Cell Stem Cell
Article

Elucidation of the Phenotypic, Functional, and Molecular Topography of a Myeloerythroid Progenitor Cell Hierarchy

Cornelis J.H. Pronk,[1,2,6] Derrick J. Rossi,[3,4,5,6] Robert Månsson,[1] Joanne L. Attema,[3,4,5] Gudmundur Logi Norddahl,[1,2] Charles Kwok Fai Chan,[3,4,5] Mikael Sigvardsson,[1] Irving L. Weissman,[3,4,5] and David Bryder[1,2,*]

[1]Lund Strategic Research Center for Stem Cell Biology and Cell Therapy
[2]Immunology Unit, Institution for Experimental Medical Science
Lund University, BMC I13, 221 84 Lund, Sweden
[3]Department of Pathology
[4]Department of Developmental Biology
[5]Institute of Stem Cell Biology and Regenerative Medicine
Stanford University School of Medicine, Stanford, CA 94305, USA
[6]These authors contributed equally to this work.
*Correspondence: david.bryder@med.lu.se
DOI 10.1016/j.stem.2007.07.005

SUMMARY

The major myeloid blood cell lineages are generated from hematopoietic stem cells by differentiation through a series of increasingly committed progenitor cells. Precise characterization of intermediate progenitors is important for understanding fundamental differentiation processes and a variety of disease states, including leukemia. Here, we evaluated the functional in vitro and in vivo potentials of a range of prospectively isolated myeloid precursors with differential expression of CD150, Endoglin, and CD41. Our studies revealed a hierarchy of myeloerythroid progenitors with distinct lineage potentials. The global gene expression signatures of these subsets were consistent with their functional capacities, and hierarchical clustering analysis suggested likely lineage relationships. These studies provide valuable tools for understanding myeloid lineage commitment, including isolation of an early erythroid-restricted precursor, and add to existing models of hematopoietic differentiation by suggesting that progenitors of the innate and adaptive immune system can separate late, following the divergence of megakaryocytic/erythroid potential.

INTRODUCTION

Hematopoiesis represents a prototype system for the study of selection, establishment, and restriction of individual developmental programs from one common precursor (Bryder et al., 2006). In this system, hematopoietic stem cells (HSCs) have the capacity to both self-propagate as well as differentiate into all blood cell lineages. Hematopoietic differentiation is a multistep process involving first the choice of HSC self-renewal versus differentiation, followed by commitment to different lineage fates. To be able to cope with the constant and varying challenges to the blood system, a considerable flexibility needs to accompany these events. Because HSCs themselves only rarely divide (Bradford et al., 1997; Cheshier et al., 1999) and are limited in numbers, it appears that the immature progeny of HSCs, which are more numerous and which harbor differential developmental and proliferative potentials, has been set up to cope with such altered demands and thereby serve to maintain a primary level of homeostatic control (Passegue et al., 2005).

Differentiation of immature progenitors into distinct hematopoietic lineages is contingent on appropriate and timely execution of specific gene expression programs. These processes are under both intrinsic and extrinsic control, which jointly serve to propagate or repress individual genes (Laiosa et al., 2006). A wide range of transcription factors are associated with lineage specificity, and the regulatory networks in which they operate are being elucidated (Blais and Dynlacht, 2005). The critical requirement of appropriate control of such factors in oligopotent—but normally not self-renewing—progenitors has been highlighted by the development of leukemias arising as a consequence of the misappropriate expression of such transcriptional regulators (Cozzio et al., 2003; Krivtsov et al., 2006). However, as such events are relatively rare, the processes governing their expression are presumably under tight regulatory control.

Insights into the cellular and molecular mechanisms underlying lineage specification have to a large extent been provided from studies utilizing in vitro assays (Brady et al., 1995; Hu et al., 1997; Iwasaki et al., 2006). Results obtained from such work are, however, limited from a physiologic point of view, emphasizing the importance of

paralleling such studies with in vivo strategies. One approach toward this goal is to determine the cellular stages that accompany development into individual hematopoietic lineages in the steady-state in vivo situation, followed by dissection of the functional and molecular properties of such cells. Using such approaches, the purification of a common lymphoid progenitor (CLP) that produces lymphoid, but not myeloid, progeny (Kondo et al., 1997) and a common myeloid progenitor (CMP) devoid of lymphoid but retaining full erythromyeloid competence (Akashi et al., 2000) suggested a separation of the lymphoid and myeloid lineages as an early branchpoint in hematopoietic lineage commitment. This model has been a useful working model but is likely an oversimplification, as suggested foremost by the low clonal frequency of mixed myeloid colonies obtainable from candidate CMP (Akashi et al., 2000; Iwasaki et al., 2005; Nakorn et al., 2003; Terszowski et al., 2005; Yoshida et al., 2006). Furthermore, Takano and colleagues provided evidence from in vitro paired daughter experiments of highly purified HSCs, where differentiation into individual myeloid lineages appeared to bypass a CMP intermediate (Takano et al., 2004). Moreover, Nutt and colleagues, using a transgenic knockin approach, separated candidate CMP based on differential expression levels of the transcription factor PU.1 and found that high-level expression of PU.1 in such cells was associated with granulocytic/macrophage offspring, whereas cells with low expression mainly produced megakaryocytic/erythroid progeny, with no evident hierarchical relationship in between those cells (Nutt et al., 2005). Consistent with this, the hematopoietic defects associated with PU.1 deficiency indicate a requirement of PU.1 for lymphoid and myeloid development while being dispensable for the generation of erythroid and megakaryocytic progenitors (Scott et al., 1994). Other studies have suggested that differentiation into the megakaryocytic/erythroid lineages may be an event upstream or in parallel to the generation of CMP (Adolfsson et al., 2005; Lai and Kondo, 2006), by a mechanism proposed to involve the transcription factor Ikaros (Yoshida et al., 2006). Understanding the cellular and molecular events underlying and accompanying such processes has broad implications, including increased understanding of aberrant hematopoiesis—such as the emergence of different types of leukemias—and for the development of effective strategies aimed at guiding differentiation toward therapeutic benefit.

Here, we demonstrate that differential cell-surface expression of signaling lymphocytic activation molecule family member 1 (Slamf1/CD150), Endoglin (Eng/CD105), and Integrin alpha 2b (Itga2b/CD41) allows for the prospective identification of a range of myeloid progenitor subsets with developmentally restricted lineage potentials, including early lineage-restricted precursors for the granulocyte/macrophage, erythroid, and megakaryocytic lineages. Genome-wide expression analyses of these subsets revealed distinct and ordered expression profiles of multiple transcription factors and growth factor receptors known to be key regulators of myeloid commitment and function, thereby illuminating the cellular and molecular steps associated with definitive restriction into distinct myeloid fates.

RESULTS

Early Hematopoietic Progenitors Exhibit Differential Expression of CD150 and Endoglin

In the mouse, Endoglin and CD150 are two cell-surface molecules associated with high expression in the primitive HSC compartment (Chen et al., 2002; Kiel et al., 2005). Because these proteins encode for cell-signaling receptors with a putative involvement in early HSC fate determination, we postulated that these markers might also exhibit differential expression patterns within other hematopoietic progenitor subsets.

To test this, we first screened for the expression of CD150 and Endoglin by using quantitative RT-PCR (qRT-PCR) in a wide range of early hematopoietic stem and progenitor cells. The minor c-kit+Lin−Sca1+ (KLS) compartment can be resolved into functionally distinct subsets based on differential expression of CD34 and flk2 (Adolfsson et al., 2001; Christensen and Weissman, 2001; Yang et al., 2005). When analyzing the three subsets within the KLS fraction (LT-HSC, CD34−flk2−; ST-HSC, CD34+flk2−; and MPPflk2+, CD34+flk2+; Figure 1A), we found highest expression of both Endoglin and CD150 (Figures 1B and 1C) in LT-HSC. Endoglin expression levels in ST-HSC and MPPflk2+ were approximately half of that of LT-HSC, whereas CD150 expression was dramatically reduced in ST-HSC and undetectable in MPPflk2+ cells (Figures 1B and 1C). Within the CLP fraction expressing flk2 (CLP^{flk2+}) (Kondo et al., 1997; H. Karsunky, T. Serwold, D. Bhattacharya, M.A. Inlay, and I.L.W., unpublished data; Figure 1A), we detected low or undetectable expression levels of Endoglin and CD150, respectively (Figures 1B and 1C). Highly differential expression of these genes was observed in the originally described myeloerythroid progenitor fractions (Akashi et al., 2000). These fractions are jointly characterized by a c-kit+Lin−Sca1− (KLS−) phenotype and can be further separated based on expression of CD34 and FcgRII/III into common myeloid progenitors (CMP, CD34+FcgRlow), granulocyte-macrophage progenitors (GMP, CD34+FcgRhigh), and megakaryocyte-erythroid progenitors (MEP, CD34−FcgRlow) (Figure 1A). Endoglin was highly expressed within the MEP fraction and at lower levels in CMP and GMP, respectively (Figure 1B). A similar expression pattern was observed for CD150, although this gene was nearly undetectable in GMP (Figure 1C). Expression levels of Endoglin and CD150 were nearly undetectable in more mature granulocytes and Ter119+ erythroid cells (Figures 1B and 1C).

Next, we phenotypically evaluated previously described myeloerythroid precursors for the cell-surface expression of CD150 and Endoglin. This analysis revealed that although GMP were uniformly low for both these markers, CMP could be subdivided into distinct CD150-negative and -positive populations, whereas MEP consisted of both CD150-positive and -negative cells as well as a large portion of cells expressing high levels of Endoglin (Figure 1D).

