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Reciprocal Activation of GATA-1 and PU.1 Marks Initial Specification of Hematopoietic Stem Cells into Myeloerythroid and Myelolymphoid Lineages

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SUMMARY

A hierarchical hematopoietic development with myeloid versus lymphoid bifurcation has been proposed downstream of the multipotent progenitor (MPP) stage, based on prospective isolation of progenitors capable of generating only myeloerythroid cells (common myeloid progenitor, CMP) or only lymphocytes (common lymphoid progenitor, CLP). By utilizing GATA-1 and PU.1 transcription factor reporters, here we identified progenitor populations that are precursors for either CMPs or CLPs. Two independent populations expressing either GATA-1 or PU.1 resided within the CD34$^+$Sca-1$^+$c-Kit$^+$ MPP fraction. The GATA-1$^+$ MPP displayed potent myeloerythroid potential without giving rise to lymphocytes, whereas the PU.1$^+$ MPP showed granulocyte/monocyte/lymphoid-restricted progenitor activity without megakaryocyte/erythroid differentiation. Furthermore, GATA-1$^+$ and PU.1$^+$ MPPs possessed huge expansion potential and differentiated into the original CMPs and CLPs, respectively. Thus, the reciprocal activation of GATA-1 and PU.1 primarily organizes the hematopoietic lineage fate decision to form the earliest hematopoietic branchpoint that comprises isolatable myeloerythroid and myelolymphoid progenitor populations.

INTRODUCTION

Understanding how multipotent cells commit to each of their terminal fate potentials is an important aspect of stem cell biology. Lineage commitment pathways from hematopoietic stem cells (HSCs) (Spangrude et al., 1991) have been proposed dependent upon the existence of prospectively isolatable lineage-restricted progenitor populations (Akashi et al., 2005; Morrison et al., 1995). In adult murine hematopoiesis, HSCs with long-term (LT) self-renewal potential reside within the Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) fraction having CD34$^-$ (Osawa et al., 1996), Thy1^{lo} (Morrison and Weissman, 1994), and Flt3/Flk2$^-$ (Adolfsson et al., 2001; Christensen and Weissman, 2001) phenotypes. The LSK cells having CD34$^+$, Thy1$^-$, and/or Flt3$^+$ phenotypes are capable of multilineage reconstitution only for a short term and therefore should contain multipotent progenitors (MPPs) (Christensen and Weissman, 2001; Iwasaki and Akashi, 2007; Osawa et al., 1996). There has been a controversy in terms of developmental steps downstream of MPPs (Iwasaki and Akashi, 2007). One model suggests that myeloerythroid and lymphoid developmental pathways are largely independent based on the fact that the earliest myeloid and lymphoid progenitors such as the common myeloid progenitor (CMP) (Akashi et al., 2000) and the common lymphoid progenitor (CLP) (Kondo et al., 1997) are prospectively isolatable outside the LSK fraction. CMPs gave rise to all myeloerythroid cells via developmental intermediates such as the granulocyte/monocyte progenitor (GMP) and the megakaryocyte/erythrocyte progenitor (MEP) (Akashi et al., 2000), while CLPs are precursors for all lymphoid cells including T, B, and NK cells (Kondo et al., 1997).

Recently, however, several studies have suggested that lineage commitment could occur at the MPP stage, preceding the proposed bifurcation of myeloid and lymphoid pathways. The MPP population contains a fraction of cells expressing the lymphoid lineage-specific recombination activation gene (rag)-1, called the earliest lymphoid progenitor (ELP), which differentiate mainly into lymphoid cells but retain a minor myeloid potential (Igarashi et al., 2002). Another study showed that a fraction of MPP expressing Flt3, especially at a high level, does not have megakaryocyte/erythroid (MegE) potential, if any, and is largely primed for the lymphoid lineage. The Flt3$^+$ MPP was therefore termed as the lymphoid-primed multipotent progenitor (LMPP) (Adolfsson et al., 2005). Because the

LMPPs also have considerable GM potential, the coupled loss of self-renewal activity and MegE potential in the early HSC commitment has been proposed (Adolfsson et al., 2005). Based on this finding, GM cells could develop without passing through the CMP stage, and the LMPP could be the major pathway for GM cells (Adolfsson et al., 2005). More recently, however, Forsberg et al. reported that the Flt3$^+$ LMPP population possesses robust MegE potential, represented by their ability to produce a significant number of erythroid cells and platelets at a relatively late phase after transplantation (day 12–15), claiming that the CMP and the CLP still constitute the major site for myeloid versus lymphoid lineage decision (Forsberg et al., 2006).

How can we reconcile the controversy in the early hematopoietic lineage map? Because uncommitted cells prime multiple lineage-affiliated developmental programs at the single-cell level prior to lineage commitment (Hu et al., 1997), the major question here is whether the GM development program is coprimed with the MegE program at the CMP stage, with the lymphoid program at the LMPP stage, or both. In fact, in previous single-cell PCR studies, a considerable fraction of single CMPs and LMPPs possess GM and MegE, and GM and lymphoid gene transcripts, respectively (Mansson et al., 2007; Miyamoto et al., 2002). The problem is that the CMP and the LMPP are still functionally heterogenous populations: the remaining MegE potential in the LMPP (Forsberg et al., 2006) raises a possibility that the LMPP contains populations with MegE potential such as MPPs, whereas CMPs possess a weak B cell potential (Akashi et al., 2000), suggesting contamination of B cell precursors. Thus, it is critical to test whether progenitor populations more strictly restricted to the GM and lymphoid lineage (granulocyte/monocyte/lymphoid progenitors, GMLP) or the CMP lacking lymphoid potential could be isolated in pure form in early hematopoiesis. In addition, it is also important to evaluate whether such populations have significant expansion potential sufficient to play a role in maintaining homeostasis of normal hematopoiesis (Spangrude, 2002).

Hematopoietic development models have been proposed simply by placing known lineage-specific progenitors in a hierarchical order, which are separated based on the difference in the expression level of antigens that may not have significant functions in lineage fate decision (i.e., Sca-1, CD34, Thy-1, and Flt3). One of reasonable approaches for separating functionally distinct populations may include the utilization of markers closely associated with lineage-instructive signaling. In establishing lineage diversities, antagonistic or cooperative effects of multiple transcription factors, called a "transcription factor network" (Orkin, 2000; Sieweke and Graf, 1998), might be critical. The Ets family transcription factor PU.1 (Spi-1) is one of the most important regulators of GM and lymphoid lineage development (Rosenbauer and Tenen, 2007) and is necessary for both HSC self-renewal and its generation of early GM and lymphoid progenitors such as CMPs, GMPs, and CLPs (Iwasaki et al., 2005b). GATA-1 is an essential transcription factor for MegE development (Fujiwara et al., 1996). Importantly, PU.1 and GATA-1 can exert instructive signals for GM and MegE lineage commitment, respectively (Heyworth et al., 2002; Iwasaki et al., 2003; Kulessa et al., 1995; Nerlov and Graf, 1998b). Furthermore, PU.1 and GATA-1 mutually inhibit each other's expression and transactivation functions (Nerlov and Graf, 1998a; Nerlov et al., 2000; Rekhtman et al., 1999; Walsh et al., 2002; Zhang et al., 1999, 2000). Thus, there is a general agreement that the antagonistic interplay of PU.1 and GATA-1 plays a critical role in early hematopoietic fate decision such as the GM or lymphoid versus the MegE lineage commitment (Iwasaki and Akashi, 2007).

These data led us to hypothesize that tracking the expression profile of PU.1 and GATA-1 in early hematopoiesis may provide critical data on developmental pathways of each lineage. By utilizing mice having GATA-1 or PU.1 transcriptional reporters, we here present a high-resolution map containing lineage-restricted progenitor populations within the MPP population of CD34$^+$ LSK phenotype. We found that MPPs upregulating either PU.1 or GATA-1 are functional GMLPs or CMPs, respectively. PU.1$^+$ GMLPs gave rise to the CLP (Kondo et al., 1997) and the GMP, while GATA-1$^+$ CMPs generated the original CMP. Accordingly, this model allows flexibility for GM development, as the GMP can be generated via either the GMLP or the CMP stages. The proposed hematopoietic developmental scheme including these new oligopotent progenitor populations reasonably reconciles a number of current controversies in early hematopoietic development.

