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Disruption of the *Cbfa2* gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis

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ABSTRACT **The *CBFA2* (*AML1*) gene encodes a DNA-binding subunit of the heterodimeric core-binding factor. The *CBFA2* gene is disrupted by the (8;21), (3;21), and (12;21) chromosomal translocations associated with leukemias and myelodysplasias in humans. Mice lacking a CBFα2 protein capable of binding DNA die between embryonic days 11.5 and 12.5 due to hemorrhaging in the central nervous system (CNS), at the nerve/CNS interfaces of cranial and spinal nerves, and in somitic/intersomitic regions along the presumptive spinal cord. Hemorrhaging is preceded by symmetric, bilateral necrosis in these regions. Definitive erythropoiesis and myelopoiesis do not occur in *Cbfa2*-deficient embryos, and disruption of one copy of the *Cbfa2* gene significantly reduces the number of progenitors for erythroid and myeloid cells.**

The core-binding factor (CBF) binds to YGYGGT (Y, pyrimidine) sites in many genes expressed in hematopoietic cells, including genes encoding cytokines, cell surface differentiation markers, and myeloid specific proteins (1). CBF consists of two unrelated subunits, a DNA-binding α subunit that contacts DNA directly, and a β subunit that forms a complex with the α subunit but does not itself bind DNA (2–4). The mammalian CBFα subunits are encoded by three distinct genes (*CBFA1, CBFA2*, and *CBFA3*), and one gene, *CBFB*, encodes the β subunit (2–8).

Two of the four genes encoding subunits of CBF are among the most frequently disrupted genes in human leukemias. The *CBFA2* gene is disrupted by chromosomal translocations associated with *de novo* acute myeloid leukemia (AML) [t(8;21), AML M2 subtype] (6, 9–11), therapy-related myeloid leukemias, myelodysplasias, and chronic myelogenous leukemia in blast crisis [t(3;21)] (12, 13), and with pediatric B-cell acute lymphoblastic leukemias [t(12;21)] (14, 15). These translocations create chimeric proteins containing various portions of the CBFα2 protein that include its DNA-binding domain, fused to sequences from another protein: ETO (MTG8) in the case of t(8;21), EAP, MDS1 or EVI1 in t(3;21), and TEL in the t(12;21). The *CBFB* gene is disrupted by inv(16) and t(16;16) in AML of the M4Eo subtype. The chimeric proteins that result from inv(16) and t(16;16) contain the heterodimerization domain in CBFβ fused to the coiled-coil tail region of a smooth muscle myosin heavy chain (8, 16). Together, translocations and inversions involving the *CBFA2* and *CBFB* genes are associated with up to 30% of *de novo* AML cases and 28% of pediatric acute lymphoblastic leukemia (ALL) (17–20).

The *Drosophila melanogaster* homologue of the *CBFA2* gene, the runt gene, is involved in several developmental pathways in *Drosophila*—segmentation, sex determination, and neurogenesis (21–24). Here we analyze the functions of *CBFA2* in mammalian development by gene disruption in mice.

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MATERIALS AND METHODS

Recombination Constructs and Targeting of Embryonic Stem (ES) Cells. The targeting vector replaces a 3.2-kb region encompassing the last exon encoding amino acids in the DNA-binding domain of the CBFα2 protein (exon 4) with the *neo* gene driven by the phosphoglycerol kinase promoter (PGKneo) and linked to PGK poly(A) sequences. A thymidine kinase gene (tk) under the regulation of the PGK promoter was incorporated at the 3′ end of the targeting vector. The PGKneo gene was flanked upstream and downstream by 4.1 and 4.3 kb of *Cbfa2* homology, respectively. The targeting vector was linearized with *Not* I and electroporated into J1 ES cells. G418^r and 1-(2-deoxy, 2-fluoro β-D-arabinofuranosyl)-5-isodouracil (Eli Lilly) (FIAU)^r ES cell colonies were picked and expanded as described (25) and screened for homologous recombination by Southern blotting. Two independent ES cell clones heterozygous for the *Cbfa2* mutation were injected into blastocysts derived from C57BL/6 and BALB/c mice. Chimeric animals were bred against the appropriate background, and *Cbfa2*$^{+/-}$ F_1 mice (129 × BALB/c or 129 × C57BL/6) were mated. No phenotypic differences were observed in progeny mice derived either from independently targeted ES cell clones or on different genetic backgrounds.

