PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis

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Although the transcription factor PU.1 is essential for fetal lymphomyelopoiesis, we unexpectedly found that elimination of the gene in adult mice allowed disturbed hematopoiesis, dominated by granulocyte production. Impaired production of lymphocytes was evident in PU.1-deficient bone marrow (BM), but myelocytes and clonogenic granulocytic progenitors that are responsive to granulocyte colony-stimulating factor or interleukin-3 increased dramatically. No identifiable common lymphoid or myeloid progenitor populations were discernable by flow cytometry; however, clonogenic assays suggested an overall increased frequency of blast colony-forming cells and BM chimeras revealed existence of long-term self-renewing PU.1-deficient cells that required PU.1 for lymphoid, but not granulocyte, generation. PU.1 deletion in granulocyte-macrophage progenitors, but not in common myeloid progenitors, resulted in excess granulocyte production; this suggested specific roles of PU.1 at different stages of myeloid development. These findings emphasize the distinct nature of adult hematopoiesis and reveal that PU.1 regulates the specification of the multipotent lymphoid and myeloid compartments and restrains, rather than promotes, granulopoiesis.
Figure 1. Conditional deletion of PU.1 in adult hematopoiesis.
(A) The genomic locus of PU.1. Exons are represented as boxes; introns are represented as black lines; coding regions are in gray; nontranslated regions are in white; arrows indicate the direction of translation. The alleles...
other key transcription factors, such as interferon regulatory factor (IRF)4, IRF8, AML-1, CCAAT/enhancer binding protein α (C/EBPα), GATA-1, and c-Jun (13). Because reported PU.1 target genes include M-CSFR, granulocyte colony-stimulating factor (G-CSF)R, GM-CSFRα (14), and IL7Rα (15), cytokine responsiveness is coupled to the action of this developmentally essential transcription factor.

Mice that bear two different PU.1 mutations have been described extensively. Both lacked granulocytes and macrophages (16, 17), but important differences were reported. The mutant of Scott et al., which died in late gestation (between E17–18), lacked lymphocytes and long-term BM repopulating activity (16, 18), whereas the mutant of McKercher et al. was born alive and could live up to 2 wk on antibiotics (17). Analysis of these pups revealed aberrant B cell development; an abnormally large population of Mac-1+ immature myeloid cells; and delayed, but normal, T lymphopoiesis. Recently, a third mutant was reported that died at 1 d and had defects in erythroid progenitor self-renewal; however, no data on its lymphoid and myeloid phenotypes were reported (19). These studies demonstrate that PU.1 is essential for normal fetal hematopoiesis, but do not address its roles in adult hematopoiesis.

We developed a conditional mutation that allows inactivation of PU.1 in adult hematopoietic cells. We find that PU.1 ablation resulted in dramatically perturbed hematopoiesis and, contrary to expectation, greatly enhanced granulopoiesis. These changes were accompanied by a marked expansion in granulocytic progenitors, and loss of macrophage-CSF (M-CSF), GM-CSF and IL-6 responsiveness, whereas blast colony-forming capacity was preserved. Analysis of the multipotent progenitors revealed that PU.1 deficiency resulted in the loss of all FACS-identifiable lymphoid and myeloid progenitor populations. These results suggest that PU.1 is essential for normal transit through the CLP and CMP/GMP stages of adult hematopoiesis where it promotes lymphopoiesis, and conversely, restricts granulopoiesis.

RESULTS

Generation and validation of a PU.1 conditional allele

The PU.1-targeting construct contained LoxP flanked PU.1 exon–5 followed by an frt flanked internal ribosome entry site (IRES)-GFP cassette (Fig. 1 A). Homologous recombination in embryonic stem (ES) cells was confirmed using Southern hybridization and PCR genotyping, producing the PU.1F0 allele (Fig. 1, A–C). Germ-line transmission was achieved with two independent ES cell clones with identical GFP expression and null phenotype. The GFP reporter allowed detailed characterization of the PU.1 expression domain, which was reported elsewhere (10). The conditional PU.1F0 allele was produced by breeding PU.1F0 with a Flp recombinase-expressing strain (Fig. 1 A).

To determine the phenotype of this PU.1 mutation, we bred the PU.1F0 or PU.1F0 allele to the Deleter-Cre strain to produce PU.1F0/+ or PU.1F0, respectively. Intercrossing of PU.1F0/+ mice resulted in no PU.1F0 individuals at day 21, whereas E18.5 PU.1F0/+ embryos were present at the expected frequency (+/+ = 13 [24%], +/Δ = 30 [57%], Δ/Δ = 10 [19%]). Timed pregnancies were allowed to continue to term, but PU.1F0/+ pups died shortly after birth (unpublished data). Analysis of PU.1F0/+ E18.5 embryos revealed an absence of myeloid (Mac-1+Gr-1−) lineage cells (Fig. 1 D). Although PU.1F0/+ embryos had populations of GFPhigh myeloid and GFPlow B cells, as well as GFP+c-kit+ progenitors, PU.1F0/+ embryos showed only a residual GFP+c-kit− population (Fig. 1 D). This phenotype was most similar to that of Scott et al. (16) and showed that PU.1 was required for the existence of virtually all PU.1-expressing cells in the fetal liver.

