AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations

Youzhong Yuan*, Liming Zhou*, Toshihiro Miyamoto†, Hiromi Iwasaki‡§, Nari Harakawa‡, Christopher J. Hetherington‡, Sebastien A. Burel*, Eric Lagasse†, Irving L. Weissman†, Koichi Akashi‡§, and Dong-Er Zhang*¶

*Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037; †Department of Pathology and Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305; ‡Department of Medicine, Harvard Medical School, Boston, MA 02115; and §Department of Cancer Immunology and AIDS, Dana–Farber Cancer Institute, Boston, MA 02115

Contributed by Irving L. Weissman, June 25, 2001

The t(8;21) is one of the most frequent chromosomal abnormalities associated with acute myeloid leukemia (AML). The translocation, which involves the *AML1* gene on chromosome 21 and the *ETO* gene on chromosome 8, generates an AML1-ETO fusion transcription factor. To examine the effect of the AML1-ETO fusion protein on leukemogenesis, we made transgenic mice in which expression of AML1-ETO is under the control of the human *MRP8* promoter (hMRP8-AML1-ETO). AML1-ETO is specifically expressed in myeloid cells, including common myeloid progenitors of hMRP8-AML1-ETO transgenic mice. The transgenic mice were healthy during their life spans, suggesting that AML1-ETO alone is not sufficient for leukemogenesis. However, after treatment of newborn hMRP8-AML1-ETO transgenic mice and their wild-type littermates with a strong DNA-alkylating mutagen, *N*-ethyl-*N*-nitrosourea, 55% of transgenic mice developed AML and the other 45% of transgenic mice and all of the wild-type littermates developed acute T lymphoblastic leukemia. Our results provide direct evidence that AML1-ETO is critical for causing myeloid leukemia, but one or more additional mutations are required for leukemogenesis. The hMRP8-AML1-ETO-transgenic mice provide an excellent model that can be used to isolate additional genetic events and to further understand the molecular pathogenesis of AML1-ETO-related leukemia.

The acute myeloid leukemia (AML)-1 gene (*AML1*, also known as *CBFA2*, *PEBP2αB*, and *RUNX1*) was initially identified as a target of chromosomal translocation in t(8;21), which is associated with ≈15% of AML (1–3). This translocation involves the *AML1* gene on chromosome 21 and the *ETO* (*MTG8*) gene on chromosome 8, and generates an AML1-ETO fusion transcription factor (4). This fusion protein consists of the N terminus of AML1 fused to a nearly full-length ETO protein (4). Native AML1 is able to form a heterodimer with CBFβ (PEBP2β) and regulate the transcription of target genes by binding to the DNA sequence TGT/cGGT through its *runt* homology domain (5–7). Subsequently, AML1 was also found to be disrupted by several other translocations, including AML1-Evi1 from t(3;21) in blast crises of chronic myeloid leukemia and in therapy-related AML (8, 9); TEL-AML1 from t(12;21), which is involved in ≈25% of childhood pre-B cell acute lymphoblastic leukemia (10); AML1-MTG16 from t(16;21) in rare cases of AML (11); and in radiation-associated AML (12). Furthermore, the function of AML1 is disrupted indirectly by the inv(16) that is found in 12–15% of AML cases (13). The inv(16) fuses *MYH11*, a smooth muscle myosin heavy chain gene, to the gene that encodes core-binding factor β (CBFβ), an AML1 heterodimeric partner. Thus, translocations targeting the AML1/CBFβ transcription factor complex are among the most frequent mutations in human acute leukemia.

Although *in vitro* studies have revealed the oncogenic potential of the *AML1-ETO* fusion gene, they do not fully represent the molecular pathogenesis of AML. Therefore, we and other groups have developed mouse models with the *AML1-ETO* fusion gene. Knocking in the *AML1-ETO* fusion gene into the *Aml1* locus has resulted in embryonic lethality and a lack of definitive hematopoiesis in the fetal liver (14, 15). These effects were strikingly similar to those seen in *AML1*$^{-/-}$ mice (16, 17). These results demonstrate that AML1-ETO is a dominant inhibitor of normal AML1/CBFβ function during early hematopoietic lineage commitment. Owing to the observed embryonic lethality, the AML1-ETO knock-in model is not informative in determining the significance of this fusion protein in leukemia. To avoid the embryonic lethality associated with AML1-ETO expression, it is essential to prevent the expression of AML1-ETO during embryogenesis and turn on its expression at a later stage of development. One such approach is to generate transgenic mice with inducible AML1-ETO expression or myeloid cell specific AML1-ETO expression. We initially developed transgenic mice with inducible AML1-ETO expression by using a tetracycline-inducible system (18). These mice did not develop leukemia despite a strong expression of AML1-ETO in their bone marrow cells upon withdrawal of tetracycline. However, the increase of replating efficiency, the delay of myeloid cell maturation, and the decrease of proliferation of progenitor cells, all of which indicate the potential effect of AML1-ETO on hematopoiesis, were observed in bone marrow *in vitro* assays. Recently, a mouse model that mimics the t(8;21) through Cre/loxP-mediated recombination has been generated (19). However, the critical role of AML1-ETO in leukemogenesis has not been directly addressed.

MRP8 is a small calcium-binding protein expressed specifically in myeloid cells of the neutrophil and monocyte lineages (20). Several transgenic mouse leukemia models that use the human *MRP8* promoter (designated as hMRP8) have been generated, including hMRP8-Bcl2, hMRP8-CBFβ-MYH11, and hMRP8-PML/RARα (21–23). In this paper, we report the generation and the analysis of hMRP8-AML1-ETO transgenic mice with leukemogenesis. Our results suggest that AML1-ETO has myeloid leukemogenic potential and needs to cooperate with additional mutations to trigger the development of leukemia.

