RESEARCH COMMUNICATION

Multipotential differentiation ability of GATA-1-null erythroid-committed cells

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GATA-1, a zinc finger transcription factor, has been believed to be indispensable for the survival of proerythroblasts. However, we found that GATA-1-null proerythroblasts could survive and proliferate on OP9 stroma cells in the presence of erythropoietin. Furthermore, myeloid and mast cells were induced from the GATA-1-null proerythroblasts by the stimulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3), respectively, but lymphoid differentiation was not achieved by in vivo transfer. Thus, without activity of the transcription factor required for terminal differentiation, even relatively mature and committed cells proliferate continuously with the differentiation capacity to other lineages. Our data suggest that GATA-1 is a critical transcription factor to fix erythroid progenitors to the erythroid lineage.

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Hematopoiesis is a cell differentiation process tightly controlled by various lineage-specific transcription factors (Cantor and Orkin 2001). Among them, GATA zinc finger transcription factors are one of the most well-studied transcription factors, and three members of the family, GATA-1, GATA-2, and GATA-3, are involved in hematopoiesis (Cantor and Orkin 2001). GATA-1, the founding member of the GATA family, participates in the expression of numerous genes involved in erythropoiesis (Morceau et al. 2004; Welch et al. 2004; Ferreira et al. 2005). Gene targeting analysis has revealed that GATA-1 is an essential transcription factor for erythropoiesis (Pevny et al. 1991, 1995; Weiss et al. 1994; Fujiwara et al. 1996). GATA-1-null embryonic stem (ES) cells gave rise to definitive erythroid precursors. But the GATA-1-null erythroid cells were arrested at the proerythroblast stage in vitro and failed to contribute to adult red blood cells in chimeric mice. Therefore, GATA-1-null mice showed complete ablation of erythropoiesis owing to the arrested maturation and apoptosis of erythroid precursors at the proerythroblast stage. Meanwhile, overexpression of GATA-1 reprograms my-

eloid cells to undergo erythroid and megakaryocytic differentiation (Kulesa et al. 1995; Iwasaki et al. 2003).

In vitro differentiation of ES cells to blood cells is a powerful tool for the analysis of hematopoietic differentiation and development because it enables us to analyze the direct consequences of the effects of gene disruption. There are basically two methods used for in vitro differentiation: the conventional embryoid body formation method and the coculture of ES cells on a stroma cell line, such as OP9. The coculture of ES cells on OP9 cells, the OP9 system, has several advantages, including the quantitative analysis of terminally differentiated blood cells by providing an appropriate hematopoietic microenvironment (Nakano et al. 1994, 1996; Kitajima et al. 2002; Tanaka et al. 2004; Zheng et al. 2006). In the OP9 system, various types of blood cells, including erythrocytes, are developed, and the time course of the development precisely recapitulates normal erythroid development (Nakano et al. 1996; Kimura et al. 2000).

In this study, we examined the erythroid differentiation from GATA-1-null ES cells using the OP9 system. Unexpectedly, GATA-1-null erythroid cells similar to proerythroblasts continued to proliferate on OP9 cells in the presence of erythropoietin (EPO). A small percentage of these presumably erythroid-committed cells came to express myeloid markers spontaneously. Furthermore, the cells differentiated to mature myeloid cells and mast cells when EPO was replaced by granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3), respectively. On the other hand, lymphoid differentiation was not detectable after in vivo transfer of the cells. In other words, the GATA-1-null proerythroblasts retained the differentiation ability to myeloid but not to lymphoid lineages. These data show that "commitment" takes place but is not feasible without the transcription factor essential for terminal differentiation.

Results and Discussion

Proliferation of erythroid cells derived from GATA-1-null ES cells

Differentiation to erythroid cells from wild-type and GATA-1-null ES cells was carried out using the OP9 system in the presence of EPO (Nakano et al. 1996; Kimura et al. 2000). Wild-type ES cells differentiated to mature erythrocytes and disappeared by completing terminal differentiation ~2 wk after the differentiation initiation. In contrast, GATA-1-null ES cells gave rise to cells that continued to proliferate for >3 mo in a logarithmic manner (Fig. 1A). Morphologically, the cells were similar to proerythroblasts in normal erythropoiesis (Fig. 1B). The GATA-1-null cells could not survive on OP9 cells without EPO or in the presence of EPO without OP9 cells, showing that the cells were strictly dependent on both EPO and OP9 stroma cells (data not shown). A significant proportion (15%–40%) of the GATA-1-null cells continuously died due to apoptosis in our culture (Fig. 1C). It has been reported that under the GATA-1-null condition, erythroid cells die at the proerythroblast stage by apoptosis (Weiss and Orkin 1995; Pan et al. 2005). Apoptosis of the GATA-1-null cells under our culture