PU.1 inactivation perturbs adult hematopoiesis

To delete PU.1 conditionally in adult hematopoietic cells, PU.1F0/B mice were crossed to the IFNαβ−inducible MxCre transgene, which can be activated efficiently by polyinosine-polycytosine (polyIC; reference 20). PU.1F0/Δ MxCre+ and MxCre− mice were injected i.p. with polyIC at days 0 and 3 and analyzed 10–14 d after injection. Exon-5 deletion frequency was monitored using a PCR assay on genomic DNA and confirmed by Western blotting (Fig. 1, E and F). Typically, at day 10 after injection, the deletion frequency approached 100% in BM and was between 70–90% in spleen, blood, and thymus (Fig. 1 E). In contrast to the loss of fetal PU.1-expressing cells, PU.1-deficient adult BM contained a population of uniformly GFPhigh cells; this suggested that PU.1 was not required for the survival of all adult hematopoietic cells (Fig. 1 G). Analysis of PU.1F0/+ mice at 2 wk after polyIC treatment revealed several hematologic abnormalities. The BM contained normal total cellularity but exhibited an increased proportion of Blast cells, and immature cells of the granulocyte...
lineage (promyelocytes and myelocytes; Table I). Staining with myeloid lineage markers showed that the mutant mice had twice as many Gr-1$^+$ cells as controls and were Mac-1$^-$ (Fig. 2 A). Genotype analysis of total BM, granulocytes, and B cells revealed virtually complete gene deletion at this time point (Fig. 2 D). Strikingly, almost all BM cells of PU.1$^{fl/fl}$ mice expressed Ly6C and c-kit, markers that are indicative of myeloid precursors. This expansion of PU.1-deleted (Mac-1$^-$) granulocytic precursors in the BM also was apparent 8 wk after the last polyIC injection. At this time point, some differentiation of PU.1 wild-type cells can be seen by the reappearance of a small number of Mac-1$^+$ cells (Fig. 2 A). Although Mac-1, F4/80, and FcγRII/III staining was lost from PU.1$^{fl/fl}$-deleted hematopoietic organs (Fig. 2 A) which indicate efficient gene deletion, morphologically recognizable monocytes remained (Table I). In contrast to the expanded granulocytic lineage population, B lymphocyte numbers were reduced variably in the PU.1$^{fl/fl}$ BM at day 14, with most cells having a preB cell phenotype (CD19$^+$B220$^+$IL7Rα$^+$c-kit$^-$) and displaying complete deletion of exon-5 (Fig. 2, B and D). PolyIC induces an IFN response that is particularly detrimental to BM B cell precursors (21). Therefore, the differences at this early time point may represent differential repopulation of the B lineage (after the IFN response) and a consequence of excessive granulopoiesis in the BM. PU.1$^{fl/fl}$ BM also lacked the nucleated erythroid cells that represented 17% of the control BM at 2 weeks (Table I). FACS analysis confirmed the PU.1$^{fl/fl}$ BM to be markedly deficient in Ter119$^+$CD71$^+$ erythroblasts (Fig. 2 C). The loss of nucleated erythroid cells also was evident in histologic sections of the mutant BM (Fig. 2 D) analyzed after 2 or 8 wk. (D) PCR analysis showing the relative PU.1 deletion of total BM and sorted cells of the indicated lineages 2 wk after polyIC treatment. B cells were identified as CD19$^+$B220$^+$, granulocytes as Gr$^1^-$. PU.1$^{fl/fl}$ tail DNA was used as a control.
3 A) and similarly apparent after 8 wk (Table I). Conversely, nucleated erythroid cells were present in spleens of both genotypes at 2 wk, but remained only in the \( P U.1^{f/f} \) spleens at 8 wk after treatment (Table I). The presence of nucleated erythrocytes in spleen at the early time point most likely reflected the response to polyIC treatment, whereas their long-term presence in the case of \( P U.1^{f/f} \) mice represented true extramedullary hematopoiesis.

The spleen of \( P U.1^{f/f} \) mice also contained increased numbers of granulocytic cells, although their frequency was variable (20–90% Gr-1+ c-kit+; Fig. 3, B and C). In more severe cases (Fig. 3, B and C), spleen histology showed homogeneous infiltration by immature granulocytic forms and loss of splenic architecture. PCR analysis confirmed complete deletion of exon-5 in splenic granulocytes (Fig. 3 C).

Absolute spleen lymphocyte numbers were relatively normal in most mice with a two- to threefold percentage decrease in individuals with the most pronounced granulocyte expansion (Table I and Fig. 3, A–C). PU.1 deletion in the splenic lymphocytes was less frequent (50–70%), although sorting of \( P U.1^{f/f} \) mature B cells based on GFP expression (that increases upon exon-5 deletion [Fig. 1 G]) showed that all GFP\textsuperscript{high} cells were PU.1 deficient (Fig. 3 C).

### Granulocyte maturation in the absence of PU.1

The expanded number of morphologically identifiable granulocytes suggested that in contrast with fetal development, PU.1 is not required for the differentiation of this lineage in vivo. To examine the maturation of \( P U.1^{-} \)-deficient granulocytes, Gr-1+ BM cells were cultured for 7 d in G-CSF before being subjected to flow cytometry, cytoospin, and molecular analysis. In agreement with the loss of Mac-1\textsuperscript{+} staining, \( P U.1^{f/f} \) granulocytes represented a pure population of deleted cells (Fig. 3 D). \( P U.1^{f/f} \) granulocytes, in contrast with wild-type controls, maintained c-kit expression and displayed some, but not complete, maturation as measured by morphologic criteria (Fig. 3)

| Parameter                  | 2 wk after induction | 8 wk after induction |
|---------------------------|----------------------|----------------------|
|                           | \( P U.1^{f/f} \) MxCre\textsuperscript{-} | \( P U.1^{f/f} \) MxCre\textsuperscript{-} | \( P U.1^{f/f} \) MxCre\textsuperscript{-} | \( P U.1^{f/f} \) MxCre\textsuperscript{-} |
|                           | \( n = 5 \)         | \( n = 5 \)         | \( n = 3 \) | \( n = 3 \) |
| **Peripheral blood**       |                      |                      |                      |
| Total cells               | 3,230 ± 1,660        | 4,350 ± 2,340        | 4,434 ± 2,759        | 8,420 ± 5,274       |
| Neutrophils               | 160 ± 280            | 850 ± 370            | 36 ± 35              | 407 ± 230           |
| Lymphocytes               | 2,910 ± 1,330        | 2,870 ± 1,340        | 3,403 ± 1,442        | 7,513 ± 4,702       |
| Monocytes                 | 100 ± 70             | 590 ± 750            | 237 ± 251            | 310 ± 185           |
| Eosinophils               | 50 ± 100             | 30 ± 50              | 0 ± 0                | 183 ± 275           |
| Platelets \( \times 10^5 \) | 6.3 ± 1.0            | 10.7 ± 3.3           | 13.7 ± 2.1           | 9.5 ± 2.1           |
| Hematocrit %              | 44 ± 3               | 43 ± 3               | 43 ± 3.6             | 46 ± 1.2            |
| **Spleen**                |                      |                      |                      |
| Weight mg                 | 149 ± 46             | 110 ± 18             | 128 ± 2.2            | 94.3 ± 15           |
| Blast cells               | 5 ± 2                | 3 ± 1                | 2 ± 0                | 4 ± 0.6             |
| Myelocytes                | 3 ± 2                | 1 ± 1                | 1 ± 1                | 0                  |
| Neutrophils               | 3 ± 1                | 5 ± 4                | 5 ± 2                | 1 ± 1               |
| Lymphocytes               | 59 ± 16              | 59 ± 14              | 60 ± 16              | 89 ± 0.6            |
| Monocytes                 | 2 ± 2                | 4 ± 3                | 1 ± 0                | 1 ± 1              |
| Eosinophils               | 1 ± 1                | 1 ± 1                | 0.3 ± 0.6            | 0.3 ± 0.6           |
| Nucleated erythroid       | 26 ± 12              | 27 ± 12              | 31 ± 13              | 3.7 ± 2.3           |
| **Bone marrow**           |                      |                      |                      |
| Total cells \( \times 10^6 \) | 44.4 ± 8.5           | 42.3 ± 6.0           | 37.9 ± 5.6           | 33.4 ± 1.5          |
| Blast cells               | 9 ± 5                | 3 ± 0                | 7 ± 1                | 4 ± 1.2             |
| Myelocytes                | 25 ± 4               | 9 ± 5                | 14 ± 0.6             | 10 ± 3.6            |
| Neutrophils               | 45 ± 5               | 48 ± 12              | 49 ± 16              | 35 ± 4.4            |
| Lymphocytes               | 14 ± 6               | 12 ± 10              | 19 ± 10              | 23 ± 2.1            |
| Monocytes                 | 7 ± 5                | 8 ± 4                | 5.7 ± 2.5            | 9.7 ± 1.2           |
| Eosinophils               | 0 ± 0                | 3 ± 2                | 0.3 ± 0.6            | 2 ± 1              |
| Nucleated erythroid       | 0.4 ± 0.6            | 17 ± 9               | 5.7 ± 9.8            | 16 ± 5.5            |