Materials and Methods

Generation of Transgenic Mice. The 2.3-kb full-length AML1-ETO cDNA was cut out from the plasmid pUHD-AML1-ETO by *Xba*I, blunt ended, and subcloned into the blunt-ended *Bgl*II site of the hMRP8 cassette in pBluescript KS(−) (18, 23). The

Abbreviations: AML, acute myeloid leukemia; CBFβ, core-binding factor β; FACS, fluorescence-activated cell sorter; RT-PCR, reverse transcription–PCR; ENU, *N*-ethyl-*N*-nitrosourea; ALL, acute lymphocytic leukemia.

¶To whom reprint requests should be addressed at: MEM-L51, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA, 92037. E-mail: dzhang@scripps.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

hMRP8-AML1-ETO transgene includes 1.5 kb of human *MRP8* gene upstream regulatory element, a 0.5 kb of human *MRP8* gene sequence (including exon 1, intron 1, and part of exon 2) upstream of the AML1-ETO cDNA, and 0.6 kb of exon 3 and the downstream flanking sequence of the human *MRP8* gene downstream of the AML1-ETO cDNA. The transgene was released from pBluescript KS(−) by digestion with *Kpn*I and *Not*I and injected into zygotes from C57BL/6J mice. Transgenic mice were generated in the transgenic facility of Beth Israel Deaconess Medical Center (Boston, MA).

Southern and Northern Blot Analyses. Genomic DNA and RNA preparation and electrophoresis were as described previously (18). The blot was hybridized with a [^{32}P]dATP-labeled, 1.8-kb ETO probe (18).

Western Blot Analysis. Bone marrow protein samples (4×10^6 cells) were electrophoresed in an SDS/8% polyacrylamide gel (acrylamide:bisacrylamide = 29:1). Cell lysate from Kasumi-1 cells was loaded as a positive control. The protein was then transferred to nitrocellulose membrane (Amersham Pharmacia). The blot was incubated with a primary polyclonal antibody against the ETO protein (Gift from S. Hiebert, Vanderbilt University, Nashville, TN) at a dilution of 1:500 and then with a secondary monoclonal antibody conjugated to horseradish peroxidase (Amersham Pharmacia). The immune complexes were visualized by chemiluminescent substrate (NEN) according to the manufacturer's recommendations.

Flow Cytometry. For lineage marker analysis, cells (1×10^6) were incubated at 4°C for 30 min in PBS containing 2% BSA with monoclonal antibodies against Gr-1, Mac-1, B220, CD3, Ter119, c-Kit, or their isotype controls (Caltag, Burlingame, CA or PharMingen). The cells were then washed twice with PBS containing 2% BSA, fixed with 1% formaldehyde in PBS, and applied for analysis on a FACSCalibur (Becton Dickinson) fluorescence-activated cell sorter (FACS). Sorting hematopoietic stem cells and different progenitor populations for reverse transcription (RT)-PCR analysis was performed as described previously (24, 25).

RT-PCR Analysis. AML1-ETO transcripts were amplified by nested RT-PCR by using primers as described previously (26). Murine MRP8 RT-PCR (final product; 234 bp) was performed with two sets of PCR primers: outer sense primer, CAATGCCGTCTGAACTGGAGAAG; outer antisense primer, CCAGCCCTAGGCCAGAAGCTCTG; inner sense primer, GAGCAACCTCATTGATGTCTAC; and inner antisense primer, GTGGCTGTCTTTGTGAGATGCCC. The glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified by using the same amount of RT product and the following primers: sense primer, GGTGCTGAGTATGTCGTGGAGTCTA, and antisense primer, CCTGCTTCACCACCTTCTTGATGTC. Murine hypoxanthine phosphoribosyltransferase cDNA was amplified by using a sense primer, GTTCTTTGCTGACCTGCTGG, and an antisense primer— TGGGGCTGTACTGCTTAACC. Five microliters of each PCR product were then electrophoresed on a 2% agarose gel and visualized by UV light.

Hematological Analysis. Two microliters of blood was diluted in 98 μl of Türk's solution (0.01% crystal violet and 3% glacial acetic acid). White blood cell counts were performed under microscopic observation. Peripheral blood and bone marrow smears or cytospin slides were stained with Wright–Giemsa staining solutions (Fisher Scientific). Differential counts of blood and bone marrow cells were obtained by counting 200 nucleated cells for each sample.

