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

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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<sup>-/-</sup>* 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 CBFβ 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 CBFβ is required for myeloid and lymphoid differentiation; but does not play a critical role in erythroid differentiation. (Blood. 2002;100:2449-2456)

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**Introduction**

Core-binding factor β (CBFβ) is a transcription factor that forms heterodimeric complexes with members of the CBFα family of proteins. 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. The genes are related by virtue of the highly conserved Runt domain, which is responsible for binding DNA and interacting with Cbfβ. 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, resulting in a complex that is a more potent transcription factor than CBFα alone.

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. 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<sup>-/-</sup>* ) mice die during midgestation from severe hemorrhages throughout the embryo. Definitive hematopoiesis is completely absent in these animals, but primitive hematopoiesis appears to be intact. The *Cbfb* and *Cbfα2* homozygous knock-out mice have identical phenotypes, providing genetic evidence of their interaction.

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). 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. 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. 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. 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
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. 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 Cbf2a or Cbf2b. In vitro, the CBFβ-MYH11 gene product, CBFβ-SMMHC, sequestered CBFβ2 in the cytoplasm. It also inhibited CBFβ2-mediated transactivation and has been shown to increase CBFβ2-mediated repression. 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 Cbf2b in normal hematopoiesis and leukemogenesis it is important to further characterize its expression in different hematopoietic cell populations. Previous studies indicated that Cbf2b is expressed in the central nervous system, cranial nerve and dorsal root ganglia, eyes, limb bud, knock-in mouse model.

Cbf2b-GFP studies indicated that fibronectin expression is considered to be controlled by the SV40 promoter. The vector also includes the arm of the targeting vector consists of a 3.5-kb (KpnI-XhoI) fragment isolated from the enhanced green fluorescence (EGFP) gene (Clontech, Palo Alto, CA). The bovine growth hormone (BGH) polyA sequence was isolated from pCDNA3.1 (Invitrogen, Carlsbad, CA) and inserted 3- bp downstream to the NotI site for the SfiI-XhoI 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 (Nhel-Nhel) fragment of Cbf2b intron 5 isolated from a 289-g DNA clone, pSKA (gift from N. A. Speck, Dartmouth College).

The targeting construct was linearized at a unique NotI site and transfected into ES cells by electroporation. Homologous recombinant clones were identified by Southern blot analysis of gDNA isolated from individual G418/FUdR-resistant ES cell colonies. The DNA was digested with either XhoI or NcoI, and the blotted DNA was hybridized with probes, one internal to the targeting DNA vector (Hygro) and one external (probe 0.2C). NcoI digestion generates a 15.7-kb band from the wild-type Cbf2b allele that is detected with the 0.2C probe. The correctly targeted Cbf2b-GFP allele generates a 6.3-kb band detected with the 0.2C probe. XhoI digestion generates a 7.4-kb band from the targeted allele that is detected with the Hygro probe.

**Genotype analysis**

The presence of Cbf2b-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 (hyg forward 5’ CACACGTGAGATCCAGACATG 3’ and hygro reverse 5’ GTGATGCTCCGATTGTGCTTG 3’). To distinguish homozygotes from hemizygotes, primers detecting the wild-type, but not the targeted allele, were used (intron 4 forward 5’ ATAAGCAGCAAAATAGG- TAGAGTG 3’ and mC5 reverse 5’ GACCTGTCTTATCCTCTAAA- TTC-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 Tie2 gene.

**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 semidy blotting system (Amersham, Piscataway, NJ). Membranes were probed with a 1:10 dilution from a monoclonal antibody specific for Cbf2b (amino acids 1-141), or a 1:5000 dilution from a polyclonal antibody specific for multiple endocrine neoplasia 1 (MEN1; gift from S. C. Chandrasekhara, 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.

**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: PE-conjugated anti-human serum albumin (HSA; M1/69), anti-CD-43 (S7); biotinylated anti-HSA (M1/69), anti-β1-6C3 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 Biologics, 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 (RM-4-5) and Cy-chrome-conjugated anti-CD8a 55-6.7 (BD Pharmingen). Appropriate isotype controls were used in each experiment. Cells were stained for flow cytometric analysis by incubating the cells for 30 minutes. After washing, cells were resuspended in 200 µL FACS buffer. The FGF 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 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. Fetial 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 with 10% fetal bovine serum (FBS; Stem Cell Technologies, Vancouver, BC, Canada). Cells were incubated in 35-mm suspension dishes in IMDM with 10% fetal bovine serum (FBS; Stem Cell Technologies, Vancouver, BC, Canada). Cells were incubated in 35-mm suspension dishes in IMDM with 10% fetal bovine serum (FBS; Stem Cell Technologies, Vancouver, BC, Canada). Cells were incubated in 35-mm suspension dishes in IMDM with 10% fetal bovine serum (FBS; Stem Cell Technologies, Vancouver, BC, Canada). Cells were incubated in 35-mm suspension dishes in IMDM with 10% fetal bovine serum (FBS; Stem Cell Technologies, Vancouver, BC, Canada).