Data are the mean values ± SD. \( n = 5 \) mice for 2-wk and \( n = 3 \) mice for 8-wk postinduction experiments. Peripheral blood values are per microliter. Total BM cells are per femur. Cellular differentials for BM and spleen are shown as percentages. Myelocytes include promyelocytes and myelocytes. Neutrophils include metamyelocytes, band neutrophils, and neutrophils.
Figure 3. Perturbed granulocytic differentiation in the absence of PU.1. (A) Histologic section of day 21 BM from mice of the indicated genotype. Insets show cytocentrifuge preparations of corresponding BM. (B) Histologic section of relatively mild and severe granulocytic infiltrations in the spleen at day 21. (C) Severe PU.1<sup>1.1A</sup> (fl/fl MxCre<sup>-</sup>) mice display splenomegaly and enhanced percentage of Gr-1<sup>-</sup>Mac-1<sup>-</sup> cells by flow cytometry. PCR showing efficient deletion of PU.1<sup>fl</sup> and PU.1<sup>gfp</sup> alleles in 2-wk spleen of a PU.1<sup>1.1A</sup> (fl/gfp MxCre<sup>-</sup>) mouse. Total, total splenocytes; Gr, Gr-1<sup>+</sup> cells; B, CD19<sup>-</sup>B220<sup>-</sup> B cells; T, CD3<sup>+</sup> T cells; Er, CD71<sup>-</sup>Ter119<sup>-</sup> nucleated erythrocytes. B cells also were gated on GFP<sup>high</sup> (BG<sup>hi</sup>) or GFP<sup>low</sup> (BG<sup>lo</sup>). The fl and gfp alleles were amplified with the primer combination b/c/d and c/e/d, respectively. (D) Control (fl/fl MxCre<sup>-</sup>) and PU.1<sup>1.1A</sup> (fl/fl MxCre<sup>-</sup>) granulocytes were grown in G-CSF for 7 d before being analyzed by flow cytometry and morphology. PCR analysis showed the complete PU.1 deletion of cultured granulocytes. (E) BM Gr-1<sup>+</sup> cells were sorted and analyzed by RT-PCR for the expression of granulocytic enzymes, myeloperoxidase (MPO), LF, and gp91.
from four mice for 7 d, stained, and counted. Experiments were performed using four separate mice and quadruplicate cultures for each stimulus. Numbers represent mean colony numbers.

Table II. In vitro analysis of bone marrow colony-forming progenitors

| Genotype (n = 4) | Stimulus | Blast | G | GM | M | Eo | Meg |
|------------------|----------|-------|---|----|---|----|-----|
| PU.1Δ/Δ MxCre+   | GM-CSF   | 2 ± 2 | 0.3 ± 0.5 | 4 ± 6 | 0 ± 0 |
| G-CSF            | 83 ± 31  | 0 ± 0 | 0 ± 0 |
| M-CSF            | 0.5 ± 1.0 | 0 ± 0 | 1.5 ± 2.4 |
| IL-3             | 12 ± 12 | 152 ± 15 | 2 ± 1 | 6 ± 5 | 0 ± 0 | 0.5 ± 1.0 |
| SCF              | 5 ± 5 | 29 ± 8 | 0 ± 0 | 0 ± 0 |
| IL-6             | 0.8 ± 0.7 | 0 ± 0 | 0 ± 0 |
| SCF + G-CSF      | 3 ± 4 | 129 ± 15 | 0 ± 0 | 0 ± 0 |
| IFNγ             | 0 ± 0 | 0.2 ± 0.2 | 0 ± 0 | 0 ± 0 |
| EPO              | 0 ± 0 | 14 ± 11 | 0 ± 0 | 0 ± 0 |
| TPO              | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| GM-CSF           | 19 ± 5 | 4 ± 4 | 31 ± 13 | 3 ± 2 |
| G-CSF            | 10 ± 3 | 0 ± 0 | 2 ± 3 |
| M-CSF            | 1 ± 1 | 5 ± 3 | 69 ± 29 |
| IL-3             | 8 ± 7 | 18 ± 6 | 11 ± 5 | 27 ± 9 | 2 ± 1 | 4 ± 3 |
| SCF              | 5 ± 3 | 14 ± 7 | 1 ± 1 | 1 ± 1 |
| IL-6             | 8 ± 4 | 1 ± 1 | 0.5 ± 0.6 |
| SCF + G-CSF      | 7 ± 3 | 18 ± 11 | 3 ± 2 | 3 ± 3 |
| IFNγ             | 2 ± 1 | 0.8 ± 0.5 | 0 ± 0 | 0.1 ± 0.3 |
| EPO              | 0 ± 0 | 3 ± 2 | 0 ± 0 | 0 ± 0 |
| TPO              | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| GM-CSF + IL-3 + EPO | 6 ± 9 | 178 ± 51 | 7 ± 5 | 4 ± 4 | 0 ± 0 | 2 ± 1 |
| saline           | 0.8 ± 1.0 | 0 ± 0 | 0 ± 0 |
| GM-CSF           | 19 ± 5 | 4 ± 4 | 31 ± 13 | 3 ± 2 |
| G-CSF            | 10 ± 3 | 0 ± 0 | 2 ± 3 |
| M-CSF            | 1 ± 1 | 5 ± 3 | 69 ± 29 |
| IL-3             | 8 ± 7 | 18 ± 6 | 11 ± 5 | 27 ± 9 | 2 ± 1 | 4 ± 3 |
| SCF              | 5 ± 3 | 14 ± 7 | 1 ± 1 | 1 ± 1 |
| IL-6             | 8 ± 4 | 1 ± 1 | 0.5 ± 0.6 |
| SCF + G-CSF      | 7 ± 3 | 18 ± 11 | 3 ± 2 | 3 ± 3 |
| IFNγ             | 2 ± 1 | 0.8 ± 0.5 | 0 ± 0 | 0.1 ± 0.3 |
| EPO              | 0 ± 0 | 3 ± 2 | 0 ± 0 | 0 ± 0 |
| TPO              | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| GM-CSF + IL-3 + EPO | 7 ± 3 | 21 ± 8 | 11 ± 6 | 24 ± 9 | 2 ± 1 | 16 ± 6 |

