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Myeloid Lineage Commitment from the Hematopoietic Stem Cell

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Prospective isolation of hematopoietic stem and progenitor cells has identified the lineal relationships among all blood-cell types and has allowed their developmental mechanisms to be assayed at the single-cell level. These isolated cell populations are used to elucidate the molecular mechanism of lineage fate decision and of its plasticity directly by stage-specific enforcement or repression of lineage-instructive signaling in purified cells. With an emphasis on the myeloid lineage, this review summarizes current concepts and controversies regarding adult murine hematopoietic development and discusses the potential mechanisms, operated by single or by multiple transcription factors, of myeloid lineage fate decision.

Introduction

Over the past four decades, much has been learned regarding the hematopoietic hierarchy that ultimately produces all mature blood-cell types from rare hematopoietic stem cells (HSCs). The identification of HSCs with $Lin^{-}Thy1.1^{lo}Sca\text{-}1^{+}$ phenotype of the mouse bone marrow (Spangrude et al., 1988) paved the way for constructing the hierarchical lineage map based on the existence of prospectively isolatable lineage-restricted progenitors downstream of HSCs. The $Thy1.1^{lo}Lin^{-}Sca\text{-}1^{+}$ HSC is found within the "LSK" ($Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$) fraction (Morrison and Weissman, 1994; Osawa et al., 1996) that is now the prevailing definition for murine HSCs.

Mature blood cells are traditionally categorized into two separate lineages: lymphoid and myeloid. The lymphoid lineage consists of T, B, and natural killer (NK) cells. The myeloid lineage includes a number of morphologically, phenotypically, and functionally distinct cell types including different subsets of granulocytes (neutrophils, eosinophils, and basophils), monocytes, macrophages, erythrocytes, megakaryocytes, and mast cells. Dendritic cells (DCs) have a unique developmental program that can be activated from either the myeloid or the lymphoid pathways (Manz et al., 2001; Traver et al., 2000). These two classes have been believed to use separate differentiation pathways (Traver and Akashi, 2004).

The successful isolation of the common lymphoid progenitor (CLP) that can generate all lymphoid types but not any myeloid cells (Kondo et al., 1997), and its counterpart, the common myeloid progenitor (CMP) that can be a source of all myeloid-cell types (Akashi et al., 2000), supports the concept that the myeloid and lymphoid developmental programs independently operate downstream of HSCs. Recent studies utilizing additional lineage-related markers, however, have provided evidences that the myeloid-versus-lymphoid divergence is more complicated. Focusing on myeloid development, this review will discuss recent progress and controversies in cellular and mechanistic aspects of lineage commitment in adult murine hematopoiesis.

Subsetting Primitive HSCs

In murine hematopoiesis, the multipotent activity resides in a small fraction of bone-marrow cells, which lacks the expression of lineage-associated surface markers (Lin) but expresses high Sca-1 and c-Kit (Ikuta and Weissman, 1992; Li and Johnson, 1995; Spangrude et al., 1988). Within the LSK fraction, several criteria have been used to isolate the most primitive self-renewing HSCs with long-term reconstituting activity (LT-HSCs). LT-HSCs reside in the $CD34^{-}$, $CD38^{+}$, or $Thy1.1^{lo}$ fraction of the LSK population (Morrison and Weissman, 1994; Osawa et al., 1996; Randall et al., 1996). In contrast, the LSK population with $CD34^{+}$, $CD38^{-}$, or $Thy1.1^{-}$ phenotype is, as a population, capable of only transient reconstitution, thereby containing short-term HSCs (ST-HSCs) or multipotent progenitors (MMPs) (Osawa et al., 1996; Randall et al., 1996). Several reports have tried to discriminate ST-HSCs and MPPs by involving low or negative expression of CD4, CD11b, or Thy-1.1 (Morrison et al., 1997; Morrison and Weissman, 1994). These studies showed some difference in duration and magnitude of reconstitution. However, no clear-cut phenotypic or functional definition for the ST-HSC and the MPP has been proposed. Furthermore, clonal multilineage differentiation activity of ST-HSCs or MPPs has never been shown at the single-cell level. Accordingly, the LSK "ST-HSC" or the "MPP" could be heterogeneous and contain a variety of transitional intermediates between LT-HSCs and oligopotent progenitors.

The Myeloid and Lymphoid Differentiation Pathways

Because the LSK population contains all multipotent precursors including LT-HSCs, ST-HSCs, and MPPs, lineage-restricted progenitor populations were first sought

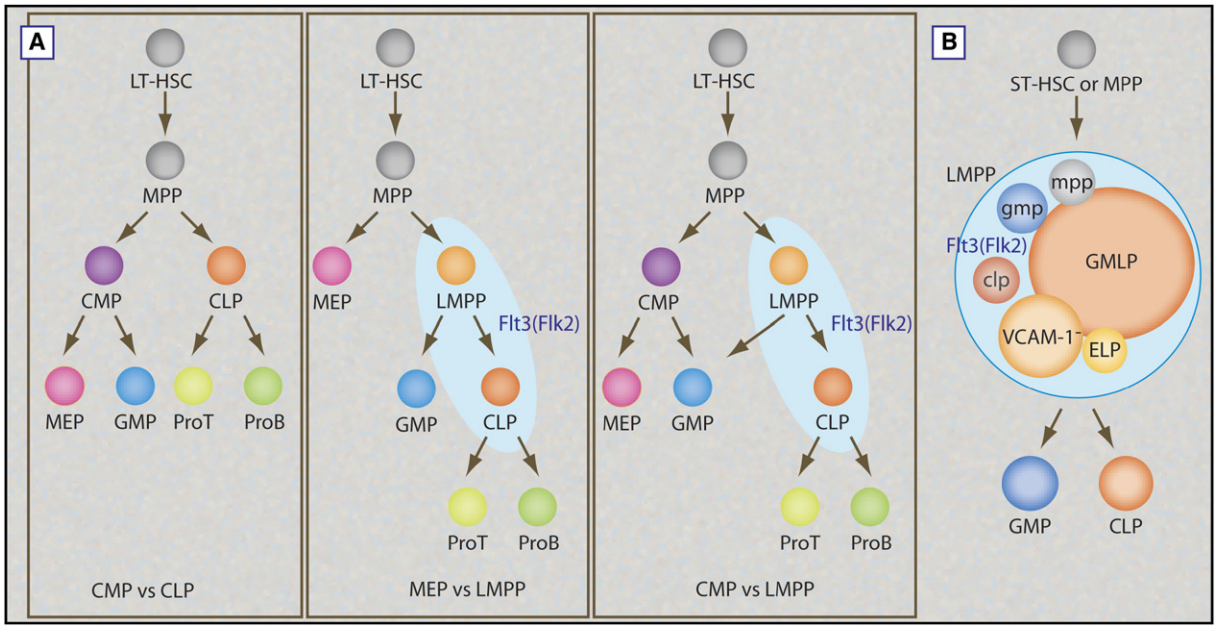

Figure 1. Cellular Pathways in Adult Murine Hematopoiesis

(A) Proposed developmental pathways based on prospective purification of lineage-restricted progenitors.

(B) The Flt3$^+$ LMPP population includes other defined progenitors such as the ELP (Igarashi et al., 2002), the VCAM-1$^-$ MPP (Lai and Kondo, 2006), and the putative GMLP. It might also include cells with a variety of myelolymphoid potential such as mpp and clp, which correspond to MPPs and CLPs, respectively. The following abbreviations are used: MPP, multipotential progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-monocyte progenitor; LMPP, lymphoid-primed multipotent progenitor; and GMLP, granulocyte-monocyte-lymphoid progenitor.

outside the LSK fraction. A series of efforts focused on the isolation of lineage-committed progenitors resulted in the successful isolation of the CLP (Kondo et al., 1997). The CLP is the earliest population that upregulates the receptor for interleukin 7 (IL-7), an essential cytokine for both T and B cell development (Bhatia et al., 1995; Peschon et al., 1994; von Freeden-Jeffry et al., 1995). The IL-7 receptor (IL-7R) is composed of the IL-7Rα chain and the common cytokine receptor γ chain (γc) (Kondo et al., 1994; Noguchi et al., 1993). Its signaling plays a critical role in thymocyte survival through maintenance of Bcl-2 (Akashi et al., 1997) and in the rearrangement of immunoglobulin heavy-chain V segments through the activation of the *Pax5* gene (Corcoran et al., 1998). The IL-7Rα^+ fraction concentrates lymphoid potential in the bone marrow, and the IL-7Rα^+c-Kit^{lo}Lin$^-$Sca-1^{lo} cells have a strong CLP activity. The IL-7Rα^+c-Kit^{lo}Lin$^-$Sca-1^{lo} CLPs possess clonogenic T, B, and NK cell potential, but lacks myelo-erythroid differentiation activity.