Cbfa2$^{-/-}$ ES cells were selected from *Cbfa2*$^{+/-}$ JI cells by growth in G418 (3.2 mg/ml) (26).

***Cbfa2* cDNA Analysis.** Total RNA was prepared from liver of embryonic day 12.5 (E12.5) embryos (27) and reverse transcribed with random hexamers (Invitrogen). Nested PCR amplifications were performed on the region spanning exons 1–4 using the following primers: 5′-TATCCCCGTAGATGCCAG-3′ and 5′-GCCGTCCACTGTGATTTTGATGG-3′ for the primary PCR; 5′-TATCCCCGTAGATGCCAG-3′ and 5′-GATGGTCAGAGTGAAGCT-3′ for the second round of PCR. Primers for β-actin (Stratagene) were used as a control. PCR amplifications (Perkin–Elmer/Cetus) were performed for 30 cycles (94°C, 2 min; 50°C, 2 min; 72°C, 2 min). The PCR product was confirmed by Southern blotting.

Histological Analysis of *Cbfa2* Mutant Embryos. Embryos at various stages of gestation were fixed in Bouin's fluid, processed, and embedded in paraffin. Transverse sections of entire embryos were cut at 8 μm thickness, mounted, and stained with hematoxylin and eosin. Yolk sacs from E10.5 embryos were teased into 1 ml of phosphate-buffered saline to liberate the hematopoietic cells, and cytospin preparations were stained with Wright–Giemsa stain.

Methylcellulose Colony-Forming Assays. Single cell suspensions of yolk sac cells were prepared by digestion in 0.1% collagenase (Sigma), 20% fetal calf serum (FCS; HyClone) at 37°C for 1 hr. Cells were washed twice with α minimum essential medium (GIBCO/BRL), 2% FCS, and were plated

Abbreviations: CBF, core-binding factor; CNS, central nervous system; ES cell, embryonic stem cell; E, embryonic day; EB, embryoid body.

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in α methylcellulose medium (0.8% methylcellulose/30% FCS/1% bovine serum albumin/2 mM L-glutamine/0.1 mM 2-mercaptoethanol) (Methocult M3230; Stemcell Technologies, Vancouver) with 1× penicillin/streptomycin (GIBCO/BRL), erythropoietin (2 units/ml) (R&D Systems), and 1.3% pokeweed mitogen-stimulated spleen cell conditioned medium (Stemcell Technologies).

***In Vitro* Differentiation of ES Cells.** *Cbfa2*$^{+/+}$ and *Cbfa2*$^{-/-}$ J1 ES cells were differentiated *in vitro* according to the protocols of Keller *et al.* (28), with the following modifications: Primary differentiation to embryoid bodies (EBs) was in Iscove's modified Dulbecco's medium (IMDM; GIBCO/BRL)/15% FCS/0.8% methylcellulose (Methocult M3100; Stemcell Technologies)/2 mM glutamine/450 μM monothioglycerol (Sigma)/1× OPI medium supplement (oxalacetic acid/sodium pyruvate/bovine insulin; Sigma). Secondary hematopoietic colonies were grown in IMDM/10% FCS/0.8% methylcellulose/2 mM glutamine/100 ng of recombinant murine stem cell factor per ml (SCF; R&D Systems)/2 units of human recombinant erythropoietin per ml (R&D Systems)/10 ng of recombinant murine interleukin 3 per ml (Stemcell Technologies)/10 ng of recombinant murine granulocyte–macrophage colony-stimulating factor per ml (Stemcell Technologies).