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Figure 1. Characterization of GATA-1-null proerythroblasts. (A) Numbers of cells during hematopoietic differentiation from wild-type (WT) and GATA-1-null (KO) mouse ES cells on OP9 stroma cells. (B) May-Giemsa staining of GATA-1-null proerythroblasts at day 20 of the culture. (C) Cell cycle analysis of the GATA-1-null proerythroblasts at day 20. (D) FACS analysis for surface markers of various hematopoietic lineages. (E) Multiparameter FACS analysis of wild-type cells at day 12 and GATA-1-null cells at day 20. (F) RT-PCR analysis of transcription factors involved in lympho-hematopoiesis. (WT) Data of day 12-induced cells; (KO) data of day 20-induced cells.

condition would reflect the apoptosis of GATA-1-null proerythroblasts *in vivo* (Fujiwara et al. 1996).

Expression of surface markers was analyzed to nail down the differentiation status of the cells (Fig. 1D,E). The GATA-1-null cells showed a high expression of CD71, transferrin receptor, and a low expression of an erythroid marker, TER119. c-Kit was expressed on the cells, but CD34 and Sca-1 were not. Megakaryocyte markers such as glycoprotein IIb and V (GPIIb and GPV) were negative, as well as lymphoid markers. Myeloid markers, Mac-1, Gr-1, and FcR γ , were negative for the majority of the cells, but a small population of the cells (~5%) expressed the myeloid markers. Multiparameter FACS analysis revealed that the majority of the CD71^{high} cells were TER119^{low/-}, c-KIT⁺, and Mac-1⁻ (Fig. 1E), and most c-KIT⁺ cells were negative for Mac-1. And four-color analysis revealed that the c-KIT⁺ cells were Sca-1⁻FcR γ ⁻CD34⁻. These data showed that the majority of the c-KIT⁺ cells were immature erythroid cells, and were neither mast cells nor myeloid progenitors. β -major globin gene and the other erythroid-specific markers, *erythroid-specific 5-aminolevulinate synthase* (ALAS-E), *5-aminolevulinate dehydratase* (ALAD), ferrochelatase (FC), and *porphobilinogen deaminase* (PBGD), all of which were GATA-1 target genes, were expressed in the GATA-1-null cells (Fig. 1F). From these data and

morphology, the vast majority of GATA-1-null cells possess erythroid characteristics and presumably belong to the stage of proerythroblasts.

Next, expression of various hematopoietic transcription factors between wild-type and GATA-1-null cells in the unsorted and sorted CD71^{high}Mac-1⁻ fractions were analyzed (Fig. 1F). For comparison, wild-type TER-119⁺ erythroid cells at day 12 after coculturing on OP9 cells in the presence of EPO were used, because day 12-induced cells were morphologically similar to the GATA-1-null erythroid cells. GATA-2 expression level of the GATA-1-null proerythroblasts was fivefold more than that of day 12 erythroid cells. Expression of the transcription factors *Pax5* and *GATA-3* involved in B- and T-cell differentiation was not observed. However, *PU.1*, a crucial regulator for macrophage, mast cells, and B cells (Scott et al. 1994; DeKoter and Singh 2000; Walsh et al. 2002), was highly expressed in the GATA-1-null proerythroblasts. In addition, a small population of the cells showed high expression levels of myeloid markers, Mac-1 and Gr-1 (Fig. 1D).