The CMP is IL-7Rα^- and is not part of the LSK population. The IL-7Rα^-c-Kit$^+$Lin$^-$Sca-1$^-$ fraction, which possesses >98% of myeloerythroid colony-forming activity in the bone marrow, can be further fractionated on the basis of the expression of FcγRII and FcγRIII (FcγRII/III) and CD34. Three distinct myeloid-progenitor subsets are isolatable: FcγRII/III^{lo}CD34$^+$ CMPs, FcγRII/III^{lo}CD34$^-$ megakaryocyte-erythrocyte progenitors (MEPs), and FcγRII/III^{hi}CD34$^+$ granulocyte-macrophage progenitors (GMPs) (Akashi et al., 2000). The CMP differentiates into the GMP and the MEP. The CMP can generate all types of myeloid colonies, whereas the GMP or the MEP produces only granulocyte macrophage (GM) or megakaryocyte erythrocyte (MegE) lineage cells, respectively. Upon in vivo transfer, these populations display short-term production of lineages consistent with their in vitro activities, indicating that they do not appreciably self-renew (Na Nakorn et al., 2002). The presence of the CMP and the CLP beyond the LSK fraction suggests that myeloid and lymphoid development start at the CMP and the CLP stages, respectively (Figure 1A, left). This simple model is widely used to analyze normal and malignant hematopoiesis. Together with the GMP and the MEP that were isolated downstream of the CMP, these prospectively isolated stem and progenitor populations have been used for targeted analysis and manipulation of cells at specific hematopoietic stages.

Early GM and Lymphoid Commitment within the LSK Fraction

Recent studies suggest that phenotypically distinct populations with skewed lymphoid potential have already emerged within the LSK population. In mice carrying GFP knocked into the *Rag1* gene locus (Igarashi et al., 2001; Kuwata et al., 1999), a fraction (~5%) of LSK cells express GFP (Igarashi et al., 2002), and this population, called the early lymphocyte precursor (ELP), displays potent T, B, and NK differentiation potential with a weak myeloid colony-forming activity (Igarashi et al., 2002).

The ELP resides within the CD34$^+$ LSK (ST-HSC or MPP) population (Osawa et al., 1996), and almost 40% of ELPs can form GM but not MegE colonies (unpublished data). Despite the fact that clonal development of GM and lymphoid cells from ELPs has not been formally proven, these data strongly suggest that the ELP is composed of cells with GM and lymphoid but not MegE lineage potential. Additionally, toward lymphoid lineage commitment, multipotent cells might lose MegE potential prior to abrogating GM potential (Akashi et al., 2005).

The existence of lymphoid progenitors retaining GM potential in early hematopoiesis has also been proposed by fractionation of the LSK population utilizing Flt3 (also known as Flk2), a HSC-specific receptor tyrosine kinase (Mackarehtschian et al., 1995), as an additional marker. According to two reports by independent groups, Flt3 is not expressed in long-term reconstituting HSCs but is upregulated in the majority (~60%) of the LSK cells incapable of self-renewal (Adolfsson et al., 2001; Christensen and Weissman, 2001). Flt3$^+$ LSK cells are CD34$^+$, satisfying the criteria of ST-HSCs or MPPs (Osawa et al., 1996). A follow-up report further showed that Flt3$^+$ LSK cells predominantly lack MegE differentiation, whereas they have clonal and robust GM and lymphoid potential and thus claimed that this population constitutes a critical developmental stage where the GM and lymphoid lineage commitment occurs (Figure 1A, middle) (Adolfsson et al., 2005).

In murine hematopoiesis, Flt3 plays a critical role in lymphoid development because mice deficient for Flt3 display loss or reduction of early T, B, and NK cells and DCs (Mackarehtschian et al., 1995; McKenna et al., 2000; Sitnicka et al., 2002) and because mice deficient for Flt3 ligand (FL) lack CLPs but possess normal numbers of CMPs (Adolfsson et al., 2001). Flt3 signaling promotes expression of IL-7R, at least in vitro (Borge et al., 1999). Taking these lymphoid functions of Flt3 into account, the Flt3$^+$ LSK population was termed as the lymphoid-primed multipotent progenitor (LMPP) (Adolfsson et al., 2005). On the basis of the presence of the LMPP within the LSK fraction, the model predicting the coupled loss of self-renewal activity and MegE potential is proposed (Figure 1A, middle) (Adolfsson et al., 2005). In this model, the CMP does not have a suitable place, and the LMPP needs to give rise to all GMPs and CLPs, whereas MEPs need to directly develop from multipotent stages (i.e., LT-HSCs, ST-HSCs, or MPPs).

Further fractionation of the MPP population has been performed (Lai and Kondo, 2006). The Thy1.1$^-$ LSK population, which is almost equal to the CD34$^+$ LSK cells, contains cells with the Flt3$^-$VCAM-1$^+$, the Flt3$^+$VCAM-1$^+$, and the Flt3$^+$VCAM-1$^-$ phenotypes. Only the Flt3$^-$VCAM-1$^+$ cells have substantial potential to give rise to CMPs, whereas MegE and GM potential are gradually lost as cells progress into the Flt3$^+$VCAM-1$^+$ and the Flt3$^+$VCAM-1$^-$ stages. Flt3$^+$VCAM-1$^-$ cells mostly display CLP activity, but similar to the ELP, approximately 10% of Flt3$^+$VCAM-1$^-$ cells give rise to GM but not MegE colonies (Lai and Kondo, 2006). Thus, like ELPs, the majority of Flt3$^+$VCAM-1$^-$ MPPs have committed to the lymphoid lineage, but a fraction of them probably still possess GM and lymphoid potential, although clonogenic studies have not been performed. This study describes the heterogeneity of the LMPP, basically supporting the idea that toward the lymphoid lineage development, the loss of myeloid potential occurs first in the MegE and then in the GM lineage (Akashi et al., 2005).

More recently, however, Forsberg et al. (2006) reported that the LMPP still possesses robust MegE potential. It is difficult to correctly evaluate donor-derived MegE cells because the expression of CD45 subclass (i.e., Ly5.1 or Ly5.2) is commonly used to label donor and recipient cells and is downregulated in the early phase of MegE development. In this paper, donor-derived platelets were visualized utilizing the actin-GFP mouse. LMPPs generate significant numbers of GFP$^+$ platelets and spleen erythroid cells at a relatively late phase after transplantation (day 12–15). These data were interpreted that the CMP and the CLP could still constitute the major site for myeloid versus lymphoid lineage decision (Forsberg et al., 2006). The Forsberg et al. (2006) report clearly shows that the LMPP contains cells with potent MegE potential. However, because only a small fraction of LMPPs could form MegE colonies in vitro (Adolfsson et al., 2005), the MegE potential of LMPPs could also be due to contaminants of ST-HSCs or MPPs within the LMPP gate. Therefore, the existence of MegE potential as a population does not exclude possibility that the putative progenitor population strictly committed to the GM-lymphoid lineage (GMLP) exists within the LMPP population.

For delineating the hematopoietic developmental pathway more clearly, it should be important to subfractionate the LMPP to purify putative GMLP (Figure 1B). The ELP is Flt3$^+$ (Igarashi et al., 2001), satisfying the criteria for the LMPP. The phenotype of ELPs substantially overlaps that of Flt3$^+$VCAM-1$^-$Thy1.1$^-$ LSK cells. LMPPs, ELPs, and the Flt3$^+$VCAM-1$^-$Thy1.1$^-$ cells are found in approximately 30%, 5%, and 15% of the CD34$^+$ or Thy1.1$^-$ LSK population, respectively, and therefore the ELP and the Flt3$^+$VCAM-1$^-$Thy1.1$^-$ cell should constitute a minor fraction of the LMPP. Although MegE potential of the LMPP in vivo may be unexpectedly robust when carefully evaluated (Forsberg et al., 2006), on the basis of the presence of the RAG1$^+$ (Flt3$^+$) ELP and the Flt3$^+$VCAM-1$^-$ cells within the LMPP population, it is highly likely that the putative GMLP exists within the Flt3$^+$ LSK LMPP fraction. The LMPP might contain the GMLP as a major population but could also contain cells with lineage potential consistent with MPPs, ELPs (Igarashi et al., 2002), VCAM-1$^+$ cells (Lai and Kondo, 2006), and even CLPs (Figure 1B).