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**Results**

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

We previously demonstrated that Cbfβ-MYH11 blocks differentiation of hematopoietic cells and promotes the development of AML in mice. To elucidate the normal role of Cbfβ in hematopoiesis, and characterize the defect caused by Cbfβ-MYH11, we generated mice expressing Cbfβ tagged with the GFP. The knock-in targeting construct that contains Cbfβ 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 Cbfβ2 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 Cbfβ (amino acids 1-141) expression construct can rescue the hematopoietic defect in a Cbfβ null ES cell line. Southern blot analysis demonstrated a 15% targeting efficiency and allowed identification of several correctly targeted ES cell clones exhibiting a 6.3-kb NcoI-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 Cbfβ-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 Cbfβ-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 Cbfβ-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 Cbfβ-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 Cbfβ-GFP.](image)

(A) Targeting scheme used to generate Cbfβ-GFP ES cells. The construct contains exon 5 (e5) of Cbfβ fused in frame to GFP. The positive selection marker is SV40-Tk. Exon 4 (e4) is in the genomic sequence 5’ to the targeting vector. Correctly targeted ES cell clones express Cbfβ-GFP under the control of the endogenous Cbfβ promoter. (B) Southern blot analysis of DNA isolated from 3 independently targeted ES cell lines. DNA was digested with either NcoI (B) or XbaI (C). The external probe (0.2C) hybridized to a 3’ genomic fragment and detected a 15.7-kb NcoI band from the wild-type allele and a 6.3-kb NcoI 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), Cbfβ-MYH11 KI no. 55 is an ES cell clone that expresses Cbfβ-MYMHC (lane 2), Cbfβ-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 Cbfβ transcripts are expressed ubiquitously in adult mice. To evaluate the expression of Cbfβ in various hematopoietic cell populations in adult mice, cells were harvested from several hematopoietic tissues in Cbfβ-GFP heterozygous animals and analyzed 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...
Cbfβ is expressed in hematopoietic stem cells and progenitors

Because the absence of definitive hematopoiesis in the fetal livers of Cbfβ 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-kithi 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-kitlo population contains only hematopoietic progenitors. Cbfβ+/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-kithi and Lin−/c-kitlo 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 × 10⁶) 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**

<table>
<thead>
<tr>
<th>Cell population</th>
<th>BFU-E (erythroid)</th>
<th>CFU-GM (granulocyte/macrophage)</th>
<th>CFU-GEM (mixed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted bone marrow</td>
<td>16.7 ± 2.4</td>
<td>68 ± 11.8</td>
<td>10 ± 4.1</td>
</tr>
<tr>
<td>GFP+</td>
<td>35 ± 10.8</td>
<td>125 ± 10.8</td>
<td>28.3 ± 6.2</td>
</tr>
<tr>
<td>GFP−</td>
<td>3.3 ± 2.4</td>
<td>5 ± 4.1</td>
<td>0</td>
</tr>
</tbody>
</table>

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 × 10⁶ cells from each population were plated in each culture.
Cbfβ is expressed in the c-kit<sup>hi</sup> 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). The GFP expression in the AGM at 11.5 dpc was found to be 1% to 2% of cells in the AGM, and all of them expressed Cbfβ-GFP (Table 2). In the fetal liver, the c-kit<sup>hi</sup> cells comprised 1% to 2% of cells in the AGM, and all of them expressed Cbfβ-GFP (Figure 4A). In the fetal liver, the c-kit<sup>hi</sup> cells included 30% to 40% of the cells, and all expressed Cbfβ-GFP, although in heterozygous animals, the distinction between GFP<sup>+</sup> and GFP<sup>-</sup> was not as clear as in the homozygous animals (Figure 4B).

