The EMBO Journal (2006) 25, 3398–3410 | © 2006 European Molecular Biology Organization | All Rights Reserved 0261-4189/06
www.embojournal.org

THE EMBO JOURNAL

Comprehensive analysis of myeloid lineage conversion using mice expressing an inducible form of C/EBPα

Yumi Fukuchi[1], Fumi Shibata[1,4], Miyuki Ito[2,4], Yuko Goto-Koshino[1], Yusuke Sotomaru[3], Mamoru Ito[3], Toshio Kitamura[1] and Hideaki Nakajima[2,*]

[1]Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [2]Center of Excellence, Institute of Medical Science, University of Tokyo, Tokyo, Japan and [3]Central Institute for Experimental Animals, Kanagawa, Japan

CCAAT/enhancer-binding protein (C/EBP) α is a critical regulator for early myeloid differentiation. Although C/EBPα has been shown to convert B cells into myeloid lineage, precise roles of C/EBPα in various hematopoietic progenitors and stem cells still remain obscure. To examine the consequence of C/EBPα activation in various progenitors and to address the underlying mechanism of lineage conversion in detail, we established transgenic mice expressing a conditional form of C/EBPα. Using these mice, we show that megakaryocyte/erythroid progenitors (MEPs) and common lymphoid progenitors (CLPs) could be redirected to functional macrophages *in vitro* by a short-term activation of C/EBPα, and the conversion occurred clonally through biphenotypic intermediate cells. Moreover, *in vivo* activation of C/EBPα in mice led to the increase of mature granulocytes and myeloid progenitors with a concomitant decrease of hematopoietic stem cells and nonmyeloid progenitors. Our study reveals that C/EBPα can activate the latent myeloid differentiation program of MEP and CLP and shows that its global activation affects multilineage homeostasis *in vivo*.
The EMBO Journal (2006) **25,** 3398–3410. doi:10.1038/sj.emboj.7601199; Published online 6 July 2006
Subject Categories: differentiation & death; immunology
Keywords: CCAAT/enhancer-binding protein α; C/EBPα; hematopoietic progenitors; plasticity; transgenic mice

Introduction

Sequential lineage specification initiating from hematopoietic stem cells (HSCs) is a fundamental characteristic of blood cell production. These processes, called commitment, are often dictated by the instructive action of lineage-specific transcription factors, which ultimately restricts their fate of differentiation (Shivdasani and Orkin, 1996). It has been thought that the commitment was an irreversible process, and cells differentiated into a certain lineage would not change their own fate. However, recent evidence suggests that many immature progenitors still sustain latent differentiation programs to other lineages than their own, which can be initiated by ectopic activation of cytokine signals or transcription factors. It was reported that enforced signals from interleukin (IL)-2 or granulocyte macrophage-colony stimulating factor (GM-CSF) could induce myeloid conversion in common lymphoid progenitor (CLP) and pro-T cells (Kondo *et al*, 2000; King *et al*, 2002; Iwasaki-Arai *et al*, 2003). In addition, ectopic expression of GATA-1 redirected lymphoid and myeloid progenitors—including CLP, pro-B, common myeloid progenitor (CMP), and granulocyte/monocyte progenitor (GMP)—to the megakaryocyte/erythroid (Meg/E) lineage (Iwasaki *et al*, 2003). CD19$^+$ B cells can also be reprogrammed into macrophages by enforced expression of CCAAT/enhancer binding protein α (C/EBPα) (Xie *et al*, 2004). These studies collectively revealed unexpected plasticity of primary lymphoid and myeloid progenitors, especially a latent multipotentiality of lymphoid cells. However, plasticity of megakaryocyte/erythroid progenitor (MEP) has not yet been reported.

C/EBPα is a member of the C/EBP family of transcription factors that contain a conserved leucine-zipper dimerization motif adjacent to a basic DNA-binding domain (Landschulz *et al*, 1988). Within the hematopoiesis, C/EBPα acts as a key factor for early granulopoiesis (Radomska *et al*, 1998) and regulates a number of myeloid genes (Tenen *et al*, 1997; Iwama *et al*, 1998). The loss of C/EBPα in mice leads to the complete absence of mature neutrophils and eosinophils in fetal liver (FL) and newborns because of the differentiation-arrest at an early myeloblast stage (Zhang *et al*, 1997). C/EBPα$^{-/-}$ FL progenitors were hyperproliferative and showed decreased differentiation potential by a block in the ability of multipotential progenitors to differentiate into bipotential granulocyte/monocyte (G/M) progenitors and their progeny (Heath *et al*, 2004). In addition, conditional disruption of C/EBPα revealed that it was necessary during the transition from CMP to GMP, but not beyond this stage, for terminal granulocyte maturation in adult bone marrow (BM) (Zhang *et al*, 2004).

Enforced expression of transcription factors by retrovirus has been utilized to address the lineage plasticity of hematopoietic progenitors (Iwasaki *et al*, 2003). However, this system cannot control the activity of the transcription factors, which hampered the detailed examination of the conversion process. In addition, the conversion cannot be investigated directly *in vivo*, as *ex vivo* manipulation of the progenitor is inevitable for virus infection. For these reasons, a novel system that does not involve virus transduction and enables conditional regulation of transcription factor activity is warranted to analyze the molecular basis of lineage conversion in more detail.

*Corresponding author. Center of Excellence, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Tel.: +81 3 5449 5759; Fax: +81 3 5449 5453; E-mail: hnakajim@ims.u-tokyo.ac.jp
[4]These authors contributed equally to this work

Received: 20 September 2005; accepted: 29 May 2006; published online: 6 July 2006

 ©2006 European Molecular Biology Organization

In this study, we established transgenic mice expressing a conditional form of C/EBPα whose activity can be regulated by 4-hydroxy tamoxifen (4-HT). Using these mice, we tested various progenitors, especially MEP and CLP, to determine if they could be redirected to myeloid lineage by C/EBPα activation *in vitro* and *in vivo*. We found that both MEP and CLP could be converted to the myeloid lineage by C/EBPα through biphenotypic intermediate cells by a clonal analysis, and surprisingly, this lineage conversion was accomplished by only a short-term activation of C/EBPα. Moreover, *in vivo* activation of C/EBPα induced an increase of mature granulocytes in peripheral blood and myeloid progenitors in bone marrow with dynamic compositional changes in HSC and nonmyeloid progenitor populations. These data establish a critical role of C/EBPα not only in the myeloid lineage but also in a whole hematopoietic system *in vivo*.

Results

Establishment of C/EBPα-ER transgenic mice

To investigate the role of C/EBPα in the various stages of hematopoietic differentiation, we generated transgenic mice (Tg) expressing an inducible form of C/EBPα (C/EBPα-ER; CEBPα fused to the ligand-binding domain of estrogen receptor (ER)). This approach enabled us to analyze directly the impact of C/EBPα activation on cell-fate conversion without culturing cells for gene transduction *in vitro*, which might induce undesired phenotypic changes of the target cells. The H-2K promoter (Domen *et al*, 1998) was used to drive C/EBPα-ER for obtaining high levels of expression in hematopoietic tissues (Figure 1A). Real-time reverse transcription (RT)–PCR analysis revealed that the expression of C/EBPα-ER was achieved in most hematopoietic tissues including spleen, bone marrow (BM), thymus, and peripheral blood (PB) in these mice (Figure 1B). Western blot analysis confirmed that C/EBPα-ER was highly expressed in spleen and thymus (Figure 1C, upper panel). Relatively low but significant expression was also detected in BM and PB. Expression levels of C/EBPα-ER protein were approximately 1/2 to 1/4 of endogenous C/EBPα in hematopoietic tissues such as spleen, BM, and thymus (Figure 1C, lower panel). Next, we isolated various hematopoietic progenitors at key differentiation branch points such as CLP, CMP, GMP, and MEP, and checked the expression of C/EBPα-ER in comparison to endogenous C/EBPα by semiquantitative RT–PCR (Figure 1D). Expression of endogenous C/EBPα was observed in CLP, CMP, GMP, and MEP, with somewhat higher expression in CMP and GMP. There was no substantial difference between Tg and non-Tg littermates in endogenous C/EBPα expression, except that a slight increase and decrease were observed in transgenic GMP and transgenic CMP, respectively. High, constitutive expression of C/EBPα-ER was detected in all progenitors examined, with approximately 2–4 times higher expression compared with the endogenous C/EBPα. These results indicate that C/EBPα-ER was expressed at high levels in CLP, CMP, GMP, and MEP and that it did not significantly affect the endogenous C/EBPα expression. There were no notable abnormalities in the complete blood cell counts (CBC), the differentiation profile of leukocytes, or FACS profile of peripheral blood in the Tg mice (data not shown).

Next, we investigated whether C/EBPα-ER could be activated by 4-HT in these mice. Gel-shift analysis revealed that there was no band shift observed in control-treated transgenic thymocytes and that 4-HT treatment induced specific binding to C/EBP oligonucleotides, which was supershifted by anti-C/EBPα and anti-ER antibodies (Figure 1E). As little as 30 min of 4-HT treatment could achieve activation at a concentration as low as 0.04 μM (data not shown). The same results were also observed in bone marrow cells from Tg mice (data not shown). These results confirmed that activity of C/EBPα-ER could be tightly regulated by 4-HT in these mice, which made them an ideal system to analyze the consequences of C/EBPα activation in various hematopoietic cells *in vitro* and *in vivo*.

Ectopic activation of C/EBPα in MEP induces myeloid differentiation

We first investigated whether C/EBPα could induce myeloid conversion in MEPs. Sorted MEPs from C/EBPα-ER Tg mice were subjected to colony assay in the presence or absence of 4-HT. As shown in Figure 2A, day 3 CFU-E was dramatically decreased from 68 to 20% by 4-HT stimulation. Moreover, Meg/E colonies such as CFU-EM, BFU-E, and CFU-MK were markedly decreased to less than 1% by 4-HT treatment. In contrast, G/M colonies were dramatically increased from 3.5 to 28% by 4-HT treatment. Of note, there was no substantial difference between MEPs from wild-type mice and control-treated MEPs from Tg mice in terms of colony composition (data not shown). To confirm myeloid conversion of MEPs is indeed the consequence of C/EBPα activation, we performed the same experiments with MEPs from wild-type C57BL/6 and H-2K-ER Tg mice. H-2K-ER Tg mice express only ER ligand-binding domain, and therefore should serve as a perfect control for C/EBPα-ER Tg mice (Supplementary Figure 1A). The results showed that there was no difference between control and 4-HT-treated MEPs in colony assays (data not shown).