RESULTS

Initial Upregulation of PU.1 and GATA-1 Independently Occurs at the CD34$^+$ LSK Stage

We first evaluated the expression of PU.1 or GATA-1 in purified conventional stem and progenitor fractions (Akashi et al., 2000; Kondo et al., 1997; Osawa et al., 1996; Spangrude et al., 1988). To this end, we used mice harboring the enhanced green fluorescent protein (GFP) knocked into the PU.1 locus (Back et al., 2004) and the transgenic GATA-1 reporter tagged with GFP (Iwasaki et al., 2005a). In these mouse lines, the level of GFP is correlated with the PU.1 or GATA-1 protein level (Back et al., 2005; Iwasaki et al., 2005a). Figure 1A shows the expression of GFP in stem and progenitor cells purified from PU.1$^{GFP/+}$ mice. LSK cells are composed of CD34$^-$ and CD34$^+$ populations that are defined as the LT-HSC and the MPP, respectively (Osawa et al., 1996). The vast majority of LSK cells expressed a low level of PU.1-GFP, as we and others have reported (Iwasaki et al., 2005b; Nutt et al., 2005). In the myeloerythroid pathway, the GFP level progressively increased as the MPP steps forward into the GMP stage, while it decreased at the MEP stage. In the lymphoid pathway, PU.1-GFP was upregulated in CLPs (Figure 1A), proT and proB cells (data not shown). The pattern of GFP expression was reversed in GATA-1-GFP reporter mice (Figure 1B). GATA-1-GFP was expressed in only a minority of the LSK population but upregulated along the MegE pathway. MEPs expressed a high level of GFP and

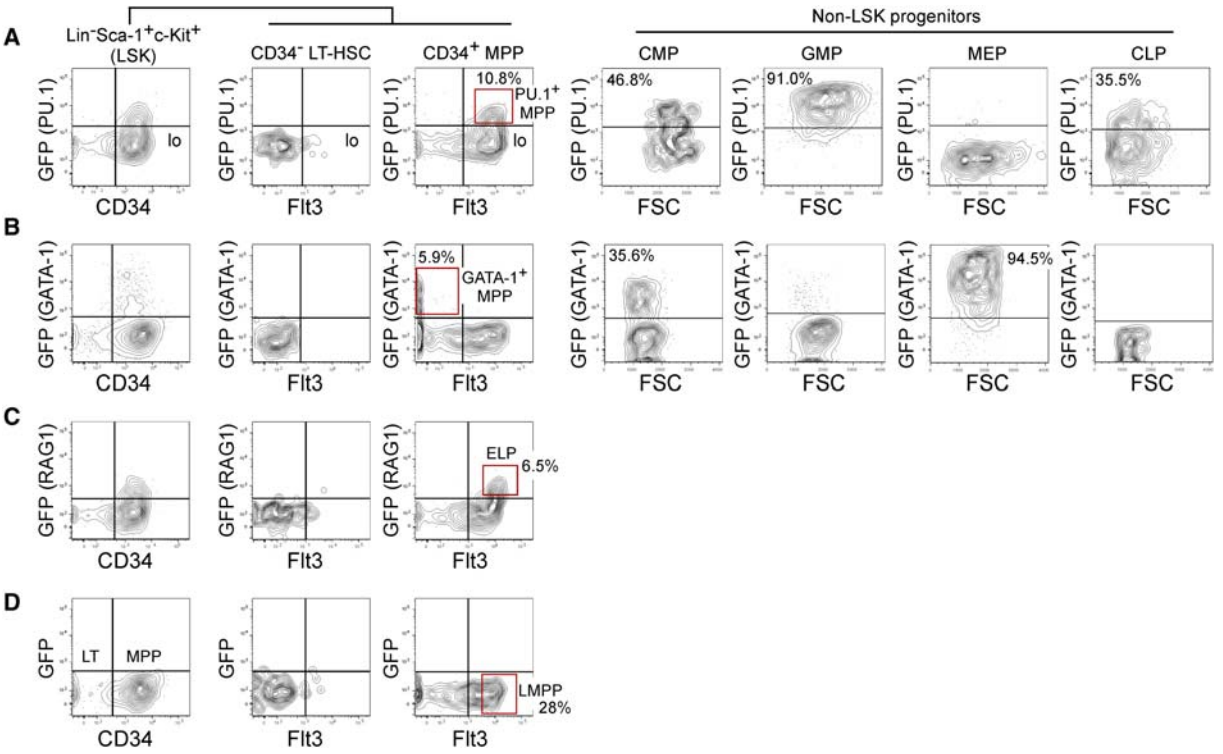

Figure 1. The Expression of the PU.1-GFP or the GATA-1-GFP Reporters within the Lin⁻Sca-1⁺c-Kit⁺ HSC Fraction

(A) The expression of GFP in the LSK fraction of bone marrow cells in *PU.1*$^{GFP/+}$ mice. Note that the upregulation of PU.1-GFP is found only in the CD34⁺Flt3⁺ LSK fraction (left panels). PU.1-GFP is upregulated in GMPs and CLPs, but not in MEPs (right panels).

(B) The expression of GFP in GATA-1-GFP reporter mice. GATA-1-GFP is detectable in the CD34⁺ MPPs and is further upregulated toward the MEP stage.

(C) The expression of RAG1-GFP within the LSK population. RAG1-GFP⁺ cells are seen within CD34⁺ MPPs, which correspond to previously defined ELPs (Igarashi et al., 2002).

(D) The control analysis of wild-type C57BL/6 mice. Representative FACS plots of more than ten mice (6–8 weeks old) analyzed in each group are shown.

CMPs expressed negative-to-low levels of GFP, whereas GMPs and CLPs were negative for GFP. Thus, the expression pattern of PU.1 and GATA-1 in early hematopoiesis appeared to be reciprocal, as expected.

We next evaluated the expression pattern of Flt3 and of PU.1 and GATA-1 reporters within the LSK fraction. Murine LT-HSCs do not express Flt3 (Adolfsson et al., 2001; Christensen and Weissman, 2001). In normal C57BL6 mice, CD34⁻ LT-HSCs were Flt3⁻, while a fraction of CD34⁺ MPPs upregulated Flt3 at a high level (Figure 1D), forming the LMPP population (Adolfsson et al., 2005). In PU.1$^{GFP/+}$ mice, LT-HSCs were entirely Flt3⁻ and PU.1-GFP^{lo} (PU.1^{lo}) (Figure 1A). In MPPs, a high level of PU.1-GFP (PU.1⁺) was seen only in a fraction of cells with a high level of Flt3, suggesting that this population represents the earliest hematopoietic stage initiating PU.1 upregulation. In GATA-1-GFP reporter mice (Figure 1B), LT-HSCs were all Flt3⁻ and GFP⁻. In marked contrast to GFP expression in PU.1$^{GFP/+}$ mice, GATA-1-GFP⁺ (GATA-1⁺) cells were found only in a fraction of MPPs that do not express Flt3 (Figure 1B). Thus, the initial upregulation of PU.1 or GATA-1 occurs independently at the MPP stage in Flt3⁺ or Flt3⁻ subpopulations, respectively. In RAG1$^{+/GFP}$ mice (Kuwata et al., 1999) (Figure 1C), GFP was expressed in a fraction of MPPs expressing a high level of Flt3. The Flt3⁺ MPPs expressing RAG1-GFP (RAG1⁺) correspond to previously defined ELPs (Igarashi et al., 2002). This population was largely committed to the lymphoid lineage retaining weak GM potential (see Figure S1A in the Supplemental Data available with this article online). These data indicate that both PU.1⁺ MPPs and RAG1⁺ ELPs reside within the LMPP population.

