacity of PU.1^-/- ES cells to establish erythropoiesis in fetal chimeras.16,17 The differing requirement for PU.1 alludes to a fundamental difference between fetal and adult erythropoiesis. One critical regulatory signal governing erythropoiesis that has been shown to change during development emanates from the stem cell factor receptor (c-kit).24,25 In vivo administration of anti-c-kit MoAbs showed that primitive erythropoiesis in the yolk sac and FL is independent of c-kit function, whereas definitive erythropoiesis depends on c-kit activity. To examine the ability of SCF to promote erythropoiesis, a serum-free progenitor assay system, was chosen to test the synergistic activity between Epo and SCF for the generation of erythroid progenitors (colony-forming unit-erythroid [CFU-E]).18 The assay was performed in duplicate on 6 different

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**Fig 2.** CRA of the hematopoietic lineages by WT and PU.1^-/- E14.5 FL cells. Irradiated Ly 5.1 adult mice (Hbb^s) were transplanted with either 70% (5 × 10^6), 80% (1 × 10^6), or 90% (2 × 10^6) E14.5 FL progenitors from donor WT (PU.1^-/-) or PU.1^-/- embryonic littermates (Ly 5.2, Hbb^d). A radioprotective dose of 2 × 10^6 normal adult bone marrow cells was included as a source of competitive, syngeneic HPC. Peripheral blood was taken from recipient animals on a monthly basis for 6 months postengraftment. (A) Contribution of donor HPC to peripheral blood erythrocytes. Cellulose acetate electrophoresis was used to separate donor from recipient hemoglobin isoforms. Relative contribution was determined by scanning densitometry and expressed as the percentage of donor contribution to total hemoglobin production. Data from 3 independent experiments are depicted on the graph. Solid lines represent animals that received WT donor HPC. Dashed lines represent animals that received PU.1^-/- donor HPC. (B) Contribution of donor HPC to the lymphoid and myeloid lineages. Contribution to the lymphoid and myeloid lineages was determined by flow cytometric analysis using an Ly 5.2^-/- specific MoAb to identify donor-derived cells and lineage-specific MoAbs to characterize the B-cell (B220^-), T-cell (CD4^-), and monocyte (CD11b^-) populations. Representative FACS profiles are shown for peripheral blood samples obtained at 2 months posttransplantation for lethally irradiated Ly 5.1^-/- adult mice receiving either pooled WT or PU.1^-/- E14.5 FL HPC (Ly 5.2^-/-).
embryos of each genotype. E16.5 WT FL cells produced 9 ± 6 CFU-E per 10^6 cells in response to Epo alone. PU.1^+/− cells produced an equivalent 7 ± 2 CFU-E with Epo alone. WT cells had a synergistic response to a combination of Epo and SCF to produce 551 ± 230 CFU-E per 10^6 cells. In contrast, PU.1^+/− cells still only produce 13 ± 1 CFU-E in response to Epo plus SCF, indicating a lack of synergy. Day E16.5 was chosen for this analysis because our previous studies quantifying FL erythroid progenitors showed normal numbers of PU.1^+/− CFU-E when assayed in the presence of serum with a cocktail of growth factors.17 Flow cytometry analysis was performed to ascertain the effect of the PU.1 mutation on c-kit expression. Very few E16.5 PU.1^+/− FL cells express detectable levels of c-kit on their surface (Fig 3). This analysis has shown that the lack of c-kit expression by E16.5 PU.1^+/− FL cells may explain the inability of PU.1^+/− erythroid progenitors to synergistically respond to Epo plus SCF.

The effect of PU.1 on homing and engraftment of FL progenitors in the adult bone marrow. One possible explanation for the lack of long-term hematopoiesis in recipient adult animals is that PU.1^+/− HPC are more prone to programmed cell death. TUNEL assay of E14.5 whole FL cell suspensions or AA4.1^+ enriched progenitor cell populations, containing a mixture of HSC and multipotent progenitor cells, showed no detectable apoptotic cells in either population (data not shown). These results suggest that an increased rate of apoptosis is an unlikely explanation for the aberrant behavior of the HPC compartment in PU.1^+/− FL. An alternative possibility to explain the inability of PU.1^+/− HSC to establish long-term reconstitution in adult animals would be a defect in the ability of progenitor cells to home to and engraft in the adult bone marrow.