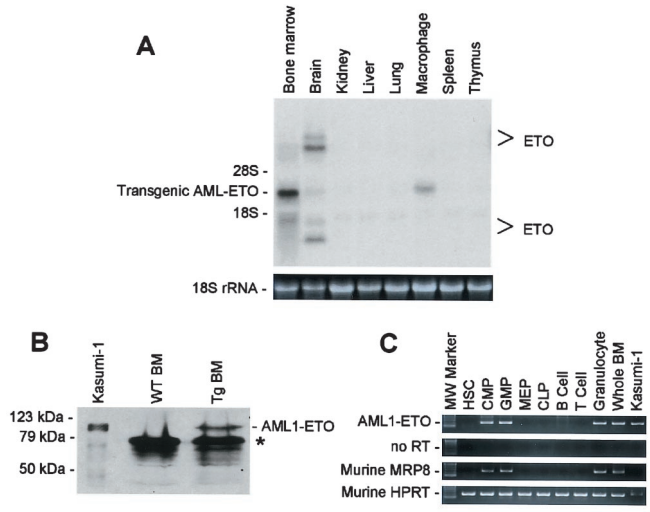

Fig. 1. Specific expression of AML1-ETO in hMRP8-AML1-ETO transgenic mice. (*A*) AML1-ETO mRNA is specifically expressed in bone marrow cells and macrophages in line no. 28. Approximately 10 μg of total RNA from different tissues was separated electrophoretically in a 1% agarose gel, transferred to nylon membrane, and hybridized with an ETO cDNA probe. The ethidium bromide staining of the 18S ribosomal RNA is presented to show the loading of the RNA samples. The positions of transcripts of endogenous ETO in the brain and transgenic AML1-ETO are marked. (*B*) AML1-ETO fusion protein is expressed in the bone marrow cells in hMRP8-AML1-ETO transgenic mice. Cell lysates from 4×10^6 bone marrow (BM) cells from wild-type mice (WT) or transgenic mice (Tg) were separated by electrophoresis on an SDS/8% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a 1:500 dilution of an anti-ETO polyclonal antibody. Lysate from Kasumi-1 cells was used as a positive control. The position of AML1-ETO is marked. The asterisk indicates nonspecific signals. (*C*) AML1-ETO mRNA is specifically expressed in the myeloid cells of hMRP8-AML1-ETO transgenic mice. Bone marrow cells from hMRP8-AML1-ETO transgenic mice were double sorted into different populations according to their surface marker expression. One thousand hematopoietic stem cells (HSC), common myeloid progenitors (CMP), granulocyte/monocyte progenitors (GMP), megakaryocyte/erythroid progenitors (MEP), common lymphoid progenitors (CLP), B cells, T cells, granulocytes, and whole bone marrow (BM) cells were subjected to RT-PCR analysis. RNA from Kasumi-1 cells was used as a positive control. PCRs without reverse transcription were used as negative controls.

***N*-ethyl-*N*-nitrosurea (ENU) Injection.** Newborn pups (less than 2 weeks old) from the breeding of transgenic mice and wild-type mice were selected for i.p. ENU injection (100 mg/kg per injection). One gram of ENU (catalog no. N3285, Sigma) was dissolved in 10 ml of 95% ethanol and then added to 90 ml of phosphate-citrate buffer (0.2 M Na_2HPO_4/0.1 M citric acid, pH 5.0). Mice were injected weekly for 3 weeks. The total ENU dosage was 300 mg/kg.

Results

Generation of hMRP8-AML1-ETO Transgenic Strains. Previous work in our laboratory by using a knock-in strategy has demonstrated that expression of AML1-ETO in mice driven by the native *AML1* promoter causes abrogation of definitive hematopoiesis and embryonic lethality (14). Okuda *et al.* (15) observed similar results. To study the role of the AML1-ETO fusion gene in hematopoiesis and leukemogenesis and to avoid AML1-ETO expression in early hematopoietic cells, we generated hMRP8-AML1-ETO transgenic mice harboring the AML1-ETO transgene driven by the human *MRP8* promoter (20). Eleven mice carrying the hMRP8-AML1-ETO transgene were identified after injection of the hMRP8-AML1-ETO transgene into C57BL/6J zygotes. Five of eleven founders gave germ-line-transmitted offspring (data not shown).

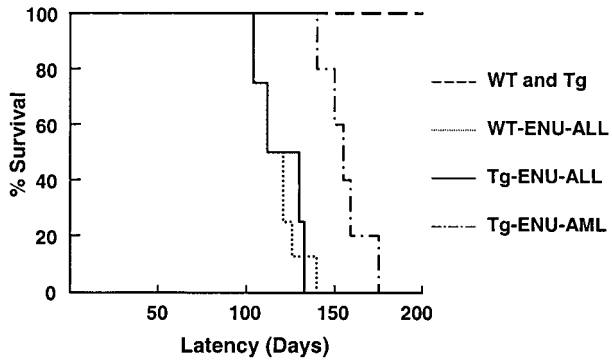

Fig. 2. Survival curves of transgenic mice (Tg) and wild- type mice (WT) after ENU treatment. Wild-type mice developed acute lymphoblastic leukemia (WT-ENU-ALL, $n = 8$). Transgenic mice developed acute lymphoblastic leukemia (Tg-ENU-ALL, $n = 4$) and AML (Tg-ENU-AML, $n = 5$). Untreated transgenic mice and wild-type mice (WT and Tg, $n = 5$, respectively) are shown as controls.

Myeloid Specific Expression of AML-ETO in Transgenic Mice. Northern blot analyses were performed to analyze AML1-ETO expression in various tissues of transgenic mice. Only one founder line (no. 28) showed bone marrow specific expression of AML1-ETO (Fig. 1*A* and data not shown). This line was used for further analysis. A relatively low level of AML1-ETO expression was also observed in the peritoneal macrophages of mice from founder line no. 28. To identify whether AML1-ETO is expressed at the protein level, bone marrow cells of transgenic and control mice were analyzed by Western blot using a polyclonal anti-ETO antibody. Protein prepared from Kasumi-1 cells was used as a positive control. As indicated in Fig. 1*B*, the AML1-ETO fusion protein was clearly detected in the bone marrow sample of the transgenic mice.

To identify bone marrow subpopulations that express AML1-ETO, bone marrow cells were sorted according to their surface markers into hematopoietic stem cells, common myeloid progenitors, common lymphoid progenitors, granulocyte/macrophage progenitors, megakaryocyte-erythroid progenitors, B cells, T cells, and granulocytes (26). One thousand cells from each population were used to perform nested RT-PCR. AML1-ETO transcripts were detected in common myeloid progenitors, granulocyte/macrophage progenitors, and granulocytes, but not in lymphoid, erythrocyte, and megakaryocyte lineages (Fig. 1*C*). This result demonstrates that AML1-ETO is expressed from the initiation of myeloid cell commitment.

Hematopoiesis in Transgenic Mice Appears Normal. To analyze the effect of AML1-ETO on hematopoiesis and its role in leukemogenesis, total white blood cell counts and differential counts of blood smears from AML1-ETO transgenic mice and their wild-type control littermates were analyzed periodically. No difference between transgenic and wild-type mice was observed (data not shown). This indicated that expression of AML1-ETO in adult mice did not result in a noticeable perturbation of hematopoiesis. The transgenic mice exhibited no outward signs of illness when they were observed for more than 12 months. *In vitro* colony assay using bone marrow cells from five transgenic mice and five wild-type control mice were also performed individually as described previously (18). The numbers of colonies from bone marrow cell cultures of both transgenic and wild-type mice were similar (data not shown). Furthermore, no difference in the distribution and the numbers of progenitors and stem cells between transgenic and normal mice can be detected with FACS analysis (data not shown).

