Cbfβ-SMMHC induces distinct abnormal myeloid progenitors able to develop acute myeloid leukemia

Ya-Huei Kuo,[1] Sean F. Landrette,[1] Susan A. Heilman,[1] Paola N. Perrat,[1] Lisa Garrett,[2] Pu P. Liu,[2] Michelle M. Le Beau,[3] Scott C. Kogan,[4] and Lucio H. Castilla[1,*]

[1] Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, Massachusetts 01605
[2] National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland 20892
[3] Section of Hematology/Oncology, University of Chicago, Chicago, Illinois 60637
[4] Department of Laboratory Medicine, University of California, San Francisco, San Francisco, California 94143
*Correspondence: lucio.castilla@umassmed.edu

Summary

The acute myeloid leukemia (AML)-associated CBFβ-SMMHC fusion protein impairs hematopoietic differentiation and predisposes to leukemic transformation. The mechanism of leukemia progression, however, is poorly understood. In this study, we report a conditional *Cbfb-MYH11* knockin mouse model that develops AML with a median latency of 5 months. Cbfβ-SMMHC expression reduced the multilineage repopulation capacity of hematopoietic stem cells (HSCs) while maintaining their numbers under competitive conditions. The fusion protein induced abnormal myeloid progenitors (AMPs) with limited proliferative potential but leukemic predisposition similar to that of HSCs in transplanted mice. In addition, Cbfβ-SMMHC blocked megakaryocytic maturation at the CFU-Meg to megakaryocyte transition. These data show that a leukemia oncoprotein can inhibit differentiation and proliferation while not affecting the maintenance of long-term HSCs.

Introduction

Acute myeloid leukemia (AML) arises from clonal expansion of hematopoietic progenitors that have acquired genetic alterations. Recurrent chromosomal rearrangements frequently found in AML create fusion proteins that alter differentiation, proliferation, and survival programs as part of the multistep process of leukemogenesis (Look, 1997). For example, the inversion of chromosome 16 inv(16)(p13.1q22) [henceforth inv(16)] breaks and joins the *CBFB* gene with the myosin gene *MYH11*, creating the *CBFB-MYH11* fusion that encodes CBFβ-SMMHC (Liu et al., 1993, 1996).

The transcription factor core binding factor (CBF) regulates a variety of hematopoietic genes associated with lymphoid and myeloid differentiation (Otto et al., 2003). CBF is a heterodimeric complex that includes the CBFβ subunit, encoded by *CBFB*, and a DNA binding α subunit, encoded by one of three *RUNX* family members (*RUNX1*, *RUNX2*, or *RUNX3*). CBFβ is critical for CBF function, as it increases the affinity of RUNX proteins for DNA (Ogawa et al., 1993; Wang et al., 1993) and seems to protect these α subunits from proteolytic degradation (Huang et al., 2001). CBF is the most common target of alterations in human AML. In addition to inv(16) that affects *CBFB*, AML samples often harbor RUNX1 alterations, including t(8;21) and loss-of-function mutations (Speck and Gilliland, 2002).

Studies using *Runx1* and *Cbfb* knockout mice have determined that the lack of either subunit abrogates the development of embryonic definitive hematopoiesis, and embryos die at midgestation (Niki et al., 1997; Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a, 1996b). Furthermore, Runx1 was shown to be critical in the emergence of hematopoietic stem cells (HSCs) from the hemogenic endothelium (Speck and Gilliland, 2002). Consistent with these studies, definitive hematopoiesis was absent in heterozygous *Cbfb-MYH11* knockin embryos, underscoring the dominant-negative effect of the fusion protein on CBF function (Castilla et al., 1996; Yergeau et al., 1997). Using the *Cbfb-MYH11* knockin chimeras, we have shown that Cbfβ-SMMHC expression alters adult multilineage hematopoietic differentiation (Castilla et al., 1999). The multistep nature of leukemia progression in inv(16) AML was also demonstrated in these mice where gain of additional mutations, induced by chemical or retroviral mutagens, was necessary for the development of AML (Castilla et al., 1999, 2004). However, the embryonic lethality observed in the heterozygous

SIGNIFICANCE

CBFβ-SMMHC is a fusion oncoprotein that is present in approximately 12% of human AML and interferes with the core binding factor transcription factor regulation of hematopoietic differentiation. Studies in the mouse have been limited due to the embryonic lethality caused by its expression. Here, we use a conditional mouse model for CBFβ-SMMHC to demonstrate that leukemia-associated fusion proteins can impair multilineage differentiation while not expanding long-term HSCs. Furthermore, the identification of CBFβ-SMMHC-induced AMPs suggests that these leukemia-prone populations may be a common theme among leukemia fusion oncogenes. Moreover, we have developed a conditional mouse model where the efficacy of novel therapeutics designed to induce differentiation of the leukemic cells can be evaluated.

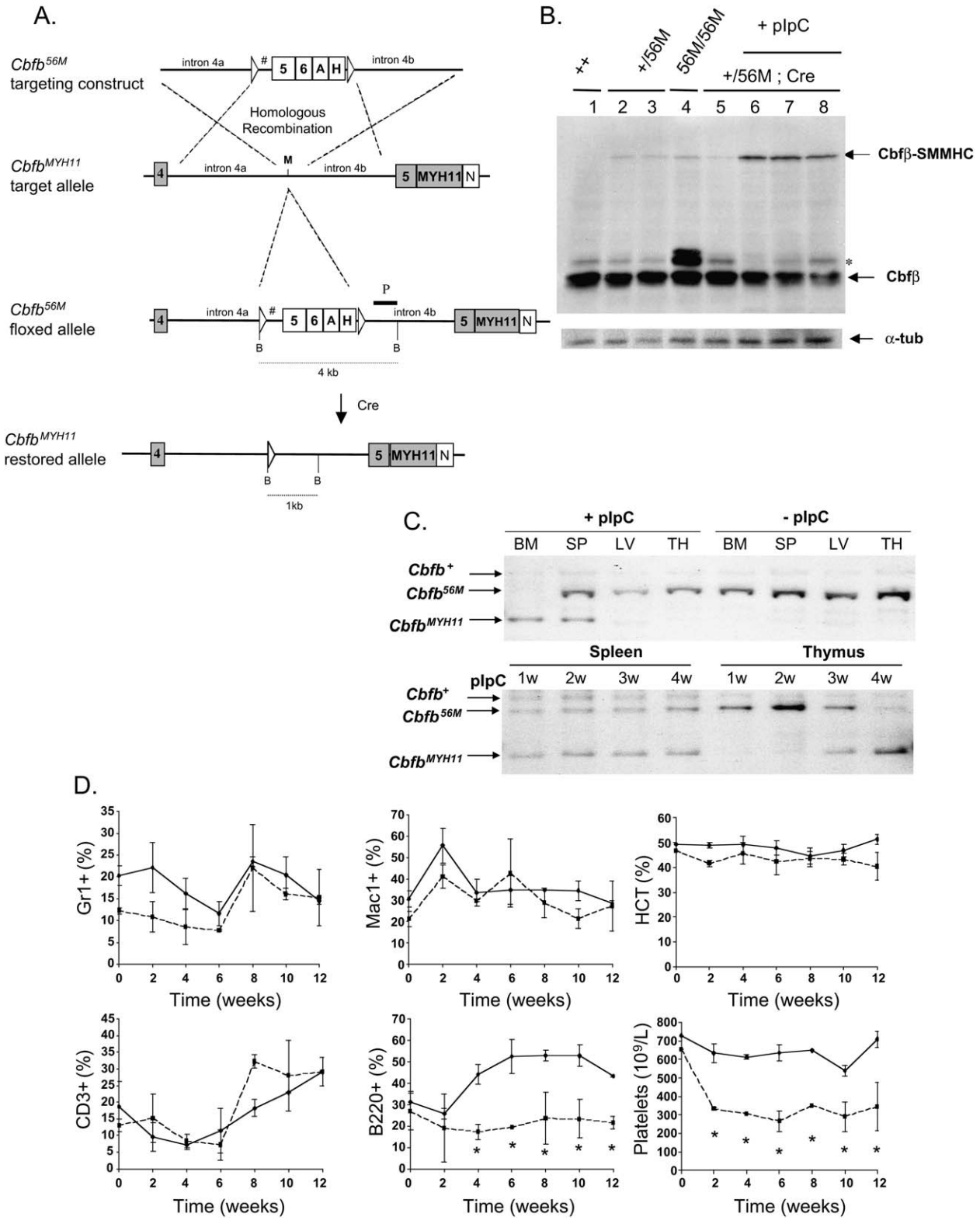

Figure 1. Generation of a conditional *Cbfb-MYH11* knockin mouse model

A: Schematic representation of targeting strategy. The *Cbfb*^{MYH11} allele (exons in black boxes; neomycin gene "N") was targeted by homologous recombination. Between loxP1 sites (triangles), the targeting construct included the 3′ end of intron 4 with the splice donor signal (#) and the beginning part of exon 5, an in-frame cDNA sequence for the second part of human exon 5 and exon 6 (open boxes), the bovine growth hormone polyadenylation signal (A), and PGK-hygromycin gene (H). This sequence was inserted in an MscI site (M) of intron 4. The *Cbfb*^{MYH11} allele is restored upon Cre-mediated deletion of the floxed sequence.

knockin mice and the lack of an inv(16) conditional knockin mouse model have limited detailed in vivo studies of Cbfβ-SMMHC effects in hematopoietic differentiation, proliferation, and survival during leukemia progression.

In this study, we created a conditional *Cbfb-MYH11* knockin mouse model to analyze the preleukemic effects of Cbfβ-SMMHC in hematopoiesis and the development of AML. Upon Cre-mediated induction of Cbfβ-SMMHC in adult mice, the numbers of B cells and platelets were markedly reduced in the peripheral blood, and an abnormal myeloid progenitor (AMP) population was detected in the bone marrow (BM). In vitro differentiation and serial replating assays demonstrated that the myeloid progenitors had a deficient proliferation capacity. These findings were replicated in vivo using BM transplantation assays, underscoring the cell-autonomous nature of the phenotype. Furthermore, competitive repopulation assays (CRA) revealed that HSCs expressing Cbfβ-SMMHC were maintained at normal levels for long periods, but interestingly, their long-term multilineage repopulation capacity was severely compromised. In addition, knockin mice expressing Cbfβ-SMMHC developed AML with a median latency of 5 months, in which disease onset varied with the number of leukemia precursors in the BM. Finally, limiting dilution transplantation assays revealed that AMPs have similar leukemic transforming capacity as HSCs. The results of this study provide in vivo evidence that leukemia fusion proteins that affect hematopoietic differentiation may maintain normal number of HSCs with deficient repopulation function, while creating preleukemic myeloid progenitors that can be targets for AML transformation.