RESULTS

Mutation of the *Cbfa2* Gene Results in Embryonic Lethality. The DNA-binding domain of CBFα2 is contained within a 128-amino acid region (aa 51–178) called the Runt domain (2, 29, 30). We replaced the last exon (exon 4) encoding amino acids in the Runt domain of CBFα2 (aa 143–178; ref. 7) with the neomycin-resistance gene (Fig. 1). Since a C-terminal truncation of CBFα2 to aa 170 was sufficient to disrupt DNA-binding and heterodimerization with CBFβ (31), loss of exon 4-encoded sequences should destroy the ability of CBFα2 to bind DNA and heterodimerize with the CBFβ subunit. Chimeric mice generated from two independently targeted ES cell clones were bred to C57BL/6 or BALB/c mice and progeny heterozygous for the *Cbfa2* mutation were interbred. *Cbfa2*$^{-/-}$ embryos died between gestational days E11.5 and

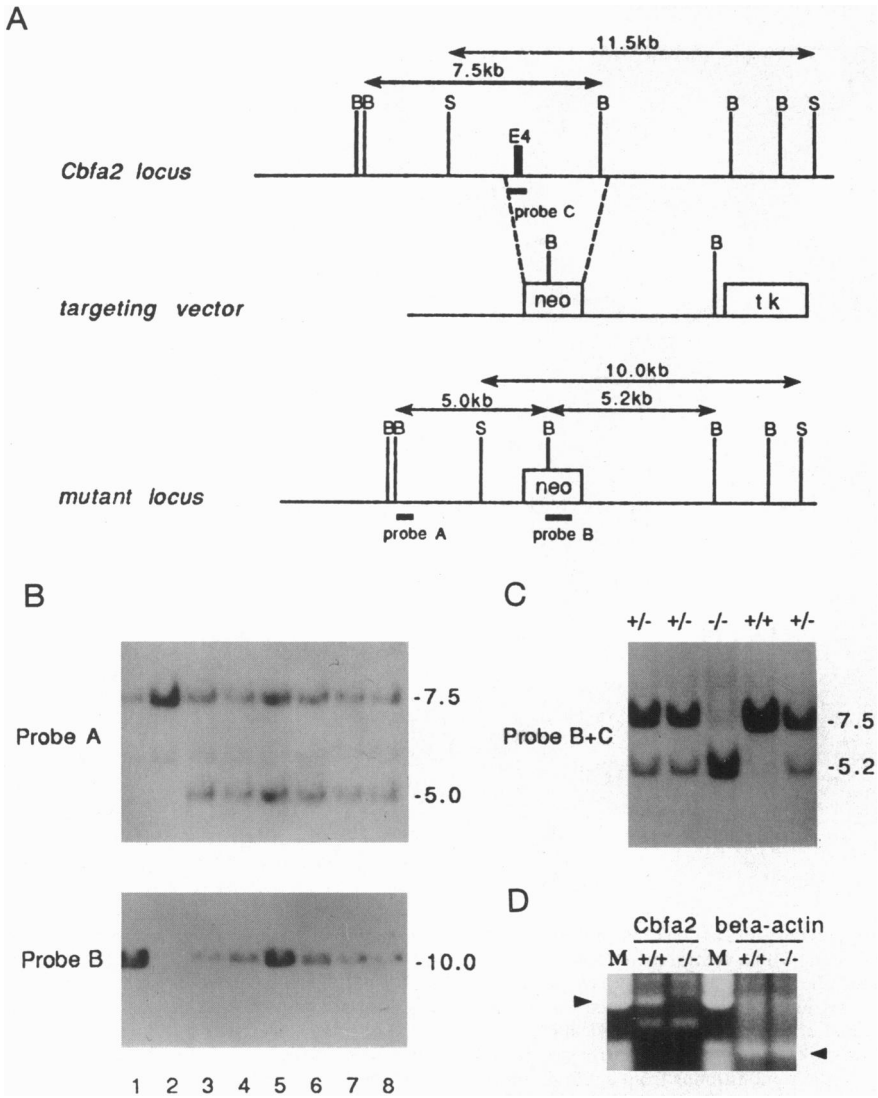

FIG. 1. Disruption of the *Cbfa2* gene by homologous recombination. (*A*) Gene-targeting strategy used to delete exon 4 (E4) of the *Cbfa2* gene. Probe A, from outside the recombination locus, was used to screen for homologous recombination events. Probe B, from the neomycin resistance gene, was used to check single integration events. Probe C is derived from the region deleted in the recombination vector. B, *Bam*HI; S, *Ssp* I. (*B*) Southern blot analysis of genomic DNA from eight selected ES cell clones. Probe A hybridizes to a 7.5- and a 5.0-kb *Bam*HI genomic fragment derived from the wild-type and the targeted *Cbfa2* alleles, respectively. Probe B hybridizes to a 10.0-kb *Ssp* I genomic fragment derived from the targeted locus. (*C*) Southern analysis of DNA from E11.5 *Cbfa2*$^{+/+}$, *Cbfa2*$^{+/-}$, and *Cbfa2*$^{-/-}$ embryos. Probe C hybridizes to a 7.5-kb *Bam*HI band from the wild-type allele and probe B hybridizes to a 5.2-kb band from the targeted allele. (*D*) Analysis of *Cbfa2* mRNA expression in F_2 embryos by reverse transcription PCR. M, markers. Arrowheads indicate expected size of the PCR products.

