PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors

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PU.1 is a unique regulatory protein required for the generation of both the innate and the adaptive immune system. It functions exclusively in a cell-intrinsic manner to control the development of granulocytes, macrophages, and B and T lymphocytes. We demonstrate that mutation of the *PU.1* gene causes a severe reduction in myeloid (granulocyte/macrophage) progenitors. *PU.1* $-/-$ myeloid progenitors can proliferate *in vitro* in response to the multilineage cytokines interleukin-3 (IL-3), IL-6 and stem cell factor but are unresponsive to the myeloid-specific cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF and M-CSF. The failure of *PU.1* $-/-$ progenitors to respond to G-CSF is bypassed by transient signaling with IL-3. In the presence of IL-3 and G-CSF, *PU.1* $-/-$ progenitors can differentiate into granulocytic precursors containing myeloperoxidase-positive granules. Thus PU.1 is not essential for specification of granulocytic precursors, but is required for their further differentiation. The failure of *PU.1* $-/-$ progenitors to respond to M-CSF is due to lack of *c-fms* gene transcription. Transduction of *c-fms* into *PU.1* $-/-$ myeloid progenitors bypasses the block to M-CSF-dependent proliferation but does not induce detectable macrophage differentiation. Therefore, PU.1 appears to be essential for specification of monocytic precursors. Importantly, retroviral transduction of *PU.1* into mutant progenitors restores responsiveness to myeloid-specific cytokines and development of mature granulocytes and macrophages. Thus PU.1 controls myelopoiesis by regulating both proliferation and differentiation pathways.

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Introduction

Hematopoiesis can be viewed as a patterning process in which a self-renewing, pluripotent stem cell (HSC) gives rise through a series of cell divisions to the eight major cell types of the blood. During its differentiation, the HSC appears to generate a hierarchical array of developmental intermediates, consisting of multipotent and lineage-committed progenitors. The latter cells give rise

to erythrocytes, megakaryocytes, mast cells, granulocytes (e.g. neutrophils), macrophages, and B and T lymphocytes. Progression through the hematopoietic developmental cascade involves tightly controlled patterns of gene expression that are orchestrated by a complex set of transcription factors. Gene targeting in mice has been used to examine the functions of these transcription factors during hematopoiesis (reviewed in Shivdasani and Orkin, 1996). Mutations in genes encoding these transcription factors, e.g. *GATA-1*, *c-myb*, *PU.1*, *Ikaros*, *E2A*, *EBF* and *NF-E2*, cause either multilineage or single lineage developmental defects in the hematopoietic system. A central issue to be resolved for many of these transcription factors is whether they regulate development of specific hematopoietic lineages by controlling the specification of progenitors or by regulating their survival and proliferation. Of course, these developmental functions are not mutually exclusive.

The transcription factor PU.1 is a tissue-specific ets-family member that is expressed in various lineages of the hematopoietic system (Klemsz *et al.*, 1990; Hromas *et al.*, 1993). It is encoded by the proto-oncogene *Spi-1*, whose deregulated expression caused by insertion of the spleen focus-forming provirus leads to erythroleukemias in the mouse (Moreau-Gachelin *et al.*, 1988). Numerous presumptive target genes have been identified for PU.1 in granulocytes, macrophages and B lymphocytes (reviewed in Moreau-Gachelin, 1994; Tenen *et al.*, 1997). Gene targeting studies have demonstrated that *PU.1* is required for the development of both myeloid (granulocyte, macrophage) and lymphoid (B, T) lineages (Scott *et al.*, 1994; McKercher *et al.*, 1996). The targeted *PU.1* allele generated by Scott *et al.* (1994) results in embryonic lethality by day 16–18 of gestation and represents a null mutation (Scott *et al.*, 1997). Fetal liver cells derived from the *PU.1* $-/-$ embryos fail to generate macrophage or granulocyte colonies or differentiate into B cells *in vitro* (Scott *et al.*, 1997). The defect in development of *PU.1* $-/-$ hematopoietic progenitors is cell-intrinsic, since these progenitors do not contribute to the lymphoid or myeloid lineages in either embryonic or bone marrow chimeras (Scott *et al.*, 1997). RT-PCR analysis of embryoid bodies generated by differentiation of *PU.1* $-/-$ embryonic stem (ES) cells indicates that some early myeloid genes can be expressed in the absence of PU.1, but expression of late myeloid genes is blocked (Olson *et al.*, 1995). However, it remains to be determined whether myeloid progenitors are generated *in vivo* in the absence of PU.1, and, if so, whether they are capable of proliferating in response to myeloid-specific cytokines. The latter issue is underscored by the presence of functional PU.1-binding sites in the promoters of genes encoding the myeloid-specific cytokine receptor subunits, granulocyte-macrophage colony-stimulating factor receptor α (GM-CSFR α)

(Hohaus *et al.*, 1995), c-Fms (Reddy *et al.*, 1994; Zhang *et al.*, 1994) and granulocyte colony-stimulating factor receptor (G-CSFR) (Smith *et al.*, 1996).

GM-CSF, G-CSF and macrophage colony-stimulating factor (M-CSF) represent a set of myeloid-specific cytokines that have been shown to be important for development of granulocytes and macrophages (reviewed in Metcalf, 1995). GM-CSF stimulates the survival, proliferation and differentiation of bipotent granulocyte-macrophage progenitors as well as unipotent macrophage or granulocyte progenitors (Metcalf *et al.*, 1986). GM-CSF acts through a heterodimeric receptor consisting of a cytokine-specific α -chain (GM-CSFR α) and a common β -chain (GM-CSFR β_c) which is shared with the interleukin-3 (IL-3) and IL-5 receptors (Gorman *et al.*, 1990; Park *et al.*, 1992). Despite the potent activity of GM-CSF *in vitro*, targeted mutations of genes encoding this cytokine or its receptor (GM-CSFR β_c) do not disrupt hematopoiesis *in vivo* (Dranoff *et al.*, 1994; Stanley *et al.*, 1994; Nishinakamura *et al.*, 1995). G-CSF stimulates survival, proliferation and maturation of granulocytic progenitors (Suda *et al.*, 1987). G-CSF acts through a single subunit receptor (the G-CSFR) which does not possess intrinsic kinase activity (Fukunaga *et al.*, 1990). In contrast to mutation of GM-CSF, targeted mutation of G-CSF or its receptor causes a selective and profound reduction in the number of circulating granulocytes in mice (Lieshke *et al.*, 1994; Liu *et al.*, 1996). Finally, M-CSF stimulates survival, proliferation and maturation of committed monocytic progenitors (Stanley, 1985). M-CSF acts through a single subunit receptor tyrosine kinase, also known as the proto-oncogene *c-fms* (Sherr *et al.*, 1985; reviewed in Sherr, 1990). M-CSF is required for development of macrophages and monocyte-derived lineages such as osteoclasts in mice, since a mutation in the M-CSF gene (*op*) results in greatly reduced numbers of tissue macrophages and osteopetrosis (Yoshida *et al.*, 1990).