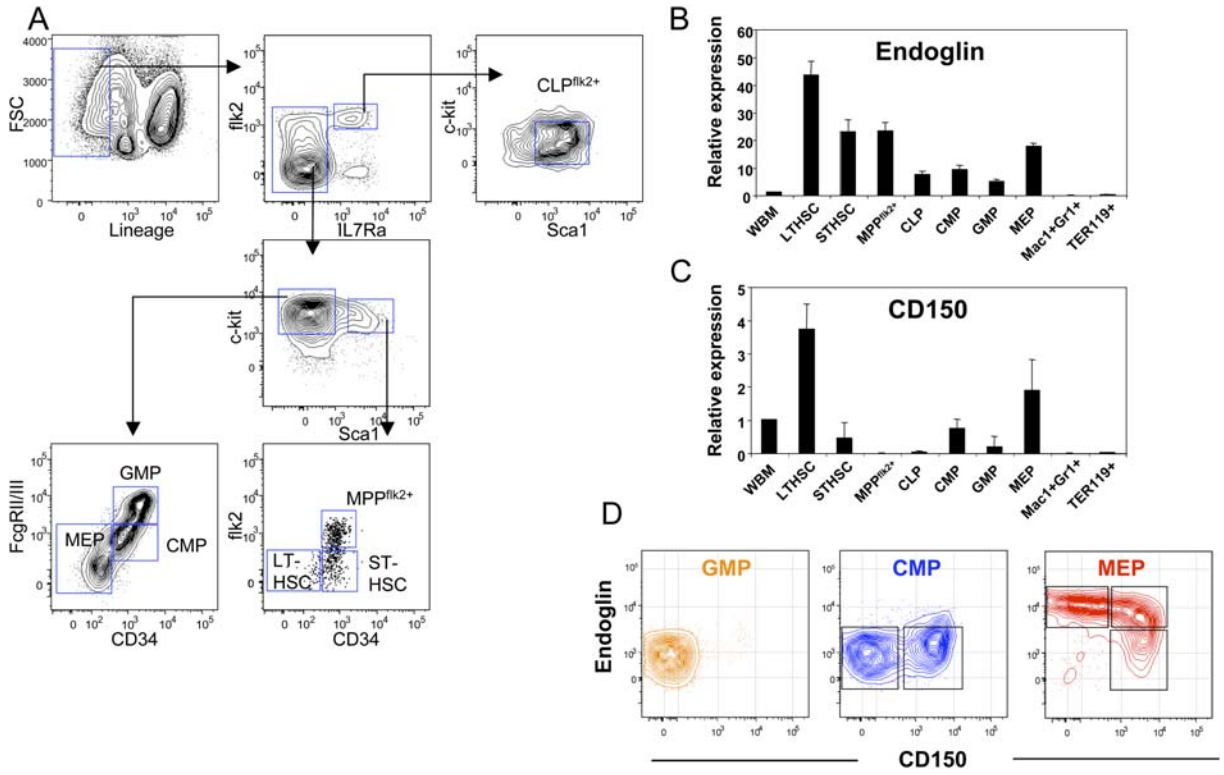

Figure 1. Endoglin and CD150 Expression Reveals Phenotypic Heterogeneity of Early Hematopoietic Progenitor Cell Compartments

(A) Phenotypic relationship of early hematopoietic precursors. c-kit-enriched BM cells were costained with antibodies against Lineage markers, Sca1, c-kit, CD34, FcgRII/III, IL7Rα, and flk2 and gated as indicated. Doublets and dead cells were excluded prior to analysis.

(B and C) RNA was extracted from sorted bone marrow LT-HSC, ST-HSC, MPPflk2+, CLP, CMP, GMP, MEP, and Mac1+Gr1+ myeloid and Ter119+ erythroid cells and subjected to quantitative RT-PCR for Endoglin (B) and CD150 (C) for each of the indicated cell types. Data show average relative expression to unfractionated bone marrow cells ± standard deviation (SD) (after normalization to β-actin).

(D) Representative cell-surface expression of Endoglin and CD150 within the CMP, GMP, and the MEP myeloid progenitor compartments.

High-Resolution Fractionation of a Myeloerythroid Progenitor Cell Compartment

The differential expression of CD150 and Endoglin in the myeloid progenitor compartment suggested that these markers might be used to subfractionate functionally distinct cell types. We therefore developed an isolation protocol that simultaneously assessed cell-surface expression of Endoglin, CD150, FcgRII/III, and CD41 in the KLS− compartment. We included CD41 in our fractionation protocol, as it has been reported that CD41-high cells within this compartment are committed to the megakaryocytic lineage (Nakorn et al., 2003). By contrast, we decided to omit the use of CD34, as preliminary experiments had demonstrated that this marker did not add further functional resolution to our seven-color protocol (D.B., unpublished data). Six resolvable cell populations within the KLS− myeloid progenitor compartment could be identified (Figure 2). These individual populations showed evident morphological differences, suggesting that they might be functionally distinct (Figure 2), and will hereafter be referred to as Pre MegE, Pre CFU-E, CFU-E, MkP, Pre GM, and GMP based on the subsequent characterization work in these studies (see below).

CD150 and Endoglin Expression on Myeloerythroid Precursors Is Associated with Distinct Lineage Outcomes In Vitro

The myeloid progenitor subsets obtainable from the isolation protocol described in Figure 2 were next functionally evaluated at the clonal level by in vitro assays. First, we determined their plating efficiencies. With the exception of CFU-E, a high proportion of cells from all other subsets could be induced to proliferate in response to a combination of early acting cytokines, with plating efficiencies ranging from 45% to 75% (Figure 3A). Lineage potential of these cell subsets was evaluated by methylcellulose and agar cultures. In these assays, MkP was exclusively associated with megakaryocyte generation (Figure 3B) and thus appears functionally equivalent to the CD9-positive megakaryocyte-committed precursor described previously (Nakorn et al., 2003). Consistent with this, MkP cells were found to express high levels of CD9 mRNA (D.B., unpublished data), and CD9-positive MkPs were found to be CD150+, with very similar in vitro lineage potentials of these subsets (Figure S1 in the Supplemental Data available with this article online).

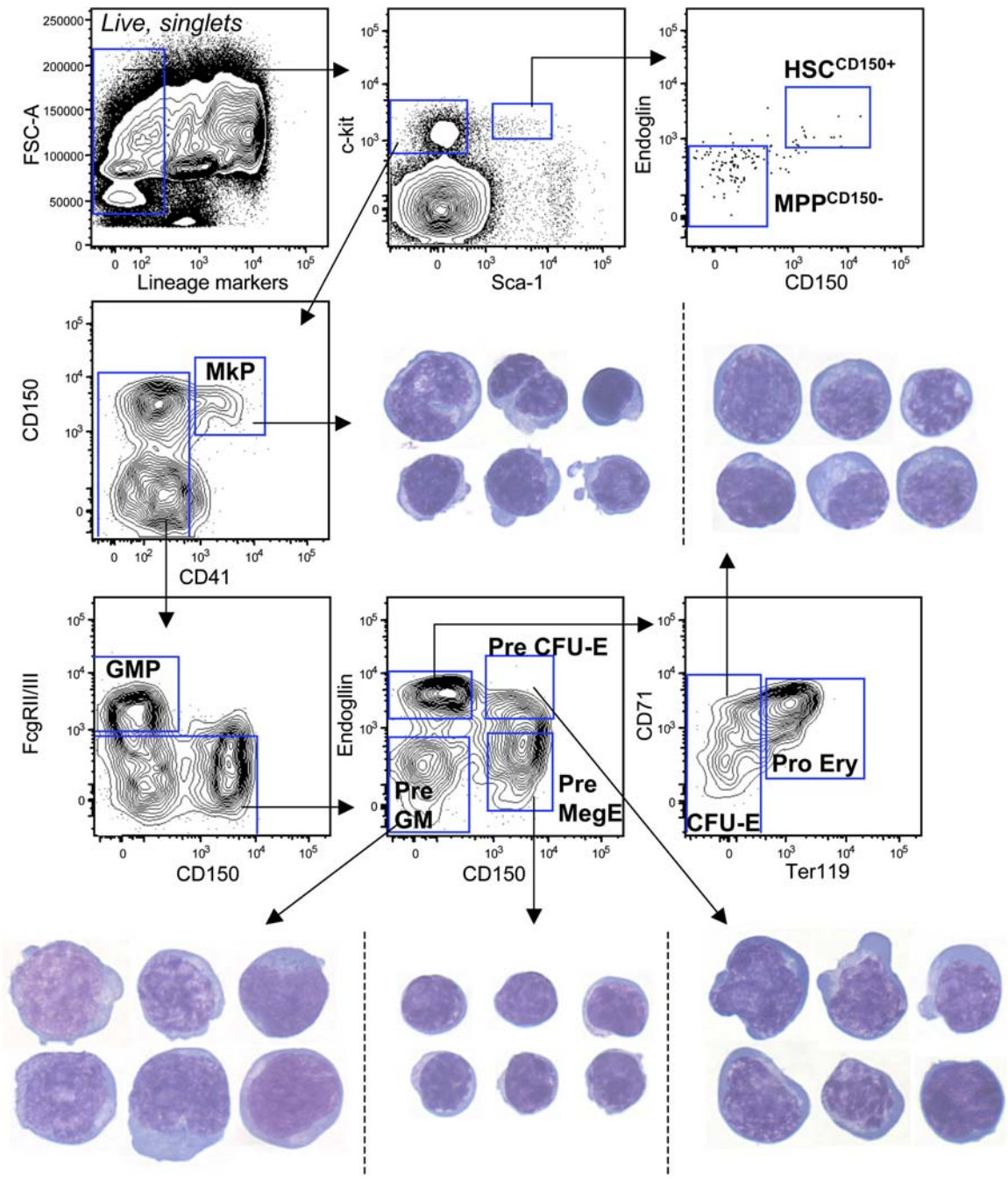

Figure 2. High-Resolution Fractionation Murine Myeloid Precursors

Murine bone marrow cells were stained with antibodies against Sca1, c-kit, CD41 (Itga2b), Endoglin (Eng/CD105), CD150 (Slamf1), FcgRII/III, Ter119, CD71, and a cocktail of mature blood cell lineage markers (Lin). Cell preparations were also stained with Propidium Iodide, and only live cells are displayed. Based on this phenotypic analysis, two populations within the KLS compartment and six populations within the KLS− compartment were defined. Morphology of KLS− cellular subsets was determined with Grunewald-Giemsa staining of cytospins from sorted fractions. Pro Ery indicates a previously described proerythroblast population (Socolovsky et al., 2001). Flow cytometric profiles are representative of more than 50 individually stained BM preparations.