Results

Creation of a conditional *Cbfb-MYH11* knockin allele

The study of Cbfβ-SMMHC function in adult hematopoiesis and leukemia in the mouse has been limited due to the embryonic lethality of the heterozygous $Cbfb^{+/MYH11}$ knockin (Castilla et al., 1996). We generated a conditional *Cbfb-MYH11* knockin mouse model, called $Cbfb^{+/56M}$, in which *Cbfb* exons 5 and 6 flanked by loxP1 sites were inserted at an Msc1 site in intron 4 (Figure 1A). Under this design, the wild-type *Cbfb* transcript is expressed from the "floxed" $Cbfb^{56M}$ allele. Upon Cre expression, the knockin allele is restored and Cbfβ-SMMHC activated. For clarity, mice and cells containing this allele will be called "restored." Heterozygous $Cbfb^{+/56M}$ and homozygous $Cbfb^{56M/56M}$ mice were born in Mendelian ratios and remained healthy over 1 year of age, indicating the expression of a functional Cbfβ from the floxed allele. *Cbfb-MYH11* function was confirmed in vivo using the *EII-Cre* transgene, in which Cre is under the control of the adenovirus *EIIa* promoter and is expressed in early embryogenesis at 2–8 cell stage (Lakso et al., 1996). $Cbfb^{+/56M}$/*EII-Cre* embryos recreated the definitive hematopoiesis deficiency and midgestation lethality phenotypes previously reported (Castilla et al., 1996; data not shown). In addition, traces of Cbfβ-SMMHC protein were detected in BM from controls (Figure 1B, lanes 2 to 5), probably due to alternative splicing that bypassed the floxed sequences. Fusion protein levels were effectively induced 12- to 15-fold in the BM from $Cbfb^{+/56M}$ mice using the *Mx1Cre* system (Kuhn et al., 1995) (Figure 1B). The efficiency of Cre-mediated deletion at the $Cbfb^{56M}$ allele in hematopoietic progenitors was assessed in hematopoietic tissues by Southern blot analysis as a function of time following polyinosinic-polycytidylic acid (pIpC) injection. After 1 week, over 80% of 5FU-treated restored BM progenitors and 50% of spleen cells had undergone Cre-mediated deletion, while no detectable deletion was observed in liver and thymus (Figure 1C, top panel). Similar analysis of untreated restored BM showed a 30% to 50% deletion (data not shown). The percentage of cells in the thymus with the deletion increased significantly after the third week, suggesting that T cell precursors expressing Cbfβ-SMMHC may have migrated into the thymus (Figure 1C, bottom panel). In addition, time course analysis of peripheral blood from restored $Cbfb^{+/56M}$/*Mx1Cre* and control $Cbfb^{+/56M}$ mice by fluorescence-activated cell sorter (FACS) analysis revealed that B cells ($B220^+$) were significantly reduced at 4 weeks and remained low throughout the experiment, while myeloid cells ($Mac\text{-}1^+$ and $Gr\text{-}1^+$) and T cells ($CD3^+$) appeared unaffected (Figure 1D). Moreover, platelet counts were reduced 50%, while hematocrits remained unaltered, suggesting that the fusion protein may interfere with megakaryocytic maturation. To examine whether expression of Cbfβ-SMMHC stimulated apoptosis of hematopoietic cells, we also performed FACS analysis of BM and peripheral blood with annexin V and lineage makers (Mac1, Gr1, CD3, and B220) from primary restored and control mice. No detectable differences between these groups were observed, suggesting that Cbfβ-SMMHC does not induce apoptosis (data not shown).

Cbfβ-SMMHC impairs competitive repopulation while not affecting maintenance of the phenotypic HSCs

Cbfβ-SMMHC impairs hematopoietic differentiation in knockin chimeric mice (Castilla et al., 1999). To our surprise, myeloid and T cell differentiation from primary restored mice (treated $Cbfb^{+/56M}$/*Mx1Cre*; Figure 2A) remained unaltered for 12 weeks after treatment (Figure 1D). To test the effects of Cbfβ-SMMHC expression in HSCs, we analyzed the phenotypic HSC compartment (Lin^-/c-Kit^+/$Sca1^+$; LKS^+) by FACS. The HSCs from primary restored mice increased 2- to 3-fold compared to similarly treated littermate controls (Figure 2B, dashed box).

To assess whether this increase was cell autonomous, we analyzed the effect of Cbfβ-SMMHC using noncompetitive repopulation assays (NCRA; Figure 2A). Lethally irradiated mice were transplanted with 1×10^6 floxed or control BM cells.

B: Western blot analysis of Cbfβ-SMMHC induction. BM protein extracts included wild-type (lane 1), $Cbfb^{+/56M}$ (lanes 2 and 3), $Cbfb^{56M/56M}$ (lane 4), $Cbfb^{+/56M}$/*Mx1Cre* (lane 5), and pIpC-treated $Cbfb^{+/56M}$/*Mx1Cre* (lanes 6–8). Bands corresponding to Cbfβ, Cbfβ-SMMHC, and α-tubulin are depicted on the right. An unspecific band corresponding to mouse IgG light chain reactive to secondary antibodies is also shown (asterisk).

C: Southern blot analysis of Cre-mediated restoration of the $Cbfb^{MYH11}$ allele in 5FU-enriched bone marrow (BM), spleen (SP), liver (LV), and thymus (TH) 1 week after pIpC treatment (top panel). Time course analysis in spleen and thymus at 1 week (1w), 2 weeks (2w), 3 weeks (3w), and 4 weeks (4w) after pIpC treatment is depicted (bottom panel).

D: Time course (2–12 weeks) FACS analysis of peripheral blood cells from pIpC-treated $Cbfb^{+/56M}$/*Mx1Cre* (squares) and littermate control (diamonds) mice. Lineage-specific ($Gr\text{-}1^+$, $Mac\text{-}1^+$, $CD3^+$, and $B220^+$) peripheral blood cells, hematocrit (HCT), and platelet counts are shown as mean + standard deviation (*$p < 0.01$; two-tailed Student's *t* test; $n = 6$).

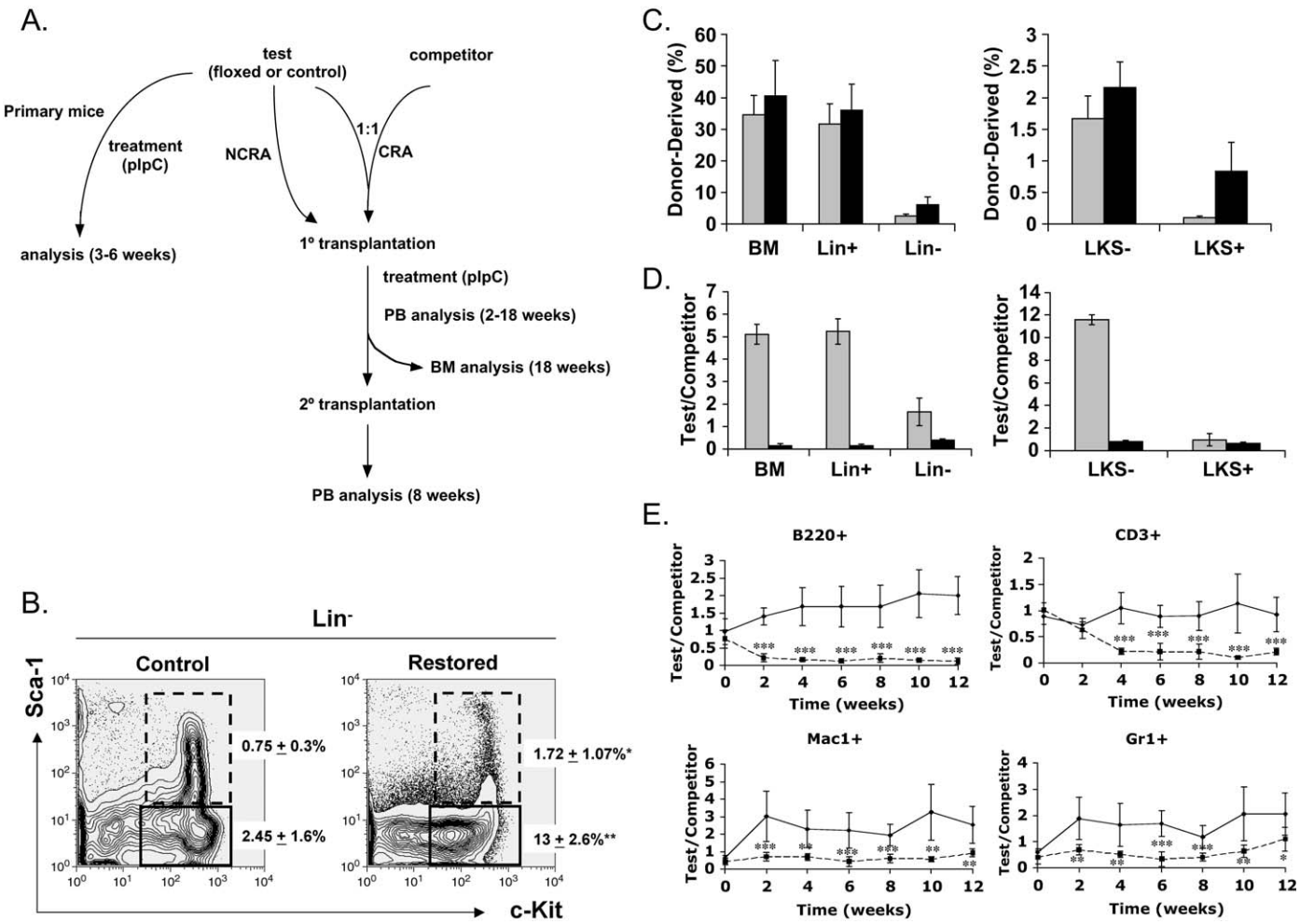

Figure 2. Cbfβ-SMMHC impairs competitive repopulation while not affecting the number of phenotypic HSCs

A: Flow chart depicting the experimental approaches used in this study. Primary *Cbfb*$^{+/56M}$*/Mx1Cre* floxed or control mice were analyzed 3–6 weeks after pIpC treatment. In noncompetitive repopulation assays (NCRA), test (floxed or control) BM cells were transplanted into lethally irradiated recipients and pIpC treated 2 weeks later. Contribution to peripheral blood was analyzed biweekly for 12 weeks. BM was analyzed at 18 weeks followed by secondary transplantation. In competitive repopulation assays (CRA), 5 × 10^5 BM test (floxed or control) and competitor cells were mixed and transplanted into lethally irradiated recipients. See Experimental Procedures for details.