Given the multilineage defects induced by the *PU.1* mutation, it is possible that PU.1 also regulates myelopoiesis by controlling the proliferation/survival of multipotential hematopoietic progenitors. The cytokines IL-3, IL-6 and stem cell factor (SCF) have been shown to act on stem cells and multipotential progenitors (reviewed in Watowich *et al.*, 1996). IL-3 has the broadest specificity of any known cytokine, as it stimulates survival, proliferation and differentiation of HSCs and progenitor cells of all known hematopoietic lineages (reviewed in Schrader, 1994). IL-3 acts through a heterodimeric receptor consisting of an IL-3-specific α -chain (IL-3R α) and either an IL-3-specific β -chain (β_{IL-3}) or a common beta chain (β_c) shared with the GM-CSF and IL-5 receptors. Targeted mutation of IL-3 or the IL-3 receptors reveals that IL-3 is not required for steady-state hematopoiesis, but is needed for proliferation of mast cells during parasitic infections (Nishinakamura *et al.*, 1996; Lantz *et al.*, 1998). IL-6 stimulates survival, proliferation and differentiation of hematopoietic stem cells, and at high concentrations can induce proliferation of committed granulocytic progenitors (reviewed in Kishimoto, 1989; Liu *et al.*, 1996). IL-6 binds to a heterodimeric receptor consisting of an IL-6-specific α subunit and a signaling subunit, gp130, which is shared with several other related cytokine receptors (Taga *et al.*, 1989). Targeted mutation of IL-6 causes

a selective reduction in granulocytic progenitors (Liu *et al.*, 1997). SCF promotes survival, proliferation and differentiation of stem cells and progenitors of multiple lineages (reviewed in Lyman and Jacobsen, 1998). SCF binds to c-Kit, a single subunit receptor with tyrosine kinase activity (Williams *et al.*, 1990). The *white-spotting* (*W*) or *steel* (*Sl*) mutations in mice represent defects in *c-kit* or the SCF gene, respectively, and cause multilineage hematopoietic defects (reviewed in Witte, 1990).

We have analyzed the effect of the *PU.1* mutation on the fate of myeloid progenitors by performing clonogenic assays in the presence of multilineage or myeloid-specific cytokines. We find that the *PU.1* mutation severely reduces but does not eliminate myeloid progenitors. These progenitors are capable of responding to the multilineage cytokines IL-3, IL-6 and SCF, but not to the myeloid-specific cytokines GM-CSF, G-CSF and M-CSF. The inability of *PU.1*^{-/-} myeloid progenitors to respond to G-CSF or M-CSF can be bypassed by transient culture in IL-3 or by retroviral transduction of *c-fms*, respectively. However, after bypass of the block to proliferation, *PU.1*^{-/-} progenitors undergo limited granulocytic differentiation but no detectable monocytic differentiation. Differentiation of *PU.1*^{-/-} myeloid progenitors into neutrophils and macrophages is restored by retroviral transduction with *PU.1* cDNA. Therefore, PU.1 controls both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors.

Results

PU.1^{-/-} hematopoietic progenitors proliferate in response to IL-3, IL-6 and SCF

Previous studies have established that *PU.1*^{-/-} fetal liver hematopoietic progenitors can give rise to normal numbers of erythroid and megakaryocytic colonies but fail to produce macrophage (M), granulocyte (G), mixed granulocyte-macrophage (GM) or mixed granulocyte-erythrocyte-megakaryocyte-macrophage (GEMM) colonies in response to conditioned medium from pokeweed mitogen-stimulated spleen cells (Scott *et al.*, 1994, 1997). To characterize the effect of the *PU.1* mutation on multipotential hematopoietic progenitors, we analyzed the ability of such progenitors to respond to the multilineage cytokines IL-3, IL-6 and SCF. Among these cytokines, IL-3 has been shown potently to induce colony formation by multipotential progenitors (Schrader, 1994). IL-6 and SCF stimulate colony formation by such progenitors weakly on their own but function synergistically with IL-3 (Bodine *et al.*, 1992; Miura *et al.*, 1993). To examine the response of *PU.1*^{-/-} hematopoietic progenitors to IL-3, we initially enriched for multipotential cells using a lineage depletion protocol. Cells positive for lineage (Lin) markers (CD4, CD8, CD3 ϵ , CD5, B220, Gr-1, Ter119) were depleted using magnetic beads (see Materials and methods). This protocol when used on wild-type or *PU.1*^{+/-} fetal liver hematopoietic cells resulted in a depletion of 80–90% of the Lin⁺ cells, producing a population that was >90% c-Kit⁺. Importantly, *PU.1*^{-/-} Lin⁻ hematopoietic progenitors were also 80–90% c-Kit⁺ and expressed the receptor at levels comparable with their heterozygous counterparts (data not shown).

We tested the response of lineage-depleted (Lin⁻) day

Fig. 1. *PU.1*^{-/-} hematopoietic progenitors form immature myeloid colonies in response to IL-3. Lin⁻ *PU.1*^{+/-} or *PU.1*^{-/-} fetal liver cells were plated in methylcellulose cultures with 10 ng/ml IL-3. After 7 days, colonies were scored and cells from individual colonies examined with Wright stain (original magnification 1000×). Macrophages (M) and neutrophils (N) are indicated.

Table I. IL-6 and SCF synergize with IL-3 to promote survival and proliferation of *PU.1*^{-/-} myeloid progenitors

Pre-stimulation condition	No. of c.f.u.
Medium alone	0
IL-3	607
IL-6	2
SCF	10
IL-3 + IL-6	1046
IL-3 + SCF	1169
IL-6 + SCF	363
IL-3 + IL-6 + SCF	2190

2×10⁴ Lin⁻ *PU.1*^{-/-} progenitors were stimulated for 96 h in the indicated combinations of 20 ng/ml IL-3, 10 ng/ml IL-6 or 100 ng/ml SCF, washed three times and plated in methylcellulose containing 10 ng/ml IL-3. IL-3-dependent colonies were scored after 7 days.

14.5 *PU.1*^{+/-} or *PU.1*^{-/-} fetal liver progenitors to recombinant murine IL-3 in methylcellulose colony-forming assays. In a representative experiment, *PU.1*^{+/-} progenitors formed 532 colonies per 2×10⁴ cells plated, while *PU.1*^{-/-} progenitors formed 42 colonies per 2×10⁴ cells plated. Both *PU.1*^{+/-} and *PU.1*^{-/-} colonies were large and contained >10⁴ cells. *PU.1*^{+/-} colonies consisted mostly of macrophages and neutrophils (Figure 1, left panel), while mast cells were detected at a lower frequency (data not shown). In contrast, *PU.1*^{-/-} progenitors gave rise to colonies of cells with immature nuclear and cytoplasmic morphology (Figure 1, right panel), suggesting that these cells are myeloid progenitors that are unable to differentiate in the absence of PU.1.

