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AML1, the Target of Multiple Chromosomal Translocations in Human Leukemia, Is Essential for Normal Fetal Liver Hematopoiesis

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Summary

The AML1–CBFβ transcription factor is the most frequent target of chromosomal rearrangements in human leukemia. To investigate its normal function, we generated mice lacking AML1. Embryos with homozygous mutations in *AML1* showed normal morphogenesis and yolk sac–derived erythropoiesis, but lacked fetal liver hematopoiesis and died around E12.5. Sequentially targeted *AML1*$^{-/-}$ ES cells retained their capacity to differentiate into primitive erythroid cells in vitro; however, no myeloid or erythroid progenitors of definitive hematopoietic origin were detected in either the yolk sac or fetal livers of mutant embryos. Moreover, this hematopoietic defect was intrinsic to the stem cells in that *AML1*$^{-/-}$ ES cells failed to contribute to hematopoiesis in chimeric animals. These results suggest that AML1-regulated target genes are essential for definitive hematopoiesis of all lineages.

Introduction

The investigation of leukemia-associated translocations has provided insights into the mechanisms of transformation in human leukemia and has led to the identification of a series of transcription factors implicated in the regulation of normal hematopoiesis (Rabbitts, 1994). The most frequent target of these translocations is the acute myeloid leukemia 1 (AML1)–core-binding factor β (CBFβ) heterodimeric transcription factor complex (Miyoshi et al., 1991; Erickson et al., 1992; Nisson et al., 1992; Nucifora et al., 1993; Mitani et al., 1994; Golub et al., 1995; Liu et al., 1993). AML1 is a member of a family of transcription factors with homology to the Drosophila pair-rule gene *runt* and directly binds the enhancer core DNA sequence TGT/cGGT, which is present in a number of different viral and cellular promoters and enhancers (Daga et al., 1992; Erickson et al., 1992; Kagoshima et al., 1993; Meyers et al., 1993; Manley et al., 1993). AML1 binds DNA via a central 118 amino acid Runt homology domain (RHD), and its DNA binding affinity is increased by heterodimerization through the RHD with CBFβ (Meyers et al., 1993; Wang et al., 1993; Ogawa et al., 1993a).

AML1 was identified through its involvement in the AML-associated t(8;21), which occurs in ~15% of de novo cases (Miyoshi et al., 1991). In this translocation, AML1, including its RHD, fuses to a zinc finger–containing putative transcription factor, ETO, on chromosome 8 to form an AML1–ETO chimeric product (Erickson et al., 1992; Nisson et al., 1992; Miyoshi et al., 1993). AML1–ETO retains the ability to interact with the enhancer core DNA sequence and has been shown to interfere with AML1-dependent transactivation (Meyers et al., 1993, 1995). Thus, transformation by AML1–ETO may result from altered transcriptional regulation of normal *AML1* target genes.

This concept was further supported by the recent characterization of several additional myeloid leukemia–associated translocations involving the AML1–CBFβ transcription factor complex. The inv(16), which accounts for 15%–18% of cases of de novo AML, results in the fusion of *CBF*β to a smooth muscle myosin heavy chain gene, *MYH11*, and produces a chimeric product that retains its ability to interact with AML1 (Liu et al., 1993; Shurtleff et al., 1995a). CBFβ–MYH11-mediated transformation has been shown to result, in part, from direct inhibition of AML1-regulated transcriptional activity (Hajra et al., 1995a, 1995b). In addition, three alternative AML1-containing fusion products, each retaining the complete RHD, are formed by the t(3;21), which occurs in rare cases of myelodysplasia and blast transformation of chronic myelogenous leukemia (Nucifora et al., 1993, 1994; Mitani et al., 1994). In this translocation, *AML1* is fused with either the *EVI1* gene, which encodes a known zinc finger–containing transcription factor, or either of two alternative genes of unknown function, *EAP* and *MDS1*, which are located adjacent to *EVI1* on chromosome 3 (for review see Nucifora and Rowley, 1995).

Although these early results suggested that *AML1* alterations were limited to leukemia of myeloid lineage, recent studies have demonstrated that *AML1* is frequently mutated in pediatric B progenitor acute lymphoblastic leukemia (ALL), the most common malignancy seen in children (Pui, 1995). Cloning of the ALL-associated t(12;21) revealed the formation of a chimeric gene that encoded a fusion protein consisting of the N-terminal helix-loop-helix domain of TEL, a member of the Ets-like family of transcription factors, fused to nearly the complete AML1 protein, including the RHD and C-terminal domains (Golub et al., 1995; Romana et al., 1995a). Analysis of a large number of pediatric ALL cases demonstrated expression of the *TEL–AML1* chimeric transcript in ~25% of cases with a B progenitor immunophenotype, despite the complete lack of cytogenetic evidence of this translocation in the majority of cases (Romana et al., 1995b; Shurtleff et al., 1995b). Thus, taken together, these data suggest that alteration of transcriptional cascades mediated by AML1 is one of the most common mechanisms of transformation in human leukemia.

AML1 binds the enhancer core motif that is present in the transcriptional regulatory region of a number of different hematopoietic-specific genes, including the T cell antigen receptors (Prosser et al., 1992; Redondo et al., 1992; Wang et al., 1993; Hsiang et al., 1993), the myeloid-specific gene myeloperoxidase (Nuchprayoon et al., 1994), and a variety of cytokines and their receptors, including granulocyte–macrophage colony-stimulating factor (GM-CSF) (Takahashi et al., 1995),

interleukin-3 (IL-3) (Shoemaker et al., 1990), and the colony-stimulating factor 1 (CSF1) receptor (Zhang et al., 1994). The enhancer core motif is necessary for the tissue-specific expression of these genes, but cannot confer tissue specificity to a heterologous promoter. In addition, this sequence is in the enhancers of the simian virus 40 (Weiher et al., 1983), polyoma virus (Kamachi et al., 1990), and all murine type C retroviruses (Manley et al., 1993; Sun et al., 1993) and has been shown to represent a critical regulatory region in several murine leukemia retroviral enhancers, where it influences tissue tropism and disease phenotype (Boral et al., 1989; Lo-Sardo et al., 1990; Speck et al., 1990). Thus, AML1 may function as a transcriptional organizer that recruits tissue-specific factors to stimulate lineage-restricted transcription. Moreover, recent data suggest that AML1-mediated transcriptional activity of cellular genes is dependent on sequences adjacent to the enhancer core motif, with activity only detected using enhancers or promoters bearing Ets-1- or c-Myb-binding sites near the *AML1* site (Hernandez-Munain and Krangel, 1995; Wotton et al., 1994).