E12.5 and exhibited extensive hemorrhages (Fig. 2 *A* and *B*; Table 1). Hemorrhaging in *Cbfa2*$^{-/-}$ embryos occurred as early as E10.5 (14%), by E11.5 most (73%) of the *Cbfa2*$^{-/-}$ embryos had hemorrhaged, and by E12.5 all live *Cbfa2*$^{-/-}$ embryos had hemorrhaged. Aside from hemorrhaging, *Cbfa2*$^{-/-}$ embryos and yolk sacs appeared to be developmentally normal by gross morphologic analysis (Fig. 2*A*).

We recognized three different types of primary hemorrhagic events: (*i*) The most extensive bleeds were those involving the central nervous system (CNS) and were specifically located in the isthmus, ventral metencephalon, and spinal cord (Fig. 2 *B–D*). The CNS acquires its vascular network from capillaries that perforate the neural tissue from the surrounding meningeal compartment (32). Early CNS hemorrhages were linear and paralleled the location and distribution of these CNS perforating capillaries (Fig. 2*D*). These bleeds often ruptured through the ependymal epithelium, causing secondary and possibly terminal intraventricular hemorrhages (Fig. 2 *B–D*). We also identified two bilateral, symmetrical foci of cellular necrosis within the ventral metencephalon (Fig. 2*C*). This necrosis appeared to involve the endothelial cells of growing

Table 1. Genotype of offspring derived from intercross of *Cbfa2*$^{+/-}$ parents

Stage	Total (litters)	Genotype +/+	+/−	−/−	Live −/−
Postnatal	122 (24)	44	78	0	0
E14.5	6 (1)	0	5	1	0
E12.5	18 (3)	3	12	3	2
E11.5	148 (16)	22	86	40	35
E10.5	78 (11)	23	41	14	14

Genotype analysis was performed by Southern blot analysis of DNA isolated from tail snips at the postnatal stage and from yolk sacs or embryos at prenatal stage. Surviving embryos were defined as those with beating hearts at the time of dissection.

CNS capillaries and not nerve tissue (not shown). (*ii*) Focal petechial (often bilateral and symmetrical) hemorrhages were seen in peripheral nerves and intersegmental regions. They were especially prominent at the nerve roots of the VII–VIII cranial nerve complex and at some spinal nerves (Fig. 2*B*). (*iii*) Mesodermal petechial hemorrhages were detected in a small