We tested whether IL-6 and SCF could synergize with IL-3 to promote survival and proliferation of IL-3-responsive *PU.1*^{-/-} progenitors. Table I shows that IL-6 and SCF functioned synergistically with IL-3 to promote survival and proliferation of *PU.1*^{-/-} progenitors. In the IL-3 + IL-6 + SCF condition, 2190 colonies were obtained compared with 42 colonies before cytokine stimulation, indicating that the progenitors had doubled approximately six times, or once every 16 h. It should be noted that after stimulation with IL-3, IL-6 and SCF, the

frequency of IL-3-responsive progenitors was equivalent in the *PU.1*^{-/-} and *PU.1*^{+/-} populations. However, after cytokine stimulation, *PU.1*^{-/-} IL-3-dependent progenitors still maintained an immature morphology and did not differentiate into macrophages or granulocytes (data not shown). These results show that the *PU.1* mutation severely reduces the frequency of myeloid progenitors but does not affect their ability to proliferate in response to the multilineage cytokines IL-3, IL-6 and SCF.

***PU.1*^{-/-} myeloid progenitors express certain myeloid-restricted genes**

RT-PCR was used to detect transcripts of myeloid genes in *PU.1*^{+/-} and *PU.1*^{-/-} Lin⁻ progenitors before and after stimulation with IL-3, IL-6 and SCF. Analysis of the genes for myeloid cytokine receptor subunits (G-CSFR, GM-CSFRα, GM-CSFRβ_c, c-Fms) revealed three types of differences between *PU.1*^{+/-} and *PU.1*^{-/-} progenitors (Figure 2). Transcripts for G-CSFR and GM-CSFRα were less abundant in the *PU.1*^{-/-} progenitors and showed an increase after cytokine stimulation. In contrast, GM-CSFRβ_c transcripts were barely detectable in *PU.1*^{-/-} progenitors before cytokine stimulation and showed a very large increase after culture with IL-3, IL-6 and SCF. Finally, c-fms transcripts were not detectable in *PU.1*^{-/-} progenitors either before or after cytokine stimulation.

In addition to subunits of the myeloid cytokine receptors, we analyzed expression of various myeloid structural genes. Myeloperoxidase (MPO) RNA is typically highly expressed in early myeloid cells and down-regulated during neutrophil development (Lubbert *et al.*, 1991; Nuchprayoon *et al.*, 1994). CD16 (FcγRIII, Ravetch *et al.*, 1986; Feinman *et al.*, 1994), CD32 (FcγRII, Hogarth *et al.*, 1987) and CD64 (FcγRI, Sears *et al.*, 1990; Eichbaum *et al.*, 1994; Perez *et al.*, 1994) are IgG Fc receptors expressed on myeloid cells. CD11b is expressed on macrophages and granulocytes and functions as an adhesion molecule (Pytela, 1988; Pahl *et al.*, 1993). Finally, the macrophage scavenger receptor (SR) functions in macrophage adhesion to pathogens, and its expression is restricted to macrophages (Moulton *et al.*, 1994; Suzuki

Fig. 2. *PU.1*^{-/-} myeloid progenitors express certain myeloid-restricted genes. RNA from *PU.1*^{+/-} and *-/-* fetal liver progenitors before (0) and after 4 days (4d) of cytokine stimulation was analyzed by RT-PCR using primers specific for G-CSFR, GM-CSFR α , GM-CSFR β_c , *c-fms*, MPO, CD16, CD32, CD64, CD11b, SR and β -actin. The control lane utilized RNA from unfractionated wild-type fetal liver cells.

et al., 1997). All of these genes have been shown to contain functional PU.1-binding sites in their promoters. MPO and CD16 gene transcripts were detectable at reduced levels in *PU.1*^{-/-} progenitors and increased upon cytokine stimulation, reflecting a pattern similar to that seen with G-CSFR and GM-CSFR α transcripts (Figure 2). In contrast, transcripts encoding CD32, CD64, CD11b and SR were not detectable in *PU.1*^{-/-} progenitors either before or after cytokine stimulation.

These results strongly suggest that *PU.1*^{-/-} myeloid progenitors can express certain myeloid genes such as those for G-CSFR, GM-CSFR α , GM-CSFR β_c , MPO and CD16. Reduced transcript levels for these genes in *PU.1*^{-/-} progenitors is probably due to a lower frequency of such progenitors in the Lin⁻ population as revealed by the IL-3 colony-forming assay. Upon cytokine stimulation, such *PU.1*^{-/-} myeloid progenitors are able to proliferate, giving rise to increased transcript levels for the aforementioned genes.

***PU.1* is required to induce G-CSF responsiveness but can be bypassed by stimulating mutant progenitors with IL-3**

Analysis of myeloid cytokine receptor subunit genes by RT-PCR (Figure 2) suggested that *PU.1*^{-/-} myeloid progenitors may respond to G-CSF and GM-CSF but not to M-CSF. We therefore tested the responses of *PU.1*^{-/-} myeloid progenitors to individual cytokines before and after stimulation with the multilineage cytokines IL-3, IL-6 and SCF. Intriguingly, freshly isolated *PU.1*^{-/-} myeloid progenitors did not form colonies in G-CSF but were able to do so after cytokine stimulation (Table

Table II. *PU.1*^{-/-} myeloid progenitors are responsive to G-CSF after cytokine stimulation

Colonies per 2 $\times$ 10 ⁴ cells plated			
Before stimulation		After stimulation	
<i>PU.1</i> ^{+/-}	<i>PU.1</i> ^{-/-}	<i>PU.1</i> ^{+/-}	<i>PU.1</i> ^{-/-}
260	0	676	258

2 $\times$ 10⁴ Lin⁻ *PU.1*^{+/-} or *-/-* progenitors were plated in methylcellulose containing 10 ng/ml G-CSF either before or after stimulation for 96 h with IL-3 + IL-6 + SCF. Colonies were scored after 7 days.

II). Although comparable numbers of G-CSF-dependent colonies were observed for *PU.1*^{+/-} and *-/-* progenitors after cytokine stimulation, the mutant colonies were consistently smaller and contained morphologically immature cells compared with the segmented neutrophils that comprised *PU.1*^{+/-} colonies (Figure 3, left and middle panels). To determine which of the three multilineage cytokines induced G-CSF responsiveness in *PU.1*^{-/-} myeloid progenitors, we incubated these cells with individual factors or combinations of factors prior to plating in G-CSF (Table III). Stimulation with IL-3 or IL-6 + SCF was sufficient to induce G-CSF responsiveness in *PU.1*^{-/-} progenitors. The combination of IL-3 + IL-6 was most potent at inducing colony formation by mutant progenitors in G-CSF. It should be noted that prior stimulation of *PU.1*^{-/-} progenitors with IL-3 + IL-6 + SCF for as little as 24 h could induce the G-CSF-responsive state (data not shown). Thus, transient signaling by IL-3 or a combination of multilineage cytokines bypasses the requirement for PU.1 in the generation of G-CSF-responsive myeloid progenitors.