Cloning of murine CBF (Wang et al., 1993) and polyoma enhancer-binding protein (PEBP2) (Ogawa et al., 1993b; Bae et al., 1993), coupled with low stringency hybridization with an *AML1* cDNA fragment (Levanon et al., 1994), revealed a family of closely related DNA-binding proteins AML1 (PEBP2-B), AML2 (PEBP2-C), and AML3 (PEBP2-A). These proteins show greater than 90% identity within the RHD and 60%–80% identity between their C-terminal sequences. *AML1* mRNA is detected in all adult tissues, with the highest levels of expression seen in the thymus, bone marrow, and peripheral blood (Zhu et al., 1994; Satake et al., 1995; Miyoshi et al., 1995). By contrast, little is known about its expression during embryogenesis or about expression of the other family members.

Although the available data suggests that AML1 is likely to play a pivotal role in the regulation of target genes involved in the function of the hematopoietic lineages, its normal cellular functions remain to be defined. To investigate directly the normal biologic function of AML1 in vivo, we generated mice carrying a disrupted *AML1* allele using gene targeting in embryonic stem (ES) cells. Mice lacking *AML1* died during midembryonic development secondary to the complete absence of fetal liver-derived hematopoiesis. Moreover, homozygous *AML1*-deficient ES cells failed to contribute to hematopoiesis in chimeric animals. These findings indicate that AML1-regulated target genes are essential for definitive hematopoiesis of all lineages.

Results

Targeted Disruption of *AML1* Results in an Embryonic Lethal Phenotype in Mice

The *AML1* gene was inactivated using a replacement vector designed to disrupt the coding sequence of *AML1* by replacing the splice acceptor and first 20 bp of the exon that encodes the central 52 amino acids of the RHD with a hygromycin B (hygrB) cassette (Figure 1A). As a consequence of this targeting strategy, termination

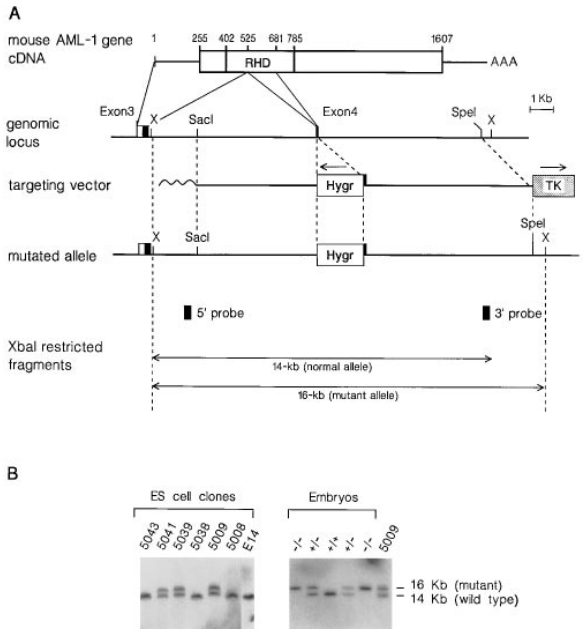

Figure 1. Targeted Disruption of Murine *AML1* Gene by Homologous Recombination

(A) Schematic representation of the *AML1* cDNA and partial restriction map of the *AML1* genomic locus with the targeting construct depicted below. The DNA-binding RHD is shown, as are SacI, SpeI, and XbaI (X) restriction sites. The arrows indicate the direction of transcription of the *hygr*^{R} (*hygr*) and TK cassettes. The positions of *AML1* exons 3 and 4 are shown as boxes, and their coding sequences are closed. Wild-type (14 kb) and targeted alleles (16 kb) could be distinguished by Southern blots of XbaI-digested DNA, hybridized with either the indicated 5′ or 3′ probes.

(B) Southern blot analysis of ES cell clones and E11.5 embryos from an *AML1*$^{+/-}$ intercross. ES cell clones with homologous targeting of one *AML1* allele contain a 16 kb restriction fragment in addition to the 14 kb fragment derived from the wild-type allele. Homozygous mutant embryos contain only the 16 kb restriction fragment.

codons are introduced into all three reading frames, and thus any AML1 protein formed will lack the RHD and will be unable to bind DNA or to interact with CBFβ. Following electroporation of the targeting construct, ES cell clones were grown in the presence of hygrB and ganciclovir (GANC), and double-resistant clones were analyzed by Southern blotting to screen for homologous recombinants (Figure 1B). Of the 80 hygrB^R/GANC^R E14 ES clones obtained, 20 (25%) contained a correctly targeted *AML1* allele. Two independently targeted clones with normal ploidy were injected into C57BL/6 blastocysts and gave germline transmission. F1 mice heterozygous for the *AML1* mutation appeared normal and had no discernible differences when compared with wild-type littermates in their hematocrits, nucleated blood cell counts, white blood cell differentials, or distribution of peripheral blood lymphocyte subsets as analyzed by fluorescence-activated cell sorting analysis (data not shown). Heterozygous mice were interbred to generate homozygous mutants, and among the 161 newborn offspring genotyped, no *AML1*$^{-/-}$ mice were identified, indicating that loss of AML1 is incompatible with normal embryonic development (Table 1).