AML Can Be Induced by ENU Treatment in Transgenic Mice. The observation of normal hematopoiesis in transgenic mice indicated the possibility that AML1-ETO itself is insufficient to trigger leukemogenesis. Additional mutations that cooperate with AML1-ETO might be necessary. To test this hypothesis, the newborn offspring from the breeding between transgenic heterozygous mice and wild-type C57BL/6J mice were injected fractionally with a total dosage of 300 mg/kg ENU, a strong DNA alkylating mutagen (see *Materials and Methods* for details). ENU has been used previously to cause mutations in the analysis of leukemogenesis (27, 28).

Four months after the ENU injections, the transgenic and wild-type mice became ill with symptoms and signs of cachexia, anemia, and labored breathing. All of the mice died or were killed because of a moribund condition within 7 months. The survival curves in Fig. 2 show the latency period after ENU treatment.

Upon postmortem examination, five of nine ENU-treated transgenic mice that had relatively longer survival times showed signs of AML, including pale femurs, enlarged spleens, and enlarged livers, but their thymuses and lymph nodes were not involved in the disease. An enlarged spleen is a very consistent feature in these leukemic mice (data not shown). Abnormal immature cells were detectable in the peripheral blood (Table 1). The total white blood cell counts and their differential counts had a large range of variation. The number of myeloblasts plus promyelocytes was significantly increased in the bone marrow (Table 2). Morphologically, these abnormally increased immature cells were characterized by a large size, oval or irregularly shaped nuclei (some with two or three nucleoli), and abundant basophilic cytoplasms with primary granules (Fig. 3). Similar cells were also observed in the spleens (Fig. 3). FACS analysis showed the increase of either the $CD11b^+Gr\text{-}1^-$ or the $CD11b^+Gr\text{-}1^+$ myeloid cell population in these leukemic mice, which indicates the accumulation of immature myeloid cells (Fig. 4). Thus, we conclude that these five transgenic mice developed AML after ENU treatment. No increase of c-Kit^+ cells was detected (data not shown). Furthermore, RT-PCR analyses indicate that AML1-ETO is continually expressed in the bone

Table 1. White blood cell and differential counts from wild-type and transgenic mice

		Differential counts, %				
Mouse type	WBC, 10^6/ml	Lymphocytes	Neutrophils	Eosinophils	Monocytes	Immature cells
WT ($n = 5$)	8.9 ± 3.8	86.3 ± 5.2	9.8 ± 4.0	0.2 ± 0.4	3.7 ± 2.0	0
Tg ($n = 5$)	10.4 ± 4.8	88.2 ± 2.8	8.0 ± 2.5	0.2 ± 0.4	3.6 ± 1.5	0
WT-ENU-ALL ($n = 5$)	35.6 ± 19.1	29.0 ± 6.8	52.8 ± 20.8	0.4 ± 0.9	3.8 ± 2.6	14.4 ± 13.9
Tg-ENU-ALL ($n = 4$)	28.1 ± 12.6	41.0 ± 0.9	34.0 ± 9.2	1.0 ± 1.4	9.5 ± 6.6	15.5 ± 14.0
Tg-ENU-AML ($n = 5$)	10.3 ± 9.7	42.2 ± 10.6	38.0 ± 19.5	0.2 ± 0.4	6.6 ± 8.0	13.0 ± 12.7

Blood was collected from healthy and leukemic wild-type (WT) and transgenic (Tg) mice. Two microliters of blood was diluted in 98 μl of Türk's solution and white blood cells (WBC) were counted manually. Blood smears were stained with Wright–Giemsa solution, and differential counts were performed under a microscope. Two hundred cells from each sample were counted. The data reported here are means ± SD.

Table 2. Differential counts of bone marrow cells from wild-type and transgenic mice

Mouse type	Differential counts, %						
	Myeloblasts + promyelocytes	Metamyelocytes + myelocytes	Neutrophils	Eosinophils	Lymphoid	Monocytes	Erythroid
WT ($n = 3$)	7.0 ± 2.0	13.7 ± 4.5	34.7 ± 5.1	1.3 ± 0.6	12.3 ± 6.5	3.0 ± 1.0	28.0 ± 16.1
WT-ENU ($n = 4$)	0	0.7 ± 1.2	1.9 ± 1.0	0	94.7 ± 1.5	0	2.7 ± 1.9
Tg-ENU-ALL ($n = 3$)	0	2.5 ± 3.5	7.0 ± 4.9	0	83.5 ± 12.0	2.5 ± 0.7	4.5 ± 0.7
Tg-ENU-AML ($n = 5$)	42.2 ± 4.4	16.5 ± 2.6	15.7 ± 3.8	3.6 ± 2.4	7.3 ± 3.8	2.8 ± 1.6	15.5 ± 8.0

Wild-type (WT) and transgenic (Tg) mice treated with ENU were killed when they were moribund. Bone marrow cells were harvested and cytospun for Wright–Giemsa staining. Differential counts were performed under a microscope. Normal wild-type mice were used as negative controls. The data reported here are means ± SD. In each bone marrow sample, 200 cells were counted.

marrow (Fig. 5*A*) and the spleen (Fig. 5*B*) of these leukemic mice.