***PU.1* can restore G-CSF-dependent differentiation of granulocytes upon transduction into mutant myeloid progenitors**

In order to examine the ability of PU.1 to restore differentiation of neutrophils from mutant myeloid progenitors, we constructed a retroviral vector based on murine stem cell virus (MSCV). This vector has been shown to express transduced genes efficiently in ES cells and HSCs and their progenitors (Beck-Engeser *et al.*, 1991; Hawley *et al.*, 1994). We modified the MSCV-pac vector by replacement of the puromycin acetylase gene with the cDNA for enhanced green fluorescent protein (EGFP) (Cormack *et al.*, 1996). *PU.1*^{+/-} and *-/-* Lin⁻ fetal liver progenitors were infected with high-titer, cell-free MSCV-EGFP or MSCV-EGFP-PU.1 retrovirus (see Materials and methods) after stimulation with IL-3 + IL-6 + SCF (Luskey *et al.*, 1992). Figure 3 (top right panel) shows an example of a green fluorescent colony generated by transduction of a *PU.1*^{-/-} myeloid progenitor with the MSCV-EGFP-PU.1 retrovirus. All of the cells in such colonies contain differentiated neutrophils, as evidenced by their segmented nuclear morphology (Figure 3, bottom right panel). *PU.1*^{-/-} myeloid progenitors transduced with the control MSCV-EGFP retrovirus were smaller and contained cells exhibiting an immature morphology, like those shown in Figure 3, bottom middle panel (data not shown). Thus,

Fig. 3. PU.1 restores G-CSF-dependent differentiation of granulocytes upon transduction into mutant progenitors. After stimulation with IL-3 + IL-6 + SCF, *PU.1* +/-, *PU.1* -/- or MSCV-EGFP-PU.1-transduced *PU.1* -/- Lin⁻ fetal liver cells were plated in methylcellulose cultures with 10 ng/ml G-CSF. After 7 days, colonies were scored and cells from individual colonies examined with Wright stain. Upper panels: phase-contrast and fluorescent micrographs of *PU.1* +/-, *PU.1* -/- and MSCV-EGFP-PU.1-transduced *PU.1* -/- G-CSF-dependent colonies (original magnification 100×). Lower panels: Wright stain of cells from *PU.1* +/-, *PU.1* -/- and MSCV-EGFP-PU.1-transduced *PU.1* -/- G-CSF-dependent colonies (original magnification 1000×).

Table III. IL-6 and SCF synergize with IL-3 to induce responsiveness to G-CSF of *PU.1* -/- myeloid progenitors

Pre-stimulation condition	No. of c.f.u.
Medium alone	0
IL-3	180
IL-6	2
SCF	2
IL-3 + IL-6	456
IL-3 + SCF	165
IL-6 + SCF	89
IL-3 + IL-6 + SCF	458

2×10⁴ Lin⁻ *PU.1* -/- progenitors were stimulated for 96 h in the indicated combinations of 20 ng/ml IL-3, 10 ng/ml IL-6 or 100 ng/ml SCF, washed three times and plated in methylcellulose containing 10 ng/ml G-CSF. G-CSF-dependent colonies were scored after 7 days.

PU.1 promotes G-CSF-dependent proliferation of myeloid progenitors as well as their differentiation into neutrophils.

PU.1 is not essential for generation of granulocytic precursors but is required for their differentiation

As described above, *PU.1* -/- progenitors are capable of extensive proliferation in response to IL-3 and can be induced to undergo limited proliferation in G-CSF. To characterize G-CSF-responsive *PU.1* -/- myeloid progenitors further, we attempted to expand such cells using a combination of G-CSF and IL-3. *PU.1* +/- or -/-

progenitors which had been stimulated with IL-3, IL-6 and SCF were washed and cultured in G-CSF alone. After 7 days, surviving cells were cultured in a combination of G-CSF + IL-3. *PU.1* +/- progenitors cultured in G-CSF for 7 days differentiated into neutrophils and subsequently failed to proliferate in G-CSF + IL-3. In contrast, *PU.1* -/- progenitors that survived selection in G-CSF responded vigorously to G-CSF + IL-3. These cells could be maintained as cell lines.

PU.1 -/- cell lines maintained in G-CSF + IL-3 were analyzed by flow cytometry. These cells do not express B220, Thy-1 or CD11b (Figure 4A and data not shown). However, the majority of the cells express CD18, the β_2 -integrin partner of CD11b (Kaufmann *et al.*, 1991), and Gr-1, a marker restricted to developing and mature granulocytes (Hestdal *et al.*, 1991). The cells also express c-Kit and varying levels of the β_{IL-3} and β_c subunits (Figure 4A), consistent with their responsiveness to IL-3. The majority of *PU.1* -/- cells expanded in G-CSF + IL-3 contain large lobular nuclei and appear to resemble myelocytes; however, rare cells contain segmented nuclei characteristic of neutrophils (Figure 4B, left panel). A significant proportion of these cells (25–50%) contain abundant granules that stain positively for MPO or chloroacetate esterase (Figure 4B, right and middle panel). Collectively, the fluorescence-activated cell sorting (FACS) and morphological analyses suggest that these cells are granulocytic precursors. Thus, under appropriate

Fig. 4. *PU.1*^{-/-} myeloid progenitors can differentiate into granulocytic precursors in the presence of G-CSF and IL-3. *PU.1*^{-/-} cell lines were established by culturing Lin⁻ progenitors with G-CSF and IL-3 (see Materials and methods). (A) FACS analysis of a *PU.1*^{-/-} granulocytic precursor cell line using antibodies recognizing CD11b, CD18, Gr-1, c-Kit, βIL-3 and βc. (B) Cytochemical analysis of the same cell line (original magnification 1000×).

conditions, *PU.1*^{-/-} myeloid progenitors can differentiate into G-CSF-responsive granulocytic precursors.

***PU.1* controls responsiveness to GM-CSF**

Previous RT-PCR analysis (Figure 2) suggested that *PU.1*^{-/-} myeloid progenitors express GM-CSFR. To determine if *PU.1*^{-/-} myeloid progenitors are responsive to GM-CSF, we performed methylcellulose colony-forming assays in the presence of GM-CSF. In contrast to the findings with G-CSF, *PU.1*^{-/-} progenitors did not form colonies in response to GM-CSF either before or after stimulation with IL-3 + IL-6 + SCF (data not shown). In order to determine if PU.1 could restore GM-

CSF responsiveness in *PU.1*^{-/-} myeloid progenitors, we infected *PU.1*^{+/-} and *-/-* Lin⁻ fetal liver progenitors with MSCV-EGFP or MSCV-EGFP-PU.1 retrovirus after cytokine stimulation. *PU.1*^{-/-} progenitors infected with the control retrovirus (MSCV-EGFP) did not form colonies in GM-CSF (Table IV). In contrast, *PU.1*^{-/-} progenitors infected with the PU.1 retrovirus (MSCV-EGFP-PU.1) formed mixed granulocyte-macrophage, pure macrophage and pure granulocyte colonies. These colonies were green-fluorescent, clearly demonstrating retroviral transduction, were similar in size to *PU.1*^{+/-} colonies and contained morphologically normal granulocytes and macrophages (Figure 5A). In addition, a fourth type of colony was