To confirm that gene targeting into the *AML1* locus

Table 1. Genotypes of Litters Obtained by Intercrossing *AML1*$^{+/-}$

Stages	Numbers of Pups	+/+	+/−	−/− Alive	−/− Dead
E10.5	25	10	9	6	0
E11.5	57	13	29	13	2
E12.5	30	10	13	2	5
E13.5	22	6	13	0	3[a]
F2 Pups	161	58	103	0	—

[a] Degenerated

had resulted in an inactivating mutation, reverse transcriptase–polymerase chain reaction (RT–PCR) analysis for *AML1* transcripts was performed on poly(A)$^+$ RNA isolated from viable embryonic day 11.5 (E11.5) whole embryos. Amplification of *AML1* mRNA was with a set of *AML1*-specific oligonucleotide primers that bracket the position of the *hygrB* cassette insertion, and PCR products were probed with an *AML1*-specific oligonucleotide after Southern blot transfer. As shown in Figure 2, no amplifiable *AML1* mRNA was detected in the *AML1*$^{-/-}$ embryos, whereas this mRNA was observed in control littermates. Thus, the targeting strategy used resulted in elimination of *AML1* mRNA encoding the RHD.

To identify the stage of embryonic development at which the *AML1* mutation is lethal, E10.5–E13.5 embryos were analyzed. At E11.5 the majority of *AML1*-deficient embryos (87%) were viable; however, by E12.5, very few *AML1*$^{-/-}$ embryos were found alive (Table 1). Morphologic evaluation of embryos at E12.5 revealed extensive hemorrhages within the ventricle of the central nervous system and vertebral canal (Figures 3A and 3B). Death appeared to occur shortly before or during embryo dissection, since mutant embryos were comparable in size and development to their wild-type and heterozygous littermates. Microscopically, the hemorrhages in the mutant animals appeared to originate within the ganglia of the cranial nerves and extended into the third and lateral ventricles. In addition, hemorrhages occurred within the pericardial space and peritoneal cavity in the majority of *AML1*-deficient animals (Figure 3C; data not shown).

At E11.5, viable *AML1*$^{-/-}$ animals were morphologically indistinguishable from their littermates except for a slight pallor to the liver (see below). The yolk sac vessels were easily visualized, and the mutant embryos showed no overt evidence of anemia. Small foci of intraparenchymal hemorrhages within the central nervous system occurred during careful dissection of several homozygous mutant live E11.5 embryos; however, all other abnormalities in the *AML1*-deficient embryos were confined to the hematopoietic system.

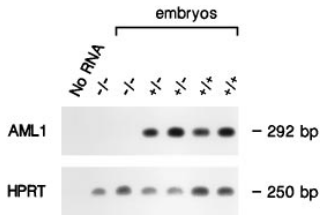

Figure 2. RT–PCR Analysis of mRNA from E11.5 Embryos

Total embryo mRNA was isolated and amplified with *AML1*-specific primers that bracket the site of targeting in exon 4. RT–PCR amplification was also performed for the ubiquitously expressed *HPRT* mRNA as a control for the presence of amplifiable RNA.

Absence of Fetal Liver Hematopoiesis in *AML1*-Deficient Embryos

Detailed microscopic analysis of the yolk sac of E11.5 embryos revealed no significant morphologic differences between *AML1*-deficient and control embryos (data not shown). During normal murine embryonic development, the major site of hematopoiesis shifts from the yolk sac to the fetal liver at around E11.5–E12.5. Microscopic examination of the livers of *AML1*-deficient embryos at E11.5 revealed the complete absence of fetal liver–derived hematopoiesis. No erythroid, myeloid, or megakaryocyte elements were identified in sections of *AML1*$^{-/-}$ livers, with only primitive nucleated erythrocytes seen within vascular channels and hepatic sinusoids (Figure 4A). Moreover, analysis of impression smears made from livers of *AML1*$^{-/-}$ embryos confirmed the lack of hematopoietic cells (Figure 4B). In addition, peripheral blood of the *AML1*-deficient embryos at both E11.5 and E12.5 contained only nucleated large primitive erythrocytes, consistent with a yolk sac origin, and lacked identifiable platelets, which were clearly visible in blood smears from wild-type and heterozygous littermates (Figure 4C).

The observed hematopoietic defect in *AML1*$^{-/-}$ embryos suggests that yolk sac–derived primitive hematopoiesis was unaffected, but that there was a complete block in the establishment of fetal liver–derived definitive hematopoiesis. To examine the hematopoietic activity further, we isolated individual yolk sacs and fetal livers from mutant and control embryos and analyzed them for in vitro hematopoietic colony formation. Yolk sacs obtained from viable E10.5 embryos were dispersed into single cell suspensions and cultured in triplicate in 1.2% methylcellulose in media containing 30% fetal calf serum (FCS) supplemented with human erythropoietin and containing murine stem cell factor, IL-3, and GM-CSF. These conditions are optimal for the differentiation of multipotential hematopoietic progenitor cells with colonies being of definitive hematopoietic origin, since at this stage of development, no primitive hematopoietic progenitors are detected in yolk sacs (Wong et al., 1986). No hematopoietic colonies of any lineage grew in the yolk sac cell cultures from *AML1*-deficient embryos, whereas 473 ± 132 and 525 ± 74 total colonies per yolk sac were detected in cultures from wild-type and heterozygous littermates, respectively (Table 2). Similarly, hematopoietic progenitors were completely absent in E11.5 fetal liver cultures from *AML1*-deficient embryos, but were readily detected in essentially equal numbers in cultures from wild-type and heterozygous controls. Thus, in the *AML1*-deficient animals, progenitors for definitive hematopoiesis of either erythroid or myeloid lineages were absent from both the yolk sac and liver. These data suggest that loss of AML1 function