To analyze the expression of lineage-affiliated genes and to see their changes by 4-HT, cells were recovered from the colonies and examined by RT–PCR (Figure 2B). Meg/E-affiliated genes such as GATA-1, FOG-1, erythropoietin receptor (EpoR), and β-globin were clearly downregulated by 4-HT stimulation. In sharp contrast, the myeloid-associated genes such as granulocyte-colony-stimulating factor receptor (G-CSF R), granulocyte/macrophage-colony stimulating factor receptor α chain (GM-CSF Rα), and macrophage-colony stimulating factor receptor (M-CSF R) were upregulated by 4-HT treatment. To investigate the early molecular events during myeloid conversion by C/EBPα, we examined MEPs treated with or without 4-HT for 16 h by RT–PCR (Supplementary Figure 2A). The data revealed that FOG-1 is clearly downregulated at this time point, whereas GATA-1 remained relatively unchanged. This suggests that the downregulation of FOG-1 in MEP is one of the key initial events for limiting erythroid/megakaryocyte differentiation by C/EBPα.

To rule out the possibility that the conversion of colony types by C/EBPα resulted from the selective expansion of contaminated myeloid progenitors, we next tried to trace the reprogrammed cell fate by surface markers in a liquid culture system (Figure 2C). Sorted MEPs were plated on an inactivated S17 stromal cell layer and cultured in the presence or absence of 4-HT. In the control culture, the majority of MEPs became CD71 (transferrin receptor; a marker of developing erythroid cells) positive and CD11b negative after 7 days of

©2006 European Molecular Biology Organization

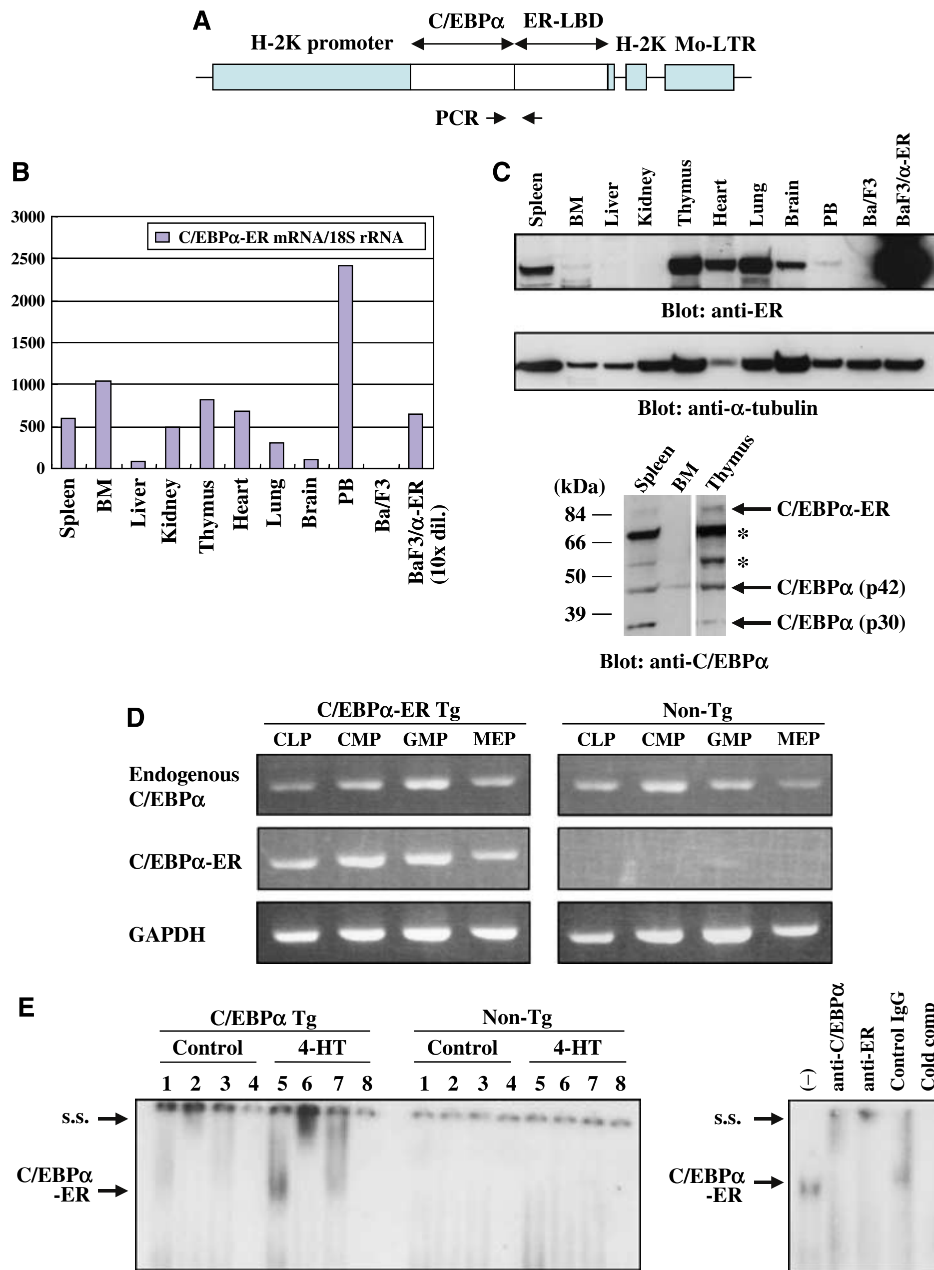


Figure 1 Establishment of C/EBPα-ER transgenic mice. (**A**) Construction of H-2K-C/EBPα-ER transgene. Arrows, primer set used in PCR-based genotyping of C/EBPα-ER Tg mice. ER-LBD, mouse estrogen receptor ligand-binding domain. (**B**) Quantification of C/EBPα-ER transcript by real-time RT–PCR. The data were normalized against 18S rRNA. (**C**) Expression of C/EBPα-ER protein. (Upper panel) Whole-cell lysates from various tissues of Tg mice and cell lines were subjected to Western blot analysis. C/EBPα-ER protein was detected by anti-ER antibody. α-Tubulin was probed as a loading control. BaF3/α-ER is BaF3 cells expressing C/EBPα-ER. (Lower panel) Comparison of expression levels for endogenous C/EBPα and C/EBPα-ER proteins by blotting with anti-C/EBPα antibody. Asterisks indicate nonspecific bands. (**D**) Comparison of endogenous C/EBPα and C/EBPα-ER transgene mRNA in hematopoietic progenitors of Tg mice. mRNA from sorted progenitors were prepared and subjected to semiquantitative RT–PCR as described in Materials and methods. (**E**) Gel-shift analysis of C/EBPα-ER protein. (Left panel) Thymocytes were prepared from Tg or non-Tg littermate mice and treated by 1 μM of 4-HT for 2 h. Lanes 1, 5: no treatment. Lanes 2, 6: supershift (s.s.) with anti-C/EBPα antibody. Lanes 3, 7: supershift with control rabbit IgG. Lanes 4, 8: cold competition. (Right panel) Same extract from 4-HT-treated Tg thymocytes was subjected to supershift reaction by anti-C/EBPα or anti-ER antibodies.

culture (gate A). Surprisingly, activation of C/EBPα converted the majority of the MEPs (50–70%, depending on the culture conditions) into $CD71^+CD11b^+$ biphenotypic cells, which were considered to be the differentiating intermediates to myeloid cells (gate C). However, the remaining MEPs (20–40%) sustained the $CD71^+$ $CD11b^-$ erythroid phenotype (gate B). $CD71^+CD11b^+$ cells that appeared in the 4-HT culture were functionally mature macrophages, as revealed by their morphology and phagocytic activity against fluorescent beads, whereas cells with the $CD71^+CD11b^-$ phenotype still retained immature erythroid morphology (Figure 2C; gate B).

Taken together, these data suggest that the ectopic induction of C/EBPα-ER activity reprograms a large fraction of MEPs into macrophages through differentiating intermediates.