Table IV. Retroviral transduction of *PU.1* cDNA restores responsiveness of *PU.1* $-/-$ myeloid progenitors to GM-CSF

	Transduced colonies per 10 ⁵ cells plated in GM-CSF			
	<i>PU.1</i> +/-		<i>PU.1</i> -/-	
	MSCV-EGFP	MSCV-EGFP-PU.1	MSCV-EGFP	MSCV-EGFP-PU.1
GM	50	18	0	60
M	180	88	0	118
G	53	8	0	55
Other	0	0	0	68

Cytokine-stimulated *PU.1* +/- and -/- progenitors were infected with cell-free MSCV-EGFP or MSCV-EGFP-PU.1 retrovirus and plated in 1 ng/ml GM-CSF. Colony types were scored after 7 days as mixed granulocyte-macrophage (GM), macrophage (M), granulocyte (G) or other. Cellular identities were confirmed by Wright staining.

Fig. 5. PU.1 controls responsiveness of myeloid progenitors to GM-CSF. (A) Retroviral transduction with *PU.1* cDNA induces *PU.1* $-/-$ myeloid progenitors to form granulocyte/macrophage colonies in response to GM-CSF. Lin⁻ *PU.1* $-/-$ fetal liver cells were transduced with MSCV-EGFP-PU.1 and plated in methylcellulose with 1 ng/ml GM-CSF. After 7 days, colonies were scored and cells from individual colonies examined with Wright stain (phase-contrast and fluorescent images, original magnification 50 $\times$; Wright stain, original magnification 1000 $\times$). (B) PU.1 protein is expressed at comparable levels in *PU.1* +/- and *PU.1* cDNA-transduced *PU.1* $-/-$ granulocytes and macrophages. Cell lysates from 10⁶ WEHI-3B myelomonocytic leukemia cells, granulocytes and macrophages from MSCV-EGFP-transduced *PU.1* +/- progenitor cells, or granulocytes and macrophages from MSCV-EGFP-PU.1-transduced *PU.1* $-/-$ progenitor cells cultured in GM-CSF for 7 days were analyzed by Western blotting with an affinity-purified anti-PU.1 antibody. Molecular weight standards (kDa) are indicated on the left.

detected which grew to very large size (10⁵ cells) and whose morphology was consistent with that of myeloid progenitors (data not shown). Western analysis using an affinity-purified anti-PU.1 antiserum (Scott *et al.*, 1997) showed that PU.1 protein was expressed at comparable levels in *PU.1* +/- or *PU.1*-transduced mutant colonies (Figure 5B). The major PU.1 protein product in transduced mutant colonies migrated faster than its counterpart in *PU.1* +/- colonies. This electrophoretic difference may be due to varying levels of phosphorylation (reviewed in Tenen *et al.*, 1997). These results establish that PU.1 controls responsiveness to GM-CSF, and that this defect is not bypassed by IL-3 signaling.

Retroviral transduction of *c-fms* restores M-CSF-dependent proliferation but not differentiation of *PU.1* $-/-$ myeloid progenitors into macrophages
As predicted by the RT-PCR analysis, *PU.1* +/- but not $-/-$ myeloid progenitors formed colonies in response to M-CSF (data not shown). To test whether retroviral transduction of the *c-fms* cDNA into *PU.1* $-/-$ progenitors is sufficient to restore M-CSF-dependent macrophage development, we infected *PU.1* +/- or $-/-$ progenitors with MSCV-EGFP, MSCV-EGFP-PU.1 or the MZen-*fms* retrovirus (Rohrschneider *et al.*, 1989), and then plated these cells in methylcellulose containing M-CSF. *PU.1* $-/-$ progenitors formed colonies in response to M-CSF after

Table V. Retroviral transduction of *PU.1* or *c-fms* cDNA restores responsiveness of *PU.1* $-/-$ myeloid progenitors to M-CSF

Retroviral vector	Transduced colonies per 10^5 cells plated in M-CSF	
	<i>PU.1</i> $+/-$	<i>PU.1</i> $-/-$
MSCV-EGFP	100	0
MSCV-EGFP- <i>PU.1</i>	200	8
MZen- <i>fms</i>	ND	12

Cytokine-stimulated *PU.1* $+/-$ and $-/-$ progenitors were transduced with MSCV-EGFP, MSCV-EGFP-*PU.1* or MZen-*fms* retrovirus and plated in 10 ng/ml M-CSF. Colonies were scored after 7 days. Total numbers of *PU.1* $+/-$ colonies per 10^5 progenitors plated were MSCV-EGFP, 1785; MSCV-EGFP-*PU.1*, 1575; and pMZen-*fms*, 2180. ND, not done.

transduction with *PU.1* or *c-fms* cDNA, but not the control construct (Table V). *PU.1*-transduced mutant colonies were green-fluorescent, were similar in size to *PU.1* $+/-$ colonies and consisted of morphologically normal macrophages (Figure 6A, upper panels). In contrast, *c-fms*-transduced colonies were smaller than control colonies and consisted of morphologically undifferentiated cells (Figure 6B, lower panels). RT-PCR was performed on RNA prepared from *PU.1*- or *c-fms*-transduced *PU.1* $-/-$ M-CSF-dependent colonies. Retroviral transduction with *PU.1* cDNA restored transcription of *c-fms* as well as the myeloid-specific genes for CD11b, CD32, CD64 and SR (Figure 6B). In contrast, retroviral transduction with *c-fms* cDNA did not restore transcription of these myeloid-restricted genes. These results demonstrate that *c-fms* transduction is sufficient to restore the M-CSF proliferation defect in *PU.1* $-/-$ myeloid progenitors but does not enable such progenitors to differentiate into macrophages. Therefore, PU.1 acts at distinct levels to control the proliferation and differentiation of macrophage progenitors.

Discussion

Myeloid progenitors are generated in the absence of *PU.1*

In this study, we present strong evidence that myeloid progenitors can be generated *in vivo* in the absence of the transcription factor PU.1. These mutant progenitors form colonies in response to IL-3, which contain cells with immature myeloid morphology. However, the number of *PU.1* $-/-$ IL-3-dependent colonies is reduced ~10-fold compared with *PU.1* $+/-$ controls. This reduction in the frequency of myeloid progenitors is consistent with our earlier observation that lymphoid-myeloid progenitors expressing the cell surface marker AA4.1 are reduced but not absent in *PU.1* $-/-$ fetal liver (Scott *et al.*, 1997). *PU.1* $-/-$ myeloid progenitors proliferate optimally in response to the multilineage cytokines IL-3, IL-6 and SCF, which previously have been shown to stimulate proliferation of stem cells and multipotential progenitors. The ability of *PU.1* $-/-$ myeloid progenitors to proliferate in response to these cytokines is consistent with the idea that the fetal stem cell compartment is unimpaired by the *PU.1* mutation, an idea proposed earlier based

on the relatively normal numbers of erythrocytes and megakaryocytes in *PU.1* $-/-$ fetal liver (Scott *et al.*, 1994).

