Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors

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PU.1 is an Ets family transcription factor that is essential for fetal liver hematopoiesis. We have generated a *PU.1^{gfp}* reporter strain that allowed us to examine the expression of PU.1 in all hematopoietic cell lineages and their early progenitors. Within the bone marrow progenitor compartment, PU.1 is highly expressed in the hematopoietic stem cell, the common lymphoid progenitor, and a proportion of common myeloid progenitors (CMPs). Based on Flt3 and PU.1 expression, the CMP could be divided into three subpopulations, Flt3$^+$ PU.1^{hi}, Flt3$^-$ PU.1^{hi}, and Flt3$^-$ PU.1^{lo} CMPs. Colony-forming assays and in vivo lineage reconstitution demonstrated that the Flt3$^+$ PU.1^{hi} and Flt3$^-$ PU.1^{hi} CMPs were efficient precursors for granulocyte/macrophage progenitors (GMPs), whereas the Flt3$^-$ PU.1^{lo} CMPs were highly enriched for committed megakaryocyte/erythrocyte progenitors (MEPs). CMPs have been shown to rapidly differentiate into GMPs and MEPs in vitro. Interestingly, short-term culture revealed that the Flt3$^+$ PU.1^{hi} and Flt3$^-$ PU.1^{hi} CMPs rapidly became CD16/32^{high} (reminiscent of GMPs) in culture, whereas the Flt3$^-$ PU.1^{lo} CMPs were the immediate precursors of the MEP. Thus, down-regulation of PU.1 expression in the CMP is the first molecularly identified event associated with the restriction of differentiation to erythroid and megakaryocyte lineages.

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Abbreviations used: AML, acute myeloid leukemia; cDC, conventional DC; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; ES, embryonic stem; GMP, granulocyte/macrophage progenitor; HSC, hematopoietic stem cell; IRES, internal ribosome entry site; Meg, megakaryocyte; MEP, megakaryocyte/erythrocyte progenitor; pDC, plasmacytoid DC; SCF, stem cell factor; TN, triple negative.

Hematopoiesis is a continuous stepwise and controlled process in which the multipotent hematopoietic stem cell (HSC) undergoes differentiation to produce all the mature blood lineages. It has been postulated that the HSC differentiates to either a clonogenic common lymphoid progenitor (CLP) that produces lymphocytes and DCs (1) or a common myeloid progenitor (CMP) capable of giving rise to the erythro-myeloid lineages. The CMP can further differentiate into either one of two more restricted progenitors, the granulocyte/macrophage progenitor (GMP) or the megakaryocyte/erythrocyte progenitor (MEP; reference 2).

The expression of a number of transcription factors is thought to orchestrate hematopoietic differentiation (for review see references 3 and 4). One of these key regulators is PU.1, a hematopoietic-specific Ets family member that is essential for fetal lymphoid and myeloid development (5–8). *PU.1*$^{-/-}$ mice die in late gestation and are devoid of fetal liver B lymphocytes, granulocytes, and macrophages. In adult hematopoiesis, recent data has suggested that PU.1 is an important tumor suppressor in murine and possibly human acute myeloid leukemia (AML; references 9–11). PU.1 regulates numerous genes within the myeloid and lymphoid lineages, including the receptors for a number of cytokines, M-CSFR, G-CSFR, GM-CSFRα (12), and IL-7Rα (13), highlighting the pivotal role this transcription factor plays in the early stages of several lineages.

It has been proposed that graded levels of PU.1 expression by hematopoietic progenitors are determinative of their lineage commitment as high PU.1 directs macrophage differentiation and lower levels are sufficient for fetal B cell development (14, 15), whereas in a more recent study, intermediate levels of PU.1 were required for granulocytes (16). However, the relevance of these results to endogenous PU.1 levels has not been demonstrated as these studies relied on overexpression systems. Further support for the concentration dependence model comes from the finding that *PU.1* is haploinsufficient when the mutation is compounded with the loss of *G-CSF* (16). Moreover, mice with a hypomorphic *PU.1* allele that express only 20% of wild-type protein develop AML at a high frequency, a malignancy thought to derive from primitive hematopoietic cells (9).

The online version of this article contains supplemental material.

These studies predict that the levels of PU.1 will be differentially regulated within the distinct BM multipotent progenitors; however, in no case has the level of PU.1 expression in myeloid and lymphoid lineage precursor populations been clearly shown. In this study, we have generated a *PU.1*^{gfp} reporter allele that enabled us to accurately determine the level and pattern of PU.1 expression at the single cell level. Using these mice, we have examined the rare BM hematopoietic progenitor populations and found that PU.1 is expressed by all HSC, CLP, GMP, Flt3$^+$ CMP, and by a proportion of Flt3$^-$ CMP. In contrast to expectations, the PU.1 levels in HSC and CLP were equivalent to those observed in the committed myeloid progenitors. The different levels of PU.1 expression within the Flt3$^-$ CMP population represented two functionally distinct precursor populations as assessed by in vitro colony-forming assays and in vivo lineage reconstitution. Therefore, the down-regulation of PU.1 in Flt3$^-$ CMP demonstrates the heterogeneity in this population and represents an early event in the restriction of the CMP to erythroid and megakaryocyte (Meg) differentiation.

RESULTS

Generation and validation of PU.1^{gfp} reporter mice

To produce a reporter of PU.1 expression, an internal ribosome entry site (IRES)-GFP cassette was inserted by homologous recombination in embryonic stem (ES) cells into the 3′ untranslated region of mouse *PU.1* (Fig. 1 A). The detailed strategy and confirmation of appropriate gene targeting will be reported elsewhere (unpublished data). The targeted allele resulted in the transcription of a bicistronic mRNA that produced wild-type PU.1 protein and GFP. The targeting strategy predicted that the IRES-GFP cassette would not affect the up-