The GMP described here produced primarily granulocytes and/or macrophage colonies (94%, Figure 3B) and are thus functionally equivalent to the previously described GMP (Akashi et al., 2000). Within the KLS−CD41lowFcgRlow/− compartment, Pre GM showed a remarkably similar clonal lineage output to the FcgR-positive GMP (82% granulocyte/macrophage clones), although rare megakaryocytic and/or erythroid elements

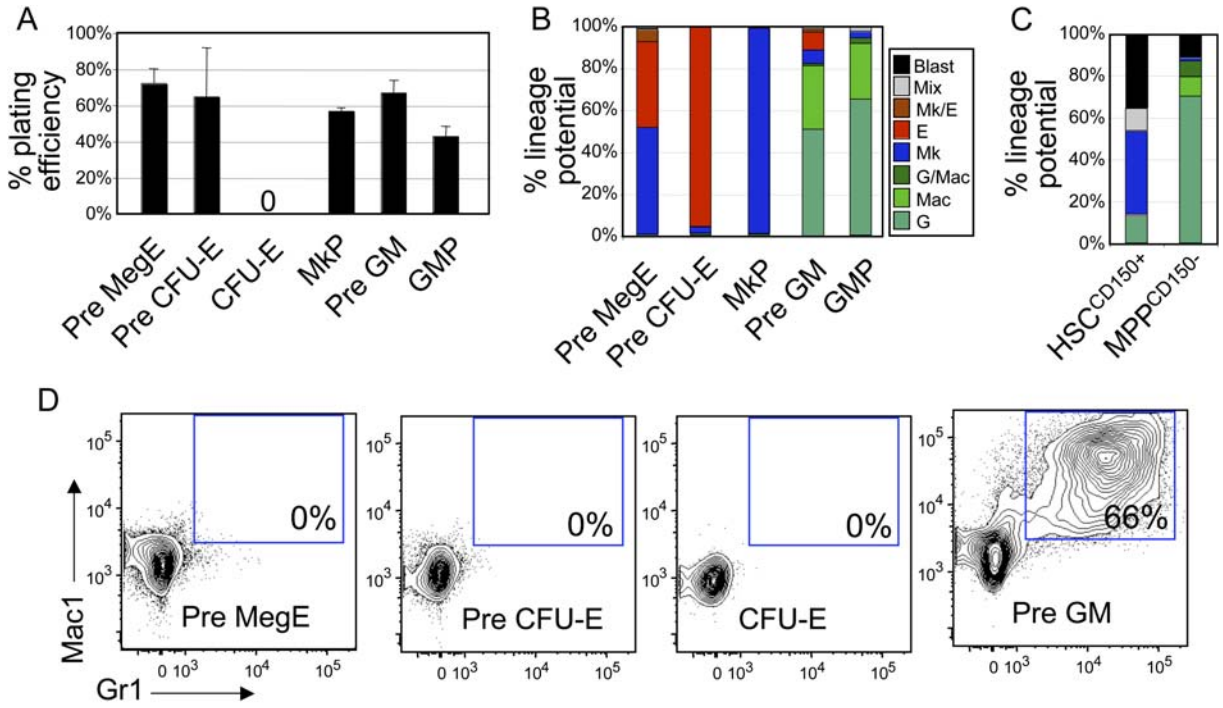

Figure 3. In Vitro Lineage Potential of Candidate Myeloid Precursor Subsets Separated Based on CD150, Endoglin, and CD41

(A) Indicated cell types were single-cell sorted by FACS into liquid cultures as described in the Experimental Procedures. Four, seven, and eleven days after seeding, cloning frequencies were evaluated of 320–480 individual cells for each population (±SD).

(B and C) To evaluate lineage potentials, indicated cell types were isolated and grown in agar cultures. Seven days after seeding, colony types and sizes were analyzed after fixation and stainings of intact cultures, as described in the Experimental Procedures. Data are from two experiments, with six or eight replicates for each population.

(D) Five-hundred cells of indicated cell type were plated in OP9 stroma-supported liquid cultures in the presence of KL, IL3, and EPO. After 5 days of culture, cells were stained with antibodies against Mac1 and Gr1 and expression was visualized by flow cytometry.

were also consistently observed from this cell population (Figure 3B and C.J.H.P. and D.B., unpublished data). Single Pre MegE cells effectively produced megakaryocytic, erythroid as well as mixed megakaryocyte/erythroid colonies. In contrast, Pre CFU-Es gave rise almost exclusively to erythroid colonies of various sizes (Figure 3B). Stroma-supported cultures confirmed that granulocyte/macrophage potential of KLS−CD41lowFcgRlow/− cells was restricted to Pre GM (Figure 3D). Lineage potential of CFU-Es had to be determined in methylcellulose cultures due to their inability to proliferate in agar cultures (see below, Figure 6), but we noted that such cells could be induced to proliferate to some extent by using OP9 stroma-supported cultures (Figure 6).

We next examined the potential of the more primitive cells upstream in the KLS compartment to see whether differential expression of CD150 within this compartment might also be predictive of lineage potential. KLSCD150+ cells, which contain all HSC activity (Kiel et al., 2005), were found to be associated with multiple colony types, including many blast colonies, supporting the notion that this fraction contains many immature cells that do not mature fully within the 7 day cell culture protocol employed here (Figure 3C). Of note was that this fraction also produced a high portion of clones containing exclusively megakaryocyte progeny (137 out of 400 cells evaluated, Figure 3C). By contrast, the KLSCD150− population, which contains multipotent progenitor but no stem cell activity (Kiel et al., 2005), essentially lacked megakaryocyte potential at a clonal level, with only 2 out of 400 cells evaluated yielding megakaryocytes (Figure 3C). These cells were nonetheless very efficient in producing granulocyte and/or macrophage progeny, which were observed in 88% of evaluated clones (Figure 3C). Taken together, these data establish that differential expression of CD150 and Endoglin of early myeloerythroid progenitors is associated with functional differences in their in vitro lineage potentials, a feature extended to a high degree also to the immature KLS compartment.

CD150 and Endoglin Expression on Myeloerythroid Precursors Is Associated with Distinct Lineage Outcomes In Vivo

We next evaluated the in vivo reconstituting activities of these cells. KLS−CD150+/− myeloid progenitors were sorted from mice where GFP expression is driven from a ubiquitously expressed *β-actin* promoter. Apart from marking donor white blood cells, this system also permits readout of mature platelets (Nakorn et al., 2003). Despite the high-clonal megakaryocyte capacity of CD150+

Table 1. Capacity of Prospectively Isolated Myeloid Subsets to Generate Platelets and Granulocytes In Vivo

Cell Type	Number of Injected Cells	Lineage Evaluated	Percentage of Donor-Derived Cells		
			Day 7	Day 14	Day 52
No cells	0	Platelets	0	0	0
		Granulocytes	0	0	0
Unfractionated BM	1,500,000	Platelets	3.5 ± 1.5	39.5 ± 3.6	49 ± 2.7
		Granulocytes	14.4 ± 10.1	78.5 ± 1.2	71.7 ± 6.2
Myeloid progenitors, CD150−	6,000	Platelets	0.15 ± 0.05	0.56 ± 0.72	0
		Granulocytes	5.6 ± 4.6	7.3 ± 11.4	0
Myeloid progenitors, CD150+	1,500	Platelets	0.23 ± 0.04	0.07 ± 0.03	0
		Granulocytes	0.1 ± 0.18	0	0
MPP, CD150−	300	Platelets	0.3 ± 0.16	3.2 ± 1.6	0
		Granulocytes	1.2 ± 0.03	23.1 ± 10.3	0.14 ± 0.02
HSC, CD150+	75	Platelets	0.43 ± 0.25	20.3 ± 2.7	42.2 ± 8.8
		Granulocytes	0.1 ± 0.18	14.8 ± 7.6	55.3 ± 10.9

Sublethally irradiated recipients (450 rad) were transplanted with 1.5×10^6 GFP expressing whole BM cells or the indicated number of isolated stem and progenitor cells estimated to be contained within 1.5×10^6 unfractionated BM cells. At days 7, 14, and 52 after transplantation, mouse PB was analyzed for the levels of donor platelets and granulocytes, respectively. n = 3 recipients receiving each cell type and show mean ± SD.

myeloid progenitors in vitro (Figure 2B), in vivo platelet reconstitution was found to be limited with such cells (Table 1). This was in striking contrast to that observed for the CD150+ HSC subset, which already showed rapid and robust platelet recovery 7–14 days after transplantation of as few as 75 cells (Table 1). Peak production of granulocytes was observed with CD150− myeloid progenitors 7 days after transplantation, a time point where CD150− myeloid progenitors reconstituted the granulocytic lineages to significantly higher degrees than observed for either of the control KLS subsets (Table 1). The failure to produce mature progeny in vivo beyond these early time points (Table 1) confirms the lack of self-renewal potential of these myeloid subsets.

To evaluate erythroid potential in vivo, we performed day 8 spleen colony forming assay (CFU-S8) (Na Nakorn et al., 2002) and also analyzed reticulocyte recovery after transplantation. In contrast to transplantation of 200 Pre MegE cells, which generated large CFU-S8 at high frequency (~1/10) (Figure 4A, right), transplantation of 200 Pre GMs gave rise to few CFU-S8 colonies (Figure 4A, left). We also transplanted lethally irradiated recipients with cells of each subset in order to gauge their in vivo erythroid potential, because rapid erythropoiesis is critical to circumvent the consequences of a lethal irradiation (Na Nakorn et al., 2002; Uchida et al., 1994) (Figure 4B). We observed substantial mortality at 12 days after transplantation; however, enhanced survival was observed in mice receiving either HSCCD150+ HSCs, Pre MegE, or Pre CFU-E cells (3/5, 4/8, and 3/8 recipients, respectively; Figure 4B). Survival above background was not detected in mice transplanted with Pre GM and GMP, and no reticulocytes were detected in peripheral blood of surviving mice in these groups (Figure 4B). By contrast, large numbers of reticulocytes were observed in mice transplanted with either Pre MegE (mean 20.7%, n = 4) or Pre CFU-E (mean 7.2%, n = 3) cells. Neither HSCCD150+ nor CFU-E produced substantial amounts of reticulocytes by 12 days (Figure 4B). For HSCs, this is consistent with the notion that extensive erythroid generation from low numbers of primitive HSCs is too slow to be detected 12 days post-transplantation (Uchida et al., 1994). The failure of CFU-E cells to generate a substantial amount of reticulocytes in vivo is consistent with the limited proliferative potential of CFU-E cells in vitro (Figure 2B).

Taken together, these data establish that the in vitro lineage potential of these subsets is recapitulated in vivo. These data also demonstrate that rapid in vivo generation of platelets is critically contingent on the transfer of immature, rather than early lineage-restricted MkPs. This is in contrast to the recoveries of neutrophils and erythrocytes, which were both rapid and high for the prospectively isolated myeloid subsets associated with these lineages.