Two other lines of evidence demonstrate the existence of myeloid progenitors in *PU.1* $-/-$ fetal liver. First, the expression of certain myeloid-restricted genes (for G-CSFR, GM-CSFR α , GM-CSFR β_c , CD16 and MPO) could be detected in freshly isolated or cytokine-stimulated *PU.1* $-/-$ hematopoietic progenitors. Finally and conclusively, retroviral transduction of *PU.1* $-/-$ myeloid progenitors with *PU.1* cDNA restored their ability to form macrophage and neutrophil colonies in response to the colony-stimulating factors G-CSF, GM-CSF and M-CSF. In conclusion, PU.1 is not required for the specification of myeloid progenitors *in vivo*. However, *PU.1* $-/-$ myeloid progenitors are reduced in number relative to controls, a finding which is likely to be accounted for by their failure to proliferate in response to myeloid-specific cytokines (see below).

PU.1 controls responsiveness of myeloid progenitors to myeloid-specific cytokines

As discussed above, myeloid progenitors could be detected in *PU.1* $-/-$ fetal liver. *PU.1* $-/-$ myeloid progenitors did not form colonies in response to the myeloid-specific cytokines G-CSF, GM-CSF and M-CSF. These results were somewhat unexpected based on the RT-PCR analysis, which showed that *PU.1* $-/-$ fetal liver cells expressed transcripts for G-CSFR and GM-CSFR but not *c-fms* (Figure 2). In the case of G-CSFR, *PU.1* $-/-$ progenitors could be induced to form small colonies in response to G-CSF after transient stimulation with IL-3. The increase in G-CSFR transcript levels detectable by RT-PCR can reasonably be accounted for by IL-3-stimulated proliferation of myeloid progenitors. Thus, IL-3 signaling is not likely to be inducing G-CSFR expression in *PU.1* $-/-$ myeloid progenitors. Therefore, the requirement for PU.1 in G-CSF signaling may be due to failed expression of a PU.1-dependent gene encoding a signal transduction molecule that couples to the G-CSFR. According to this scenario, transient signaling with IL-3 would either induce expression of this gene or activate a functional counterpart.

In the case of GM-CSFR, *PU.1* $-/-$ myeloid progenitors did not respond to GM-CSF even though both receptor subunits could be detected by RT-PCR. *PU.1* $-/-$ progenitors were unresponsive to GM-CSF both before and after cytokine stimulation. It is possible that the levels of GM-CSFR α transcripts detected by RT-PCR are insufficient for adequate receptor expression on the cell surface. In this regard, we note that *PU.1* $-/-$ progenitors can express GM-CSFR β_c on the cell surface (Figure 4A). Thus, if GM-CSFR α protein is also expressed on the cell surface at levels sufficient for signal transduction, then PU.1 must regulate GM-CSFR signaling by a mechanism distinct from G-CSFR signaling. Transient stimulation of *PU.1* $-/-$ progenitors by IL-3 restores G-CSF- but not GM-CSF-dependent proliferation.

In the case of *c-fms*, *PU.1* $-/-$ myeloid progenitors do not express detectable transcripts, demonstrating that transcription of the *c-fms* gene is critically dependent on PU.1. Retroviral transduction of *c-fms* cDNA is sufficient to restore responsiveness to M-CSF. However, *c-fms*-transduced colonies were invariably small in size (<500 cells) as was also the case for G-CSF-dependent *PU.1* $-/-$

Fig. 6. *PU.1* controls M-CSF-dependent proliferation as well as differentiation of myeloid progenitors into macrophages.

(A) Transduction with *PU.1* but not *c-fms* cDNA induces macrophage colony formation from *PU.1*^{-/-} myeloid progenitors. Lin⁻ *PU.1*^{-/-} fetal liver cells transduced with MSCV-EGFP-*PU.1* or MZen-*fms* were plated in methylcellulose cultures with 10 ng/ml M-CSF. After 7 days, colonies were scored and cells from individual colonies examined with Wright stain (phase-contrast, original magnification 50×; Wright stain, original magnification 1000×). (B) Retroviral transduction with *PU.1* but not *c-fms* cDNA induces macrophage-specific gene expression. RNA from MSCV-EGFP-transduced *PU.1*^{+/-} colonies, MSCV-EGFP-*PU.1*-transduced *PU.1*^{-/-} colonies or MZen-*fms*-transduced *PU.1*^{-/-} colonies grown for 7 days in M-CSF was analyzed by RT-PCR using primers specific for *PU.1*, *c-fms*, CD11b, CD32, CD64, SR or β-actin. The control lane utilized RNA from unfractionated wild-type fetal liver cells.

colonies. The poor proliferative response of *c-fms*-transduced *PU.1*^{-/-} progenitors may also be due to failed expression of a *PU.1*-dependent gene encoding a signal transduction component that couples to *c-Fms*.

In conclusion, *PU.1* controls responsiveness of myeloid progenitors to the myeloid-specific cytokines G-CSF, GM-CSF and M-CSF. The results suggest distinct types of signaling defects in these three cytokine receptor systems induced by the *PU.1* mutation. Although functional *PU.1*-binding sites are found in the promoters of genes encoding G-CSFR, GM-CSFRα and *c-Fms*, our results show that only *c-fms* gene transcription is critically dependent on *PU.1* in myeloid progenitors. Mutational analysis has

shown that G-CSF and M-CSF are important for generating normal numbers of neutrophils and macrophages, respectively (Yoshida *et al.*, 1990; Lieshke *et al.*, 1994). The reduction, but not elimination of myeloid cells by these mutations is probably due to redundancy in cytokine signaling systems. Although crosses of several of these cytokine or cytokine receptor mutations have been generated (reviewed in Lieshke, 1997), it remains to be determined how myelopoiesis would be affected by the combined loss of G-CSF, GM-CSF and M-CSF signaling systems. We suggest that the combined loss of these signaling systems may entirely account for the severe reduction in myeloid progenitors in *PU.1*^{-/-} fetal liver.

Fig. 7. Model for distinct functions of PU.1 in regulating granulocyte and macrophage development. (1) PU.1 is required for proliferation/survival of myeloid progenitors, for example in response to GM-CSF signaling. (2) PU.1 is required for G-CSF-dependent proliferation of granulocytic precursors. This function can be bypassed by IL-3 signaling. (3) PU.1 is not essential for specification of granulocytic precursors but is required for their differentiation into neutrophils. (4 and 5) PU.1 appears essential for the generation of monocytic precursors that can proliferate in response to M-CSF.