Because both the LMPP and the CMP independently exist in normal hematopoiesis, they should constitute a critical diverging point from the multipotent stage (Figure 1A, right). In this composite model, GMPs should be derived from both CMPs and LMPPs. The role of Flt3 signaling in myeloid development is noted for its constitutive

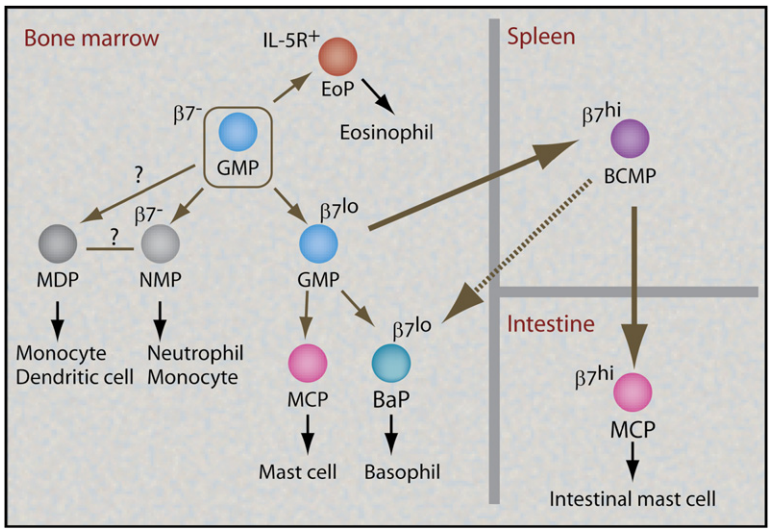

Figure 2. Developmental Pathways downstream of GMPs

Isolatable progenitor populations for each granulocyte lineage are shown. The GMP is divided into integrin $\beta7^-$ and $\beta7^{lo}$ populations. The latter is primed to the basophil and the mast-cell lineages: $\beta7^{lo}$ GMPs presumably migrate into the spleen to become $\beta7^{hi}$ BMCPs that give rise to $\beta7^{hi}$ intestinal MCPs. $\beta7^{lo}$ GMPs may also give rise to $\beta7^{lo}$ BaPs and $\beta7^{hi}$ MCPs in the bone marrow. In contrast, $\beta7^-$ GMPs differentiate into $\beta7^-$ EoPs as well as MDPs or putative NMPs. The lineal relationships among these progenitor populations remain unclear. The following abbreviations are used: MCP, mast-cell progenitor; BaP, basophil progenitor; BMCP, basophil/mast-cell progenitor; EoP, eosinophil progenitor; NMP, neutrophil-monocyte progenitor; and MDP, macrophage-dendritic-cell progenitor.

active mutants in human myeloid leukemias (Nakao et al., 1996; Stirewalt and Radich, 2003), but unlike human hematopoiesis where Flt3 is expressed in the entire LT-HSC and GMP populations as well as in CLPs (unpublished data), murine Flt3 is not expressed in GMPs or in the vast majority of CMPs (Karsunky et al., 2003). Therefore, the LMPP may include only a part of early GM potential within the LSK population. Flt3 is not a perfect marker to exclude early progenitors with MegE potential such as MPP or ST-HSCs. Furthermore, the relative contribution of CMPs and LMPPs in GM lineage development and the progenitor-progeny relationships among LMPPs, GMPs, and CLPs remain obscure.

Conversely, CMPs may also be contaminated with lymphoid progenitors because they possess a weak B cell potential (Akashi et al., 2000) within a minor CMP fraction expressing Flt3 (Karsunky et al., 2003). CMPs are purified within the c-$Kit^+Lin^-Sca\text{-}1^-$ fraction on the basis of the expression pattern of CD34 and FcγRII/III: CMPs were defined as the $CD34^+Fc\gamma RII/III^{lo}$ population (Akashi et al., 2000). Of note, the $Flt3^+$ LSK LMPP has the $CD34^+Fc\gamma RII/III^{lo}$ profile, which is indistinguishable to that of the CMP. Thus, the phenotypic distinction between the $Flt3^+$ CMP and the LMPP is only dependent upon the expression level of Sca-1. The amounts of fluorescence detected by FACS could be affected by many factors such as the efficiency of fluorescent labeling for each antibody and the sensitivity of fluorescent channels (Akashi et al., 2005). Perhaps additional markers are required to purify progenitors with more rigorous lineage restriction, including GMLPs and CMPs that are devoid of MegE and lymphoid potential, respectively.

In summary, the currently available data strongly suggest that the MPP go through either the CMP or the LMPP (or GMLP) stages to give rise to myeloid and lymphoid lineages (Figure 1A, right). The CLP might be downstream of the LMPP (or GMLP). Further fractionation of the LSK population by use of additional markers including transcription factor reporters (see below) might help delineate myeloid and lymphoid developmental pathways from HSCs.

Developmental Pathways downstream of the GMP and the MEP

Purified GMPs can generate mainly neutrophils, monocytes, and macrophages and a minor population of eosinophils, basophils, and mast cells as well. Progenitor populations restricted to such lineages have been prospectively isolated (Arinobu et al., 2005; Iwasaki et al., 2005a). A developmental scheme downstream of GMPs is shown in Figure 2.

Macrophages and DCs play critical roles in antigen presentation in immune and inflammatory responses. Both cell types are the progeny of GMPs, although DCs are also generated from lymphoid progenitors such as CLPs and pro-T cells (Manz et al., 2001; Shigematsu et al., 2004). Bipotent macrophage-dendritic-cell progenitors (MDPs) are isolatable from the bone marrow in a mouse carrying a GFP reporter knocked into the *Cx3cr1* locus (Fogg et al., 2006). The Lin^-c-$Kit^{lo}CX_3CR1$-GFP^+ MDP displays macrophage- and DC-restricted potential, and single MDPs may give rise to both macrophages and DCs. The origin of MDPs is not clear, but MDPs express CD34 and FcRγII/III at amounts indistinguishable from those of GMPs, suggesting that MDPs are derived from GMPs and therefore represent precursors for myeloid DCs.

Eosinophils, basophils, and mast cells are multifunctional hematopoietic effectors that cooperate to mount a variety of allergic and innate immune responses (Galli, 2000; Rothenberg, 1998; Wedemeyer et al., 2000). Eosinophils and basophils normally constitute only 1%–2% of circulating blood cells, whereas mast cells circulate as progenitors and finalize their maturation after migration into peripheral tissues such as the skin, heart, lung, and the gastrointestinal mucosa. The progenitor populations committed to each of these lineages have recently been isolated downstream of GMPs.

Eosinophil lineage-committed progenitors (EoPs) are isolatable within the mouse bone marrow expressing high IL-5 receptor α chain (Iwasaki et al., 2005a). EoPs have the IL-5Rα$^+$Lin$^-$Sca-1$^-$CD34$^+$c-Kit^{lo} phenotype. Although EoPs constitute only 0.05% of the steady-state bone-marrow cells, they can expand substantially in response to helminth infection, suggesting that the EoP stage is physiologically critical for eosinophil production.