Single-Cell Multiplex PCR Fails to Demonstrate Robust Evidence of Mixed Myeloid Potential at a Clonal Level

Given the apparently defined lineage potentials of these fractions, we next performed multiplex single-cell RT-PCR of Pre MegE, Pre CFU-E, MkP, and Pre GM cells in order to investigate whether the lineage restriction of these subsets could be traced also at a molecular level. We chose to investigate the simultaneous expression of *myeloperoxidase* (*Mpo*) due to its association with the granulocyte/macrophage lineages, *Mpl* (Thrombopoietin receptor) and *Willebrand factor* (*Vwf*) for the megakaryocytic

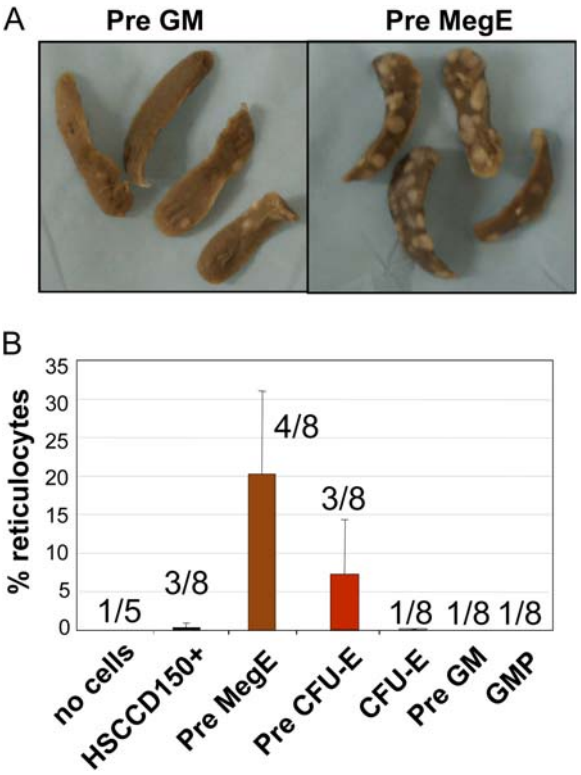

Figure 4. In Vivo Erythroid Repopulation Activities of Progenitors Separated Based on CD150 and/or Endoglin Expression

(A) Two-hundred Pre GM or Pre MegE cells were transplanted into lethally irradiated hosts, and 8 days after transplantation, spleens were harvested and fixed, followed by macroscopic evaluations of generated colonies.

(B) Two-hundred-fifty candidate HSC or 2500 of the indicated myeloid progenitor type were transplanted into lethally irradiated hosts, and after 12 days, peripheral blood was analyzed for reticulocyte levels in surviving lethally irradiated recipients. For each cell type, eight recipients were transplanted, and the number of surviving mice is indicated in addition to levels of reticulocytes in peripheral blood at day 12 (mean ± SD).

lineage, and *Erythropoietin receptor* (*Epor*) and *Kruppel-like factor 1* (*Klf1*) as erythroid lineage markers. *c-kit* was included in experiments as an internal PCR control, as all these subsets are characterized by high *c-kit* expression.

Ninety percent of single MkP cells expressed *Mpl* and most also coexpressed *Vwf* (Figure 5). Coexpression of *Epor* was noted for many (59%) of these cells, consistent with Erythropoietin being a potent stimulator of megakaryocyte development (Metcalf et al., 2002). By contrast, only 7% of single MkP cells expressed *Klf1*. These data are consistent with our functional data, indicating that MkP cells are restricted to a megakaryocyte fate (Figure 3B). All Pre CFU-E cells were found to express *Epor*, and most of these cells (73%) also coexpressed *Klf1*. Some Pre CFU-E cells exhibited *Vwf* expression (15%); however, no cells of this fraction were found to express either *Mpl* or *Mpo*, consistent with their restriction to the erythroid lineage. By contrast, Pre MegE cells, which

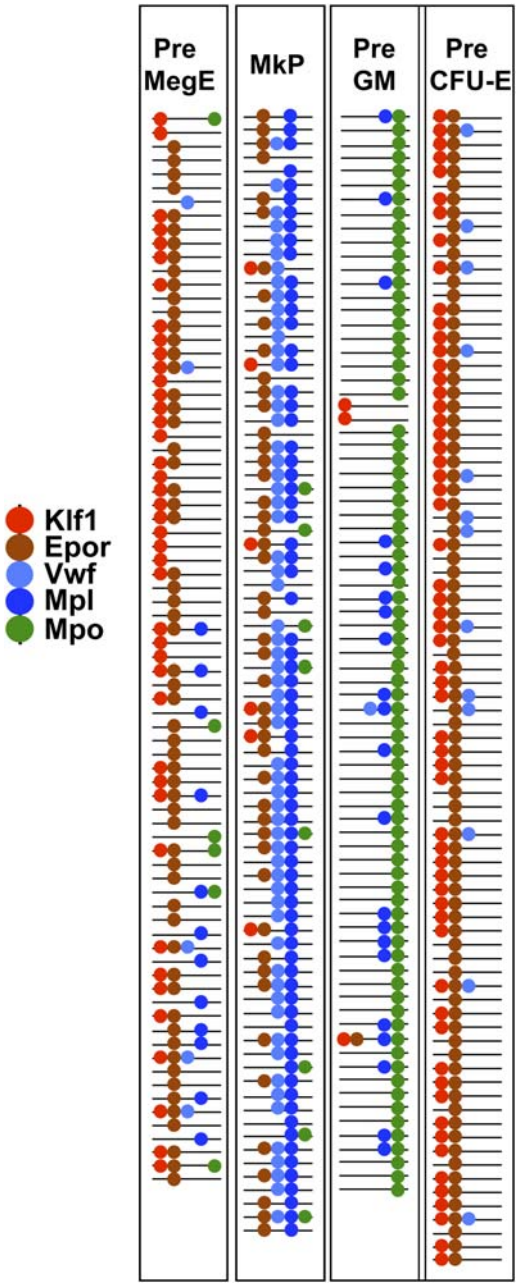

Figure 5. Multiplex Single-Cell Gene Expression of Lineage-Affiliated Genes of Myeloid Precursors Prospectively Separated Based on Differential Expression of CD150, Endoglin, and CD41

Indicated cell types were single-cell sorted directly into lysis buffer in 96-well PCR plates. After reverse transcription and a two-step nested PCR approach, c-kit expressing cells (positive control, data not shown) were analyzed for presence or absence of indicated gene expression by using a multiplex single-cell RT-PCR approach. Each line indicates a single cell, and presence of transcripts in individual cells is shown by uniquely colored dots for indicated genes. In these experiments, three to six cells of each fraction were positive for c-kit but negative for all other genes; these cells have been omitted from analysis.

produce both erythroid megakaryocytic as well as a few mixed megakaryocyte/erythroid clones (Figure 3B), expressed *Epor*, *Mpl*, and *Klf1* in various combinations but only rarely (8% of cells) expressed *Mpo*, consistent with their commitment to a megakaryocyte/erythroid fate. In striking contrast, 97% of single Pre GM cells expressed *Mpo*, 27% of which also coexpressed *Mpl*. Of all the cell types investigated, only a few scattered cells were found to simultaneously express granulocyte/macrophage with megakaryocytic- and erythroid-oriented genes, and no cell (0/323 cells scored) was found to simultaneously express all of the investigated genes.

Taken together, these data are in agreement with the functional properties associated with each of the investigated cell types. Importantly, these experiments provide little evidence that a substantial fraction of the candidate myeloid progenitors evaluated here displayed transcriptional properties associated with mixed myeloid lineage potential (megakaryocytic/erythroid/granulocytic/macrophage).

Proliferation and Differentiation In Vitro Suggest a Hierarchical Structure of Murine Myeloid Progenitors

In order to delineate the hierarchical relationship between these myeloid progenitors, we analyzed the burst sizes of colonies generated from the described progenitor fractions, reasoning that higher proliferative activity correlates with primitiveness (Baines et al., 1982). These experiments demonstrated that clone sizes obtained from Pre GM cells were larger than those obtained from GMP (Figure 6A). Moreover, in short-term liquid cultures, Pre GM cells rapidly generated phenotypic GMP, whereas GMP could not generate phenotypic Pre GM (C.J.H.P. and D.B., unpublished data). These results are consistent with the interpretation that Pre GM lie developmentally upstream of GMP.

Similarly, erythroid clone sizes were larger from Pre MegE than for Pre CFU-E, suggesting a hierarchical progression from Pre MegE to Pre CFU-E (Figure 6B). Consistent with this, clones obtained from Pre MegE were found to contain CFU-E-like clusters at day 7 (D.B., unpublished data). As clonal liquid culture assays did not support growth of CFU-E (Figure 2B), we compared proliferative potentials of Pre CFU-E and CFU-E with methylcellulose cultures. CFU-E gave primarily rise to single-cluster CFU-Es, whereas Pre CFU-E produced not only CFU-Es (many which comprised two to three clusters) but also some larger sized BFU-Es (Figure 6C). KLS− cells with robust erythroid potential were also cultured in stroma-supported liquid media. After 4 days of culture, 9.3% of cells generated from Pre MegE still exhibited an immature Ter119-c-kit+ phenotype, whereas these cells were detected at progressively decreased levels from cultures with Pre CFU-E and CFU-E cells (Figure 6D). These experiments established that Pre MegE can generate phenotypic Pre CFU-E and CFU-E, and that Pre CFU-E can generate CFU-E, and thereby suggest a developmental progression of early erythropoiesis wherein Pre MegE give rise to Pre CFU-E, which in turn differentiates into CFU-E.

We next assessed the proliferative capacities of cells with megakaryocyte potential. MkPs generated single megakaryocytes in 85% of wells (Figure 6E) and only rarely larger clones, indicating their proximity and restriction to a mature megakaryocytic fate. In contrast, megakaryocyte colonies generated from Pre MegE generated two or more cells in 40% of the wells, suggesting a hierarchical progression from Pre MegE to MkP. Consistent with these observations, 85% of megakaryocyte containing clones generated from single CD150+ HSCs gave rise to two or more cells and generated a substantially higher number of large megakaryocyte clones compared to both Pre MegE and MkP (Figure 6E).

