Role of *Cbfb* in hematopoiesis and perturbations resulting from expression of the leukemogenic fusion gene *Cbfb-MYH11*

Mondira Kundu, Amy Chen, Stacie Anderson, Martha Kirby, LiPing Xu, Lucio H. Castilla, David Bodine, and Pu Paul Liu

Core-binding factor β (CBFβ) and CBFα2 form a heterodimeric transcription factor that plays an important role in hematopoiesis. The genes encoding either CBFβ or CBFα2 are involved in chromosomal rearrangements in more than 30% of cases of acute myeloid leukemia (AML), suggesting that CBFβ and CBFα2 play important roles in leukemogenesis. Inv(16)(p13; q22) is found in almost all cases of AML M4Eo and results in the fusion of *CBFB* with *MYH11*, the gene encoding smooth muscle myosin heavy chain. Mouse embryos heterozygous for a *Cbfb-MYH11* knock-in gene lack definitive hematopoiesis, a phenotype shared by *Cbfb*$^{-/-}$ embryos. In this study we generated a *Cbfb-GFP* knock-in mouse model to characterize the normal expression pattern of Cbfβ in hematopoietic cells. In midgestation embryos, Cbfβ was expressed in populations enriched for hematopoietic stem cells and progenitors. This population of stem cells and progenitors was not present in mouse embryos heterozygous for the *Cbfb-MYH11* knock-in gene. Together, these data suggest that *Cbfb-MYH11* blocks embryonic hematopoiesis at the stem-progenitor cell level and that *Cbfb* is essential for the generation of hematopoietic stem and progenitor cells. In adult mice, Cbfβ was expressed in stem and progenitor cells, as well as mature myeloid and lymphoid cells. Although it was expressed in erythroid progenitors, Cbfβ was not expressed during the terminal stages of erythropoiesis. Our data indicate that *Cbfb* is required for myeloid and lymphoid differentiation; but does not play a critical role in erythroid differentiation. (Blood. 2002;100:2449-2456)

Introduction

Core-binding factor β (CBFβ) is a transcription factor that forms heterodimeric complexes with members of the CBFα family of proteins.[1] The α subunit includes 3 family members, each encoded by a unique gene: *CBFA1 (RUNX2, AML3, PEBP2αA)*, *CBFA2 (RUNX1, AML1, PEBP2αB)*, and *CBFA3 (RUNX3, AML2, PEBP2αC)*. *CBFA1* is required for osteoblast differentiation and bone formation; *CBFA2* is required for hematopoiesis; the function of *CBFA3* is currently unknown.[2-8] The genes are related by virtue of the highly conserved Runt domain, which is responsible for binding DNA and interacting with Cbfβ.[9] CBFβ is encoded by a single gene, *CBFB*. It stabilizes the flexible C-terminal loop of the Runt domain (CBFα) that interacts with the minor groove of DNA,[10] resulting in a complex that is a more potent transcription factor than CBFα alone.[11,12]

Although CBFβ interacts with all 3 Cbfα family members in vitro, mouse models have only shown evidence for a role for Cbfβ in hematopoiesis. In mouse embryos, there are 2 stages of hematopoiesis: primitive and definitive.[13] The yolk sac is the major site for the generation of primitive hematopoietic cells, which include nucleated red blood cells and primitive macrophages. Primitive erythrocytes are found in the yolk sac beginning at 7 days postcoitus (dpc). Definitive hematopoietic cells, which give rise to mature lineages commonly found in adults, originate in the yolk sac, para-aortic splanchnopleura and in hematopoietic clusters of the aorta-gonad-mesonephros (AGM). By 11 dpc, the fetal liver becomes the major site for definitive hematopoiesis. Homozygous *Cbfb* knock-out *(Cbfb*$^{-/-}$*)* mice die during midgestation from severe hemorrhages throughout the embryo.[14,15] Definitive hematopoiesis is completely absent in these animals, but primitive hematopoiesis appears to be intact. The *Cbfb* and *Cbfa2* homozygous knock-out mice have identical phenotypes, providing genetic evidence of their interaction.[6,7]

The crucial role of the CBF complex in hematopoiesis is underscored by the observation that *CBFB* or *CBFA2* are targeted by chromosomal rearrangements in nearly 30% of individuals with acute myeloid leukemia (AML).[16] The primary chromosomal rearrangement involving *CBFB* is inv(16)(p13q22). Inv(16) is associated with almost all cases of AML subtype M4Eo and results in the fusion of *CBFB* with *MYH11*, the gene for smooth muscle myosin heavy chain.[17] Previously, we used a knock-in strategy to generate a mouse model in which *Cbfb-MYH11* is expressed under the control of the endogenous mouse *Cbfb* gene.[18] Chimeric mice derived from embryonic stem (ES) cells targeted with the knock-in *Cbfb-MYH11* gene were used to assess the leukemogenic potential of the fusion gene.[19] Although the *Cbfb-MYH11* knock-in chimeras did not develop leukemia naturally in the first year of life, most of the animals developed AML within 3 to 5 months after treatment with the chemical mutagen, *N*-ethyl-*N*-nitroso-urea (ENU). The dose of ENU used was not sufficient to induce leukemia in wild-type chimeras. The leukemia in the *Cbfb-MYH11* chimeras

From the Genetics and Molecular Biology Branch and Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, and Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester.

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Reprints: Pu Paul Liu, Room 3A18, Building 49, National Institutes of Health, 49 Convent Dr, Bethesda, MD 20892; e-mail: pliu@nhgri.nih.gov.

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was characterized by the presence of myelomonocytic blasts and occasional eosinophils, very similar to patients with AML M4Eo. These observations suggested that although expression of *Cbfb-MYH11* is not sufficient for leukemogenesis, it is a necessary event in the multistep process that gives rise to leukemias associated with inv(16).

Analysis of the contribution of ES cells with the *Cbfb-MYH11* knock-in gene in chimeric animals provided evidence that *Cbfb-MYH11* blocks differentiation of the myeloid and lymphoid cells at the level of the c-kit$^+$ progenitors, but does not affect erythroid maturation in adults.[19] Expression of the *Cbfb-MYH11* knock-in gene in heterozygous embryos results in a severe defect in definitive hematopoiesis, a phenotype similar to that observed in embryos containing homozygous knock-out of either *Cbfa2* or *Cbfb*. In vitro, the *CBFB-MYH11* gene product, CBFβ-SMMHC, sequestered CBFα2 in the cytoplasm.[20,21] It also inhibited CBFα2-mediated transactivation and has been shown to increase CBFα2-mediated repression.[21,22] Together, these data provide evidence that expression of *Cbfb-MYH11* blocks hematopoietic differentiation in a dominant-negative manner by inhibiting the normal function of CBF.

