Dysplastic definitive hematopoiesis in *AML1/EVI1* knock-in embryos

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The *AML1/EVI1* chimeric gene is created by the t(3;21)(q26;q22) chromosomal translocation seen in patients with leukemic transformation of myelodysplastic syndrome or blastic crisis of chronic myelogenous leukemia. We knocked-in the *AML1/EVI1* chimeric gene into mouse *Aml1* genomic locus to explore its effect in developmental hematopoiesis in vivo. *AML1/EVI1/+* embryo showed defective hematopoiesis in the fetal liver and died around embryonic day 13.5 (E13.5) as a result of hemorrhage in the central nervous system. The peripheral blood had yolk-sac–derived nucleated erythroblasts but lacked erythrocytes of the definitive origin. Although E12.5 fetal liver contained progenitors for macrophage only, E13.5 fetal liver contained multilineage progenitors capable of differentiating into dysplastic myelocyte and megakaryocyte. No erythroid progenitor was detected in E12.5 or E13.5 fetal liver. Hematopoietic progenitors from E13.5 *AML1/EVI1/+* fetal liver were highly capable of self-renewal compared with those from wild-type liver. Maintained expression of *PU.1* gene and decreased expression of *LMO2* and *SCL* genes may explain the aberrant hematopoiesis in *AML1/EVI1/+* fetal liver. (Blood. 2005;106:2147-2155)

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Introduction

The t(3;21)(q26;q22) chromosomal translocation occurs in patients with aggressive transformation of myelodysplastic syndrome (MDS) or chronic myelogenous leukemia (CML).[1-4] In the joining region of t(3;21)(q26;q22), the *AML1* gene on 21q22 is fused with the *EVI1* (ecotropic viral integration site-1) gene on 3q26.[5] The resultant *AML1/EVI1* fusion gene is translated in frame to generate a chimeric transcription factor in which the N-terminal of AML1, including its DNA-binding Runt domain, is fused to almost the entire portion of EVI1. This chimeric transcription factor could be a molecular culprit for the leukemic progression of stem-cell malignancies caused by t(3;21)(q26;q22).

AML1 is involved in transcriptional regulation of a number of hematopoietic cell-specific genes. The Runt domain of AML1 binds to a specific DNA consensus sequence named polyomavirus enhancer binding protein 2 (PEBP2; ACCRCA), together with a non–DNA-binding β subunit (core-binding factor β [CBFβ]/PEBP2β) to form a heterodimeric active transcription factor complex.[6-10] *AML1*- or *CBFβ*-deficient mice are embryonic lethal at embryonic day 12.5 (E12.5) and show massive hemorrhage in the central nervous system (CNS) with lack of hematopoiesis in the fetal liver.[11-15] A recent study demonstrated that inactivation of AML1 in adult mice results in megakaryocyte maturation arrest, defect in T- and B-lymphocyte development, and increase in hematopoietic precursor cells.[16]

The *EVI1* gene was initially identified as the frequent retrovirus integration site in myeloid tumors in AKXD mice.[17] EVI1 is less detected in normal hematopoietic cells but highly expressed in some patients of MDS or acute myelogenous leukemia (AML).[18,19]

EVI1 has 2 zinc finger domains, one in the N-terminal and the other in the C-terminal region. EVI1 is reported to interfere with the transforming growth factor β (TGFβ) signaling and to antagonize its growth inhibitory effect through targeting an intracellular signal transducer Smad3.[20] EVI1 is also known to enhance activator protein 1 (AP-1) activity[21] or block c-Jun N-terminal kinase (JNK) activity.[22] These findings suggest the versatile nature of this molecule in malignant transformation of hematopoietic cells.

The molecular characterization of AML1/EVI1 points to 2 major mechanisms of its leukemogenic effect; one is the dominant suppression of the functions of wild-type AML1, and the other is the ectopic expression of EVI1 molecule in the hematopoietic cells.[23] AML1/EVI1 binds to the PEBP2 site through its Runt domain and suppresses the expression of AML1 target genes by recruiting corepressor C-terminal binding protein by the EVI1 portion.[24,25] This dominant-negative effect is a common feature among AML1-related chimeric molecules, including AML1/ETO (eight-twenty-one) in t(8;21)(q22;q22) and translocation ets leukemia (TEL)/AML1 in t(12;21)(p13;q22).[9,23,26-29] By suppressing the expression of canonical AML1 target genes, AML1/EVI1 distorts the normal function of AML1 and may lead to leukemia development. The second mechanism of leukemogenesis is brought by the EVI1 part; inhibition of TGFβ signaling,[30] stimulation of AP-1 activity,[31] or repression of JNK activity.[22] Thus, EVI1 portion also contributes to the oncogenicity of AML1/EVI1.

Of the AML1-fusion proteins, AML1/EVI1 and AML1/ETO are of particular interest. They show a similar structure; N-terminal half of AML1, including the Runt domain, is fused to almost the

entire of EVI1 or ETO, both of which are the zinc finger protein.[23,28,29] Moreover, AML1/EVI1 and AML1/ETO mediate dominant-negative effects over wild-type AML1 by recruiting corepressors via EVI1[23] and ETO portions,[28,29] respectively. However, despite such similarities in molecular structure and function, AML1/EVI1 and AML1/ETO are differentially associated with the disease phenotype. AML1/EVI1 aggravates MDS or CML,[1-4] while AML1/ETO develops de novo AML subtype M2 of the French-American-British classification.[29] A possible explanation for such different disease settings is that AML1/EVI1 and AML1/ETO perturb different signaling pathway. Thus, exploring the molecular functional differences between AML1/EVI1 and AML1/ETO in vivo would advance our understanding toward differential role of the chimeric transcription molecules in causing different forms of leukemia.