Analysis of Global Gene Expression Patterns Reveals Specific Genetic Programs Associated with Lineage Restriction within the Early Myeloerythroid Compartment

Having established the distinct functional properties associated with subsets of early myeloid progenitors, we undertook a global gene expression approach in order to objectively evaluate their molecular identities and to evaluate the transcriptional changes accompanying early unilineage megakaryocytic and erythroid restriction.

To this end, we FACS purified 2,000–10,000 Pre MegE, Pre GM, MkP, and Pre CFU-E cells, followed by RNA extraction, reverse transcription, linear amplification, and hybridization to Affymetrix 430.2 arrays, which contain ~46,000 probe sets representing ~36,000 genes. In addition, we also generated an expression signature of CLP^{flk2+} cells (H. Karsunky, T. Serwold, D. Bhattacharya, M.A. Inlay, and I.L.W., unpublished data). With this approach, we were aiming at simultaneously exposing such genes that are involved in early lymphoid versus myeloid fate decisions.

After filtration (see Experimental Procedures), 3184 genes remained and were subjected to hierarchical clustering. This analysis confirmed the distinct gene expression characteristics of the various progenitor subsets and suggested an internal relationship of these cell types based on their transcriptional similarities (Figure 7A).

Next, we focused our analyses more directly on the transcriptional changes associated with early lineage establishment. To this end, we investigated the genes passing the above filtering criterion and investigated genes with a 2-fold or higher expression change between CLP, Pre GM, and Pre MegE (with a 90% confidence interval). These analysis criteria revealed that only 623 probe sets that were differentially regulated within these cell types (298 associated with Pre MegE, 147 with Pre GM, and 178 with CLP^{flk2+}) (Figure 7B, full gene lists from these analyses can be found in Table S2). In order to evaluate the quality of these data by independent means, we performed quantitation real-time PCR (qRT-PCR) of several of the genes identified by our microarray approaches, including *Gata1*, *Gata2*, *EpoR*, *granulocyte-colony stimulating factor 3 receptor 1* (*G-csfr*), *macrophage colony*

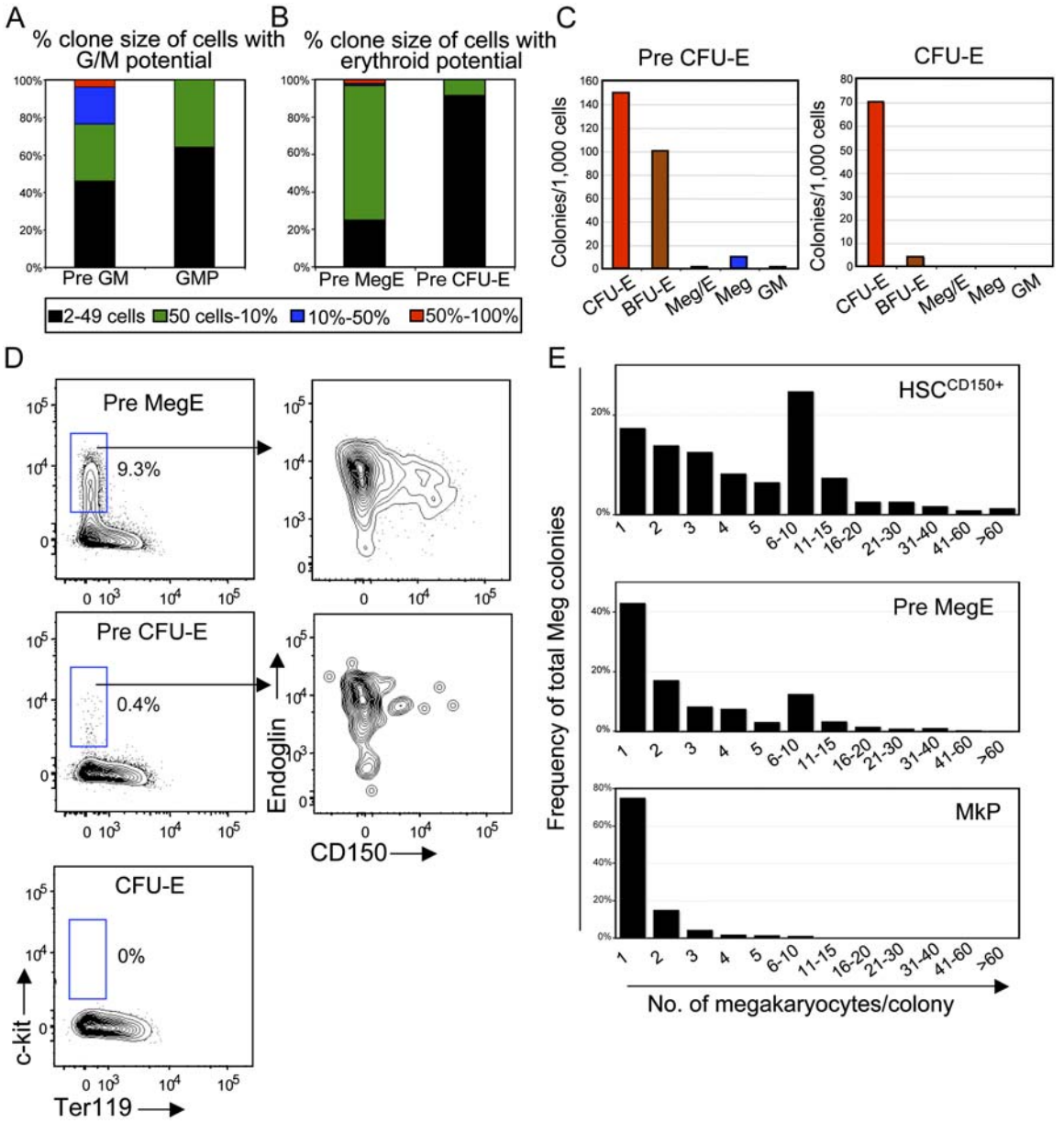

Figure 6. Hierarchical Structure and Proliferative Capacities of Candidate Myeloid Precursors Prospectively Separated Based on Differential Expression of CD150, Endoglin, and CD41

(A and B) Single Pre GM, GMP, Pre MegE, and Pre CFU-E cells were plated in IMDM supplemented with FCS, KL, IL3, and EPO in 60-well Terasaki plates by using a flow cytometry coupled single-cell depositor. Seven days after initiation of cultures, clone sizes were determined from cells generating either (A) granulocyte/macrophage or (B) erythroid progeny, respectively, and scored according to criteria as described previously (Bryder and Jacobsen, 2000).

(C) Pre CFU-E and CFU-E were plated at low densities in methylcellulose. CFU-Es were enumerated at day 3 and BFU-E at day 7.

(D) Five-hundred cells of indicated cell type were plated in stroma (OP9)-supported liquid cultures in the presence of kit ligand, Interleukin 3, and Erythropoietin. After 5 days of culture, cells were stained with antibodies against c-kit, Ter119, Endoglin, and CD150, and expression of these markers on generated progeny was visualized by flow cytometry.

(E) HSC, Pre MegE, and MkP were plated at low densities in agar cultures. After 7 days of culture, the number of acethylcholinesterase-positive megakaryocytes was determined in colonies containing such cells.

stimulating factor 1 (*M-csfr*), *CCAAT/enhancer binding protein alpha* (*Cebpa*), *Terminal deoxynucleotidyl transferase* (*Dntt*), *immunoglobulin heavy chain 6* (*Igh-6/sterile IgM*), and *Notch 1*, which showed that the qRT-PCR data were in close agreement with the microarray data (Figure 7C).

When exploring the differentially expressed genes associated with early lineage restriction, we could identify

multiple well-known regulators of such processes in addition to a range of genes associated with lineage specificity. For instance, for the Pre MegE cluster, we observed high expression of *Glycoprotein 1* (both α and β polypeptides), *von Willebrand factor* (*Vwf*), *Thrombin receptor*, and *Glycoprotein 5*: genes with nonredundant functions specific to platelet function. Expression levels of these genes were further elevated in MkP (Table S2), in agreement with the notion that the transition of Pre MegE into lineage-restricted MkP is accompanied by a strengthening of the genetic program associated with terminal differentiation. Also, well-characterized transcriptional regulators of megakaryocyte development, including *Gata2* and *Zfpm1* (*Fog1*), exhibited this pattern of expression, although significant expression was noted for both of these genes also in the Pre CFU-E population. Similarly, multiple genes associated with erythroid specification, including *Gata1*, *Tal1* (*Scl*), *Gfi1b*, *Kruppel–like factor 1* (*Klf1*), and *Epor* were robustly expressed in Pre MegE cells and further elevated in Pre CFU-E (Table S2).

Of the genes associated with high expression in Pre GM cells, we observed several growth factor receptors associated with neutrophil/macrophage production, including *G-csfr*, *M-csfr*, and the β common chain for Interleukin-3, Interleukin-5, and GM-CSF (Figures 7B and 7C). Elevated expression of the transcription factor *Cebpa* was also associated with this subset, as was expression of *lysozyme* and *Mpo*, all well-characterized neutrophil and macrophage effector molecules (Borregaard and Cowland, 1997). A somewhat intriguing expression pattern was observed for a number of the genes associated with the Pre GM subset, including for instance *M-csfr* and *G-csfr*; as we could observe clear expression of such genes also in CLP^{flk2+}. Conversely, several genes with high expression in CLP^{flk2+} cells, including *Dntt*, *Notch 1*, and *sterile IgM*, were expressed also in Pre GM cells (Figure 7C). These data are consistent with the lineage proximity of CLP^{flk2+} and Pre-GM cells that emerged from the hierarchical clustering analysis (Figure 7A).

Based on the large number of genes previously shown to be associated with myeloid lineage restriction and various myeloid effector cell functions, we conclude that the global expression patterns obtained here are in striking accordance with the functional behavior of these subsets. Taken together, these data illuminate the molecular circuitry underlying the specification of distinct myeloid fates and provide previously unknown insights into the lineage relationship existing between different progenitors. Moreover, in addition to the many genes with well-established roles in fate specification, these data reveal a large number of candidate myeloid specification genes that have not been previously implicated in such fate decisions.