Figure 2A shows the quantitative real-time PCR analysis of PU.1 and GATA-1 mRNA in purified MPP subpopulations. In PU.1$^{GFP/+}$ mice (upper panels), LT-HSCs and PU.1^{lo} MPPs had a similar low level of PU.1 transcript, whereas PU.1 ⁺ MPPs possessed an ~2-fold higher level of PU.1 transcripts as compared to those in PU.1^{lo} MPPs. In all of these subpopulations, GATA-1 transcripts were below the measurable level. CLPs expressed PU.1 at a level similar to that of PU.1⁺ MPPs. The PU.1 mRNA level progressively increased along the GM differentiation pathway: CMPs and GMPs expressed 5- and 8-fold higher levels of PU.1 transcripts as compared to those in PU.1^{lo}

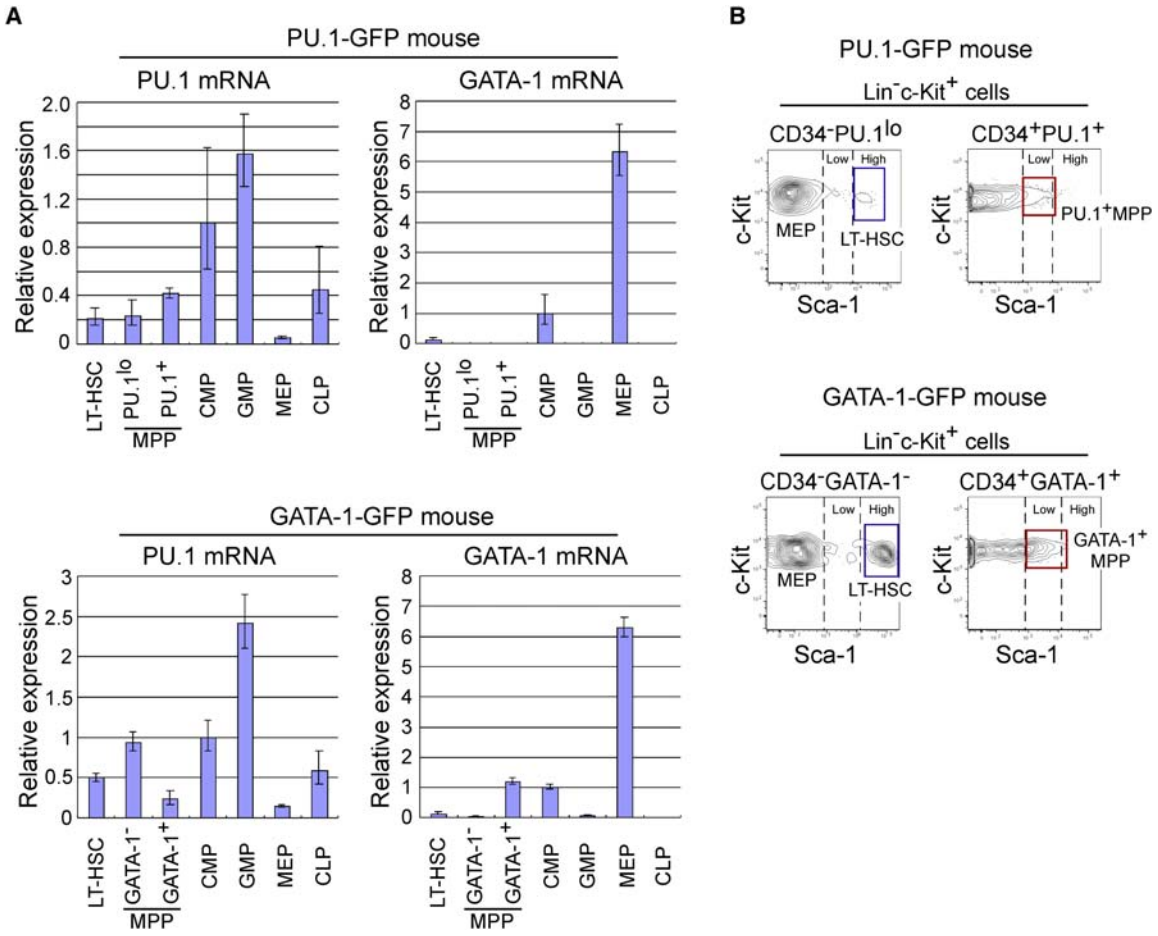

Figure 2. The Expression Level of PU.1 mRNA, GATA-1 mRNA, and Sca-1 Antigen in the PU.1-GFP- or GATA-1-GFP-Based LSK Subfractions

(A) Quantitative real-time PCR assays of PU.1 and GATA-1 mRNA in stem and progenitors purified from *PU.1*$^{GFP/+}$ or GATA-1-GFP mice. PCR reactions were triplicated and performed at least twice. Data were compensated with the internal control β2-MG value and shown as mean ± standard deviation (error bars).

(B) The analysis of the expression levels of Sca-1 in the LSK subpopulations. Both PU.1$^+$ and GATA-1$^+$ MPPs express Sca-1 at a lower level as compared to that of LT-HSCs. Representative FACS plots from three mice analyzed are shown.

MPPs, respectively. In contrast, MEPs did not express a measurable level of PU.1 mRNA but had a high level of GATA-1 mRNA.

In GATA-1-GFP mice (bottom panels), GATA-1 mRNA was not found in the LT-HSCs or GATA-1$^-$ MPPs, while GATA-1$^+$ MPPs had a considerable level of GATA-1 transcripts whose levels were almost equal to those in CMPs. Interestingly, GATA-1$^+$ MPPs possessed an almost 4-fold lower level of PU.1 mRNA as compared to that in GATA-1$^-$ MPPs. MEPs possessed GATA-1 transcript at a level ~6-fold higher than that in GATA-1$^+$ MPPs, whereas GMP or CLPs did not express GATA-1. Thus, the levels of PU.1 or GATA-1 mRNA fairly corresponded to the GFP levels in each subpopulation, and the expression patterns of PU.1 and GATA-1 mRNA were generally reciprocal during hematopoietic development, consistent with the previous finding that transcription of PU.1 and GATA-1 genes is mutually exclusive.

Upregulation of PU.1 and GATA-1 within the CD34$^+$ LSK Stage Occurs with Decrease in the Expression Level of Sca-1

The "gold standard" definition for murine HSC is the "LSK" (Ikuta and Weissman, 1992; Osawa et al., 1996), while the myeloid progenitors such as CMPs, GMPs, and MEPs were defined within the Lin$^-$Sca-1$^-$c-Kit$^+$ population (Akashi et al., 2000), suggesting that Sca-1 is gradually downregulated during the early phase of myeloerythroid commitment. We thus carefully evaluated the expression level of Sca-1 in PU.1$^+$ and GATA-1$^+$ MPPs. In both PU.1$^{GFP/+}$ and GATA-1-GFP reporter mice, LT-HSCs expressed Sca-1 at the highest level (Figure 2B). Notably, the PU.1$^+$ and GATA-1$^+$ MPP populations did not express a high level of Sca-1 but expressed Sca-1 only at a low level (Sca-1^{lo}). These data clearly show that the upregulation of either PU.1 or GATA-1 within the LSK fraction occurs along with the decline in the level of Sca-1.

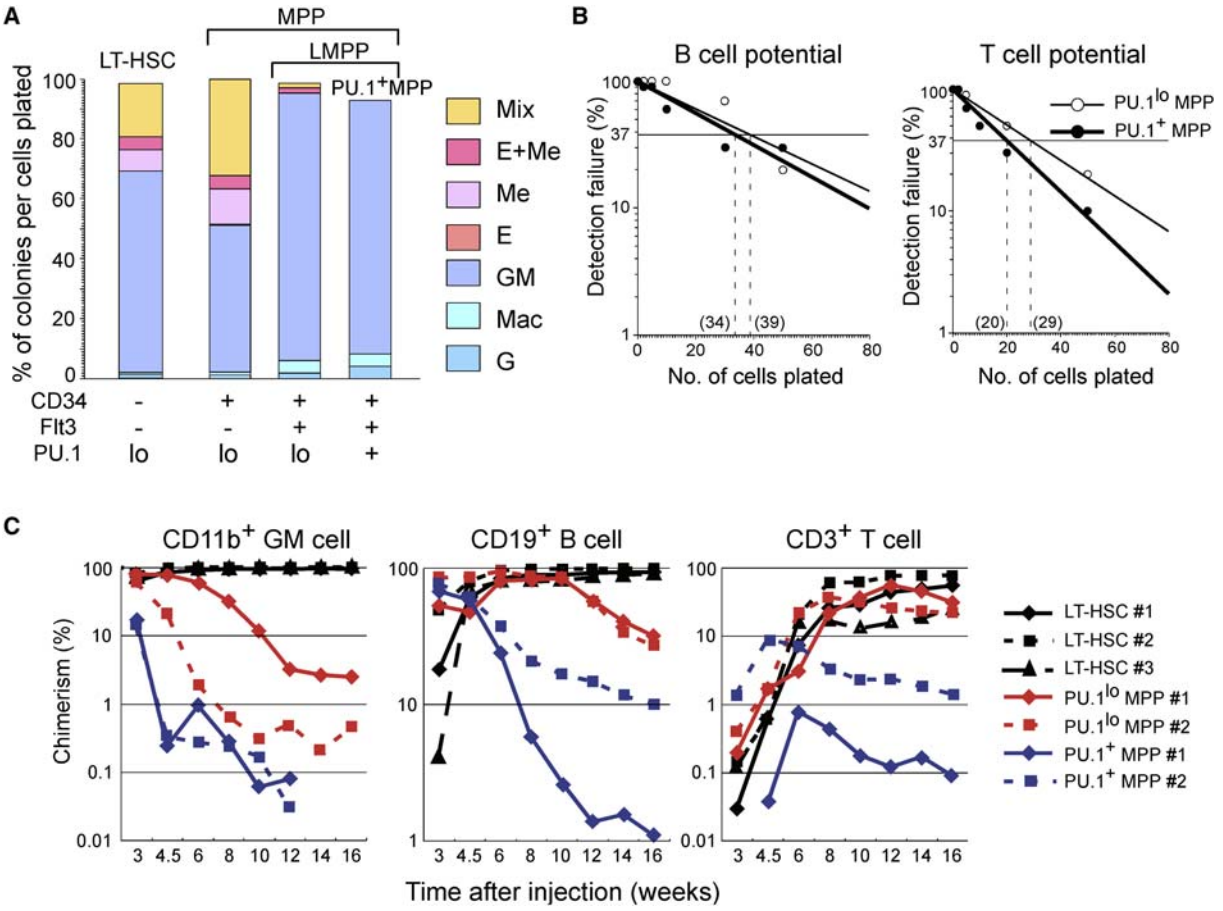

Figure 3. PU.1$^+$ MPPs Possess Differentiation Potential Restricted to the GM and the Lymphoid Lineages

(A) Clonogenic myeloerythroid colony assays for LSK subfractions in *PU.1*$^{GFP/+}$ mice. Types of colonies were determined morphologically and cytochemically by May-Giemsa staining.