For a direct comparison of AML1/EVI1– and AML1/ETO-expressing animals, we created *AML1/EVI1* knock-in mice and compared its phenotype with that of *AML1/ETO* knock-in mice described previously.[32,33] *AML1/EVI1/+* embryos died around E13.5 resulting from hemorrhage in the brain and the spinal cord as a result of impaired definitive hematopoiesis in the fetal liver. However, E13.5 fetal liver contained hematopoietic progenitors with enhanced replating efficiency. These phenotypes are shared between *AML1/EVI1/+* and *AML1/ETO/+* embryos. On the other hand, unique to *AML1/EVI1/+* mice was that hematopoietic progenitors in the fetal liver cannot develop to erythroid cells but can develop into dysplastic myeloid and megakaryocytic cells, whereas hematopoietic progenitors in the *AML1/ETO/+* fetal liver produce cells of all 3 lineages with normal morphology. Expression analysis for the crucial genes revealed that *AML1/EVI1/+* fetal liver maintained normal level of the *PU.1* gene expression, but lacked expression of the *LMO2* and *SCL* genes. Normal expression of the *PU.1* gene may support maintenance of the progenitor cells capable of differentiating into the myeloid and megakaryocytic lineages, and the low levels of *LMO2* and *SCL* genes expression could lead to blockade of erythroid differentiation in *AML1/EVI1/+* fetal liver. Dysplastic hematopoiesis seen in *AML1/EVI1/+* embryo partly recapitulates that observed in patients with the t(3;21)(q26;q22) translocation, indicating the pathogenic role for this chimeric protein in t(3;21)(q26;q22)-related leukemia.

Materials and methods

Construction of AML1/EVI1 knock-in targeting vector

Murine *Aml1* genome derived from a genomic library was amplified by polymerase chain reaction (PCR) method to produce a DNA fragment that includes intron 4 and the first 94 base pair (bp) of exon 5 with silent mutations at codons 519 and 522 of exon 5 creating a *Sac*II cleavage site. In the same fashion, another DNA fragment including the last 16 bp of human *AML1* exon 5 and 5′ part of subsequent *EVI1* sequence was also amplified using human *AML1/EVI1* cDNA[5] as a template to create a *Sac*II site by introducing silent mutations at identical codons. These 2 PCR products were subcloned into the human *AML1/EVI1* expression plasmid, pME18S-AML1/EVI1,[5] to give an in-frame fusion of the first 94 bp of murine *Aml1* exon 5 to the following portion of human *AML1/EVI1* cDNA (Figure 1A). The Neo-resistant gene derived from *pBK-Neo*[32] was added downstream of the *AML1/EVI1* sequence. A 4.1-kb (kilobase) *Xba*I-*Avr*II fragment from intron 4 and a 4.3-kb *Eco*RI fragment from intron 5 of murine *Aml1* genome were used for homologous recombination. To create the final targeting vector, the DNA fragment containing the previously mentioned sequences was inserted into the *Not*I and *Cla*I sites of pBluescript-DTA (diphtheria toxin-A gene), in which the *Dta* gene from pMC1DTApolyA (Stratagene, La Jolla, CA) was subcloned into the *Sal*I site of pBluescript. The construct was verified by restriction endonuclease mapping and DNA sequencing.

Production of chimeric mice

The linearized targeting vector (50 μg) was electroporated into TT2 embryonic stem (ES) cells.[34] G418-resistant clones were analyzed for homologous recombination by Southern analysis hybridizing *Eco*RI-digested DNA with a probe 5′ to the 5′ homologous sequence in the targeting vector and a *Neo* gene probe (Figure 1B). Heterozygous *AML1/EVI1/+* clones with undifferentiated morphology were injected into morulas of the ICR mouse strain. Chimeric males were bred with C57BL/6 females, and whole-body specimens of resultant embryos were analyzed for the presence of the knock-in allele by Southern analysis.

Visual inspection and histologic analysis

Embryos were removed from the uterus, dissected free from the fetal membranes, and inspected under a dissecting microscope for evidence of gross abnormalities. Embryos were fixed in formaldehyde solution and embedded in paraffin. The sections were stained with hematoxylin and eosin solution. Peripheral blood was collected in 10 mM EDTA (ethylenediaminetetraacetic acid), and smears were stained with Wright-Giemsa solution.

Histologic images were obtained on either an Olympus AX80 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera, a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with an Olympus DP12 digital camera, or an Olympus IMT-2 microscope equipped with an Olympus DP12 digital camera. Each microscope was equipped with a 10 ×/.22 ocular lens. Images were cropped in Adobe Photoshop CS (Adobe Systems, San Jose, CA) and composed in Canvas 9 (Polaroid, Waltham, MA).

Cell culture, transfection, and induction of megakaryocytic differentiation

K562 cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum. To obtain bulk transfectant of mock or the *AML1/EVI1* cDNA, 1×10^7 K562 cells were electroporated with 50 μg pCXN2 or pCXN2-AML1/EVI1 at 380 V and 975 μF using Gene Pulser (Bio-Rad, Hercules, CA) and selected with 0.4 mg/mL G418 (Sigma-Aldrich, St Louis, MO). Resistant cells were screened for expression of AML1/EVI1 chimeric
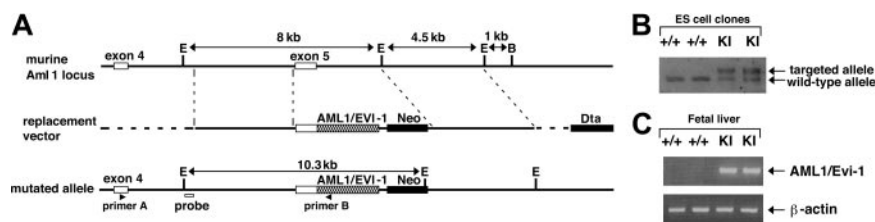

Figure 1. Gene targeting strategy to introduce AML1/EVI1 chimeric gene into the murine AML1 locus. (A) Schematic presentation of partial murine *Aml1* genomic locus (top line), replacement vector containing a partial human *AML1/EVI1* cDNA, a *neomycin resistance* cassette (Neo) for positive selection and a *diphtheria toxin-A* cassette (Dta) for negative selection (middle line), and the targeted allele (bottom line). (B) Southern analysis of wild-type (+/+) and *AML1/EVI1* knock-in (KI) ES cell clones. For detecting homologous recombination, the murine *Aml1*-specific probe indicated in the lower line of panel A was used. (C) RT-PCR analysis on E12.5 fetal liver cells from wild-type (+/+) and KI embryos. *AML1/EVI1* mRNA was amplified using primers A and B indicated in the lower line of panel A. Amplification was also performed for β-actin mRNA as a control for the presence of amplifiable RNA.

proteins by Western analysis using anti-AML1 Ab-1 antibody (Oncogene Research Products, San Diego, CA). To induce megakaryocytic differentiation, 100 nM staurosporine (Sigma-Aldrich) was added to the culture.

DNA content analysis

Each mock or AML1/EVI1–expressing K562 bulk population treated with 100 nM staurosporine was washed, fixed, and stained with 50 mg/mL propidium iodide using Cycle TEST PLUS (BD Biosciences, San Jose, CA). DNA content of nuclei was determined by fluorescence-activated cell sorting analysis.

