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

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ABSTRACT

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 over 30% of cases of acute myeloid leukemia, 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 mid-gestation 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 indicates that Cbfβ is required for myeloid and lymphoid differentiation; but does not play a critical role in erythroid differentiation.

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INTRODUCTION

CBFβ is a transcription factor that forms heterodimeric complexes with members of the CBFα family of proteins. The α-subunit includes three 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 three Cbfα family members in vitro, mouse models have only shown evidence for a role for Cbfβ in hematopoiesis. In mouse embryos, there are two 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 post-coitus (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 Chfb knock-out (Chfb−/−) mice die during mid-gestation from severe hemorrhages throughout the embryo. Definitive hematopoiesis is completely absent.
in these animals, but primitive hematopoiesis appears to be intact. The \textit{Cbfb} and \textit{Cbfa2} homozygous knock-out mice have identical phenotypes, providing genetic evidence of their interaction\textsuperscript{6,7}.

The crucial role of the CBF complex in hematopoiesis is underscored by the observation that \textit{CBFB} or \textit{CBFA2} are targeted by chromosomal rearrangements in nearly 30\% of acute myeloid leukemia (AML)\textsuperscript{16}. The primary chromosomal rearrangement involving \textit{CBFB} is inv(16)(p13q22). Inv(16) is associated with almost all cases of AML subtype M4 Eo and results in the fusion of \textit{CBFB} with \textit{MYH11}, the gene for smooth muscle myosin heavy chain\textsuperscript{17}. Previously, we used a knock-in strategy to generate a mouse model in which \textit{Cbfb-MYH11} is expressed under the control of the endogenous mouse \textit{Cbfb} gene\textsuperscript{18}. Chimeric mice derived from ES cells targeted with the knock-in \textit{Cbfb-MYH11} gene were used to assess the leukemogenic potential of the fusion gene\textsuperscript{19}. Although the \textit{Cbfb-MYH11} knock-in chimeras did not develop leukemia naturally in the first year of life, most of the animals developed AML within 3-5 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 \textit{Cbfb-MYH11} chimeras was characterized by the presence of myelomonocytic blasts and occasional eosinophils, very similar to patients with AML M4 Eo. These observations suggested that while expression of \textit{Cbfb-MYH11} is not sufficient for leukemogenesis, it is a necessary event in the multi-step process that gives rise to inv(16)-associated leukemias.

Analysis of the contribution of ES cells with the \textit{Cbfb-MYH11} knock-in gene in chimeric animals provided evidence that \textit{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 \textit{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 \textit{Cbfa2} or \textit{Cbfb}. In vitro, the \textit{CBFB-MYH11} gene product, CBF\(\beta\)-SMMHC, sequestered CBF\(\alpha2\) in the cytoplasm\(^{20,21}\). It also inhibited CBF\(\alpha2\)-mediated transactivation, and has been shown to increase CBF\(\alpha2\)-mediated repression\(^{21,22}\). Together, these data provide evidence that expression of \textit{Cbfb-MYH11} blocks hematopoietic differentiation in a dominant negative manner by inhibiting the normal function of CBF.

Considering the critical role of \textit{Cbfb} in normal hematopoiesis and leukemogenesis it is important to further characterize its expression in different hematopoietic cell populations. Previous studies indicated that \textit{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 \textit{in situ} hybridization\(^{1,14}\). In adults, \textit{Cbfb} expression is considered to be ubiquitous since 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 \textit{Cbfb} in embryonic and adult hematopoietic tissues and dissect the specific hematopoietic defects associated with \textit{CBFB-MYH11} expression, using a newly created \textit{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 (KpnI-XhoI) fragment of genomic DNA that contains Cbfb intron 4 and the first 56 bp of exon 5. The genomic DNA was isolated from a 129J genomic clone, pSKA (gift of NA Speck, Dartmouth College, Hanover, NH). Exon 5 sequences were fused in-frame to the SalI-XbaI fragment isolated from the enhanced green fluorescent protein (EGFP) gene (Clontech). The bovine growth hormone (BGH) polyA sequence was isolated from pCDNA3.1 (Invitrogen) and inserted 3’ to EGFP. The 3’ arm of the targeting vector consists of a 4.7 kb (NheI-NheI) fragment of Cbfb intron 5 isolated from a 129J genomic DNA clone, pSKB (gift of NA Speck, Dartmouth College, Hanover, NH).