Bone marrow of PU.1Δ/Δ (fl/fl MxCre+) and control (fl/fl MxCre–) mice was analyzed on day 14 after induction. 25,000 cells were cultured in agar with the indicated cytokines for 7 d, stained, and counted. Experiments were performed using four separate mice and quadruplicate cultures for each stimulus. Numbers represent mean colony numbers from four mice ± SD. Values in bold highlight examples of increased numbers of committed colony-forming progenitors responsive to selected growth factors or growth factor combinations after PU.1 deletion.

Eo, eosinophil colony; Fl, Flt3 ligand; G, granulocyte colony; GM, granulocyte/macrophage colony; M, macrophage colony; Meg, megakaryocyte colony; TPO, thrombopoietin.

D). RT-PCR analysis of the sorted Gr-1hi cells revealed that the primary granule component, myeloperoxidase, as well as the secondary granule component, lactoferrin (LF), were expressed normally, whereas the putative PU.1 target gene and component of nicotinamide adenine dinucleotide phosphate oxidase, gp91 (22), was down-regulated (Fig. 3 E). The normal expression of LF contrasted with the absence of this transcript in PU.1Δ/Δ fetal liver-derived cultures (30). These data suggest that PU.1Δ/Δ granulocytes were capable of aberrant, and possibly delayed, differentiation to relatively mature cells (Fig. 3, A inset, D, E), despite the reported requirement for PU.1 in the transcription of a variety of genes that is required for granulocyte function.

**Expanded clonogenic granulocyte progenitors in the absence of PU.1**

Progenitor cells in the BM and spleen of PU.1Δ/Δ mice were analyzed at 2 and 8 wk after PU.1 ablation, using agar cultures and a range of stimulating factors. At the earlier time point, the mutant BM was grossly depleted in cells that were able to respond to GM-CSF or M-CSF by forming colonies or clusters (Table II). In sharp contrast, PU.1Δ/Δ cultures that were stimulated by G-CSF developed greatly elevated numbers of granulocytic colonies, the cells of which showed some abnormal maturation (Fig. 3 D). Excessive numbers of granulocytic colonies also developed in cultures that were stimulated by IL-3 or stem cell factor (SCF; c-kit L), although a few macrophage-containing colonies did develop in IL-3–stimulated cultures. In contrast with the excessive granulocyte colony numbers that developed with G-CSF, virtually no colonies arose in response to IL-6. Moreover, the PU.1Δ/Δ BM lacked eosinophil colony-forming cells that were responsive to GM-CSF, IL-3, or IL-5 (Table II). In cultures that were stimulated by the optimal growth factor combination for megakaryocyte colony formation (SCF +
IL-3 + erythropoietin (EPO), few or no megakaryocyte colonies developed in cultures of PU.1Δ/Δ BM cells. PCR analysis of BM (Fig. 2 D) and individual colonies (n = 81) showed the nearly total deletion of exon-5; this confirmed that any colony formation was the result of PU.1-deficient progenitors (unpublished data).

Analysis of clonogenic BM progenitors 8 wk after polyIC treatment showed a similar trend toward excess granulocytic progenitor numbers. However, G-CSF–responsive progenitors were present in only slightly higher numbers (fl/fl 8.6 ± 3.8, Δ/Δ 16 ± 4.7), whereas IL-3 (fl/fl 15 ± 10, Δ/Δ 95 ± 22), SCF/G-CSF (fl/fl 20 ± 5.0, Δ/Δ 48 ± 13), and SCF/IL-3/EPO (fl/fl 16 ± 3.6, Δ/Δ 97 ± 8.9) cultures generated increased numbers of granulocytic colonies (mean ± SD; n = 3 for each genotype).

At 2 wk after polyIC treatment, the spleen in control mice was enlarged and contained elevated numbers of colony-forming cells. Strikingly, mutant spleen cells generated vastly increased numbers of granulocytic colonies (Table III), excessive numbers of megakaryocytic colonies, and, interestingly, IL-3–responsive macrophage-containing colonies. These data demonstrate that PU.1 deficiency induced extramedullary hematopoiesis that, similarly to BM, resulted in excessive granulopoiesis. The spleen also contained significant numbers of IL-3, but not M-CSF–responsive macrophage colony–forming cells. This was despite the fact that the deletion frequency was high and no Mac-1 cells were present (Fig. 3 C; Table III). This effect largely was transient because many fewer clonogenic cells were found in the spleen at the 8-wk time point. However, increased SCF/IL-3/EPO–responsive granulocyte (fl/fl 1 ± 1, Δ/Δ 25 ± 7.2) and megakaryocyte (fl/fl 3.7 ± 2.9, Δ/Δ 12 ± 7.5) colonies were still present in the mutant spleens; this was indicative of ongoing hematopoiesis (mean ± SD; n = 3 for each group). Collectively, these data demonstrate that PU.1 inactivation resulted in rapid and dramatic changes in the responsiveness, number, and anatomic location of hematopoietic progenitors.