(B) Limiting dilution analyses of B or T cell potential tested on the OP9 or the OP9-DL1 stromal cell layer, respectively. Both PU.1^{lo} and PU.1$^+$ MPPs differentiated into B and T cells at a similar frequency.

(C) In vivo reconstitution potential of LSK subfractions in *PU.1*$^{GFP/+}$ mice. Percentages of donor-derived cells in blood leukocytes from two representative recipients per group of five mice are shown. PU.1$^+$ MPPs displayed significant but transient reconstitution for GM, B, and T cells.

PU.1$^+$ MPPs Lack MegE Potential but Possess GM- and Lymphoid-Restricted Differentiation Potential

We evaluated the differentiation potential of PU.1$^+$ MPPs. Single cells from each LSK subfraction based on CD34 and PU.1 expression gave rise to colonies at high plating efficiencies (>95%) in vitro (Figure 3A). LT-HSCs gave rise to a variety of myeloerythroid colonies. Within the MPP population, Flt3$^+$ LMPP gave rise mainly to GM colonies with only a few percent of MegE colonies, consistent with the previous report that the exclusion of MegE lineage potential has already started with the upregulation of Flt3 (Adolfsson et al., 2005). PU.1$^+$ MPPs formed only GM colonies, suggesting that the vast majority of them have lost MegE potential. Purified PU.1^{lo} or PU.1$^+$ MPPs were then cultured in vitro on OP9 or OP9-DL1 stromal layers to test B or T cell potential, respectively. As shown in Figure 3B, both populations gave rise to B and T cells at similar high frequencies, indicating that the lymphoid potential is preserved during the upregulation of PU.1 within the MPP stage. Around 80% of CD34$^+$Flt3$^+$ LSK cells possessed clonogenic GM/T/B potential in vitro (Adolfsson et al., 2005). We also found that at least >20% of single PU.1$^+$ MPPs (Ly5.2) gave rise to GM, T, and B cells when they were injected directly into the congenic (Ly5.1) thymi (Figure S1B).

We then transplanted 500 cells each of LT-HSC, PU.1^{lo} MPP, and PU.1$^+$ MPP populations (Ly5.2) into congenic hosts (Ly5.1) competitively with 300 recipient-type LSK cells (Ly5.1). LT-HSCs displayed strong and LT multilineage reconstitution activity, as expected (Figure 3C). Reconstitution by PU.1^{lo} MPPs was observed only for a short term. Percentages of donor-derived cells reached >80% at peaks in all GM, B, and T cell lineages at 3, 6, and 12 weeks posttransplantation, respectively, but all of these lineage cells progressively decline thereafter. PU.1$^+$ MPPs displayed transient reconstitution that was even shorter than that by PU.1^{lo} MPPs. The peak percentages of GM and B cell progeny were seen at 3 weeks

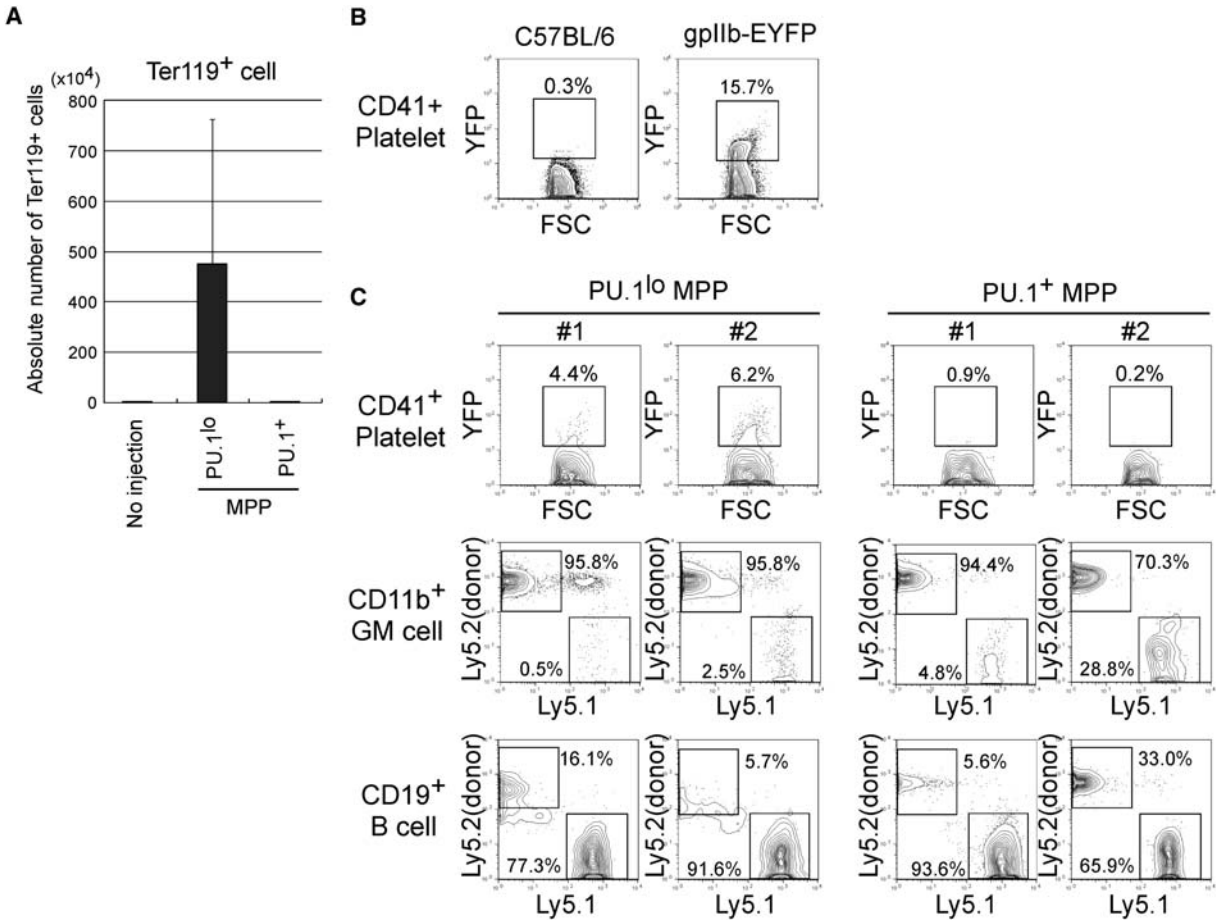

Figure 4. PU.1$^+$ MPPs Lack MegE Potential In Vivo

(A) The absolute number of Ter119$^+$ erythroid progeny in the spleen 12 days after injection of 1000 cells each of PU.1^{lo} and PU.1$^+$ MPPs. Representative data of an experiment containing three recipients per group are shown as mean ± standard deviation (error bars).

(B) A fraction of platelets expressed the YFP reporter in *gpIIb*$^{+/YFP}$ mice.

(C) The analysis of donor-derived platelets, GM cells, and B cells after injection of 500 cells each of PU.1^{lo} and PU.1$^+$ MPPs purified from *gpIIb*$^{+/YFP}$ × *PU.1*$^{GFP/+}$ mice. Analyses were performed 15 days after transplantation. Two representative recipients per group of ten mice are shown.

posttransplantation, while donor-derived T cells reached a peak at ~6 weeks posttransplantation. Donor-type spleen TER119$^+$ erythroid cells were found 1 or 2 weeks after transplantation in mice injected with LT-HSCs or PU.1^{lo} MPPs, but not in mice with PU.1$^+$ MPPs at these time points (data not shown). These data collectively suggest that the PU.1$^+$ MPP possesses the GM/lymphoid-restricted lineage potential.