In contrast, all of the wild-type mice and the remaining four transgenic mice showed lymphoma or acute lymphocytic leukemia (ALL) after ENU treatment. Such ENU-induced ALL has been reported (29, 30). Enlarged thymuses were evident in these mice in addition to pale femurs, enlarged spleens, and enlarged livers. Some of these mice died suddenly of heart failure due to compression of the heart by an enlarged thymus. In wild-type mice with ALL, the bone marrow was hypercellular, infiltrated with more than 90% lymphoblast cells (Table 2 and Fig. 3). Normal hematopoiesis was severely suppressed in these wild-type mice. Few myeloid cells were found in the bone marrow. FACS analysis of the representative bone marrow samples showed that the lymphoma/leukemia is of T cell origin because they express CD3 on the cell surface (Fig. 4). In transgenic mice with ALL, although the majority of cells in bone marrow were lymphoblasts, a certain percentage of myeloid cells, including neutrophils, could still be found in the bone marrow, indicating that myeloid hematopoiesis was not totally suppressed (Table 2 and data not shown).

Some of the newborn transgenic and wild-type mice were also treated with lower doses of ENU (100–200 mg/kg). The pathological phenotypes appeared much later in these mice. Furthermore, not all of these mice developed leukemia. However, AML was detected only in hMRP8-AML1-ETO transgenic mice. Hematopoietic cells of these AML mice showed the similar morphology and surface marker expression to high-dose ENU-injected AML mice (Figs. 3 and 4). We analyzed the distribution of hematopoietic progenitors in the bone marrow of one AML mouse in this group (Fig. 6). Non-ENU-treated, healthy transgenic mice exhibited normal distribution of $Lin^-Sca\text{-}1^-c\text{-}Kit^+$ myeloid progenitors that include $Fc\gamma RII/III^{lo}CD34^+$ common myeloid progenitors, $Fc\gamma RII/III^{hi}CD34^+$ granulocyte/macrophage progenitors, and $Fc\gamma RII/III^{lo}CD34^-$ megakaryocyte/erythroid progenitors. In contrast, the $Lin^-Sca\text{-}1^-c\text{-}Kit^+$ bone marrow in the AML mice was mostly occupied by an $Fc\gamma RII/III^{lo\text{-}hi}CD34^+$ population, which represents the transition from the common myeloid progenitor to the granulocyte/macrophage progenitor stages. These data strongly suggest that in the AML mouse bone marrow, leukemic transformation occurred after the commitment to granulocyte/macrophage lineages, and the proliferating leukemia clone inhibited the population of megakaryocytes and erythroid cells.

Discussion

Following the analyses of AML1-ETO knock-in mice and transgenic mice with tetracycline-inducible AML1-ETO expression (14, 18), we report here another mouse model that has AML1-ETO expression under the control of a myeloid-specific promoter from the human *MRP8* gene. An apparent correlation of

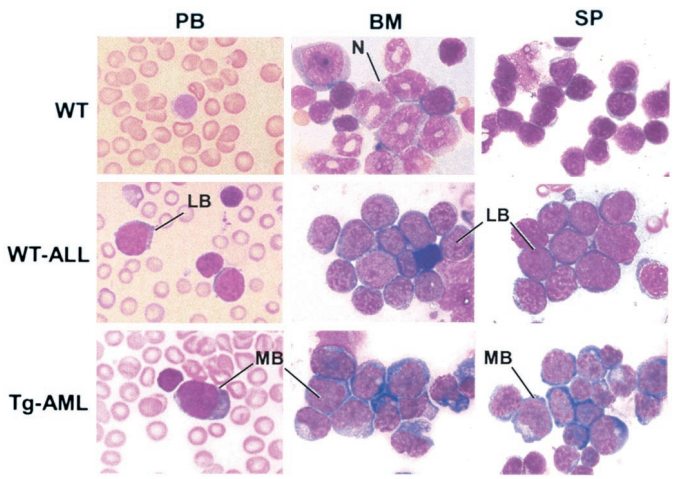

Fig. 3. Development of leukemia in transgenic mice and wild-type littermates after ENU treatment. Wright–Giemsa staining of peripheral blood smears (PB), bone marrow cytospins (BM), and spleen cytospins (SP) from representative leukemic and wild-type mice. N, neutrophil; LB, lymphoblast; MB, myeloblast. (Original magnification, ×1,000.)

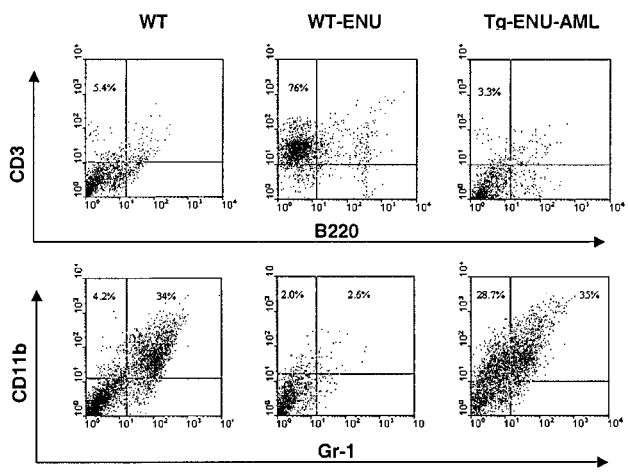

Fig. 4. Flow cytometry analysis of bone marrow cells from leukemic transgenic mice. Healthy wild-type mice (WT), wild type with signs of disease (WT-ENU), and AML1-ETO-transgenic mice with signs of disease (Tg-ENU) were killed and single-cell suspensions were made from the bone marrow. Cells were double stained with anti-CD3 [phycoerythrin (PE)-labeled] and anti-B220 (FITC-labeled) or anti-CD11b-1 (PE) and anti-Gr-1 (FITC) antibodies. The percentages of $CD3^+/B220^-$ cells, $CD11b^+/Gr\text{-}1^-$, and $CD11b^+/Gr\text{-}1^+$ double-positive cells are indicated. Consistent results were obtained from the analysis of four WT-ENU mice and six Tg-ENU-AML mice.