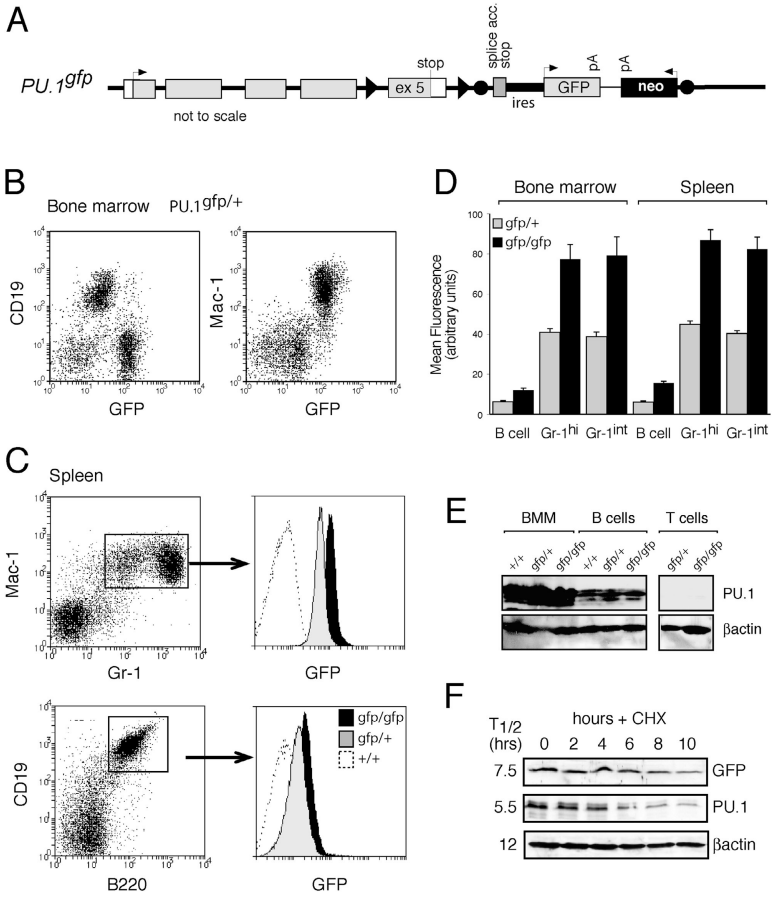

Figure 1. Generation and validation of a *PU.1*^{gfp} reporter strain. (A) The targeted *PU.1*^{gfp} locus is shown with the exons indicated as boxes and the introns as black lines. Coding regions are in gray and nontranslated regions are white. Arrows indicate direction of translation from initial methionine. pA, polyadenylation signal sequence; circles, frt sites; triangles, loxP sites; stop, stop codon; splice acc., splice acceptor. The targeted allele translates full-length *PU.1* and GFP from the same mRNA transcript. (B) GFP expression in *PU.1*$^{gfp/+}$ BM. Cells were stained for CD19 (B cells) and Mac-1 (myeloid cells). (C) GFP expression in B cells and macrophage/granulocytes of adult spleen of +/+, *PU.1*$^{gfp/+}$, and *PU.1*$^{gfp/gfp}$ mice. (D) Quantitation of mean fluorescence of B cells (CD19$^+$ B220$^+$), granulocytes (Mac-1$^+$ Gr.1^{high}), and immature myeloid cells (Mac-1$^+$ Gr-1–intermediate [Gr.1^{int}]) from BM and spleen. n = 4–10 mice per group. Relative mean fluorescence was determined relative to identically gated C57BL/6 cells and is shown in arbitrary units. (E) Western blotting for PU.1 in BM Mac-1$^+$/Gr.1$^+$ myeloid cells (BMM), CD19$^+$ B220$^+$ spleen B cells, and CD4$^+$ T lymphocytes. β actin was a loading control. (F) Determination of PU.1 and GFP stability in splenocytes. Cells were cultured for up to 12 h in the protein synthesis inhibitor cyclohexamide, and equivalent cell numbers were assayed for PU.1, GFP, and β actin levels by Western blotting. The calculated half-life of the proteins is indicated (left).

stream *PU.1* mRNA transcript. To confirm this, homozygous *PU.1*$^{gfp/gfp}$ mice were generated. In contrast to the embryonic or postnatal lethality of *PU.1*$^{-/-}$ pups (5, 6), *PU.1*$^{gfp/gfp}$ mice were indistinguishable in survival, hematopoietic cellularity, and lineage composition from C57BL/6 controls (unpublished data). As predicted, PU.1 protein level in B lymphocytes and myeloid cells was not affected by the host genotype (Fig. 1 E).

PU.1^{gfp} expression by mature hematopoietic lineage cells

PU.1 expression by mature myeloid and lymphoid lineage cells has been previously examined at mRNA and/or protein levels (14, 17). However, the results obtained from these studies could not distinguish whether all, or only a proportion, of cells within a given population express *PU.1*. The *PU.1*^{gfp} reporter mice provided an excellent tool to clarify this issue. We examined the GFP fluorescence of different hematopoietic cell populations from BM and spleen as defined by flow cytometry. The levels of *PU.1* expression were quantified as the mean fluorescence of GFP expression by these cells. PU.1 is expressed at significantly higher levels in macrophages as compared with B cells (14). Analysis of the lymphoid organs of adult *PU.1*$^{gfp/+}$ mice confirmed these lineage-specific expression levels with approximately eightfold higher GFP observed in all Mac-1$^+$ myeloid cells compared with CD19$^+$ B cells (Fig. 1, B and C). The Mac-1$^+$ fraction contains immature granulocytes/monocytes (Gr-1^{int}) and mature granulocytes (Gr-1^{hi}), all of which displayed similar GFP fluorescence, indicating relatively uniform *PU.1* transcription throughout granulocytic/monocytic differentiation (Fig. 1, C and D). A similar uniformity was observed for B lineage cells (Fig. 1, C and D). Analysis of B cell and macrophage/granulocyte populations revealed an exquisite gene dosage sensitivity of the reporter allele, with *PU.1*$^{gfp/gfp}$ cells containing almost exactly twice the GFP fluorescence of heterozygous cells (Fig. 1, C and D). Moreover, determination of the half-life of the proteins revealed relatively similar turnover rates (5.5 h for PU.1 and 7.5 h for GFP), indicating that GFP loss is also an accurate reporter for PU.1 down-regulation (Fig. 1 F). The lineage-specific and gene dosage–sensitive levels of GFP in the *PU.1*^{gfp} mice validate the allele as an accurate reporter of endogenous transcription and enabled full characterization of PU.1 expression in a number of cell types that have not been fully characterized, including DCs, NK cells, and erythrocyte lineages.

The role of PU.1 in DC development is not clear. PU.1 has been reported to be required for the differentiation of all DCs (18) or more specifically, for myeloid-derived DCs (19), with no data available for plasmacytoid DCs (pDCs). We examined the PU.1^{gfp} expression by freshly isolated thymic and splenic CD11c$^+$ CD45RA$^-$ conventional DCs (cDCs) and the type I IFN–producing CD11c^{int} CD45RA$^+$ pDCs. As shown in Fig. 2, A and B, all of the cDCs from the thymus and spleen expressed levels of GFP comparable to myeloid cells. In contrast, all of the pDCs displayed moderate levels of GFP similar to B cells (Fig. 2, A–C). As both CMPs and CLPs can produce all DC types in vivo, these data indicate that *PU.1* expression in cDCs is unrelated to their developmental origin (20).