Nevertheless, sorting the c-kit<sup>hi</sup> cells from a heterozygous embryo into GFP<sup>+</sup> and GFP<sup>-</sup> 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<sup>hi</sup> cells in the fetal liver and AGM of wild-type, heterozygous, and homozygous embryos (Figure 4A,B). There were very few cells expressing Cbfβ-GFP in the Cbfβ<sup>GFP/MIT11</sup> embryos, confirming the absence of cells expressing Cbfβ (and presumably Cbfβ-MYH11). In the AGM, the c-kit<sup>hi</sup> population represents the cells in the hematopoietic clusters that give rise to the hematopoietic stem cells and progenitors. This population of cells was also absent from embryos expressing Cbfβ-MYH11 (Figure 4C), 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

<table>
<thead>
<tr>
<th>Cell population</th>
<th>BFU-E (erythroid)</th>
<th>CFU-GM (granulocyte/macrophage)</th>
<th>CFU-GEM (mixed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted fetal liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cbfβ&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>5.0 ± 1.0</td>
<td>21.5 ± 1.5</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Cbfβ&lt;sup&gt;+/GFP&lt;/sup&gt;</td>
<td>4.5 ± 0.5</td>
<td>25.0 ± 0.0</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>Cbfβ&lt;sup&gt;GFP/GFP&lt;/sup&gt;</td>
<td>3.5 ± 0.5</td>
<td>19.0 ± 1.0</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>Sorted Cbfβ&lt;sup&gt;+/GFP&lt;/sup&gt; fetal liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-kit&lt;sup&gt;+/GFP&lt;/sup&gt;</td>
<td>42.0 ± 11.1</td>
<td>153.0 ± 28.2</td>
<td>26.2 ± 6.7</td>
</tr>
<tr>
<td>c-kit&lt;sup&gt;+/GFP&lt;/sup&gt;</td>
<td>6.3 ± 2.9</td>
<td>43.3 ± 3.1</td>
<td>6.0 ± 3.6</td>
</tr>
</tbody>
</table>

Cells were isolated from fetal liver of 14.5-dpc Cbfβ<sup>+/+</sup>, Cbfβ<sup>+/GFP</sup>, and Cbfβ<sup>GFP/GFP</sup> embryos. Cells from Cbfβ<sup>+/GFP</sup> embryos were sorted into c-kit<sup>+/GFP</sup> and c-kit<sup>-/GFP</sup> 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 Cbfβ<sup>+/+</sup>, Cbfβ<sup>+/GFP</sup>, Cbfβ<sup>GFP/GFP</sup>) and sorted cells (n = 3); 1 × 10<sup>5</sup> fetal liver cells were plated in each culture.
Discussion

The importance of Cbfβ 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 Cbfβ expression is marked by GFP, which is easily detected by FACS. To preserve the normal function of Cbfβ, while tagging it with GFP, exon 5 of Cbfβ 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 Cbfβ, suggesting that most of exon 5 and all of exon 6 are dispensable for the normal function of Cbfβ in hematopoiesis.25

Because the Cbfβ–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 Cbfβ–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. However, 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 Cbfβ-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 Cbfβ-MYH11 blocks hematopoiesis at the level or upstream of the c-kit+ progenitors. Together, our results suggest that Cbfβ 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 CBA2 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 Runx 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: Cbfβ-MYH11–targeted ES cells contribute to the c-kit+/Ter119+, c-kit+/Ter119+, and terminally differentiated erythrocyte populations in chimeric animals.19 Because Cbfβ-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 Cbfβ-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.23 In this study, we demonstrated that the entire population of c-kit+ 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 Cbfβ-MYH11. The c-kit+ 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 Cbfβ-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 Cbfa2+) hematopoietic clusters.26 In adult Cbfβ-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.

This study provides a detailed analysis of Cbβ 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 Cbβ in the development of hematopoietic stem cells and progenitors in adults and during embryogenesis, it provides the first evidence of a role for Cbβ 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 Cbβ-GFP expression through maturation of different lineages, as observed in erythroid cells and B cells. The Cbβ-GFP ES cells and animals presented in this study should continue to provide a valuable resource for furthering our knowledge of Cbβ expression and function in hematopoiesis as well as other organ systems.

Note added in proof. Two articles recently described mouse Runx3 (Cbfα3) knock-out models. The data showed that Runx3 may regulate proliferation and apoptosis of gastric epithelial cells, and may also act as a tumor suppressor in human gastric cancer. In addition, Runx3 plays a critical role in the development of neurons in the cranial and dorsal root ganglia.

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References

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

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