To monitor migration, pooled FLs from WT (PU.1^+/+,+/−) and PU.1^−/− E14.5 embryos were fluorescently labeled with either CFDASE or FITC. After fluorescent labeling, tagged cell populations were enriched for AA4.1^+ progenitors by magnetic bead selection and injected retro-orbitally into lethally irradiated adult mice. Recipient animals were killed at 5, 16, or 48 hours posttransplant. Bone marrow, spleen, thymus, and liver were examined for the presence of fluorescent cells (Table 1). The results for the spleen and thymus were essentially constant for all time points, with approximately 2-fold fewer PU.1^+/− AA4.1^+ HPC migrating to these tissues. A striking difference was observed between the WT and PU.1^+/− AA4.1^+ FL progenitors in the bone marrow. At 5 hours posttransplant, there was a 2.5-fold reduction in PU.1^+/− HPC migrating to the bone marrow. By 16 hours posttransplant, there were 8.5-fold fewer PU.1^+/− HPC in the marrow. The gap widened to an 11-fold decrease in the marrow by 48 hours. In contrast, after 48 hours, there was a 9-fold increase in PU.1^+/− AA4.1^+ progenitors detected in the adult liver when compared with WT. The data presented in Table 1 represent a single homing assay that has been repeated 4 independent times with similar results. Therefore, HPC from PU.1^+/− FLs are severely impaired in their ability to home to and engraft the adult bone marrow. The altered migration of the mutant progenitor cells results in their abnormal accumulation in the liver after transplantation.

### Table 1. Quantification of Fluorescently Tagged E14.5 WT and PU.1^+/− AA4.1^+ FL Cells Migrating to the Bone Marrow, Spleen, Thymus, and Liver

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bone Marrow</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Liver</th>
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<tr>
<td>(A) 5 Hours posttransplant</td>
<td></td>
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<tr>
<td>WT (n = 3)</td>
<td>149 ± 14 151 ± 22</td>
<td>61 ± 11 117 ± 33</td>
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<tr>
<td>PU.1^+/− (n = 3)</td>
<td>67 ± 17 82 ± 7</td>
<td>42 ± 8 201 ± 30</td>
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<tr>
<td>(B) 16 Hours posttransplant</td>
<td></td>
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<tr>
<td>WT (n = 3)</td>
<td>184 ± 25 85 ± 7</td>
<td>160 ± 60 45 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PU.1^+/− (n = 3)</td>
<td>22 ± 12 54 ± 13</td>
<td>72 ± 22 310 ± 60</td>
<td></td>
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<tr>
<td>(C) 48 Hours posttransplant</td>
<td></td>
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<tr>
<td>WT (n = 2)</td>
<td>98 ± 20 265 ± 50 147 ± 3</td>
<td>39 ± 7</td>
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<tr>
<td>PU.1^+/− (n = 2)</td>
<td>9 ± 4 138 ± 21</td>
<td>70 ± 15 349 ± 35</td>
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Approximately 1 to 2 × 10^6 fluorescently labeled, AA4.1^+ FL cells were transplanted into lethally irradiated adult recipients. At 5, 16, or 48 hours posttransplantation, recipient bone marrow, spleen, thymus, and liver were examined for the number of fluorescently tagged cells. Results are depicted as the mean number (±SD) of fluorescent cells per 10^6 cells counted.

Adhesion molecule profile of hematopoietic progenitors isolated from PU.1^+/− E14.5 FL. The observed defect in the ability of PU.1^+/− progenitors to home to the bone marrow prompted an evaluation of a number of adhesion molecules thought to mediate hematopoietic progenitor cell migration. Initial flow cytometry experiments demonstrated that the integrin CD11b was missing and suggested that integrins α4 and α5 were affected on HPC, but not in the FL as a whole (data not shown). Integrins α4/α6/4-α5/VLA-5 have been strongly implicated in the attachment of hematopoietic progenitors to bone marrow stroma and associated extracellular matrix.26-28 To more closely examine integrin α4 and α5 expression, RT-PCR analysis was undertaken on total cellular RNA isolated from either whole FLs or AA4.1^+ enriched cell populations from WT and PU.1^+/− embryos. RT-PCR followed by Southern blotting was chosen as the most sensitive method to examine expression given the difficulty of obtaining large numbers of PU.1^+/− HPC.
Figure 4 shows that the integrin CD11b/αM is not expressed by PU.1<sup>-/-</sup> AA4.1<sup>+</sup> cells, whereas expression of its heterodimeric binding partner, integrin β2/CD18, is reduced. Both the α4 and α5 integrins were expressed in whole PU.1<sup>-/-</sup> FLs, but not in the AA4.1<sup>+</sup> PU.1<sup>-/-</sup> FL HPC, even after Southern blot analysis of the PCR reactions products (Fig 4). Expression of the integrin β1, which has been demonstrated to affect HSC migration to the FL, was unaffected by the PU.1 mutation. Another integrin, β7, possessing a role in lymphocyte homing, showed a minor reduction in expression. These results are consistent with the flow cytometric characterization of FL cells from PU.1<sup>-/-</sup> embryos. Thus, a number of functionally important integrins are not properly expressed in the absence of PU.1, which may explain the aberrant homing and engraftment properties of PU.1<sup>-/-</sup> HPC.