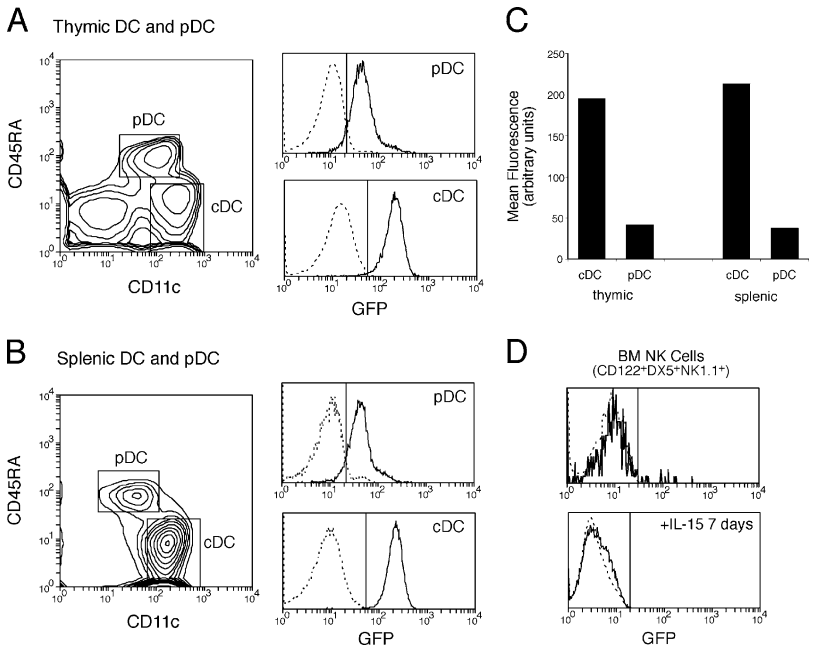

Figure 2. PU.1^{gfp} expression in DCs and NK cells. (A) The thymic and (B) splenic cDCs and pDCs were prepared from the *PU.1*$^{gfp/gfp}$ mice. The GFP fluorescent intensities of cDCs and pDCs were analyzed on gated cDC (CD11c^{hi} CD45RA$^-$) and pDC (CD11c^{int} CD45RA$^+$) populations and presented as histograms. (C) PU.1 expression by cDCs and pDCs were quantified as the mean fluorescence of GFP. (D) PU.1^{gfp} expression in mature NK cells isolated from BM (CD122$^+$ DX5$^+$ NK1.1$^+$). NK cells were also generated from BM cultures in the presence of IL-15 for 7 d and assessed for GFP. The solid lines represent the PU.1^{gfp} levels and the dotted lines represent the background from wild-type mice.

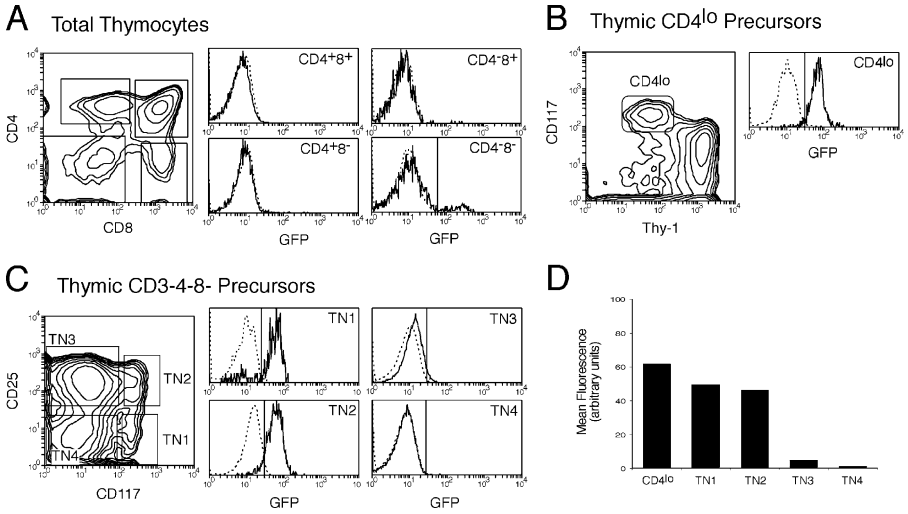

Figure 3. PU.1^{gfp} expression during T cell development. (A) Total thymocytes from *PU.1$^{gfp/gfp}$* mice were analyzed for CD4, CD8, and GFP. The PU.1^{gfp} expression by each major thymocyte populations was analyzed on gated cells and is presented as histograms. (B) PU.1^{gfp} expression by the earliest intrathymic precursor population (CD4^{lo}, CD3^{-}8^{-} Thy-1^{lo} CD117^{+}). (C) CD3^{-}4^{-}8^{-} (TN) thymic precursor populations were isolated and PU.1^{gfp} expression by each TN precursor population was analyzed on gated cells. (D) The level of PU.1 expression was quantified as the mean fluorescence of GFP by each thymic precursor population. The solid lines represent the PU.1^{gfp} and the dotted lines represent the background of each cell population in *+/+* mice.

It was also reported by an earlier study that NK cells express *PU.1* mRNA (21). However, we have not observed any GFP fluorescence in mature NK cells either freshly isolated from mouse BM (CD122^{+} DX5^{+} NK1.1^{+}) or obtained in culture with IL-15 (Fig. 2 D). *PU.1* might be expressed in pro–NK cells (CD122^{+} DX5^{-} NK1.1^{-}) and down-regulated upon maturation; however, a definitive analysis has not been possible as we have not been able to exclude PU.1-expressing myeloid cells from this population (unpublished data).

PU.1 was originally isolated from a virally induced erythroleukemia (22) and is expressed in developing erythroid progenitors from fetal liver (7, 23). In contrast, adult BM erythrocytes, neither mature (Ter-119^{+} CD71^{-}) nor immature (Ter-119^{+} CD71^{+}), showed expression of GFP, indicating that PU.1 is silenced at an early stage of erythropoiesis (unpublished data).

In summary, the *PU.1^{gfp}* allele described here has allowed the rapid and quantitative determination of *PU.1* expression levels in a variety of hematopoietic lineages and revealed a complex and dynamic expression pattern throughout adult hematopoiesis.

PU.1^{gfp} expression during thymocyte development

Analysis of the PU.1^{gfp} during T lineage cell development revealed that the majority of thymocytes, including CD4^{+}8^{+}, CD4^{+}8^{-}, and CD4^{-}8^{+} were GFP^{-} (Fig. 3 A). In contrast, a small fraction of the CD4^{-}8^{-} thymocytes was GFP^{+}, suggesting that the T cell precursors express *PU.1*. The earliest intrathymic precursor population (CD4^{lo} precursors) displayed intermediate levels of GFP, whereas the majority of the CD3^{-}4^{-}8^{-} CD25^{-} CD117^{+} (triple negative [TN]1) pro–T cells expressed GFP at a slightly lower level than that of the CD4^{lo} precursors (Fig. 3, B–D). GFP expression was maintained in the CD25^{+} CD117^{+} (TN2) precursors before being markedly down-regulated at the CD25^{+} CD117^{-} (TN3) stage, coinciding with the onset of TCR gene rearrangement (Fig. 3, C and D). These results were consistent with a previous study in which the *PU.1* mRNA expression by these T cell precursor populations was examined (24). This loss of PU.1 was permanent as mature peripheral T cells were GFP^{-} (Fig. 1 E).

PU.1^{gfp} expression by BM hematopoietic progenitor populations

The graded levels of PU.1 reported here and observed by others, has led to a model whereby distinct PU.1 levels arise in multipotent progenitors and are deterministic of lineage choice (25). Some of these studies have shown that *PU.1* mRNA was expressed at different levels by different hematopoietic progenitor populations (2, 26). These data are problematic because of technical limitations of amplifying PU.1 from these rare populations. These assays did not indicate if the protein levels were of functional significance, and finally, they are not able to distinguish whether all of the cells or only a subset of the cells within a given population expressed *PU.1*. The *PU.1^{gfp}* reporter mice enabled us to examine the *PU.1* expression by different rare hematopoietic progenitors at the single cell level.