Considering the critical role of *Cbfb* in normal hematopoiesis and leukemogenesis it is important to further characterize its expression in different hematopoietic cell populations. Previous studies indicated that *Cbfb* is expressed in the central nervous system, cranial nerve and dorsal root ganglia, eyes, limb bud, somites, and ribs of mouse embryos, as assessed by in situ hybridization.[1,14] In adults, *Cbfb* expression is considered to be ubiquitous because it has been detected in most adult tissues and various cell lines by Northern blot analysis.[11,12] In this paper we characterize the expression of *Cbfb* in embryonic and adult hematopoietic tissues and dissect the specific hematopoietic defects associated with *CBFB-MYH11* expression, using a newly created *Cbfb-GFP* knock-in mouse model.

Materials and methods

Generation of *Cbfb-GFP* knock-in mice

The targeting construct was assembled in the plasmid vector, pPNT-Hygro, which includes the positive selection marker, hygromycin, expressed under control of the SV40 promoter. The vector also includes the *HSV thymidine kinase* gene expressed from the *pgk* promoter for negative selection. The 5′ arm of the targeting vector consists of a 3.5-kb (*Kpn*I-*Xho*I) fragment of gDNA that contains *Cbfb* intron 4 and the first 56 bp of exon 5. The gDNA was isolated from a 129J genomic clone, pSKA (gift from N. A. Speck, Dartmouth College, Hanover, NH). Exon 5 sequences were fused in-frame to the *Sal*I-*Xba*I fragment isolated from the enhanced green fluorescent protein (*EGFP*) gene (Clontech, Palo Alto, CA). The bovine growth hormone (BGH) polyA sequence was isolated from *pCDNA3.1* (Invitrogen, Carlsbad, CA) and inserted 3′ to *EGFP*. The 3′ arm of the targeting vector consists of a 4.7-kb (*Nhe*I-*Nhe*I) fragment of *Cbfb* intron 5 isolated from a 129J gDNA clone, pSKB (gift from N. A. Speck, Dartmouth College).

The targeting construct was linearized at a unique *Not*I site and transfected into ES cells by electroporation. Homologous recombinant clones were identified by Southern blot analysis of gDNA isolated from individual G418/FIAU-resistant ES cell colonies. The DNA was digested with either *Xba*I or *Nco*I, and the blotted DNA was hybridized with probes, one internal to the targeting DNA vector (Hygro) and one external (probe 0.2C).[18] *Nco*I digestion generates a 15.7-kb band from the wild-type *Cbfb* allele that is detected with the 0.2C probe. The correctly targeted *Cbfb-GFP* allele generates a 6.3-kb band detected with the 0.2C probe. *Xba*I digestion generates a 7.4-kb band from the targeted allele that is detected with the Hygro probe.

Genotype analysis

The presence of *Cbfb-GFP* was analyzed by polymerase chain reaction (PCR) from DNA isolated from tail biopsies or yolk sac. Fifty nanograms template DNA was amplified by PCR using primers specific for hygromycin (hygro forward 5′ CCATCGTCGAGATCCAGACATG 3′ and hygro reverse 5′ GTATATGCTCCGCATTGGTCTTG 3′). To distinguish heterozygotes from homozygotes, primers detecting the wild-type, but not the targeted allele, were used (intron 4 forward 5′ ATAAGCAGCAAATAGGTAGAGTG 3′ and mC5 reverse 5′ GACCTGTCTCTATCCTCAAATTC-3′). The PCR samples were initially denatured at 94°C for 2 minutes, followed by 30 cycles of amplification (30 seconds each at 94°C, 60°C, and 72°C), and a final extension step at 72°C. The quality of the template DNA was confirmed in parallel amplification with primers specific for the *Trp53* gene.[18]

Western blot analysis

Lysates from adult tissues or ES cells were prepared by resuspending 1×10^6 cells in NuPage lithium dodecyl sulfate (LDS) sample buffer with reducing agent (Invitrogen) and boiling the samples for 15 minutes. The proteins were separated by electrophoresis on NuPage 4% to 12% bis-tris gels in 2-N-morpholino ethane sulfonic acid (MES) running buffer and transferred onto nitrocellulose membranes using the semidry blotting system (Amersham, Piscataway, NJ). Membranes were probed with a 1:10 dilution from a monoclonal antibody specific for Cbfβ (amino acids 1-141),[14] or a 1:5000 dilution from a polyclonal antibody specific for multiple endocrine neoplasia 1 (MEN1; gift from S. C. Chandrasekharappa, National Institutes of Health, Bethesda, MD), followed by a secondary antibody conjugated to horseradish peroxidase (HRP). Enhanced chemiluminescence (ECL; Amersham) was used to detect the antibody complexes.

Ter119$^+$ and Ter119$^-$ cells were separated from adult mouse bone marrow using Ter-119 microbeads and the AutoMACS sorting system (Miltenyi Biotech, Auburn, CA). Then, 2.7×10^6 cells from each population were resuspended in LDS buffer and analyzed for Cbfβ expression by Western blot analysis.

Cell staining and flow cytometry

Peripheral blood was obtained from anesthetized animals by cardiac puncture. Bone marrow was obtained by flushing femur and tibia with fluorescence-activated cell sorter (FACS) buffer (5% fetal calf serum [FCS] in phosphate-buffered saline [PBS]), followed by trituration through a 25-gauge needle. Bone marrow, spleen, and peripheral blood samples were incubated in ACK lysing buffer (Biowhittaker, Walkersville, MD) to lyse the erythrocytes prior to staining with antibodies. Bone marrow and peripheral blood were stained with phycoerythrin (PE)-conjugated antibodies to CD3 (17A2), B220 (RA3-6B2), Mac1 (M1/70), Gr-1 (RB6-8C5), Ter119 (Ly 76), and c-kit (2B8; BD Pharmingen, San Diego, CA). Additional B-cell staining was performed using the following antibodies purchased from BD Pharmingen as described previously[23]: PE-conjugated anti-human serum albumin (HSA; M1/69), anti–CD-43 (S7); biotinylated anti-HSA (M1/69), anti–BP-1 6C3 and anti-IgM; and allophycocyanin (APC)-conjugated B220 (RA3-6B2). For staining of megakaryocytes, unlysed bone marrow was resuspended in PBS containing 5% donkey serum. Two hundred nanograms sheep anti-human platelet glycoprotein (GP) IIb-IIIa antibody (Affinity Biologicals, Hamilton, ON, Canada) was used for staining 1×10^6 cells. The secondary antibody was PE-conjugated donkey anti-sheep immunoglobulin (1:200 dilution). Cells were isolated from lymph node, thymus, and spleen of 3- to 6-month-old mice by passage through a nylon mesh. Cells were stained with PE-conjugated antibodies to CD4 (RM4-5) and Cy-chrome–conjugated anti-CD8α 53-6.7 (BD Pharmingen). Appropriate isotype controls were used in each experiment. Cells were stained for flow cytometric analysis by incubating with 0.2 μg to 1 μg antibody per 1 million cells in ice-cold FACS buffer for 30 minutes. After washing, cells were resuspended in 200 μL FACS buffer. The GFP signal was detected on FL-1 channel of FACScan (BD Biosciences, San Diego, CA) or FACSCalibur (BD Biosciences). PE was detected on FL-2, and Cy-chrome on FL-3 on the FACScan. For 4-color experiments, APC was detected on FL-7 of FACSCalibur.