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 genomic DNA isolated from individual G418/FIAU-resistant ES cell colonies. The DNA was digested with either XbaI or NcoI, and the blotted DNA was hybridized with probes, one internal to the targeting DNA vector (Hygro) and one external (probe 0.2C)\(^\text{18}\). NcoI 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. XbaI 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 PCR from DNA isolated from tail biopsies, or yolk sac. Fifty nanograms of template DNA were amplified by PCR using primers specific for hygromycin (hygro forward 5’ CCATCGTCGAGATCCAGACATG 3’ and hygro reverse 5’ GTATATGCTCCGCATTGGTCTTTT 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 min, followed by 30 cycles of amplification (30s 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.

Western Blot Analysis

Lysates from adult tissues or ES cells were prepared by resuspending 1X10⁶ cells in NuPage LDS sample buffer with reducing agent (Invitrogen, Carlsbad, CA) and boiling the samples for 15 min. The proteins were separated by electrophoresis on NuPage 4-12% bis-tris gels in MES running buffer and transferred onto nitrocellulose membranes using the semi-dry blotting system (Amersham, Piscataway, NJ). Membranes were probed with a 1:10 dilution of a monoclonal antibody specific for Cbfβ (a.a. 1-141), or a 1:5000 dilution of a polyclonal antibody specific for MEN1 (gift of SC Chandrasekharappa, National Institutes of Health, Bethesda MD); followed by a
secondary antibody conjugated to horseradish peroxidase (HRP). 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). 2.7 X10⁶ 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 anaesthetized animals by cardiac puncture. Bone marrow was obtained by flushing femur and tibia with FACS buffer (5% FCS in PBS), followed by trituration through 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 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-HSA (M1/69), anti-CD-43 (S7); biotinylated anti-HSA (M1/69), anti-BP-1 (6C3) and anti-IgM; and APC-conjugated B220 (RA3-6B2). For staining of megakaryocytes, unlysed bone marrow was resuspended in PBS containing 5% donkey serum. Two hundred nanograms of Sheep anti-human platelet glycoprotein IIb-IIIa antibody (Affinity Biologicals, Hamilton, ON, Canada) was used for staining 1X10⁶ cells. The secondary antibody was PE-conjugated donkey anti-sheep Ig (1:200 dilution). Cells were isolated from lymph node, thymus, and spleen of 3-6 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-CD8alpha (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 ug to 1 ug of antibody per 1 million cells in ice-cold FACS buffer for 30 min. After washing, cells were resuspended in 200 ul 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 four-color experiments, APC was detected on FL-7 of FACSCalibur.

Lineage depletion and cell sorting of bone marrow was performed as described previously\textsuperscript{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 pharmentigen) were used to stain bone marrow cells after lineage depletion. Fetal liver and aorta-gonad-mesonephros (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 Iscoves MDM (Invitrogen) with 10% FBS (StemCell 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 ug/ml bovine pancreatic insulin, 200 ug/ml human transferring, $10^{-4}$ M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/mL rm SCF, 10 ng/ml rm IL-3, 10 ng/ml rm IL-6, and 3 units/ml rh erythropoietin
(MethoCult GF M3434 StemCell 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. 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 Green Flourescent Protein (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 ability to interact with Cbfα2 in vitro and exhibited a sub-cellular localization pattern that was identical to wild-type Cbfβ in cultured cells (data not shown). We anticipated that Cbfb-GFP should function normally, at least with respect to hematopoiesis, since a Cbfβ (aa1-141) expression construct can rescue the hematopoietic defect in a Cbfb 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 Nco1-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 (#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 germ-line. 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<sup>+</sup>GFP compared to wild type adults (data not shown). The studies in adult mice were performed using heterozygous animals, while 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<sup>GFP/GFP</sup> 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<sup>GFP/GFP</sup> pups (data not shown). These data suggest that hematopoiesis is relatively normal and does not account for the lethality of the newborn pups.