B: FACS analysis of primary (restored and control) HSCs (dashed box; n = 6; *p = 0.042) and myeloid progenitors (solid box; n = 6; **p = 0.0007).

C and **D:** Histogram representations of donor-derived BM contribution in NCRA (**C**) and test/competitor ratio in CRA (**D**), including control (gray) and restored (black) cells. Left: contribution to whole BM, lineage-positive (Lin$^+$), and lineage-negative (Lin$^-$) progenitors. Right: contribution to HSCs (LKS$^+$) and progenitors (LKS$^-$). NCRA (mean + SD; n = 4); CRA (mean ratio of test to competitor + SD; n = 3; *p = 0.05).

E: Time-lapse contribution of restored (squares) or control (diamonds) cells to peripheral blood in CRA (n = 8–10; *p < 0.05; **p < 0.01; ***p < 0.001).

Similar engraftment of floxed and control BM was confirmed prior to induction of Cbfβ-SMMHC. Transplanted recipients (floxed and control) were then treated with seven doses of pIpC to ensure maximum induction of fusion protein expression. FACS analysis of peripheral blood 2–12 weeks after pIpC treatment showed similar lineage contribution as in primary restored mice (data not shown). Analysis of BM 18 weeks posttreatment indicated similar contribution between restored and control donor cells in whole BM, and lineage-restricted (Lin$^+$) and myeloid progenitor (LKS$^-$) compartments (Figure 2C, left panel). However, restored donor-derived long-term phenotypic HSCs (LKS$^+$) were increased 2- to 8-fold (Figure 2C right panel), confirming that Cbfβ-SMMHC-induced HSC expansion is cell autonomous.

To test the proliferative capacity of BM progenitors expressing Cbfβ-SMMHC, we analyzed their expansion in CRA (Figure 2A). Equal numbers of test (control or floxed; 129 SvEv strain; Ly9.1$^+$) and competitor (wild-type C57BL/6-SJL strain; Ly5.1$^+$) cells were mixed and transplanted into lethally irradiated recipients (C57BL/6-SJL strain; Ly5.2$^+$). Similar engraftment between restored and control donors was confirmed before pIpC treatment (see Figure 2E, 0 time point). The contribution of restored donors to whole BM, lineage-restricted (Lin$^+$), and myeloid progenitors (Lin$^-$/c-Kit$^+$/Sca1$^-$; LKS$^-$) was significantly reduced under competitive conditions (Figure 2D, left panel). In contrast, restored and control donors were maintained at similar numbers in the long-term HSC (LKS$^+$) compartment (Figure 2D, right panel). Biweekly FACS analysis of peripheral blood revealed a significant reduction of restored donors in all lineages analyzed (Figure 2E). Furthermore, restored BM cells engrafted into secondary transplants and showed a deficient multilineage repopulation at 8 weeks, similar to that found in primary transplants. Secondary transplants developed AML between weeks

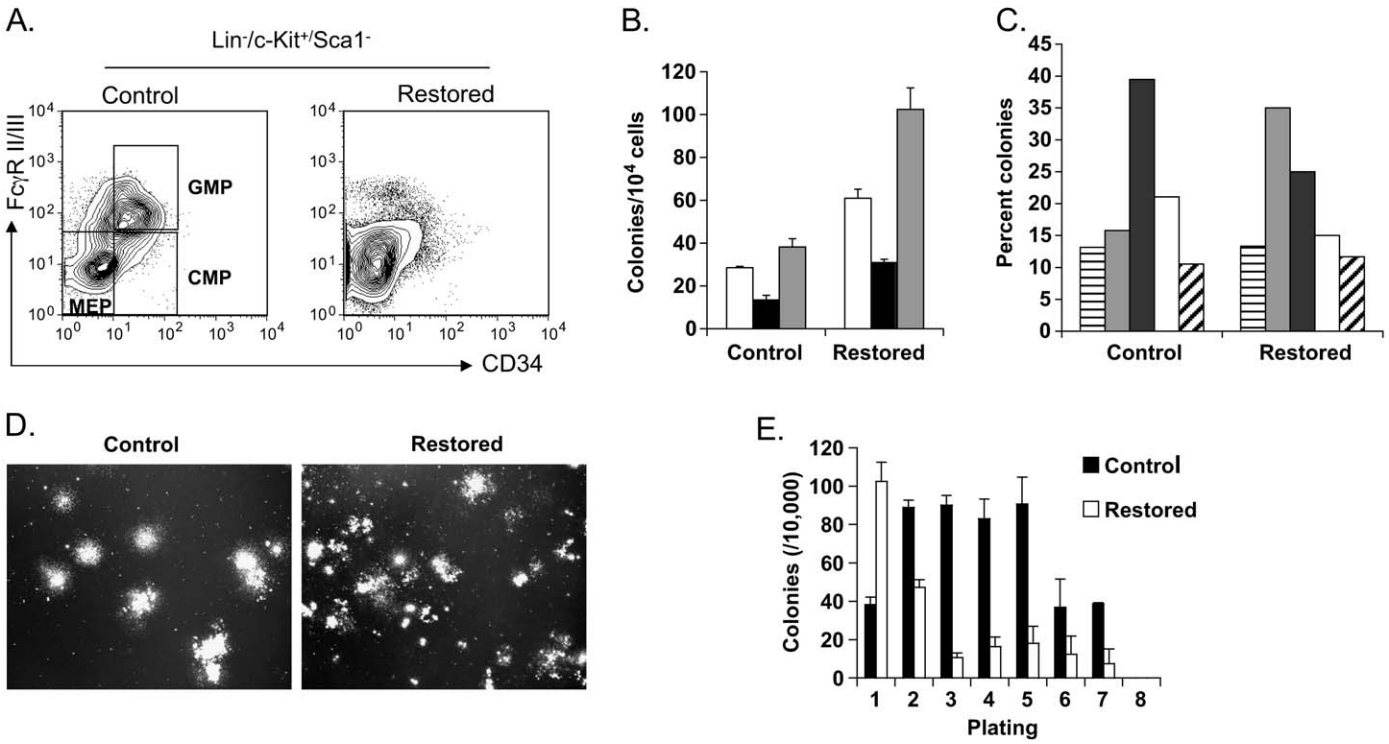

Figure 3. Cbfβ-SMMHC induces an AMP compartment

A: FACS analysis of the myeloid compartment (LKS$^-$; solid box in Figure 2B) in primary mice. Distinct CMP (CD34$^+$/FcγRII/III^{low}), MEP (CD34$^-$/FcγRII/III$^-$), and GMP (CD34$^+$/FcγRII/III^{hi}) subpopulations are shown in control mice (left panel).

B and **C:** In vitro erythroid and myeloid differentiation assays of primary restored and control cells in methylcellulose. **B:** White bars represent CFU-E, black bars represent BFU-E, and gray bars represent CFU-myeloid (mean + SD; n = 4 in duplicates). **C:** Percentage of each type of colony determined based on morphology of cytospin preparations scored from 60 colonies from each of four independent assays (striped bar, CFU-blast; gray bar, CFU-GEMM; black bar, CFU-GM; white bar, CFU-G; diagonally striped bar, CFU-M; mean + SD; n = 4 in duplicates).

D: Representative images of myeloid colonies at day 7 depicting an increased number of smaller colonies in restored cultures.

E: Serial replating assays of 1 × 10^4 pooled restored or control cells at day 7 (mean + SD; n = 3 in duplicates).

12 to 16, probably due to acquisition of transforming mutations (data not shown). Together, these results show that HSCs expressing Cbfβ-SMMHC can be maintained for long periods in transplanted mice, although their multilineage repopulation capacity is markedly compromised.

Expression of CBFβ-SMMHC generates an AMP compartment

To assess the role of Cbfβ-SMMHC in the myeloid progenitor compartment, we analyzed the LKS$^-$ fraction from primary BM by FACS (Akashi et al., 2000). Restored myeloid progenitors increased 3- to 9-fold when compared to littermate controls (Figure 2B, solid box). Notably, the myeloid progenitor compartment included a predominant AMP population that could not be further separated into distinct common myeloid progenitor (CMP; CD34$^+$/FcγRII/III^{low}), myeloid-erythroid progenitor (MEP; CD34$^-$/FcγRII/III$^-$) and granulocyte-macrophage progenitor (GMP; CD34$^+$/FcγRII/III^{hi}) subpopulations (Figure 3A). Due to their apparent MEP-like immunophenotype (LKS$^-$/CD34$^-$/FcγRII/III$^-$), we tested whether AMPs could differentiate into myeloid and erythroid lineages in vitro. Methyl-cellulose cultures from restored BM consistently generated a 1.5- to 2.5-fold increase in erythroid and myeloid colonies (Figure 3B). Analysis of myeloid colonies revealed an increase in CFU-GEMM and reduction of CFU-GM, indicating that AMPs have a CMP-like differentiation capacity (Figure 3C). Furthermore, restored BM cultures showed a significant increase of smaller colonies when compared to controls (Figures 3D and 3E, plating 1). Approximately 90% of these colonies contained the Cre-mediated deletion when tested by single-colony PCR (data not shown), thus confirming Cbfβ-SMMHC expression. Moreover, serial replating assays of BM cells from primary restored mice showed that the replating capacity of AMP-derived CFUs was markedly lower than that of wild-type controls (Figure 3E). These results show that the fusion protein induces the creation of AMPs with an MEP-like immunophenotype, a CMP-like differentiation potential, and reduced proliferation capacity.