©2006 European Molecular Biology Organization

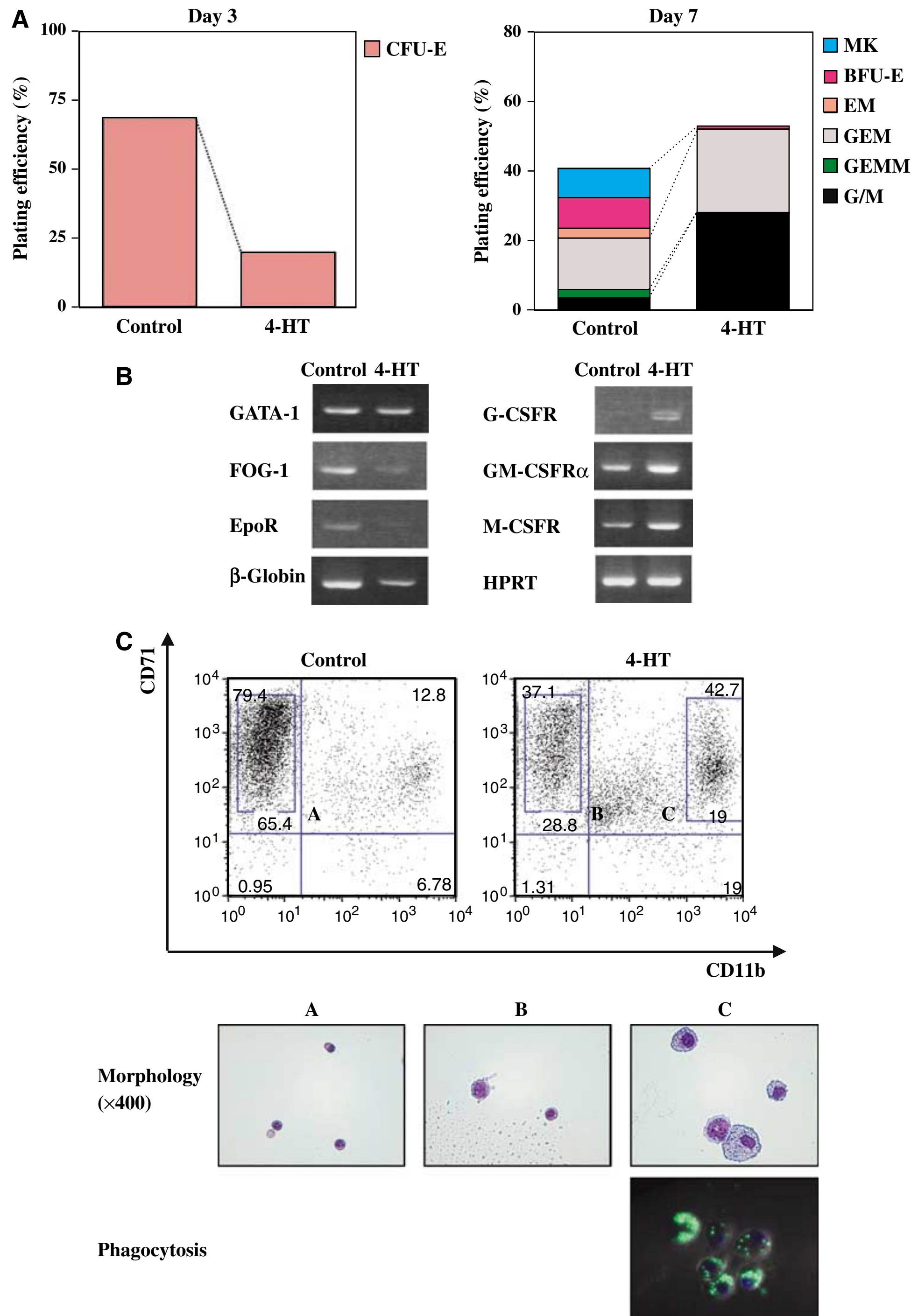


Figure 2 Conversion of MEPs into myeloid lineage by C/EBPα. (**A**) Colony assay. Sorted MEPs were cultured in methylcellulose in the absence or presence of 4-HT, and the colony formation was assessed at day 3 for CFU-E and day 7 for other progenitors. MK, CFU-MK; EM, CFU-EM; GEM, CFU-GEM; GEMM, CFU-GEMM; G/M, CFU-GM + CFU-G + CFU-M. (**B**) Expression of lineage-specific genes. Cells were recovered from the colonies and RT–PCR was performed as described in Materials and methods. (**C**) Changes of surface-antigen expression and cellular morphology during myeloid conversion. Sorted MEPs were cocultured on an S17 stromal layer with SCF, IL-3, EPO, TPO, and G-CSF in the absence or presence of 4-HT. After 7 days, cells were analyzed for surface-antigen expression by FACS. Cells in the gates A, B, and C were sorted, cytospun onto glass slides, and stained with Wright–Giemsa solution (magnification ×400). For cells from gate C, phagocytic activity was examined as described in Materials and methods. Gate A, $CD71^{+}CD11b^{-}$; gate B, $CD71^{+}CD11b^{-}$; gate C, $CD71^{+}CD11b^{+}$.

C/EBPα converts CLP into the myeloid lineage

Next, we investigated whether ectopic activation of C/EBPα activity in CLP could convert these cells into the myeloid lineage. CLPs from C/EBPα-ER Tg mice were subjected to colony assay in the presence or absence of 4-HT (Figure 3A). Although the plating efficiency was low, composition of the colonies was strikingly different between control and 4-HT cultures. Most colonies appearing in the 4-HT-treated plate were G/M colonies, whereas only lymphoid colonies were observed in the control culture. Cytospin preparation of the

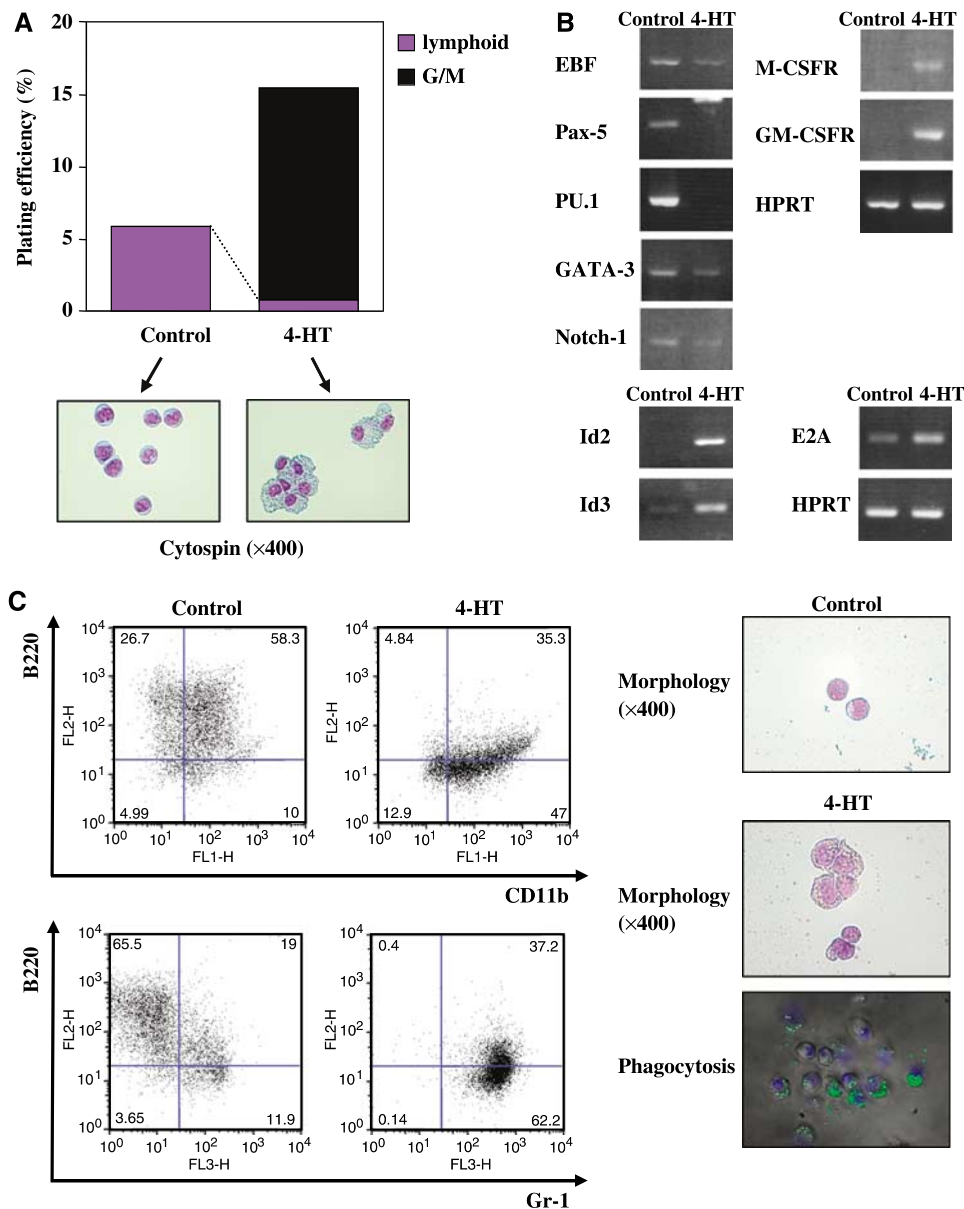


Figure 3 C/EBPα converts CLPs into the myeloid lineage. (**A**) Colony assay. Day 8 colonies derived from C/EBPα-ER Tg CLPs in the absence or presence of 4-HT. Cytospin preparations of the colonies were stained with Wright–Giemsa solution (magnification ×400). (**B**) Expression of lineage-specific genes. Cells recovered from the colonies were subjected to RT–PCR as described in Materials and methods. (**C**) FACS analysis of surface-antigen expression during myeloid conversion. Sorted CLPs were cocultured on an S17 stromal layer with SCF, IL-3, IL-7, FL, and G-CSF in the absence or presence of 4-HT. After 4 days, cells were analyzed for B220, CD11b, and Gr-1 expressions by FACS. Cells were also sorted for analysis of morphology and phagocytic activity.

colonies showed morphological differentiation of CLP into macrophages by 4-HT stimulation, whereas cells from the control culture showed typical lymphocyte morphology (Figure 3A). Control experiments by treating CLPs from wild-type C57BL/6 or ER-Tg mice with 4-HT did not induce any myeloid conversion by colony assays (data not shown). RT–PCR analysis revealed downregulation of lymphoid-associated genes, such as EBF, Pax-5, PU.1, GATA-3, and Notch-1, and upregulation of myeloid-specific genes, such as M-CSF R and GM-CSF Rα, in 4-HT-treated cells (Figure 3B). In addition, clear induction of Id2 and Id3, well-known inhibitors for E2A proteins (Busslinger, 2004), with a slight increase of E2A were observed by 4-HT treatment (Figure 3B). These data suggest that inhibition of E2A activity by Id2 and Id3 combined with a decrease of EBF, Pax-5, PU.1, GATA-3, and Notch-1 expressions limit the differentiation capacity of CLP into B or T cells during myeloid conversion by C/EBPα. The analysis of early molecular events in C/EBPα-induced myeloid conversion of CLPs revealed that Id2 and Id3 genes are still not upregulated after 5 h of 4-HT stimulation. In contrast, Notch-1 is clearly downregulated at this time point, suggesting that downregulation of Notch-1 is one of the early events induced by C/EBPα in CLPs (Supplementary Figure 2B).