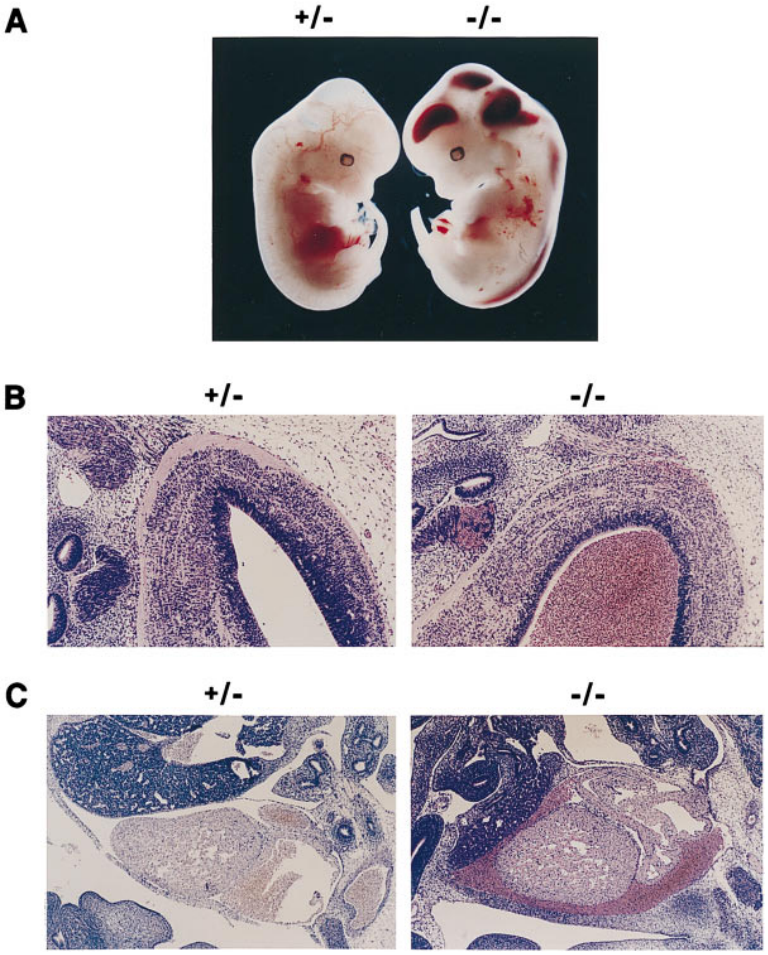

Figure 3. Phenotypes of E12.5 Control and *AML1*$^{-/-}$ Embryos

(A) Appearance of a mutant mouse embryos (−/−) compared with their control littermate (+/−). The mutant embryo is equal in size compared with the control and appears to have developed normally. The mutant embryo is distinguished, however, by the presence of hemorrhage within the ventricle of the brain and vertebral canal.

(B) Sagittal sections of the brains show a comparable level of development in the control and mutant embryos; however, in the *AML1*$^{-/-}$ embryo, hemorrhages can be seen within the ganglia of the cranial nerves with extension into the ventricles.

(C) Sagittal section through the thoracic cavity demonstrates a hemorrhage within the pericardial space of the *AML1*$^{-/-}$ embryo.

results either in an intrinsic defect in stem cells or multipotential progenitors responsible for establishing definitive hematopoiesis or in a defect in the yolk sac and fetal liver microenvironment that prevents normal in vivo development of these cells.

AML1$^{-/-}$ ES Cells Differentiate into Primitive Erythrocytes In Vitro, but Fail to Contribute to Hematopoietic Lineages In Vivo

To define further the hematopoietic defect induced by loss of AML1, we generated ES cells with both *AML1* alleles disrupted. An *AML1*$^{+/-}$ ES cell clone was subjected to a second round of gene targeting using the replacement vector pKO4-4-*neo*, which was similar to that used in the initial targeting of *AML1* except that the *hygrB*^{R} gene was replaced by a neomycin resistance (*neo*^{R}) coding sequence. *AML1*$^{+/-}$ ES cells were electroporated with this construct and selected in media containing hygrB, G418, and GANC, and triply resistant clones were analyzed by Southern blotting for evidence of homologous recombination. Results of this analysis revealed that 20% of triply resistant clones had undergone targeted inactivation of the wild-type allele. The undifferentiated *AML1*$^{-/-}$ ES cell clones were morphologically indistinguishable from *AML1*$^{+/-}$ ES cells and had similar growth rates. Four independent *AML1*$^{-/-}$ ES cell clones with normal ploidy and single vector insertions in the targeted *AML1* alleles and three nonhomologous recombinants (*AML1*$^{+/-}$ ES cell clones) with only random integrations of the second targeting vector were selected for in vitro and in vivo differentiation studies.

The hematopoietic differentiation capacity of these ES cell lines were assayed by culturing them in vitro as embryoid bodies. When ES cells are cultured as embryoid bodies, cells of both the myeloid and erythroid series develop, and the erythroid cells express embryonic hemoglobins and thus represent primitive erythropoiesis (Wiles and Keller, 1991). Analysis of 8- to 10-day-old ES cell cultures from both control and *AML1*-deficient ES cells revealed numerous hemoglobinized embryoid bodies that contained nucleated erythrocytes (Figure 5). Thus, *AML1*-deficient ES cells retain the ability to undergo primitive erythroid differentiation in vitro. By contrast, after 16 days in culture no monocytes or macrophages were seen in the embryoid bodies from *AML1*-deficient cells, whereas numerous differentiated monocytes were present in the control cultures. These data suggest that under these in vitro conditions, loss of AML1 also impairs monocytic differentiation.