Mast cells and basophils share their origin at the basophil-mast-cell bipotent progenitor (BMCP) stage (Arinobu et al., 2005). In adult mice, the intestine is the main peripheral tissue harboring mast-cell colony-forming activity (Gurish et al., 2001). The β7-integrin (β7) is an essential molecule for formation of the intestinal mast-cell pool and plays a role in tissue-specific homing of putative precursors for intestinal mast cells (Gurish et al., 2001). BMCPs are present within the β7^{hi} fraction of the spleen and have the Lin$^-$CD13^{lo}CD34$^+$β7^{hi}FcεRIα$^-$FcγRII/III$^+$c-Kit$^+$Thy-1$^+$ phenotype. This population, consisting only 0.005% of the total spleen cells, gives rise to basophils and mast cells at the single-cell level and can reconstitute mucosal and tissue mast cells after transplantation into mast-cell-deficient W/W^v mice. The monopotent mast-cell progenitor (MCP) is also isolatable in the intestine. Intestinal MCPs are Lin$^-$CD34$^+$β7^{hi}FcεRIα^{lo}FcγRII/III$^+$c-Kit^{lo}Thy-1$^-$ cells. They bear blastic morphology with a few scattered metachromatic granules and can give rise exclusively to pure mast-cell colonies. The monopotent basophil progenitor (BaP) is also present in the bone marrow and has the Lin$^-$CD34$^+$FcεRIα^{hi}c-Kit$^-$ phenotype (Arinobu et al., 2005).

In vitro, purified GMPs can give rise to EoPs, BMCPs, MCPs, and BaPs, whereas BMCPs generate MCPs and BaPs (Arinobu et al., 2005). The lineal relationship among these populations is schematized in Figure 2. Differentiation of spleen BMCPs into monopotent progeny may lead to their selective migration, BaPs to the bone marrow or MCPs to peripheral tissues. This progenitor allocation may be critical for their distinct lineage functions and their development. In the bone marrow, the basophil-mast-cell potential was enriched in the β7^{lo} GMPs (Arinobu et al., 2005), and a fraction expressing high T1/ST2, a mast-cell marker (Moritz et al., 1998), is mast-cell lineage committed (Chen et al., 2005). The spleen BMCP could be derived from the β7^{lo} GMPs. Relationships between the progenitor allocation and the physiological pathway of these cells are still unclear.

The monopotent megakaryocyte lineage-committed progenitor (MKPs) has been isolated downstream of MEPs by CD9, a megakaryocyte-associated surface protein. MKPs have the CD9$^+$IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$ Thy1.1$^-$ phenotype and represent only 0.01% of the total bone-marrow cells (Nakorn et al., 2003). MKPs give rise exclusively to various sizes of megakaryocyte colonies. MEPs represent the majority of day 8 CFU-S activity, but MKPs do not have CFU-S activity, generate only megakaryocytes in vitro, and could give rise to platelets for approximately 3 weeks when transplanted into mice. Erythroid-committed progenitors, a putative counterpart of MKPs, however, have not been identified.

Granulocyte and Monocyte Lineage Commitment by Single Transcription Factors

These prospectively isolatable progenitor populations emerge in normal hematopoiesis as a result of lineage specification. Therefore, understanding the mechanism of lineage commitment should in turn be useful to delineate the hematopoietic developmental pathway. Lineage commitment and subsequent differentiation of multipotent cells is likely to involve the selective activation and silencing of a set of genes. Such programs could be triggered by extrinsic signals (Kondo et al., 2000; Metcalf, 1998), intrinsic signals (Akashi et al., 1997; Fairbairn et al., 1993; Maraskovsky et al., 1997; Suda et al., 1983), or by both at different developmental stages, which are ultimately controlled by transcription factors. Transcription factors can play a key role in activating lineage-specific programs dependent upon their expression levels (Dahl et al., 2003; Iwasaki et al., 2003; Laslo et al., 2006) and timing (Iwasaki et al., 2006), and multiple transcription factors exert collaborative or competitive actions for cell fate decision (Laslo et al., 2006). The function of each transcription factor is usually analyzed by the loss-of-function and force-to-express studies. For detailed function of each transcription factor, readers should refer to recent reviews (Laiosa et al., 2006a; Rosenbauer and Tenen, 2007).

The disappearance of specific myeloid progenitors by disruption of single transcription factors is one of the strongest piece of evidence that they are absolutely required to form and maintain a certain stage of hematopoietic development. Such results have been clearly shown in either PU.1- or C/EBPα-deficient mice. These factors cooperate in the regulation of a number of GM-related genes (Rosenbauer and Tenen, 2007; Tenen, 2003). Of note, PU.1 also has a function for lymphoid development (Singh and Pongubala, 2006; Warren and Rothenberg, 2003). PU.1-deficient mice die at a late embryonic stage or shortly after birth and have impaired GM and B cell development (McKercher et al., 1996; Scott et al., 1994). T and NK cell development is also severely impaired in PU.1-deficient mice (Colucci et al., 2001; Spain et al., 1999), but MegE development is intact (McKercher et al., 1996; Scott et al., 1994). Conditional disruption of PU.1 in adult bone marrow results in a complete loss of CMPs, GMPs, and CLPs but retains a slightly increased number of MEPs (Dakic et al., 2005; Iwasaki et al., 2005b). Thus, PU.1 is necessary for multipotent progenitors to proceed to the CMP and the CLP stages. In contrast, mice deficient in C/EBPα lack neutrophils and eosinophils (Zhang et al., 1997). Mice conditionally disrupted with C/EBPα do not have granulocytes but have normal numbers of mature lymphoid and MegE cells (Zhang et al., 2004). In the bone marrow, GMPs disappear, but the development of CMPs, MEPs, or CLPs is normal, indicating that C/EBPα is necessary for CMPs (or LMPPs) to become GMPs (Zhang et al., 2004).

C/EBPβ-deficient mice show normal hematopoiesis under steady-state conditions, suggesting that myeloid-progenitor distribution should be normal. Mice having C/EBPβ knocked into the *Cebpa* gene locus show normal hematopoietic development (Jones et al., 2002), indicating that C/EBPβ can replace C/EBPα function. The difference between phenotype of C/EBPα- and C/EBPβ-deficient hematopoiesis might be due to their distribution: C/EBPα is gradually upregulated in the GM pathway during HSC, CMP to GMP development, whereas the expression of C/EBPβ declines, particularly at the CMP stage (Hirai et al., 2006). Therefore, CMPs could be maintained by C/EBPα under steady-state hematopoiesis. C/EBPβ is important in neutrophil production under stress hematopoiesis on the basis of the fact that C/EBPα-deficient mice can develop neutrophils in the presence of a high concentration of cytokines in association with rapid upregulation of C/EBPβ in myeloid progenitors, whereas C/EBPβ-deficient mice are incapable of emergent neutrophil production in response to exogenous cytokines or infection (Hirai et al., 2006). The requirement for late GM lineage development is also different between PU.1 and C/EBPs: The GMP deficient for PU.1 disrupted by retrovirally transduced Cre recombinase displays severe differentiation arrest at the myeloblastic stage (Iwasaki et al., 2005b), whereas the disruption of C/EBPα at the GMP stage does not prevent their terminal differentiation (Zhang et al., 2004). This may be because C/EBPα function is substituted by C/EBPβ (Jones et al., 2002) after the GMP stage. Thus, PU.1 but not C/EBPα or C/EBPβ is absolutely required for GMPs to differentiate into mature GM cells.

Interestingly, these major myeloerythroid transcription factors are expressed in the LSK HSC population (Miyamoto et al., 2002). Although the expression of these genes is considered to reflect myeloid lineage priming as discussed in the next section, the low expression of transcription factors such as PU.1 and C/EBPα might also control self-renewal activity of HSCs. The disruption of PU.1 at birth induced a rapid loss of HSCs (Iwasaki et al., 2005b), whereas that of C/EBPα induced expansion of HSCs (Zhang et al., 2004). Although the mechanism of these phenomena is still unclear, it has been suggested that they could act as a positive or negative regulator for HSC self-renewal, respectively, and these factors may competitively interact with each other to control HSC self-renewal, as they do at the granulocyte versus monocyte divergence (Dahl et al., 2003).