DISCUSSION

Unraveling the stepwise differentiation processes that lead from multipotent stem cells to mature effector cells is critically important for understanding both normal and neoplastic hematopoiesis. The isolation of progenitors with well-defined functional and molecular properties is an important step toward this goal. In this work, we have explored the processes of myeloid cell differentiation and reveal a number of novel intermediate progenitors (Figure 7D). Our data showing the potent in vivo reconstituting activities of several of these subsets in the short-term transplantation setting highlight their potential utility for cellular therapy, although the limited in vivo platelet reconstitution potential obtained from committed MkPs suggest a unique requirement for transplantation of more immature precursors to ensure robust in vivo generation of this lineage.

Our studies demonstrate distinct transcription patterns associated with the progenitor subsets identified in this work. In accordance with their functional properties, such patterns are characterized by differential expression of multiple established regulators for the individual myeloid lineages. In addition to the well-characterized lineage specification genes we identified, it is likely that many of the lesser known genes we found to be enriched in each of the progenitor subsets will likely play important roles in lineage specification, which may be borne out of future studies.

The possibility of generating genome-wide transcriptional signatures of prospectively isolatable and well-defined cellular subsets has provided a means through which lineage relationships can be correlated to an entire transcriptome. Such approaches uncovered an unexpectedly close relationship between progenitors committed toward lymphoid and granulocyte/macrophage lineages as opposed to cell populations containing merely megakaryocytic/erythroid potentials. The clustering of the Pre MegE and CFU-E against MkP cells at this branch could be explained by the presence of a higher number of erythroid-primed or -committed cells within the heterogeneous Pre MegE, giving this population more of an erythroid than a megakaryocytic global transcription signature. In addition to genome-wide clustering analysis, the close lineage relationship between CLP and Pre-GM progenitors was supported by the expression of individual genes such as *sterile IgM* and Dntt (*TdT*) transcripts in Pre GM cells, transcripts traditionally associated with lymphopoiesis (Medina et al., 2001). In parallel, significant expression of myeloid-oriented growth factor receptors such as *M-csfr* and *G-csfr* were highly expressed in CLP^{flk2+}. Evidence for a developmental relationship between the lymphoid and granulocyte/macrophage lineages was recently provided by observations of residual granulocyte/macrophage capacity of isolated lymphoid progenitors (Balciunaite et al., 2005; Rumfelt et al., 2006) and by the identification of early progenitors with robust lymphomyeloid but little megakaryocyte/erythroid potential (Adolfsson et al., 2005; Lai and Kondo, 2006). Those studies suggested that the megakaryocytic and/or erythroid lineages can diverge prior to a lymphoid/myeloid branchpoint (CMP/CLP), a view that is in accordance with the data presented here and proposed more than 10 years ago based primarily on the hematopoietic defects in *PU.1*-deficient mice (Singh, 1996). On the other hand, one must be

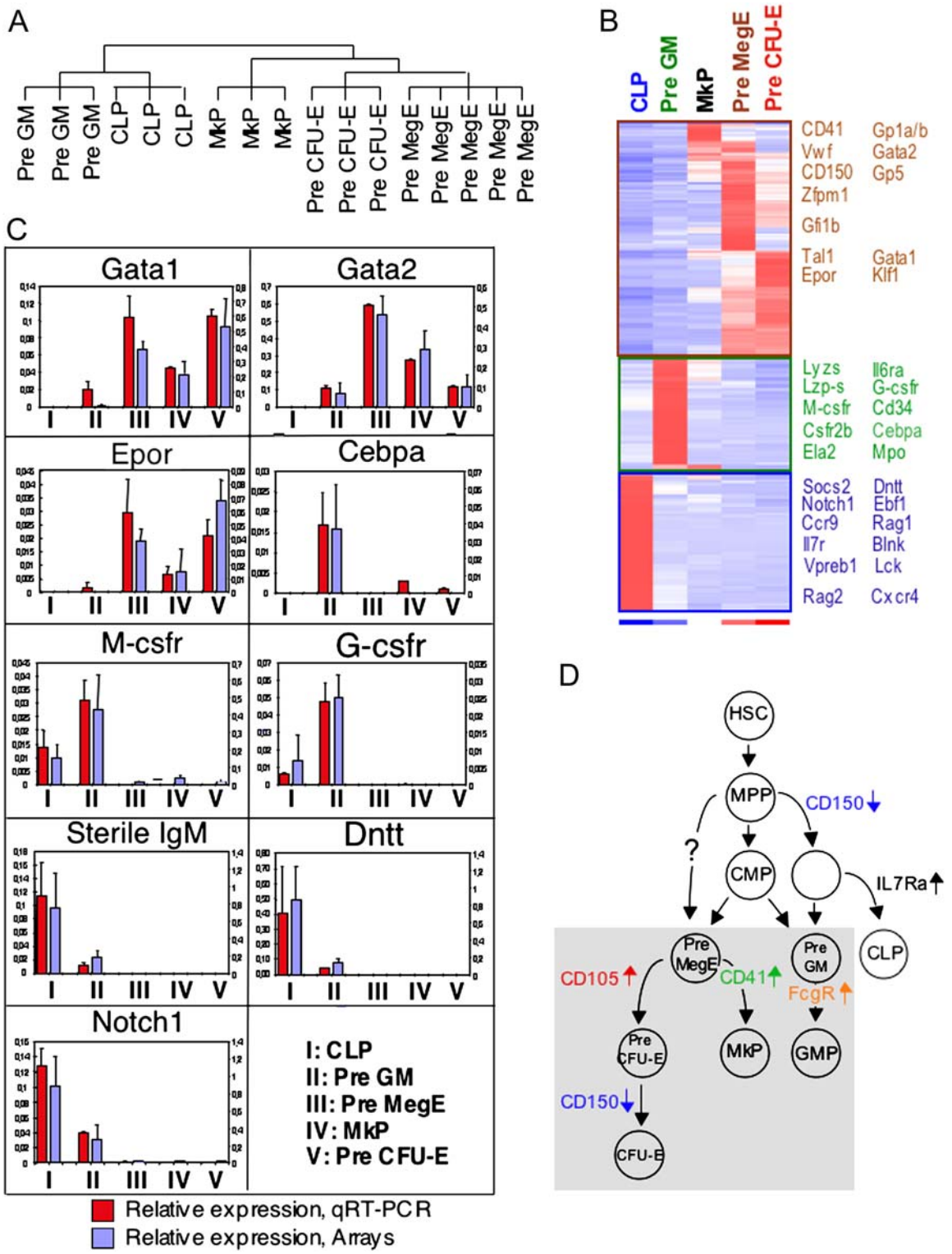

Figure 7. Genome-wide Expression Signatures of Early Myeloid Progenitors and Developmental Position of Precursors Described in This Work

RNA was extracted from 2,000–10,000 cells of each indicated fraction, followed by linear amplification, and hybridized to Affymetrix 430A 2.0 arrays. (A) Clustering of cell fractions based on total transcripts on arrays. Replicate arrays were pooled, followed by gene filtering (0.5 < SD/mean < 1000 and present calls in at least one subset) to eliminate noise and genes not differing in expression in between samples. Filtering resulted in 3184 probe sets, on which overall clustering was performed.

mindful of the absoluteness of such pathways, as we and others do observe rare cells in the myeloid progenitor compartment investigated here that possess mixed myeloid lineage potential. In fact, it appears likely to us that the frequency of such cells might be even higher, as improvements in marker detection (i.e., for Sca1) do not justify direct comparison to original studies of CMP.

Several reports have demonstrated that lineage conversion can occur in already lineage committed cells, most convincingly evidenced by the presence of immunoglobulin or T cell receptor rearrangements in myeloid progeny (Drynan et al., 2005; Kondo et al., 2000; Xie et al., 2004). Such studies have implicated the possibility of lineage plasticity to accompany hematopoietic progenitor biology. When seeded in vitro, lineage switching of CLP into the granulocyte/macrophage lineages could only be induced within the first 2 days after plating, but not at later time points (Kondo et al., 2000). Similar observations were recently made for *Cebpa*-mediated conversion of CLP and MEP, with a notably wider time window through which conversion could be achieved for megakaryocyte precursors (Fukuchi et al., 2006). Such data suggest that varying degrees of lineage plasticity accompany initial phases of lineage commitment and may relate to the presence of residual transcripts or transcript accessibility. However, it is at this point unclear whether such developmental plasticity represents a physiologic-utilized mechanism for regulating homeostasis or if it is strictly an experimentally introduced phenomenon.

Although correlative, the finding of genetic similarities between early lymphoid and early granulocytic/macrophage precursors could help to explain the origin of certain lymphomyeloid biphenotypic leukemias, which do not appear to involve the erythroid and/or megakaryocytic lineages (Imamura et al., 2002; Wang et al., 2005). Our data would suggest that such leukemias, rather than reflecting reactivation of previously repressed genetic programs, might be a consequence of transformation of normally occurring precursors with limited or no megakaryocytic/erythroid potential. The finding of common transcriptional features at several stages of differentiation (either CLP, early granulocytic/macrophage precursors, or an early bipotent precursor with the ability to generate both adaptive and innate immune components) broadens the developmental window where immortalization events might display such biphenotypic properties.

In conclusion, the results presented herein have defined a panel of myeloid progenitor cell intermediates that offer an unprecedented resolution of early myelopoiesis. These studies set the stage for their further exploration in the study of mechanisms governing both normal and aberrant myelopoiesis and also should provide valuable insight for the identification of corresponding subsets in the human system. This might be directly applicable for markers such as CD150 and Endoglin, as development into mature platelets from HSCs appears to be characterized by continuous CD150 expression, and human platelets are also CD150 positive (Nanda et al., 2005), whereas subsets of early human erythroid progenitors are characterized by Endoglin expression (Rokhlin et al., 1995). The identification of the first lineage-restricted precursors of the erythroid, granulocytic/monocytic, and megakaryocytic lineages should be useful to dissect the processes governing entry, proliferation, and maturation into these lineages.

EXPERIMENTAL PROCEDURES

Mice

Two- to three-month-old β actin GFP transgenic mice (Wagers et al., 2002) or C57BL/6 mice (either CD45.2 or CD45.1) were used throughout these studies. Mice were maintained at the Lund University and Stanford University animal facilities. All mice procedures were performed with consent from the local ethics committees.