Differentiation from GATA-1-null proerythroblasts to myeloid and mast cells

High *PU.1* expression and the small population of cells expressing myeloid markers imply that GATA-1-null proerythroblasts could retain the differentiation ability to myeloid lineages, and a small proportion of the cells could spontaneously differentiate to myeloid cells. To examine this possibility, we first analyzed the expression of various cytokine receptors in the unsorted cells and the FACS-purified CD71^{high}Mac-1⁻ GATA-1-null cells by RT-PCR (Fig. 2A). mRNA of not only *EPO receptor*, but also myeloid lineage receptors *GM-CSF receptor- α* and *IL-3 receptor- α* , were expressed in the GATA-1-null erythroid, namely CD71^{high}Mac-1⁻ cells. The expression of *c-fms*, *G-CSF receptor*, and *IL-7 receptor- α* were detected in the unsorted GATA-1-null cells but not in the sorted GATA-1-null CD71^{high}Mac-1⁻ cells. These results showed that *c-fms*, *G-CSF receptor*, and *IL-7 receptor- α* were expressed in the GATA-1-null non-erythroid cells.

Responsiveness of the cells to the ligands of these receptors was analyzed (Fig. 2B). The cells responded to GM-CSF and IL-3 up until 1 wk after changing the culture from an EPO-containing medium to a GM-CSF- or IL-3-containing medium. Notably, the growth curves after changing media were similar to that of the EPO-containing medium. The GATA-1-null cells did not respond to M-CSF, G-CSF, or IL-7, which was consistent with the mRNA expression of the receptors of these cytokines (Fig. 2A). The cells cultured in the presence of GM-CSF differentiated to neutrophils and macrophages in a week, which was confirmed by morphological, phagocytic activity, and surface marker analyses (Fig. 2C,D). In contrast, IL-3 stimulation induced the differentiation to mast cells (Fig. 2E).

GATA-1-null proerythroblasts did not express *c-mpl*, thrombopoietin (TPO) receptor, and did not respond to TPO (Fig. 2A,B). However, erythroid and megakaryocytic cells have been reported to have closely related lineages (Debili et al. 1996; Era et al. 1997; Akashi et al. 2000; Vannucchi et al. 2000). Then, we examined whether reconstituted TPO/*c-mpl* signaling induced the differentiation to megakaryocytes after retroviral expression of

Figure 2. Responsiveness of GATA-1-null proerythroblasts to various cytokines. (A) RT-PCR analysis of cytokine receptors. (B) Number of cells after altering the condition from an EPO-containing medium to the media containing cytokines as described. Data are shown as mean $\pm$ standard deviation ($n = 6$). (C) May-Giemsa staining and latex phagocytosis analysis of cells after culturing in the presence of GM-CSF for 7 d. (D) FACS analysis of cells after culturing in the presence of GM-CSF for 7 d. (E) May-Giemsa and toluidine blue staining of cells after culturing in the presence of IL-3 for 7 d. (F) Number of cells infected with control EGFP and *c-mpl* retrovirus 1 wk after culturing in the presence of EPO or TPO as described. (G) Expression of a megakaryocyte marker GPV on the *c-mpl*-expressing cells 1 wk after culturing in the presence of EPO or TPO as described.

c-mpl. Although expression of *c-mpl* induced TPO-dependent cell proliferation, no megakaryocytic differentiation took place (Fig. 2F,G). These data suggest that the GATA-1-null proerythroblasts have lost the differentiation capacity to megakaryocytic lineage. To analyze the in vivo differentiation activity to myeloid lineage cells, transplantation of the GATA-1-null proerythroblasts to sublethally irradiated NOD/SCID mice was carried out. GATA-1-null proerythroblasts did not contribute to lymphoid, erythroid, or myeloid repopulation in the mice 4 wk after the transplantation (data not shown). Considering that the survival of the GATA-1-null cells required the cytokine stimulation of EPO, GM-CSF, or IL-3 (Fig. 2B), the defective myeloid differentiation in vivo would probably be due to some inappropriate microenvironment in the transplanted mice.

Differentiation dynamics to myeloid cells

When the cells were stained with anti-Mac-1 and anti-CD71 antibodies, the vast majority of GATA-1-null cells

belonged to the CD71^{high}Mac-1⁻ population. A significant percentage of the cells other than this major population expressed Mac-1. There are two possibilities to explain the existence of these two populations. One is that both CD71^{high}Mac-1⁻ cells and Mac-1⁺ cells proliferate simultaneously, and the other is that one population is the progeny of the other. When sorted CD71^{high}Mac-1⁻ cells were cultured in the presence of EPO, the cells gave rise to both populations (Fig. 3A). In contrast, CD71^{low}Mac-1⁺ cells did not produce CD71^{high}Mac-1⁻ cells (data not shown).