Lineage depletion and cell sorting of bone marrow was performed as described previously[24] using purified antibodies to CD4, CD8, B220, Mac1, GR1, and Ter119 (Caltag Laboratories, Burlingame, CA). Biotinylated c-kit (ACK4-biotin) antibody and streptavidin-PE (BD Pharmingen) were used to stain bone marrow cells after lineage depletion. Fetal liver and AGM were dissected from 11.5- and 12.5-dpc embryos using standard techniques. The tissues were dissociated by trituration using a 25-gauge needle and passed through a nylon mesh.

Methylcellulose colony-forming assays

Adult bone marrow and 11.5-dpc fetal liver cells were washed and resuspended in Iscove modified Dulbecco medium (IMDM; Invitrogen) with 10% fetal bovine serum (FBS; Stem Cell Technologies, Vancouver, BC, Canada). Cells were incubated in 35-mm suspension dishes in IMDM containing 0.9% methylcellulose, 15% FBS, 1% bovine serum albumin, 10 μg/mL bovine pancreatic insulin, 200 μg/mL human transferring, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/mL recombinant murine stem cell factor (rmSCF), 10 ng/mL recombinant murine interleukin 3 (rmIL-3), 10 ng/mL rmIL-6, and 3 U/mL recombinant human erythropoietin (MethoCult GF M3434; Stem Cell Technologies). Colonies were visualized and counted after 10 days in culture.

Results

Generation of the *Cbfb-GFP* knock-in mouse model

We previously demonstrated that *Cbfb-MYH11* blocks differentiation of hematopoietic cells and promotes the development of AML in mice.[18,19] To elucidate the normal role of *Cbfb* in hematopoiesis, and characterize the defect caused by *Cbfb-MYH11*, we generated mice expressing Cbfβ tagged with the GFP. The knock-in targeting construct that contains *Cbfb* exon 5 (amino acids 1-151) fused in-frame to GFP cDNA is shown in Figure 1A. The fusion protein generated by this construct maintained the ability to interact with Cbfα2 in vitro and exhibited a subcellular localization pattern that was identical to wild-type Cbfβ in cultured cells (data not shown). We anticipated that Cbfβ-GFP should function normally, at least with respect to hematopoiesis, because a *Cbfb* (amino acids 1-141) expression construct can rescue the hematopoietic defect in a *Cbfb* null ES cell line.[25] Southern blot analysis demonstrated a 15% targeting efficiency and allowed identification of several correctly targeted ES cell clones exhibiting a 6.3-kb *Nco*I-digested band detected with the external probe 0.2C (Figure 1B). To verify that the targeting vector was integrated only once, we used a probe directed against the *hygromycin* gene that is unique to the targeting vector to demonstrate a single 7.4-kb band (Figure 1C). Western blot analysis demonstrated expression of both the endogenous 25-kDa Cbfβ and the 47-kDa Cbfβ-GFP fusion protein in targeted ES cells (Figure 1D). Three targeted ES cell clones (nos. 44, 52, and 74) heterozygous for the knocked-in allele were injected into C57BL/6-derived host blastocysts. Injection of ES cell clone 44 gave rise to low percentage chimeras. Chimeric male mice from ES clones 52 and 74 were crossed with 129/Sv females and passed the targeted *Cbfb-GFP* allele through the germline. All phenotypes were identical in adults and embryos derived from either of the independently targeted clones. Mice derived from both clones were used in these studies. There was no significant difference in cell number or percentage of any hematopoietic lineage in $Cbfb^{+/GFP}$ compared with wild-type adults (data not shown). The studies in adult mice were performed using heterozygous animals, whereas those in embryos were done using both heterozygous and homozygous embryos. Homozygous embryos died shortly after birth. The reason for the neonatal lethality is unclear, but apparently unrelated to hematopoiesis. The presence of functional stem/progenitor cells in $Cbfb^{GFP/GFP}$ embryos was confirmed by flow cytometric analysis and methylcellulose colony assays of stem/progenitor cells (Figure 4 and Table 2) and long-term repopulation assays using 14.5-dpc fetal liver (data not shown). The presence and normal distribution of all mature lineages was confirmed by flow cytometric analysis of 16.5-dpc fetal liver and peripheral blood smear of newborn $Cbfb^{GFP/GFP}$ pups (data not shown). These data suggest that hematopoiesis is relatively normal and does not account for the lethality of the newborn pups.