Megakaryocyte and Erythroid Lineage Commitment by Single Transcription Factors

For MegE differentiation, GATA factors and their cofactors such as a friend of GATA-1 (FOG-1) are indispensable (Fujiwara et al., 1996; Tsai and Orkin, 1997; Tsang et al., 1998). GATA-1-deficient mice die between embryonic day 10.5 and 11.5 of gestation because of severe anemia. Maturation of erythroid cells is arrested at an early proerythroblast-like stage in GATA-1-deficient embryos (Fujiwara et al., 1996). In another mutant line with modification of the DNase I-hypersensitive region upstream of the GATA-1 locus (Shivdasani et al., 1997), GATA-1 expression is impaired specifically in the megakaryocyte lineage. The platelet production of this mouse is markedly reduced, whereas in the bone marrow, megakaryocytes with severely impaired cytoplasmic maturation are increased (Shivdasani et al., 1997). Collectively, GATA-1 is indispensable for the MegE differentiation, whereas the MegE lineage commitment occurs in the absence of GATA-1. GATA-1 possesses strong MegE lineage-instructive effects (Iwasaki et al., 2003) as discussed in the next section. This discrepancy might be explained by a functional redundancy between GATA factors. The enforced expression of either GATA-2 or GATA-3 transgenes can rescue the erythroid lineage defect in GATA-1-deficient mice (Takahashi et al., 2000). Furthermore, primitive erythropoiesis is completely absent in the GATA-1 and GATA-2 double-deficient mice (Fujiwara et al., 2004).

GATA-2-deficient mouse embryos also die at the early stage of gestation because of severe anemia. Impaired expansion of HSCs and progenitors is the cause of the anemia, whereas the early erythroid maturation is normal in the GATA-2-deficient yolk sac (Tsai and Orkin, 1997), suggesting that GATA-1 but not GATA-2 is primarily critical for early erythroid differentiation. Interestingly, GATA factors are expressed in granulocyte subclasses such as eosinophils, basophils, and mast cells. Deletion of a high-affinity GATA-binding site in the GATA-1 promoter, an essential element for positive autoregulation of GATA-1 expression, leads to selective loss of the eosinophil lineage (Yu et al., 2002), suggesting that GATA-1 is indispensable for eosinophil development. In the GATA-2-deficient yolk sac, mast-cell development is severely impaired (Tsai and Orkin, 1997).

FOG-1 binds to GATA factors and cooperatively regulates transactivation of their target genes (Tsai and Orkin, 1997). FOG-1-deficient embryos also die between embryonic day 10.5 and 12.5 of gestation because of impaired erythroid maturation comparable to that of GATA-1-deficient mice (Tsang et al., 1998). FOG-1-deficient embryos completely lack megakaryocytes (Tsang et al., 1998), whereas GATA-1 deficiency leads to an increase of megakaryocytes (Shivdasani et al., 1997). FOG-1 absolutely requires the binding of GATA factor for its indispensable role in megakaryopoiesis because mice carrying mutant GATA-1 and GATA-2, both of which are unable to bind to FOG-1, display megakaryocyte deficiency, the phenotype similar to FOG-1-deficient mice (Chang et al., 2002). No conditional knockout systems have been developed for GATA factors or FOG-1. Therefore, requirement of these factors in adult hematopoiesis has not been thoroughly evaluated. GATA-1, GATA-2, and FOG-1 are also expressed in a fraction of LSK cells (Iwasaki et al., 2003; Miyamoto et al., 2002), but it is unknown whether they play a role in HSC function.

Plasticity of Lineage Determination via Ectopic Transcription-Factor Expression

The myeloid transcription factors including GM-related PU.1 and C/EBPα and MegE-related GATA factors play

a role in cell fate decision of uncommitted progenitor cells (Laiosa et al., 2006a; Traver and Akashi, 2004). Although HSCs prime all of these factors at a low level, enforcement of each factor by retroviral transduction into purified HSCs results in their specification into a lineage. HSCs transduced with PU.1 or C/EBPα formed GM but not MegE colonies, whereas those enforced with GATA-1 or GATA-2 mainly formed MegE or eosinophil colonies, respectively (Iwasaki et al., 2006; Iwasaki et al., 2003). The enforced FOG-1 does not change the colony-forming activity of HSCs (Iwasaki et al., 2003), suggesting that FOG-1 can play a role in the presence of an appropriate amount of GATA-1. These data strongly suggest that the increment of the expression of single factors is sufficient to abrogate self-renewal activity of HSCs and to instruct them into specific lineages.

In these experiments, it is difficult to exclude the possibility that these effects result from a selection of a particular lineage, which is dependent upon "permissive" signals that simply support survival or expansion of target cells. The most clear-cut evidence for lineage instructive action of transcription factors is to prove their ability to reprogram cells into a different lineage because this phenomenon reflects two important components for lineage instruction: perturbation of natural cell fate and reestablishment of a new program. A number of reports have shown that lymphoid-cell lines could be reprogrammed into macrophages by a variety of external ectopic signals (Graf, 2002). CLPs are one of the most useful cell types for testing myeloid instructive signals: They are primary cells purified from normal mice and do not have any myeloid potential nor express any major myeloid genes even at a genome-wide level (Akashi et al., 2000), whereas they possess plasticity for all myeloerythroid lineages, which could be triggered by ectopic transcription factors or cytokine signaling (Hsu et al., 2006; Iwasaki et al., 2006; Iwasaki et al., 2003; Iwasaki-Arai et al., 2003; King et al., 2002; Kondo et al., 2000).

The enforced expression of C/EBPα reprograms CLPs into the GM lineage (neutrophils and monocytes but not eosinophils or basophils) (Hsu et al., 2006; Iwasaki et al., 2006), whereas enforced GATA-1 or GATA-2 reprograms CLPs into either the MegE or the mast-cell lineage, respectively (Iwasaki et al., 2006; Iwasaki et al., 2003). Importantly, the clonal efficiencies of single-plated cells are $\geq$80%, and this reprogramming occurs at the expense of lymphoid potential of CLPs. CLPs with enforced FOG-1 normally develop B cells in vitro (unpublished data), again suggesting that FOG-1 has an effect only in the presence of GATA factors.

Even more committed T or B cell precursors can be reprogrammed into the myeloid lineage by the enforced expression of C/EBPα, C/EBPβ, or PU.1. Graf and colleagues have extensively analyzed the myeloid-reprogramming processes from monopotent T or B cell precursor stages (Laiosa et al., 2006b; Xie et al., 2004). They used ROSA26-EYFP mice with lck-cre or CD19-cre genes to perform in vivo labeling of cells having expressed T cell-related lck or B cell-related CD19, respectively. Lck-EYFP$^+$ cells are mostly DN3 and DN4 pre-T cells. The retroviral expression of C/EBPα and C/EBPβ or of PU.1 reprograms EYFP$^+$ pre-T cells into macrophages or dendritic cells, respectively. CD19-EYFP$^+$ pro B and even spleen B cells are also converted into macrophages by C/EBPs. Unlike CLPs, the lineage conversion from T or B cell precursors appears to occur in a fraction of transfected cells, suggesting that the lineage plasticity may decline as cells become further committed. It is important to note that at these committed T or B cell stages, cells are converted only into macrophages or DCs but not into other myeloid classes such as MegE cells or granulocytes. When GATA-1 is enforced in proB cells, cells immediately undergo apoptotic cell death, whose process cannot be rescued by the enforced expression of Bcl-2 (Iwasaki et al., 2003). Thus, plasticity for lineage specification is preserved until monopotent T or B cell progenitor stages, but their destination is predominantly limited to the macrophage lineage.

A series of force-to-express studies have thus shown that single-myeloid transcription factors can establish the myeloid developmental program in uncommitted progenitors or even in lymphoid progenitors. It is critical to understand how transcription factors override the ongoing lymphoid program and ultimately reprogram cells into the myeloid lineage in a cell-context-dependent manner. The reprogramming event might at least be dependent upon the ability of transcription factors to remodel chromatin (Bonifer, 2005; Muller and Leutz, 2001). Reprogramming from the myeloid into the lymphoid lineage, however, has never been achieved. Lymphoid development may require more complex regulatory processes governed by multiple lymphoid transcription factors (Laiosa et al., 2006a).

For lymphoid commitment, because HSCs naturally prime myeloid transcription factors, some mechanisms that can abrogate or repress preceding myeloid programs might be required. The OP9 expressing Notch ligand (OP9-DL1) (Schmitt and Zuniga-Pflucker, 2002) induces T cell differentiation from HSCs via CLPs, suggesting that Notch signaling is critical for multipotent cells to initiate the T cell program. Interestingly, the myeloid potential as well as the PU.1- or C/EBPα-dependent myeloid reprogramming of thymic precursors (pro-T and pre-T cells) can be blocked by active Notch signaling (Franco et al., 2006; Laiosa et al., 2006b; Rothenberg, 2007 [this issue of *Immunity*]). In this context, the myeloid fate could be a default commitment pathway for multipotent HSC, and lymphoid lineage commitment may be dependent upon successful perturbation of the preceding myeloid program by myeloid-repressing Notch signals.