Next, we performed liquid culture of CLP on S17 stromal cells, which allows B-lymphoid differentiation, and examined B220, CD11b, and Gr-1 expression by FACS (Figure 3C). Strikingly, cells differentiated into $B220^{low}CD11b^{+}Gr\text{-}1^{+}$ myeloid cells upon C/EBPα activation by 4-HT. These cells

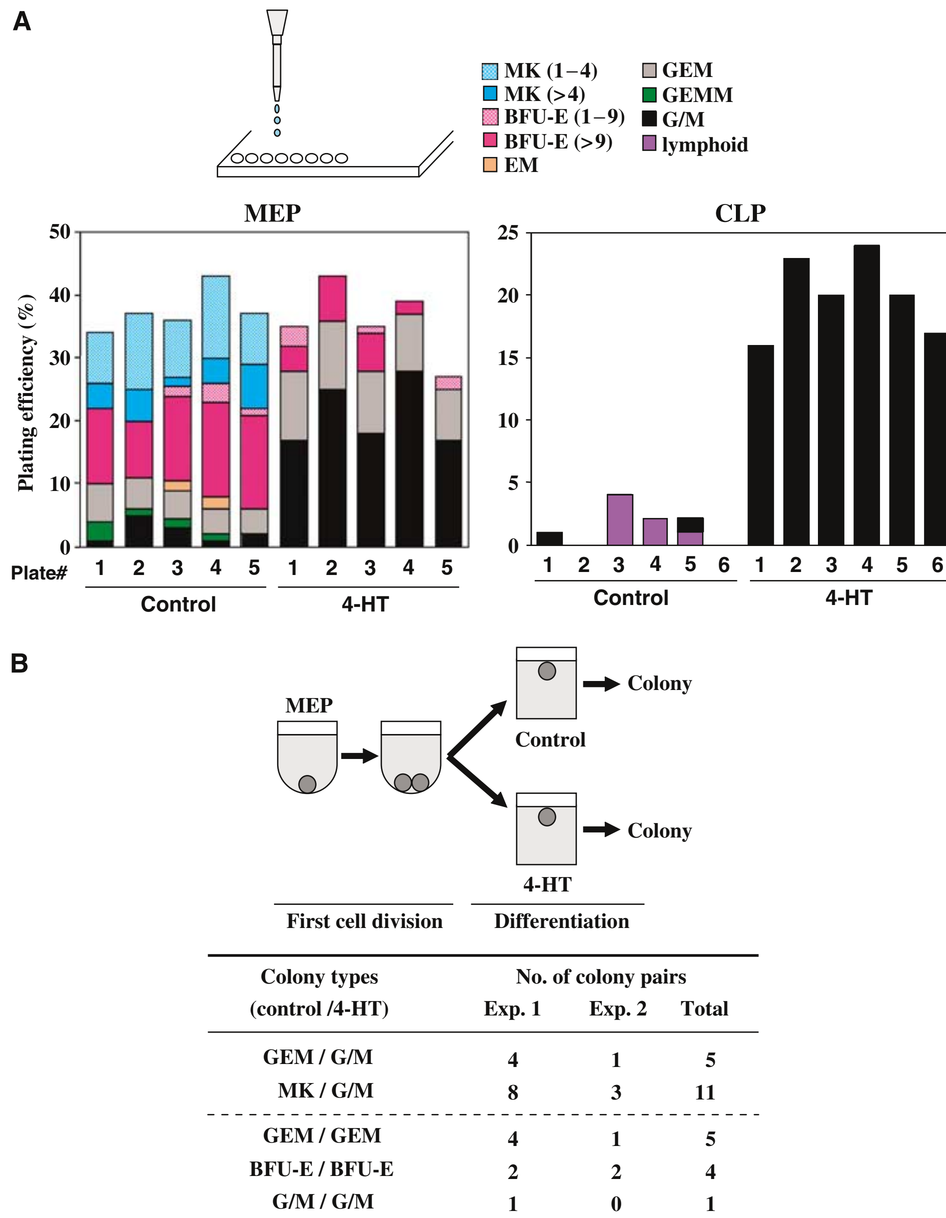
©2006 European Molecular Biology Organization

had macrophage-like morphology and exhibited phagocytic activity *in vitro*. In contrast, the control-treated cells differentiated into B-lymphoid phenotype ($B220^{+}CD11b^{low}Gr\text{-}1^{low/-}$), and these cells retained lymphocyte morphology.

These data clearly indicate that ectopic activation of C/EBPα reprograms CLPs into functionally mature macrophage-like cells.

Conversion of MEPs and CLPs into G/M lineage occurs in cell-autonomous manner

Given that ectopic activation of C/EBPα in MEP and CLP can redirect their colony-forming potential to the myeloid lineage, we next asked whether these changes could occur in a cell-autonomous manner or in a manner influenced by the coexisting cells. To answer this question, we performed a single-cell, 'clonal' colony assay. MEPs and CLPs were clonally deposited into methylcellulose in a 96-well plate by single-cell FACS sorting, and cultured in the presence or absence of 4-HT. As shown in Figure 4A, the plating efficiency and the composition of the colony types in both control and 4-HT-treated plates were similar to those from regular 'bulk' colony assays for both MEPs and CLPs. These results strongly suggest that C/EBPα-induced conversion of MEPs and CLPs into the G/M lineage occurs in a cell-



Colony types (control /4-HT)	No. of colony pairs		
	Exp. 1	Exp. 2	Total
GEM / G/M	4	1	5
MK / G/M	8	3	11
GEM / GEM	4	1	5
BFU-E / BFU-E	2	2	4
G/M / G/M	1	0	1

Figure 4 Clonal analysis of cell-fate conversion. (**A**) Single-cell colony assay. MEPs and CLPs were clonally sorted and deposited into methylcellulose containing vehicle or 4-HT in 96-well plates. Five plates for MEP and six plates for CLP for each culture condition (as numbered from 1 though 6) were analyzed. The colonies were evaluated at day 5 for MEP and at day 6 for CLP. Colony composition and the plating efficiency for each plate were plotted individually in the figure. (**B**) Paired daughter-cell colony assay. Clonally sorted MEPs were cultured in the presence of SCF, IL-11, IL-6, and IL-3. When a single MEP is divided to generate two daughter cells, cells were separately deposited into methylcellulose containing vehicle control or 4-HT by micromanipulation. Colonies from each daughter-cell pair were evaluated at day 5 to compare the fate of differentiation. Data from two experiments (Experiment 1 and Experiment 2) and the total numbers of colony pairs are shown.

©2006 European Molecular Biology Organization

autonomous manner, but not by the secondary effect such as cytokine secretion from the contaminated cells (i.e., T cells or monocytes).

Cell-fate conversion of daughter-cell pairs from MEP by C/EBPα

Although the colony data presented above strongly indicate that C/EBPα can redirect MEP and CLP to myeloid lineage, it is still possible that C/EBPα or 4-HT itself permits the survival of the contaminated myeloid progenitors that otherwise do not survive to form colonies in the control culture. To further confirm the cell-fate conversion at clonal levels, we performed a paired daughter-cell colony assay using MEPs. In brief, sorted MEPs were clonally deposited into 96-well plates and allowed to divide once to generate a daughter-cell pair in the liquid culture. Cells were then separated by micromanipulation and transplanted into control or 4-HT containing methylcellulose, respectively, and the fate of differentiation of each cell was compared (Figure 4B). A total of 768 MEPs successfully gave rise to 230 daughter-cell pairs, which were separated and transferred either into control- or 4-HT-methylcellulose. Out of these, 26 assessable colony pairs were obtained from two consecutive experiments (Figure 4B). Interestingly, the pairs could be classified clearly into two populations with regard to their response to 4-HT. In 16 daughter-cell pairs, one daughter cell in 4-HT chose to follow the myeloid path, whereas the other in the control culture formed GEM or MK colonies. In contrast, in the remaining 10 pairs, 4-HT did not induce myeloid differentiation, and both of the daughter cells followed the same differentiation pathways either in control or 4-HT. Of note, BFU-E was exclusively found in the latter group, indicating that BFU-E no longer retains plasticity having once gone through a cell division. In contrast, all MK colonies were found in the former group, indicating that MK-progenitor was sensitive to myeloid-converting stimuli.

These data clearly demonstrate that C/EBPα converts approximately 60% of MEPs to myeloid lineage at the clonal level. However, the remaining population was not sensitive for myeloid conversion by C/EBPα, and thus MEPs could be divided into two subpopulations with regard to the plasticity for myeloid lineage.

Short-term activation of C/EBPα is sufficient to convert MEP and CLP into myeloid lineage

We next attempted to determine the minimum time required for C/EBPα to convert MEPs and CLPs into myeloid lineage. Sorted MEPs or CLPs were first placed in the initial culture with 4-HT, and then 4-HT was washed off at various time points to assess further differentiation (Figure 5). As for MEPs, conversion seemed to occur gradually from 12 h through day 2, but there was a clear window between day 2 and day 4 in which most (80–90%) of the cells converted to the myeloid lineage with concomitant loss of their Meg/E fate (Figure 5A). In contrast, CLPs required only 12–24 h to lose lymphoid potential and fully switch to the myeloid lineage (Figure 5B and C). It is noteworthy that, like MEP, CLP converts into myeloid lineage through differentiating intermediates that have both B-cell and myeloid markers ($B220^+CD11b^+Gr\text{-}1^+$).

Collectively, both MEP and CLP could be converted into myeloid lineage by a short, limited-term activation of C/EBPα (2–4 days for MEP and 12–24 h for CLP). In addition, CLP is clearly more sensitive to converting stimuli than MEP, indicating that the threshold for lineage conversion varies depending on the cell types.