Table III. In vitro analysis of spleen colony-forming progenitors

| Genotype  | Stimulus    | Blast | G  | GM | M  | Eo | Meg |
|-----------|-------------|-------|----|----|----|----|-----|
| PU.1fl/fl | G-CSF       | 118 ± 96 | 2 ± 2 | 1 ± 1 |     |     |     |
| IL-3      | 26 ± 26     | 269 ± 200 | 27 ± 18 | 59 ± 38 | 0 ± 0 | 19 ± 14 |
| SCF + IL-3 + EPO | 16 ± 21 | 247 ± 154 | 28 ± 20 | 60 ± 47 | 0 ± 0 | 74 ± 52 |

At 2 wk after polyIC treatment, the spleen in control mice was enlarged and contained elevated numbers of colony-forming cells. Numbers represent the mean ± SD of colony counts from four mice of each genotype. Values in bold highlight examples of increased numbers of committed colony-forming progenitors responsive to selected growth factors or growth factor combinations after PU.1 deletion. Eo, eosinophil colony; G, granulocyte colony; GM, granulocyte/macrophage colony; M, macrophage colony; Meg, megakaryocyte colony.

PU.1 is required for the balanced production of multipotent bone marrow progenitors

To determine directly the effect of PU.1 deletion on defined BM progenitors, we isolated lineage-negative (lin−) cells 2 wk after Cre activation, a time point when gene deletion is essentially complete (Fig. 4 B). PU.1 deficiency resulted in a dramatic loss of any readily identifiable lymphoid and myeloid progenitor or stem cell populations (Fig. 4 A). Lin− cells were c-kit+/Sca1+/IL-7Rα+/CD34+/FcγRII/IIIlow, a phenotype that is not defined by the existing progenitor scheme (Fig. 4 A). Morphologic analysis of sorted lin− cells revealed a high proportion of blast cells in either genotype with a slightly higher proportion in the S+G2+M phases of the cell cycle in the PU.1Δ/Δ preparations (Fig. 4 B). All PU.1Δ/Δ lin− cells expressed high levels of GFP, which indicated that such cells can survive in the absence of PU.1 (Fig. 4 A) and contrasts with a report that PU.1 positively regulates its own promoter (23).

RT-PCR analysis of lin− BM cells revealed that PU.1-deficient cells had little M-CSFR, GM-CSFRA, or IL-5Rα expression, and also lacked IL-6Rα and gp130, the common components of the IL-6R and leukemia inhibitory factor (LIF) receptor (Fig. 4 C). It also was of note that PU.1Δ/Δ cells showed decreased expression of a variety of important transcription factors that have been shown to interact functionally with PU.1, including GATA-1 and IRF-8 (Fig. 4 C). The levels of GATA-1 and IRF-8 most likely reflected the expansion of the granulocytic lineage and absence of nucleated erythrocytes in BM. The possibility that the Ets family members, Spi-B or Spi-C, compensate for some PU.1 function was excluded because neither was expressed in PU.1Δ/Δ lin− BM. Several other critical genes, such as GM-CSFRIA, IL-2Rγ, GATA-2, and ikaros, were similarly expressed in cells of both genotypes (Fig. 4 C). Thus, in agreement with the altered in vitro growth characteristics, PU.1Δ/Δ progenitors displayed specific alterations in their transcriptional profile as compared with wild-type cells.

The apparent loss of FACS-identifiable progenitors simply could be the consequence of the loss of expression of pu-
tative PU.1 target genes, such as IL-7Rα (15) and FcγRIIIa (24). To overcome this uncertainty and to determine the source of the excess granulocytic cells that were observed, we sorted myeloid progenitors at short time intervals after PU.1 deletion. Mice were injected with polyIC 36 h and 24 h before the isolation of the defined myeloid and erythroid progenitors. This short time frame was sufficient for efficient deletion of exon-5 in CMP and GMP, but not for the loss of the critical cell surface markers (Fig. 5). At this stage, cre+–sorted CMP and GMP populations were morphologically indistinguishable from their cre− counterparts (Fig. 5). Analysis of the in vitro clonogenic potential of sorted CMPs and GMPs confirmed the efficient PU.1 inactivation, because virtually no M-CSF–responsive colonies were obtained from PU.1 fl/fl progenitors (Fig. 5). Although the number of granulocyte colonies was relatively similar from PU.1+ and PU.1 fl/fl CMPs (see G-CSF, IL-3, or IL-3/SCF/EPO), the corresponding cultures of GMPs showed a pronounced enhancement of PU.1−deleted granulocyte colonies. As expected, sorted MEP gave rise to very few myeloid colonies in these assays (unpublished data). These data suggest that the enhanced granulopoiesis that was observed in the absence of PU.1 is derived from increased/deregulated differentiation capacity of the GMP, but not the more multipotent CMP.

These experiments gave two additional unexpected findings. First, in contrast with the loss of GM-CSF–responsive

Figure 4. PU.1 is essential for multipotent lymphoid and myeloid progenitors. (A) BM lin− cells from 2-wk PU.1 fl/fl (fl/fl MxCre−) and control (fl/fl MxCre+) polyIC-treated mice were assayed for HSC and lymphoid and myeloid progenitors. For GFP analysis, PU.1 gfp/fl (PU.1gfp/fl MxCre−) or control (PU.1gfp/fl MxCre+) mice were treated as above. (B) Cytocentrifuge preparation and cell cycle analysis of lin− cells. Inset: PCR showing complete deletion of exon-5 in lin− cells. Range of cycling cells (S+G2+M) observed from two experiments is indicated. (C) RT-PCR for key hematopoietic regulators and PU.1 target genes in lin− and total BM. HPRT was used as a control for cDNA input. −RT, no reverse transcriptase was used in the reaction. Samples were taken at four cycle intervals. Ikaros isoforms 1 and 2 are indicated.
colonies 2 wk after Cre induction, short-term PU.1-deleted GMPs generated excess granulocyte colonies when stimulated with GM-CSF (Fig. 5). A similar trend was seen with total BM cells at this short time point (fl/fl 21.2 ± 6.1, Δ/Δ 32.9 ± 12; mean ± SD; n = 3). We interpret this responsiveness to GM-CSF to indicate that PU.1 may be critical for the expression of the GM-CSFRα between HSC and CMP stages (as assayed at the 2-wk time point), but not for the maintenance of its expression in the more mature GMPs (which only can be assayed with short time points before new GM-CSFRα-negative GMPs are generated from HSC/CMP). Second, despite the lack of M-CSF responsiveness, macrophage colonies developed in IL-3 or IL-3/SCF from PU.1Δ/Δ CMPs, but not GMPs; this suggests that PU.1 loss may have resulted in an earlier than normal switch to granulocyte commitment in this sequence of amplifying myeloid progenitors.