Growth of the cells after altering the cytokines from EPO to GM-CSF was quite similar to the growth in the presence of EPO (Fig. 2B), suggesting that the majority of the cells responded to GM-CSF. This indicates that CD71^{high}Mac-1⁻ cells responded to GM-CSF. It is unlikely that only the minor population that did not belong to the CD71^{high}Mac-1⁻ population responded to GM-CSF, because the minor population was <10% and the kinetics of proliferation after changing the cytokines was very fast. To confirm that the small fraction of CD71^{low}Mac-1⁺ cells was not propagated preferentially in the GM-CSF culture, the sorted CD71^{high}Mac-1⁻ cells were cultured in the presence of GM-CSF. As shown in Figure 3B, the CD71^{high}Mac-1⁻ cells decreased gradually, and the CD71^{low}Mac-1⁺ cells reciprocally increased after switching the cytokines. The sorted CD71^{high}Mac-1⁻ cells exhibited the characteristics of macrophage and neutrophil after culturing in the presence of GM-CSF (data not shown). Next, we carried out cloning experiments using a limiting dilution method. CD71^{high}Mac-1⁻ cells were sorted, and cloned cells were propagated in the presence of EPO. The cells were then transferred to the culture containing GM-CSF. The data of two representative clones are shown in Figure 3, C and D. Again, EPO-responsive CD71^{high}Mac-1⁻ clonal cells gave rise to CD71^{low}Mac-1⁺ cells in the presence of EPO, and differentiated to neutrophils and macrophages in the presence of GM-CSF.

Figure 3. Differentiation from CD71^{high}Mac-1⁻ cells to myeloid cells. (A) Expression of CD71 and Mac-1 on unsorted cells, sorted cells, and the cells cultured for 1 wk in the presence of EPO after sorting. (B) Transition from the sorted CD71^{high}Mac-1⁻ cells to myeloid cells in the presence of GM-CSF. (C) Expression of CD71 and Mac-1 on the cloned GATA-1-null proerythroblasts in the presence of EPO or GM-CSF.

Figure 4. Terminal differentiation induced by retroviral gene expression. (A) Expression of CD71 and Mac-1 on the unsorted cells and the sorted cells. (B) Terminal erythroid differentiation using GATA-1-expressing retrovirus. Benzidine staining is shown. (C) CD71 and TER-119 expression after the infection of GATA-1 retrovirus. (D) Expression of CD71 and Mac-1 after retroviral introduction of C/EBP α and C/EBP β . (E) May-Giemsa staining of the cells infected with control, C/EBP α , and C/EBP β retrovirus and cultured for 1 wk in the presence of EPO.

Terminal differentiation by forced expression of GATA-1 and C/EBP

We analyzed the effect of transcription factors on cell differentiation using retroviral gene transduction. The first experiment is the recapitulation of GATA-1 expression in the GATA-1-null proerythroblasts. The CD71^{high}Mac-1[−] cells were purified (Fig. 4A) and infected with the retroviruses. Two days after the infection of retrovirus expressing GATA-1 and EYFP, EYFP-positive cells were sorted and analyzed. Benzidine-positive cells appeared, and more than half of the infected cells exhibited a high expression of Ter-119 (Fig. 4B,C). In addition, enucleated erythrocytes appeared 4 d after the infection (data not shown). These data show that only a relatively short period was necessary to complete the terminal erythroid differentiation of GATA-1-null proerythroblasts. When the retroviral-infected cells were cultured in the presence of TPO, none of the megakaryocyte markers such as GPIIb, GPV, and acetylcholine esterase was expressed (data not shown), suggesting that megakaryocytic differentiation was not recapitulated by the expression of GATA-1.

C/EBP α or C/EBP β are crucial transcription factors in myeloid differentiation, and overexpression of the factors can induce myeloid differentiation (Heavey et al. 2003; Xie et al. 2004). Retroviral overexpression of C/EBP α and C/EBP β induced the differentiation of the GATA-1-null CD71^{high}Mac-1[−] proerythroblasts to CD71^{low}Mac-1⁺ neutrophils in the presence of EPO (Fig. 4D). These data show that EPO signaling is not inhibitory to granulocytic differentiation under the GATA-1-null condition. Although the GATA-1-null CD71^{high}Mac-1[−] cells expressed GATA-2, Fog-1, and Scl

(Fig. 1F), all of which were implicated in the regulation of erythropoiesis, expression of those factors was not sufficient for the definite commitment to the erythroid lineage.