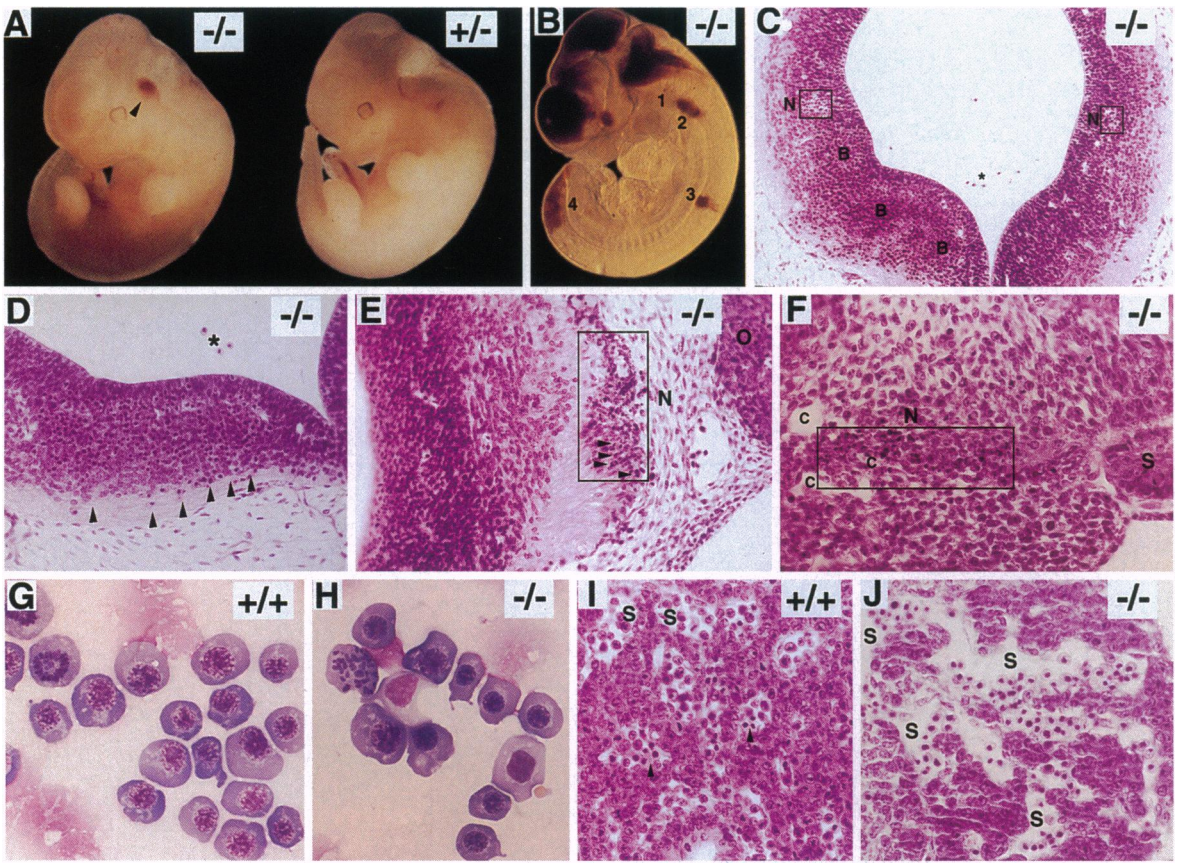

FIG. 2. Composite figure showing gross and microscopic views of the observed pathologic lesions. (*A*) Gross appearance of two E11.5 littermates showing a *Cbfa2*$^{-/-}$ embryo (*Left*) with a ventral metencephalic hemorrhagic area (arrowhead) roughly located at the level of the VII–VIII cranial nerve complex and close to the otic placode (arrowhead). (*Right*) Unaffected *Cbfa2*$^{+/-}$ embryo. (*B*) Gross appearance of an E11.5 *Cbfa2*$^{-/-}$ embryo with several hemorrhagic lesions, including intraventricular (lateral, 3rd, infundibulum, aqueduct, and 4th ventricle), and segmental bleeds in various regions: at the VII–VIII cranial nerve complex (*1*), cervical (*2*), thoracic (*3*), and caudal (*4*) regions. (*C*) Cross section of the CNS isthmus of an E11.0 *Cbfa2*$^{-/-}$ embryo showing extensive CNS hemorrhages (B) and bilateral symmetrical areas of vascular necrosis (N). There is also blood in the ventricle (*). (*D*) Detail of the same isthmus from a margin of the hemorrhage in *C*, showing several separate lineal and vertical hemorrhages (arrowheads), paralleling the location and distribution of the CNS perforating vessels. Also shown is intraventricular blood (*). (*E*) Detail of the CNS/nerve interface of the VII–VIII cranial nerve complex of an E10.5 *Cbfa2*$^{-/-}$ embryo showing extensive necrosis (N) involving neural crest and endothelial cells. Note a few cells in mitosis (arrowheads), indicating the great cellular activity of these CNS/nerve interfaces. This corresponds to the area of bleeding shown at E11.5 (*A*, arrowhead; *B, 1*). Also shown is the edge of the otic (O) placode. (*F*) Detail of the somitic (S) intersomitic necrosis (N) around and probably involving segmental vessels (c) in an E10.5 *Cbfa2*$^{-/-}$ embryo. (See *B*, areas 2–4.) (*G* and *H*) Details of cytospin preparations from E10.5 yolk sacs showing blood precursor cells and nucleated primitive erythrocytes from *Cbfa2*$^{+/+}$ and *Cbfa2*$^{-/-}$ embryos. (*I* and *J*) Detail of liver sections showing many blood precursor cells in the liver sinusoids (S) of an E11.0 *Cbfa2*$^{+/+}$ embryo and their absence in the liver sinusoids (S) of the *Cbfa2*$^{-/-}$ embryo, which essentially contains only circulating nucleated primitive erythrocytes. Arrowheads in *I* point to examples of nucleated primitive erythroid cells; remaining cells in the sinusoids of *Cbfa2*$^{+/+}$ livers are less differentiated hematopoietic cells.