Mouse BM hematopoietic progenitor populations were isolated as described previously (20). The enriched BM HSCs were defined as Lin^{-} c-kit^{+} Sca-1^{+} cells and were uniformly PU.1^{gfp} high (PU.1^{hi}; Fig. 4 A), suggesting a role of PU.1 in the earliest stage of hematopoiesis. Interestingly, although the mature B lymphoid cells were low for PU.1^{gfp}, almost all of the CLPs were PU.1^{hi} (Fig. 4, A and C). The PU.1 decrease appeared to correlate with B lineage commit-

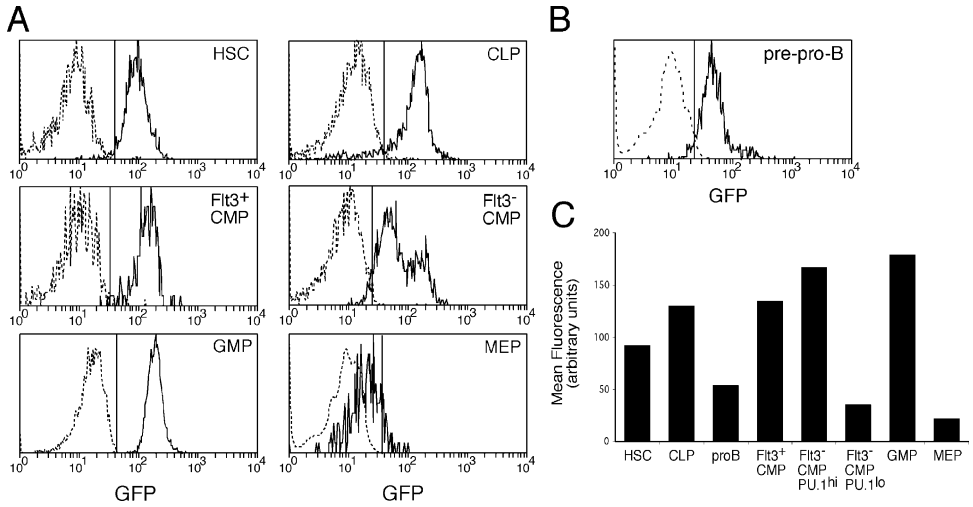

Figure 4. PU.1^{gfp} expression by BM HSCs and multipotent progenitors. (A and B) The BM HSCs and indicated hematopoietic progenitor populations from *PU.1$^{gfp/gfp}$* and wild-type mice were isolated as described in Materials and methods. The GFP fluorescence intensity of each progenitor population is presented as histograms. The solid lines are from *PU.1$^{gfp/gfp}$* cells and the dotted lines from +/+ mice. (C) Quantitation of the mean fluorescence of GFP expression by each cell population.

ment as pre-pro–B cells (defined as CD19$^-$ B220$^+$ CD43$^+$ c-kit$^+$) had already decreased the PU.1^{gfp} expression to a level comparable with mature B cells (Fig. 4, B and C).

We previously reported that the CMP population could be divided into two fractions based on the surface Flt3 expression (20). As shown in Fig. 4 A, all of the Flt3$^+$ CMPs were PU.1^{hi}, but the Flt3$^-$ CMPs could be further divided into two fractions based on differing GFP expression. Approximately 30–40% of Flt3$^-$ CMPs expressed high levels of PU.1^{gfp} compared with the Flt3$^+$ CMPs, whereas the remaining Flt3$^-$ CMPs (~60–70%) were PU.1^{gfp} low (PU.1^{lo}; Fig. 4, A and C). The correlation between *PU.1* and GFP expression was further confirmed using RT-PCR (Fig. 5 C). Therefore, the CMP, originally described as a homogenous clonogenic population, contains at least three subsets, i.e., Flt3$^+$ PU.1^{hi}, Flt3$^-$ PU.1^{hi}, and Flt3$^-$ PU.1^{lo}. Of the more downstream committed progenitors, the GMP contained the strongest PU.1^{gfp} fluorescence of any population, whereas the committed MEP expressed the lowest (Fig. 4, A and C).

Down-regulation of PU.1 expression is associated with the restriction of CMPs to erythroid and Meg differentiation

The majority of CMPs (>90%) had the morphology of large undifferentiated blast cells (Fig. 5 A, a and b) and exhibited mitotic figures. GMPs were generally similar in size but frequently contained small numbers of large granules in the cytoplasm resembling those of promyelocytes (Fig. 5 A, c and d). In contrast, MEPs often had dark cytoplasm and some of these cells resembled early erythroblasts (Fig. 5 A, e and f). The fractionated CMP populations had a generally similar morphology to one another except that some CMP Flt3$^-$ PU.1^{hi} cells had some cytoplasmic granules and some CMP Flt3$^-$ PU.1^{lo} cells had dark cytoplasm (Fig. 5 B).

Table I. Colony-forming potential of the CMP subpopulations

CMP		Mean number of colonies[a]					
Fractions	Stimulus	Blast	G	GM	M	Meg[b]	E
	SCF	3.0 ± 1.6	7.5 ± 1.9	1.2 ± 0.6	1.2 ± 0.8	0	0
Flt3$^+$	IL-3	4.7 ± 0.9	6.0 ± 1.4	9.0 ± 2.9	24.3 ± 8.3	0	0
PU.1^{hi}	SCF+IL-3+EPO	6.0 ± 2.8	11.0 ± 1.6	13.3 ± 4.2	16.3 ± 6.2	0	0
	SCF	2.6 ± 1.2	30.3 ± 11.3	0.3 ± 0.5	0.3 ± 0.5	0	0
Flt3$^-$	IL-3	3.3 ± 1.2	29.7 ± 6.1	11.7 ± 4.1	18.0 ± 4.9	2.3 ± 0.6	0
PU.1^{hi}	SCF+IL-3+EPO	4.3 ± 1.9	29.0 ± 10.0	13.0 ± 2.2	14.0 ± 3.3	5.7 ± 3.7	0.3 ± 0.6
	SCF	0.3 ± 0.5	0.4 ± 0.4	0	0.4 ± 0.1	0	0
Flt3$^-$	IL-3	1.3 ± 0.8	0.3 ± 0.4	0.2 ± 0.2	0.2 ± 0.2	3.7 ± 0.8	0
PU.1^{lo}	SCF+IL-3+EPO	1.3 ± 0.9	1.5 ± 0.7	0.3 ± 0.5	1.0 ± 1.4	30.9 ± 3.5	4.0 ± 3.0

100 purified cells of each fraction were cultured for 7 d in 0.3% agar with the growth factors indicated.
[a]The numbers of colonies are the mean number of colonies ± SD from three separate experiments.
[b]15–27% of these Meg colonies also contained erythroid cells.
Blast, blast cell colony; G, granulocytic colony; GM granulocyte-macrophage colony; M, macrophage colony; Meg, megakaryocytic colony; E, erythroid colony.