**Cbfbβ 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<sup>11,12</sup>. In order to evaluate the expression of Cbfbβ in various hematopoietic cell populations in adult mice, cells were harvested from several hematopoietic tissues in Cbfb-<i>GFP</i> 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 three 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.**

In order 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+ and/or GR1+) in bone marrow (Figure 2B, middle panels) and peripheral blood (data not shown) revealed single peaks of GFP-expressing 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. However, as shown in Figure 2C, as erythroid cells matured from c-kit+ progenitors (R2) to Ter119hi 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 Cbfβ+/GFP bone marrow as assessed by western blot analysis (Figure 2D, lane 1).

GFP expression analysis in B220+ B-lymphocytes in the bone marrow revealed two populations (see Figure 2E, left panel). The various stages of B-cell differentiation in the bone marrow and corresponding markers are reviewed in. 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, B220+ cells 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 Chfβ-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 Chfβ 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, while the Lin\(^-\)/c-kit\(^{lo}\) population contains only hematopoietic progenitors\(^{22}\). Cbf\(\beta^{+/GFP}\) 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 Cbf\(\beta\)-GFP (Figure 3A, right panel). This suggests that a population enriched for long-term repopulating hematopoietic stem cells and hematopoietic progenitors expresses Cbf\(\beta\). A methylcellulose colony assay was used as an additional method of examining the expression of Cbf\(\beta\)-GFP in progenitors. Bone marrow cells from heterozygous animals were sorted into GFP\(^+\) and GFP\(^-\) populations (Figure 3B). Equal numbers of cells (5X10\(^4\)) from each population were plated in methylcellulose cultures containing SCF, IL-3, IL-6, and erythropoietin. There was a >10-fold enrichment in BFU-E, CFU-GM and CFU-GEM in the GFP\(^+\) population compared to the GFP\(^-\) population, suggesting that most, if not all, of the hematopoietic progenitor cells express Cbf\(\beta\) (Table 1). It is interesting to note that the greatest enrichment was observed in the CFU-GEM, which originates from a more immature progenitor that gives rise to both erythroid and myeloid cells.

**Cbf\(\beta\) is expressed in the c-kit\(^{hi}\) cells in the embryonic sites of definitive hematopoiesis.**
In order 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\textsuperscript{hi} cells in the AGM at 11.5 dpc comprise 1-2% cells in the AGM, and all of them expressed Cbfβ-GFP (Figure 4A). In the fetal liver, the c-kit\textsuperscript{hi} cells included 30-40% of the cells, and again, all expressed Cbfβ-GFP, although in heterozygous animals, the distinction between GFP\textsuperscript{+} and GFP\textsuperscript{−} was not as clear as in the homozygous animals (Figure 4B). Nevertheless, sorting the c-kit\textsuperscript{hi} cells from a heterozygous embryo into GFP\textsuperscript{+} and GFP\textsuperscript{−} populations (Figure 4C) resulted in a significant enrichment of erythroid (6-7 fold), myeloid (3-4 fold), and mixed (4-5 fold) colony-forming units, 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\textsuperscript{hi} cells in the fetal liver and AGM of wild-type, heterozygous, and homozygous embryos (Figure 4A and 4B); 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\textsuperscript{hi} population of cells is absent from AGM and fetal liver of embryos expressing \textit{Cbfb-MYH11}.