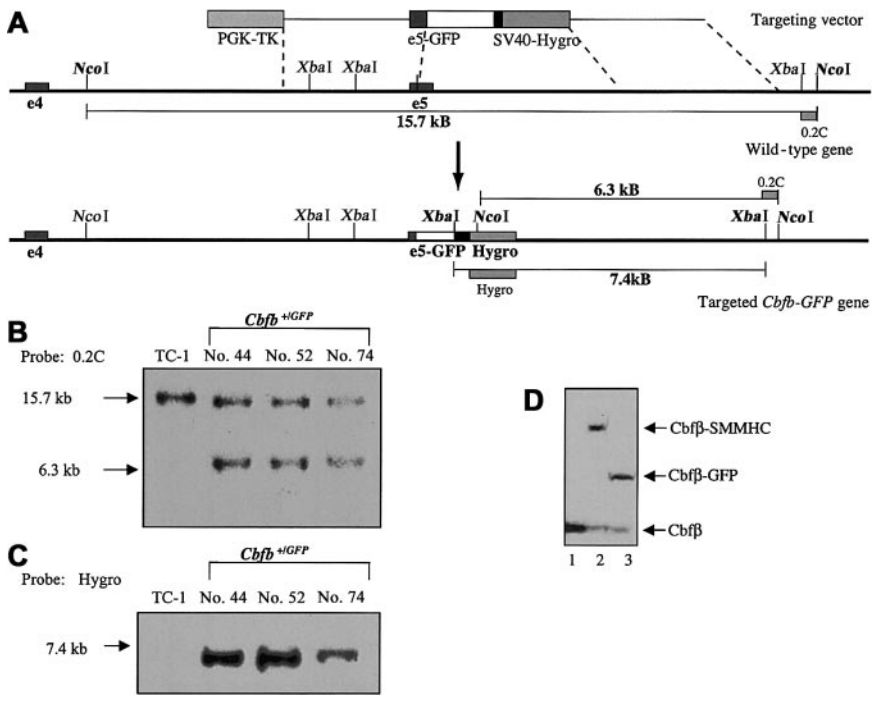

Figure 1. Generation of knock-in ES cells expressing *Cbfb-GFP*. (A) Targeting scheme used to generate $Cbfb^{+/GFP}$ ES cells. The construct contains exon 5 (e5) of *Cbfb* fused in frame to GFP. The positive selection marker is SV40-Hygro; the negative selection marker is PGK-TK. Exon 4 (e4) is in the genomic sequence 5′ to the targeting vector. Correctly targeted ES cell clones express *Cbfb-GFP* under the control of the endogenous *Cbfb* promoter. (B,C) Southern blot analysis of DNA isolated from 3 independently targeted ES cell lines. DNA was digested with either *Nco*I (B) or *Xba*I (C). The external probe (0.2C) hybridized to a 3′ genomic fragment and detected a 15.7-kb *Nco*I band from the wild-type allele and a 6.3-kb *Nco*I band from the targeted allele (B). The internal probe (Hygro) hybridized to the *hygromycin* gene and detected a single 7.4-kb band in the targeted allele (C). (D) Western blot analysis using a monoclonal antibody against Cbfβ (1-141) demonstrated expression of endogenous Cbfβ (22 kDa) or the Cbfβ fusion proteins in 3 ES cell lines. TC-1 is the wild-type ES cell line (lane 1); *Cbfb-MYH11* KI no. 55 is an ES cell clone that expresses Cbfβ-SMMHC (lane 2); *Cbfb-GFP* no. 52 is one of the correctly targeted ES cell clones expressing Cbfβ-GFP (lane 3).

Cbfβ is expressed in all of the major hematopoietic tissues in adult mice

Previous studies have suggested that *Cbfb* transcripts are expressed ubiquitously in adult mice.[11,12] To evaluate the expression of Cbfβ in various hematopoietic cell populations in adult mice, cells were harvested from several hematopoietic tissues in *Cbfb-GFP* heterozygous animals and analyzed for GFP expression by flow cytometry. FACS analysis showed a single peak of GFP-expressing cells in the thymus, lymph nodes, spleen, and peripheral blood, suggesting that most of the cells in these tissues express Cbfβ (Figure 2A). By contrast, in the bone marrow there were consistently 3 populations of nucleated cells that expressed different levels of Cbfβ-GFP, ranging from no expression to high levels of expression (Figure 2B, left panel). This was the first indication that Cbfβ may not be expressed in all hematopoietic cell populations.

Cbfβ expression is uniformly expressed in myeloid cells, but decreases during erythroid and B-lymphocyte maturation

To more closely examine the significance of the different GFP-expressing populations in the bone marrow, we analyzed Cbfβ-GFP expression in various lineages by flow cytometry. Analysis of GFP expression in monocytes and granulocytes (Mac1$^+$ or GR1$^+$ or both) in bone marrow (Figure 2B, middle panels) and peripheral blood (data not shown) revealed single peaks of GFP-expressing