Ultrastructural study

E13.5 fetal livers of *AML1/EVI1/*+ heterozygous embryos and their wild-type littermates were examined with a JEOL 1200CX electron microscope (JEOL, Tokyo, Japan). Each mock or AML1/EVI1–expressing bulk population treated with 100 nM staurosporine for 48 hours was also examined.

In vitro culture of hematopoietic cells

Fetal livers from E12.5 or E13.5 embryos were disrupted by passing through 21-gage needles and then 26-gage needles for 3 times each. Single-cell suspensions with 1×10^4 cells were plated in triplicate in 1.2% methylcellulose in Iscoves modified Dulbecco medium containing 30% fetal calf serum, 0.1 mM β-mercaptoethanol, 2 mM glutamine, and 1% bovine serum albumin, 5 U/mL human erythropoietin, 100 ng/mL murine stem cell factor, 5 ng/mL murine interleukin 3 (IL-3; Kirin Brewery, Tokyo, Japan). Cell aggregates containing more than 50 cells were counted as colonies. Cytocentrifuge preparations of hematopoietic colonies were stained with Wright-Giemsa for morphologic examination, benzidine for the presence of erythroblasts, and choline esterase for the presence of megakaryocytes. In replating experiments, all of the cells from the preceding culture were collected, washed, and then replated at 1×10^4 cells per methylcellulose plate under the same condition as in the preceding culture. Colonies were scored as previously mentioned.

Quantitative reverse transcriptase (RT)–PCR analysis.

E12.5 and E13.5 live embryos were selected on the basis of the presence of heartbeats. Total RNA was isolated from liver cells using RNAeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Random hexamer–primed cDNA was prepared from 2 µg total RNA with mouse mammary leukemia virus reverse transcriptase (Gibco BRL, Grand Island, NY) in a total volume of 25 µL. An oligonucleotide primer set containing murine *Aml1* exon 5–derived sense (5′-AAGAGCTTCACTCT-GACCAT-3′) and human *EVI1* noncoding region–derived antisense (5′-CCTTTCACCTACTTCGATCT-3′) sequences was used for amplification of the *AML1/EVI1* fusion sequence. Amplifications of various hematopoietic regulator transcripts were performed with primer sets described previously[35-39] as well as that for murine *CD11b*, sense (5′-CAGATCAA-CAATGTGACCGTATGG-3′) and antisense (5′-CATCATGTCCTTGTACT-GCCGC-3′) primers, using CYBR Green PCR Master Mix and ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster, CA). Parallel reactions were carried out using a β-actin–amplifying primer set to ensure the integrity of the RNA samples.

Quantification of mRNA level was performed by measuring the cycle threshold (CT) that is defined as the fractional cycle number at which the fluorescence encounters a fixed threshold. The CT value of each gene was normalized to that of β-actin (ΔCT; CT value of target gene minus CT value of β-actin). Results were expressed as difference of ΔCT between *AML1/EVI1/*+ versus wild-type or *AML1*−/− versus wild-type animals, and fold expression.

Results

Generation of an *AML1/EVI1* fusion allele

The t(3;21) breakpoint on chromosome 21 in human leukemic cells occurs in the fifth intron of the *AML1* gene, which is 3′ to the exons

encoding the Runt DNA-binding domain.[5] To mimic in the mouse genome the mutant *AML1/EVI1* chimeric gene at the human t(3;21) allele, we made a targeting construct that replaces murine *Aml1* exon 5 with human *AML1* exon 5 fused in frame to the human *EVI1* sequence (Figure 1A). This targeting strategy renders generation of the murine/human hybrid *AML1/EVI1* gene whose expression is controlled by the endogenous mouse *Aml1* promoter. Using electroporation, we obtained 8 ES cell clones targeted correctly (no. 31, no. 35, no. 37, no. 42, no. 47, no. 50, no, 70, and no. 97), as determined by the presence of both *AML1/EVI1* allele and endogenous murine *Aml1* allele in Southern analysis (Figure 1B). These *AML1/EVI1/*+ ES cell clones were injected into mouse morulas, and 2 chimeric mice (no. 31 and no. 97) transmitted the mutant allele through the germ line. These chimeric mice were largely healthy up to 7 months but showed sudden death thereafter. The cause of death is unknown despite anatomic examination performed immediately after the death of the 5 chimeric mice; there were no signs of hepatosplenomegaly or lymphadenopathy, excluding at least the leukemic cause of death.

Knock-in expression of *AML1/EVI1* fusion gene results in embryonic lethality with CNS hemorrhage and a lack of fetal-liver hematopoiesis

Genotyping analysis of 42 delivered F1 pups of *AML1/EVI1* chimeric male and wild-type C57BL/6 female found no *AML1/EVI1/*+ genotypes, suggesting that *AML1/EVI1/*+ embryos died in the uteri. *AML1*+/+ agouti mice appeared normal; thus, designated major mutations did not occur in the ES cells. To examine the timing and cause of embryonic death in *AML1/EVI1/*+ heterozygous embryos, we killed pregnant females at various time points between E10.5 and E14.5. The *AML1/EVI1* chimeric mRNA was detected in the fetal liver of the heterozygous mice by RT-PCR (Figure 1C). Between E10.5 and E11.5, all the *AML1/EVI1/*+ embryos were viable and showed no significant morphologic abnormalities (Table 1). However, 10% of the embryos was found dead at E12.5 and 100% by E14.5, as judged by the absence of heart beats. The size of the *AML1/EVI1/*+ embryos between E12.5 and E13.5 was comparable to that of the control littermates (Figure 2A). However, they had noticeable white livers, and signs of massive hemorrhage in the CNS and intersegmental regions of the presumptive spinal cord. Microscopic analysis revealed that the hemorrhage occurred as early as E12.5 in the cerebral ventricle and the dorsal root ganglia (Figure 2B-E), and such hemorrhagic sites were somewhat conserved in *AML1/EVI1/*+ embryos analyzed (n = 58). This hemorrhage and lethality of *AML1/EVI1/*+ heterozygous embryos were observed in 2 independent ES cell lines (no. 31 and no. 97). These data indicate that *AML1/EVI1/*+ embryos die around E13.5 from the massive hemorrhage in the CNS. We focused our further analysis on embryos derived from line no. 97

Table 1. Genotype and phenotype of embryos derived from AML1/EVI1 chimeric males mated with normal females