***PU.1*^{−/−} myeloid progenitors partially differentiate along the granulocytic pathway in response to G-CSF + IL-3**

As discussed above, transient signaling with IL-3 allowed a limited proliferative response of *PU.1*^{−/−} myeloid progenitors to G-CSF. Induction of this response was exploited to select for G-CSF-responsive progenitors in liquid culture, which were then expanded and maintained as cell lines in G-CSF + IL-3. Under these conditions, *PU.1*^{−/−} cell lines displayed limited neutrophil differentiation by several criteria. First, expression of the G-CSFR is restricted to neutrophils and their progenitors (reviewed in Nagata and Fukunaga, 1993). Secondly, these cells express the myeloid-restricted cell surface marker CD18 and the granulocyte-restricted marker Gr-1. Finally, they accumulate granules in their cytoplasm containing chloroacetate esterase and MPO. It should be noted that the majority of these cells are unable to differentiate into neutrophils, as seen by their immature nuclear morphology and expression of c-Kit. Rarely, cells have segmented nuclear morphology characteristic of neutrophils. In conclusion, G-CSF-responsive *PU.1*^{−/−} myeloid progenitors can be expanded and maintained as cell lines in G-CSF + IL-3, and show clear evidence of granulocytic differentiation as revealed by several lineage-specific markers. However, these *PU.1*^{−/−} granulocytic precursors are unable to differentiate into c-Kit[−] neutrophils with segmented nuclear morphology. Thus, PU.1 is not required for the specification of granulocytic precursors *in vitro*. It should be noted that cells expressing Gr-1 or MPO are not detectable in the fetal liver of *PU.1*^{−/−} embryos (Scott *et al.*, 1994). This is probably due to the severe reduction in myeloid progenitors as well as the inability of such progenitors to respond to G-CSF signaling *in vivo*.

***PU.1* controls monocytic development at distinct levels of proliferation and differentiation**

PU.1^{−/−} myeloid progenitors do not form colonies in response to M-CSF, as predicted by the lack of detectable *c-fms* transcription. *c-fms*-transduced *PU.1*^{−/−} progenitors respond weakly to M-CSF, forming small colonies of cells which show no overt morphological differentiation and do not express any macrophage-specific genes. In contrast, *PU.1*-transduced *PU.1*^{−/−} progenitors form robust macrophage colonies in which macrophage-specific gene transcripts can be readily detected. Thus, PU.1 regulates macrophage development at the distinct levels of M-CSF-dependent proliferation, as well as by controlling the transcription of macrophage-specific structural genes. PU.1 appears to play a more profound role in regulating both the proliferation and differentiation of monocytic compared with granulocytic cells. This is demonstrated by the failure of *PU.1*^{−/−} myeloid progenitors developmentally to activate transcription of *c-fms* and various macrophage-specific structural genes.

Model for distinct functions of PU.1 in regulating granulocyte and macrophage development

The ability to analyze *PU.1*^{−/−} hematopoietic progenitors *in vitro* using recombinant cytokines, and to restore their development by retroviral transduction with *PU.1* cDNA, has provided insight into several distinct functions of PU.1 in the generation of granulocytes and macrophages (Figure 7). First, granulocyte/macrophage progenitors can be generated in the absence of PU.1; however, their numbers are significantly reduced. Although these progenitors readily proliferate *in vitro* in response to IL-3, IL-6 and SCF, the results suggest a proliferative/survival defect *in vivo*. Secondly, although *PU.1*^{−/−} myeloid progenitors

are defective in responding to G-CSF, transient signaling with IL-3 partially bypasses this block. In the presence of G-CSF and IL-3, *PU.1*^{-/-} myeloid progenitors differentiate into granulocytic precursors. Thus, *PU.1* is not essential for specification of granulocytic precursors. However, it is required for further differentiation of these precursors into mature neutrophils. In contrast to its role in granulocytic development, *PU.1* appears to be more critically required for various steps in the macrophage developmental pathway. In its absence, neither M-CSF-dependent proliferation nor differentiation are observed. This is due to a failure developmentally to induce transcription of the *c-fms* gene and various macrophage-specific structural genes. Thus, *PU.1* appears to be required for specification of monocytic precursors. These results are consistent with a model for hematopoiesis which invokes a sequential and ordered emergence of lineage fates (Singh, 1996). According to this model, granulocytic precursors arise in a developmental sequence before monocytic and lymphoid precursors. Thus we speculate that the lineage specification functions of *PU.1* may be executed in a multipotential progenitor that can give rise to macrophages and lymphocytes.

Materials and methods

Mouse strains

Male and female *PU.1*^{+/+} mice (Scott *et al.*, 1994) were mated to produce *PU.1*^{+/+}, *+/+* and *-/-* embryos for these studies. The presence of a vaginal plug on the morning after matings was taken as day 0.5 of gestation. All embryos were genotyped by PCR analysis of genomic DNA using primers detecting the intact *PU.1* gene (sense 5'-CGG-ATGTGCTTCCCTTATCAAC-3', antisense 5'-TGACTTCTTACC-TCCCTGTC-3') or the neomycin resistance cassette (sense 5'-TTT-CGTTGGTGGTCAATC-3', antisense 5'-GATGGATTGCACGC-AGGTTTC-3').

Isolation of fetal hematopoietic progenitors

Day 14.5 *PU.1*^{+/+} and *-/-* embryos were obtained from timed matings as described above. Embryos were phenotyped by FACS analysis for fetal liver CD11b expression as previously described by Scott *et al.* (1997). Genotypes were confirmed by PCR analysis. Fetal liver cell suspensions were prepared by repeated passage of tissue through a 25 gauge needle. These cell suspensions were depleted of erythrocytes using a ficoll gradient (Lympholyte-M, Cedarlane Labs). Fetal liver cell suspensions were depleted of lineage-positive (Lin⁺) cells by first incubating with biotinylated monoclonal antibodies to CD4, CD5, CD8a, CD3e, Gr-1, Ter119 and B220 (PharMingen), then incubating with streptavidin magnetic beads and passing through a MiniMACS or VarioMACS column (Miltenyi Biotec). Lineage-depleted hematopoietic progenitors were stimulated for 2–4 days in complete Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS), 5 × 10⁻⁵ M β-mercaptoethanol, 1 U/ml L-glutamine, 1 U/ml penicillin-streptomycin, 100 ng/ml recombinant murine SCF (Biosource), 20 ng/ml recombinant murine IL-3 (Biosource) and 10 ng/ml recombinant murine IL-6 (R&D Systems).

Establishment of G-CSF- and IL-3-dependent *PU.1*^{-/-} cell lines

Cell lines were established by culturing *PU.1*^{-/-} cytokine-stimulated hematopoietic progenitors as isolated above in complete IMDM medium containing 10 ng/ml recombinant G-CSF (R&D Systems) for 7 days. Surviving cells were washed and maintained in 10 ng/ml G-CSF and 5 ng/ml IL-3 at cell densities between 10⁵ and 10⁶ cells/ml.