DISCUSSION

Phenotypic and functional analysis of PU.1<sup>-/-</sup> embryos demonstrated severe abnormalities in fetal hematopoiesis occurring in both the FL and yolk sac. This analysis showed a cell-intrinsic defect in lymphoid and myeloid development. In contrast, both primitive and definitive erythropoiesis, along with megakaryopoiesis, appeared normal in the yolk sac and FL. A second targeted mutation of the PU.1 allele demonstrates a similar phenotype with respect to fetal hematopoiesis.

However, mice containing the second PU.1 mutation can be kept alive for 2 weeks by high-dose antibiotics. During those 2 weeks, T cells and abnormal neutrophils can develop. Recent work with our mutation demonstrates that similar neutrophils and T cells<sup>32</sup> can be cultured. Therefore, with respect to hematopoietic development, the two PU.1 mutations appear almost identical. Strain differences may contribute to the variable onset of lethality. Rescue of PU.1<sup>-/-</sup> animals in utero by WT HSC (Fig 1) demonstrates that mutant embryos die from hematopoietic deficiencies. Furthermore, PU.1<sup>-/-</sup> microenvironments can foster proper development of WT hematopoietic progenitors. These data suggest a role for PU.1 in a multipotential lymphoid-myeloid progenitor population in the FL. Our prediction is that one or more PU.1-regulated genes are required for efficient commitment and/or differentiation of multipotent progenitors to either the B-lymphoid or myeloid lineage. This hypothesis has recently been supported by work that demonstrates that PU.1 can irreversibly commit retroviral transformed avian multipotent progenitor cells to the myeloid lineage. The normal levels of erythroid and megakaryocytic progenitors found in PU.1<sup>-/-</sup> embryos suggests that PU.1<sup>-/-</sup> HSC form in the AGM region and are able to colonize the yolk sac and FL. However, PU.1<sup>-/-</sup> HSC do not provide radioprotection for lethally irradiated recipients. In addition, PU.1<sup>-/-</sup> ES cells contribute to fetal but not adult erythropoiesis in chimeric animals. These data suggest that PU.1<sup>-/-</sup> HSC may be functionally impaired and point to a possible role for PU.1 during adult erythropoiesis.

The competitive reconstitution experiments were designed to allow donor-derived hematopoiesis to be evaluated over the course of several months. In the presence of a radioprotective dose of competitor adult bone marrow cells, there were significant levels of PU.1<sup>-/-</sup> derived erythrocytes detectable for 1 month (Fig 2A). These results show that PU.1<sup>-/-</sup> FL HPC can migrate to the adult bone marrow and contribute to erythropoiesis, but they are quickly out-competed by syngeneic WT cells. This suggests that, on a per cell basis, PU.1<sup>-/-</sup> fetal HPC are much less efficient than WT bone marrow cells. The absence of donor-derived PU.1<sup>-/-</sup> erythrocytes beyond 2 months in radiation chimera is entirely consistent with the lack of contribution to the erythroid lineage by PU.1<sup>-/-</sup> ES cells in adult chimeras. When reconstitution of the lymphoid and myeloid lineages was examined, no contribution was seen from PU.1<sup>-/-</sup> HSC (Fig 2B). FL HSC should be highly favored in these competitive reconstitutions due to their 4- to 5-fold greater long-term repopulating capacity. Furthermore, the lack of any detectable Ly<sup>5.2<sup>+</sup></sup> hematopoietic cells beyond 2 months in the recipients indicates the importance of PU.1 for the function of HSC in the adult bone marrow. It is still unclear exactly how far along the lymphoid and/or myeloid differentiation pathways PU.1<sup>-/-</sup> HSC can proceed in a competitive microenvironment.

In situ immunohistochemical expression analysis of the murine bone marrow has documented PU.1 protein expression in immature erythroblasts. PU.1 expression is downregulated upon terminal differentiation into erythrocytes. Continued expression of PU.1 in erythroblasts leads to growth immortalization and subsequent conversion to an erythroblastic state. One key regulatory cascade operative during definitive erythropoiesis requires synergy between the cytokines Epo and SCF.
Both Epo and SCF have been shown to be necessary for erythroid differentiation, proliferation, and survival. Serum-free clonogenic assays can directly measure synergistic and/or additive effects of growth factors. It is important to examine growth factor responsiveness using a defined assay system due to wide degree of functional redundancies between cytokines and cytokine receptors. For example, thrombopoietin can rescue CFU-E colonies in EpoR knockout mice. Serum-free clonogenic assays of E16.5 PU.1−/− FL erythroid progenitors showed their lack of synergy to Epo plus SCF. Flow cytometric analysis of PU.1−/− E16.5 FL cells showed little or no expression of c-kit (Fig 3). The possibility of PU.1-dependent expression of c-kit in erythroid progenitors is supported by recent analysis that has shown an ets family member, in conjunction with c-myc, is involved in the regulation of the c-kit promoter. One postulate is that, during repopulation, PU.1−/− erythroid progenitors are at a competitive disadvantage in the bone marrow due to their inability to respond to differentiative, proliferative, and survival signals emanating from EpoR and c-kit.