Transcriptional Regulatory Network that Governs Hierarchical Hematopoietic Development

If hematopoietic development is dependent upon gradual accumulation of sequentially activated lineage-promoting signals, it is reasonable to assume that the primitive HSCs or MPPs do not express any lineage-related genes.

Previous studies, however, have provided evidences supporting the concept that the developmental potential is engraved in the chromatin of cells at the multipotent stage. For lineage-specific genetic programs to be activated, local chromatin must become accessible to the transcription machinery (Berger and Felsenfeld, 2001; Felsenfeld et al., 1996). The activation of chromatin remodeling can occur prior to substantial expression of genes in the region of interest (Kontaraki et al., 2000; Weintraub, 1985). An open chromatin structure is maintained in early hematopoietic progenitors, enabling multilineage differentiation programs to be readily accessible (Cross and Enver, 1997), and multipotent cells "prime" multiple lineage-affiliated programs of gene activity (i.e., transcription factors, cytokine receptors, and genes encoding lineage-exclusive function) at a low level, prior to being specified into each lineage (Hu et al., 1997). In fact, a number of GM- and MegE-related genes are coexpressed in single HSCs, MPPs, or CMPs (Miyamoto et al., 2002), even at the genome-wide level (Akashi et al., 2003; Mansson et al., 2007). Because most myeloid transcription factors possess lineage-instructive functions as discussed, the subtle change in the expression of these factors at the HSC stage should lead to an early myeloid lineage fate decision.

Uncommitted Progenitor and Stem Cells Primes Multiple Lineage-Affiliated Genes and Transcription Factors

The first evidence for the hematopoietic lineage priming was shown in a multipotent cell line, FDCP-mix (Hu et al., 1997). This seminal study showed that single multipotent FDCP-mix cells coexpress GM- and MegE-related and some lymphoid-related genes by single-cell RT-PCR assays. The ability to prospectively isolate lineage-restricted progenitor subsets has enabled the similar analysis at specific stages in normal hematopoiesis. In the myeloid lineage, the majority (>60%) of CMPs coexpress GM- (i.e., G-CSFR, myeloperoxidase, and PU.1) and MegE-related genes (i.e., EpoR, β-globin, and GATA-2) at the single-cell level, whereas virtually all GMPs and MEPs express only GM- or MegE-related genes, respectively (Miyamoto et al., 2002). In mice harboring a GFP reporter knocked into the murine lysozyme M (LysM) locus (Faust et al., 2000), ~60% of CMPs express substantial amounts of LysM^{GFP}. Both LysM^{GFP+} and LysM^{GFP-} CMPs express erythroid β-globin and can display MegE lineage differentiation at equal efficiencies (Miyamoto et al., 2002). Similarly, in the lymphoid pathway, single CLPs coexpress genes that encode B (i.e., λ5 and Pax-5) and T lymphoid (GATA-3 and CD3δ) cells (Miyamoto et al., 2002). A fraction of CLPs express pTα receptor mRNA, represented by a T cell-affiliated pTα reporter (Reizis and Leder, 1999), but their frequency for coexpression of other B and T lymphoid genes and B cell potential is equal irrespective of the pTα reporter expression. Collectively, transcription of LysM or pTα at a low level does not predict their GM or T cell fates. These studies suggest that promiscuous gene priming is likely to play a key role in maintaining flexibility in oligopotent precursors and that the level of gene expression may fluctuate at multipotent or oligopotent stages. The priming of genes affiliated with multiple lineages would afford flexibility in cell fate decisions and would allow multipotent precursors to rapidly respond to environmental cues (Hu et al., 1997). In this context, CMPs and CLPs have the molecular signature to assure their common myeloid and lymphoid potency, respectively. Thus, lineage commitment and subsequent differentiation of multipotent cells involve the upregulation of genes associated with the appropriate lineage, whereas the concomitant downregulation of inappropriate genes makes such lineage decision stable (Enver and Greaves, 1998).

At the genome-wide level, HSCs express a large number of myeloid but not lymphoid genes (Akashi et al., 2003; Mansson et al., 2007). The myeloid gene expression in the HSC fraction might not be due to committed contaminants. This was confirmed by tracing of the fate of HSCs with lysM activation (Ye et al., 2003). In mice harboring a LysM Cre-knockin together with ROSA-EYFP reporter alleles, cells activating LysM transcription are permanently marked by EYFP. In this mouse, EYFP is expressed not only in GM cells but also in a minority of MegE and lymphoid cells. A fraction of LT-HSCs also express EYFP, and these cells are capable of long-term reconstitution after transplantation with nearly 100% of the reconstituted cells being EYFP^{+}. This provides formal proof that the lysM gene is physiologically primed at the HSC stage (Ye et al., 2003). Thus, HSCs are ready to commit to the myeloid lineage presumably as a default, although some other internal or external cues might be necessary for them to initiate the lymphoid developmental program. A fraction of the LMPP coexpresses both GM and early lymphoid genes such as *Rag1* and *Il7r* (encoding IL-7Rα), again suggesting that progenitor cells at the stage common to GM and lymphoid lineages exist within the LMPP population (Mansson et al., 2007). If the lineage priming of transcription factors at low levels is a natural property of multipotent cells such as hematopoietic stem and progenitors, both the upregulation and downregulation of single or multiple transcription factors should be critical to turn on lineage-specification programs.

Transcription-Factor Dosage Determines Lineage Fate at Binary Branchpoints

The amount of transcription factor might play an important role in binary lineage fate decision. B cell and macrophage differentiation can be restored in PU.1 (encoded by *Sfpi1*)-deficient fetal-liver hematopoietic progenitors by retroviral transduction of *Sfpi1* with a GFP expression reporter. After culture on OP9 stromal layer, the *Sfpi1*$^{-/-}$ progenitor gives rise mainly to macrophages with a high expression of GFP and rare B cell progeny expressing only a low amount of GFP. In contrast, *Sfpi1*$^{-/+}$ fetal-liver progenitors naturally give rise to B cells and rare macrophages on OP9. When they are infected with the *Sfpi1*-GFP retrovirus, the *Sfpi1*$^{-/+}$ fetal-liver progenitor with a high expression of GFP gives rise mainly to macrophages at the expense of

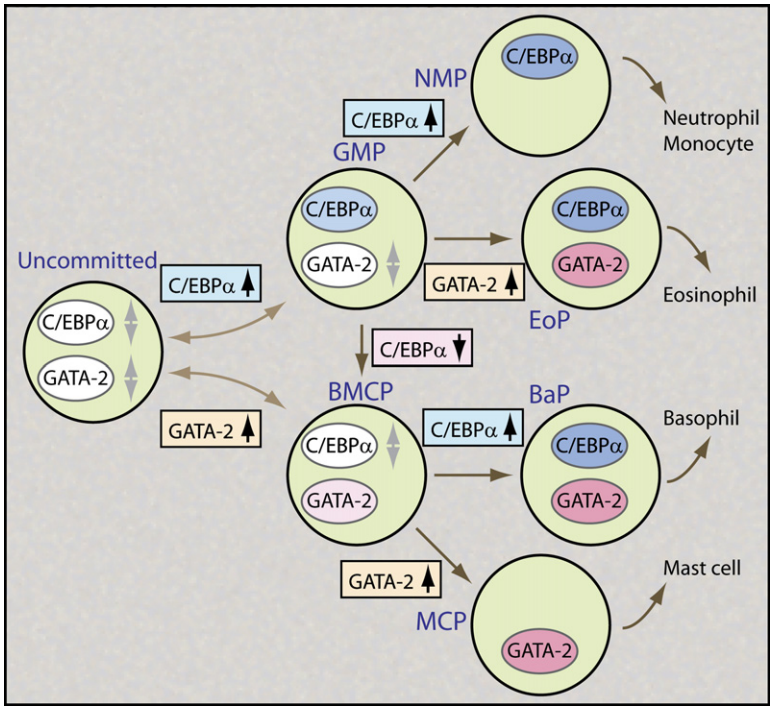

Figure 3. Timing-Based Interplay of C/EBPα and GATA-2 in Lineage Decision of Granulocyte Lineages

If uncommitted cells upregulate C/EBPα first, cells became GMPs. Further upregulation of C/EBPα should lead to the formation of putative NMP that gives rise only to neutrophils and monocytes that are the major products of GMPs. If GATA-2 is upregulated at the GMP stage, cells commit into the eosinophil lineage. In contrast, if uncommitted cells upregulate GATA-2 first, they become the BMCP. Further upregulation of GATA-2 should result in the generation of MCP, whereas if C/EBPα is upregulated at the BMCP stage, cells commit into the basophil lineage (Iwasaki et al., 2006). These results suggest that GATA-2 instructs eosinophil lineage commitment in the presence of a sufficient level of C/EBPα, whereas C/EBPα activates the basophil lineage program in the presence of GATA-2. Collectively, the order of the expression of C/EBPα and GATA-2 can be a critical lineage determinant for the eosinophil, the basophil, the neutrophil, and the mast-cell lineages.