To determine whether these cells have the capacity to differentiate into hematopoietic lineages within the context of an intact animal, *AML1*$^{-/-}$ ES cells were in-

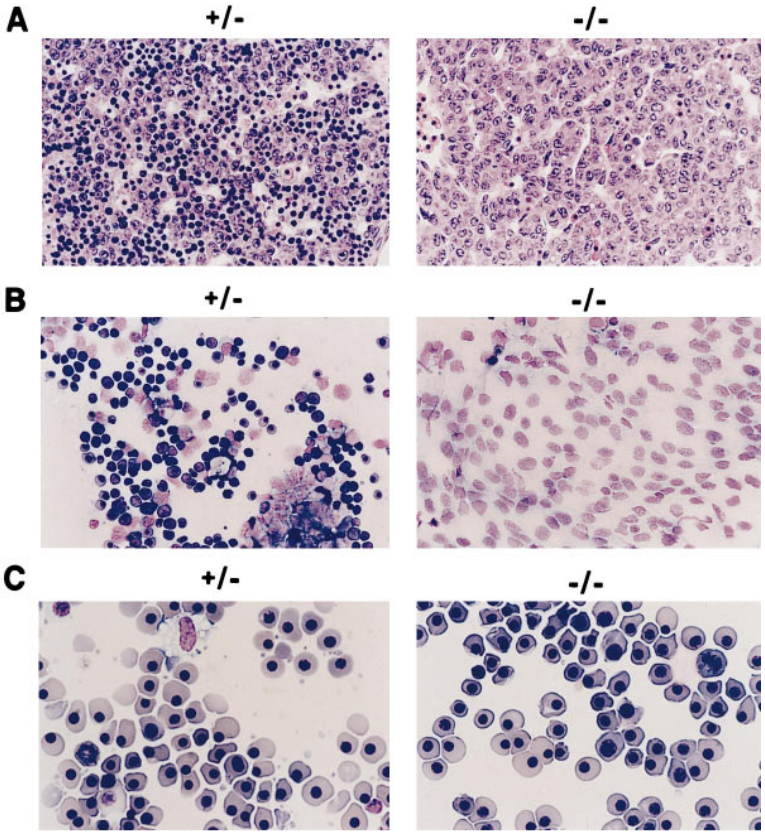

Figure 4. Histologic Analysis of the Hematopoietic System of a Control and Mutant Embryos at E11.5–E12.5.

(A) Sections of livers from an E11.5 control (+/−) embryo contained numerous dark-staining hematopoietic elements. In contrast, sections of the *AML1*$^{-/-}$ mutant (−/−) embryo revealed the complete absence of hematopoietic precursors, with only rare primitive nucleated erythrocytes seen within vessels and sinusoids.

(B) Touch preparation of liver from a control embryo contained numerous immature to midmature erythroid cells, along with scattered macrophages, immature myeloid cells, and rare megakaryocytes (data not shown). In contrast, liver touch preparation from *AML1*-deficient mice contained primarily hepatocytes, with only scattered primitive nucleated erythrocytes seen.

(C) Cytocentrifuge preparations of peripheral blood from a viable E12.5 control embryo contained primitive nucleated erythrocytes, rare nonnucleated definitive erythrocytes, immature myeloid elements, and numerous platelets. Cytocentrifuge preparations from peripheral blood of a viable E12.5 *AML1*$^{-/-}$ embryo consisted exclusively of nucleated primitive erythrocytes with a complete absence of identifiable platelets.

jected into blastocysts, and chimeric animals were analyzed for the contribution of the ES cells to the tissues of the mouse. The relative contribution of *AML1*$^{-/-}$ and *AML1*$^{+/-}$ ES cells to chimeric animals, as estimated by agouti coat color, was similar and typically ranged from 50% to 90%. The extent of contribution of the injected ES cells to individual organs was determined by use of glucose phosphate isomerase (GPI) isoform analysis (Williams et al., 1994). The E14 ES cells contain the GPI-1A isoform, whereas the C57BL/6 cells of the host embryo are GPI-1B isoform specific. We examined 13 different tissues from 1-month-old chimeric mice, and representative results are illustrated in Figure 6. *AML1*$^{-/-}$ ES cells failed to contribute to bone marrow, spleen, thymus, or peripheral blood, whereas they had considerable contribution to all of the nonhematopoietic organs examined. By contrast, *AML1*$^{+/-}$ ES cells contributed to all tissues, including sites of hematopoiesis. These data suggest that *AML1*$^{-/-}$ ES cells have a selective defect in their ability to contribute to definitive hematopoiesis and that this deficit is intrinsic to hematopoietic stem cells or multipotential progenitors.

Discussion

Molecular analyses of leukemia-associated chromosomal rearrangements have indicated that the genes encoding the AML1–CBFβ transcription factor complex are the most frequent target of translocations and inversions in human leukemia (for reviews see Nucifora and Rowley, 1995; Liu et al., 1995; Meyers and Hiebert, 1996). AML1–CBFβ directly binds the enhancer core motif and has been shown to regulate expression of a variety of hematopoietic specific genes, suggesting that it is likely to play an important role in normal hematopoietic development (Meyers and Hiebert, 1996). Using *AML1*-deficient mice, we now demonstrate that expression of

Table 2. Hematopoietic Progenitors in Yolk Sac and Fetal Liver

Yolk Sac [a] (E10.5)				Fetal Liver[b] (E11.5)			
AML1 (n)	E	M	Mix	*AML1* (n)	E	M	Mix
+/+ (5)	114 ± 34	296 ± 83	62 ± 27	+/+ (9)	11 ± 4	92 ± 28	18 ± 3
+/− (5)	152 ± 48	280 ± 47	93 ± 37	+/− (14)	11 ± 5	92 ± 23	17 ± 6
−/− (4)	0	0	0	−/− (7)	0	0	0

Abbreviations: n, numbers of embryos analyzed; E, erythroid colonies; M, myeloid colonies; Mix, erythroid–myeloid mixed colonies.
[a] Numbers represent colonies per yolk sac, mean ± SD.
[b] Numbers represent colonies per 5×10^3 fetal liver cells, mean ± SD.