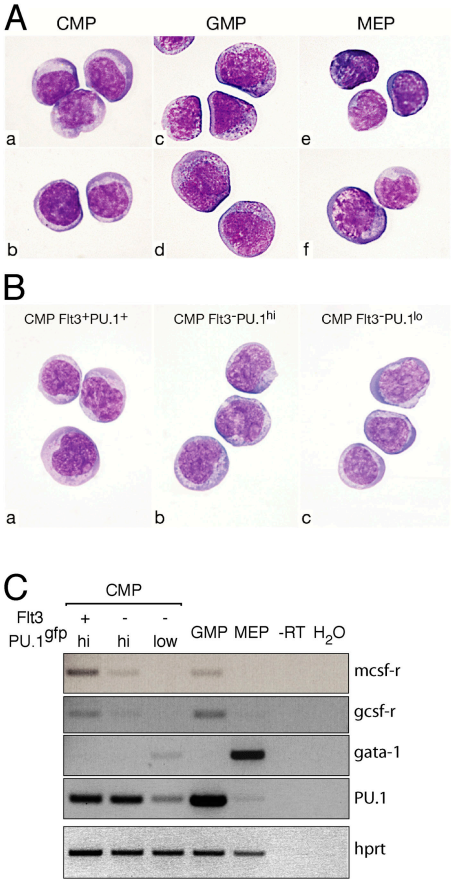

Figure 5. Morphology and differential gene expression by the BM progenitor populations. (A) Morphology of unseparated CMP (a and b), GMP (c and d), and MEP (e and f) populations. (B) Morphology of the indicated three CMP subpopulations. BM progenitor populations were purified by flow cytometric cell sorting and cytocentrifuged onto a glass slide and stained with May-Grunwald-Giemsa solution. (C) RT-PCR for the expression of the indicated genes was performed with the cDNA prepared from each progenitor populations. *hprt* was used as a control for cDNA input.

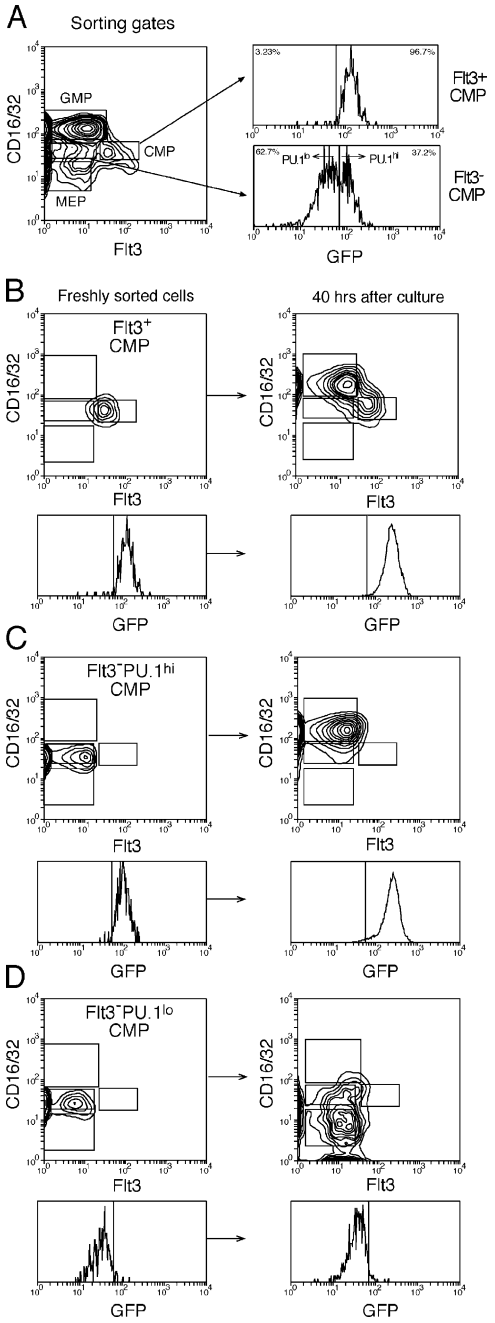

Figure 6. In vitro differentiation of the CMP subpopulations. The three CMP populations, defined as CD16/32^{int} Flt3^{+}, CD16/32^{int} Flt3^{-} PU.1^{hi}, and CD16/32^{int} Flt3^{-} PU.1^{lo}, were sorted using the gates shown in A and cultured on S17 stromal cells in the presence of SCF for 40 h. The levels of the PU.1^{gfp}, CD16/32, and Flt3 on freshly isolated precursor populations are shown. Flt3^{+} CMP (B), Flt3^{-} PU.1^{hi} CMP (C), and Flt3^{-} PU.1^{lo} CMP (D) are shown in the left panels. The phenotypes of the progeny of each precursor population after 40 h in culture are shown in the right panels. The results shown are representative of three experiments.

To examine the correlation of different levels of Flt3/PU.1 and cell differentiation potential, in vitro colony-forming assays were performed (Table I). In the presence of stem cell factor (SCF), which stimulates the formation of blast and granulocytic colonies, the Flt3^{+} PU.1^{hi} and Flt3^{-} PU.1^{hi} CMPs formed small numbers of blast colonies and a significant number of granulocytic colonies. In contrast, few Flt3^{-} PU.1^{lo} CMPs exhibited blast or granulocytic colony-forming potential. Similarly, in the presence of IL-3, which stimulates the colony formation of all cell types, both Flt3^{+} PU.1^{hi} and Flt3^{-} PU.1^{hi} CMPs formed significant numbers of granulocytic, granulocyte-macrophage, and macrophage colonies, whereas the Flt3^{-} PU.1^{lo} CMPs lacked this potential. Interestingly, the Flt3^{-} PU.1^{hi} CMPs expressed slightly higher levels of GFP and were more efficient in generating granulocytic colonies than the Flt3^{+} PU.1^{hi} CMPs (Table I).

Most importantly, when a combination of SCF, IL-3, and erythropoietin, the most potent stimulus for Meg colony formation, was used, the Flt3^{+} PU.1^{hi} and Flt3^{-} PU.1^{hi} CMPs virtually lacked clonogenic Meg progenitors, whereas, strikingly, >30% of the Flt3^{-} PU.1^{lo} CMP cells formed Meg colonies, from 15–27% of which also contained erythroid

Table II. Generation of myeloid cells and DCs in vivo by CMP subpopulations

CMP subpopulations	Donor-derived cells ($\times 10^4$)		
Injected ($\times 10^4$)	Mac-1$^+$ Gr-1$^+$ cells in BM[a]	Mac-1$^+$ Gr-1$^+$ cells per spleen	CD11c$^+$ DCs per spleen
Flt3$^+$ PU.1^{hi}	3.1–16.0	52.0–78.1	26.5–59.2
Flt3$^-$ PU.1^{hi}	7.9–8.5	34.4–52.9	3.1–8.5
Flt3$^-$ PU.1^{lo}	0.4–0.6	2.9–15.9	0.1–0.5

Purified BM progenitor populations from *PU.1$^{gfp/gfp}$* mice (C57BL/6 Ly5.2) were intravenously injected into lethally irradiated Ly5.1 recipient mice. For myeloid cell generation, the recipient mice were analyzed 10 d after injection. For DC generation, recipient mice were analyzed 14 d after injection. The values in this table are the ranges of donor-derived cell numbers obtained from two experiments. Each experiment included two to three recipients for each precursor population.
[a]Donor-derived cells in the BM of two femur and two tibia collected from each mouse.

cells. In addition, cells of this type formed small numbers of pure erythroid colonies (Table I). Thus, the Flt3$^-$ PU.1^{lo} CMPs showed the lowest capacity to form myeloid lineage colonies, but the highest capacity for megakaryo-erythropoiesis. These results demonstrate that the down-regulation of PU.1 expression is closely associated with loss of myeloid lineage potential and restriction to Meg and erythroid (MegE) differentiation.