number of embryos. Pericardiac and peritoneal hemorrhages, for which we could not identify a source, were also found in some embryos. These latter bleeds may represent late events resulting from terminal embryonic deterioration and/or handling at the time of dissection.

To gain insight into the cause of bleeding, and to identify events that precede hemorrhaging, we analyzed younger *Cbfa2*$^{-/-}$ embryos. We observed foci of cellular necrosis, often bilateral and symmetrical, at the nerve/CNS interfaces of cranial and spinal nerves of E10.5 embryos (Fig. 2*E*) and somitic/intersomitic necrosis in the lower thoracic and lumbar regions (Fig. 2*F*). Their locations paralleled the segmental bleeds observed in E11.5–E12.5 embryos (Fig. 2*B*). The necrosis in these regions appears to involve neural crest cells and endothelial cells. Substantial areas of necrosis were found in only three regions (CNS capillaries, nerve/CNS interfaces, and somitic/intersomitic regions). However, scattered microscopic foci of perineural necrosis were detected throughout the mesoderm and retroperitoneal regions of E10.5 embryos that seemed to be more common in *Cbfa2*$^{-/-}$ embryos than in *Cbfa2*$^{+/+}$ littermates.

Mutation of the *Cbfa2* Gene Blocks Definitive Erythropoiesis and Myelopoiesis. Mutation of the *Cbfa2* gene caused no gross differences in either the number or morphology of primitive erythrocytes in E10.5 *Cbfa2*$^{-/-}$ yolk sacs (Fig. 2 *G* and *H*), but fetal liver hematopoiesis was impaired. Liver sinusoids from E11.0 +/+ embryos contained primitive nucleated erythrocytes plus numerous, less differentiated cells (Fig. 2*I*). Immature granulocytes and monocytes/macrophages were also found in liver smears from E12.5$^{+/+}$ embryos stained by Wright–Giemsa or for myeloperoxidase activity (not shown). In contrast, only primitive nucleated erythroid cells could be detected either in liver sections from E11.0 *Cbfa2*$^{-/-}$ embryos (Fig. 2*J*) or in liver smears from E12.5 embryos (not shown).