Systemic activation of C/EBPα-ER induces myeloid expansion with a concomitant decrease of hematopoietic stem and nonmyeloid progenitor cells in vivo

We have shown that induction of C/EBPα activity induced myeloid conversion of MEP and CLP *in vitro*. To confirm this result and to test the impact of C/EBPα activation on the whole hematopoietic differentiation system *in vivo*, we next performed serial systemic administration of 4-HT to C/EBPα-ER Tg mice. 4-HT was given intraperitoneally every other day for 4 weeks, and then mice were killed to examine the stem or progenitor cell fractions in BM and thymus (Figure 6A). As expected, mature granulocytes (seg + stab) in peripheral blood clearly increased from 27 to 41%, and lymphocytes decreased from 72 to 58% by 4-HT treatment (Figure 6B), while CBC did not show significant changes (data not shown). Similarly, the numbers of G/M progenitors increased by approximately two-fold in 4-HT-treated bone marrow and spleen (Figure 6C). Of note, a slight increase (about 1.5-fold) of Meg/E progenitors (CFU-EM, BFU-E, and CFU-MK) and CFU-GEM was also observed in spleen and bone marrow, respectively.

FACS profile of bone marrow cells and thymocytes revealed striking changes in the differentiation pattern of hematopoietic progenitors in 4-HT-treated mice (Figure 6D). As for lymphoid lineage, CLP, PreB, ProB, TN1, TN2, and TN3 cells all decreased by 4-HT treatment, whereas TN4 fractions were relatively increased. Transdifferentiating intermediate cells that have both lymphoid and myeloid markers, such as $CD11b^+B220^+$ or $CD11b^+CD3^+$ cells, were present in those mice. These cells had mature myeloid morphology and retained the rearranged immunoglobulin or T-cell receptor genes, indicating that these cells were indeed converted from lymphoid to myeloid lineage (Supplementary Figure 3). Interestingly, the HSC fraction ($c\text{-}Kit^+Sca\text{-}1^+Lineage^-$ cells) also decreased from 3.0 to 0.4%, indicating that C/EBPα works negatively on HSCs. Another intriguing change induced by C/EBPα was found in the differentiation pattern of MEP (Figure 6E). In 4-HT treated mice, MEPs as a whole (shown as a $CD34^-$ population) expressed higher levels of FcγRII/III compared to control-treated MEP (several-fold higher than control), shown as a bulk 'upwards' shift of the MEP population in the FACS profile. Surprisingly, the newly appeared fraction of cells with $CD34^-Fc\gamma RII/III^{mid\text{-}high}$ phenotype had enlarged cytoplasm with convoluted nuclei, a typical morphology of differentiating myeloid cells (pictured as '4-HT G-MEP' in Figure 6E). Based on morphology and high FcγRII/III expression, we considered these cells to be MEPs transdifferentiating to the myeloid lineage, and thus termed these cells 'G-MEP.' In addition, classical MEPs ($CD34^-Fc\gamma RII/III^{low}$) were also slightly enlarged and had irregular nuclei, suggesting that these cells are on the way to myeloid conversion. As expected, clonogenic assays of sorted populations revealed that 4-HT-treated G-MEPs had increased myeloid progenitors and decreased Meg/E progenitors (Figure 6E).

©2006 European Molecular Biology Organization

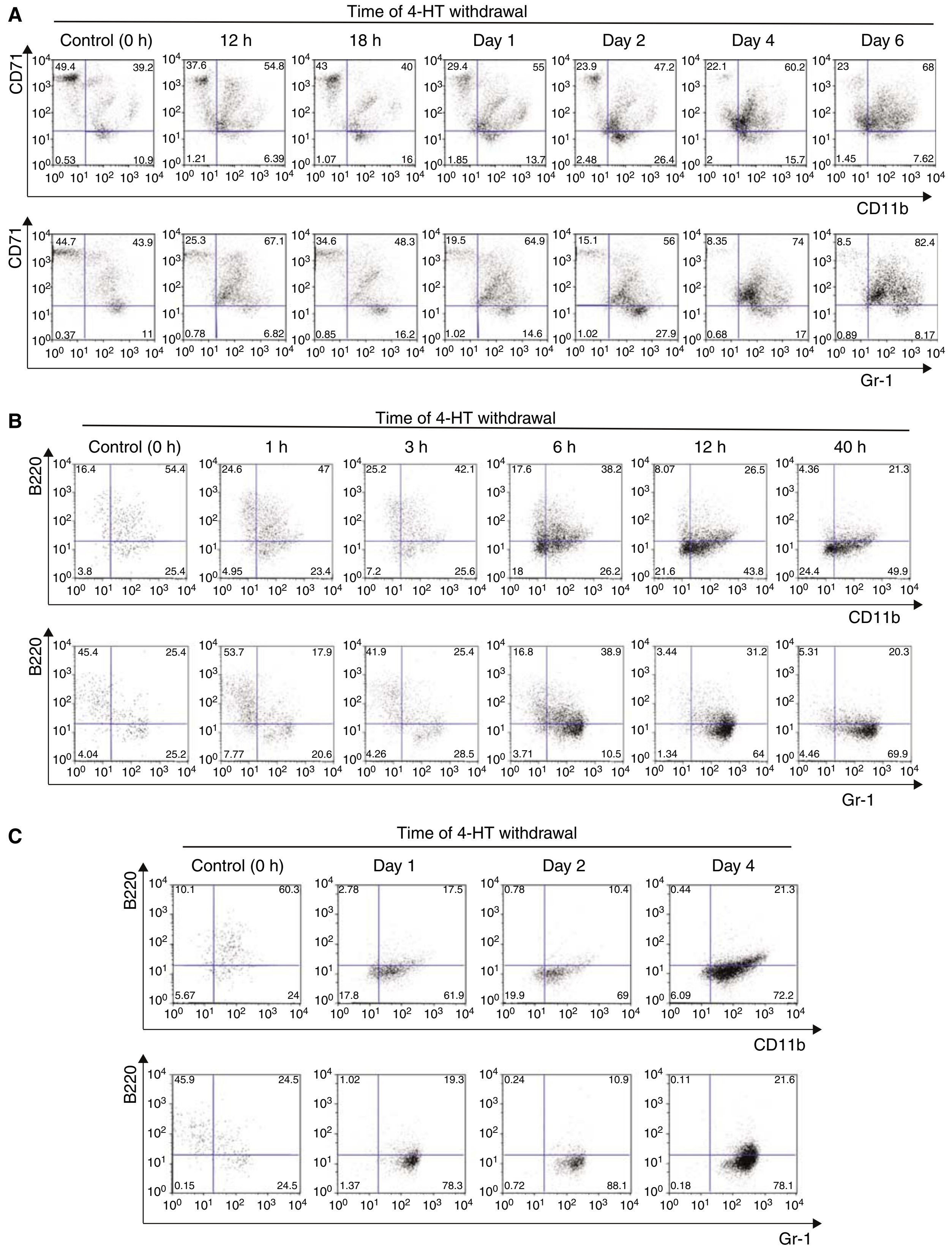


Figure 5 Time-course analysis of myeloid conversion of MEP and CLP. (A, B, C) Sorted MEPs (**A**) and CLPs (**B, C**) were cultured on S17 stromal cells with 4-HT, as described in Materials and methods. At the indicated time points, cells were recovered and washed with PBS twice. Washed cells were replated on new S17 stromal cell layer and further cultured without 4-HT. Cells were harvested at day 6 for MEP or day 4 for CLP, and expression of cell-surface antigens was analyzed by FACS.