In support of these clonogenic assays, sorted Flt3$^+$ PU.1^{hi} and Flt3$^-$ PU.1^{hi} CMPs and GMPs expressed the mRNA for *m-csfr* and *g-csfr*, whereas the Flt3$^-$ PU.1^{lo} CMPs lacked these transcripts and in contrast expressed low levels of the MegE regulator *gata-1* (Fig. 5 C). This gene expression profile suggests that the Flt3$^-$ PU.1^{lo} CMP exhibits the initial activation of the MegE differentiation pathway.

We have also performed in vivo cell transfer and lineage reconstitution assays with these sorted populations. Purified progenitor populations from *PU.1$^{gfp/gfp}$* (Ly5.2$^+$) mice were intravenously injected together with 5×10^4 recipient-type BM cells into lethally irradiated Ly5.1$^+$ recipient mice. The potential of these cells to generate myeloid cells and DCs was analyzed at 10 and 14 d after transfer. Both the Flt3$^+$ PU.1^{hi} and Flt3$^-$ PU.1^{hi} CMPs were able to efficiently produce the Mac-1$^+$ Gr-1$^+$ myeloid cells and CD11c$^+$ DCs in vivo, with the Flt3$^+$ PU.1^{hi} CMPs being slightly more efficient in generating these cells (Table II). In comparison, the Flt3$^-$ PU.1^{lo} CMPs gave rise to only a small number of myeloid cells and very few DCs. These results were consistent with that of colony-forming assays and again demonstrated that the Flt3$^-$ PU.1^{lo} CMPs had the lowest potential to generate myeloid lineage cells. The CMP has previously been shown to rapidly differentiate into GMPs and MEPs upon in vitro culture (2). In an attempt to reveal the developmental relationship amongst the three CMP populations, an identical short-term culture system was used. Purified Flt3$^+$ PU.1^{hi}, Flt3$^-$ PU.1^{hi}, or Flt3$^-$ PU.1^{lo} CMPs were cultured on S17 stromal cells in the presence of SCF (Fig. 6). After 40 h, the cultured cells were analyzed for CD16/32, Flt3, and c-kit expression. The majority of the Flt3$^+$ PU.1^{hi} cells had developed into CD16/32^{hi} Flt3$^-$PU.1^{hi}, a phenotype of GMP (Fig. 6 B). Similarly, the Flt3$^-$ PU.1^{hi} cells also developed into CD16/32^{hi} Flt3$^-$ PU.1^{hi} GMP (Fig. 6 C). In contrast, most of the Flt3$^-$ PU.1^{lo} cells developed into CD16/32$^{-/lo}$ Flt3$^-$ PU.1^{lo}, a phenotype of MEP (Fig. 6 D). Therefore, based on the levels of Flt3 and PU.1, the originally defined "CMP" population contains three separate populations that did not display any precursor–product relationship. Moreover, the combination of three approaches to determine the developmental potential of these newly identified CMP fractions demonstrated that PU.1 down-regulation is a very early event in the divergence of the myeloid and MegE lineages.

DISCUSSION

One model of hematopoietic lineage commitment proposes that the relative levels of key transcription factors, including PU.1, influence cell fate decisions (4, 27). The multiple lineages and developmental stages of hematopoietic cells and the rarity of the multipotent progenitors have made testing this model using endogenous expression levels in primary cells problematic. Therefore, most studies have focused on model cell lines and/or overexpression systems. To study the function of PU.1 in adult hematopoietic cell development, we generated a *PU.1^{gfp}* reporter allele that has allowed us to determine accurately the levels of PU.1 expression in all hematopoietic cell types and their early progenitors.

The analysis of GFP expression by mature hematopoietic cells of adult *PU.1$^{gfp/gfp}$* mice confirmed the previous findings that monocytes/granulocytes expressed significantly higher levels of PU.1 (approximately eightfold) as compared with B cells (14, 17). The strikingly uniform expression of PU.1 in both lineages, the relatively similar protein stability between GFP and PU.1, and the exquisite sensitivity of the fluorescence (heterozygous cells contained exactly 50% GFP levels of homozygous cells) demonstrated that the reporter would enable the quantitative analysis of the mean GFP fluorescence in defined cell populations. A broader analysis revealed that PU.1 is silenced at an earlier stage in erythrocytes, NK cells, and T cells. Within the DC lineages, PU.1 showed specific expression levels, with the cDC populations having uniformly high levels of PU.1 comparable to that of myeloid cells, whereas the pDCs expressed moderate levels of PU.1 similar to that of B cells. cDC ontogeny is complex with at least three distinct subsets, CD8$^+$, CD4$^+$, and double negative, which are derived from both lymphoid and myeloid progenitors (for review see reference 28). However, GFP expression was uniform within all cDCs, suggesting that PU.1 levels are not related to the phenotype or origins of the lineage. The similar levels of GFP in pDCs compared with B

cells may reflect the shared genetic program between these cell types, regardless of lymphoid or myeloid origin, resulting in *D-J*$_H$ recombinations at the *IgH* locus (29) and the expression of common transcriptional regulators, including Spi-B (30, 31).

The analysis of expression of *PU.1* in multipotent BM progenitors has to date been restricted to RT-PCR (2, 26, 32). These approaches have suggested that PU.1 is expressed in all progenitor fractions but are problematic due to the difficulties inherent in controlling for sorting purity, generating cDNA from these rare cells, and the interpretation of the data due to the reported promiscuous low level transcriptional priming of noncommitted progenitor cells (32). For example, the original description of *PU.1* expression in defined erythro-myeloid progenitors suggested equally low expression in all populations (2), whereas a subsequent study has suggested that *PU.1* mRNA levels are higher in GMPs than MEPs (26). The heterogeneity of the CMP reported here makes such a population level analysis uninformative. In contrast, the *PU.1*^{gfp} reporter mice enabled us to quantify the levels of PU.1 expression at a single cell level. Overexpression studies have shown that the lineage fate of *PU.1*$^{-/-}$ fetal liver progenitors can be directed by the ectopically expressed PU.1(14–16). These experiments have led to the prediction that PU.1 will be lowly expressed in most primitive progenitors, up-regulated in the CMP, and remain low in the CLP (15). Our results suggest an alternate model as we found that PU.1 was already expressed at high levels in the HSC. Moreover, we found that the CLP and CMP were comparably GFP fluorescent, suggesting that the PU.1 level was not the determining factor of lympho-myeloid lineage commitment. In contrast, the high level PU.1 expression in this early progenitor stage and undetectable CLPs and CMPs in the BM of mice with induced deletion of PU.1 (unpublished data) support a requirement for PU.1 in the transition of HSCs to the CLP or CMP stages of adult hematopoiesis.