Cbfβ-SMMHC blocks megakaryocytic differentiation

Previous studies have implicated CBF as a key regulator of terminal megakaryocytic maturation. Patients with familial platelet disorder have been linked to heterozygous *RUNX1* mutations and are predisposed to AML (Song et al., 1999). Recent studies using *Runx1* conditional knockouts reported that *Runx1* null or haploinsufficient mice exhibited deficient terminal megakaryocytic differentiation (Growney et al., 2005; Ichikawa et al., 2004; Sun and Downing, 2004). Our findings that Cbfβ-SMMHC alters the myeloid progenitor compartment and reduces platelet counts prompted us to assess its effect on megakaryocytic maturation. Analysis of primary restored BM sections 3–4 weeks

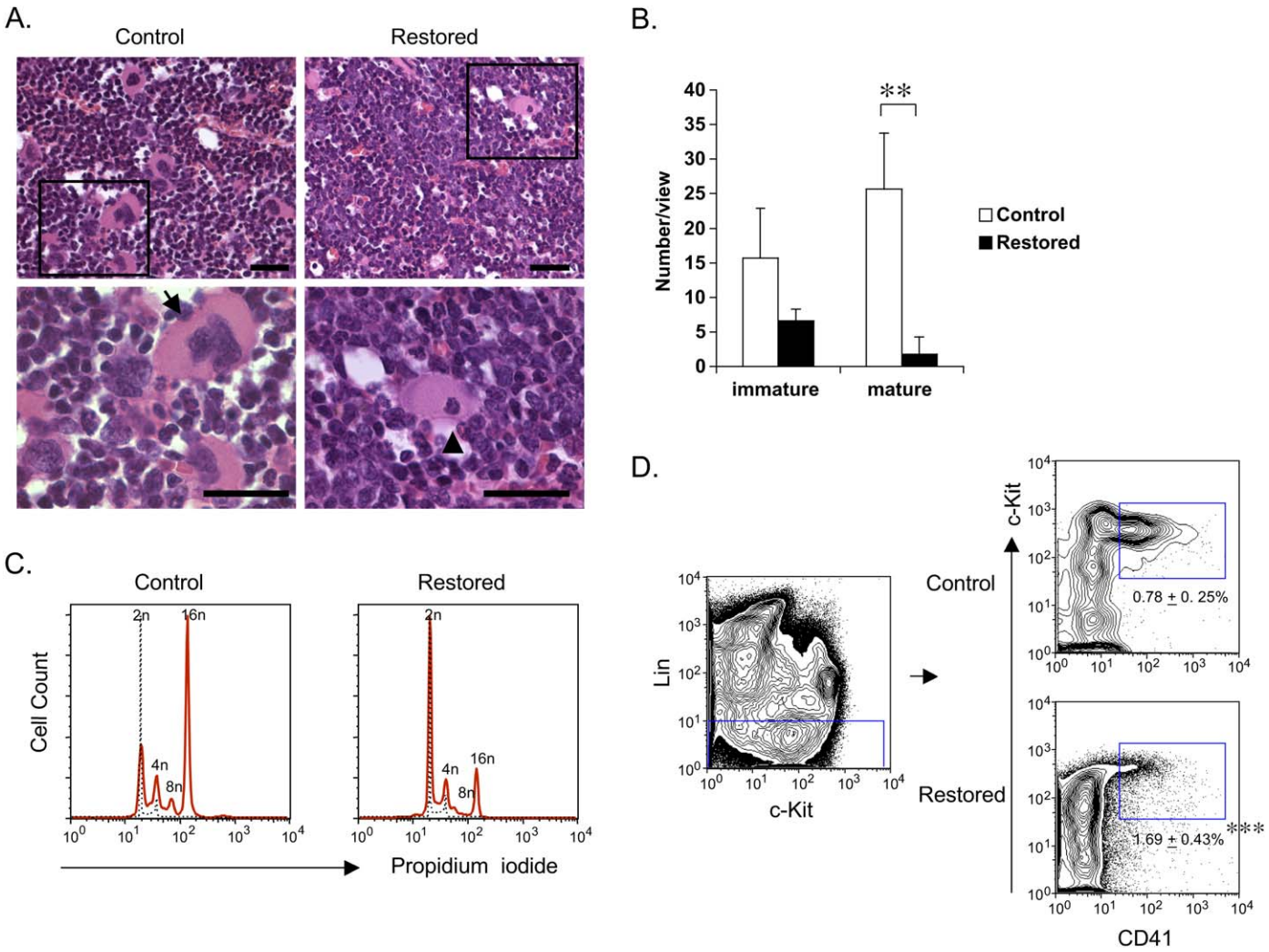

Figure 4. Cbfβ-SMMHC impairs megakaryocytic maturation

A: H&E-stained BM histology sections of restored and control mice. Higher magnification of the boxed area in the top panels is shown in the bottom panels. Mature megakaryocytes (arrow) and immature megakaryocytes (arrowhead) are shown. Scale bars, 50 μm.

B: Differentials of immature and mature megakaryocytes in BM (mean + SD; n = 4; *p < 0.02).

C: DNA ploidy of $CD41^+$ BM cells (red solid lines) and $CD41^-$ factions (black dashed lines). Typical results are shown (n = 2–6).

D: Flow cytometric analysis of CFU-Meg ($Lin^-/c\text{-}Kit^{hi}/CD41^{hi}$) fraction (n = 6 ; **p < 0.013).

after induction revealed a 14-fold reduction in mature megakaryocytes (abundant cytoplasm and lobulated nucleus; Figure 4A, arrow; Figure 4B) and 2.3-fold reduction of immature megakaryocytes (large cytoplasm with regular nucleus; Figure 4A, arrowhead; Figure 4B). The deficiency in mature megakaryocytes was also evident by flow cytometric measurement of DNA content, as $CD41^+$ restored BM presented a marked reduction in 16N cells concomitant with an increase in 2N cells (Figure 4C). In addition, we observed a 2 fold increase of CFU-Meg progenitors ($Lin^-/c\text{-}Kit^{hi}/CD41^{hi}$ fraction), which give rise to immature megakaryocytes (Figure 4D; Hodohara et al., 2000). These results demonstrate that Cbfβ-SMMHC impairs megakaryocyte maturation at the CFU-Meg to immature megakaryocyte transition.

Cbfβ-SMMHC induces spontaneous AML

We have previously shown that Cbfβ-SMMHC is necessary but not sufficient to cause leukemia (Castilla et al., 1999, 2004). Surprisingly, 90% of primary restored mice developed AML between 11 and 24 weeks of age when treated with three doses of pIpC (Figure 5A, solid line). We reasoned that the observed difference in AML development between the restored mice and the knockin chimeras may reside on the number of Cbfβ-SMMHC-expressing progenitors in the BM. We tested this hypothesis using two independent assays. First, when mice were induced with only one dose of pIpC, AML latency was delayed to 21 weeks with 15% efficiency by 24 weeks (Figure 5A, dashed line). The proportion of restored cells between three (17% ± 4.8) and one (6% ± 2.9) pIpC doses was approximately 2:1, 1 week after treatment when tested by qPCR (Figure S1A in the Supplemental Data available with this article online). Second, when titrating numbers of restored BM cells (3×10^6, 1×10^6, or 0.5×10^6) were transplanted into irradiated recipients, AML was observed only in the first group (3×10^6), with 70% efficiency at 26 weeks, while recipients harboring 1×10^6 or 0.5×10^6 restored BM cells

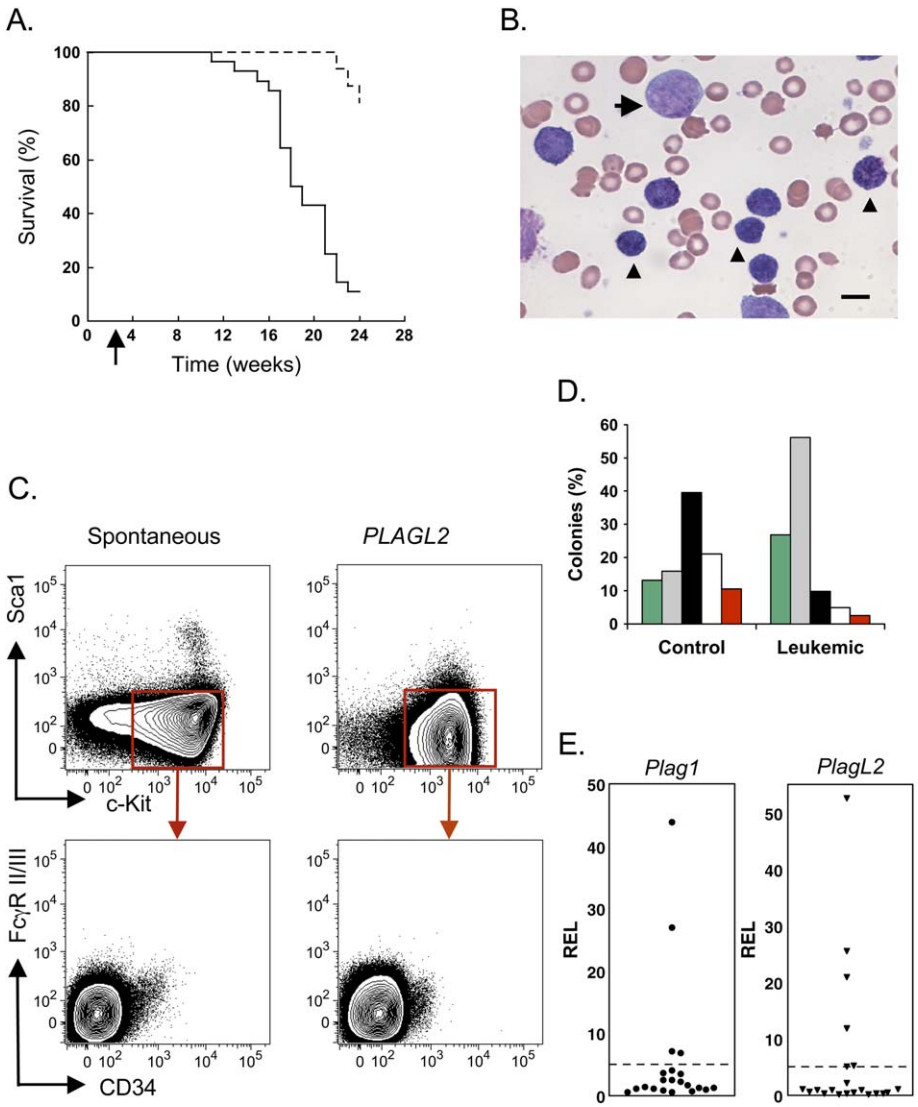

Figure 5. Cbfβ-SMMHC-associated AMPs progress to leukemia

A: Kaplan-Meier survival curve of *Cbfb*$^{+/56M}$/*Mx1Cre* mice induced at 3 weeks (arrow) with one (dashed line, n = 16) or three (solid line, n = 28) doses of plpC.