Mutation of the *Cbfa2* gene could affect either homing of hematopoietic progenitor cells to the liver or the ability of the fetal liver microenvironment to support hematopoiesis. To assess the differentiation capacity of progenitor cells isolated from yolk sacs of *Cbfa2*$^{+/+}$, *Cbfa2*$^{+/-}$, and *Cbfa2*$^{-/-}$ embryos, we performed *in vitro* methylcellulose colony-forming assays (Table 2) (33). Definitive erythroid, myeloid, and mixed erythroid/myeloid colonies differentiated from *Cbfa2*$^{+/+}$ and *Cbfa2*$^{+/-}$ yolk sac cells. Significantly fewer colonies differentiated from yolk sac cells isolated from *Cbfa2*$^{+/-}$ embryos compared to those from *Cbfa2*$^{+/+}$ embryos (Table 2). Essentially no erythroid or myeloid colonies appeared when yolk sac cells from *Cbfa2*$^{-/-}$ embryos were cultured in the same manner.

In vitro differentiation of *Cbfa2*$^{-/-}$ ES cells (28) also indicated that this gene plays a role in either the commitment or the differentiation of definitive erythroid and myeloid progenitor cells. In primary differentiation cultures, both *Cbfa2*$^{+/+}$ and *Cbfa2*$^{-/-}$ ES cells formed hemaglobinized EBs. Primitive erythroid cells were seen in cytospin preparations of the disaggregated cells from both *Cbfa2*$^{+/+}$ and *Cbfa2*$^{-/-}$ EBs (not shown). However, after disaggregation of the EBs and secondary culture of the cells in the presence of hematopoietic growth factors, only cells derived from *Cbfa2*$^{+/+}$ EBs, and not those from *Cbfa2*$^{-/-}$ EBs, were able to form definitive erythroid and myeloid colonies (Table 2).

DISCUSSION

Lack of a CBFα2 protein capable of binding specific DNA target sequences and dimerizing with the CBFβ subunit leads to extensive hemorrhaging and ultimately embryonic death. The hemorrhaging occurred at specific sites in the embryo—at nerve/CNS interfaces, in the CNS vasculature in the metencephalon, and in somitic/intersomic zones. This localized hemorrhaging differs from the more generalized impairment of vasculogenesis and hemorrhage seen upon disruption of the *tek, Flk-1, Flt-1, Tie-1, Tie-2*, and *Gap* genes (34–38). Hemorrhaging in E11.5–E12.5 *Cbfa2*$^{-/-}$ embryos was preceded by cellular necrosis at E10.5 in these same regions. The symmetrical, bilateral nature of the necroses indicates that there is a specific developmental defect caused by mutation of the *Cbfa2* gene.

The *Cbfa2* gene is widely expressed in the mouse embryo (39). Expression starts at E9.2, and by E10.5 *Cbfa2* transcripts are found in the neural tube, sensory ganglia (notably cranial nerves V, VII–VIII, and IX and the dorsal root ganglia of spinal nerves), specialized sensory epithelial structures, chondrogenic centers of both neural crest and mesodermal origin, and the genital system (39). Necrosis and hemorrhaging in the E10.5–12.5 *Cbfa2*$^{-/-}$ embryos is found in a subset of those areas where the gene is normally expressed. Since the nerve/CNS interfaces and CNS capillaries undergo considerable cellular growth and migration at this stage of embryonic development (32, 40–42), this may render them particularly susceptible to damage. An alternative explanation for the limited distribution of the necrosis and hemorrhaging relative to *Cbfa2* expression is that the first lesions that develop cause embryonic death. Additional lesions might have appeared at later stages of development had the embryos survived longer. We note that two places where focal necrosis was observed, the VII–VIII cranial nerve complex and the dorsal root ganglia of spinal nerves, correspond to areas where substantial expression of *Cbfa2* was seen in E10.5–E12.5 embryos (39).