Previous studies revealed that heterozygous (\textit{Cbfb\textsuperscript{+/MYH11}}) embryos expressing \textit{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 Cbfβ^{+/MYH11} embryos. In the fetal liver at 11.5 dpc, we found a complete absence of the c-kit^{hi} (CD34-positive and –negative) population suggesting that expression of Cbfβ-MYH11 prevented the formation and/or migration of the stem/progenitor cells (Figure 5A and 5B). There were very few cells expressing Cbfβ-GFP in the Cbfβ^{GFP/MYH11} embryos, confirming the absence of cells expressing Cbfβ (and presumably Cbfβ-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 Cbfβ-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.
DISCUSSION

The importance of Chfβ 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 Chfβ expression is marked by GFP, which is easily detected by FACS. In order to preserve the normal function of Chfβ, while tagging it with GFP, exon 5 of Chfβ 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 a.a. of exon 5) are sufficient to rescue the defect in definitive myeloid and erythroid differentiation in vitro in cells lacking Chfβ, suggesting that most of exon 5 and all of exon 6 are dispensable for the normal function of Chfβ in hematopoiesis. Since the ChfβGFP/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 Chfβ-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, suggests 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 Cbfβ-MYH11 gene contribute to the population of cells containing erythroid and myeloid progenitors, but does 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 Cbfα 2 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. 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-E 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 analysis28. 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: Chfb-MYH11-targeted ES cells contribute to the c-kit+/Ter119+, c-kit-/Ter119+ and terminally differentiated erythrocyte populations in chimeric animals19. Since Chfb-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 Chfb-MYH11, histological 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 30-100 fold reduction in the number of myeloid and erythroid colonies18. In this study, we demonstrated that the entire population of c-kithi hematopoietic stem cells and progenitors in the AGM and fetal liver express Cbfβ and that both of these populations are absent in heterozygous embryos expressing Chfb-MYH11. The c-kithi cells in the AGM have been shown to express Chfa2 and form intraortic hematopoietic clusters, which contain the hematopoietic stem cells that are capable of repopulating lethally-irradiated recipients long-term. The absence of these cells in Chfb-MYH11 heterozygotes suggests that the defect in hematopoiesis occurs at the level of the hematopoietic stem cell. A similar defect is observed in Chfa2-/+ embryos, which appear
to lack the c-kit$^+$ (and Cbfα2$^+$) hematopoietic clusters$^{36}$. 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$^{19}$.

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 adult 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 Cbfβ-GFP ES cells and animals presented in this study should continue to provide a valuable resource for furthering our knowledge of Cbfβ expression and function in hematopoiesis as well as other organ systems.

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REFERENCES


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 BM</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 three populations (unsorted heterozygous bone marrow, GFP-positive [GFP+], and GFP-negative [GFP-]) shown in Figure 3B were assessed by methylcellulose colony assay. The table shows the mean and standard deviation of the data collected from three independent experiments (n=3). 5 X 10^4 cells from each population were plated in each culture.
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 FL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cbfb&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>5.0 ± 1.0</td>
<td>21.5 ± 1.5</td>
<td>2.0 ± 0</td>
</tr>
<tr>
<td>Cbfb&lt;sup&gt;+/GFP&lt;/sup&gt;</td>
<td>4.5 ±0.5</td>
<td>25.0 ± 0</td>
<td>3.0 ±1.0</td>
</tr>
<tr>
<td>Cbfb&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 Cbfb&lt;sup&gt;+/GFP&lt;/sup&gt; FL</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>
<tr>
<td>c-kit&lt;sup&gt;+/GFP&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells were isolated from fetal liver of 14.5dpc Cbfb<sup>+/+</sup>, Cbfb<sup>+/GFP</sup>, and Cbfb<sup>GFP/GFP</sup> embryos. Cells from Cbfb<sup>+/GFP</sup> embryos were sorted into c-kit<sup>+/GFP</sup> and c-kit<sup>+/GFP</sup> populations by FACS (see 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<sup>+/+</sup>, Cbfb<sup>+/GFP</sup>, Cbfb<sup>GFP/GFP</sup>) and sorted cells (n=3). 1 X 10⁴ fetal liver cells were plated in each culture.
FIGURE LEGENDS