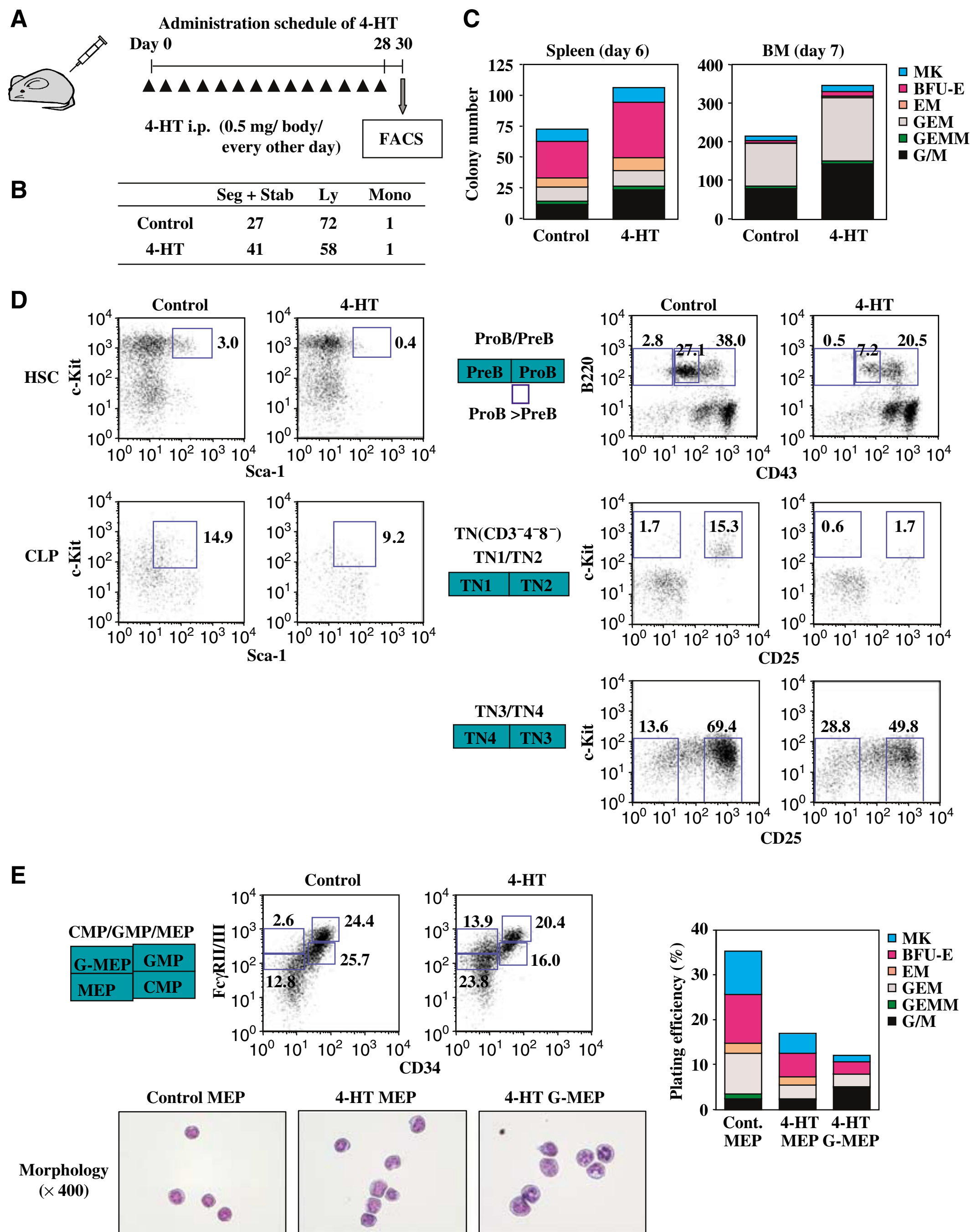


Figure 6 Systemic activation of C/EBPα induces myeloid conversion *in vivo*. (**A**) Administration schedule of 4-HT. A vehicle control and 4-HT were intraperitoneally injected according to the schedule shown in the figure. Mice were killed at day 30 and hematopoietic stem cells/progenitors were analyzed. (**B**) Differential count of white blood cells in peripheral blood (%). Seg: segmented neutrophil, Stab; band neutrophil, Ly; lymphocyte, Mono; monocyte. (**C**) Colony assays of spleen and bone marrow cells from control- or 4-HT-treated mice. (**D**) FACS analysis of hematopoietic stem/progenitor cells after 4 weeks of treatment. Percentages of each stem cell/progenitor fraction against gated parental cell population are shown in each figure. The staining and the gating protocols for FACS are described in Materials and methods. (**E**) FACS profiles of CMP/GMP/MEP in control- or 4-HT-treated mice. Percentages of each progenitor fraction against gated parental population are shown in each figure. Morphology and clonogenic activity of sorted cell population are also shown.

Total number of BM mononuclear cells and thymocytes in 4-HT-treated mice clearly decreased by 60 and 55%, respectively. Absolute cell numbers decreased by C/EBPα activation in all progenitors except G-MEPs, although the extent of reduction varied among progenitors. In contrast, the number of G-MEP increased about two-fold by 4-HT treatment (Supplementary Figure 4).

Administration of 4-HT to control C57BL/6 or ER-Tg mice did not induce any changes in FACS profiles of HSC, CLP, and CMP/GMP/MEP compared with nontreated animals, indicating that the observed effects were indeed the consequence of C/EBPα activation (Supplementary Figure 1B).

Two weeks of 4-HT administration (Supplementary Figure 5A) induced a similar decrease in cell numbers of BM and

 ©2006 European Molecular Biology Organization

thymus, as well as in fractions of HSC and lymphoid progenitors (Supplementary Figure 5B and C). However, no significant changes were observed in the CMP/GMP/MEP profile after 4-HT treatment for 2 weeks (Supplementary Figure 5D).

These results suggest that systemic activation of C/EBPα in mice leads to the increase of peripheral granulocytes and myeloid progenitors *in vivo*. This increase is accompanied by a global reduction of various immature progenitors, including CLPs, B-cell precursors, and T-cell precursors, as well as HSCs, probably by a facilitated transdifferentiation to myeloid lineage. Furthermore, a remarkable increase of the G-MEP fraction strongly suggests that redirection of MEP into G/M lineage does not occur by tracing back the differentiation pathway to CMP to redifferentiation through GMP, but rather progresses directly through a novel intermediate, G-MEP.

Discussion

Transgenic mice versus retrovirus system

The retrovirus system has been commonly used to transduce primary hematopoietic progenitors with a variety of transcription factors in order to analyze their lineage plasticity (Heyworth *et al*, 2002; Souabni *et al*, 2002; Iwasaki *et al*, 2003; Xie *et al*, 2004). However, a major drawback of this system is that the differentiation or unrecognizable changes of progenitor cells could be induced during the *in vitro* culture period, which requires at least 2 days with a cocktail of cytokines for virus infection. In addition, transduced genes are constitutively expressed and therefore cannot be regulated. To overcome these potential problems, we decided to generate transgenic mice expressing an inducible form of C/EBPα (C/EBPα-ER). Using this system, we were able to investigate the consequence of C/EBPα activation in the isolated progenitors without taking time for virus transduction. Furthermore, conditional regulation of C/EBPα activity allowed us a detailed kinetic analysis. In these mice, the induction of C/EBPα activity occurs as quickly as 30 min by 4-HT stimulation with full activation achieved within 2 h, and the activity is quickly shut down by 4-HT withdrawal. In addition, C/EBPα activity was tightly regulated by a mutated estrogen receptor ligand-binding domain (ER-LBD), which specifically responds to 4-HT, but not to endogenous estrogen (Littlewood *et al*, 1995). Another advantage of this system is that C/EBPα activity can be induced *in vivo* by 4-HT administration into mice, which enables us to analyze the effect of C/EBPα in the live animal.

In this study, only one transgenic founder line was available for the analysis, and it was possible that the integration site of the transgene might have affected the conclusion. To rule out this possibility, we transduced C/EBPα-ER into CLPs and MEPs by retrovirus, which should have random integration sites in multiple clones, and examined the effect of C/EBPα activation *in vitro*. In this experiment, induction of C/EBPα activity in MEPs and CLPs resulted in clear increase of myeloid colonies with a reduction of erythroid/megakaryocyte or lymphoid colonies (data not shown), which perfectly mimics the observation in C/EBPα-ER Tg mice. These data strongly suggest that the myeloid conversion by C/EBPα-ER occurs irrelevantly of the integration site of the transgene.

Plasticity of MEP and CLP

The present study clearly demonstrated that MEP and CLP have a latent myeloid differentiation program, which can be activated by C/EBPα. To our knowledge, this is the first work to demonstrate differentiation plasticity of MEP and a myeloid conversion of CLP by C/EBPα. A previous study demonstrated that MEP was not converted to myeloid lineage by ectopic GM-CSF signals (Iwasaki-Arai, Iwasaki *et al*, 2003). This finding indicates that signals from the GM-CSF receptor are not sufficient to activate the latent myeloid differentiation program in MEP, probably because C/EBPα does not lie downstream of GM-CSF. Our data on CLP or other lymphoid progenitors are consistent with the previous reports showing that CLPs and pro-T cells could be reprogrammed to myeloid cells by ectopic cytokine signals (Kondo *et al*, 2000; King *et al*, 2002; Iwasaki-Arai *et al*, 2003). There are also other reports on lineage plasticity, such as conversion of CD19^{+} B-cell progenitors into macrophages by C/EBPα (Xie *et al*, 2004) and CLP/CMP/GMP into Meg/E lineages by GATA-1 (Iwasaki *et al*, 2003). Collectively, these data indicate that virtually all of the immature progenitors such as CMP, MEP, GMP, CLP, pro-B, and pro-T cells are not fully committed to their specific lineages and can be redirected to other lineages by appropriate stimuli.

It is of note that we demonstrated the lineage plasticity of various progenitors not only *in vitro* but also *in vivo* by administering 4-HT directly into mice. To date, lineage infidelity of the progenitors has been shown by experiments involving *in vitro* culture, and we confirmed, for the first time, lineage conversion of the hematopoietic progenitors by directly inducing C/EBPα activity *in vivo*. These data suggest a possibility that the cell-fate conversion might also occur in the physiological setting if appropriate conditions are met.

Myeloid commitment can be achieved by a short-term activation of C/EBPα

It is well known that lineage-specific transcription factors can instruct cell-fate decision at the differentiation branch points. However, the duration of their activation required for inducing commitment to the given lineage is totally unknown. We have shown that MEPs and CLPs required C/EBPα to be active only for 2–4 days and 12–24 h, respectively, to be committed to myeloid lineage. These data suggest that C/EBPα is required only for the initial commitment step, but not for the entire differentiation process. This observation is consistent with a previous report showing that C/EBPα is not required after the GMP stage of myeloid differentiation (Zhang *et al*, 2004). In addition, our data indicate that the sensitivity for converting stimuli varies depending on the cell types and that CLP has a lower threshold for myeloid conversion compared to MEP.

Generation of macrophages from MEP and CLP

It is well known that C/EBPα is a critical regulator for early granulocyte development, as its absence results in complete elimination of mature granulocytes and eosinophils (Zhang *et al*, 1997). However, the present study demonstrates that only macrophages are generated from MEP and CLP by ectopic C/EBPα activation. Previous reports have also made similar observations that C/EBPα could induce macrophage differentiation in B-cell progenitors, T-cell progenitors, PU.1$^{-/-}$ PreB cells, and Pax-5$^{-/-}$ ProB cells (Heavey *et al*, 2003; Laiosa

et al, 2004; Xie *et al*, 2004). One possible explanation for this discrepancy is that the cellular context in which C/EBPα is expressed affects the final output of lineage conversion; physiological expression in GMP leads to granulocyte differentiation, and ectopic expression in MEP or CLP leads to macrophage differentiation. Alternatively, C/EBPα might be capable of inducing macrophage differentiation by itself. In fact, it has recently been reported that C/EBPα deficiency impairs macrophage development *in vitro* and *in vivo* (Heath *et al*, 2004). The other possibility is that the culture conditions used in these studies, in which progenitors are cocultured on stromal cells producing a plenty of myeloid-acting cytokines (i.e., GM-CSF and M-CSF), might have skewed the differentiation program to macrophages. Considering that activating C/EBPα *in vivo* increased only mature granulocytes, but not monocytes, experimental conditions *in vitro* might have affected the output of differentiation. Regardless, further studies will be required to reveal the precise molecular mechanisms of macrophage induction by C/EBPα.