We further evaluated the MegE activity of these populations. One thousand PU.1^{lo} and PU.1$^+$ MPPs were transplanted into lethally irradiated hosts, and the absolute number of Ter119$^+$ erythroid cells was enumerated in the spleen on day 12. As shown in Figure 4A, while spleens of mice transplanted with PU.1^{lo} MPPs had abundant Ter119$^+$ cells, those in mice with PU.1$^+$ MPPs did not contain measurable numbers of Ter119$^+$ cells. Because platelets do not express Ly5 antigens, Forsberg et al. used actin-GFP mice (Wright et al., 2001) as a donor to mark donor-derived platelets as GFP$^+$ and showed that LMPPs could generate significant numbers of GFP$^+$ platelets ~2 weeks after transplantation (Forsberg et al., 2006). To visualize donor-derived platelets in our system, we crossed the PU.1$^{GFP/+}$ mouse with a line harboring the enhanced yellow fluorescent protein (YFP) knocked into the gpIIb locus, whose platelets possess YFP (Schulze et al., 2006). In gpIIb$^{+/YFP}$ mice, almost 16% of platelets were labeled with YFP (Figure 4B). Five hundred cells of PU.1^{lo} or PU.1$^+$ MPPs (Ly5.2) were purified from PU.1$^{GFP/+}$ × gpIIb$^{+/YFP}$ mice and were injected into lethally irradiated congenic hosts (Ly5.1). Two weeks after transplantation, 4%–6% of platelets were labeled with YFP in mice transplanted with PU.1^{lo} MPPs. In contrast, YFP$^+$ platelets were never found in mice transplanted with PU.1$^+$ MPPs, while GM and B cell lineages were reconstituted at a level comparable to mice with PU.1^{lo} MPPs (Figure 4C). Thus, the PU.1$^+$ MPP fails to produce erythrocytes and platelets, but it develops GM, T, and B cells at the single-cell level, providing a formal proof that the GMLP exists within the LMPP fraction.

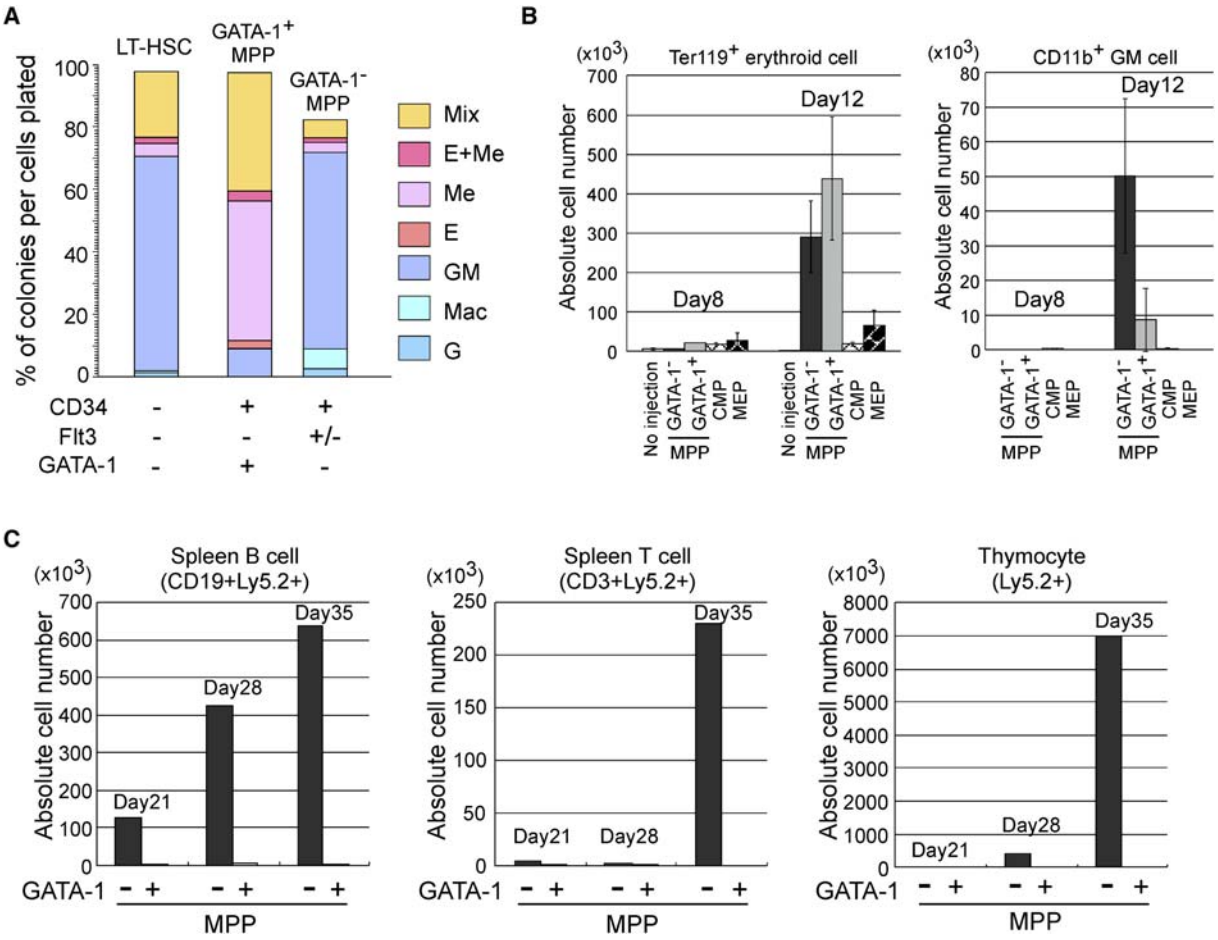

Figure 5. GATA-1$^+$ MPPs Possess GM and MegE but Not Lymphoid Potential

(A) Clonogenic myeloerythroid colony assays for LSK subpopulations in GATA-1-GFP mice. GATA-1$^+$ MPPs gave rise to GM and MegE colonies in vitro. Note that ~40% of their colonies were CFU-Mix that contained both GM and MegE cells.

(B) In vivo myeloerythroid potential of LSK subfractions and progenitors. Five hundred cells each of listed populations were injected into lethally irradiated hosts, and the absolute numbers of Ter119$^+$ erythroid and CD11b$^+$ GM progeny were evaluated in the spleen on days 8 and 12. Three recipient mice were analyzed for each group. Results are shown as mean ± standard deviation (error bars).

(C) In vivo lymphoid potential in GATA-1$^-$ and GATA-1$^+$ MPPs. Five hundred cells each were transplanted into congenic hosts together with 300 *RAG2*$^{-/-}$ LSK (Ly5.1) cells. Absolute numbers of lymphocytes was enumerated in the spleen and the thymus on days 21, 28, and 35. GATA-1$^+$ MPPs never gave rise to lymphocyte progeny in these time points. Four or five mice were analyzed for each experiment.

GATA-1$^+$ MPPs Lack Lymphoid Potential but Possess a Strong Common Myeloid Progenitor Activity

LT-HSCs are GATA-1$^-$, and a fraction of Flt3$^-$ but not Flt3$^+$ MPPs (LMPPs) express GATA-1-GFP (Figure 1B). Figure 5A shows the myeloerythroid differentiation activity of MPP subpopulations. LT-HSCs gave rise to a variety of myeloerythroid colonies, including 20% of colonies containing both MegE and GM lineage cells (CFU-Mix). Interestingly, the CFU-Mix activity was most concentrated in the GATA-1$^+$ MPPs, in which ~40% of single cells formed mixed colonies. In vivo analyses showed that GATA-1$^+$ MPPs possess very potent myeloerythroid potential (Figure 5B). Purified 500 cells each of GATA-1$^-$ MPPs, GATA-1$^+$ MPPs, and Lin$^-$Sca-1$^-$c-Kit$^+$ CMPs and MEPs (Akashi et al., 2000) were injected into lethally irradiated congenic hosts. On day 8, GATA-1$^+$ MPPs produced Ter119$^+$ cells whose numbers were almost equal to those from the same number of CMPs or MEPs. On day 12, however, GATA-1$^+$ MPPs gave rise to a much higher number of Ter119$^+$ cells as compared to CMPs and MEPs. Furthermore, although only CMPs produced a detectable level of CD11b$^+$ GM cells on day 8, GATA-1$^+$ MPPs produced >20-fold higher numbers of GM cells as compared to those from CMPs on day 12, indicating that GATA-1$^+$ MPPs possess potent GM potential.