MEDICAL SCIENCES

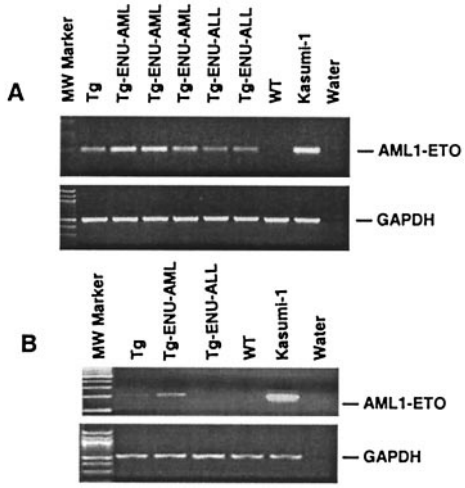

Fig. 5. Detection of AML1-ETO expression in the spleen and bone marrow cells from transgenic mice without ENU treatment (Tg), leukemic transgenic mice (Tg-ENU-AML or Tg-ENU-ALL), and wild-type (WT) mice. Representative results are shown. Kasumi-1 cell line RNA was used as a positive control. (*A*) RT-PCR analysis of bone marrow cells from three AML and two ALL transgenic mice. (*B*) RT-PCR analysis of spleen cells from one AML and one ALL transgenic mice. PCR fragments were not detectable in the absence of an RT reaction (data not shown). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight.

endogenous murine *MRP8* gene expression and AML1-ETO expression driven by the human *MRP8* promoter in myeloid cells of early and late developmental stages was demonstrated by using cell sorting and RT-PCR assays. Although AML1-ETO expression is clearly detected in the myeloid progenitors of these mice, they do not develop leukemia. This result further supports our previous conclusion from studies of transgenic mice with tetracycline-inducible AML1-ETO expression that AML1-ETO alone is not sufficient to cause leukemia. The *MRP8* promoter has been used successfully by several other groups to establish mouse models with abnormal myelopoiesis (21–23). These reports indicate that human *MRP8* is an excellent regulatory element to direct expression of oncogenes in analysis of their functions in myelopoiesis and that it is achievable to generate human leukemia models in mice. Furthermore, transgenic mouse models of leukemia that use other promoters for fusion proteins, such as BCR/ABL (31, 32), PML/RARα (33–35), PLZF-RARα (36, 37), and E2A-HLF (38), have been reported. The difficulties of generating AML1-ETO-associated leukemia mouse models may be due to the unique function of AML1-ETO in leukemogenesis. AML1-ETO expression in knock-in mice blocks the development of definitive hematopoietic cells (14, 15). Furthermore, inducible expression of AML1-ETO in the myeloid cell line U937 has shown that AML1-ETO expression not only blocks cell differentiation but also delays the cells cycle (39). The hypothesis that AML1-ETO alone is insufficient to cause leukemia is also supported by studies in remission patients. AML1-ETO expression can often be detected by RT-PCR in patients remaining in clinical remission years after treatment and in nonleukemic stem cells from remission patients (26). Furthermore, no previous data have demonstrated that AML1-ETO is directly involved in the development of AML.

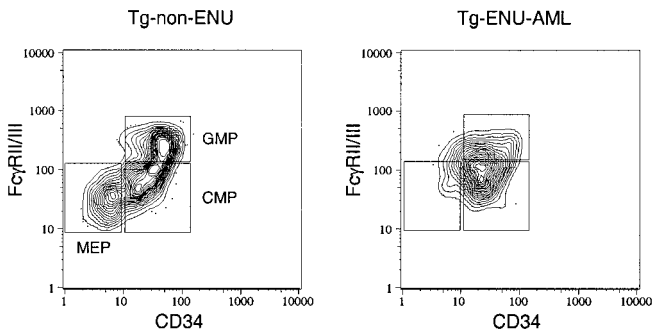

Fig. 6. Identification of myeloid progenitor populations in transgenic mice. FACS analysis of bone marrow cells from a non-ENU-treated healthy transgenic mouse (*Left*) and an ENU-treated AML transgenic mouse (*Right*). The distribution of $Lin^-/Sca\text{-}1^-/c\text{-}Kit^+$ bone marrow myeloid progenitors is presented based on the expression of CD34 and Fcγ receptors II/III (FcγRII/III). CMP, common myeloid progenitors; GMP, granulocyte/monocyte progenitors; MEP, megakaryocyte/erythroid progenitors.

To demonstrate that AML1-ETO expression is necessary but not sufficient for the development of myeloid leukemia, we have used ENU treatment to induce further mutations in hMRP8-AML1-ETO mice. With ENU treatment, 55% of hMRP8-AML1-ETO transgenic mice developed AML; the other 45% of hMRP8-AML1-ETO transgenic mice and 100% of wild-type littermates developed acute lymphoblastic leukemia. Our results provide direct evidence that AML1-ETO plays an important role in the *in vivo* development of myeloid leukemia. However, expression of AML1-ETO alone is insufficient to induce leukemia. ENU treatment results in one or more additional mutations that cooperate with AML1-ETO to induce AML. Our conclusion is supported by observations in chimeric AML1 heterodimer partner CBFβ fusion CBFβ-MYH11 knock-in mice (28). These mice do not develop leukemia in the first year of their lives. Chimeric CBFβ-MYH11 knock-in mice developed leukemia within 6 months of ENU treatment, indicating that CBFβ-MYH11 predisposes these mice to leukemia but that one or more other mutations are required to progress to the development of leukemia. Taken together, most of the CBF-subunit-containing fusion proteins may have very similar oncogenic potential to predispose leukemia *in vivo*, with the exception of the AML1-Evi1 fusion protein, which alone induces AML in mice by retroviral transduction (40). Indeed, Evi1 is the only fusion partner of AML1 that has been implicated by itself in the development of myeloid leukemia in mice (41).