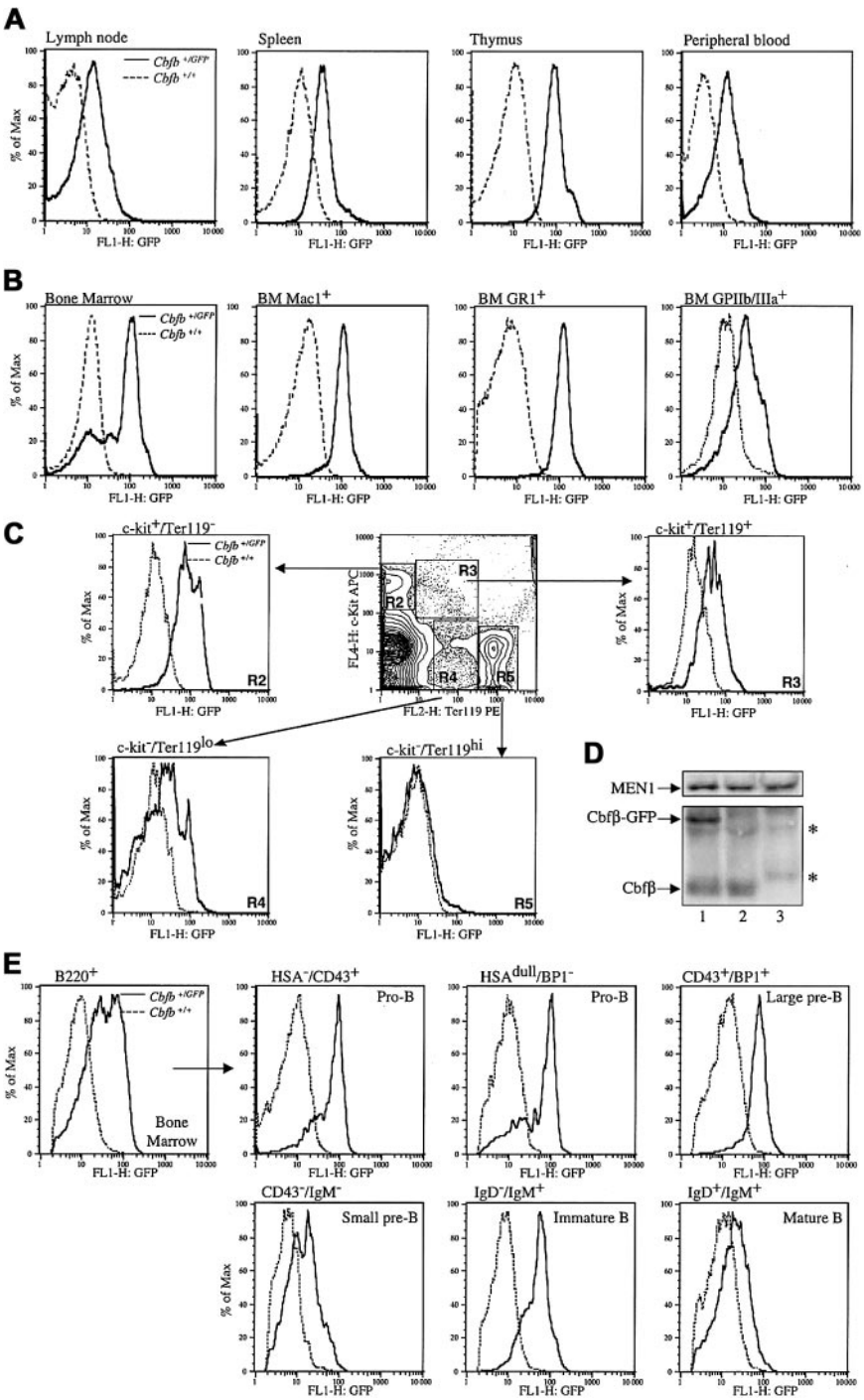

Figure 2. Cbfβ is expressed at uniform levels in most hematopoietic tissues of *Cbfb*$^{+/GFP}$ mice, but shows differential expression in hematopoietic lineages isolated from bone marrow. Cells were isolated from adult (6-month-old) *Cbfb*$^{+/GFP}$ and *Cbfb*$^{+/+}$ mice and analyzed by FACS. Representative histograms show the distribution of cells with respect to GFP fluorescence. Dashed line (– –) represents *Cbfb*$^{+/+}$ autofluorescence; solid line (—) represents fluorescence from *Cbfb*$^{+/GFP}$ animals. (A) Expression of Cbfβ-GFP in the indicated tissues. (B) Cells were isolated from bone marrow (BM) of adult *Cbfb*$^{+/+}$ and *Cbfb*$^{+/GFP}$ mice, enucleated cells were lysed, and the remaining cells were analyzed for GFP expression (left panel). Bone marrow cells were also stained with PE-conjugated antibodies against Mac1, GR1, or GPIIb/IIIa. The positively stained cells were gated and analyzed for GFP expression. (C) Cells were isolated from bone marrow of adult *Cbfb*$^{+/+}$ and *Cbfb*$^{+/GFP}$ mice, lysed in ACK lysing buffer, and stained with APC-conjugated anti–c-kit and PE-conjugated anti-TER119. A representative contour plot (c-kit–APC versus Ter119-PE) is shown. Cells from both wild-type and heterozygous mice were gated into the following populations and analyzed for GFP expression: c-kit$^+$/Ter119$^-$ (R2 = 2.3%), c-kit$^+$/Ter119$^+$ (R3 = 0.2%), c-kit$^-$/Ter119^{lo} (R4 = 0.6%), c-kit$^-$/Ter119^{hi} (R5 = 2.4%). (D) Nucleated cells from *Cbfb*$^{+/GFP}$ and *Cbfb*$^{+/+}$ bone marrow were separated into Ter119-enriched and Ter119-depleted populations by magnetic sorting using Ter119 microbeads. Cells from each population were analyzed by Western blot: Ter119-depleted cells from *Cbfb*$^{+/GFP}$ (lane 1) and *Cbfb*$^{+/+}$ (lane 2) bone marrow, and the Ter119-enriched population from *Cbfb*$^{+/+}$ bone marrow (lane 3). MEN1 indicates multiple endocrine neoplasia 1; *, nonspecific bands. (E) Cells were isolated from bone marrow of adult *Cbfb*$^{+/+}$ and *Cbfb*$^{+/GFP}$ mice and stained with APC-conjugated anti-B220 and the markers indicated above each histogram. The particular B-cell population being examined is also indicated in the upper right hand corner of the graphs. Cells from wild-type and heterozygotes were gated appropriately and analyzed for GFP expression.

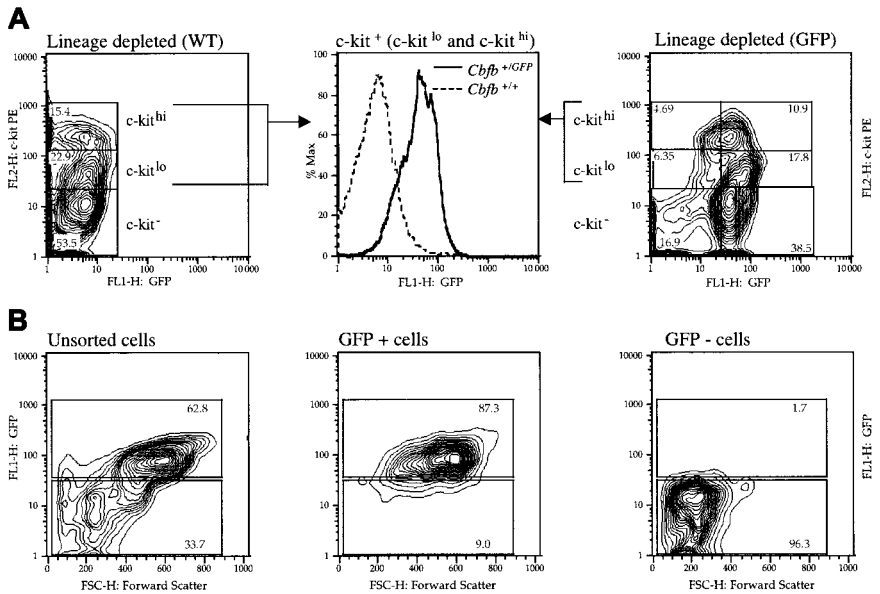

Figure 3. Cbfβ is expressed in adult hematopoietic stem cells and progenitors. (A) Cells were isolated from bone marrow of adult *Cbfb*$^{+/+}$ (left panel, WT) and *Cbfb*$^{+/GFP}$ (right panel, GFP) mice and depleted of cells expressing lineage markers (CD3, CD4, CD8, B220, Mac1, GR1, Ter119). Lin$^-$ bone marrow cells were stained for c-kit and analyzed by flow cytometry. Representative contour plots (left and right panels) show the distribution of cells with respect to GFP and c-kit PE fluorescence. The c-kit$^+$ (c-kit^{lo} and c-kit^{hi}) cells from wild-type and heterozygotes were gated and plotted on a histogram to allow comparison of the GFP fluorescence in the 2 populations (middle panel). (B) Bone marrow cells from *Cbfb*$^{+/GFP}$ adults were incubated in ACK lysis buffer to eliminate the enucleated erythrocytes and were assessed for GFP expression. The cells were sorted into GFP$^+$ and GFP$^-$ populations by FACS. Representative contour plots show forward scatter versus GFP profiles of unsorted bone marrow (left panel), and sorted populations (middle and right panels). Progenitors in all 3 populations (unsorted heterozygous bone marrow, GFP$^+$, and GFP$^-$) were assessed by methylcellulose colony assay. The results are shown in Table 1.

cells, indicating uniform expression of Cbfβ-GFP. Megakaryocytes (GP IIb-IIIa$^+$) also expressed a uniform level of Cbfβ-GFP (Figure 2B, right panel). Nucleated Ter119$^+$ erythroblasts in the bone marrow did not express Cbfβ-GFP. However, as shown in Figure 2C, as erythroid cells matured from c-kit$^+$ progenitors (R2) to Ter119^{hi} erythroblasts (R5), there was a progressive loss of Cbfβ-GFP expression. The majority of Ter119$^+$ cells in the bone marrow did not express Cbfβ-GFP. We confirmed that the Cbfβ-GFP signal was representative of the normal distribution of Cbfβ by examining endogenous Cbfβ expression in Ter119-enriched and Ter119-depleted populations by Western blot analysis (Figure 2D, lanes 2 and 3). In a population that contained approximately 90% Ter119$^+$ cells, we were unable to detect endogenous Cbfβ by Western blot analysis. By contrast, there was abundant Cbfβ expression in the Ter119$^-$ population, as predicted by FACS analysis. In addition, the levels of wild-type Cbfβ and Cbfβ-GFP were comparable in the Ter119-depleted population from adult *Cbfb*$^{+/GFP}$ bone marrow as assessed by Western blot analysis (Figure 2D, lane 1).