We believe that cellular necrosis, and not lack of circulating platelets, is the cause of hemorrhaging for two reasons: First, the necrosis seen at E10.5 precedes the onset of definitive liver hematopoiesis (43) and expression of prothrombin (44). Second, other mutations that selectively disrupt definitive eryth-

Table 2. Colonies arising from yolk sac progenitors and *in vitro* differentiation of ES cells

	E10.5				E11.5				ES cells	
	Total no. of yolk sacs analyzed	Colony no. per yolk sac*			Total no. of yolk sacs analyzed	Colony no. per yolk sac*			Primary EBs†	Secondary colony no.‡
Genotype		E	M	Mix		E	M	Mix		
+/+	11	221 (61)	224 (64)	54 (24)	6	139 (54)	196 (54)	37 (20)	135 (51)	20 (2)
+/−	19	166 (92)	135 (45)	24 (14)	25	55 (22)	108 (47)	13 (10)	ND	ND
−/−	5	0	0	1	13	0	0	0	94 (19)	0

*Colonies (>30 cells in size) were scored on day 7 of incubation. E, erythroid colonies; M, myeloid colonies; Mix, erythroid–myeloid mixed colonies. Average number of colonies per yolk sac is indicated, followed by standard deviation in parentheses. The difference between the numbers of colonies from *Cbfa2*$^{+/-}$ and *Cbfa2*$^{+/+}$ yolk sacs is significant at $P < 0.01$, except for the numbers of erythroid colonies from E10.5 yolk sacs, which is significant at $P < 0.1$.

†Primary EBs per 1000 ES cells plated were counted after 6 days of differentiation. Numbers represent means of three plates, each obtained in two independent experiments. ND, not determined.

‡EBs were collected and disaggregated after 6 days of primary differentiation. Numbers of myeloid, erythroid, and mixed colonies per 10^5 cells plated were counted after 7 days of secondary differentiation. Numbers represent means of three plates, each obtained in two independent experiments.

ropoiesis and myelopoiesis, including mutations in c-*myb*, and the genes encoding the c-kit receptor (W) and its ligand (SI), do not cause hemorrhaging in the CNS (45, 46). Likewise, mice homozygous for disruption of the NF-E2 gene, which selectively blocks platelet formation, are born alive with birth-induced abdominal hemorrhages, and a small percentage of the mice survive into adulthood (47).

Homozygous disruption of the *Cbfa2* gene completely blocks definitive erythropoiesis, myelopoiesis, and lymphopoiesis (this paper and ref. 48), suggesting a defect in an early progenitor cell for all these lineages. Primitive erythropoiesis is largely intact, although we do discern subtle differences in the morphology and staining intensity of primitive erythroid cells from $Cbfa2^{-/-}$ embryos relative to $Cbfa2^{+/+}$ embryos, which could indicate a minor impairment in the development of this lineage. We also find that disruption of one copy of the *Cbfa2* gene decreases the number of erythroid and myeloid colonies that can be differentiated *in vitro*. This could result either from the presence of smaller numbers of progenitor cells or from an impairment in their capacity to differentiate. The targeted disruption has either decreased the levels of functional CBFα2 protein or caused a truncated protein to be produced that functions as a transdominant inhibitor of hematopoiesis. Assuming the disruption is a loss-of-function mutation, we predict that hemizygosity of the *CBFA2* gene may contribute to leukemogenesis in humans. Translocations in the *CBFA2* gene could block differentiation by two mechanisms—by creating chimeric proteins with altered properties and by decreasing the level of functional CBFα2 protein in the leukemic cells.

Cbfa2, like its *Drosophila* homologue runt, appears to be involved in multiple developmental pathways. The *Drosophila* runt gene plays a vital role in neurogenesis and, like *Cbfa2*, is expressed in the CNS (23). However, some mutant alleles of the runt gene cause defects at very early stages of *Drosophila* development, segmentation and sex determination (21, 22, 24), whereas *Cbfa2* appears to play no role in these processes. Although participation in multiple developmental pathways is an evolutionarily conserved property of these genes, the particular pathways in which they function have apparently diverged.

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