ENU is a strong carcinogenic mutagen. ENU transfers its ethyl group to oxygen or nitrogen radicals in DNA, resulting in mispairing and base pair substitution. ENU predominantly modifies A·T base pairs, with 44% A·T to T·A transversions and 38% A·T to G·C transitions. When translated into a protein product, these changes result in 64% missense mutations, 10% nonsense mutations, and 26% splicing errors (42). ENU has been successfully used for inducing mutations in mice (27). We injected newborn offspring from crosses of heterozygous transgenic mice and wild-type littermates with ENU by using fractioned doses. A high dose and a fractionated injection are used because mutations are induced with a higher efficiency compared with a single dose (43). The high incidence of T cell lymphoma/leukemia in nontransgenic mice and nearly 50% of the transgenic mice is consistent with another study in which ENU was used to induce a high incidence of lymphoma in mice (29). However, the high percentage of AML in AML1-ETO transgenic mice after a high dose of ENU indicates that AML1-ETO could cooperate with additional mutation(s) in leukemogenesis and that AML1-ETO has a critical role in deciding the lineage of leukemia cells. It has been reported recently that AML1-ETO activates transcription of the granulocyte colony-stimulating factor receptor (44). The enhanced granulocyte colony-stimulating factor signal transduction due to the increase of its receptor may affect lineage commitment. Furthermore, AML1-ETO expression might lead to the down-regulation of currently unknown critical factors

related to T cell lineage commitment. These factors may block normal cell differentiation toward T cells.

The nature of the particular mutation(s) that can cooperate with AML1-ETO remains to be defined. However, one would predict that these should include genes in granulocyte/macrophage-related pathways involved in growth factors regulating cell proliferation, differentiation, and cell survival. Supporting this interpretation is our recent data from tetracycline-inducible AML1-ETO-expressing myeloid cell lines U937T-A/E (39). U937T-A/E cell lines are generated in the absence of AML1-ETO expression. Upon AML1-ETO expression, the proliferation of AML1-ETO-positive cells is severely reduced and most of the cells eventually undergo apoptosis, indicating that AML1-ETO has a negative effect on cell proliferation. Furthermore, AML1-ETO expression also blocks neutrophilic differentiation in U937T-A/E cells. In addition, the *in vitro* replating assay using bone marrow cells collected from tetracycline-inducible AML1-ETO-expressing transgenic mice also shows a defect in cell proliferation with decreased colony number and colony size (18). These data indicate that although AML1-ETO can block cell differentiation, its inhibitory effect on cell proliferation does not favor a solitary role in leukemogenesis. Additional mutations that are associated with an increase in cell proliferation and a block of apoptosis might trigger the development of leukemia in combination with AML1-ETO-blocked cell differentiation (45). In fact, the activation mutations in the c-Kit receptor tyrosine kinase associated with core-binding factor-related leukemias suggest that activated growth factor signals may increase cell proliferation, enhance cell survival, and cooperate with AML1-ETO to induce transformation (46).

Thus, our data strongly suggest that an acquisition of AML1-ETO is not sufficient for cells to become leukemic, but AML1-ETO can cooperate with mutated gene(s) in granulocyte/macrophage-committed progenitors for transformation into AML-M2. Ultimately, our transgenic mice provide a valuable tool for further exploring the mechanisms of AML1-ETO action, for isolating the additional genetic events that collaborate with AML1-ETO to induce AML, and for exploring therapeutic approaches that may improve the survival of patients with t(8;21) and other leukemias.

We thank Scott Hiebert for providing the Anti-ETO polyclonal antibody, Joel Lawitts for technical assistance in generating transgenic mice, and Daniel Tenen, David Traver, and members in the laboratory of D.-E.Z. for valuable discussions. This work was supported by National Institutes of Health Grant CA72009. D.-E.Z. is a Leukemia and Lymphoma Society Scholar. S.A.B. is supported by a fellowship from the Lady Tata Memorial Trust. The Stein Endowment Fund has partially supported the Departmental Molecular Biology Service Laboratory for DNA Sequencing and Oligonucleotide Synthesis. This is manuscript 13974-MEM from The Scripps Research Institute.