B: Wright-Giemsa staining of peripheral blood from a leukemic mouse depicting immature cells with predominant monocytic (arrow) and blastlike (arrowheads) progenitors; scale bars, 5 μm.

C: FACS analysis of myeloid progenitor compartment of spontaneous leukemia (left panel) and *PLAGL2*-induced leukemia (right panel). Typical results are shown (n = 4–7).

D: In vitro differentiation assay of leukemic cells and untreated controls. Myeloid colonies (green bar, CFU-blast; gray bar, CFU-GEMM; black bar, CFU-GM; white bar, CFU-G; red bar, CFU-M) were identified based on morphology of cytospin preparations scored from 60 colonies from each of four independent assays.

E: Quantitative RT-PCR of *Plag1* and *PlagL2* in a panel of 20 spontaneous AML samples. The threshold of *Plag1* and *PlagL2* leukemic function was defined at relative expression levels (RELs) of 5, as indicated by the dashed line. RELs shown represent the average values of two independent experiments.

as well as wild-type controls remained disease free (Figure S1B). In all cases tested, the leukemic nature of these cells was confirmed in secondary transplantations. Together, these results support the hypothesis that the latency and penetrance of disease depend on the size of progenitor population at risk to develop full-blown disease after acquiring cooperating mutations.

The pathology of the disease was consistent with AML developed in knockin chimeras, including splenomegaly, infiltration in spleen and liver, leukocytosis, and anemia (see Figure S2; Castilla et al., 1999). Multiparameter analysis of these spontaneous AMLs with myeloid progenitor compartment markers showed that the predominant leukemic population presented an AMP-like immunophenotype (LKS$^-$/CD34$^-$/FcγRII/III$^-$; Figure 5C, left panel). Furthermore, immunophenotype analysis using 28 hematopoietic markers and cytology examination determined that leukemic cells included two main subpopulations. A predominant blastlike population represented approximately 85% of AML cells and was characterized as c-kit$^+$, CD45^{lo}, and CD71^{mod} (Figure 5B, arrowheads; and Table S1). A second monocytic population represented approximately 15% of AML and was Gr1$^+$, Mac1$^+$, Ly71(F4/80)$^+$, CD45$^+$, CD24$^+$, CD16/32$^+$, CD31$^+$, CD59$^+$, CD71^{lo}, and CD86$^+$ (Figure 5B, arrows; and Table S1). Spectral karyotyping analysis of leukemic samples (n = 5) revealed no clonal chromosomal abnormalities (data not shown), consistent with the hypothesis that Cbfβ-SMMHC does not induce genomic instability. Analysis of the in vitro differentiation potential of spontaneous AMLs revealed a predominance of immature colonies (CFU-blast and CFU-GEMM; Figure 5D). In addition, the serial replating capacity of AML cells was sustained up to plating 5 (data not shown). These data show that the deficient proliferation potential observed in AMPs is restored in leukemic cells. However, leukemic cells do not show in vitro immortalization, probably due to their dependency on cytokines and/or growth factors that are not present in these cultures.

To test whether *Cbfb-MYH11*-mediated spontaneous AML samples present deregulated expression of cooperating genes, we analyzed the expression levels of *Plag1* and *Plagl2* by qPCR in a panel of 20 spontaneous AML samples. First, we defined the threshold of *Plag1* and *PlagL2* leukemic function based on their transcript levels in *MIG-Plag1*- and *MIG-PLAGL2*-induced AML samples (relative expression levels [RELs] $\geq$ 4.5; unpublished

Table 1. Limiting dilution transplantation of sorted HSCs and myeloid progenitors

Starting population	Gene alteration	Transduction efficiency (%)	Transplanted cells	Estimated transduced cells	Transplanted animals	Incidence of AML (%)	Latency (weeks ± SD)
HSC (LKS^+)	*Cbfb-MYH11* + *PLAGL2*	30	10,000	3000	4	100	12.8 ± 3.9
			1000	300	4	75	15.7 ± 3.8
			100	30	5	20	11
HSC (LKS^+)	wild-type + *PLAGL2*	30	2000	600	5	0	na
AMPs (LKS^-)	*Cbfb-MYH11* + *PLAGL2*	30	10,000	3000	4	100	12.8 ± 3.9
			1000	300	4	75	15.7 ± 3.8
			100	30	5	20	11
Myeloid progenitors (LKS^-)	wild-type + *PLAGL2*	30	200,000	60,000	5	0	na
			20,000	6000	5	0	na

na, not applicable.

data; and Landrette et al., 2005). Spontaneous AMLs showed significant *Plag1* overexpression in 20% (4/20) of the samples and *PlagL2* in 30% (6/20) of the samples (Figure 5E). These results highlight the deregulation of *Cbfb-MYH11* cooperating genes in spontaneous AMLs. Furthermore, considering that *PLAG1* and *PLAGL2* were found overexpressed in 20% of human AML samples, these observations underscore the relevance of this model to human disease.

It is thought that leukemia rises from the accumulation of mutations in the HSCs (Reya et al., 2001). Our finding that Cbfβ-SMMHC creates a unique myeloid progenitor (AMPs) prompted us to test whether this restricted progenitor behaves as a leukemic precursor at similar frequency as HSCs upon the acquisition of other mutations. We have previously reported that while PLAGL2 and Cbfβ-SMMHC cooperate in AML development, expression of either transcription factor independently is insufficient to cause AML (Landrette et al., 2005). On this basis, we compared the ability of HSCs (LKS^+) and AMPs (LKS^-) to induce AML using limiting dilution BM transplantation assays. Sorted LKS^+ and LKS^- cells from primary restored BM were transduced with *MIG-PLAGL2* and transplanted into sublethally irradiated recipients at various diluted concentrations (1×10^4, 1×10^3, and 1×10^2 cells). Mice transplanted with either LKS^+ or LKS^- cells developed AML with similar latency (9–12 weeks), and the disease penetrance correlated with the number of progenitors transplanted (Table 1). Control groups confirmed that PLAGL2 is not sufficient to induce AML when transduced into wild-type HSCs or myeloid progenitors. The disease was readily transplantable to secondary recipients with short latency. In addition, the leukemic cells were GFP^+, indicating the expression of *MIG-PLAGL2* (data not shown). The pathology of disease was similar in both groups, and resembled that of spontaneous AML. Likewise, multiparameter flow cytometry analysis of these AML cells showed an AMP-like immunophenotype irrespective of the initial transduced population (Figure 5C, right panel). Together with the marked expansion observed in the primary restored mice, these results show that AMPs represent a unique Cbfβ-SMMHC-expressing progenitor at risk for leukemia development.

Discussion

In this study, we used a *Cbfb-MYH11* conditional knockin mouse model to analyze the effect of Cbfβ-SMMHC in leukemia progression. We show that AML-associated fusion proteins that impair differentiation may not alter maintenance of long-term HSC, while severely compromising their repopulation function. In addition, we define a preleukemic myeloid progenitor population induced by Cbfβ-SMMHC that is a target of AML transformation.

A hallmark of HSCs is their ability to undergo symmetric (perpetuating HSCs) and asymmetric (differentiating into progenitors that can replenish multilineage hematopoiesis) replication programs (Weissman, 2000). We show that alterations in these programs caused by Cbfβ-SMMHC closely mimic those resulting from Runx1 deficiency (Growney et al., 2005; Ichikawa et al., 2004). First, Cbfβ-SMMHC does not alter the maintenance of long-term HSCs (called PSCs; see Figure 6) and may remain in this compartment asymptomatic for long periods. Our findings are in agreement with clinical observations in human AML. For example, studies in inv(16) samples have shown that HSCs ($CD34^+$, $CD38^-$) harboring *CBFB-MYH11* can be present prenatally and remain undetected for years before progressing to AML (McHale et al., 2003), and can be detected after diagnosis (Haase et al., 1995; Mehrotra et al., 1995). In addition, inv(16) patients in remission can remain *CBFB-MYH11* positive 2 years after allogenic BM transplantation (Costello et al., 1997; Tobal et al., 1995). Interestingly, the other frequent CBF leukemia fusion protein RUNX1-ETO has been shown to expand HSCs and hematopoietic progenitors in mouse and human cells (de Guzman et al., 2002; Higuchi et al., 2002; Mulloy et al., 2002; Tonks et al., 2003). This contrast suggests that Cbfβ-SMMHC and RUNX1-ETO may affect different programs during leukemia progression.

Second, as PSCs undergo asymmetric replication, differentiation programs exhibit lineage-dependent sensitivity to Cbfβ-SMMHC. Under noncompetitive conditions, circulating B cells and platelet counts were drastically reduced, whereas T cells and myeloid cells seemed unaffected. These results suggest that small changes in CBF levels may significantly affect the regulation of B cell and megakaryocytic differentiation. Interestingly, multilineage repopulation was limited under competitive conditions, providing in vivo demonstration that Cbfβ-SMMHC expression impairs differentiation and proliferation capacity of multipotent progenitors.