B cell differentiation (DeKoter and Singh, 2000). Thus, the dosage of PU.1 might play a critical role in macrophage versus B cell differentiation when the commitment process is started at an experimental stage completely deficient for PU.1: Cultured *Sfpi1*$^{-/-}$ fetal-liver cells are not normal multipotent progenitors because they are incapable of differentiation into myeloerythroid cells except for macrophages. This phenomenon was not evaluated at the single-cell level, and therefore, it is still unclear whether different concentrations of PU.1 can specify macrophage or B cell fates or can differentially support maturation of each lineage.

The quantity of the transcription factor is also important at the site of mast-cell generation from GMPs. Graded reduction of C/EBPα by transduction of a set of anti-C/EBPα RNAi shows that the frequency of mast-cell development is inversely correlated with the amount of C/EBPα (Iwasaki et al., 2006). In contrast, GMPs retrovirally transduced with C/EBPα lose mast-cell (and basophil) potential, suggesting that the amount of C/EBPα is critical also for GMPs to choose neutrophil versus mast-cell fates (Iwasaki et al., 2006).

Interestingly, downstream of GMPs, C/EBPα is a critical determinant for the mast-cell versus basophil lineage fate decision. The depletion of C/EBPα in BMCPs results in their exclusive differentiation into mast cells (Iwasaki et al., 2006). In contrast to GMPs, retroviral transduction of C/EBPα into BMCPs results in their exclusive generation of basophils, and MCPs enforced with C/EBPα are reprogrammed into basophils (Arinobu et al., 2005). These data collectively suggest that the dosage of single transcription factors is able to specify the lineage in a cell-context-dependent manner.

Lineage Diversification by Timing-Based Interplay of Myeloid Transcription Factors

If the expression oscillates at the priming stage, it is likely to randomly reach the threshold at which it can activate a lineage-specification program. Thus, the timing of activation of each transcription factor could be "stochastic." Recent findings show that eosinophil versus basophil lineage specification could be determined by the order of expression of C/EBPα and GATA-2 (Iwasaki et al., 2006) (Figure 3).

BaPs and EoPs expressed both GATA-2 and C/EBPα, whereas upstream GMPs expressed only C/EBPα (Arinobu et al., 2005; Iwasaki et al., 2005a). All single GMPs with the enforced expression of GATA-2 generate pure eosinophil colonies, indicating that GATA-2 can instruct GMPs to become EoPs (Iwasaki et al., 2006). In contrast, MCPs express GATA-2 but not C/EBPα, and all single MCPs with the enforced expression of C/EBPα form pure basophil colonies (Arinobu et al., 2005). These results suggest that GATA-2 instructs eosinophil lineage commitment in the presence of C/EBPα, whereas C/EBPα activates the basophil lineage program in the presence of GATA-2. Furthermore, C/EBPα and GATA-2 can reprogram CLPs differentially into eosinophil and basophil lineages by changing the timing of their expression (Iwasaki et al., 2006). When CLPs are retrovirally introduced with C/EBPα and then GATA-2 within a 24 hr interval, CLPs transduced with C/EBPα alone generate neutrophil and monocyte/macrophage colonies, whereas those transduced with C/EBPα plus GATA-2 give rise to eosinophil colonies. In contrast, when the order of retroviral infection is switched, CLPs introduced with GATA-2 alone or GATA-2 plus C/EBPα generate mast-cell or basophil

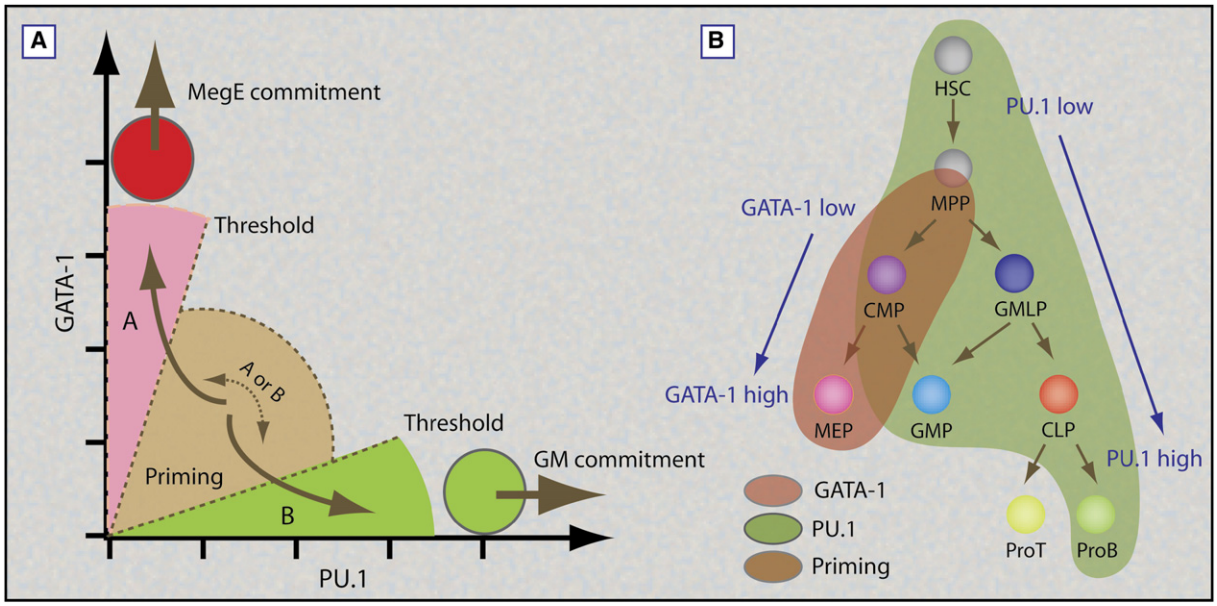

Figure 4. Schematic Representation of Promiscuous Gene Priming and Lineage Commitment in GATA-1 and PU.1

(A) GATA-1 and PU.1 expression is mutually exclusive, but each factor has a positive autoregulatory loop. In this model, commitment occurs if cells express more than threshold amounts (dashed line) of either of transcription factors. GATA-1 and PU.1 are simultaneously expressed at low amounts at bipotent stages (lineage priming), and their expression may fluctuate. When commitment occurs, the dominant transcription factor further upregulates in an autoregulatory manner, whereas the other transcription is shut down.

(B) The distribution of GATA-1 and PU.1 in normal hematopoiesis according to the result of transcriptional reporter mouse systems. LT-HSCs have a low amount of PU.1 but not GATA-1, and at least a low amount of PU.1 is required in all early hematopoietic stages except for MEPs. GATA-1 is upregulated at the CMP stage where both GATA-1 and PU.1 are primed to have both GM and MegE potential. The upregulation of GATA-1 and PU.1 in a fraction of MPPs results in generation of the CMP and the putative GMLP, respectively. Further GATA-1 upregulation suppresses PU.1 transcription in CMPs to give rise to MEPs excluding the GM potential, whereas PU.1 upregulation in the GMLP inhibit the GATA-1 upregulation to produce GMPs or CLPs excluding the MegE potential. The diagram suggests that the competitive interplay of GATA-1 and PU.1 might be the key for hierarchical hematopoietic development.

colonies, respectively. Thus, by simply changing the order of the expression of C/EBPα and GATA-2, CLPs can activate at least four myeloid developmental programs for neutrophil/monocyte, eosinophil, basophile, and mast-cell lineages. These data show that diversity of lineage choices can be orchestrated by the timing of activation of multiple transcription factors. The expression of these primed genes might oscillate independently, and therefore the order of expression of each transcription factor could be random, resulting in the "stochastic" behavior of lineage fate decisions (Ogawa, 1999).