Purification of Hematopoietic Stem and Progenitor Cells

Hematopoietic stem and myeloid progenitor cells were isolated by staining of unfractionated bone marrow cells with unconjugated antibodies against CD4, CD5, CD8, B220, Ter119, Gr1, and Mac1 and visualized with fluorochrome-conjugated goat and rat antibodies. Lineage-stained cells were next c-kit-enriched by using c-kit-conjugated magnetic beads (Miltenyi, Bergisch Gladbach, Germany). c-kit-enriched cells were subsequently stained with antibodies against Sca1, c-kit, FcgRII/III (FcgR), CD41 (Itga2b), CD150 (Slamf1), and Endoglin (Eng/CD105) or for investigation of CD150 and Endoglin expression on previously described myeloid progenitor cell fractions, with antibodies against lineage markers, Sca1, c-kit, CD34, FcgRII/III, CD150, and Endoglin. Propidium Iodide (Molecular Probes) or 7-Amino-Actinomycin D (7-AAD; Sigma-Aldrich Co., St. Louis, MO) was used to exclude dead cells. Common lymphoid progenitor cells were obtained by sorting cells with a lineage-negative, Sca1 low, ckit low, IL7Ra positive, flt3 positive phenotype. Antibody clones and suppliers are indicated in Table S1. Cells were maintained on ice when possible through all procedures and were sorted on FACS Aria cell sorters (Becton Dickinson). All flow cytometry and FACS data were analyzed with FlowJo software (Tree Star, Ashland, OR). In some experiments, indicated cell populations were transferred on slides by using a cytospin centrifuge and subsequently stained with May-Grünwald/Giemsa-staining (Sigma-Aldrich) for morphological examination.

In Vitro Culture Assays

Freshly isolated unfractionated BM cells and sorted hematopoietic stem and progenitor cells were cultured with liquid and semisolid culture conditions (at 37°C, 98% humidity, and 5% CO2) to evaluate single-cell clonogenic activity and lineage potentials of isolated cell populations. For clonogenic liquid cultures, single cells (using a single-cell depositor coupled to a FACS Aria) were sorted into Terasaki plates in 20 ml medium (IMDM) supplemented with 20% prescreened

(B) A heat map is displayed with genes that were 2-fold or higher differentially expressed between Pre MegE versus Pre GM and CLP, Pre GM versus Pre MegE and CLP, and CLP versus Pre GM and Pre MegE. Red indicates high expression; white, intermediate; and blue, low-level gene expression. A selection of individual genes found in each gene cluster is presented next to the heat map display.
(C) qRT-PCR was performed on selected genes identified by our microarray approach. To be able to directly correlate qRT-PCR data with those expression patterns on arrays, array data was also normalized internally to β-actin (± SD). When genes were called absent from one or more population on arrays, the corresponding expression value for such arrays was subtracted from all other samples.
(D) Proposed myeloid developmental scheme and cell surface marker changes associated with the precursors studied in detail in this work (gray box).

fetal calf serum (FCS; Hyclone, Logan, UT), 0.1 mM β-mercaptoethanol and recombinant growth factors c-kit Ligand (KL; 50 ng/ml PeproTech Inc, Rocky Hill, NJ), Interleukin-3 (IL-3; 10 ng/ml, PeptroTech) and Erythropoietin (EPO; 2 U/ml, Janssen-Cilag), and in some cases addition of granulocyte-colony stimulating factor (G-CSF;10 ng/ml, Amgen). Wells were evaluated for colony size and type by light microscopy after 4, 7, and 11 days in culture. Lineage potentials were evaluated in semisolid agar cultures (Metcalf, 2004) or methylcellulose. Indicated cell numbers and cell types were seeded in either serum containing methylcellulose-based medium (M3434, StemCell Technologies Inc., Vancouver, Canada) or agar-based medium supplied with growth factors as indicated above. Colonies generated in methylcellulose were scored as CFU-E (3 days after seeding) or BFU-E, GM, Meg, mixed Meg, and E or GM by morphologic criteria 7 days after seeding. Agar cultures were performed as described (Metcalf, 2004) with some minor modifications. In short, 0.6% BactoAgar 2X (Becton Dickinson) was mixed with preheated DMEM 2X (Chemicon), supplemented with sodium pyruvate (Sigma) and sodium bicarbonate (Sigma), FCS, and cytokines as above and kept at 37°C. Sorted cells were added, and 1 ml was plated in quadruplicates in 25 mm Petri dishes. After 7 days, cultures were fixed with glutaraldehyde (Fluka) and thereafter floated in a water bath and dried onto a glass slide and subsequently stained for (I) 4 hr for acethylcholinesterase (components: trisodium citrate [Sigma], copper sulfate pentahydrate [Sigma], potassium ferricyanide [Sigma], disodiumhydrogen orthophosphate [Fluka], sodiumdihydrogen orthophosphate [Fluka] and acethylthiocholine iodide [Sigma]), (II) 1.5 hr luxol fast stain (Luxol fast blue powder [Sigma], Urea [Fluka], 70% alcohol), followed by (III) 2 min of hematoxylin staining (hematoxylin [Sigma], sodium iodide [Sigma], potassium aluminum sulfate [Sigma], chloral hydrate [Sigma], and citric acid [Sigma]). Slides were mounted with coverslips using DePex. Thereafter, numbers and types of generated colonies were determined by light microscopy. Sorted progenitor cells were cultured in stroma-supported cultures (OP9) and analyzed for lineage potentials. Briefly OP9 cells (kindly provided by Drs. A. Cumano, Paris) were maintained in OptiMEM with L-Glutamine (Invitrogen, Carlsbad, California), supplemented with 10% FCS. Cell lines were trypsinized and plated at a density of $2 \times 10-4$ cells/ml 4–5 hr prior to addition of cells. Immediately prior to addition of cells, medium was removed from stroma and indicated sorted progenitor cells (500 of each type) were plated in quadruplicates in 250 ml medium supplemented with cytokines as above. After 5 days of culture, cells were harvested, washed, and stained with indicated antibodies and analyzed by flow cytometry.

Transplantation Experiments

Cells were transplanted at indicated cell numbers into sublethally irradiated recipients to evaluate their in vivo capacities to generate granulocytes or platelets. We transplanted 1.5×10^6 whole bone marrow cells or the estimated number of sorted hematopoietic stem and progenitor cells contained within 1.5×10^6 whole bone marrow cells. At indicated time points, blood samples were evaluated by flow cytometry for the levels of GFP+CD150+ positive platelets, and GFP-positive granulocytes were identified by their distinct forward and side scatter profile. For granulocyte analysis, peripheral blood was obtained from the tail vein and red blood cells sedimented with 2% Dextran T-500 (Pharmacia, Uppsala, Sweden), and remaining red blood cells were lysed with ammonium chloride. For platelet analysis, whole peripheral blood was shortly centrifuged and platelet-containing supernatant was stained with antibodies against CD150 (Nanda et al., 2005) and evaluated based on their small size, CD150 expression, and donor/host origin (GFP/non-GFP). Erythroid potential was determined by transplantation of lethally irradiated recipients with indicated cell populations (2500 cells or 250 cells for HSC containing fraction). Reticulocyte counts were determined at the Stanford University laboratory animal facilities. For generation of CFU-S8, an assay that primarily detects erythroid production (Na Nakorn et al., 2002), macroscopic evaluation of spleens obtained from lethally irradiated mice and transplanted with indicated cell fractions were performed after fixation in Tellesniczky's fixative.

Quantitative RT-PCR and Multiplex Single-Cell RT-PCR

For quantitative (q)RT-PCR, total RNA was isolated with an RNeasy mini kit (QIAGEN) or by Trizol reagent (Invitrogen) from purified hematopoietic cells, digested with DNase I to remove genomic DNA contamination, and used for reverse-transcription with random hexamers according to the manufacturer's instructions (SuperScript II kit, Invitrogen). Real-time PCR reactions were performed with 200–500 cell equivalents of RNA in an ABI 7000 sequence detection system using the 2XSYBR green master mix according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). Fold expression relative to unfractionated BM cells after β-actin normalization was calculated by using the comparative Ct (2-DDCt) method. For the quantitative RT-PCR (qRT-PCR) analyses in Figure 7C, we used ABI (Foster City, CA) Taqman assay on demand primers and probes. Triplicate real-time PCR reactions were performed with 100 cell equivalents of RNA and normalized to β-actin (for comparative purposes to array data, array data were also normalized to β-actin). Multiplex single-cell RT-PCR was performed as described by Hu et al. (1997). Single hematopoietic cells were deposited into 96-well plates containing 4 ml lysis buffer (0.4% Nonidet P-40, 65 mol/l dNTPs, 25 mol/l dithiothreitol, and 0.5 U/ml RNaseOUT) (Invitrogene Corporation, California). Cell lysates were reverse transcribed with six pairs of gene-specific reverse primers (against c-kit, Mpo, Mpl, Vwfh, Epor, and Klf1) and 50 U MMLV-RT per reaction in the buffer provided by the supplier (Invitrogen Corporation, California). The first-round PCR was performed by addition of 40 ml PCR buffer containing dNTPs, forward outside primers, and Taq polymerase (TaKaRa Bio Inc., Shiga, Japan) and run for 35 cycles. One-microliter aliquots of first-round PCRs were further amplified with fully nested gene-specific primers. Primer sequences are available upon request. Aliquots of second-round PCR products were subjected to gel electrophoresis and visualized by ethidium bromide staining on ordinary agarose gels or E gels (Invitrogen). c-kit gene product was used as positive internal control.

Affymetrix Gene Expression and Data Analysis

RNA was extracted from purified adult BM subsets with an RNeasy mRNA purification kit (QIAGEN). Subsequent handling was performed at the SweGene Affymetrix unit at Lund University (http://www.lth.se/index.php?id=11754). Briefly, RNA (arrays in triplicate for all progenitor fractions except for Pre MegE for which five arrays were produced) was labeled and amplified according to Affymetrix Small Sample Labeling Protocol v.2, with the exception that the second round of in vitro transcription (IVT) was performed with an Affymetrix GeneChip Expression 3′-Amplification kit. Hybridization and washing was performed according to Affymetrix GeneChip Expression analysis technical manual. Chips were scanned with an Affymetrix GeneChip Scanner 3000 and scaled to a median intensity of 100. For subsequent analysis, probe level expression values were extracted by using RMA (Irizarry et al., 2003), and subsequent analyses were performed with dChip software (http://biosun1.harvard.edu/complab/dchip/) after filtering ($0.5 < SD/mean < 1,000$).

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/4/428/DC1/.