Importantly, our work also shows that Cbfβ-SMMHC induced distinct phenotypes that cannot be explained by loss of Runx1 function. First, spontaneous AML efficiently developed 4–6 months after induction of the fusion protein. The pathology of disease resembled that of our previous knockin model induced

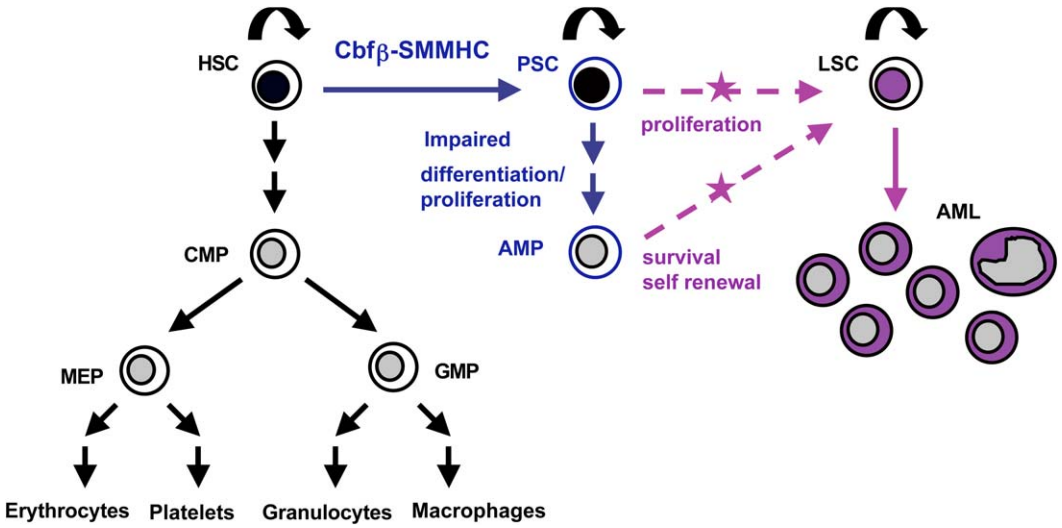

Figure 6. Proposed model of Cbfβ-SMMHC-associated leukemia progression

HSCs have the potential for multilineage differentiation (straight arrows) and self-renewal (curved arrow). HSCs expressing Cbfβ-SMMHC generate a preleukemia stem cell (PSC), in which differentiation and proliferation ability are deficient, and create a distinct abnormal myeloid progenitor (AMP) with limited proliferation potential. Acute myeloid leukemia (AML) can emerge from either PSCs or AMPs after gaining additional mutations, presumably affecting proliferation and survival programs. Leukemic stem cells (LSC) can generate blastlike and monocytic leukemic cells.

by chemical or retroviral mutagenesis (Castilla et al., 1999, 2004), emphasizing that despite its pleiotropic effect in differentiation, Cbfβ-SMMHC seems to determine a consistent myeloid leukemia phenotype. The efficiency of AML in our model also contrasted with the conditional Runx1-Eto knockin, which developed granulocytic sarcomas and T cell lymphomas only after ENU treatment (Higuchi et al., 2002). The use of these two models in parallel comparative in vivo studies may unravel the mechanism underlying the identified phenotypic differences between the CBF oncogenes. Second, the preleukemic myeloid compartment presented distinct AMPs, in contrast to the neutrophilic expansion observed in Runx1 null mice. The AMPs presented the following: (1) a CMP-like function, generating myeloid and erythroid colonies in vitro; (2) low proliferation capacity, as colonies were significantly smaller; (3) deficient replating capacity; (4) immunophenotype similar to MEPs.

We have used two independent assays to demonstrate that the efficiency of AML development in this conditional model is dependent on the size of the Cbfβ-SMMHC-expressing progenitor pool at risk for transformation. Consistent with this notion, it is likely that *Cbfb*$^{+/MYH11}$ HSCs in the knockin chimeras were diluted below a threshold of detectable ''spontaneous'' disease. Alternatively, it is possible that, unlike the inducible model, ES cell-derived *Cbfb*$^{+/MYH11}$ HSCs had undergone a selection process during embryonic development, and only a subgroup of cells with reduced oncogenic potential were present in the BM of the chimeras.

A critical open question is what distinct CBFβ-SMMHC function directs the predisposition to AML development. Myeloid-erythroid differentiation is tightly regulated by transcription factors (Tenen, 2003). Recently, it has been suggested that low expression levels, rather than the absence of some transcription factors (such as GATA-1, PU.1, and CEBP-α), may induce leukemia (Rosenbauer et al., 2005). Likewise, AMPs may emerge from CBFβ-SMMHC-induced downregulation of myeloid transcription factors. Alternatively, CBFβ-SMMHC may effect an altered balance of the expression and function of the RUNX family (RUNX1–3), since RUNX binding sites are present in the promoter of the three *RUNX* genes (Otto et al., 2003). For example, the expression of *RUNX3*, a tumor suppressor lost in gastric cancer (Li et al., 2002), is consistently downregulated in inv(16)$^{+}$ AML samples (Gutierrez et al., 2005; Valk et al., 2004).

The striking immunophenotypic similarity between AMPs and leukemic cells suggest that AMPs may represent a preleukemic intermediate step toward AML. It has been proposed that HSCs (and noncommitted progenitors) are targets of transforming mutations, since AML cells, like HSCs, retain self-renewal function (Reya et al., 2001). Alternatively, committed progenitors can progress to leukemia when transduced with leukemia fusion oncogenes, suggesting that oncogenic function (and effects of viral insertions) may provide self-renewal and transforming properties to a short-lived myeloid progenitor (Cozzio et al., 2003; Huntly et al., 2004). In our study, limiting dilution BM transplantation analysis demonstrated that LKS^{-} progenitors expressing Cbfβ-SMMHC (enriched with AMPs) can trigger AML with similar efficiency as LKS^{+} progenitors (enriched for PSCs), underscoring the significance of AMPs as an important target population in this mouse model.

Based on this study, we propose a model for Cbfβ-SMMHC-associated leukemia progression (Figure 6). Expression of Cbfβ-SMMHC in HSCs creates a preleukemic population in the BM composed of PSCs able to both perpetuate and remain asymptomatic in the HSC compartment for long periods. PSCs undergoing asymmetric replication have deficient multilineage repopulation capacity and produce AMPs with reduced proliferative ability. Both preleukemic progenitors can gain mutations (such as activated Ras, c-kit, or upregulation of *PlagL2*), presumably enhancing proliferative and survival programs, to trigger AML. It is expected that (at least) some leukemic cells (the leukemia stem cells [LSC]) could retain or regain self-renewal capacity and produce leukemic cells with blastlike and monocytic forms.

Finally, this conditional knockin mouse model allows the inducible allelic switch from *Cbfb* to *Cbfb-MYH11* in the adult mouse. This strategy bypasses the embryonic lethality that is associated with CBFβ-SMMHC expression and enables its activation in the adult BM. This model will be key for the study of early *Cbfb-MYH11* target genes in hematopoietic progenitors and the in vivo validation of inv(16) cooperating oncogenes, as well as for the evaluation of candidate drugs for improved treatment of AML.

Experimental procedures

Targeting experiments

The previously generated *Cbfb*$^{+/MYH11}$ embryonic stem cell ES55 (Castilla et al., 1996) was retargeted to insert the floxed *Cbfb* exons 5 and 6 at the Msc1 restriction site of the *Cbfb*$^{+/MYH11}$ intron 4 by homologous recombination (Figure 1A). The pPNT-based targeting construct included a 5.4 kb Xho1-Msc1 fragment of *Cbfb* intron 4, a loxP1 site, a 120 bp Hinf1-Xho1 genomic fragment including the 3′ end of intron 4 and part of exon 5, an XhoI-XhoI sequence with the in-frame cDNA sequence for the second part of human *CBFB* exon 5 and exon 6, a 300 bp Pst1-Pst1 fragment from pCDNA3.1 (Invitrogen, Carlsbad, CA) including the bovine growth hormone polyadenylation signal, a PGK-hygromycin gene, a second loxP1 site, and a 3.5 kb Msc1-Xho1 genomic fragment of *Cbfb* intron 4. Three of 75 hygromycin-resistant ES cells (ES 28, 32, 56) presented the expected bands by Southern blot analysis of ES cell genomic DNA cut with NcoI and using an internal (hygromycin probe) and an external probe (*MYH11*) for hybridization. Chimeric mice for *Cbfb*$^{+/56M}$ were backcrossed into 129/SvEv background to obtain heterozygous *Cbfb*$^{+/56M}$ and homozygous *Cbfb*$^{56M/56M}$ mice.

Induction of Cbfβ-SMMHC expression

To induce Cbfβ-SMMHC expression in adult mice, heterozygous *Cbfb*$^{+/56M}$ or homozygous *Cbfb*$^{56M/56M}$ mice were crossed with the *Mx1-Cre* transgenic mice (generously provided by Klaus Rajewsky, Harvard University; Kuhn et al., 1995). For induction, floxed primary mice (*Mx1Cre*$^{+}$/*Cbfb*$^{+/56M}$ double heterozygous) were injected every other day with three doses (unless otherwise indicated) of 200 μg/dose pIpC (Sigma, St. Louis, MO) intraperitoneally at 3 weeks of age. BM cells from restored *Cbfb*$^{+/56-M}$/*Mx-cre* and similarly treated *Cbfb*$^{+/56M}$ control littermates were analyzed 3 to 6 weeks after induction.

Genotype analysis

The presence of the floxed *Cbfb*^{56M} allele in mice was determined routinely by PCR using tail-snip DNA. PCRs included 50 ng of template DNA and 0.2 μM gene-specific primers (hyg-f 5′-CCATCGTCGAGATCCAGACATG-3′ and hygr-r 5′-GTATATGCTCCGCATTGGTCTTG-3′; Cre-f 5′-CCGGGCTGC CACGACCAA-3′ and Cre-r 5′-GGCGCGGCAACACCATTTTT-3′). The PCR samples were denatured at 94ºC for 2 min, followed by 30 cycles of amplification (94ºC for 30 s, 56ºC for 30 s, 72ºC for 45 s), and a final extension step at 72ºC for 5 min.

Southern blot analysis

The efficiency of Cre-mediated deletion was analyzed using standard procedures. Briefly, 10 μg genomic DNA from BM, spleen, liver, and thymus were digested with BamHI, electrophoresed in 1% agarose gel, transferred onto Hybond-XL membrane (Amersham Bioscience, Piscataway, NJ), and hybridized with an P^{32}-labeled EcoRI-BamHI fragment of *Cbfb* intron 4. For visualization, membranes were exposed to Kodak Bio Max X-ray film at −80ºC.