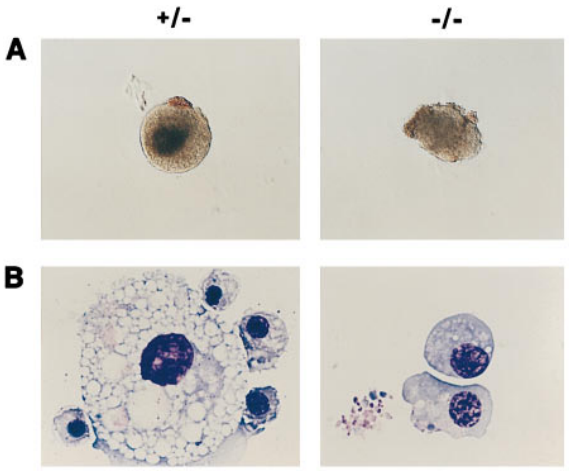

Figure 5. Embryoid Bodies Derived from Control and *AML1*-Deficient ES Cells

(A) Hemoglobinized embryoid bodies were observed in cultures of both $AML1^{+/-}$ and $AML1^{-/-}$ ES cells.

(B) Cytocentrifuge preparations of single embryoid bodies from control (+/−) and mutant (−/−) derived cultures.

AML1 is essential for the establishment of definitive hematopoiesis of all lineages. *AML1*-deficient embryos showed normal morphogenesis and had active yolk sac–derived primitive erythropoiesis; however, they lacked definitive hematopoietic progenitors in both the yolk sac and fetal liver and failed to develop fetal liver hematopoiesis. As a result of this hematopoietic defect, the $AML1^{-/-}$ embryos died during midembryonic development, secondary to hemorrhages within the brain, pericardium, and peritoneum that presumably resulted from an absence of circulating platelets, compounded by an apparently evolving anemia resulting from an inability to produce definitive nonnucleated erythrocytes. Moreover, the underlying hematopoietic defect resulting from the loss of AML1 was cell autonomous in that $AML1^{-/-}$ ES cells failed to contribute to any hematopoietic tissues in the normal microenvironment of chimeric mice.

The presence of normal primitive yolk sac hematopoiesis along with the complete absence of fetal liver–derived hematopoiesis within the *AML1*-deficient embryos suggests a primary defect in the developmental program of stem cells responsible for establishing definitive hematopoiesis. Although the presented results do not directly address the nature of this defect, possibilities include an inability of the *AML1*-deficient animals to generate stem cells capable of establishing definitive hematopoiesis or, alternatively, an inability of stem cells or multipotential progenitors to activate AML1–CBFβ target genes that are crucial for proper differentiation or proliferation. A number of putative AML1-regulated targets have been identified, including the hematopoietic growth factors IL-3 and GM-CSF (Shoemaker et al., 1990; Takahashi et al., 1995) and the growth factor receptor for CSF1 (Zhang et al., 1994); however, it is unlikely that the loss of expression of any of these genes is mechanistically involved in the observed phenotype. Mice carrying a targeted mutation in the GM-CSF gene have normal steady-state hematopoiesis but develop pulmonary alveolar proteinosis (Dranoff et al., 1994; Stanley et al., 1994). An autosomal recessive inactivating mutation in the gene encoding the CSF1 receptor ligand is the underlying defect in osteopetrotic (*op/op*) mice (Yoshida et al., 1990). These animals are characterized by impaired mononuclear phagocyte development and a deficiency of osteoclasts that results in an increase in bone density and occlusion of the marrow cavity. Moreover, mice with mutations in both the GM-CSF and *CSF1* genes present with features corresponding to mice deficient in either factor alone, with the predominant hematopoietic defect being a deficiency of mature macrophages (Lieschke et al., 1994). Similarly, IL-3 is not thought to play a major role in the regulation of embryonic hematopoietic stem cells and is expressed primarily by activated T cells, which appear well after establishment of fetal liver hematopoiesis (for review see Ihle, 1992). Therefore, it is likely that additional AML1-regulated target genes remain to be identified and that these target genes will function as components in signal transduction pathways critical for the normal survival, proliferation, or differentiation of hematopoietic stem cells that are responsible for establishing definitive hematopoiesis.

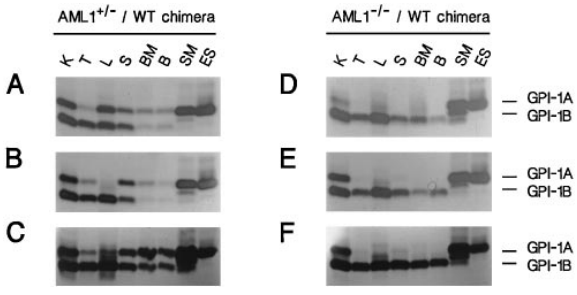

Figure 6. Contribution of $AML1^{+/-}$ and $AML1^{-/-}$ ES cells to Tissues in Chimeric Animals

GPI analysis of chimeric animals derived from three $AML1^{+/-}$ (A–C) and three $AML1^{-/-}$ (D–F) ES cell clones. ES cells contain the GPI-1A isoform, whereas the host contains the GPI-1B isoform. Results from the following tissues are illustrated: kidney (K), thymus (T), liver (L), spleen (S), bone marrow (BM), peripheral blood (B), and skeletal muscle (SM). WT, wild type.

The phenotype resulting from the loss of AML1 is distinct from the consequences of targeted mutations in a number of other genes implicated in hematopoietic development (Orkin, 1995). Mice with null mutations in either *tal1/SCL* (Shivdasani et al., 1995) or *rbtn2* (Warren et al., 1994) die during early embryonic development from severe anemia that results from the complete absence of primitive yolk sac–derived erythropoiesis. Similarly, *GATA1*-deficient ES cells do not contribute to primitive erythropoiesis in chimeric animals or in vitro (Pevny et al., 1991; Simon et al., 1992; Weiss et al., 1994). By contrast, mutations in *GATA2* lead to an early embryonic death as a result of impairment of both primitive and definitive erythropoiesis (Tsai et al., 1994).