Differentiation status and potential of GATA-1-null proerythroblasts

It is difficult to precisely determine to which differentiation stage the GATA-1-null erythroid cells belong, because the GATA-1-null erythroid cells could have resulted from some aberrant differentiation. However, it is conceivable that the cells are similar to proerythroblasts for the following reasons: (1) the morphology and erythroid marker expression, and (2) the short period for the completion of the terminal differentiation. Completion of erythroid differentiation in 2–4 d after the recapitulation of GATA-1 verifies that the GATA-1-null cells are the committed, relatively mature erythroid cells (Fig. 4B).

Characteristics of the GATA-1-null proerythroblastic cells are summarized in Figure 5. The erythroid-committed cells possess the differentiation ability to become macrophages, neutrophils, and mast cells, but cannot differentiate to lymphoid cells or megakaryocytes. It is possible that GATA-1-null proerythroblasts could not give rise to lymphoid cells, since erythroid and lymphoid lineages are distantly separated from the standpoint of hematopoietic lineages. It is intriguing that megakaryocytic differentiation cannot be achieved even when TPO/*c-mpl* signaling was restored in the GATA-1-null erythroid cells. Even though erythroid and megakaryocytic lineages are considered quite close and share various molecular mechanisms in differentiation, there may exist some specific molecular mechanisms prohibiting differentiation to an adjacent cell lineage. Recently, Weiss and colleagues (Stachura et al. 2006) found that common megakaryocyte–erythroid precursor (MEP) cells could be obtained by the differentiation of GATA-null ES cells with the OP9 system. They added TPO instead of EPO during the differentiation induction and obtained MEP cells. In both cases, additional mutation(s) does not seem to be necessary. It remains to be elucidated how the different cytokine signals induce different multipotent progenitor cells with different phenotypes.

In zebrafish, hematopoietic differentiation alteration under the GATA-1-null condition was analyzed during ontogeny (Galloway et al. 2005). Loss of GATA-1 transforms primitive blood precursors into myeloid cells in vivo and brought about subsequent massive expansion of granulocytic neutrophils and macrophages at the expense of red blood cells. Although the study did not define whether the conversion to myeloid lineage cells oc-

Figure 5. Diagram of the differentiation activity of GATA-1-null proerythroblasts.

curred via committed erythroid cells, our present data of multipotential proerythroblasts is consistent with the phenotype of zebrafish GATA-1-null hematopoiesis. Thus, in both fish and mammals, the absence of erythroid terminal differentiation brought about differentiation capacity to the myeloid lineages.

Lineage instability of the cells lacking the transcription factor essential for differentiation

B-cell commitment and development requires the stepwise activation of multiple transcription factors, including the basic helix-loop-helix (bHLH) E2A proteins and the paired homeodomain-containing protein Pax5 (Urbánek et al. 1994; Zhuang et al. 1994). Pro-B cells lacking Pax5 or E2A have been reported to function as pluripotent hematopoietic progenitors (Mikkola et al. 2002; Ikawa et al. 2004). These pro-B cells exhibit promiscuous gene expression, such as myeloid and erythroid lineages, and possess the capability to differentiate to T, NK, and myeloid lineages. Since wild-type pro-B cells can be propagated with the stimulation of IL-7 and SCF without further differentiation, it is conceivable that Pax5- and E2A-null pro-B cells could be maintained similarly.

Differentiation-blocked pro-B cells lacking the transcription factors essential for differentiation could have been exceptional cases. Our present study, however, indicates that such lineage infidelity would be a more general rule when the transcription factors essential for subsequent differentiation are nullified. The other important point is that not only very immature and potentially propagative cells such as pro-B cells but also the committed cells still possess the ability to differentiate to other lineages.