GATA-1$^+$ MPPs were also cultured in vitro on OP9 or OP9-DL1 stromal layers to test their lymphoid potential. Repeated culture of 1000 GATA-1$^+$ MPPs in this condition, however, never gave rise to B or T cells (data not shown). We then transplanted 500 cells each of GATA-1$^-$ and GATA-1$^+$ MPPs (Ly5.2) into congenic C57BL6 (Ly5.1) mice competitively with 300 RAG2$^{-/-}$ LSK cells (Ly5.1). The number of progeny was evaluated on days 21, 28,

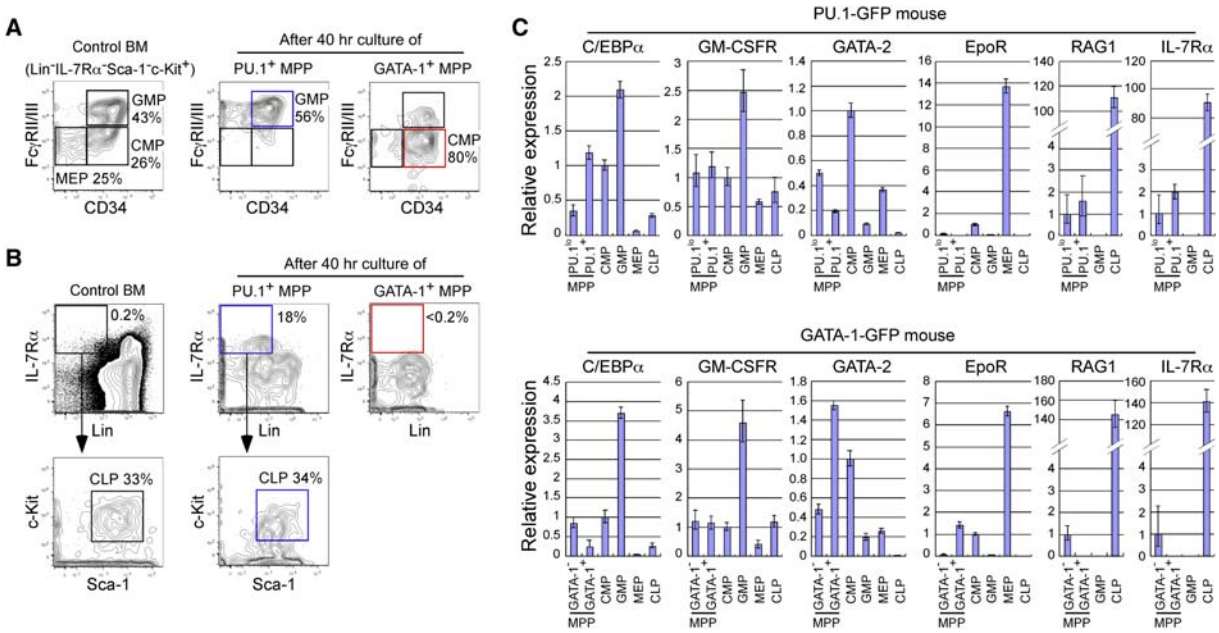

Figure 6. Lineal Relationships between PU.1⁺ and GATA-1⁺ MPPs and Conventional Myeloid and Lymphoid Progenitor Populations

Short-term differentiation of PU.1⁺ and GATA-1⁺ MPPs on the OP9 stromal layer. After a 40 hr culture, cells were subjected to myeloerythroid or lymphoid progenitor analysis.

(A) The analysis of the Lin⁻Sca-1⁻c-Kit⁺ fraction for the conventional myeloerythroid progenitors such as CMPs, GMPs, and MEPs (Akashi et al., 2000). Note that PU.1⁺ MPPs give rise to GMPs, but not CMPs, while GATA-1⁺ MPPs gave rise mainly to CMPs.

(B) The analysis for cells with CLP phenotype. Note that PU.1⁺ MPPs, but not GATA-1⁺ MPPs, generated CLPs.

(C) Real-time PCR analyses for lineage-related genes in purified progenitor populations. PCR reactions were triplicated and performed at least twice. Results are shown as mean ± standard deviation (error bars).

and 35. Again, GATA-1⁺ MPPs did not gave rise to detectable numbers of T or B cells in the spleen or the thymus, while GATA-1⁻ MPPs progressively generated T and B cells (Figure 5C).

Thus, at least ~40% of GATA-1⁺ MPPs possess potent and clonal GM and MegE potential but lack lymphoid differentiation activity, and the appearance of GM and MegE progeny in vivo was significantly delayed as compared to those progeny from CMPs. These data indicate that GATA-1⁺ MPPs possess strong CMP potential and also suggest that they are upstream of the original Lin⁻Sca-1⁻c-Kit⁺ CMP (Akashi et al., 2000).

The PU.1⁺ and the GATA-1⁺ MPPs Are Precursors for Conventional Hematolymphoid Progenitors

To analyze the lineal relationship between PU.1⁺ or GATA-1⁺ MPPs and conventional hematolymphoid progenitors such as CMPs, GMPs, MEPs, and CLPs (Akashi et al., 2000; Kondo et al., 1997), we tracked changes in surface phenotype of PU.1 ⁺ or GATA-1⁺ MPPs in vitro. Figure 6 shows progeny from PU.1⁺ or GATA-1⁺ MPPs after a 40 hr culture on the OP9 stromal layer.

PU.1^{lo} MPPs and GATA-1⁻ MPPs differentiated into all myeloerythroid progenitors such as CMPs, GMPs, and MEPs, as well as into CLPs (data not shown). Strikingly, PU.1⁺ MPPs gave rise to GMPs, but not MEPs or CMPs (Figure 6A), while this population also generated CLPs (Figure 6B), correctly reflecting their GM/lymphoid potential in vivo. In marked contrast, GATA-1⁺ MPPs differentiated mainly into CMPs as well as small numbers of GMPs and MEPs (Figure 6A), while they did not generate CLPs (Figure 6B). These data directly show that the PU.1⁺ MPPs are immediate precursors for the original GMP and CLP, whereas GATA-1⁺ MPPs are precursor for the original Lin⁻Sca-1⁻c-Kit⁺ CMP.

To evaluate whether gene priming status in PU.1⁺ or GATA-1⁺ MPPs reflects each of their lineage potential, we performed the real-time PCR analysis of representative lineage-related genes in purified PU.1⁺ or GATA-1⁺ MPPs (Figure 6C). PU.1⁺ MPPs expressed GM-related C/EBPα and GM-CSFRα transcripts, whose levels were almost equal to the original CMPs, but did not express a detectable level of erythropoietin receptor (EpoR). PU.1⁺ MPPs also expressed a low level of lymphoid genes including RAG1 and IL-7Rα. Thus, as in case of LMPPs (Mansson et al., 2007), PU.1⁺ MPPs appear to prime both GM and lymphoid developmental programs. This pattern of gene expression well correlates with their GM and lymphoid lineage-restricted potential. In contrast, GATA-1⁺ MPPs expressed C/EBPα only at a low level, as in the case of PU.1 expression in this population (Figure 2A). They expressed GM-CSFRα, EpoR, and GATA-2 at levels equivalent to those in the original CMPs, whereas the lymphoid RAG1 or IL-7Rα genes