Inactivation of two genes implicated in myeloid differentiation, c-*myb* and *PU.1*, results in phenotypes that more closely resemble the effects observed in *AML1*-deficient mice (Mucenski et al., 1991; Scott et al., 1994). Homozygous loss of c-*myb*, like *AML1*, results in normal

yolk sac–derived erythropoiesis, but a severe defect in fetal liver hematopoiesis (Mucenski et al., 1991). The major difference from the phenotypic consequences resulting from the loss of AML1 is that livers from c-*myb*-deficient mice retain hematopoietic progenitors, although at markedly reduced numbers compared to controls. In addition, mice lacking c-*myb* have normal megakaryocytic development and hence do not die from bleeding, but succumb to complications of anemia somewhat later in development. Similarly, loss of PU.1, a member of the Ets-like family of transcription factors, results in impaired fetal liver hematopoiesis; however, this defect also appears to spare megakaryocytes and to be less severe in the erythroid lineage (Scott et al., 1994). These mice die from anemia at an even later stage of development than the c-*myb*-deficient mice. The close relationship among the phenotype of mice that lack *AML1*, c-*myb*, or *PU.1* is interesting given that AML1 cooperates with c-Myb- and Ets-like family members in regulating the tissue-specific expression of several hematopoietic-specific genes (Wotton et al., 1994; Hernandez-Munain and Krangel, 1995). It is likely that AML1, c-Myb, and Ets-like proteins coordinately regulate partially overlapping sets of genes. The observed phenotypes suggest that target genes regulated by AML1 appear to be essential for the development of definitive hematopoiesis of all lineages, whereas c-Myb and PU.1 target genes are necessary in a more restricted set of lineages.

More recently, targeted disruption of the *GATA3* transcription factor was shown to result in a phenotype that is, in part, similar to that seen in the *AML1*$^{-/-}$ embryos (Pandolfi et al., 1995). *GATA3*-deficient embryos die between E11 and E12 from massive internal bleeding and have markedly suppressed fetal liver hematopoiesis; however, these mice display normal yolk sac definitive hematopoiesis, and their fetal livers contain erythroid precursors and normal megakaryocytes. In addition, *GATA3*$^{-/-}$ embryos show marked growth retardation and severe deformities of the brain and spinal cord. These data suggest that GATA3 is required for later stages of hematopoietic development than is AML1 and that it has critical functions outside of hematopoietic tissues. Thus, among the transcription factors implicated in hematopoietic development (Orkin, 1995), AML1 appears to provide unique functions in the establishment of definitive hematopoiesis of all lineages.

The fact that loss of AML1 resulted in a phenotype confined to the hematopoietic system at E12.5 was somewhat surprising given the high degree of homology of *AML1* to the Drosophila gene *runt* (Erickson et al., 1992). *runt* is a pair-rule gene involved in establishing the positional identities of individual blastoderm cells during Drosophila embryogenesis, and its mutation leads to segmentation defects and lethality (Gergen and Wieschaus, 1985; Gergen and Wieschaus, 1986). In addition, it has been shown to function in Drosophila sex determination and neural development (Duffy and Gergen, 1991; Kania et al., 1990). Moreover, murine *AML1* is widely expressed in a large number of tissues and cell types (Miyoshi et al., 1995). The absence of a more widespread phenotype in the *AML1*-deficient mice could in part be the result of noncritical functions for AML1 in these tissues, or, alternatively, its function is redundant and can be complemented by other AML1 family members. Along these lines, it is curious that bleeding appears to initiate within the cranial nerve ganglia of the *AML1*-deficient embryos. Either these structures are uniquely susceptible to bleeding at this stage of embryonic development or AML1 provides an essential function within these cells. No morphologic abnormalities were observed in cells of the nervous system of the *AML1*-deficient mice, and thus we are unable at present to address this latter issue. Detailed analysis of the expression pattern of *AML1*, *AML2*, and *AML3* during embryogenesis in both wild-type and mutant animals should help to clarify these questions.

In summary, our results demonstrate that the AML1–CBFβ transcription factor complex plays a pivotal role in regulating the transcription of genes that are essential for definitive hematopoiesis. The frequent targeting of this complex by leukemia-associated chromosomal rearrangements suggests that alteration of its activity leads to the disruption of AML1-mediated signals, which are critical for normal growth control or differentiation. We speculate that AML1 functions as a master switch that controls the ability of hematopoietic stem cells to differentiate into definitive hematopoietic elements. Alterations in its activity may result in a block in differentiation that leads to an increase in proliferating hematopoietic progenitors that are prone to acquire secondary mutations that eventually result in malignant transformation.

Experimental Procedures

Construction of *AML1* Targeting Vectors

To construct pKO4-4-*hygrB*, overlapping *AML1* genomic clones were isolated from a phage library made from strain 129–derived CCE ES cell DNA (van Deursen et al., 1992), and an 8 kb fragment containing exon 4 was subcloned into pBluescript (Stratagene). A 2 kb *hygrB*^{R} cassette (van Deursen et al., 1991) was used to replace a 28 bp unique AatII fragment containing 8 bp of intron 3 and 20 bp of exon 4 and was oriented in the opposite transcriptional orientation to the *AML1* gene. An additional 3 kb fragment that included intron 3 sequence was then ligated to the 5′ end of the construct. Finally, a 2 kb HSV–thymidine kinase (*tk*) cassette (van Deursen and Weiringa, 1992) was added to the 3′ end of the construct. The vector was linearized at a unique SalI site, 3′ to the *tk* sequences prior to introduction in ES cells. The construct for the second round of gene targeting, pKO4-4-*neo*, was constructed as described above except that the *hygrB*^{R} gene was replaced by a *neo*^{R} gene obtained from pRC/RSV (Invitrogen). This substitution introduced an XbaI restriction site, 5′ to the *neo*^{R} cassette, which was used to discriminate the targeted *AML1* alleles by Southern blot analysis.