Conversion of MEP and CLP through differentiating intermediates, not by retrograde differentiation

Detailed FACS analysis of myeloid conversion of MEP and CLP by C/EBPα indicates that redirection occurs through differentiating intermediate cells, such as G-MEP. In other words, myeloid conversion does not accompany the retrograde differentiation back to the myeloid branch point and to redifferentiation through GMP. This process closely resembles a myeloid reprogramming of B-cell precursors, in which conversion occurs through $CD19^{+}Mac\text{-}1^{+}$ biphenotypic intermediates (Xie *et al*, 2004). These data indicate that trans-differentiation between distinct lineages does not follow classical differentiation pathways—either orthograde or retrograde—but takes a 'shortcut' through biphenotypic intermediate cells.

MEP is classified into two subpopulations according to the sensitivity to C/EBPα

In contrast to CLPs, most of which could be converted to myeloid lineage by FACS analysis (Figure 3C), a fraction of MEPs (approximately 20–40%) were clearly resistant to converting stimuli by C/EBPα (Figure 2C). According to the results of colony assays (Figure 2A), CFU-GEM is the most resistant to the myeloid-converting stimuli by C/EBPα. CFU-GEM could be derived from contaminated CMP or a transient cell population between CMP and MEP. Paired daughter-cell analysis revealed that half of CFU-GEM could be redirected to G/M lineage by 4-HT, whereas the other half sustained GEM potential. The molecular basis why a part of CFU-GEM does not respond to C/EBPα is not clear. It could be that the activity of C/EBPα is suppressed in CFU-GEM because of the lack of protein modification (i.e., phosphorylation) or essential cofactors. Along this line of thinking, it is interesting to note that so-called 'lineage priming' occurs in multipotent progenitors such as CMP, in which master-regulator genes for several lineages are coexpressed before they commit to a single lineage (Miyamoto *et al*, 2002). In this model, those master regulators must be silenced either by protein modification or trans-repression between the transcription factors in order to maintain their multipotentiality. If this is the case, the same repression mechanism might also act on the C/EBPα-ER proteins, and thus the protein cannot exert converting activity.

On the other hand, differentiated progenitors such as CFU-MK and BFU-E were extremely sensitive to G/M-converting stimuli (Figure 2A), indicating that these progenitors still sustain high degree of plasticity. However, BFU-E is no longer plastic once it divides to generate daughter-cell pairs (Figure 4B). In contrast, CFU-MK still can be reprogrammed to the G/M lineage even after cell division. These data show the differentiation stages at which the progenitors lose their plasticity differ between megakaryocytic and erythroid lineages.

In summary, this study revealed, for the first time, that MEP as well as CLP could be converted to the myeloid lineage through novel biphenotypic intermediate cells (summarized in Figure 7) by a short-term activation of C/EBPα. Combined with previous reports, this study indicates that lineage plasticity is not restricted to the specific progenitor, but rather is a universal characteristic common to most of the immature progenitors. Finally, these Tg mice will also be useful in investigating the role of C/EBPα in embryonic hematopoiesis, as well as in leukemogenesis and adipogenesis, in future studies.

Materials and methods

Mice

Full-length human C/EBPα fused to a mutated ligand-binding domain of the mouse estrogen receptor (Littlewood *et al*, 1995) (C/EBPα-ER) or a mutated ligand-binding domain of the mouse ER only was inserted into the H-2K-I-LTR cassette vector (gift from Dr IL Weissman). C/EBPα-ER or ER transgenic mice were generated by injecting *Hin*dIII fragment of pH-2K-C/EBPα-ER or pH-2K-ER into fertilized mouse eggs as described previously (Kaneda *et al*, 1991; Kobayashi *et al*, 1992). Integration of the transgene into the murine genome was confirmed by Southern blot using *Bgl*II-digested pH-2K-C/EBPα-ER or pH-2K-ER plasmid as a probe, and was screened by PCR in later generations. Mice, 8–12 weeks old, were used in all experiments. All mice were bred and maintained in specific pathogen-free environment, and all animal experiments were carried out according to the guidelines for animal use issued by the Committee of Animal Experiments, the Institute of Medical Science, the University of Tokyo.

4-Hydroxytamoxifen

4-Hydroxytamoxifen (4-HT, Sigma) was dissolved in ethanol (EtOH) at 5 mM and was used at the final concentration of 1 μM.

Western blot analysis

Mouse tissues were homogenized and the protein extracts were prepared as previously described (Nakajima *et al*, 2001). The extracts (30 μg/lane) from each tissue were separated by SDS–PAGE and transferred to PROTRAN BA85 membrane (Schleicher & Schuell). The membranes were probed sequentially with anti-ERα (MC-20; SantaCruz) or anti-C/EBPα (14AA; SantaCruz) antibodies and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit Ig polyclonal antibody (Amersham), or anti-α-tubulin monoclonal antibody (Sigma) and HRP-conjugated sheep anti-mouse Ig polyclonal antibody (Amersham). Bound antibodies were visualized by enhanced chemiluminescence (ECL; Amersham).

RT–PCR and PCR analysis for rearrangements of immunoglobulin and T-cell receptor genes

Total RNA was extracted using Trizol reagent (Invitrogen), and then treated with RNase-free DNase I (Invitrogen) to remove contaminating genomic DNA. Messenger RNA (mRNA) was isolated using Micro-FastTrack 2.0™ mRNA Isolation Kit (Invitrogen). First-strand cDNA was synthesized with Super Script II reverse transcriptase (Invitrogen). For real time RT–PCR of C/EBPα-ER, cDNA quantity was normalized by 18S rRNA using Light Cycler Fast Start DNA

©2006 European Molecular Biology Organization

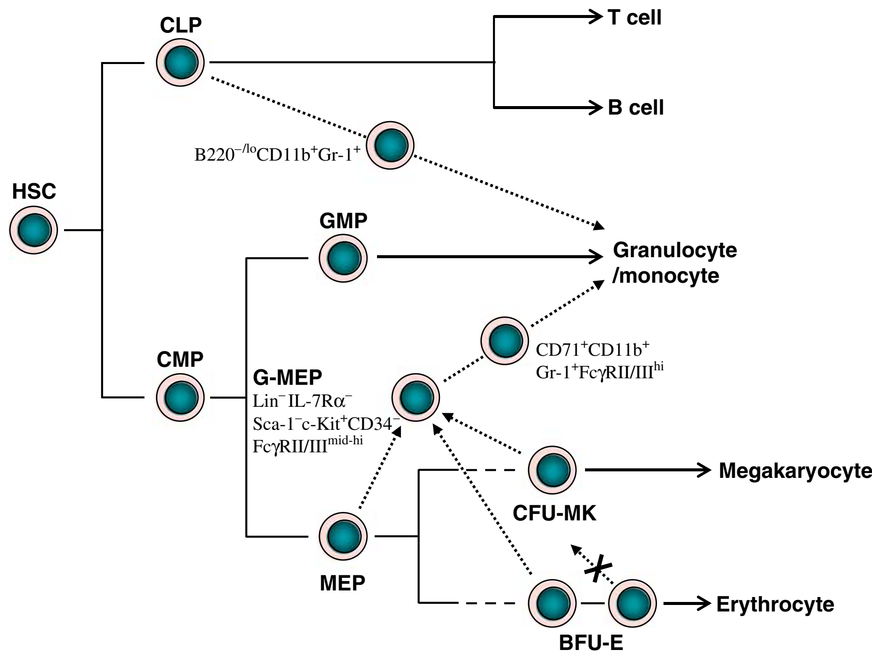


Figure 7 Lineage conversion of hematopoietic progenitors by C/EBPα. Lineage conversions by C/EBPα shown in this study are indicated by broken arrows.

SYBR Green I kit (Roche), and then PCR was carried out using C/EBPα-ER primer set, which was also used for mouse genotyping. For endogenous C/EBPα or lineage-specific genes, concentration of cDNAs was normalized by GAPDH or HPRT by real-time PCR and then semiquantitative RT–PCR was performed using Ex Taq-HS (TaKaRa). Primer sets for lineage-specific genes and GAPDH were reported previously (Pan *et al*, 1999; Becker-Herman *et al*, 2002; Iwasaki *et al*, 2003). Other primer sequences were shown in Supplementary Table 1. PCR analyses for rearrangements of immunoglobulin and T-cell receptor genes were carried out as previously described (Xie *et al*, 2004).

Gel-shift assay

Nuclear extracts were made from bone marrow cells and thymocytes of C/EBPα-ER Tg mice or non-Tg littermates stimulated with or without 4-HT at various times and concentrations. Cell extracts (10 μg of total protein) were incubated with 2 μg of poly dI-dC for 15 min, and then with double-stranded C/EBP consensus oligonucleotide probes (TGCAGATTGCGCAATCTGCA) labeled with γ^{32}P-ATP using T4 polynucleotide kinase for 30 min on ice. Cold competition and a super-shift reaction were carried out by adding 40-fold excess of cold C/EBP consensus oligo or 1.5 μg of anti-C/EBP (14AA)X antibody (Santa Cruz), respectively. The resulting complexes were resolved on 4.5% polyacrylamide gel in 1 × TBE buffer. Gels were dried and visualized by autoradiography.

Cell staining and sorting

For sorting HSC and CLP, BMMNCs were stained with lymphoid lineage antibody mixture: purified rat anti-mouse CD3 (CT-CD3), CD4 (GK1.5), CD8 (53–6.7), B220 (RA3-6B2), Gr-1 (8C5), TER119 (TER-119), CD19 (1D3), IgM (R6-60.2), CD11b (M1/70). Lin^+ cells were removed with sheep anti-rat IgG-conjugated magnetic beads (Dynabeads M-450; Dynal Biotech), and the remaining cells were stained sequentially with lymphoid lineage antibody mixture and PE-Cy5-goat anti-rat IgG (H+L) polyclonal antibody $F(ab')_2$ (Caltag). After washing, cells were stained with anti-IL-7R α chain-PE (A7R34), anti-Thy1.1-FITC (HIS51), anti-c-Kit-APC (2B8), and anti-Sca-1-biotin monoclonal antibodies (D7), followed by PE-Cy7-conjugated streptavidin (Beckman Coulter). CLPs were sorted as Lin^-IL-7$R\alpha^+$c-Kit^{lo}Sca-1^{lo}Thy1.1^- population.