The analysis of GFP expression in B220$^+$ B lymphocytes in the bone marrow revealed 2 populations (Figure 2E, left panel). The various stages of B-cell differentiation in the bone marrow and corresponding markers are reviewed by Hendriks et al.[23] Figure 2E demonstrates that Cbfβ was expressed at high levels in pro-B (B220$^+$/HSA$^-$/CD43$^+$; B220$^+$/HAS^{dull}/BP1$^-$) and large pre-B cells (B220$^+$/CD43$^+$/BP1$^+$), and decreased in small pre-B cells (B220$^+$/CD43$^-$/IgM$^-$). Mature B cells (B220$^+$/IgM$^+$/IgD$^+$) in bone marrow (Figure 2E) and spleen (data not shown) expressed only low levels of Cbfβ, whereas B220$^+$ cells in peripheral blood expressed slightly higher levels (data not shown).

The number and percentage of CD4/8 T cells were normal in the thymus, lymph nodes, and spleen of heterozygote *Cbfb-GFP* animals, as was the percentage of CD3$^+$ T lymphocytes in the peripheral blood (data not shown). All of the populations expressed uniform levels of GFP, suggesting that T lymphocytes express Cbfβ-GFP (data not shown).

Cbfβ is expressed in hematopoietic stem cells and progenitors

Because the absence of definitive hematopoiesis in the fetal livers of *Cbfb* homozygous knock-out embryos suggests an early defect in hematopoietic differentiation, we wanted to determine whether or not Cbfβ is expressed in hematopoietic stem cells and progenitors. Previous studies have demonstrated that the lineage-negative (Lin$^-$) c-kit^{hi} population of cells in adult mice is significantly enriched for stem cells that can support long-term repopulation of lethally irradiated animals, whereas the Lin$^-$/c-kit^{lo} population contains only hematopoietic progenitors.[24] *Cbfb*$^{+/GFP}$ mice had comparable numbers of Lin$^-$ cells as wild-type animals. Lineage depletion enriched for GFP$^+$ cells as evidenced by the increased ratio of GFP$^+$ to GFP$^-$ cells in the Lin$^-$ population (3:1) compared to that in total bone marrow (2:1; Figure 3A). Closer examination revealed that the entire population of Lin$^-$/c-kit^{hi} and Lin$^-$/c-kit^{lo} cells expressed Cbfβ-GFP (Figure 3A, right panel). This suggests that a population enriched for long-term repopulating hematopoietic stem cells and hematopoietic progenitors expresses Cbfβ. A methylcellulose colony assay was used as an additional method of examining the expression of Cbfβ-GFP in progenitors. Bone marrow cells from heterozygous animals were sorted into GFP$^+$ and GFP$^-$ populations (Figure 3B). Equal numbers of cells (5×10^4) from each population were plated in methylcellulose cultures containing SCF, IL-3, IL-6, and erythropoietin. There was a more than 10-fold enrichment in erythroid burst-forming units (BFU-Es), granulocyte-macrophage colony-forming units (CFU-GMs) and granulocyte-erythrocyte-macrophage colony-forming units (CFU-GEMs) in the GFP$^+$ population compared with the GFP$^-$ population, suggesting that most, if not all, of the hematopoietic progenitor cells express Cbfβ (Table 1). It is interesting to note that the greatest enrichment was observed in the CFU-GEMs, which originate from a more immature progenitor that gives rise to both erythroid and myeloid cells.

Table 1. Methylcellulose colony assay using adult bone marrow

Cell population	BFU-E (erythroid)	CFU-GM (granulocyte/macrophage)	CFU-GEM (mixed)
Unsorted bone marrow	16.7 ± 2.4	68 ± 11.8	10 ± 4.1
GFP$^+$	35 ± 10.8	125 ± 10.8	28.3 ± 6.2
GFP$^-$	3.3 ± 2.4	5 ± 4.1	0

Progenitors in all 3 populations (unsorted heterozygous bone marrow, GFP$^+$, and GFP$^-$) shown in Figure 3B were assessed by methylcellulose colony assay. The table shows the mean and SD of the data collected from 3 independent experiments (n = 3); 5×10^4 cells from each population were plated in each culture.

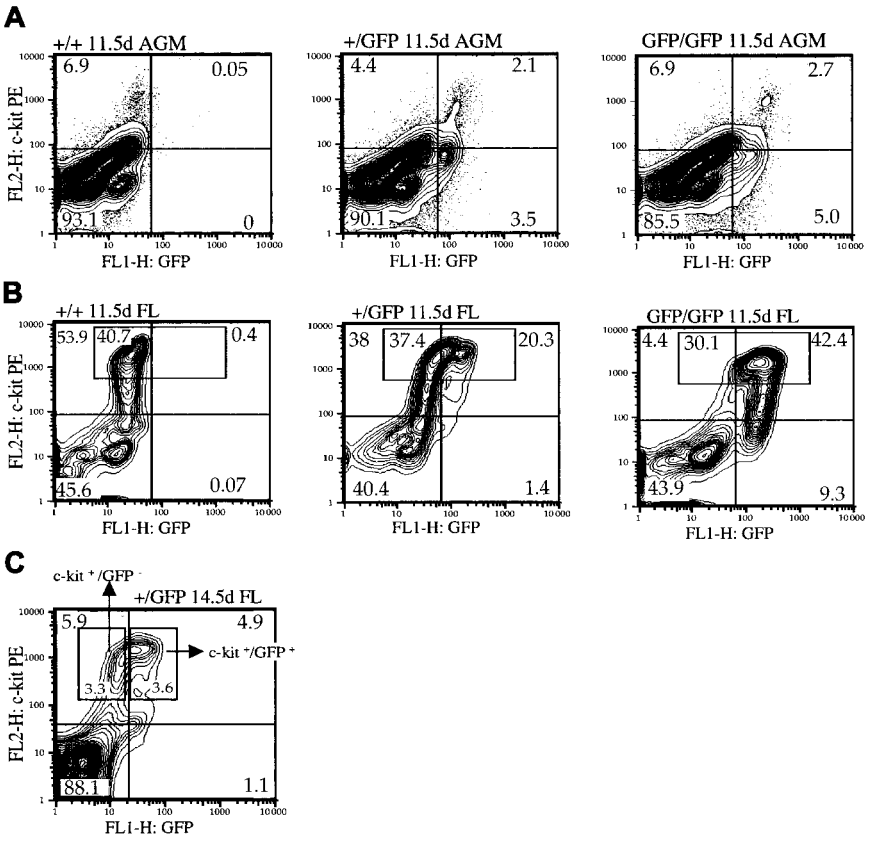

Figure 4. Cbfβ is expressed in c-kit^{hi} cells in AGM and fetal liver embryos. Cells were isolated from 11.5-dpc AGM (A) and fetal liver (B) of *Cbfb*$^{+/+}$ (+/+), *Cbfb*$^{+/GFP}$ (+/GFP), and *Cbfb*$^{GFP/GFP}$ (GFP/GFP) embryos and stained with c-kit PE. Representative contour plots show the distribution of cells with respect to GFP and c-kit fluorescence. (C) Cells were isolated from fetal liver of 14.5 dpc *Cbfb*$^{+/GFP}$ embryos and sorted into c-kit^{+}/GFP^{-} and c-kit^{+}/GFP^{+} populations by FACS. Progenitors in each of these populations (sorted and unsorted) were assessed by methylcellulose colony assay. The data from the methylcellulose colony assays are shown in Table 2.