Lineage Diversification by Antagonistic Interplay of Myeloid Transcription Factors

The antagonistic interplay between primary lineage-determining transcription factors is also critical in initiating and resolving priming states (Graf, 2002; Laiosa et al., 2006a; Orkin, 2000). This model was originally proposed by studies of avian progenitors transformed with the E26 virus, which expresses the Myb-Ets oncogene. The MEP^{E26} cells differentiate into either erythrocytes or thrombocytes when the oncoprotein is inactivated, whereas they differentiate into myeloblastic macrophage precursors by activation of the Ras pathway (Graf et al., 1992). Enforced expression of GATA-1 in myeloblasts induces restoration of multipotent MEP^{E26} cells (Kulessa et al., 1995), whereas enforced PU.1 reprograms MEP^{E26} cells into myeloblasts (Nerlov and Graf, 1998). Thus, the balance of PU.1 and GATA-1 can decide multipotent versus macrophage precursor stages of MEP^{E26} cells, and importantly, this process is reversible, suggesting that the expression of priming genes may oscillate. Subsequent mechanistic analyses of GATA-1 and PU.1 functions have shown that these factors physically interact and can functionally antagonize one another in transactivation and differentiation assays (Nerlov et al., 2000; Rekhtman et al., 1999; Zhang et al., 1999). Furthermore, each factor has a positive autoregulatory loop (Chen et al., 1995; Okuno et al., 2005; Tsai et al., 1991) (Figure 4A).

A similar competitive interplay of transcription factors also exists in macrophage versus neutrophil cell fate decision. Macrophage and neutrophil development require the transcription factors PU.1 and C/EBPα, respectively (Scott et al., 1994; Zhang et al., 1997). IL-3-dependent PU.1-deficient myeloid progenitors differentiate into macrophage in the presence of a high PU.1 induction, whereas they differentiate into neutrophils with a low amount of PU.1. C/EBPα inhibits the ability of PU.1 to activate transcription directed by a minimal promoter containing multiple PU.1-binding sites (Reddy et al., 2002). As such, enforced C/EBPα represses PU.1-induced macrophage

differentiation, simultaneously enhancing neutrophil differentiation. Because G-CSF increases the C/EBPα:PU.1 ratio in IL-3-dependent PU.1-deficient myeloid progenitors, G-CSF signaling could be an external cue for granulocyte differentiation by increasing the relative concentration of C/EBPα at the macrophage versus neutrophil binary cell fate decision (Dahl et al., 2003).

It is well known that either a mutually inhibitory-feedback or positive-feedback loop of two factors can produce bistability that plays like a toggle switch between two discrete states (Angeli et al., 2004). This might be the case for both GATA-1 versus PU.1, and C/EBPα versus PU.1 cross-antagonisms (Figure 4A). In addition to the cross-antagonism, GATA-1 and PU.1 have positive autoregulatory loops. A mathematical model integrating both cross antagonism and positive autoregulation shows that in addition to classical bistable states, there is another stable region corresponding to the priming stage (Figure 4A) (Enver and Huang, 2005, ASH, abstract). In this model, the priming stage constitutes a stage where cells can stably express a low level of two transcription factors. For lineage-specific programs to be activated, cells need to upregulate either of them beyond the threshold (Enver and Huang, 2005, ASH, abstract). These mathematical models represent a small network module, and regulation of hematopoietic development might consist of multiple modules that may further interact with one another. For example, the secondary cross-antagonism composed of Egr/Nab and Gfi-1 has been proposed downstream of C/EBPα and PU.1 (Laslo et al., 2006). These models are likely to capture essential features of the hematopoietic hierarchy, and they should ultimately be useful to predict the genome-scale behavior of multipotent cells in lineage specification.

Lineage Tracing by Utilizing Transcription-Factor Reporters

If the hierarchical lineage fate decision is operated by single or a collaboration of multiple transcription factors, tracing the timing and the amount of transcription-factor expression should in turn be useful to delineate early hematopoietic developmental pathways.

In two mouse lines having a GFP reporter knocked into the PU.1 locus (Back et al., 2004; Nutt et al., 2005), $CD34^-$ LSK HSCs express low PU.1-GFP (Iwasaki et al., 2005b; Nutt et al., 2005). A fraction of $CD34^+$ MPPs begins to express a high PU.1-GFP, which is further upregulated in CMPs, GMPs, and CLPs, but not MEPs (Iwasaki et al., 2005b; Nutt et al., 2005). In contrast, in mice having a transgenic GFP reporter for GATA-1 (Iwasaki et al., 2005a), $CD34^-$ LSK HSCs do not express GATA-1-GFP, but a fraction of $CD34^+$ MPPs express GATA-1-GFP at a low level. GATA-1-GFP is further upregulated in CMPs and MEPs but not in GM or lymphoid progenitors. In Figure 4B, the distribution of PU.1 and GATA-1 reporters in normal hematopoiesis is overlaid on the cellular-evidence-based developmental scheme. In this developmental model, HSCs express low PU.1, perhaps to maintain self-renewal activity (Iwasaki et al., 2005b), and the subsequent upregulation of GATA-1 occurs in a fraction of $CD34^+$ MPPs and CMPs to establish the bipotent GATA-1 versus PU.1 priming state. Then, when PU.1 becomes dominant, the CMP and the putative GMLP (or the LMPP) give rise to GMPs plus CLPs, whereas if GATA-1 becomes dominant, CMPs produce MEPs at the expense of GMPs. In fact, $CD34^+$ MPPs expressing a high level of PU.1-GFP possess predominantly the GM- and lymphoid-restricted developmental potential (unpublished data). This diagram nicely fits the concept that the competitive interplay of PU.1 and GATA-1 might play a critical role in early hematopoietic lineage fate decision (Graf, 2002; Laiosa et al., 2006a; Orkin, 2000).

Ikaros is a transcription factor that plays a critical role in T and B cell development. $Ikaros^{-/-}$ mice lack all B cells and have only a small number of T cell precursors in the thymus (Wang et al., 1996) with an increased number of myeloid cells (Nichogiannopoulou et al., 1999). In a transgenic mouse strain expressing the GFP reporter under control of an Ikaros promoter-enhancer (Kaufmann et al., 2003), the LSK population is subdivided into $GFP^{-/lo}$ and GFP^+ cells, and the latter is exclusively found within the $Flt3^+$ population (Yoshida et al., 2006) that is an equivalent of the LMPP (Adolfsson et al., 2005). Ikaros-GFP^+ LSK cells differentiate mainly into GM cells as well as into T and B cells but form only a few percent of mixed colonies containing both GM and MegE cells (Yoshida et al., 2006). These data suggest that the graded upregulation of Ikaros is associated with progression of restriction into the GM and lymphoid lineages. Thus, both Ikaros and PU.1-reporter analyses again support the existence of the putative GMLP in early hematopoiesis (Figure 1).

By tracking the expression of transcription factors or genes capable of lineage instruction, we should be able to visualize the stage at which each developmental program turns on. Accordingly, the lineage-instructive signal-based fractionation studies utilizing transcription-factor reporter systems should be useful to further understand the developmental pathway and mechanisms in early hematopoietic development in future studies.

Conclusions

The ability to prospectively isolate lineage-restricted progenitors has greatly helped us understand the mechanism of lineage commitment from HSCs. It is now clear that murine HSCs prime myeloid but not lymphoid genes as their natural property. On a cellular basis, however, we have not reached a general agreement concerning where the myeloid and lymphoid branching occurs. On a molecular basis, cooperative and antagonistic interplays between transcription factors as well as timing of their expression might play a critical role in hematopoietic lineage commitment. It is still unclear, however, how such transcription factors are activated or repressed in a cell-context-dependent manner. Future studies on epigenetic and posttranscriptional regulation of these lineage determinants are critical to further understand the mechanism of hematopoietic development from multipotent HSCs.

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