Colony-forming assays

PU.1^{+/+} or *-/-* hematopoietic progenitors were plated in 1.5 ml of 0.9% methylcellulose mixture (MethoCult, StemCell Technologies) in 35 mm Petri dishes (Falcon). MethoCult was supplemented with 1% penicillin-streptomycin, 1% L-glutamine, 5 × 10⁻⁵ M β-mercaptoethanol, and either 10 ng/ml recombinant murine IL-3, 1 ng/ml recombinant

murine GM-CSF (R&D Systems), 10 ng/ml recombinant murine M-CSF (R&D Systems) or 10 ng/ml recombinant murine G-CSF. After 7 days of incubation, colonies were counted and analyzed by Wright staining. Colonies generated by transduction of progenitors with EGFP-encoding retroviruses were examined using a Zeiss Axiovert inverted fluorescent microscope.

Cytochemical analysis

Cells from individual colonies or *PU.1*^{-/-} cell lines (up to 10⁵ cells) were cytocentrifuged onto glass slides, fixed for 30 s in methanol and examined by Wright stain (Sigma). Chloroacetate esterase or MPO were detected as described (Yam *et al.*, 1971). Cells were photographed using a Zeiss Axiovert microscope.

Retroviral production

MSCV-pac (Hawley *et al.*, 1994) was modified by replacement of the puromycin resistance gene with the gene for EGFP (Cormack *et al.*, 1996) (Clontech). EGFP was amplified by PCR to introduce *Hind*III and *Clal* sites, then directionally ligated into the *Hind*III and *Clal* sites of MSCV-pac to produce MSCV-EGFP. HA-tagged *PU.1* cDNA (Brass *et al.*, 1996) was blunt-end ligated into the *Hpa*I site of MSCV-EGFP to produce MSCV-EGFP-*PU.1*.

φNX-Eco retroviral packaging cells (Kinsella and Nolan, 1996) were maintained in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. φNX-Eco cells at >80% confluence on 15 cm dishes were transiently transfected with 50 μg of DNA using the calcium phosphate method. Transfections were done in the presence of 25 μM chloroquine to increase viral titer as previously described (Pear *et al.*, 1993). After changing the medium once, retroviral supernatants were collected at 18 and 36 h and pooled. pMZEn-*fms* virus was produced as a supernatant from stably transfected ψ2 packaging cells as described (Rohrschneider *et al.*, 1989). Retroviral supernatants were concentrated 20-fold using a 500 kDa cut-off filter in a stirred-cell ultrafiltration apparatus (Amicon). Concentrated virus was frozen in aliquots and titered using NIH 3T3 cells.

Infection of fetal hematopoietic progenitors with retroviral constructs

Cell-free retroviral stocks were titered by infection of NIH 3T3 cells for 4 h in the presence of 8 μg/ml polybrene (Sigma), after which the medium was changed. After 2 days to allow retroviral integration and protein expression, FACS analysis for EGFP fluorescence was performed to measure viral titer. For the MZEn-*fms* retrovirus, titer was measured by FACS analysis for the c-*Fms* extracellular domain as described (Bourette *et al.*, 1997). Titers of viruses used in this study were >1 × 10⁷ infectious particles/ml.

Lin⁻ fetal liver progenitors that had been cultured in IL-3, IL-6 and SCF for 2 days were infected by resuspension in high-titer, cell-free retroviral supernatants for 4 h in the presence of 25 μg/ml polybrene. Infected cells were washed three times in complete medium, then cultured for 2 days in complete medium containing IL-3, IL-6 and SCF to allow retroviral integration and expression. Infected cells were washed three times before plating in methylcellulose to assess colony-forming potential.

RT-PCR

Total RNA was prepared from cells using RNAzol B reagent according to the manufacturer's instructions (Tel-Test Inc.). Random-primed cDNA was generated using a first-strand cDNA synthesis kit (Pharmacia). PCR was performed using 2 pM primers, 0.1 mM dNTPs and 0.1 U/ml *Taq* DNA polymerase (Boehringer Mannheim). Buffer and pH conditions were optimized for each primer pair using a PCR optimization kit (Opti-Prime, Stratagene). PCR was performed using 30 cycles. The following primers were used: G-CSFR, (sense 5'-CTCAAACCTAT-CCTGCCTCATG-3', antisense 5'-TCCAGGCAGAGATGAGCGAATG-3'); GM-CSFRα (sense 5'-GAGGTCCACAGGTCAAGGTG-3', antisense 5'-GATTGACAGTGGCAGGCTTC-3'); GM-CSFRβ (sense 5'-TTTCCATCACAACGAAGACT-3', antisense 5'-AATGATAGTA-AGCCATCTT-3'); c-*fms* (sense 5'-GCGATGTGTGAGCAATGGCAGT-3', antisense 5'-AGACCGTTTTCGCTAAGACCTG-3'); MPO (sense 5'-ATGCAGTGGGGACAGTTTCTG-3', antisense 5'-GTCGTTGTAG-GATCGGTACTG-3'); CD16 (sense 5'-ATGTTTTCAGATGCACACT-CT-3', antisense 5'-CAGAAATCACTCCAGATCTA-3'); CD32 (sense 5'-TGCAAAGGAAGTCTAGGAAGG-3', antisense 5'-GCAGAAGA-GTCTTGAGTTGGG-3'); CD64 (sense 5'-ATTCGGAGGTCGCCATT-TGA-3', antisense 5'-CCATCGCTTCTAAGTGTGTA-3'); CD11b (sense 5'-AAACCACAGTCCCGCAGAGAGC-3', antisense 5'-GCCA-

GGTCCATCAAGCCATCCA-3'); SR (sense 5'-GTACTAATACCTG-TTGTGGA-3', antisense 5'-CGTGCCTTGTCTTCTTCA-3'); β -actin (sense 5'-CCTAAGGCCAACCGTGAAG-3', antisense 5'-TCTTCATGGTGCTAGGAGCCA-3').

Western blot analysis of PU.1 protein

Proteins from whole cell lysates were resolved by SDS-PAGE and electrotransferred to a supported nitrocellulose membrane (Gibco-BRL). The membrane was incubated with an affinity-purified anti-PU.1 antibody (Scott *et al.*, 1997). Immunoreactive proteins were detected using HPRT-conjugated anti-rabbit antibody and the ECL system (Amersham).

Antibodies/FACS analysis

Flow-cytometric analysis was performed on single-cell suspensions of cells washed in phosphate-buffered saline (PBS) containing 0.05 M EDTA and 0.5% BSA. Cells were stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies and analyzed on a FACSscan (Becton-Dickinson). Propidium iodide uptake and sense scatter gating for cell size were used to exclude dead cells from analysis. Anti-mouse monoclonal antibodies used in this study included H129.19 (CD4), 53-6.7 (CD8a), 53-7.3 (CD5), 145-2C11 (CD3e), C71/16 (CD18), M1/70 (CD11b, Mac-1), RBC-8C5 (Ly-6G, Gr-1), Ter119, RA3-6B2 (B220, CD45R) and 2B8 (c-Kit, CD117), and were used according to the instructions of the supplier (PharMingen). HA (β IL-3, AIC2A) and HB antibodies (β c, AIC2B) were obtained from Medical and Biological Research Laboratories Co. Ltd (MBL).

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