Flow cytometry and cell sorting

For FACS analysis and cell sorting, peripheral blood was extracted from the retro-orbital sinus, followed by lysis of red blood cells with RBC lysis solution (Puregene, Gentra Systems, Minneapolis, MN), and the white blood cell pellet was resuspended in PBS with 0.5% bovine serum albumin. BM cells isolated from femurs and tibias were resuspended in staining media A (PBS; 0.5% BSA) or staining media B (biotin-, flavin-, and phenol red-deficient RPMI 1640 [Invitrogen, Carlsbad, CA], 10 mM HEPES [pH 7.2], 0.02% sodium azide, 1 mM EGTA, 3% newborn calf serum) and treated for 10 min on ice with 2.4G2 Fc block (BD Biosciences, San Jose, CA). Cells were incubated for 20 min on ice with primary antibodies, and washed and biotin-stained cells were incubated with secondary reagents for 15 min on ice. After two washes, cells were resuspended in 1 μg/ml propidium iodide to exclude dead cells. Primary antibodies included c-kit (2B8)-allophycocyannin (APC), Sca-1 (E13-161.7) fluorescein isothiocyanate (FITC) or -phycoerythrin (PE), IL-7R PE-Cy7 or PE, CD34 (RAM34)-PE or APC, CD16/32 FcγRIII/II (2.4G2)-FITC, CD41 (MWReg30)-PE, CD229.1 (Ly-9.1) (30C7)-FITC, and CD45.1 (Ly-5.1) (A20)-PE. (BD Biosciences, San Jose, CA; eBiosciences, San Diego, CA). In addition, B220 (RA3-6B2), CD19, IgM (clone 331), CD3 (17A2), CD4 (L3T4), CD8 (53-6.7), Gr-1 (R86-8C5), Mac-1 (M1/70), Ter119 (Ly-76) conjugated with biotin, FITC, PE, APC, or PerCP-Cy5.5 (also from BD Biosciences). Secondary reagents used were streptavidin (SA)-APC or PerCP-Cy5.5 (BD Biosciences, San Jose, CA). Flow cytometry was performed on a 3 laser, 9 detector LSR II (BD Biosciences, San Jose CA) or a 3 laser, 7 detector DIVA FACSVantage and analyzed with FlowJo software (Tree Star, Ashland, OR). For sorting experiments, Lin^+ cells were partially removed by negative selection magnetic capture following the procedure suggested by the manufacturer (EasySep, StemCell Technologies, Vancouver BC). Cells were sorted using antibodies as previously described (Akashi et al., 2000; Morrison et al., 1997; Morrison and Weissman, 1994). Phenotypic populations were defined as HSC (Lin^-/c-kit^{hi}/Sca-1^{hi}), CMP (Lin^-/c-kit^{hi}/Sca-1^-/$CD34^+$/$CD16/32^{lo}$), GMP (Lin^-/c-kit^{hi}/Sca-1^-/$CD34^+$/$CD16/32^{hi}$), and MEP (Lin^-/c-kit^{hi}/Sca-1^-/$CD34^-$/$CD16/32^{lo}$). The purity of the sorted populations was approximately 91%–98% by FACS analysis.

Ploidy analysis of megakaryocytes

BM cells isolated in CATCH media were treated 10 min on ice with 2.4G2 Fc block (BD Biosciences, San Jose, CA) before staining with CD41-FITC antibody (BD Biosciences, San Jose, CA) on ice for 30 min. After 2 washes, cells were incubated with propidium iodide (50 μg/ml) in hypotonic buffer (0.1% sodium citrate; 0.1% Triton X-100 and 50 μg/ml RNaseA) for 30 min at room temperature. Flow cytometric analysis was performed as previously described (Growney et al., 2005; Ichikawa et al., 2004; Jackson et al., 1984).

Transplantation experiments

NCRA

BM cells (1×10^6) from *Cbfb*$^{56-M/+}$/*Mx-cre* (floxed) or control mice (test mice: 129Sv/Ev; $Ly9.1^+$) were transplanted into lethally irradiated (1300 rads) wild-type C57BL/6 ($Ly9.2^+$/$Ly5.2^+$) mice. Engraftment of test cells was confirmed before the administration of seven doses of pIpC every other day, 2 weeks after transplantation. Isotype-specific antibody FITC-Ly9.1 (BD Biosciences, San Jose, CA) was used to determine the donor-derived population by FACS analysis. Peripheral blood was analyzed every 2 weeks for 12 weeks. BM was analyzed at 18 weeks, at which time 1×10^6 cells were transplanted into secondary recipients.

CRA

Test (floxed or wild-type control; 129SvEv; $Ly9.1^+$/$Ly5.2^+$) BM cells (5×10^5) were mixed with equal numbers of wild-type competitor (C57BL/6-SJL; $Ly9.2^+$/$Ly5.1^+$) BM cells and injected intravenously into lethally irradiated (1300 rads) C57BL/6 ($Ly9.2^+$/$Ly5.2^+$) mice. Engraftment of test cells was confirmed before the administration of seven doses of pIpC every other day 2 weeks after transplantation. The isotype-specific antibodies FITC-Ly9.1 and PE-Ly5.1 (BD Biosciences, San Jose, CA) were used to determine the origins of repopulating cells by FACS analysis. Peripheral blood was analyzed before induction and biweekly afterwards by Wright-Giemsa staining and FACS analysis. BM cells were analyzed 18 weeks after induction; 1×10^6 cells were transplanted into lethally irradiated recipients.

Limited dilution transplantation assays

LKS^+ and LKS^- populations from primary restored mice were sorted and transduced with *MIG-PLAGL2* as previously described (Cozzio et al., 2003). Sublethally irradiated (650 rads) wild-type mice were transplanted with the indicated number of cells (10^4, 10^3, 10^2). The percentage of *PLAGL2*-transduced GFP-positive cells was measured after 2 days of in vitro culture and was used to estimate the number of transduced cells. Mice were monitored for a period of 6 months for signs of leukemia.

Western blot analysis

BM cells were solubilized in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0]) containing a cocktail of protease inhibitors (Calbiochem, San Diego, CA). Protein concentrations

were determined by the Lowry assay-based *DC* protein assay (Bio-Rad, Hercules, CA). Twenty micrograms of protein lysate were electrophoresed in 12% SDS-polyacrylamide gel and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Blots were probed with one of the following antibodies: mouse monoclonal anti-Cbfβ-Ab (β141, kindly provided by Nancy Speck, Dartmouth Medical School) or mouse anti-α-tubulin (Sigma, St. Louis, MO) followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-mouse Ig-Ab (Jackson ImmunoResearch Labs, West Grove, PA). Immunoblots were developed using luminol-based enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ) and autoradiography.

In vitro differentiation assays

BM cells (1×10^4) were isolated from primary, control, or leukemic mice and cultured in methylcellulose (M3434, StemCell Technologies, Vancouver BC). CFU-E and BFU-E were scored after 2 days and 3 days of culture, respectively. The total numbers of myeloid and mixed colonies were counted after 7 days of culture in 5% CO_2 at 37ºC. Single colonies were isolated for PCR genotyping and cytology analyses. Myeloid CFUs (blast, GEMM, GM, G and M) were identified based on the morphology of cytospin preparations from 70 to 100 individual colonies. In serial replating experiments, the colony number was scored at day 7, cells were pooled, and 1×10^4 cells were plated for subsequent culture.

Histology and cytology analyses

Tissue samples (spleen, liver, tibia, lung, thymus, lymph nodes, brain, epithelium) collected from leukemic mice were fixed in 10% buffered formalin and embedded in paraffin, and sectioned specimens were stained with H&E. For cytological and morphological analysis, cyto-centrifuged preparations were stained with Wright-Giemsa (Fisher Scientific, Pittsburgh, PA). Other stains included Sudan Black B, Naphthol AS-D Chloroacetate Esterase (CAE), and α-Naphthyl Acetate Esterase (all from Sigma Aldrich, St. Louis, MO). Stains were performed according to the manufacturer's instructions, except that CAE-stained slides were incubated 30 min.

Quantitative RT-PCR

RNA from leukemic samples was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First-strand cDNA was generated using 2 μg total RNA, 200 U SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), and 0.5 μg oligo dT primer in a 20 μl reaction. QPCR was performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA) containing 0.2 μM gene-specific primers and detected in ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Primers for *Plag1* were mP1x1 (5′-GGTTCACTCCTTCTCTCACACG-3′) and mP1X2 (5′-TGAG TAGCCATGTGCCTTTGTA-3′), and primers for *Plagl2* were mPL2x1 (5′-TA TAGGCACATGGCCACCCACT-3′) and hL2R3 (5′-GTACTCTCAAAGGTCTG CAGG-3′). Samples were normalized to *β-actin* expression levels (forward primer, 5′-CGAGGCCCAGAGCAAGAGAG-3′; reverse primer, 5′-CGGTTGG CCTTAGGGTTCAG-3′), and the RELs were determined by the standard curve method.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, two supplemental figures, and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/1/57/DC1/.

Acknowledgments

The authors wish to thank Richard Konz, Jr., Weibo Li, and Mei Lin Maunakea for their technical assistance. This work was supported by National Institutes of Health grants R01-CA096983 (to L.H.C.) and CA84221 (to S.C.K. and M.M.L.), and by a Specialized Center of Research (SPOR) grant (7019-04 to M.M.L.) from the Leukemia & Lymphoma Society; Y-H. K. was partly supported by the Our Danny Cancer Fund grant and by Ruth L. Kirschstein National Research Service Award (F32CA101571).

Received: July 6, 2005
Revised: October 7, 2005
Accepted: December 12, 2005
Published: January 16, 2006

References

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

Castilla, L.H., Wijmenga, C., Wang, Q., Stacy, T., Speck, N.A., Eckhaus, M., Marin-Padilla, M., Collins, F.S., Wynshaw-Boris, A., and Liu, P.P. (1996). Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11. Cell *87*, 687–696.

Castilla, L.H., Garrett, L., Adya, N., Orlic, D., Dutra, A., Anderson, S., Owens, J., Eckhaus, M., Bodine, D., and Liu, P.P. (1999). The fusion gene Cbfb-MYH11 blocks myeloid differentiation and predisposes mice to acute myelomonocytic leukaemia. Nat. Genet. *23*, 144–146.