Within the lymphoid lineages, the earliest progenitor, the CLP, expressed high levels of *PU.1*, which was down-regulated during the transition from CLPs to committed T or B cells. All B cells expressed low levels of PU.1, whereas *PU.1* is silenced at the TN3 stage of T lymphopoiesis, a finding consistent with previous RT-PCR studies (24). This down-regulation is required for progression in the T cell lineage because enforced constitutive expression of PU.1 during T cell development results in growth inhibition and an arrest at the pro–T cell (TN2) stage (24). These findings suggest that the high PU.1 expression in the CLP is repressed upon B/T cell commitment to the characteristic low B cell expression state and completely repressed to allow T cell development.

In contrast to the uniform expression of PU.1^{gfp} in the HSC and CLP, we found clear evidence of heterogeneity in the CMP. The CMPs were originally reported as clonogenic myeloid precursors (2). However, recent studies of ours (20) and others (33) demonstrated that the CMP could be divided into two fractions based on the Flt3 expression. The Flt3$^+$ CMPs were shown to be more efficient progenitors for myeloid cells and DC populations than the Flt3$^-$ CMPs (20, 33). The Flt3$^+$ CMPs also contain precursors of B cells (20). In this study, we showed that the different levels of PU.1 expression further subdivided the Flt3$^-$ CMP into two populations, namely the Flt3$^-$ PU.1^{hi} and the Flt3$^-$ PU.1^{lo} CMPs. These populations were morphologically very similar but in vitro colony formation and the in vivo precursor transfer assays demonstrated the differences in progenitor potentials of these three CMP populations, with the Flt3$^+$ PU.1^{hi} cells as the most efficient progenitors for myeloid cells and DCs, the Flt3$^-$ PU.1^{hi} cells as efficient progenitors for myeloid cells but not for DCs, and the Flt3$^-$ PU.1^{lo} cells as containing progenitors mainly for MegE. CMPs have been demonstrated to be direct precursors of the GMP and MEP populations (2). Here we have shown that the Flt3$^+$ PU.1^{hi} and Flt3$^-$ PU.1^{hi} CMPs directly differentiated into GMP-like cells, whereas the Flt3$^-$ PU.1^{lo} cells differentiated to MEPs. The lack of true bipotent cells in these fractions in this assay suggests that the true CMP is either a relatively small proportion of the defined gate or confined to an as yet unidentified earlier stage. In summary, we have demonstrated that the CMP contains at least three phenotypically, functionally, and developmentally distinct cell subsets.

The fact that the Flt3$^-$ PU.1^{lo} cells were highly enriched for clonogenic MegE progenitors together with the very low levels of *PU.1* expression by the MEP and the induction of the MegE regulator *gata-1* by these cells suggests that down-regulation of *PU.1* is one of the first events associated with the restriction to MegE differentiation. Although it is at present not definitively known whether this down-regulation is essential for erythroid commitment, studies using viral integration or transgenic overexpression demonstrate that PU.1 is incompatible with normal erythropoiesis as ectopic PU.1 blocks early erythroid differentiation, resulting in erythroleukemia (22, 34). In contrast, forced *gata-1* expression in vivo reprograms CLP and GMP to the MegE lineages (26). It has been proposed that the interactions of these proteins are direct and result in functional antagonism of either partner (35–38). These results emphasize the importance of considering the functionality of PU.1 as well as its expression level. PU.1 can be serine phosphorylated and interacts with a variety of other transcription factors (8). Although the *PU.1*^{gfp} model does not allow us to discern such posttranslation influences, the transcriptional down-regulation or PU.1 in the Flt3$^-$ PU.1^{lo} CMP and MegE lineages allows us to propose that the primary determinant of PU.1 versus GATA-1 stoichiometry and lineage determination occurs via transcriptional regulation as few or no progenitors coexpress high levels of both transcripts.

The genetic elements underlying this dynamic expression pattern of PU.1 have not been determined. Deletion of a distal enhancer ~14-kb upstream of the start of transcription was recently shown to reduce expression to 20% that of wild-type cells (9). However, that study did not ascertain if the re-

duction in PU.1 was uniform or lineage/differentiation-stage specific. Interestingly, these mice developed AML with a high frequency, indicating that the regulation of PU.1 expression is an essential process in controlling hematopoietic malignancies. PU.1 has also been proposed to autoregulate its own transcription with *PU.1*$^{-/-}$ fetal liver cells lacking the truncated *PU.1* mRNA (5, 39). Therefore, antagonizing PU.1 function would break this autoregulatory loop and provide a simple method to reduce expression. The *PU.1*^{gfp} mice will provide an excellent tool to address this question.

This study has revealed a complex and dynamic expression pattern of *PU.1* throughout adult hematopoiesis. We propose that *PU.1* transcription is controlled at multiple points in hematopoiesis. *PU.1* is induced in the most primitive HSC and maintained at this high level in lymphoid and myeloid progenitors. In contrast, PU.1 down-regulation is an early event in the loss of myeloid differentiation capacity associated with commitment to megakaryo-erythropoiesis. Upon unilineage commitment, *PU.1* expression is further modified to result in the characteristic high levels in macrophages, low levels in B cells, and transcriptional silencing in a number of other cell types.

MATERIALS AND METHODS

Generation of PU.1^{gfp} mice. The pKW11 vector consists of a splice acceptor, stop codons in all reading frames, an IRES, *eGFP* cDNA, and a SV40 polyadenylation signal, and a *PGK-Neo*^{r} gene was introduced into the 3′ untranslated region of PU.1 by homologous recombination in C57BL/6 ES cells. Targeted ES cell clones were injected into BALB/c blastocysts to obtain chimeric founders. Germline transmission was achieved with two clones that gave identical patterns and levels of GFP fluorescence. Mice were bred and maintained at the Walter and Eliza Hall Institute under animal ethics guidelines.

Antibodies. The following mAbs were used as supernatants for immunomagnetic bead depletion of lineage marker$^+$ BM cells: CD3 (KT-3.1), CD8 (53–6.7), CD2 (RM2-1), B220 (RA3-6B2), Mac-1 (M1/70), Gr-1 (RA6-8C5), and Ter-119. The following supernatants were used for depletion in splenic DC and pDC preparations: CD3, CD19 (ID3), CD90 (T24/31.7), Gr-1, and Ter-119. For thymic DC and pDC preparation, CD11b (M1/70) and F4/80 were also added to the depletions. Note that the use of Gr-1 did not cause depletion of pDCs (40).