Cbfβ is expressed in the c-kit^{hi} cells in the embryonic sites of definitive hematopoiesis

To examine the expression of Cbfβ in embryonic hematopoietic cells, we dissected the major sites of hematopoiesis including the AGM, fetal liver, and yolk sac from 11.5-dpc embryos. The GFP signal in wild-type yolk sac cells was indistinguishable from heterozygous and homozygous embryos (data not shown). In the AGM and fetal liver, c-kit marks the hematopoietic stem-progenitor cells. The c-kit^{hi} cells in the AGM at 11.5 dpc comprise 1% to 2% of cells in the AGM, and all of them expressed Cbfβ-GFP (Figure 4A). In the fetal liver, the c-kit^{hi} cells included 30% to 40% of the cells, and again, all expressed Cbfβ-GFP, although in heterozygous animals, the distinction between GFP^{+} and GFP^{-} was not as clear as in the homozygous animals (Figure 4B). Nevertheless, sorting the c-kit^{hi} cells from a heterozygous embryo into GFP^{+} and GFP^{-} populations (Figure 4C) resulted in a significant enrichment of erythroid (6- to 7-fold), myeloid (3- to 4-fold), and mixed (4- to 5-fold) CFUs, suggesting that myeloid and erythroid progenitor cells express high amounts of Cbfβ (Table 2).

There was no significant difference in the percentage of c-kit^{hi} cells in the fetal liver and AGM of wild-type, heterozygous, and homozygous embryos (Figure 4A,B) nor was there any significant difference in the colony-forming potential of the fetal livers isolated from these animals (Table 2), suggesting that the hematopoietic stem cells and progenitors in homozygous embryos are intact.

The c-kit^{hi} population of cells is absent from AGM and fetal liver of embryos expressing *Cbfb-MYH11*

Previous studies revealed that heterozygous (*Cbfb*$^{+/MYH11}$) embryos expressing *Cbfb-MYH11* exhibited a complete absence of definitive hematopoiesis in the fetal liver. To further characterize the defect in these embryos, we examined the expression of c-kit and Cbfβ-GFP in the *Cbfb*$^{+/MYH11}$ embryos. In the fetal liver at 11.5 dpc, we found a complete absence of the c-kit^{hi} (CD34^{+} and CD34^{-}) population suggesting that expression of *Cbfb-MYH11* prevented the formation or migration of the stem-progenitor cells (Figure 5A,B). There were very few cells expressing Cbfβ-GFP in the *Cbfb*$^{GFP/MYH11}$ embryos, confirming the absence of cells expressing *Cbfb* (and presumably *Cbfb-MYH11*). In the AGM, the c-kit^{hi} population represents the cells in the hematopoietic clusters that give rise to the hematopoietic stem cells and progenitors.[26] This population of cells was also absent from embryos expressing *Cbfb-MYH11* (Figure 5C), suggesting that the defect occurs very early in hematopoietic differentiation, prior to migration of hematopoietic stem cells and progenitors from the AGM to the fetal liver.

Table 2. Methylcellulose colony assay using 14.5-dpc fetal liver

Cell population	BFU-E (erythroid)	CFU-GM (granulocyte/macrophage)	CFU-GEM (mixed)
Unsorted fetal liver			
Cbfb$^{+/+}$	5.0 ± 1.0	21.5 ± 1.5	2.0 ± 0
Cbfb$^{+/GFP}$	4.5 ± 0.5	25.0 ± 0	3.0 ± 1.0
Cbfb$^{GFP/GFP}$	3.5 ± 0.5	19.0 ± 1.0	3.5 ± 0.5
Sorted *Cbfb*$^{+/GFP}$ fetal liver			
c-kit^{+}/GFP^{+}	42.0 ± 11.1	153.0 ± 28.2	26.2 ± 6.7
c-kit^{+}/GFP^{-}	6.3 ± 2.9	43.3 ± 3.1	6.0 ± 3.6

Cells were isolated from fetal liver of 14.5-dpc *Cbfb*$^{+/+}$, *Cbfb*$^{+/GFP}$, and *Cbfb*$^{GFP/GFP}$ embryos. Cells from *Cbfb*$^{+/GFP}$ embryos were sorted into c-kit^{+}/GFP^{+} and c-kit^{+}/GFP^{-} populations by FACS (Figure 4C). Progenitors in each of these populations (sorted and unsorted) were assessed by methylcellulose colony assay. The table shows the average data collected from cultures of unsorted cells (from *Cbfb*$^{+/+}$, *Cbfb*$^{+/GFP}$, *Cbfb*$^{GFP/GFP}$) and sorted cells (n = 3); 1×10^4 fetal liver cells were plated in each culture.

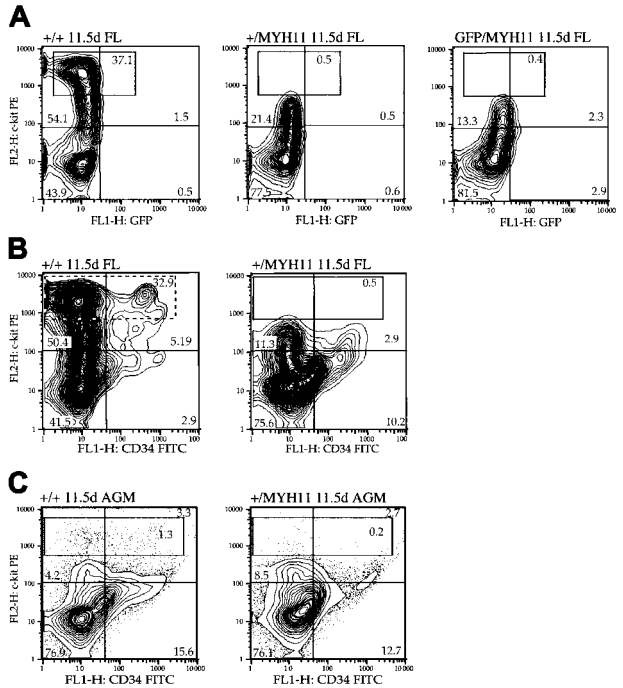

Figure 5. The c-kit^{hi} population is absent from the fetal liver and AGM of embryos heterozygous for the knock-in *Cbfb-MYH11* (*Cbfb*$^{+/MYH11}$). (A) Cells were isolated from 11.5-dpc fetal liver of *Cbfb*$^{+/+}$ (+/+), *Cbfb*$^{+/MYH11}$ (+/MYH11), and *Cbfb*$^{GFP/MYH11}$ (GFP/MYH11) embryos and stained with anti–c-kit PE. Representative contour plots show the distribution of cells with respect to GFP and c-kit fluorescence. Cells were isolated from (B) fetal liver and (C) AGM of *Cbfb*$^{+/+}$, and *Cbfb*$^{+/MYH11}$ 11.5-dpc embryos and stained with PE-conjugated anti–c-kit and FITC-conjugated anti-CD34. Representative contour plots show the distribution of cells with respect to FITC and c-kit fluorescence.