Compared with B lymphoid cells, it is extremely difficult to culture myeloid and erythroid lineages maintaining a specific differentiation stage. It is surprising that relatively mature erythroid-committed proerythroblasts gained long-term proliferation ability when the transcription factor necessary for terminal differentiation was eliminated. Notably, GATA-1-null proerythroblasts differentiated to granulocytes, macrophages, and mast cells, but not to megakaryocytes or lymphoid cells. It is conceivable that GATA-1-null proerythroblasts are relatively mature, compared with Pax5- and E2A-null pro-B cells, from the view of the differentiation process. Therefore, the relatively limited differentiation ability of the GATA-1-null erythroblasts could be attributed to the maturation level of the cells. Meanwhile, the GATA-1-null cells could not differentiate to megakaryocytes in spite of the close lineage relationship between erythroid and megakaryocytic lineages. There could be some active mechanisms to inhibit the differentiation to the closely related lineage.

Recently, it has been reported that the fate of committed hematopoietic progenitors can be altered by cytokine signaling or enforced expression of a transcription factor (Kondo et al. 2000; Iwasaki et al. 2003; Iwasaki-Arai et al. 2003; Xie et al. 2004). Moreover, the cells that once expressed a lineage marker possess the ability to differentiate into the other lineages. Our data strongly support the novel notion of the differentiation flexibility of the committed cells. Namely, although commitment proceeds gradually and the differentiation ability is reduced along the differentiation progression, even the commit-

ted cells possess the differentiation ability into lineages other than the committed lineage.

Materials and methods

Cells

Cultures of wild-type E14tg2a (WT) and GATA-1-null (KO) ES cells (Zheng et al. 2006) were maintained as previously described (Kitajima et al. 2002). The hematopoietic differentiation induction of ES cells on OP9 stromal cells has been described (Nakano et al. 1994, 1996). Human EPO (2 U/mL), murine GM-CSF (10 ng/mL), murine IL-3, murine IL-7 (10 ng/mL), human TPO (10 ng/mL), human M-CSF (10 ng/mL), and G-CSF (10 ng/mL) were added in the culture as indicated. Phagocytosis was examined by incubation of cells with the microsphere (Fluoresbrite carboxylate 0.75- μ m microspheres, Polysciences, Inc.).

Antibodies, staining, and analysis

Biotinylated TER-119 antibody was a kind gift from Dr T. Kina (Kyoto University, Kyoto, Japan) (Ikuta et al. 1990). PE-conjugated anti-CD71 (e-Bioscience), biotinylated anti-GPV (Seikagaku Corp.) (Takada et al. 1995), anti-GPIIb, biotinylated Mac-1, biotinylated or PE-conjugated GR-1, FITC-conjugated Fc γ , biotinylated B220, biotinylated Thy-1.2, biotinylated or APC/Cy7-conjugated anti-c-KIT, PE-conjugated anti-CD34, and biotinylated or APC-conjugated SCA-1 antibodies (Pharmingen) were used for FACS analysis. The biotinylated antibodies were visualized by PE- or Cychrome-conjugated streptavidin (Pharmingen), and the GPIIb antibody was visualized by PE-conjugated anti-Rat IgG antibody (Pharmingen). The cells were stained with the antibodies as previously described (Kitajima et al. 2002). The analysis of stained cells and cell sorting were done by FACSCalibur and FACSAria (Becton Dickinson). Cell cycle was analyzed by staining cells with propidium iodide.

RT-PCR analysis

Total RNA was isolated using RNeasy (Qiagen) and reverse transcribed by the ThermoScript RT-PCR system (Invitrogen). The cDNA was diluted in series, and PCR was performed for 25–30 cycles using specific primers whose sequences will be provided on request.

Retrovirus transduction

Retrovirus plasmid vector pMY.IRES.EGFP, human c-mpl cDNA, and PLAT-E ecotropic packaging cell line (Morita et al. 2000) were kind gifts from Dr. T. Kitamura (Tokyo University, Tokyo, Japan). pMY.IRES.EYFP was constructed by replacing the EGFP gene of pMY.IRES.EGFP to EYFP gene purchased from Clontech. GATA-1, C/EBP α , and C/EBP β cDNAs were inserted into the multicloning site (MCS) of pMY.IRES.EYFP, and c-mpl cDNA was inserted into the MCS of pMY.IRES.EGFP. The retrovirus supernatant was obtained by a transient transfection of the retrovirus plasmid vectors to the PLAT-E packaging cell line using Lipofectamine 2000 (Invitrogen). The infection was done by spinoculation (2 h, 25°C, 2500 rpm). Retroviruses expressing GATA-1, C/EBP α , and C/EBP β were infected to the GATA-1-null CD71^{high}MAC-1⁺ erythroid cells.

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