ACKNOWLEDGMENTS

We thank L. Jerabek for laboratory management, C. Richter for antibody production, L. Hildalgo for animal care, and Anabel Kearney at the Swegene Affymetrix Unit at Lund University for preparation of microarrays. Gerd Sten is acknowledged for maintenance of OP9 cells. We thank Susan Prohaska for sharing unpublished observations. This work was supported by grants from Cancerfonden, the Swedish Strategic Research Foundation (SSF), and Crafoordska Stiftelsen to D.B. C.J.H.P. was supported by an ALF training position. D.J.R. was supported by the Damon Runyon Cancer Foundation. Lund Stem

Cell Center is a Center of Excellence in the Life Sciences and is supported by grants from SSF.

Received: March 16, 2007
Revised: June 11, 2007
Accepted: July 10, 2007
Published: October 10, 2007

REFERENCES

Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S.E. (2001). Up-regulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity *15*, 659–669.

Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., et al. (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell *121*, 295–306.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193–197.

Baines, P., Bol, S.J., and Rosendaal, M. (1982). Physical and kinetic properties of haemopoietic progenitor cell populations from mouse marrow detected in five different assay systems. Leuk. Res. *6*, 81–88.

Balciunaite, G., Ceredig, R., Massa, S., and Rolink, A.G. (2005). A B220+ CD117+ CD19- hematopoietic progenitor with potent lymphoid and myeloid developmental potential. Eur. J. Immunol. *35*, 2019–2030.

Blais, A., and Dynlacht, B.D. (2005). Constructing transcriptional regulatory networks. Genes Dev. *19*, 1499–1511.

Borregaard, N., and Cowland, J.B. (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. Blood *89*, 3503–3521.

Bradford, G.B., Williams, B., Rossi, R., and Bertoncello, I. (1997). Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. Exp. Hematol. *25*, 445–453.

Brady, G., Billia, F., Knox, J., Hoang, T., Kirsch, I.R., Voura, E.B., Hawley, R.G., Cumming, R., Buchwald, M., and Siminovitch, K. (1995). Analysis of gene expression in a complex differentiation hierarchy by global amplification of cDNA from single cells. Curr. Biol. *5*, 909–922.

Bryder, D., and Jacobsen, S.E. (2000). Interleukin-3 supports expansion of long-term multilineage repopulating activity after multiple stem cell divisions in vitro. Blood *96*, 1748–1755.

Bryder, D., Rossi, D.J., and Weissman, I.L. (2006). Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am. J. Pathol. *169*, 338–346.

Chen, C.Z., Li, M., de Graaf, D., Monti, S., Gottgens, B., Sanchez, M.J., Lander, E.S., Golub, T.R., Green, A.R., and Lodish, H.F. (2002). Identification of endoglin as a functional marker that defines long-term repopulating hematopoietic stem cells. Proc. Natl. Acad. Sci. USA *99*, 15468–15473.

Cheshier, S.H., Morrison, S.J., Liao, X., and Weissman, I.L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc. Natl. Acad. Sci. USA *96*, 3120–3125.

Christensen, J.L., and Weissman, I.L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc. Natl. Acad. Sci. USA *98*, 14541–14546.

Cozzio, A., Passegue, E., Ayton, P.M., Karsunky, H., Cleary, M.L., and Weissman, I.L. (2003). Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. Genes Dev. *17*, 3029–3035.

Drynan, L.F., Pannell, R., Forster, A., Chan, N.M., Cano, F., Daser, A., and Rabbitts, T.H. (2005). Mll fusions generated by Cre-loxP-mediated de novo translocations can induce lineage reassignment in tumorigenesis. EMBO J. *24*, 3136–3146.

Fukuchi, Y., Shibata, F., Ito, M., Goto-Koshino, Y., Sotomaru, Y., Ito, M., Kitamura, T., and Nakajima, H. (2006). Comprehensive analysis of myeloid lineage conversion using mice expressing an inducible form of C/EBP alpha. EMBO J. *25*, 3398–3410.

Hu, M., Krause, D., Greaves, M., Sharkis, S., Dexter, M., Heyworth, C., and Enver, T. (1997). Multilineage gene expression precedes commitment in the hemopoietic system. Genes Dev. *11*, 774–785.

Imamura, T., Morimoto, A., Takanashi, M., Hibi, S., Sugimoto, T., Ishii, E., and Imashuku, S. (2002). Frequent co-expression of HoxA9 and Meis1 genes in infant acute lymphoblastic leukaemia with MLL rearrangement. Br. J. Haematol. *119*, 119–121.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., and Speed, T.P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics *4*, 249–264.

Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E.A., Iwasaki-Arai, J., Mizuno, S., Arinobu, Y., Geary, K., Zhang, P., Dayaram, T., et al. (2005). Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. Blood *106*, 1590–1600.

Iwasaki, H., Mizuno, S., Arinobu, Y., Ozawa, H., Mori, Y., Shigematsu, H., Takatsu, K., Tenen, D.G., and Akashi, K. (2006). The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. Genes Dev. *20*, 3010–3021.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Yilmaz, O.H., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109–1121.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661–672.

Kondo, M., Scherer, D.C., Miyamoto, T., King, A.G., Akashi, K., Sugamura, K., and Weissman, I.L. (2000). Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. Nature *407*, 383–386.

Krivtsov, A.V., Twomey, D., Feng, Z., Stubbs, M.C., Wang, Y., Faber, J., Levine, J.E., Wang, J., Hahn, W.C., Gilliland, D.G., et al. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature *442*, 818–822.

Lai, A.Y., and Kondo, M. (2006). Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. J. Exp. Med. *203*, 1867–1873.

Laiosa, C.V., Stadtfeld, M., and Graf, T. (2006). Determinants of lymphoid-myeloid lineage diversification. Annu. Rev. Immunol. *24*, 705–738.

Medina, K.L., Garrett, K.P., Thompson, L.F., Rossi, M.I., Payne, K.J., and Kincade, P.W. (2001). Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. Nat. Immunol. *2*, 718–724.

Metcalf, D. (2004). Blood Lines - an Introduction to Characterizing Blood Diseases of the Post-Genomic Mouse (Durham, NC: AlphaMed Press).

Metcalf, D., Di Rago, L., and Mifsud, S. (2002). Synergistic and inhibitory interactions in the in vitro control of murine megakaryocyte colony formation. Stem Cells *20*, 552–560.

Na Nakorn, T., Traver, D., Weissman, I.L., and Akashi, K. (2002). Myeloerythroid-restricted progenitors are sufficient to confer radioprotection and provide the majority of day 8 CFU-S. J. Clin. Invest. *109*, 1579–1585.

Nakorn, T.N., Miyamoto, T., and Weissman, I.L. (2003). Characterization of mouse clonogenic megakaryocyte progenitors. Proc. Natl. Acad. Sci. USA *100*, 205–210.

Nanda, N., Andre, P., Bao, M., Clauser, K., Deguzman, F., Howie, D., Conley, P.B., Terhorst, C., and Phillips, D.R. (2005). Platelet

aggregation induces platelet aggregate stability via SLAM family receptor signaling. Blood *106*, 3028–3034.

Nutt, S.L., Metcalf, D., D'Amico, A., Polli, M., and Wu, L. (2005). Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. J. Exp. Med. *201*, 221–231.

Passegue, E., Wagers, A.J., Giuriato, S., Anderson, W.C., and Weissman, I.L. (2005). Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. J. Exp. Med. *202*, 1599–1611.

Rokhlin, O.W., Cohen, M.B., Kubagawa, H., Letarte, M., and Cooper, M.D. (1995). Differential expression of endoglin on fetal and adult hematopoietic cells in human bone marrow. J. Immunol. *154*, 4456–4465.

Rumfelt, L.L., Zhou, Y., Rowley, B.M., Shinton, S.A., and Hardy, R.R. (2006). Lineage specification and plasticity in CD19- early B cell precursors. J. Exp. Med. *203*, 675–687.

Scott, E.W., Simon, M.C., Anastasi, J., and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science *265*, 1573–1577.

Singh, H. (1996). Gene targeting reveals a hierarchy of transcription factors regulating specification of lymphoid cell fates. Curr. Opin. Immunol. *8*, 160–165.

Socolovsky, M., Nam, H., Fleming, M.D., Haase, V.H., Brugnara, C., and Lodish, H.F. (2001). Ineffective erythropoiesis in Stat5a(−/−)5b(−/−) mice due to decreased survival of early erythroblasts. Blood *98*, 3261–3273.

Takano, H., Ema, H., Sudo, K., and Nakauchi, H. (2004). Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J. Exp. Med. *199*, 295–302.

Terszowski, G., Waskow, C., Conradt, P., Lenze, D., Koenigsmann, J., Carstanjen, D., Horak, I., and Rodewald, H.R. (2005). Prospective isolation and global gene expression analysis of the erythrocyte colony-forming unit (CFU-E). Blood *105*, 1937–1945.

Uchida, N., Aguila, H.L., Fleming, W.H., Jerabek, L., and Weissman, I.L. (1994). Rapid and sustained hematopoietic recovery in lethally irradiated mice transplanted with purified Thy-1.1lo Lin-Sca-1+ hematopoietic stem cells. Blood *83*, 3758–3779.

Wagers, A.J., Allsopp, R.C., and Weissman, I.L. (2002). Changes in integrin expression are associated with altered homing properties of Lin(-/lo)Thy1.1(lo)Sca-1(+)c-kit(+) hematopoietic stem cells following mobilization by cyclophosphamide/granulocyte colony-stimulating factor. Exp. Hematol. *30*, 176–185.

Wang, G.G., Pasillas, M.P., and Kamps, M.P. (2005). Meis1 programs transcription of FLT3 and cancer stem cell character, using a mechanism that requires interaction with Pbx and a novel function of the Meis1 C-terminus. Blood *106*, 254–264.

Xie, H., Ye, M., Feng, R., and Graf, T. (2004). Stepwise reprogramming of B cells into macrophages. Cell *117*, 663–676.

Yang, L., Bryder, D., Adolfsson, J., Nygren, J., Mansson, R., Sigvardsson, M., and Jacobsen, S.E. (2005). Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. Blood *105*, 2717–2723.

Yoshida, T., Ng, S.Y., Zuniga-Pflucker, J.C., and Georgopoulos, K. (2006). Early hematopoietic lineage restrictions directed by Ikaros. Nat. Immunol. *7*, 382–391.

Accession Numbers

Microarray files of purified subsets described in Figure 7 (three to five replicates/cell type) were deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE8407).