Stages	No. of Pups	Genotype		Phenotype of KI* mice	
		+/+	+/KI*	Hemorrhage	Death
E10.5	9	1	8	0	0
E11.5	14	11	3	0	0
E12.5	98	67	31	27	3
E13.5	78	62	16	16	10
E14.5	32	21	11	11	11

Genotype analysis was performed by Southern analysis of DNA extracted from embryos. Surviving embryos were defined as those with beating hearts at the time of dissection.
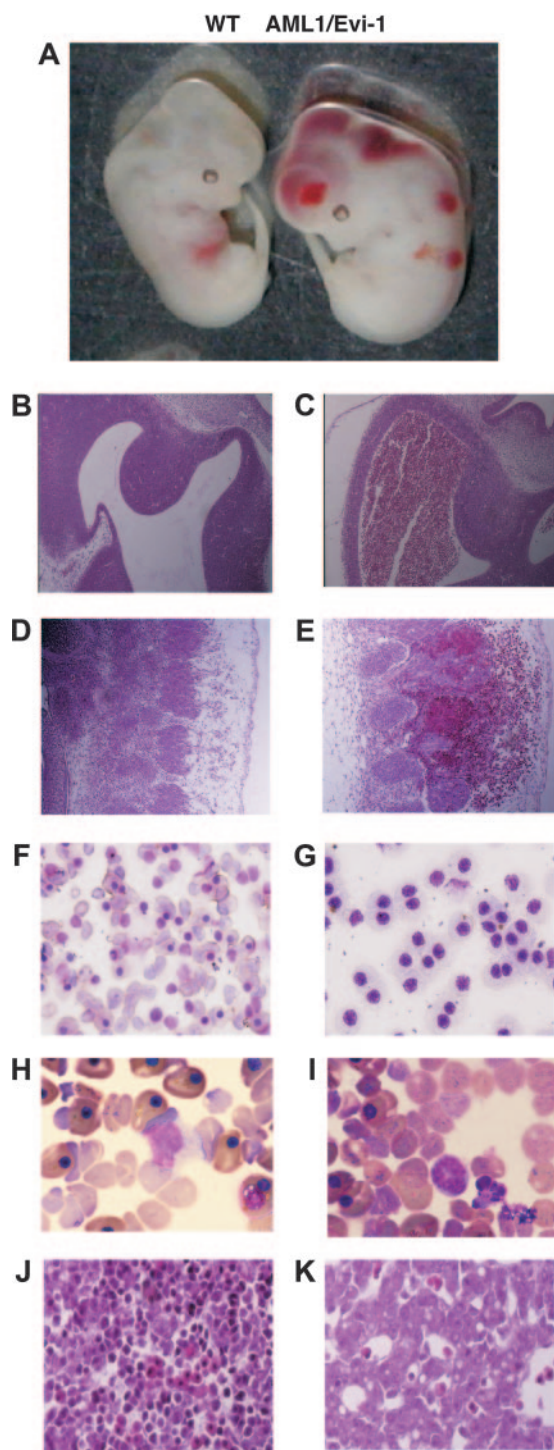
*KI indicates AML1/EVI1 knock-in.

Figure 2. Morphologic comparison of E12.5 wild-type (WT) and AML1/EVI1/+ heterozygous embryos. (A) External appearance of WT and *AML1/EVI1/+* littermates. *AML1/EVI1/+* embryo on the right is similar in size to WT littermate on the left but is clearly identifiable by the presence of fetal liver pallor and massive hemorrhage within the CNS and soft tissues in the back (objective lens [OL], 2 ×/0.05; original magnification [OL], × 20). (B-C) Sections of the cerebral ventricle from WT (B) and *AML1/EVI1/+* (C) embryos. *AML1/EVI1/+* embryo shows massive hemorrhage into the ventricle (OL, 10 ×/0.40; OM, × 100). (D-E) Sections of the dorsal root ganglia from WT (D) and *AML1/EVI1/+* (E) embryos. *AML1/EVI1/+* embryo shows hemorrhage in the ganglia (OL, 10 ×/0.40; OM, × 100). (F-G) Smears of the peripheral blood from WT (F) and *AML1/EVI1/+* (G) embryos. *AML1/EVI1/+* embryo shows the absence of definitive erythrocytes, while WT littermate shows numerous enucleated definitive erythrocytes (OL, 40 ×/0.65; OM, × 400). (H-I) Smears of the peripheral blood from WT embryo. Only WT littermate shows monocyte (H) and neutrophil (I) (OL, 100 ×/1.40; OM, × 1000). (J-K) Sections of the fetal liver from WT (J) and *AML1/EVI1/+* (K) embryos. *AML1/EVI1/+* embryo shows a near complete absence of hematopoietic precursors, while WT littermate shows numerous hematopoietic precursor cells (OL, 20 ×/0.70; OM, × 200) .
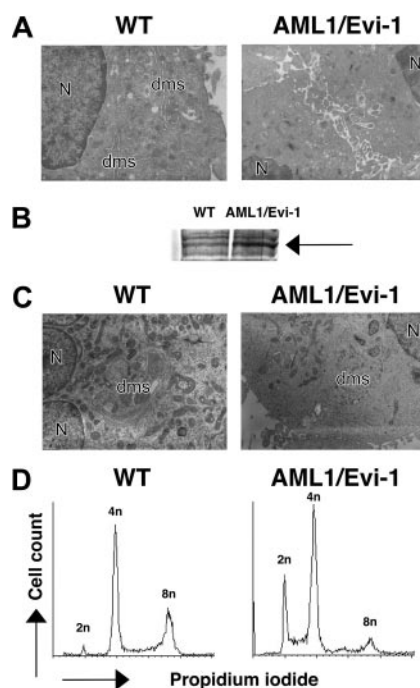

Figure 3. Morphology and DNA content of megakaryocytic cells overexpressing AML/EVI1. (A) Electron micrographs of megakaryocytes in E13.5 fetal liver. N indicates nucleus; dms, demarcation membrane (OM, × 3000). (B) Expression of AML1/EVI1 chimeric protein in transfected K562 cells. An arrow indicates overexpressed AML1/EVI1 proteins. (C) Electron micrographs of mock or AML1/EVI1–overexpressing K562 cells after staurosporine treatment for 48 hours. N indicates nucleus; dms, demarcation membrane (OM, × 3000). (D) DNA content of mock or AML1/EVI1–overexpressing K562 cells after staurosporine treatment for 48 hours.

because of the efficient germ line transmission and propagation relative to those from line no. 31.