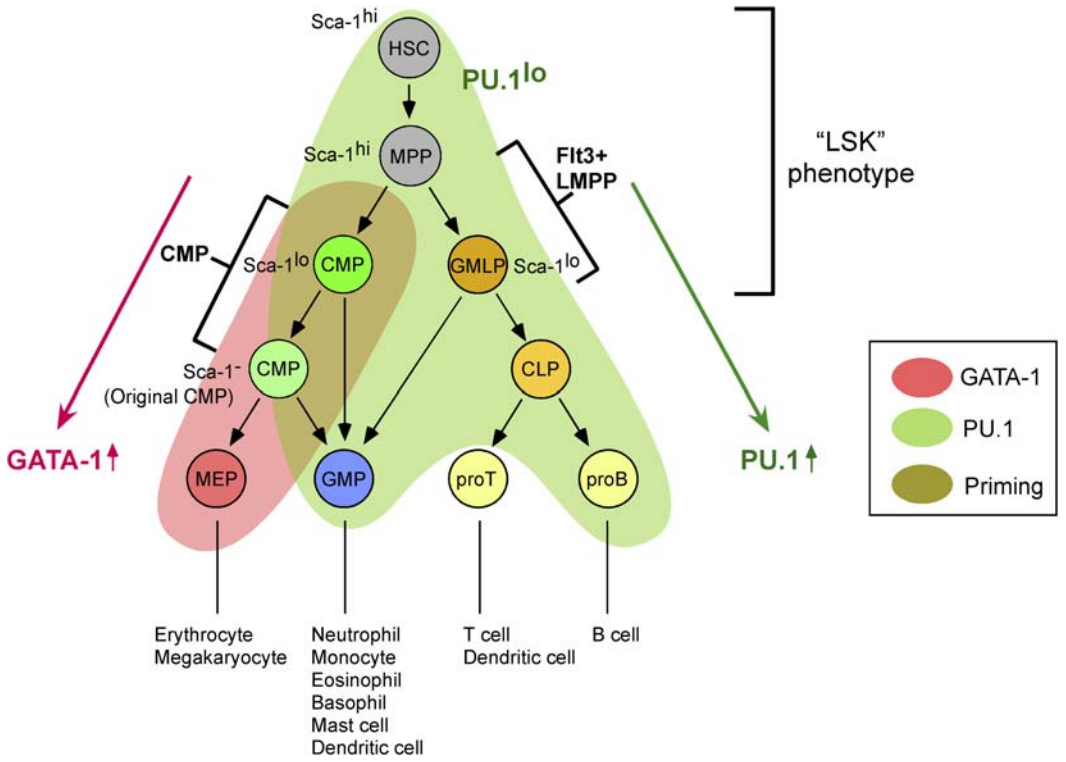

Figure 7. The Proposed Hematopoietic Developmental Scheme Mapped by PU.1 and GATA-1 Transcriptional Reporters

A low level of PU.1 is expressed at the Sca-1^{hi} LT-HSC stage. PU.1 and GATA-1 upregulation initiate at the Sca-1^{lo} MPP stage, resulting in generation of the GMLP and the CMP, respectively. The GATA-1^{+}Sca-1^{lo} CMP and the PU.1^{+}Sca-1^{lo} GMLP are mutually independent populations and give rise to conventional Sca-1^{-} CMPs and CLPs, respectively. The major CMP activity resides in the GATA-1^{+}Sca-1^{lo} CMP stage. Note that GMPs can develop from both CMPs and GMLPs, suggesting flexible developmental capability of GM lineage cells. MPP, multipotential progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythrocyte progenitor; GMP, granulocyte/monocyte progenitor; LMPP, lymphoid-primed multipotent progenitor; GMLP, granulocyte/monocyte/lymphoid progenitor.

were undetectable. Thus, GATA-1^{+} MPPs are likely to immediately respond to both myeloerythroid cytokines such as GM-CSF and Epo but do not prime lymphoid programs represented by RAG1 and IL-7Rα expression. These priming patterns of lineage-affiliated genes appear to reflect their developmental potential.

DISCUSSION

In this study, by utilizing either PU.1 or GATA-1 transcription factor reporters, we isolated hematolymphoid progenitor populations within the adult bone marrow LSK fraction that was originally defined as the most primitive HSC population (Ikuta and Weissman, 1992; Osawa et al., 1996; Spangrude et al., 1988). The upregulation of these transcription factors was found exclusively in the CD34^{+} MPP fraction. The PU.1^{+} MPP represents the GMLP capable of reconstitution restricted to the GM and the lymphoid lineage. On the other hand, the GATA-1^{+} MPP is the functional CMP with clonal and robust differentiation activity for both the GM and the MegE lineages without reading out lymphoid differentiation. Both populations express lower levels of Sca-1 as compared to that of LT-HSCs (Figure 2B). Newly isolated PU.1^{+}Sca-1^{lo} MPPs and GATA-1^{+}Sca-1^{lo} MPPs are therefore termed here as PU.1^{+}Sca-1^{lo} GMLPs and GATA-1^{+}Sca-1^{lo} CMPs, respectively. These populations develop independently with or without the upregulation of Flt3 within the LSK fraction. Because both the PU.1^{+}Sca-1^{lo} GMLP and the GATA-1^{+}Sca-1^{lo} CMP possess potent in vivo expansion capability, these progenitors should constitute critical physiological stages for hematolymphoid development. The proposed hematolymphoid developmental scheme is shown in Figure 7.

There has been a controversy regarding differentiation activity of LMPPs (Adolfsson et al., 2005; Forsberg et al., 2006) that express a high level of Flt3 within the CD34^{+} MPP population (Figure 1D). In agreement with the report by Adolfsson et al., only rare LMPPs displayed MegE differentiation (Figure 3A), suggesting that the vast majority of LMPPs should have committed to the GM and lymphoid lineages. The rare MegE potential of LMPPs could be due to a small number of multipotent cells resides within the LMPP definition. In contrast, the PU.1^{+}Sca-1^{lo} GMLP constitutes ~40% of the LMPP and appears to be more strictly committed to the GM/lymphoid lineage (Figure 4). Furthermore, ~20% of LMPPs have activated RAG1 transcription (Figure 1C), sufficing the ELP definition (Igarashi et al., 2002). Only a fraction of ELPs possess GM potential but lack MegE potential (Figure S1A), while the majority of ELPs express a high level of PU.1 mRNA and a low level of

Sca-1 (Y.A. and K.A., unpublished data), suggesting that at least a fraction of ELPs should be included in the PU.1^{+}Sca-1^{lo} GMLP. Accordingly, a series of GM/lymphoid progenitors such as the LMPP, the PU.1^{+}Sca-1^{lo} GMLP, and the RAG1^{+} ELP exist within the MPP fraction expressing Flt3. Considering the lineage potential of each population, they may represent continuous steps for HSCs to differentiate into the lymphoid lineage where cells progressively lose MegE and then GM potential (Akashi et al., 2005).

It is important to note that the newly identified PU.1^{+}Sca-1^{lo} GMLP differentiated exclusively into CLPs (Kondo et al., 1997) and GMPs, whereas the GATA-1^{+}Sca-1^{lo} CMP gave rise to the original Sca-1^{-} CMPs (Akashi et al., 2000), which is a precursor for both GMPs and MEPs. Furthermore, the PU.1^{+}Sca-1^{lo} GMLP has potent GM and lymphoid reconstitution activity (Figure 3), and the GATA-1^{+}Sca-1^{lo} CMP had greater expansion potential to give rise to erythrocytes and GM cells as compared to the original Sca-1^{-} CMP in vivo (Figure 5B). Since the PU.1^{+}Sca-1^{lo} GMLP and the GATA-1^{+}Sca-1^{lo} CMP populations do not overlap (Figures 1A and 1B), our data provide formal evidence that the GM lineage could develop via two independent pathways initiating from each of these progenitor populations (Figure 7). This evidence strongly suggests that hematolymphoid lineage commitment has already initiated within the LSK MPP fraction. It should be critical to estimate the relative contribution of the PU.1^{+}Sca-1^{lo} GMLP and the GATA-1^{+}Sca-1^{lo} CMP toward GM lineage development in parallel. In our system, however, such analysis is currently difficult, since the PU.1$^{GFP/+}$ mouse lacks one allele of PU.1 that causes a slight decrease in the number of GM cells in vivo (Iwasaki et al., 2005b).

The successful separation of functionally distinct progenitor populations in early hematopoiesis by PU.1 or GATA-1 reporters suggests that early lineage commitment is organized at least by the antagonistic interplay of lineage-instructive signals. In line with the "priming" concept (Enver and Greaves, 1998; Iwasaki and Akashi, 2007), the original CMP coexpresses a low level of GM- and MegE-related genes including PU.1 and GATA-1 at the single-cell level (Miyamoto et al., 2002). PU.1 is expressed at a low level in CD34^{-} LT-HSCs and CD34^{+} MPPs to play a critical role in their maintenance in vivo (Iwasaki et al., 2005b), continues to be expressed at the CMP and CLP stages (Iwasaki et al., 2005b; Miyamoto et al., 2002), and is indispensable for their development (Iwasaki et al., 2005b). In contrast, GATA-1 is not expressed in CD34^{-} LT-HSCs or the vast majority of CD34^{+} MPPs (Miyamoto et al., 2002) (Figures 1B and 2A). Thus, the slight upregulation of GATA-1 at the MPP stage may result in the coexpression of PU.1 and GATA-1 at the single-cell level to form the competitive transcriptional regulatory circuit for GM versus MegE lineage fate decision, which might be required to maintain the CMP function (Iwasaki and Akashi, 2007) (Figure 7). In turn, the upregulation of PU.1 at the MPP stage up to a high level may suppress GATA-1 transcription, excluding the MegE potential to generate GMLPs. Collectively, the timing (Iwasaki et al., 2006) and the level (DeKoter and Singh, 2000; Kulessa et al., 1995) of PU.1 and GATA-1 expression might be critical in organizing the early hematopoietic lineage fate decision (Figure 7).