Figure 5. PU.1 regulates granulocyte production from the GMP. BM lin− cells from 36-h post-polyIC treatment, PU.1Δ/Δ (fl/fl MxCre−) and control (fl/fl MxCre+) mice were assayed for erythromyeloid progenitors. Sorted CMP and GMP using the indicated gates were subjected to PCR for PU.1 exon-5 deletion. Cytocentrifuge preparations were stained with May-Grunwald-Giemsa. Sorted cells were cultured in the indicated cytokines for 7 d, stained, and counted. 100 cells were plated in quadruplicate for each stimulus. Numbers represent the mean ± average deviation from three independent experiments.
PU.1-deficient stem cells do not generate lymphoid progeny

It has been reported that PU.1/H11002/fetal liver stem cells are unable to reconstitute adult hematopoiesis (18). However, given our data showing the abundance of PU.1-deficient cells in adult organs, we reinvestigated this issue using BM chimeras. Ly5.1 mice were reconstituted with PU.1fl/fl/MxCre (Ly5.2) and competing Ly5.1 BM cells. After 6 wk, relative reconstitution was assessed by analysis of peripheral blood. PU.1 inactivation was then induced by polyIC and mice were analyzed at 6, 12, and 24 wk after deletion. Because PU.1 is required for expression of CD45 (Ly5) (25, 26) and Mac-1 (27) in myeloid cells, we used the absence of these markers to determine the efficiency of PU.1 deletion in the reconstituting donor cells (Fig. 6 A). A small number of nondeleted PU.1fl/fl/Ly5.2 cells could be distinguished easily from the other populations (Fig. 6 A). PCR analysis on DNA from total hematopoietic tissues or sorted Ly5.1 donor and Ly5.1/WT competitor BM cells for WT, fl, and fl tail DNA was used as a control. (+) Competitor Ly5.1+ DNA. Note that the synthesis of + (WT) allele PCR product is more efficient that that of Δ allele and cannot be used for relative quantification of host/donor cellular contribution to chimerism. PU.1ΔΔ was amplified from the BM, whereas no PU.1-deleted cells were found in the thymus, spleen, or blood. (C) The percentage of PU.1ΔΔ cells in hematopoietic organs was determined at 6, 12, and 24 wk after polyIC injection. Total PU.1ΔΔ cells (Ly5.1+) are shown relative to Ly5.1+ cells. The numbers have been normalized for the predeletion reconstitution frequency. Because cells lose CD45 expression in some lineages, mutant cells were defined as Ter119−Ly5.1−.

Figure 6. Competitive BM reconstitution. (A) PU.1fl/fl MxCre+ BM was mixed with Ly5.1 BM (2:1 ratio) and used to reconstitute lethally irradiated Ly5.1 mice. The degree of reconstitution was assessed from peripheral blood after 6 wk, followed by the induction of PU.1 deletion by polyIC. Flow cytometric analysis of spleen from chimeric mice at 24 wk. Ly5.1− cells were almost entirely PU.1-deficient granulocytes (Gr-1−Mac-1−), whereas Ly5.1+ competitors were Gr-1+Mac-1+ (macrophages/granulocytes) and CD19+Ly5.2− (B cells). c-kit+CD71+ erythroid progenitors were present in both populations. (B) BM, thymus, spleen, and blood cells and sorted Ly5.1− and Ly5.1+ BM cells were subjected to PCR for PU.1 exon-5 deletion. PU.1ΔΔ, PU.1Δ/Δ, and PU.1+/Δ tail DNA was used as a control. (+)
in the absence of wild-type cells to repopulate blood and spleen; this effect also was observed in the peripheral blood of nonchimeric PU.1-deficient animals (Table I). Thus, in contrast to reconstitution of irradiated recipients with PU.1<sup>−/−</sup> fetal liver (18) or PU.1<sup>fl/fl</sup> BM deleted stem cells (unpublished data), PU.1 deletion in steady-state hematopoiesis produced mutant stem cells with long-term erythromyeloid cell production capacity that is biased toward the granulocyte lineage.

### DISCUSSION

**PU.1 deficiency results in enhanced granulopoiesis**

The most striking abnormality of PU.1-deficient BM was the overwhelming granulopoiesis that virtually excluded other lineages. This phenotype was surprising as it contrasted with the lack of granulocytes in PU.1<sup>−/−</sup> fetal livers (16, 17). The cells expressed markers indicative of an immature granulocyte (c-kit, Ly6C) but not the PU.1 target Mac-1 (Fig. 2 A) and appeared to undergo perturbed maturation in vivo (Fig. 3 A). Previous studies on PU.1-deficient fetal liver (28) or neonates (29–31) indicated that the hematopoietic progenitors lacked G-CSF responsiveness ex vivo, but could be grown in IL-3. These studies are complicated by the distinct phenotypes observed in these strains that have not been satisfactorily explained (32). The germ-line mutation reported here most resembles the more severe phenotype of Scott et al. (16), in that the mice die at birth and lack granulocytes, whereas the phenotype of McKercher et al. is relatively less severe and results in postnatal lethality and the development of some immature granulocytes (17). The granulocytes shown here, although displaying some morphologic maturation and expression of G-CSFR and primary and secondary granules, clearly were aberrant in their differentiation in that they maintained c-kit expression. This phenotype was strikingly different than the lack of fetal liver granulocytes, and suggested that in adults, PU.1 suppressed the formation of granulocytic progenitors, and subsequently, was required for their final functional maturation. Heightened in vitro granulocyte response and a corresponding BM neutrophilia also were reported for mice with conditional deletions of SOCS3 (33) and STAT3 (34), in contrast to the PU.1 mutation, the cells in both of these mutants were hyperresponsive to G-CSF in vitro.