Yolk sac hematopoiesis appeared largely intact in *AML1/EVI1/+* embryos, with normal number and morphology of the yolk sac–derived nucleated erythroblasts circulating in the peripheral vessel (Figure 2G). Erythroblasts in E12.5 *AML1/EVI1/+* embryos looked similar to those in E11.5 wild-type littermates, suggesting that they normally developed in the yolk sac. In contrast, fetal liver hematopoiesis was severely impaired, as indicated by markedly whitish liver resulting from a lack of red cells. The microscopic examination showed near complete absence of the erythroid, myeloid, or megakaryocytic progenitors of the definitive origin (Figure 2K). Postenucleated erythrocytes were absent in the peripheral blood from E12.5 *AML1/EVI1/+* embryos, whereas it was abundantly observed in the peripheral blood of E12.5 wild-type littermates (Figure 2F-G). Also, monocytes and neutrophils were absent in the smears from *AML1/EVI1/+* embryos, whereas they were easily observed in the smears from wild-type littermates (Figure 2H-I). These findings indicate that *AML1/EVI1/+* heterozygous mice fail to establish definitive hematopoiesis in the fetal liver but maintain primary hematopoiesis in the yolk sac, recapitulating hematopoietic defects observed in *AML1*[−/− 11,12] or *AML1/ETO/+* mice.[32,33] We further performed electron microscopic examination to search for hematopoietic progenitors that would have been missed by histologic microscopic examination in the E13.5 fetal liver. In addition to decreased erythroid and myeloid progenitors, we observed megakaryocytes that were defective in their demarcation membrane (Figure 3A), which resemble those in *AML1* conditional knock-out mice.[16] Thus, although there is massive defect in fetal liver hematopoiesis, some hematopoietic progenies do exist, although dysplastic, in the *AML1/EVI1/+* fetal liver.

Table 2. Hematopoietic progenitors in E12.5 and E13.5 fetal liver

Stages/genotype	No. of Pups	BFU-E	CFU-GM	CFU-GEMM	CFU-M
E12.5					
+/+	13	10 ± 3	32 ± 8	20 ± 9	8 ± 2
AML1/EVI1/+	6	0	0	0	25 ± 11
E13.5					
+/+	9	8 ± 3	58 ± 14	25 ± 11	10 ± 3
AML1/EVI1/+	5	0	0	154 ± 54	4 ± 3

Numbers represent colonies per 1×10^4 fetal liver cells, mean ± SD.

BFU-E indicates erythroid burst-forming unit; CFU-GM, granulocyte/macrophage colony-forming unit; CFU-GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte colony-forming unit; CFU-M, macrophage colony-forming unit.

To test for the direct effect of AML1/EVI1 molecule in megakaryocyte morphology, we expressed *AML1/EVI1* cDNA in human leukemic cell line K562 (Figure 3B) and differentiated one bulk population into the megakaryocytic lineage by exposing to staurosporine.[40] The AML1/EVI1–expressing K562 cells treated with staurosporine showed poorly developed demarcation membrane and lower level of polyploidy as compared with control mock cells (Figure 3C), indicating that the expression of AML1/EVI1 molecule is causative for the aberrant maturation of megakaryocytes in *AML1/EVI1/+* mice.

AML1/EVI1 generates dysplastic hematopoietic progenitors in the fetal liver

To analyze for the defective hematopoiesis in the *AML1/EVI1/+* embryos, we performed methylcellulose colony-forming assay using fetal liver cells from E12.5 or E13.5 embryos. Because the total number of cells recovered from E12.5 or E13.5 *AML1/EVI1/+* fetal livers was 20-fold less than that recovered from wild-type fetal livers, we adjusted the number of plated fetal liver cells (1×10^4 cells) to assess for the precursor frequencies. In day 10 of culture, wild-type fetal liver gave rise to mutilineage colonies, whereas E12.5 *AML1/EVI1/+* fetal liver cells generated macrophage colonies only, with no detectable erythroid, myeloid, and mixed colonies (Table 2). The E13.5 *AML1/EVI1/+* cell culture looked similar to that of E12.5, except for some mixed-like colonies that were 5-fold more numerous than those from wild-type cell culture in microscopic examination. These data indicate that expression of AML1/EVI1 leads to severe defects in definitive hematopoiesis, but the fetal liver contains some progenitors capable of differentiating into multilineage hematopoietic progenies.

To gain more insights into the mixed–lineage-like colonies derived from E13.5 *AML1/EVI1/+* fetal liver, we performed time-course microscopic analysis of the methylcellulose culture. On day 7 of culture, colonies from E13.5 *AML1/EVI1/+* fetal liver consisted mainly of myelocytic blastoid cells, while those from wild-type fetal liver contained numerous erythroblasts, hypergranular myeloid cells, and megakaryocytes (Figure 4). Benzidine staining confirmed the absence of erythroblasts in *AML1/EVI1/+* cell-derived colonies. However, choline esterase–positive megakaryocytes were observed in both types of the colonies. On day 10, colonies from *AML1/EVI1/+* fetal liver contained a large number of dysplastic myelocytes with bilobulated or multilobulated nuclei along with scanty cytoplasmic granulation. These findings suggest that hematopoietic progenitors in the *AML1/EVI1/+* fetal liver are completely blocked for differentiation into the erythroid lineage but are capable of differentiating into the myeloid and megakaryocyte lineages, although immature and dysplastic.

Hematopoietic progenitor in the *AML1/EVI1/+* fetal liver is highly capable of self-renewal

To examine self-renewal capacity of hematopoietic progenitors in the E13.5 *AML1/EVI1/+* fetal liver, we performed a serial methylcellulose replating assay using bulk population of fetal liver–derived colonies under the conditions optimal for the development of multipotential hematopoietic progenitors. Cells from *AML1/EVI1/+* fetal livers were able to replate beyond 7 passages, whereas cells from wild-type fetal livers lost their ability to form colonies after 5 passages (Table 3). During each replating, AML1/EVI1–expressing colonies showed increase in size and number (Figure 5, left). The cytospin samples from the fourth passage colony identified a polymorphic population of dysplastic cells, including agranular myelocytes and megakaryocytes (Figure 5, right). We conclude that expression of AML1/EVI1 in the fetal liver produces multilineage potential progenitors that are capable of more self-renewal than the wild-type progenitors and have a dysplastic maturation tendency. However, even after amplification of the cells through passage, we failed to demonstrate expression of AML1/EVI1 chimeric proteins in the colony-forming cells by Western analysis with anti-AML1 antibody, possibly because of low expression and high molecular weight (190 kDa) of the proteins.