Generation of *AML1* Mutant Mice

E14 ES cells were cultured in 60% BRL-conditioned medium (Smith and Hooper, 1987) supplemented with 1000 U/ml mouse recombinant LIF (GIBCO BRL); 50 μg/ml SalI-linearized targeting vector, pKO4-4-*hygrB* or pKO4-4-*neo*, was electroporated into 1.5×10^7 ES cells in 2 ml of phosphate-buffered saline (PBS) at 230 V and 500 μF. Selection was initiated 24 hr later by adding 90 U/ml hygrB (Calbiochem) and 2 μM GANC (Syntex) or, in the second round of targeting, by the further addition of 125 μg/ml G418 (GIBCO BRL), and selection was continued for 8 days. Doubly or triply resistant clones were analyzed by Southern blot analysis for evidence of homologous recombination. Southern blots of XbaI-digested genomic DNA were hybridized with a 0.4 kb 5′ or a 0.5 kb 3′ *AML1*

genomic fragment that corresponded to genomic sequences outside of the vector. ES cell clones with normal karyotypes were used for injections into C57BL/6 blastocysts. Male chimeras were bred with C57BL/6 females, and germline transmission of the ES cell–derived genotype was assessed by coat color. Tail biopsies of agouti offspring were screened for the disrupted *AML1* gene by Southern blot analysis as previously described (Okuda et al., 1995).

RT–PCR Analysis

Poly(A)$^+$ RNA was isolated from mouse embryos and cDNA synthesized with random hexamers as previously described (Downing et al., 1993). Amplification of *AML1* was performed with a set of oligonucleotide primers that bracketed the site of targeting within the RHD and corresponded to sequences in exons 3 and 4, respectively (Bae et al., 1993; Miyoshi et al., 1995). Parallel reactions were performed using hypoxanthine phosphoribosyl transferase (*HPRT*) primers to ensure the integrity of the RNA samples (Keller et al., 1993). Amplified PCR products were size fractionated by electrophoresis through an agarose gel, transferred to nylon membrane, and hybridized with oligonucleotides specific for either *AML1* or *HPRT*. The sequence of the oligonucleotides used were as follows: forward *AML1*, 5′-CCA GCA AGC TGA GGA GCG GCG-3′; reverse *AML1*, 5′-CCG ACA AAC CTG AGG TCG TTG-3′; *AML1* detection oligonucleotide, 5′-GTG GTG GCA CTG GGG GAC GT-3′.

Isolation and Histologic Analysis of Embryos

Embryos were removed from the uterus and inspected for gross abnormalities, and fetal membranes or embryonic tissue were taken for genotyping. Embryos were then fixed in Bouin's solution for 18 hr and embedded in paraffin, and 7 μm sections were cut and stained with hematoxylin and eosin. Peripheral blood was collected in PBS containing 50% FCS and in 10 mM EDTA for cytocentrifuge preparations. Slides of peripheral blood and impression smears of fetal livers were stained with Wright–Giemsa.

In Vitro Hematopoietic Colony Assays

Yolk sacs from E10.5 embryos were dissected and treated with 0.1% collagenase (Wong et al., 1986). Following digestion, single cell suspensions were plated in triplicate in 1.2% methylcellulose in Iscove's modified Dulbecco's medium (IMDM) containing 30% FCS, 0.1 mM β-mercaptoenthanol, 2 mM glutamine, and 1% bovine serum albumin and were supplemented with 2 U/ml human erythropoietin (Amgen), 10 ng/ml murine stem cell factor (Genzyme), 1.8 ng/ml murine IL-3 (R & D Systems), and 10 ng/ml murine GM-CSF (Genzyme). Similarly, livers from E11.5 embryos were dispersed into single cell suspensions and cultured under identical conditions. Cultures were maintained at 37°C under humidified conditions with 5% CO_2. Colonies that contained more than 50 cells were counted on day 7, and myeloid, erythroid, and mixed colonies were defined based on their morphology (Sonoda et al., 1990).

Embryoid Body Cultures

The procedure for in vitro differentiation of embryoid bodies was modified from previously published methods (Wiles and Keller, 1991; Simon et al., 1992). In brief, ES cell clones were adapted to grow in media that contained 1000 U/ml murine recombinant LIF but lacked BRL-conditioned media. Triplicate cell suspensions of 3×10^2 adapted ES cells were plated in 1 ml of IMDM containing 1.2% methylcellulose, 15% FCS, 2 mM glutamine, and 450 μM monothioglycerol. The cultures were supplemented with appropriate combinations of cytokines as described above. Embryoid bodies were scored for the presence of blood islands by visual examination. The presence of erythroid cells was confirmed by microscopic examination of cytocentrifuge preparations of single dispersed embryoid bodies.

GPI Analysis

Frozen tissue samples were thawed, homogenized in 50 mM Tris–HCl containing 0.1% Triton X-100 (Sigma), subjected to three cycles of freeze/thawing, and centrifuged, and the supernatant was used for analysis. Aliquots of appropriately diluted samples were electrophoretically separated on cellulose acetate membranes (Helena Laboratories) in a 25 mM Tris–glycine buffer (pH 8.0) for 1.5 hr at 300 V at 4°C. Following electrophoresis, membranes were stained with 10 ml of a 0.9% agarose solution that contained 5 mM magnesium acetate, 15 mg of fructose-6-phosphate, 2 mg of methylthiazolium tetrazolium, 0.36 mg of phenazine methosulfate, 2 mg of nicotinamide adenine dinucleotide phosphate, and 10 U of glucose-6-phosphate dehydrogenase, and bands appeared in 10–15 min (Williams et al., 1994).

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