The analysis of clonogenic progenitors from mutant BM and spleen demonstrated that multiple dramatic changes occurred in the absence of PU.1, paralleling the hematologic aberrations that were observed in vivo. These included a marked loss of clonogenic cells that were responsive to GM-CSF or M-CSF. In sharp contrast, granulocyte progenitors occurred at abnormally high frequency in the BM and spleen; this provided a basis for the enhanced granulopoiesis that was observed in these tissues. Although the granulocytic population exhibited full dependency on growth factors for proliferation, the clonogenic cells exhibited several abnormalities, including heightened responsiveness to IFNγ and a failure to respond to IL-6, an agent with actions similar to G-CSF when acting on normal granulocytic progenitor cells. In contrast to granulopoiesis, the loss of cells that were responsive to M-CSF and GM-CSF was identical to that reported previously and is likely due to the induced failure of existing and newly-formed cells to express the cognate receptors, as was noted previously in fetal PU.1<sup>−/−</sup> cells (28). Although GM-CSF and M-CSF are the only known stimuli for macrophage formation in vivo (35), we observed the growth of IL-3–stimulated macrophage colonies from PU.1-deficient cells; this indicated that macrophage development may not be lost. This finding also was supported by the presence of almost normal numbers of BM monocytes 8 wk after the induction of PU.1 deletion (Table I).

Within the erythroid and megakaryocytic cell lineages, the PU.1-deficient adult phenotype was complex because the pronounced loss of clonogenic cells and nucleated erythrocytes from the BM was mirrored by increased erythromegakaryopoiesis in the mutant spleen (Table III). Such extramedullary hematopoiesis has been observed in other situations in which granulocyte cells become dominant in the BM (36, 37). The presence of erythromegakaryocytic colonies in the spleen contrasted with the loss of responsiveness to EPO + SCF or IL-3 (38) and the premature terminal differentiation (19) that was reported for PU.1<sup>−/−</sup> fetal liver erythroid progenitors; this highlighted another difference between fetal and adult hematopoiesis.

B lymphocyte numbers were reduced, but not eliminated, in the BM after gene inactivation which suggests a role for PU.1 in B lymphopoiesis. However, the interpretation of these results is problematic because polyIC treatment results in a transient-type I IFN response that was demonstrated to reduce BM B cell numbers by >80% (21). After 8 wk, B and T cells were still identifiable in BM and spleen; however, we were unable to determine if these were surviving mature lymphocytes and progeny of their homeostatic proliferation; lymphocytes that were generated from a wave of intermediate precursors, such as those preB cells seen 2 wk after PU.1 deletion; or lymphocytes that developed from early lymphoid progenitors. To better assess lymphoid development, we performed BM reconstitution in the presence of wild-type competitors (Fig. 6). These experiments demonstrate that PU.1-deficient lymphopoiesis was incapable of producing B or T cells in this environment. Therefore, we propose that the decrease in B cells in PU.1-deficient BM reflected a defect in the production of early lymphoid progenitors, potentially via the regulation of the IL-7Ra (15) and not a defect in mature B cell survival. This conclusion is supported by the normal development of PU.1<sup>fl/fl</sup> B cells when Cre expression was driven from the
committed B cell–specific CD19 regulatory sequences (unpublished data). In summary, we propose that PU.1 regulates B and T lymphopoiesis by controlling the commitment of multipotent progenitors and not through the regulation of lymphopoiesis directly.

**PU.1 regulates the differentiation of multipotent lymphoid and myeloid progenitors**

The enhanced BM granulopoiesis and highly aberrant in vitro clonogenic responses that were observed in the absence of PU.1 suggested that PU.1 regulated the balanced generation of multipotent myeloid progenitors (CMP and GMP) or significantly changed their developmental potential. Analysis of mutant BM indicated that the lin− compartment contained a uniform population of c-kit+ cells with blast-like morphology that lacked all other commonly used markers to isolate multipotent progenitor cells prospectively (Fig. 4 A). This observation, combined with the presence of the most primitive blast colony-forming cells in BM (Table II) and spleen (Table III), the enhanced numbers of day 12 CFU-spleen (not depicted) and the ability of PU.1−/− BM to reconstitute granulo- and erythropoiesis for at least 24 wk (Fig. 6), suggested that PU.1 was not required for stem cell function, but for the balanced commitment of the immediate downstream progenitors. In vitro analysis of defined myeloid progenitor populations that were isolated immediately after PU.1 inactivation supported this model because CMPs gave rise to relatively normal numbers of colonies, whereas GMPs had markedly increased granulocytic potential. Taken together, these data demonstrate that the neutrophilia that was observed in the PU.1−/− mice arose as a result of deregulated production of GMP-derived granulocytes. A mechanical explanation for this observation could be that after loss of PU.1, a switch to granulocytic commitment occurs earlier in the pathway of transient amplifying myeloid progenitors that favors the granulocytic lineage over the monocyte fate. Immediately after PU.1 deletion, CMPs can still support macrophage development in response to IL-3, whereas this potential is lost prematurely in GMPs (Fig. 5). A switch from macrophage to granulocytic differentiation also has been observed in the absence of the PU.1-interacting partner IRF-8 (IFN consensus sequence–binding protein); this suggests that this interaction also is important in vivo (13, 39).