Expression of *PU.1* is maintained in *AML1/EVI1/+* fetal liver cells but significantly decreased in *AML1$^{-/-}$* cells

To get the molecular underpinning for the dysregulated hematopoiesis caused by AML1/EVI1, we assessed the expression of various genes implicated in definitive hematopoiesis. The total RNA was extracted from E12.5 fetal liver cells from wild-type, *AML1$^{-/-}$*, and *AML1/EVI1/+* embryos, and mRNA was quantified using semiquantitative RT-PCR analysis. The level of expression in

Figure 4. Morphology of dysplastic mixed-like hematopoietic colonies in the AML1/EVI1$^{/+}$ fetal liver culture. On days 7 and 10 of culture, cytocentrifuge preparations of cells from mixed-like colonies were stained with Wright-Giemsa for morphologic examination (OL, 100 ×/1.40; OM, × 1000), benzidine for the presence of erythroblasts (OL, 10 ×/0.30; OM, × 100), or choline esterase for the presence of megakaryocytes (OL, 60 ×/1.40; OM, × 600). *AML1/EVI1/+* mixed-like colonies in the bottom panels show retarded maturation and hypogranulation of myeloid cells as well as the absence of erythroid cells. In contrast, WT mixed colonies in the top panels show normal trilineage differentiation.
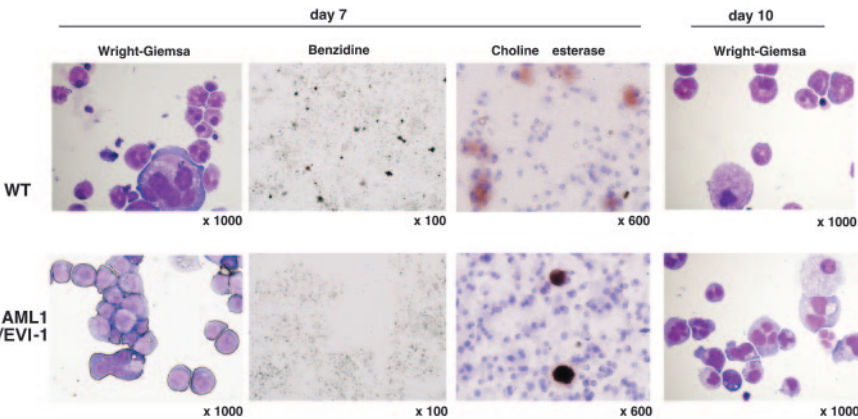
Table 3. Serial in vitro replating assays of E13.5 fetal liver hematopoietic progenitors

Genotype	In vitro passage						
	1	2	3	4	5	6	7
+/+	99 ± 23	384 ± 86	164 ± 41	59 ± 76	11 ± 9	0	0
AML1/EVI1/+	162 ± 59	388 ± 131	365 ± 150	403 ± 89	436 ± 104	407 ± 114	626 ± 3

Cells from methylcellulose culture were disaggregated, washed, and replated at 1×10^4 cells per methylcellulose plate under the same condition every 7 days. Numbers represent colonies per 1×10^4 cells, mean ± SD.

$AML1^{-/-}$ and $AML1/EVI1/^+$ fetal liver cells was compared with that in wild-type cells. Table 4 revealed the calculated fold expression of the indicated genes in $AML1^{-/-}$ and $AML1/EVI1/^+$ embryos relative to that of wild-type littermates along with the cycle threshold differences. The expression of the *PU.1*[41] gene in $AML1/EVI1/^+$ fetal liver cells was maintained to the normal, whereas its expression level was markedly decreased in $AML1^{-/-}$ fetal liver cells, as has been reported previously.[42] Consistent with this, the expression of the *CD11b* gene, one of the target genes of PU.1 (an ets transcription factor family member), was maintained in $AML1/EVI1/^+$ knock-in liver, whereas markedly decreased in $AML1^{-/-}$ liver. The expression of the critical transcription factor such as PU.1 in $AML1/EVI1/^+$ fetal liver may support multilineage hematopoietic progenitors up to E13.5 and also aids the monocyte/macrophage lineage differentiation. The myeloid lineage-specific transcription factor *C/EBPA*[43] was expressed several fold higher in $AML1/EVI1/^+$ fetal liver cells than in wild-type cells, and this may bolster the myeloid-lineage differentiation along with PU.1. The expression levels of cytokine receptors transcripts such as G-CSF receptor and GM-CSF receptor were largely unchanged across wild-type, $AML1^{-/-}$ and $AML1/EVI1/^+$ fetal liver cells. On the other hand, the expression of erythroid lineage–specific genes such as *LMO2*[44] and *SCL*[45] was approximately 10-fold lower in $AML1/EVI1/^+$ fetal liver cells than in wild-type cells. The decreased expression of *LMO2* and *SCL* could impinge on successful differentiation along the erythroid lineage in $AML1/EVI1/^+$ fetal liver cells, and the similar decrease was observed in $AML1^{-/-}$ fetal liver cells that lack erythroid cells as well. Other erythroid lineage–specific transcripts such as δ-aminolevulinic acid synthase-erythroid *(ALASE)* and β-globin were more decreased in $AML1/EVI1/^+$ fetal liver cells than in $AML1^{-/-}$ cells, despite equivalent expression of their master regulator GATA binding protein 1 (GATA-1) in both cell types. The net GATA-1 activity was presumably repressed in $AML1/EVI1/^+$ fetal liver cells because of

maintained expression of PU.1 that inhibits DNA binding of GATA-1.[46] The suppressed expression of *ALASE* and β-globin could also contribute to the complete defect of erythroid differentiation in $AML1/EVI1/^+$ progenitor cells. These expression analyses provide a logical explanation for the distinct hematopoietic capacity intrinsic to $AML1^{-/-}$ and $AML1/EVI1/^+$ fetal liver cells. Our data point that the sufficient expression of the *PU.1* gene may be the first critical prerequisite for the definitive hematopoiesis to be set off in the fetal liver.

Discussion

AML1-CBFβ transcription factor complex is one of the critical regulators of the hematopoietic system. A substantial amount of evidence highlights the central role for this transcription factor system in normal hematopoiesis as well as in abnormal hematopoietic disorders. Four gene-ablated animals including ours, *AML1*-null,[11,12] *CBFB*-null,[13-15] *AML1/ETO* knock-in,[32,33] and *AML1/EVI1* knock-in mice, show normal hematopoiesis in the yolk sac but share common defects in definitive hematopoiesis in the fetal liver. These 4 embryos also show massive hemorrhage in the CNS and spinal cord and die during midgestation. A series of biochemical analyses in AML1-CBFβ transcription factor, or AML1/ETO or AML1/EVI1 fusion molecules[23,28,29] indicate that such in vivo phenotypes arise from shutting off or suppressing AML1 function in the early hematopoietic tissues. We speculate that such phenotype in *AML1/EVI1* knock-in mice also comes from dominant-negative effect against wild-type-AML1 in vivo.