The following mAbs were used for cell staining and sorting: Gr-1, CD19, Ter-119, CD49b (HMα2), CD8, CD45RA (14.8), and Flt3 (A2F10.1) used as a PE conjugate; c-kit (CD117) (2B8), Thy1.2 (30H12), and NK1.1 (PK136) used as an allophycocyanin (APC) conjugate; Sca-1 (E13-161-7), CD4, CD11c (N418), and IL–7Rα (A7R34) used as Alexa 594 conjugates; and Ly 5.2 (AL1-4A2), FcγRII/III (CD16/32(2.4G2)), IL-7Rα (A7R34), CD25 (PC61), B220, Mac-1, CD71 (C2), CD43 (S7), CD122 (Tm-β1), and CD34 (RAM34) were biotinylated. mAbs were purified from hybridoma supernatants with the exception of CD71, CD43, CD122, c-kit, and CD34, which were from BD Biosciences. Anti–rat immunoglobulin–Texas red, PE-avidin, or PerCp–Cy5.5-avidin (all from BD Biosciences) were used for second-stage staining.

Isolation of BM precursor populations. The early intrathymic lymphoid precursors (41) and BM precursor populations (42) were purified as described previously. In brief, HSC and CLP populations from BM were purified by immunomagnetic bead depletion of lineage marker$^+$ cells, followed by staining with c-kit–APC, Sca-1–Alexa 594, and IL-7Rα–biotin, followed by PE-avidin. The HSC was identified as Lin$^-$ IL-7Rα$^-$ Sca-1^{hi} c-kit^{hi}. The CLP was identified as Lin$^-$ IL-7Rα$^+$ Sca-1^{int} c-kit^{int} cells. The myeloid precursor populations from BM were isolated by first depleting lineage marker$^+$ cells by means of immunomagnetic beads. The remaining cells were then stained with goat anti–rat immunoglobulin–Texas red, Sca-1–Alexa 594, IL-7Rα–Alexa 594, c-kit–APC, Flt3-PE, FcRγII/III (CD16/32)–biotin, and followed by PerCP–Cy5.5-avidin. The previously described CMP population was identified as Lin$^-$ Sca-1$^-$ IL-7Rα$^-$ c-kit$^+$ CD16/32^{low} cells. The CMPs can be further divided into three populations based on Flt3 and PU.1^{gfp} expression, namely Flt3$^+$ PU.1^{hi}, Flt3$^-$ PU.1^{hi}, and Flt3$^-$ PU.1^{lo}. Because of limitations in the available fluorescent channels, our gating for the CMP populations differed from that previously published in that it did not include CD34 (2). We believe that the parameters used in this study identify the same CMP population as those defined previously (for details see Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041535/DC1). The GMP was identified as Lin$^-$ Sca-1$^-$ IL-7Rα$^-$ c-kit$^+$ CD16/32$^+$ CD34$^+$ cells and the MEP was Lin$^-$ Sca-1$^-$ IL-7Rα$^-$ c-kit$^+$ CD16/32$^-$ CD34$^-$. The stained cells were analyzed or sorted using a FACStar^{PLUS} or a DiVa instrument (BD Biosciences). The BM pre-pro–B cells were purified by immunomagnetic bead depletion of lineage marker$^+$ (except B220$^+$) cells, and then stained with CD19-PE, B220-Cy5, and CD43-biotin (revealed with Alexa 594 avidin). Pre-pro–B cells were defined as CD19$^-$ B220$^+$ CD43$^+$. The purity of sorted cells was determined by reanalyzing a small sample of the collected cells and was usually >97%. Fractionated BM progenitors were cytocentrifuged onto slides and stained with May-Grunwald-Giemsa solution.

Determination of PU.1^{gfp} expression. The PU.1^{gfp} expression by different hematopoietic cell populations was examined by flow cytometric analysis. The level of PU.1^{gfp} was determined by the relative mean fluorescence, i.e., the mean fluorescence of a defined cell population of *PU.1*$^{gfp/+}$ or *PU.1*$^{gfp/gfp}$ mice subtracted with the mean fluorescence of the same cell population of the control C57Bl/6 mice. As the fluorescence intensity of equivalent cell populations varied between the analytical flow cytometer (Fig. 1, LSR; BD Biosciences) and the DiVa instrument (Figs. 2–4; BD Biosciences), only relative fluorescence in arbitrary units is indicated for each histogram.

Western blotting. Total protein extracts were produced from equivalent numbers of cells and Western blotting was performed as described previously (43). Rabbit anti-PU.1 (T21), rabbit anti-GFP (FL), and goat anti–β actin (I-19) were from Santa Cruz Biotechnology, Inc. Specific protein signals were determined by densitometry of the resulting X-ray film.

In vitro cell culture. For the analysis of protein stability, erythrocyte-depleted splenocytes were cultured in IMDM and 10% FCS with 50 μg/ml cyclohexamide added at appropriate time points before the completion of the 12-h culture. Sorted progenitor cell populations were seeded at 10,000 cells/100 μl in IMDM, 10% FCS, and 100 μg/ml SCF on a semiconfluent layer of S17 stroma as described previously (2). Cells were analyzed after 40 h. CD49b$^+$ TCRβ$^-$ NK cells were FACS sorted from the spleen and cultured in 50 ng/ml IL-15 for 7 d as described previously (44).

In vivo hematopoietic cell lineage reconstitution. The CMP populations were purified from the BM of *PU.1*$^{gfp/gfp}$ (C57BL/6 Ly5.2) mice and then intravenously injected together with 5×10^4 recipient-type BM cells into lethally irradiated (550 rads, twice) C57BL/6 Ly5.1 recipient mice. 10 d after injection, the donor-derived cells in the recipient thymus, spleen, and BM were analyzed by flow cytometry. Donor-derived myeloid cells were identified as Ly5.2$^+$ Mac-1$^+$ or Gr-1$^+$. The donor-derived B and T cells were identified as Ly5.2$^+$ CD19$^+$ B220$^+$ and Ly5.2$^+$ CD4$^+$ or CD8$^+$, respectively. For DC production, the recipient mice were analyzed 14 d after precursor transfer. The splenic DCs were prepared and stained as described elsewhere (45), and the donor-derived DCs were identified as Ly5.2$^+$ CD11c$^+$.

Semisolid culture of BM progenitors. BM cells were cultured in 0.3% agar cultures and analyzed as described previously (46). The recombinant

cytokines were used at the following concentrations: 10 ng/ml IL-3, 100 ng/ml SCF, and 2 IU/ml erythropoietin. Differential colony counts were performed on fixed preparations stained for acetylcholinesterase, Luxol fast blue, and hematoxylin.

RT-PCR analysis. Semiquantitative RT-PCR was performed as described previously (47). cDNA concentrations were normalized to *hprt* by dilution analysis. PU.1 primers were as follows: PU1 number 1: GTTTTCCCTCACCGCCCCTCCAT; PU1 number 2: CTGCCTCTCACCCTCCTCCTCATC. All other primer sets have been described (48).

cDNA. Amplification products all spanned introns and were visualized on 2% agarose gels.

Online Supplemental Material. Fig. S1 shows the parameters used for the sorting and analysis of PU.1^{gfp} expression by BM progenitor populations and compares CMP populations defined in this and previous studies. Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20041535/DC1.

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