Prospective isolation strategy has been largely dependent upon differences in cell surface markers that correlate with lineage commitment. To separate a particular population, one needs to draw lines between populations of different expression levels for each antigen, whereas the change in the level of antigen expression in cells should occur in a continuum manner. Setting the standard for each gate is currently difficult, which could compromise accurate comparisons of data between laboratories (Akashi et al., 2005). Lineage tracing by utilizing transcription factor reporters should be a significant addition for future improvement of purification of cells with functional homogeneity. For example, we originally separated CMPs within the Lin^{-}Sca-1^{-}c-Kit^{+} fraction based upon the expression pattern of CD34 and FcγRII/III (Akashi et al., 2000). Interestingly, both PU.1^{+}Sca-1^{lo} GMLPs and GATA-1^{+}Sca-1^{lo} CMPs possess the CD34 and FcγRII/III profile indistinguishable to those in the original Sca-1^{-} CMPs (Figure S1C). Since their discrimination on FACS in the conventional staining protocol should rely only on the level of Sca-1, both the PU.1^{+}Sca-1^{lo} GMLP and the GATA-1^{+}Sca-1^{lo} CMP could fall into the original Sca-1^{-} CMP gate. This could explain the heterogeneity of the original CMP (Karsunky et al., 2003; Nutt et al., 2005): the original CMP possesses weak B cell potential (Akashi et al., 2000) that is found exclusively in its Flt3^{+} fraction (Karsunky et al., 2003). The "Flt3^{+} CMPs" with lymphoid potential could be ascribed to PU.1^{+}Sca-1^{lo} GMLP (Flt3^{+}) that fell into the Sca-1^{-} gate. In fact, the Flt3^{+} CMP possessed a higher level (Nutt et al., 2005) and copy number (Warren et al., 2006) of PU.1 mRNA as compared to the "Flt3^{-} CMP." Given the potent myeloerythroid activity observed in the GATA-1^{+}Sca-1^{lo} CMP as compared to the original Sca-1^{-} CMP, we should now redefine that the main CMP activity resides in Sca-1^{lo}GATA-1^{+} CMP (Figure 7). However, to purify the Sca-1^{lo} CMP in normal mice, we need to identify an additional Sca-1^{lo} CMP-specific antigen that can replace the GATA-1 reporter.

In summary, tracking hematopoietic development utilizing reporters for lineage-instructive transcription factors provided us with a high-resolution map within the LSK HSC population. The upregulation of GATA-1 or PU.1 at the previously defined MPP stage results in generation of the functional CMP or the GMLP with potent expansion potential in vivo. These data reasonably reconcile current controversies including the issue of myeloerythroid versus lymphoid divergence (Akashi et al., 2005; Hock and Orkin, 2005; Iwasaki and Akashi, 2007) and of possible heterogeneity of the LMPP (Adolfsson et al., 2005) and of the original CMP (Akashi et al., 2000) purified by conventional protocols. Based on the mutually exclusive interplay between PU.1 and GATA-1, HSCs might be able to lose lymphoid potential to give rise to the CMP and could also discard MegE potential to generate the GMLP during early hematopoietic development. These early LSK progenitors are

precursors for conventional hematolymphoid progenitors including the original CMP (Akashi et al., 2000) and CLP (Kondo et al., 1997). Prospective isolation of current and yet-to-be discovered stem and progenitor cell subsets by a lineage-instructive signal-based approach should thus be useful to create high-resolution maps for all blood cell lineages in future studies.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J (Ly5.2) and congenic B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (Ly5.1) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Rag2-deficient mice (B6.SJL[129S6]-*Ptprc*^{a}/BoyCrTac-*Rag2*^{tm1Fwa} N10) were purchased from Taconic Farms. *PU.1*$^{GFP/+}$ knockin (Back et al., 2004), GATA-1-GFP transgenic (Iwasaki et al., 2005a), *RAG1*$^{+/GFP}$ knockin (Igarashi et al., 2002) (kindly provided by Dr. Sakaguchi), and *gpIIb*$^{+/YFP}$ knockin (Schulze et al., 2006) mouse lines were bred and maintained in the Research Animal Facility at the Dana-Farber Cancer Institute in accordance with the guidelines.

Antibodies, Cell Staining, and Sorting

Sorting of LSK subfractions was accomplished by staining bone marrow cells with biotinylated anti-CD34 (RAM34) (PharMingen, San Diego, CA) monoclonal antibodies, PE-conjugated anti-Flt3 (A2F10), APC-conjugated anti-Sca-1 (D7), APC-Cy7-conjugated anti-c-Kit (2B8), and PE-Cy5-conjugated rat antibodies specific for IL-7Rα (A7R34) (eBioscience, San Diego, CA) and the following lineage markers: CD3 (CT-CD3), CD4 (RM4-5), CD8 (5H10), B220 (6B2), Gr-1 (8C5), and CD19 (6D5) (Caltag, Burlingame, CA), followed by avidin-PE-Cy7 (eBioscience, San Diego, CA). For myeloid progenitors sorting, bone marrow cells were stained with biotinylated anti-CD34 (RAM34) monoclonal antibodies, PE-conjugated anti-FcγRII/III (93) (eBioscience), APC-conjugated anti-Sca-1 (D7), APC-Cy7-conjugated anti-c-Kit (2B8), and PE-Cy5-conjugated rat antibodies specific for IL-7Rα (A7R34) and the lineage markers described above, followed by avidin-PE-Cy7. Myeloid progenitors were sorted as IL-7Rα^{-}Lin^{-}Sca-1^{-}c-Kit^{+}CD34^{+}FcγRII/III^{lo} (CMPs), IL-7Rα^{-}Lin^{-}Sca-1^{-}c-Kit^{+}CD34^{+}FcγRII/III^{hi} (GMPs), and IL-7Rα^{-}Lin^{-}Sca-1^{-}c-Kit^{+}CD34^{-}FcγRII/III^{lo} (MEPs) as described previously (Akashi et al., 2000). CLP staining was performed by using PE-conjugated anti-IL-7Rα (A7R34) (eBioscience), APC-conjugated anti-Sca-1 (D7), APC-Cy7-conjugated anti-c-Kit (2B8), and PE-Cy5-conjugated rat antibodies specific for the lineage markers described above. CLP was defined as a Lin^{-}IL-7Rα^{+}Sca-1^{lo}c-Kit^{lo} population (Kondo et al., 1997). All of these stem and progenitor cells were double sorted using BD FACSAria cell-sorting system (BD Biosciences, San Jose, CA).

In Vitro Assays to Evaluate Lineage Potential

To test myeloerythroid potential, single cells were sorted directly into 60-well Terasaki plates with IMDM containing 20% fetal bovine serum (FBS). Cytokines such as murine Slf (20 ng/ml), IL-3 (20 ng/ml), IL-11 (10 ng/ml), GM-CSF (10 ng/ml), Epo (4 unit/ml), and Tpo (10 ng/ml) (R&D Systems, Minneapolis, MN) were added at the initiation of cultures. Cell components of each cultures were determined morphologically and cytochemically by May-Giemsa staining. To evaluate B cell differentiation potential, cells were sorted onto irradiated (2500 cGy) OP9 stromal layers in the presence of IL-7 (20 ng/ml) and Slf (20 ng/ml). To test T cell differentiation potential, cells were sorted onto irradiated OP9-DL1 stromal layers in the presence of IL-7 (5 ng/ml) and Flt3L (5 ng/ml) (Schmitt and Zuniga-Pflucker, 2002). To evaluate the lineal relationships among LSKs, CMPs, GMPs, MEPs, and CLPs, 10,000 cells of each population were cultured for 40 hr on OP9 stromal layers in 24-well plates with IMDM containing 10% FBS in the presence of Slf (10 ng/ml). All cultures were incubated at 37°C in a humidified chamber under 5% CO_2.

In Vivo Reconstitution Assays

Five hundred cells of each population (Ly5.2) were intravenously transplanted into congenic mice (Ly5.1) after lethal (950 rad) irradiation. In some experiments, 300 LSKs purified from recipient-type (Ly5.1) mice or Rag2-deficient mice (Ly5.1) were cotransplanted to rescue transplanted mice.

Analysis of Gene Expression from Total RNA

Total RNA extracted from 200 cells each of target populations was subjected to real-time PCR analyses as described previously (Iwasaki et al., 2005a). Primer and probe sequences used in this assay are described in Table S1.

Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/4/416/DC1/.

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