The microscopic analysis of the fetal liver of the 4 mutant embryos shows common lack of visible hematopoietic cells.

Figure 5. Morphology of serially replated AML1/EVI1/+ hematopoietic colonies. (Left) WT and typical dysplastic multilineage *AML1/EVI1/+* colonies after the fourth passage. The size of *AML1/EVI1/+* hematopoietic colonies is significantly larger than that of WT colonies. (Right) Wright-Giemsa–stained cytocentrifuge preparations of the WT and *AML1/EVI1/+* colony. *AML1/EVI1/+* hematopoietic colonies maintain the dysplastic nature. Scales bars equal 300 μm (OL, 100 ×/1.40; OM, × 1000).

Table 4. Expressional changes of various hematopoietic regulators in E12.5 *AML1/EVI1/+* and *AML1⁻/⁻* fetal liver cells

	$\Delta CT_{KI} - \Delta CT_{WT}$	Fold (KI/WT)	$\Delta CT_{KO} - \Delta CT_{WT}$	Fold (KO/WT)
C/EBPA	−1.4 ± 0.1	2.7 ± 0.1	−2.9 ± 0.0	7.2 ± 0.1
LMO2	3.5 ± 0.3	0.1 ± 0.6	3.3 ± 0.1	0.1 ± 0.3
SCL	3.9 ± 0.8	0.1 ± 1.7	2.4 ± 0.2	0.2 ± 0.3
c-MYB	−0.5 ± 0.2	1.4 ± 1.5	−2.4 ± 0.3	5.4 ± 0.6
GATA1	−0.4 ± 0.7	1.4 ± 1.3	−1.6 ± 0.5	3.0 ± 1.0
PU.1	−0.4 ± 0.1	1.3 ± 0.1	8.2 ± 0.1	0.0 ± 0.3
NFE2	2.9 ± 0.5	0.1 ± 1.0	0.3 ± 0.2	0.6 ± 0.2
CD11b	1.2 ± 0.4	0.4 ± 0.8	5.0 ± 1.8	0.0 ± 3.6
MCSFR	0.1 ± 0.2	0.9 ± 0.4	−1.5 ± 0.9	2.8 ± 1.8
GCSFR	−0.6 ± 0.1	1.5 ± 0.2	0.0 ± 0.0	1.0 ± 0.0
GMCSFR	0.1 ± 0.2	1.1 ± 0.4	−0.6 ± 0.1	1.5 ± 0.2
MPO	10 ± 1.1	0.0 ± 2.2	7.0 ± 0.7	0.0 ± 1.3
β-*globin*	3.9 ± 0.9	0.1 ± 1.7	1.0 ± 0.4	0.5 ± 0.8
FOG1	2.3 ± 0.2	0.2 ± 0.3	2.0 ± 0.2	0.3 ± 0.3
ALASE	5.6 ± 0.4	0.0 ± 0.7	1.9 ± 0.4	0.3 ± 0.8

ΔCT indicates mean CT of indicated gene − mean CT of β-actin; KI, AML1/EVI1 knock-in; KO, AML1 knock-out; fold, fold difference relative to that of WT litters calculated by $2^{-(\Delta CT_{KI \text{ or } KO} - \Delta CT_{WT})}$. Data are means ± standard deviation.
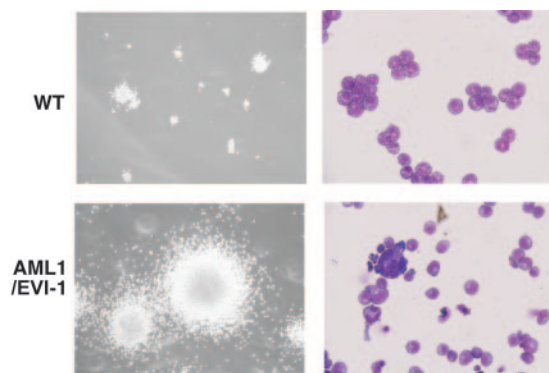
Despite such gross similarity, close examination of the hematopoiesis reveals some intriguing differences. First, although *AML1*[−/−11,12] or *CBFB*[−/−13-15] fetal liver has no hematopoietic progenitors that would have produced colonies, *AML1/EVI1/*[+] and *AML1/ETO/*[+32,33] fetal livers have progenitors that can give rise to macrophage colonies. *AML1/EVI1/*[+] fetal liver (E12.5) contains 3-fold more progenitors than wild-type fetal liver capable of differentiating into the monocyte/macrophage lineage, and a similar finding is reported for *AML1/ETO/*[+] yolk sac where Yergeau et al[32] observed a marked increase in the number of macrophage colonies. Therefore, the capacity to differentiate along the monocyte/macrophage lineage seems a characteristic feature of hematopoietic progenitors expressing AML1/EVI1 or AML1/ETO in the fetal hematopoietic system. Second, in contrast to *AML1*^{−/−} or *CBFB*^{−/−} fetal liver, *AML1/EVI1/*[+] and *AML1/ETO/*[+33] fetal livers (E13.5) can also give rise to colonies committed to erythrocyte/megakaryocyte and myelocyte lineages. The appearance of megakaryocytic cells in the colony assay would suggest some production of platelets in these embryos, and this might contribute to 1 day–longer survival of *AML1/EVI1* and *AML1/ETO* knock-in embryos (lethal at E13.5) than *AML1* or *CBFB* knock-out embryos (lethal at E12.5). Third, the serial colony-forming assay demonstrates that AML1/EVI1– or AML1/ETO[33]-expressing progenitors show more self-renewal than those of the wild-type animals, although these progenitors cannot establish functional hematopoiesis in the fetal liver. These differences between AML1/EVI1– or AML1/ETO-expressing cells versus *AML1* or *CBFB* knock-out cells may arise from either incomplete suppression of wild-type-AML1 or additional functions brought by the individual fused molecules.