1. Downing, J. R., Higuchi, M., Lenny, N. & Yeoh, A. E. (2000) *Semin. Cell Dev. Biol.* **11,** 347–360.
2. Friedman, A. D. (1999) *Leukemia* **13,** 1932–1942.
3. Lutterbach, B. & Hiebert, S. W. (2000) *Gene* **245,** 223–235.
4. Miyoshi, H., Shimizu, K., Kozu, T., Maseki, N., Kaneko, Y. & Ohki, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10431–10434.
5. Meyers, S., Lenny, N. & Hiebert, S. W. (1995) *Mol. Cell. Biol.* **15,** 1974–1982.
6. Kagoshima, H., Shigesada, K., Satake, M., Ito, Y., Miyoshi, H., Ohki, M., Pepling, M. & Gergen, P. (1993) *Trends Genet.* **9,** 338–341.
7. Wang, S., Wang, Q., Crute, B. E., Melnikova, I. N., Keller, S. R. & Speck, N. A. (1993) *Mol. Cell. Biol.* **13,** 3324–3339.
8. Mitani, K., Ogawa, S., Tanaka, T., Miyoshi, H., Kurokawa, M., Mano, H., Yazaki, Y., Ohki, M. & Hirai, H. (1994) *EMBO J.* **13,** 504–510.
9. Nucifora, G., Birn, D. J., Espinosa, R., Erickson, P., LeBeau, M. M., Roulston, D., McKeithan, T. W., Drabkin, H. & Rowley, J. D. (1993) *Blood* **81,** 2728–2734.
10. Golub, T. R., Barker, G. F., Bohlander, S. K., Hiebert, S. W., Ward, D. C., Bray-Ward, P., Morgan, E., Raimondi, S. C., Rowley, J. D. & Gilliland, D. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4917–4921.
11. Gamou, T., Kitamura, E., Hosoda, F., Shimizu, K., Shinohara, K., Hayashi, Y., Nagase, T., Yokoyama, Y. & Ohki, M. (1998) *Blood* **91,** 4028–4037.
12. Hromas, R., Shopnick, R., Jumean, H. G., Bowers, C., Varella-Garcia, M. & Richkind, K. (2000) *Blood* **95,** 4011–4013.
13. Liu, P., Tarle, S. A., Hajra, A., Claxton, D. F., Marlton, P., Freedman, M., Siciliano, M. J. & Collins, F. S. (1993) *Science* **261,** 1041–1044.
14. Yergeau, D. A., Hetherington, C. J., Wang, Q., Zhang, P., Sharpe, A. H., Binder, M., Marin-Padilla, M., Tenen, D. G., Speck, N. A. & Zhang, D. E. (1997) *Nat. Genet.* **15,** 303–306.
15. Okuda, T., Cai, Z., Yang, S., Lenny, N., Lyu, C., van Deursen, J. M. A., Harada, H. & Downing, J. R. (1998) *Blood* **91,** 3134–3143.
16. Okuda, T., van Deursen, J. M. A., Hiebert, S. W., Grosveld, G. & Downing, J. R. (1996) *Cell* **84,** 321–330.
17. Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H. & Speck, N. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3444–3449.
18. Rhoades, K. L., Hetherington, C. J., Harakawa, N., Yergeau, D. A., Zhou, L., Liu, L. Q., Little, M. T., Tenen, D. G. & Zhang, D. E. (2000) *Blood* **96,** 2108–2115.
19. Buchholz, F., Refaeli, Y., Trumpp, A. & Bishop, J. M. (2000) *EMBO Rep.* **1,** 133–139.
20. Lagasse, E. & Weissman, I. L. (1992) *Blood* **79,** 1907–1915.
21. Brown, D., Kogan, S., Lagasse, E., Weissman, I., Alcalay, M., Pelicci, P. G., Atwater, S. & Bishop, J. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2551–2556.
22. Kogan, S. C., Lagasse, E., Atwater, S., Bae, S. C., Weissman, I., Ito, Y. & Bishop, J. M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 11863–11868.
23. Lagasse, E. & Weissman, I. L. (1994) *J. Exp. Med.* **179,** 1047–1052.
24. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. (2000) *Nature (London)* **404,** 193–197.
25. Kondo, M., Weissman, I. L. & Akashi, K. (1997) *Cell* **91,** 661–672.
26. Miyamoto, T., Weissman, I. L. & Akashi, K. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7521–7526.
27. Breuer, M., Slebos, R., Verbeek, S., van Lohuizen, M., Wientjens, E. & Berns, A. (1989) *Nature (London)* **340,** 61–63.
28. Castilla, L. H., Garrett, L., Adya, N., Orlic, D., Dutra, A., Anderson, S., Owens, J., Eckhaus, M., Bodine, D. & Liu, P. P. (1999) *Nat. Genet.* **23,** 144–146.
29. Frei, J. V. & Lawley, P. D. (1980) *J. Natl. Cancer Inst.* **64,** 845–856.
30. Breuer, M., Wientjens, E., Verbeek, S., Slebos, R. & Berns, A. (1991) *Cancer Res.* **51,** 958–963.
31. Heisterkamp, N., Jenster, G., ten Hoeve, J., Zovich, D., Pattengale, P. K. & Groffen, J. (1990) *Nature (London)* **344,** 251–253.
32. Huettner, C. S., Zhang, P., Van Etten, R. A. & Tenen, D. G. (2000) *Nat. Genet.* **24,** 57–60.
33. He, L. Z., Tribioli, C., Rivi, R., Peruzzi, D., Pelicci, P. G., Soares, V., Cattoretti, G. & Pandolfi, P. P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5302–5307.
34. Grisolano, J. L., Wesselschmidt, R. L., Pelicci, P. G. & Ley, T. J. (1997) *Blood* **89,** 376–387.
35. Early, E., Moore, M. A., Kakizuka, A., Nason-Burchenal, K., Martin, P., Evans, R. M. & Dmitrovsky, E. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7900–7904.
36. Cheng, G. X., Zhu, X. H., Men, X. Q., Wang, L., Huang, Q. H., Jin, X. L., Xiong, S. M., Zhu, J., Guo, W. M., Chen, J. Q., *et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6318–6323.
37. He, L., Bhaumik, M., Tribioli, C., Rego, E. M., Ivins, S., Zelent, A. & Pandolfi, P. P. (2000) *Mol. Cell* **6,** 1131–1141.
38. Smith, K. S., Rhee, J. W., Naumovski, L. & Cleary, M. L. (1999) *Mol. Cell. Biol.* **19,** 4443–4451.
39. Burel, S. A., Harakawa, N., Zhou, L., Pabst, T., Tenen, D. & Zhang, D. E. (2001) *Mol. Cell. Biol* **21,** 5577–5590.
40. Cuenco, G. M., Nucifora, G. & Ren, R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 1760–1765. (First Published February 4, 2000, 10.1073/pnas.030421197)
41. Mucenski, M. L., Taylor, B. A., Ihle, J. N., Hartley, J. W., Morse, H. C., III, Jenkins, N. A. & Copeland, N. G. (1988) *Mol. Cell. Biol.* **8,** 301–308.
42. Justice, M. J., Noveroske, J. K., Weber, J. S., Zheng, B. & Bradley, A. (1999) *Hum. Mol. Genet.* **8,** 1955–1963.
43. Hitotsumachi, S., Carpenter, D. A. & Russell, W. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6619–6621.
44. Shimizu, K., Kitabayashi, I., Kamada, N., Abe, T., Maseki, N., Suzukawa, K. & Ohki, M. (2000) *Blood* **96,** 288–296.
45. Zhang, D. E., Burel, S., Zhou, L., Hetherington, C. J. & Yuan, Y. (2001) *Blood Cells Mol. Dis.* **27,** 368–376.
46. Beghini, A., Peterlongo, P., Ripamonti, C. B., Larizza, L., Cairoli, R., Morra, E. & Mecucci, C. (2000) *Blood* **95,** 726–727.