Discussion

The importance of *Cbfb* in hematopoiesis and leukemogenesis prompted us to investigate the expression pattern of Cbfβ in hematopoietic cells. Analysis of hematopoietic cells is simplified due to the ease of analysis by flow cytometry and the extensive array of well-established cell surface markers available for characterization. To take advantage of this feature of hematopoietic cells, we developed a knock-in mouse model in which *Cbfb* expression is marked by GFP, which is easily detected by FACS. To preserve the normal function of *Cbfb*, while tagging it with GFP, exon 5 of *Cbfb* was fused in-frame to GFP. Previous studies using in vitro differentiation of ES cells demonstrated that amino acids 1-141 (exon 1-4 plus 8 amino acids of exon 5) are sufficient to rescue the defect in definitive myeloid and erythroid differentiation in vitro in cells lacking *Cbfb*, suggesting that most of exon 5 and all of exon 6 are dispensable for the normal function of *Cbfb* in hematopoiesis.[25] Because the *Cbfb*$^{GFP/GFP}$ embryos have no apparent defect in hematopoiesis, it appears that Cbfβ-GFP is able to function in a manner similar to endogenous Cbfβ. However, the early lethality of homozygous pups suggests that in other tissues the function of Cbfβ may be partially disrupted by fusion with GFP.

In this study, adult *Cbfb-GFP* heterozygotes were used to analyze the expression of Cbfβ in various populations of hematopoietic cells. Our data, especially the comparable expression pattern and levels of Cbfβ and Cbfβ-GFP in Ter119$^+$ and Ter119$^-$ cells by FACS and Western blot, suggest that analysis of Cbfβ-GFP by FACS provides an accurate reflection of endogenous Cbfβ expression. We cannot, however, rule out the possibility that there is a difference in the half-life of the proteins, which may influence interpretation of our FACS results. With this potential caveat in mind, we found that Cbfβ is expressed in hematopoietic stem cells and progenitors, megakaryocytes, and in mature myeloid and lymphoid cells. Cbfβ is expressed in all myeloid cells and T lymphocytes, but exhibits a biphasic expression pattern in B lymphocytes. In adult bone marrow, the pro-B and large pre-B cells express more Cbfβ than the small pre-B and mature B cells. These results suggest that although a low level of Cbfβ expression is maintained in all adult B cells, its expression decreases as B lymphocytes differentiate. In adult chimeric animals, ES cells targeted with the dominant-negative *Cbfb-MYH11* gene contribute to the population of cells containing erythroid and myeloid progenitors, but do not contribute to differentiated myeloid and lymphoid cells, suggesting that *Cbfb-MYH11* blocks hematopoiesis at the level or upstream of the c-kit$^+$ progenitors. Together, our results suggest that *Cbfb* is required for early steps of hematopoietic differentiation. The continued expression of Cbfβ in mature myeloid and lymphoid cells suggests that it may also be required for later stages of myeloid and lymphoid differentiation.

The importance of Cbfα2 and Cbfβ in megakaryocyte development has been suspected because of the linkage between heterozygous mutations in the *CBFA2* gene and a human disease that is characterized by thrombocytopenia.[27] The observation that Cbfβ-GFP is expressed in megakaryocytes, however, is the first evidence that Cbfβ may play a direct role in megakaryocyte development.

The only hematopoietic cells that do not express Cbfβ are erythroid cells starting from the c-kit$^-$/Ter119$^+$ erythroblast stage. Cbfβ is expressed in the erythroid progenitors that give rise to BFU-Es in methylcellulose colony assays and in c-kit$^+$/Ter119$^+$ cells, but not in c-kit$^-$/Ter119$^+$ erythroblasts and enucleated red cells. A previous study demonstrated the absence of any Runt domain–containing proteins in Ter119$^+$ cells by Western blot analysis.[28] Together, these results demonstrate that expression of the CBF complex decreases during erythroid maturation and suggest that CBF is not required for terminal differentiation of erythroid cells. Even in c-kit$^+$/Ter119$^+$ progenitors, CBF function is probably not critical: *Cbfb-MYH11*–targeted ES cells contribute to the c-kit$^+$/Ter119$^+$, c-kit$^-$/Ter119$^+$, and terminally differentiated erythrocyte populations in chimeric animals.[19] Because *Cbfb-MYH11* functions in a dominant-negative manner, the CBF complex is probably not required for differentiation of erythroid cells at the c-kit$^+$/Ter119$^+$ stage.

In heterozygous embryos expressing knocked-in *Cbfb-MYH11*, histologic analysis of fetal liver prior to death of the embryos by hemorrhaging revealed an absence of definitive hematopoiesis. In vitro differentiation of fetal liver from these animals resulted in a 30- to 100-fold reduction in the number of myeloid and erythroid colonies.[18] In this study, we demonstrated that the entire population of c-kit^{hi} hematopoietic stem cells and progenitors in the AGM and fetal liver expresses Cbfβ and that both of these populations are absent in heterozygous embryos expressing *Cbfb-MYH11*. The c-kit^{hi} cells in the AGM have been shown to express *Cbfa2* and form intra-aortic hematopoietic clusters, which contain the hematopoietic stem cells that are capable of repopulating lethally irradiated recipients long-term. The absence of these cells in *Cbfb-MYH11* heterozygotes suggests that the defect in hematopoiesis occurs at the level of the hematopoietic stem cell. A similar defect is observed in *Cbfa2*$^{-/-}$ embryos, which appear to lack the c-kit$^+$ (and Cbfα$_2^+$) hematopoietic clusters.[26] In adult *Cbfb-MYH11* chimeras, it appears that at least some hematopoietic stem cells are able to survive, perhaps as a result of the microenvironment provided by

the normal cells. These cells, which are arrested early in myeloid differentiation, can then be targeted by additional mutations and give rise to leukemia.[19]

This study provides a detailed analysis of Cbfβ expression in hematopoietic cells from stem cells and progenitors to mature cells of all lineages. In addition to providing supporting evidence of a role for Cbfβ in the development of hematopoietic stem cells and progenitors in adults and during embryogenesis, it provides the first evidence of a role for Cbfβ in later stages of myeloid and lymphoid differentiation, and in megakaryocytes. Flow cytometric assays have allowed us to isolate small populations of cells and detect variations in Cbfβ-GFP expression through maturation of different lineages, as observed in erythroid cells and B cells. The *Cbfb-GFP* ES cells and animals presented in this study should continue to provide a valuable resource for furthering our knowledge of *Cbfb* expression and function in hematopoiesis as well as other organ systems.

Note added in proof. Two articles recently described mouse Runx 3 (Cbfa3) knock-out models.[29,30] The data showed that Runx 3 may regulate proliferation and apoptosis of gastric epithelial cells, and may also act as a tumor suppressor in human gastric cancer. In addition, Runx 3 plays a critical role in the development of neurons in the cranial and dorsal root ganglia.

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