Figure 1. Generation of “knock-in” ES cells expressing Cbfb-GFP. (A) Targeting scheme used to generate Cbfb<sup>+/GFP</sup> 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 and C) Southern blot analysis of DNA isolated from three 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) and/or the Cbfβ fusion proteins in three ES cell lines. TC-1 is the wild type ES cell line (lane 1); Cbfb-MYH11 KI #55 is an ES cell clone that expresses Cbfβ-SMMHC (lane 2); Cbfb-GFP #52 is one of the correctly targeted ES cell clones expressing Cbfβ-GFP (lane 3).

Figure 2. Cbfβ is expressed at uniform levels in most hematopoietic tissues of Cbfb<sup>+/GFP</sup> mice, but shows differential expression in hematopoietic lineages isolated from bone marrow. Cells were isolated from adult (6-month old) Cbfb<sup>+/GFP</sup> and Cbfb<sup>+/+</sup> mice and analyzed by FACS. Representative histograms show the distribution of cells
with respect to GFP fluorescence. Dashed line (---) represents Chfb^{+/+} autofluorescence; solid line (—) represents fluorescence from Chfb^{+/GFP} animals. (A) Expression of Chfβ-GFP in the indicated tissues. (B) Cells were isolated from bone marrow of adult Chfb^{+/+} and Chfb^{+/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 GPIb/IIIa. The positively stained cells were gated and analyzed for GFP expression. (C) Cells were isolated from bone marrow of adult Chfb^{+/+} and Chfb^{+/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 Chfb^{+/GFP} and Chfb^{+/+} 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 Chfb^{+/GFP} (lane 1) and Chfb^{+/+} (lane 2) bone marrow, and the Ter119-enriched population from Chfb^{+/+} bone marrow (lane 3). MEN1: multiple endocrine neoplasia 1. *: non-specific bands. (E) Cells were isolated from bone marrow of adult Chfb^{+/+} and Chfb^{+/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 graph. 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 Cbfβ+/+ (left panel, WT) and Cbfβ+/GFP (right panel, GFP) mice and depleted of cells expressing lineage markers (CD3, CD4, CD8, B220, Mac1, GR1, Ter119). Lineage negative 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-kitlo and c-kithi) cells from wild-type and heterozygotes were gated and plotted on a histogram to allow comparison of the GFP fluorescence in the two populations (middle panel). (B) Bone marrow cells from Cbfβ+/GFP adults were incubated in ACK lysis buffer to eliminate the enucleated erythrocytes and assessed for GFP expression. The cells were sorted into GFP-positive and negative 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 three populations (unsorted heterozygous bone marrow, GFP+, and GFP-) were assessed by methylcellulose colony assay. The results are shown in Table 1.

Figure 4. Cbfβ is expressed in c-kithi cells in AGM and fetal liver embryos. Cells were isolated from 11.5 dpc AGM (A) and fetal liver (B) of Cbfβ+/+ (+/+), Cbfβ+/GFP (+/GFP), and CbfβGFP/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 14.5dpc Cbfβ+/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 is shown in Table 2.

**Figure 5. The c-kit\textsuperscript{hi} population is absent from the fetal liver and AGM of embryos heterozygous for the knock-in Chfb-MYH11 (Chfb\textsuperscript{+/MYH11}).** (A) Cells were isolated from 11.5 dpc fetal liver of Chfb\textsuperscript{+/+} (+/+), Chfb\textsuperscript{+/MYH11} (+/MYH11), and Chfb\textsuperscript{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 Chfb\textsuperscript{+/+}, and Chfb\textsuperscript{+/MYH11} 11.5 dpc embryos and stained with PE-conjugated anti-c-kit and FITC-conjugate anti-CD34. Representative contour plots show the distribution of cells with respect to FITC and c-kit fluorescence.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Role of Cbfb in hematopoeisis and perturbations resulting from expression of the leukemogenic fusion gene, Cbfb-MYH11

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