Recently, it was reported that the targeted deletion of regulatory enhancer sequences that reduced PU.1 expression by 80% resulted in the development of AML (11). In this model, preleukemic BM had some features that were reminiscent of those reported here, including enhanced granulopoiesis and loss of M-CSFR and GM-CSFR expression. In contrast, these cells continued to express PU.1 targets Mac-1 and FcγRII/III and had normal numbers of G-CSF–responsive progenitors; this suggests that distinct processes are occurring (11). The rapid onset of the neutrophilia (Figs. 2 and 3), the erythromyeloid differentiation capacity of PU.1−/− stem cells in chimeric mice in the absence of detectable disease (Fig. 6), and the appearance of excess growth factor–dependent granulocyte colonies in vitro shortly after PU.1 deletion (Fig. 5) suggested that in our model, the deregulated differentiation occurred independently of any subsequent transformation event. Our preliminary studies of aging PU.1−/− mice revealed that PU.1−/− mice do develop myeloid leukemia at a high frequency (unpublished data). However, the resulting leukemias are heterogeneous in terms of their in vitro growth properties and ability to transfer the disease to secondary recipients; this suggests that the role of PU.1 as a tumor suppressor is more complex than currently appreciated (11, 12). In an attempt to provide further molecular insights into the role that PU.1 plays in the multipotent progenitors, we analyzed gene expression in BM lin− cells. PU.1−/− progenitors had reduced expression of M-CSFR, GM-CSFRα, and IL-7Rα, but not G-CSFR. The normal expression of G-CSFR contrasted with the absence of this receptor in PU.1−/− from fetal liver (28, 29, 31), and provided a potential explanation of the increased granulopoiesis. However, PU.1−/− BM also showed increased granulocyte colonies in the presence of IL-3 or IL-3/SCF/EPO which indicated that the expression of G-CSFR was not solely responsible for the phenotype. Moreover, at longer time points after PU.1 inactivation, the relative contribution of G-CSF in driving the proliferation and differentiation of clonogenic progenitors decreased compared with IL-3/SCF; this suggested that these factors were the more important. Unexpectedly, PU.1 also was required for the expression of the IL-6 and LIF receptor components (IL-6Rα and gp130) and IL-5Rα expression (Fig. 4); this further supported the proposition that PU.1 regulates the response of progenitors to extrinsic regulatory signals. We also assessed the expression of a cohort of key transcriptional regulators of early hematopoiesis, several of which— including IRF-8 and GATA-1—were expressed at lower levels in the absence of PU.1 (Fig. 4 C). In contrast, C/EBPα, whose expression is essential for the CMP to GMP transition (40), was increased in PU.1−/− lin− BM cells (Fig. 4 C). A recent study in PU.1−/−−/− cell lines demonstrated that C/EBPα is induced by G-CSF signaling and that the ratio of C/EBPα to PU.1 is crucial to commitment to the granulocyte cell fate, potentially providing a candidate mechanism for the excess granulopoiesis observed in the PU.1−/− tissues (41). G-CSFR expression already is detectable in the HSC (42) which makes this G-CSF–C/EBPα proposal in the absence of balancing PU.1 action an even more attractive hypothesis. In addition to these important regulatory genes, it was striking that PU.1 also was required for the expression of several myeloid markers, including Mac-1 (27), FcγRII/III (24), CD45 (25), and Fcγ/III (24–25). This makes interpretation of flow cytometric data problematic without using the in vitro clonogenic progenitor approach that is outlined here.

The prevailing models of PU.1 function in hematopoiesis have been derived almost exclusively from studies of fetal
liver cells where lymphoid versus macrophage lineage commitment depends on the graded levels of PU.1 (15, 43). In contrast, PU.1 expression in myeloid progenitors was proposed not to be required for cell commitment but for the response to extrinsic signals once a cell is restricted to a particular lineage (28, 29, 44, 45). Although this latter model is consistent with the requirement for PU.1 for the expression of a number of lineage-specific cytokine receptors, the grossly deregulated granulopoiesis; absence of de novo lymphopoiesis; and the loss of discernable CLP, CMP, GMP, or MEP populations along with long-term self-renewing hematopoietic activity suggests that the predominant developmental role of PU.1 in the adult is to control the balanced transition of the stem cell through the progressively committed multipotent progenitor stages.

**Monoclonal antibodies and flow cytometry.** mAbs against the following antigens were used as fluorescent or biotin conjugates for cell staining and sorting: Ly 5.2 (AL1-4A2), Ly5.1 (A20-1-1), Mac-1 (M1/70), Gr-1 (RB6-8C5), FcyRI/III (2.4G2), B220 (RA3-6B2), CD19 (BD3), Ter119, CD4 (GK1.5), and CD8 (YTS 169.4) were purified from hybridoma supernatants and conjugated to fluorochromes. c-kit (2B8), Sca-1 (E13-161-7), IL7Rα (A7R34), CD34 (RAM34), CD71 (C2), and Ly6C (5075-3.6) were from BD Biosciences. Anti-γ-δ T lymphoid immunoglobulin—Texas red and phycoerythrin- and PerCP-Cy5.5-streptavidin (BD Biosciences) were used as secondary detection reagents. Sorting and analysis was performed on a FACStar-Plus or a DiVa high-speed flow cytometer (BD Biosciences). For cell cycle status, sorted cells were fixed in 70% ethanol overnight, washed, and stained in cell cycle buffer (0.1% sodium acetate, 0.2% Triton X-100, 10 μg/ml RNase A, 50 μg/ml propidium iodide).

**Isolation of bone marrow precursor populations.** The BM precursor populations were isolated by procedures that were described in detail elsewhere (49). The CMP population was identified as Lin−Sca-1−IL-7Rα−c−kit−CD34−FcyR++ cells. GMPs were defined as Lin−Sca-1−IL-7Rα−c−kit− and CLPs as Lin−Sca-1−IL-7Rα−c−kit++ cells.

**Western blotting.** Total protein extracts were produced from equivalent numbers of cells and Western blotting was performed as described previously (49). Rabbit anti–PU.1 (T21) and goat anti–β-actin (I-19) were obtained from Santa Cruz Biotechnology, Inc.

**Bone marrow reconstitution.** For long-term competitive assays, lethally irradiated Ly5.1 mice were reconstituted with PU.1fl/fl MxCre+ BM mixed at 3:1 ratio with Ly5.1 competitor. The degree of reconstitution was determined after 6 wk by analysis of peripheral blood with Ly5.1 and Ly5.2 mAbs. PU.1 deletion was induced by polyI:C injection as above and the chimeras were analyzed 6, 12, and 24 wk later. Because PU.1 regulates CD45 in some lineages, donor cells were defined as Ly5.1+ after erythroid (Ter119+)

**In vitro clonogenic cultures.** BM (2.5 × 10⁶) and spleen (5 × 10⁵) cells were cultured in 1 ml of Dulbecco’s modified Eagle medium containing 0.3% agar and analyzed as described previously (50). Sorted granulocytes (Gr-1+) were similarly cultured in liquid medium plus G-CSF for 7 d. The recombiant cytokines were used at the following final concentrations GM-CSF, G-CSF, IL-3, M-CSF, LIF (10 ng/ml), SCF and IL-6 (100 ng/ml), and flt3L (500 ng/ml). IFNγ (2 × 10⁴ U/ml), thrombopoietin (50 ng/ml), and EPO (20 U/ml). After 7 d of incubation, differential colony counts were performed on fixed whole mount preparations that were stained for acetylcholinesterase, Luxol fast blue, and hematoxylin.

**RT-PCR analysis.** Semi-quantitative RT-PCR was performed as previously described (51). For each primer set, a sample of the PCR reaction was taken at three cycle numbers; each sample was four cycles apart. Amplification products all spanned introns and were visualized on 2% agarose gel. Primer sequences are available upon request.

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