For sorting myeloid progenitors (CMP, GMP, MEP), bone marrow mononuclear cells (BMMNCs) were stained with myeloid lineage antibody mixture; lymphoid lineage antibody mixture plus IL-7R α chain, and Sca-1. Lin^+IL-7$R\alpha^+$Sca-1^+ cells were depleted, and the remaining cells were stained sequentially with myeloid lineage antibody mixture and PE-Cy5-goat anti-rat IgG (H+L) polyclonal antibody $F(ab')_2$. After washing, cells were stained with anti-FcγRII/III-PE (2.4G2), anti-CD34-FITC (RAM34), and anti-c-Kit-APC monoclonal antibodies. MEPs were sorted as Lin^-IL-7$R\alpha^-$Sca-1^-c-$Kit^+$$CD34^-$FcγRII/$III^{lo}$ population.

B-cell progenitors and triple-negative (TN; $CD3^-CD4^-CD8^-$) T-cell progenitors were stained as previously described (Kondo *et al*, 1997; King *et al*, 2002).

All antibodies were purchased from e-Bioscience and BD PharMingen. Cell sorting was performed by FACS Aria (BD Bioscience), and data were analyzed with FlowJo software (Tree Star).

Colony assay and paired daughter-cell assay

MEPs (150 cells) were cultured in MethoCult GF M3434 (Stem Cell Technologies) supplemented with 50 ng/ml of human (h) TPO, 10 ng/ml of mouse (m) GM-CSF, with or without 4-HT. CLPs (200 cells) were cultured in MethoCult M3630 (Stem Cell Technologies) supplemented with 50 ng/ml of mSCF, 20 ng/ml of hFLT3-ligand (FL), with or without 4-HT. For the single-cell colony assay, MEPs or CLPs were clonally sorted into 96-well plates filled with methylcellulose described above.

For the paired daughter-cell colony assay, single sorted MEPs were cultured in S-Clone SF-03 (Sanko Junyaku) supplemented with 10% FBS, 100 ng/ml of mSCF, 100 ng/ml of IL-11, 50 ng/ml of mIL-6, 10 ng/ml of IL-3, and 50 μM of 2-mercaptoethanol until a single cell divided into two daughter cells. The daughter cells were separated by micromanipulation techniques (Suda *et al*, 1984; Takano *et al*, 2004) and deposited into methylcellulose. All cytokines were purchased from R&D Systems.

Colonies were counted under an inverted microscope from day 3 to day 8. Types of colonies were determined by Wright–Giemsa staining of cells that were picked from individual colonies.

Coculture on S17 stromal cells

Sorted progenitors were cocultured on irradiated (3000 rad) S17 stromal layers (a gift from Dr K Akashi) in 24-well plate (<1000 cells/well) in S-Clone SF-03 supplemented with 10% FBS, 20 ng/ml of mSCF, 10 ng/ml of mIL-3, 1 U/ml of hEPO, 50 ng/ml of hTPO, 100 ng/ml of mG-CSF for MEPs, or mSCF, mIL-3, 10 ng/ml of mIL-7, and 20 ng/ml of hFL, mG-CSF for CLPs, with or without 4-HT. After 4–7 days of coculture, MEP- or CLP-derived cells were stained with anti-c-Kit-APC, anti-CD11b-FITC, anti-Gr-1-biotin, and anti-CD71-PE

(C2) or anti-B220-PE monoclonal antibodies, followed by PerCP-Cy5.5-conjugated streptavidin. Cells were analyzed by FACS.

For turning off C/EBPα-ER activity, 4-HT-treated cells were collected at various time points, washed twice with PBS, and replated on a newly prepared S17 stromal cell layer. Cells were further cocultured in the same media minus 4-HT.

Phagocytosis assay

Sorted $CD71^+CD11b^+$ or $B220^{-/low}CD11b^+$ cells were incubated with 1 μm yellow-green fluorescent carboxylated microspheres (Molecular Probes) for 2–12 h at 37°C in the same media used for coculture with S17 cells. After incubation, cells were stained with DAPI, and photographed by fluorescent microscope (Olympus). Images were captured by IP Lab software (Scanalytics).

In vivo administration of 4-HT

4-HT or EtOH were mixed with sunflower oil (Sigma) (final concentration 0.5 mg) before injection. These were injected intraperitoneally every other day for 2 weeks or 4 weeks to C/EBPα-ER Tg mice.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We acknowledge Dr A Iwama (Chiba University) for valuable advice and his expertise in the micromanipulation. We thank Drs N Watanabe, T Shibata, and S Saito (FACS Core Laboratory, Institute of Medical Science, University of Tokyo) for FACS sorting. We also thank Dr IL Weissman (Stanford University School of Medicine) for providing the H-2K-I-LTR cassette vector, and Dr K Akashi (Dana-Farber Center Institute, Harvard Medical School) for providing S17 stromal cells. This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Becker-Herman S, Lantner F, Shachar I (2002) Id2 negatively regulates B cell differentiation in the spleen. *J Immunol* **168:** 5507–5513

Busslinger M (2004) Transcriptional control of early B cell development. *Annu Rev Immunol* **22:** 55–79

Domen J, Gandy KL, Weissman IL (1998) Systemic overexpression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. *Blood* **91:** 2272–2282

Heath V, Suh HC, Holman M, Renn K, Gooya JM, Parkin S, Klarmann KD, Ortiz M, Johnson P, Keller J (2004) C/EBPalpha deficiency results in hyperproliferation of hematopoietic progenitor cells and disrupts macrophage development *in vitro* and *in vivo*. *Blood* **104:** 1639–1647

Heavey B, Charalambous C, Cobaleda C, Busslinger M (2003) Myeloid lineage switch of Pax5 mutant but not wild-type B cell progenitors by C/EBPalpha and GATA factors. *EMBO J* **22:** 3887–3897

Heyworth C, Pearson S, May G, Enver T (2002) Transcription factor-mediated lineage switching reveals plasticity in primary committed progenitor cells. *EMBO J* **21:** 3770–3781

Iwama A, Zhang P, Darlington GJ, McKercher SR, Maki R, Tenen DG (1998) Use of RDA analysis of knockout mice to identify myeloid genes regulated *in vivo* by PU.1 and C/EBPalpha. *Nucleic Acids Res* **26:** 3034–3043

Iwasaki H, Mizuno S, Wells RA, Cantor AB, Watanabe S, Akashi K (2003) GATA-1 converts lymphoid and myelomonocytic progenitors into the megakaryocyte/erythrocyte lineages. *Immunity* **19:** 451–462

Iwasaki-Arai J, Iwasaki H, Miyamoto T, Watanabe S, Akashi K (2003) Enforced granulocyte/macrophage colony-stimulating factor signals do not support lymphopoiesis, but instruct lymphoid to myelomonocytic lineage conversion. *J Exp Med* **197:** 1311–1322

Kaneda N, Sasaoka T, Kobayashi K, Kiuchi K, Nagatsu I, Kurosawa Y, Fujita K, Yokoyama M, Nomura T, Katsuki M, Nagatsu T (1991) Tissue-specific and high-level expression of the human tyrosine hydroxylase gene in transgenic mice. *Neuron* **6:** 583–594

King AG, Kondo M, Scherer DC, Weissman IL (2002) Lineage infidelity in myeloid cells with TCR gene rearrangement: a latent developmental potential of proT cells revealed by ectopic cytokine receptor signaling. *Proc Natl Acad Sci USA* **99:** 4508–4513

Kobayashi K, Sasaoka T, Morita S, Nagatsu I, Iguchi A, Kurosawa Y, Fujita K, Nomura T, Kimura M, Katsuki M, Nagatsu T (1992) Genetic alteration of catecholamine specificity in transgenic mice. *Proc Natl Acad Sci USA* **89:** 1631–1635

Kondo M, Akashi K, Domen J, Sugamura K, Weissman IL (1997) Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice. *Immunity* **7:** 155–162

Kondo M, Scherer DC, Miyamoto T, King AG, Akashi K, Sugamura K, Weissman IL (2000) Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature* **407:** 383–386

Laiosa CV, Xie H, Ye M, Graf T (2004) T to myeloid lineage reprogramming by the synergistic action of C/EBP and PU.1. *Blood, the Journal of The American Society of Hematology* **104:** 68a

Landschulz WH, Johnson PF, Adashi EY, Graves BJ, McKnight SL (1988) Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev* **2:** 786–800

Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI (1995) A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* **23:** 1686–1690

Miyamoto T, Iwasaki H, Reizis B, Ye M, Graf T, Weissman IL, Akashi K (2002) Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell* **3:** 137–147

Nakajima H, Brindle PK, Handa M, Ihle JN (2001) Functional interaction of STAT5 and nuclear receptor co-repressor SMRT: implications in negative regulation of STAT5-dependent transcription. *EMBO J* **20:** 6836–6844

Pan L, Sato S, Frederick JP, Sun XH, Zhuang Y (1999) Impaired immune responses and B-cell proliferation in mice lacking the Id3 gene. *Mol Cell Biol* **19:** 5969–5980

Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG (1998) CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol* **18:** 4301–4314

Shivdasani RA, Orkin SH (1996) The transcriptional control of hematopoiesis. *Blood* **87:** 4025–4039

Souabni A, Cobaleda C, Schebesta M, Busslinger M (2002) Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. *Immunity* **17:** 781–793

Suda T, Suda J, Ogawa M (1984) Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. *Proc Natl Acad Sci USA* **81:** 2520–2524

Takano H, Ema H, Sudo K, 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

Tenen DG, Hromas R, Licht JD, Zhang DE (1997) Transcription factors, normal myeloid development, and leukemia. *Blood* **90:** 489–519

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

Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG (1997) Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci USA* **94:** 569–574

Zhang P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, Owens BM, Shigematsu H, Levantini E, Huettner CS, Lekstrom-Himes JA, Akashi K, Tenen DG (2004) Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity* **21:** 853–863

©2006 European Molecular Biology Organization