Castilla, L.H., Perrat, P., Martinez, N.J., Landrette, S.F., Keys, R., Oikemus, S., Flanegan, J., Heilman, S., Garrett, L., Dutra, A., et al. (2004). Identification of genes that synergize with Cbfb-MYH11 in the pathogenesis of acute myeloid leukemia. Proc. Natl. Acad. Sci. USA *101*, 4924–4929.

Costello, R., Sainty, D., Blaise, D., Gastaut, J.A., Gabert, J., Poirel, H., Buzyn-Veil, A., and Macintyre, E. (1997). Prognosis value of residual disease monitoring by polymerase chain reaction in patients with CBFβ/MYH11-positive acute myeloblastic leukemia. Blood *89*, 2222–2223.

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

de Guzman, C.G., Warren, A.J., Zhang, Z., Gartland, L., Erickson, P., Drabkin, H., Hiebert, S.W., and Klug, C.A. (2002). Hematopoietic stem cell expansion and distinct myeloid developmental abnormalities in a murine model of the AML1-ETO translocation. Mol. Cell. Biol. *22*, 5506–5517.

Growney, J.D., Shigematsu, H., Li, Z., Lee, B.H., Adelsperger, J., Rowan, R., Curley, D.P., Kutok, J.L., Akashi, K., Williams, I.R., et al. (2005). Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype. Blood *106*, 494–504.

Gutierrez, N.C., Lopez-Perez, R., Hernandez, J.M., Isidro, I., Gonzalez, B., Delgado, M., Ferminan, E., Garcia, J.L., Vazquez, L., Gonzalez, M., and San Miguel, J.F. (2005). Gene expression profile reveals deregulation of genes with relevant functions in the different subclasses of acute myeloid leukemia. Leukemia *19*, 402–409.

Haase, D., Feuring-Buske, M., Konemann, S., Fonatsch, C., Troff, C., Verbeek, W., Pekrun, A., Hiddemann, W., and Wormann, B. (1995). Evidence for malignant transformation in acute myeloid leukemia at the level of early hematopoietic stem cells by cytogenetic analysis of CD34+ subpopulations. Blood *86*, 2906–2912.

Higuchi, M., O'Brien, D., Kumaravelu, P., Lenny, N., Yeoh, E.J., and Downing, J.R. (2002). Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. Cancer Cell *1*, 63–74.

Hodohara, K., Fujii, N., Yamamoto, N., and Kaushansky, K. (2000). Stromal cell-derived factor-1 (SDF-1) acts together with thrombopoietin to enhance the development of megakaryocytic progenitor cells (CFU-MK). Blood *95*, 769–775.

Huang, G., Shigesada, K., Ito, K., Wee, H.J., Yokomizo, T., and Ito, Y. (2001). Dimerization with PEBP2β protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. EMBO J. *20*, 723–733.

Huntly, B.J., Shigematsu, H., Deguchi, K., Lee, B.H., Mizuno, S., Duclos, N., Rowan, R., Amaral, S., Curley, D., Williams, I.R., et al. (2004). MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell *6*, 587–596.

Ichikawa, M., Asai, T., Saito, T., Yamamoto, G., Seo, S., Yamazaki, I., Yamagata, T., Mitani, K., Chiba, S., Hirai, H., et al. (2004). AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat. Med. *10*, 299–304.

Jackson, C.W., Brown, L.K., Somerville, B.C., Lyles, S.A., and Look, A.T. (1984). Two-color flow cytometric measurement of DNA distributions of rat megakaryocytes in unfixed, unfractionated marrow cell suspensions. Blood *63*, 768–778.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427–1429.

Lakso, M., Pichel, J.G., Gorman, J.R., Sauer, B., Okamoto, Y., Lee, E., Alt, F.W., and Westphal, H. (1996). Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc. Natl. Acad. Sci. USA *93*, 5860–5865.

Landrette, S.F., Kuo, Y.H., Hensen, K., Barjesteh van Waalwijk van Doorn-Khosrovani, S., Perrat, P.N., Van de Ven, W.J., Delwel, R., and Castilla, L.H. (2005). Plag1 and Plagl2 are oncogenes that induce acute myeloid leukemia in cooperation with Cbfb-MYH11. Blood *105*, 2900–2907.

Li, Q.L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X.Z., Lee, K.Y., Nomura, S., Lee, C.W., Han, S.B., et al. (2002). Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell *109*, 113–124.

Liu, P., Tarle, S.A., Hajra, A., Claxton, D.F., Marlton, P., Freedman, M., Siciliano, M.J., and Collins, F.S. (1993). Fusion between transcription factor CBFβ/PEBP2β and a myosin heavy chain in acute myeloid leukemia. Science *261*, 1041–1044.

Liu, P.P., Wijmenga, C., Hajra, A., Blake, T.B., Kelley, C.A., Adelstein, R.S., Bagg, A., Rector, J., Cotelingam, J., Willman, C.L., and Collins, F.S. (1996). Identification of the chimeric protein product of the CBFB-MYH11 fusion gene in inv(16) leukemia cells. Genes Chromosomes Cancer *16*, 77–87.

Look, A.T. (1997). Oncogenic transcription factors in the human acute leukemias. Science *278*, 1059–1064.

McHale, C.M., Wiemels, J.L., Zhang, L., Ma, X., Buffler, P.A., Feusner, J., Matthay, K., Dahl, G., and Smith, M.T. (2003). Prenatal origin of childhood acute myeloid leukemias harboring chromosomal rearrangements t(15;17) and inv(16). Blood *101*, 4640–4641.

Mehrotra, B., George, T.I., Kavanau, K., Avet-Loiseau, H., Moore, D., II, Willman, C.L., Slovak, M.L., Atwater, S., Head, D.R., and Pallavicini, M.G. (1995). Cytogenetically aberrant cells in the stem cell compartment (CD34+lin-) in acute myeloid leukemia. Blood *86*, 1139–1147.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity *1*, 661–673.

Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E., and Weissman, I.L. (1997). Identification of a lineage of multipotent hematopoietic progenitors. Development *124*, 1929–1939.

Mulloy, J.C., Cammenga, J., MacKenzie, K.L., Berguido, F.J., Moore, M.A., and Nimer, S.D. (2002). The AML1-ETO fusion protein promotes the expansion of human hematopoietic stem cells. Blood *99*, 15–23.

Niki, M., Okada, H., Takano, H., Kuno, J., Tani, K., Hibino, H., Asano, S., Ito, Y., Satake, M., and Noda, T. (1997). Hematopoiesis in the fetal liver is impaired by targeted mutagenesis of a gene encoding a non-DNA binding subunit of the transcription factor, polyomavirus enhancer binding protein 2/core binding factor. Proc. Natl. Acad. Sci. USA *94*, 5697–5702.

Ogawa, E., Inuzuka, M., Maruyama, M., Satake, M., Naito-Fujimoto, M., Ito, Y., and Shigesada, K. (1993). Molecular cloning and characterization of PEBP2β, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2α. Virology *194*, 314–331.

Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G., and Downing, J.R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell *84*, 321–330.

Otto, F., Lubbert, M., and Stock, M. (2003). Upstream and downstream targets of RUNX proteins. J. Cell. Biochem. *89*, 9–18.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105–111.

Rosenbauer, F., Koschmieder, S., Steidl, U., and Tenen, D.G. (2005). Effect of transcription factor concentrations on leukemic stem cells. Blood *106*, 1519–1524.

Sasaki, K., Yagi, H., Bronson, R.T., Tominaga, K., Matsunashi, T., Deguchi, K., Tani, Y., Kishimoto, T., and Komori, T. (1996). Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor β. Proc. Natl. Acad. Sci. USA *93*, 12359–12363.

Song, W.J., Sullivan, M.G., Legare, R.D., Hutchings, S., Tan, X., Kufrin, D., Ratajczak, J., Resende, I.C., Haworth, C., Hock, R., et al. (1999). Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat. Genet. *23*, 166–175.

Speck, N.A., and Gilliland, D.G. (2002). Core-binding factors in haematopoiesis and leukaemia. Nat. Rev. Cancer *2*, 502–513.

Sun, W., and Downing, J.R. (2004). Haploinsufficiency of AML1 results in a decrease in the number of LTR-HSCs while simultaneously inducing an increase in more mature progenitors. Blood *104*, 3565–3572.

Tenen, D.G. (2003). Disruption of differentiation in human cancer: AML shows the way. Nat. Rev. Cancer *3*, 89–101.

Tobal, K., Johnson, P.R., Saunders, M.J., Harrison, C.J., and Liu Yin, J.A. (1995). Detection of CBFB/MYH11 transcripts in patients with inversion and other abnormalities of chromosome 16 at presentation and remission. Br. J. Haematol. *91*, 104–108.

Tonks, A., Pearn, L., Tonks, A.J., Pearce, L., Hoy, T., Phillips, S., Fisher, J., Downing, J.R., Burnett, A.K., and Darley, R.L. (2003). The AML1-ETO fusion gene promotes extensive self-renewal of human primary erythroid cells. Blood *101*, 624–632.

Valk, P.J., Verhaak, R.G., Beijen, M.A., Erpelinck, C.A., Barjesteh van Waalwijk van Doorn-Khosrovani, S., Boer, J.M., Beverloo, H.B., Moorhouse, M.J., van der Spek, P.J., Lowenberg, B., and Delwel, R. (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. N. Engl. J. Med. *350*, 1617–1628.

Wang, S., Wang, Q., Crute, B.E., Melnikova, I.N., Keller, S.R., and Speck, N.A. (1993). Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol. Cell. Biol. *13*, 3324–3339.

Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A.H., and Speck, N.A. (1996a). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc. Natl. Acad. Sci. USA *93*, 3444–3449.

Wang, Q., Stacy, T., Miller, J.D., Lewis, A.F., Gu, T.L., Huang, X., Bushweller, J.H., Bories, J.C., Alt, F.W., Ryan, G., et al. (1996b). The CBFβ subunit is essential for CBFα2 (AML1) function in vivo. Cell *87*, 697–708.

Weissman, I.L. (2000). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science *287*, 1442–1446.

Yergeau, D.A., Hetherington, C.J., Wang, Q., Zhang, P., Sharpe, A.H., Binder, M., Marin-Padilla, M., Tenen, D.G., Speck, N.A., and Zhang, D.E. (1997). Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. Nat. Genet. *15*, 303–306.