Although *AML1/EVI1/*[+] and *AML1/ETO/*[+] fetal liver showed similar results in colony counts, there are some striking differences in their contents of colonies that are unlikely to result from minor differences in culture conditions. We used higher concentrations of erythropoietin and stem cell factor than those reported in *AML1/ETO/*[+] fetal liver culture.[32,33] Nonetheless, *AML1/EVI1/*[+] hematopoietic progenitors fail to generate erythroid cells, whereas *AML1/ETO/*[+] hematopoietic progenitors give rise to mixed colonies containing numerous erythroblasts.[33] The observed resistance to the erythroid-lineage differentiation is highly characteristic of *AML1/EVI1/*[+] hematopoietic progenitors. This is consistent with the reported retroviral expression of EVI1 in hematopoietic progenitor cells that blocks erythroid differentiation in vitro[47] or the transgenic expression of EVI1 impairing erythropoiesis in vivo.[48] Therefore, the expression of EVI1, either in canonical form or as the fusion chimeric protein, is detrimental to erythroid-lineage differentiation. Another distinct finding between the 2 cell types is the more severe dysplasticity in *AML1/EVI1/*^{+} cells. In *AML1/EVI1/*[+] progenitors, the arising myeloid cells arrest at the myelocyte stage with no granulation, and the arising megakaryocytes show ill-developed demarcation membrane in the cytoplasm. In contrast, *AML1/ETO/*[+] progenitors exhibited far more differentiated morphologies, although reported dysplastic in the text.[33] Overexpressed EVI1 protein in G-CSF–treated 32Dcl3 cells completely blocks myeloid differentiation.[49] Thus, the adverse effect of EVI1 in myeloid differentiation may contribute to the more severe defect in the myeloid maturation of the AML1/EVI1–expressing progenitors than that of the AML1/ETO-expressing cells. Finally, the abnormal expression of the *AML1/EVI1* gene causes disturbance of megakaryocytic maturation, which is not observed in AML1/ETO-expressing animals. This effect seems direct as evi-

dent by AML1/EVI1–expressing K562 cells showing poor demarcation membrane and lower DNA ploidy when treated with staurosporine. In this case, however, the result seems to be caused by the dominant-negative effect over wild-type-AML1, as *AML1* conditional knock-out mice show similar maturation abnormality in megakaryocytes.

AML1/EVI1 causes broad perturbation of gene expression that is key to normal hematopoietic cell development. The first key gene is *PU.1* that is known to play a pivotal role in B cell/monocyte development[50-54] and the maintenance of hematopoietic stem cell in the fetal liver.[55] *PU.1* transcripts are markedly decreased in *AML1*^{−/−} fetal liver cells, as reported previously,[42] whereas its expression is normal in *AML1/EVI1/*[+] fetal liver cells, and this expression pattern is paralleled by one of its direct target gene *CD11b*. The maintained expression of PU.1 may afford *AML1/EVI1/*[+] hematopoietic progenitors a capacity to produce macrophage and mixed colonies. Importantly, our data suggest that the reported complete lack of hematopoietic progenitors in *AML1*^{−/−} fetal liver may result from the absence of PU.1, and the recent finding underscores the critical role for PU.1 in supporting hematopoietic stem cell maintenance in the fetal liver.[55] Thus, PU.1 seems to be the key transcription factor that explains the phenotypic difference between *AML1/EVI1/*[+] and *AML1*^{−/−} animals. The second key gene *CEBPA* is involved in early granulocytic differentiation.[56] The expression of *CEBPA* is increased in both *AML1/EVI1/*[+] and *AML1*^{−/−} fetal livers. In *AML1/EVI1/*[+] liver, the increased expression of C/EBPα may enhance myeloid/monocytic differentiation of the existing hematopoietic progenitor supported by PU.1. In *AML1*^{−/−} fetal liver, however, the lack of PU.1 expression dissipates the hematopoietic progenitor; thus, there is no myeloid/monocytic progeny arising from even the increased expression of C/EBPα. Third, the expression of *LMO2* and *SCL* is severely repressed in *AML1/EVI1/*[+] fetal liver. The *LMO2* and *SCL* genes are essential for erythroid lineage differentiation,[44,45] and low expression of these genes could account for the absence of erythroid cells in *AML1/EVI1/*[+] fetal liver, even though the hematopoietic progenitor cells are supported by the presence of PU.1. The lack of *LMO2* and *SCL* expression is also observed in *AML1*^{−/−} fetal liver. This suggests that the defective expression of *LMO2* and *SCL* in *AML1/EVI1/*^{+} cells is due to dominant-negative effect of AML1/EVI1 over wild-type AML1, and that *LMO2* and *SCL* might be candidate target genes for wild-type AML1. This possibility might be worth exploring, although no PEBP2 binding sites are found at least in the proximal regulatory region of the *LMO2* or *SCL* gene.[57,58] Finally, it is worth emphasizing that *GATA1* expression is kept comparable across wild-type, *AML1*^{−/−}, and *AML1/EVI1/*[+] fetal livers, and yet its target genes *ALASE* and β-globin are much more repressed in *AML1/EVI1/*[+] fetal liver cells than in *AML1*^{−/−} cells. These differences in expression of *ALASE* and β-globin genes could be due to the interplay between PU.1 and GATA-1,[46] providing a case that some of the altered gene expressions are the consequences of the combinatory effect of involved transcription factors.

In summary, we have demonstrated that *AML1/EVI1* knock-in mice almost completely lack effective hematopoiesis in the fetal liver, but they retain progenitors with increased self-renewal capacity and potential to differentiate into the myeloid/monocytic and megakaryocytic lineages with some dysplasticity. We provide correlative evidence that PU.1 plays a critical role in the maintenance of hematopoietic progenitors in the *AML1/EVI1/*[+] fetal liver. Collectively, our study suggests that AML1/EVI1 is not only a

dominant-negative suppressor of wild-type–AML1 but also has more a proliferative and dysplastic effect added on by the fusing EVI1 part. Clinical evidence shows that the emergence of *AML1/EVI1* is associated with the progression of hematopoietic stem cell disorder, and our results explain the aggressive and dysplastic transformation of the disease course observed in patients with AML1/EVI1–expressing leukemia. Inducible expression of AML1/ETO in mouse hematopoietic tissue shows that the expression of AML1/ETO per se is not leukemogenic[59,60] and requires a second hit introduced by alkylating agents to cause leukemia.[60,61] It certainly needs to be tested whether the expression of AML1/EVI1 is sufficient to cause leukemia when expressed in an inducible fashion in adult mice. Our prediction would be that AML1/EVI1 expression is leukemogenic on its own, or at least more prone to leukemia when a second hit comes in. A conditional project of *AML1/EVI1* knock-in expression is in progress in our laboratory.

Acknowledgments

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