We next examined the homing potential of PU.1−/− HPC with a quantitative homing assay. PU.1−/− HPC were 11-fold less efficient at homing/colonizing to the adult bone marrow after 48 hours (Table 1). There was an accompanying 9-fold increase in the frequency of PU.1−/− HPC found in the adult liver. Cell migration through the bone marrow microenvironment has been hypothesized to be a 2-step process of initial cell binding followed by transendothelial migration into the hematopoietic compartment. For both steps, the importance of cell-cell interactions is clear. This prompted an evaluation of adhesion molecule expression by HPC isolated from PU.1−/− E14.5 FLs (Fig 4). PU.1−/− HPC lack expression of a number of integrins, including CD11b, α4/VLA-4, and α5/VLA-5, but retain expression of β1, β2, and β7.

Of particular interest was the absence of α4/VLA-4. Considerable data have implicated α4/VLA-4 as a key regulatory surface molecule mediating HPC/stromal cell contact in vitro and controlling HPC homing in vivo. The lack of α4/VLA-4 expression in PU.1−/− HPC is supported by a previous study that identified a functional ets regulatory element in the α4/VLA-4 promoter. However, the investigators in this study failed to determine which ets family member bound this element. Flow cytometric and RT-PCR analyses have shown that α4/VLA-4 is still expressed outside the AA4.1+ HPC population in PU.1−/− FL (Fig 4). Thus, these data suggest a direct role for PU.1 in regulating α4/VLA-4 expression in HPC. Another ets family member may be involved in α4/VLA-4 expression outside the HPC compartment. However, the lack of α4 is not sufficient to explain the PU.1−/− homing defect, because HSC migration to the bone marrow during fetal development appears to be normal in the absence of α4 expression. The ability of a recombinant fibronectin fragment containing binding sites for α4, α5, and CD44 to inhibit multilineage engraftment has indicated a potential role for these adhesion molecules in interactions with the bone marrow stroma. Therefore, the 11-fold decrease in bone marrow homing/colonization of PU.1−/− HPC is most likely the aggregate effect of several missing adhesion molecules. The decrease may also reflect a reduction in the absolute number of HPC in AA4.1+ population of PU.1−/− animals that are capable of homing to any tissue. Accumulation of PU.1−/− HPC in the adult liver raises the interesting possibility of an active tropic mechanism to explain this observation. Both PU.1−/− embryos and fetal chimeras made with PU.1−/− ES cells indicate that mutant HSC home to and function in the FL microenvironment. β1 integrin has been shown to be required for proper homing of HSC to the FL. Without β1, cells fail to enter the FL and remain in the circulation, suggesting that β1 may be directly involved in liver homing. PU.1−/− progenitors retain β1 expression, but lack expression of other integrins. Perhaps this absence of other integrins causes PU.1−/− progenitors to retain their liver tropism resulting in their abnormal accumulation in the adult liver after transplantation. The data presented in this study demonstrate that PU.1 is required for fetal HSC to establish and maintain long-term hematopoiesis in the bone marrow. In addition, we have shown that PU.1 is necessary for long-term definitive erythropoiesis. Therefore, the PU.1−/− mouse provides an ideal model system to study the mechanism of hematopoietic progenitor migration to and multilineage engraftment of the adult bone marrow. We are currently in the process of reintroducing missing integrin molecules into PU.1−/− HPC via retroviral transduction to ascertain their effect on progenitor cell function in the bone marrow. These future studies should provide insight into adhesion molecule-dependent events that control hematopoietic stem and progenitor cell migration and subsequent multilineage repopulation in the bone marrow microenvironment.

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REFERENCES


10. Medvinsky A, Dzierzak E: Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 86:897, 1996


32. DeKoter R, Walsh J, Singh H: PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocytemacrophage progenitors. EMBO J 17:4456, 1998


34. Rebel VI, Miller CL, Eaves CJ, Lansdorp PM: The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts. Blood 87:3500, 1996


A Critical Role for PU.1 in Homing and Long-Term Engraftment by Hematopoietic Stem Cells in the Bone Marrow

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