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Plag1 and *Plagl2* are oncogenes that induce acute myeloid leukemia in cooperation with *Cbfb-MYH11*

Sean F. Landrette, Ya-Huei Kuo, Karen Hensen, Sahar Barjesteh van Waalwijk van Doorn-Khosrovani, Paola N. Perrat, Wim J. M. Van de Ven, Ruud Delwel, and Lucio H. Castilla

Recurrent chromosomal rearrangements are associated with the development of acute myeloid leukemia (AML). The frequent inversion of chromosome 16 creates the *CBFB-MYH11* fusion gene that encodes the fusion protein CBF β -SMMHC. This fusion protein inhibits the core-binding factor (CBF), resulting in a block of hematopoietic differentiation, and induces leukemia upon the acquisition of additional mutations. A recent genetic screen identified *Plag1* and *Plagl2* as

CBF β -SMMHC candidate cooperating proteins. In this study, we demonstrate that *Plag1* and *Plagl2* independently cooperate with CBF β -SMMHC in vivo to efficiently trigger leukemia with short latency in the mouse. In addition, *Plag1* and *Plagl2* increased proliferation by inducing G₁ to S transition that resulted in the expansion of hematopoietic progenitors and increased cell renewal in vitro. Finally, *PLAG1* and *PLAGL2* expression was increased in 20% of human AML samples.

Interestingly, *PLAGL2* was preferentially increased in samples with chromosome 16 inversion, suggesting that *PLAG1* and *PLAGL2* may also contribute to human AML. Overall, this study shows that *Plag1* and *Plagl2* are novel leukemia oncogenes that act by expanding hematopoietic progenitors expressing CbF β -SMMHC. (Blood. 2005;105:2900-2907)

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Introduction

The core-binding factor (CBF) is the most common target of chromosomal translocations in acute myeloid leukemia (AML). Frequent rearrangements affecting CBF include the chromosome 16 inversion inv(16)(p13;q22) [hereafter inv(16)] and the translocation t(8;21)(q22;q22).¹ CBF is a heterodimeric transcription factor composed of 1 of 3 DNA-binding α subunits (encoded by *RUNX1*, *RUNX2*, and *RUNX3*) and a non-DNA binding β subunit (encoded by *CBFB*). In hematopoiesis, the RUNX1:CBF β complex is a key regulator of lymphoid and myeloid differentiation. For example, studies using knock-out mice demonstrated that embryonic definitive hematopoiesis is defective in the absence of CbF β or Runx1.²⁻⁵ In adult hematopoiesis, *Runx1* knockouts show deficient differentiation of T cells, B cells, and myeloid cells.⁶

The inv(16) breaks and joins *CBFB* with the smooth muscle myosin heavy chain (*MYH11*) gene, creating the *CBFB-MYH11* fusion gene, which encodes the CBF β -SMMHC fusion protein.⁷ Interestingly, *Cbfb*^{+/MYH11} knock-in embryos expressing this fusion protein lack definitive hematopoiesis, as shown in *Cbfb* and *Runx1* knock-out embryos, thus indicating that CbF β -SMMHC is a dominant inhibitor of CBF function.⁸ Normal lymphoid and myeloid differentiation was also impaired in hematopoietic stem cells from *Cbfb*^{+/MYH11} knock-in chimeras expressing CbF β -SMMHC. In addition, these mice developed AML after induction of additional mutations.⁹ However, little is known about the genes and pathways that cooperate with CBF β -SMMHC in leukemogen-

esis. We have recently performed a genetic screen, by means of retroviral insertional mutagenesis (RIM), in *Cbfb*^{+/MYH11} knock-in chimeras to identify genes that could synergize with CbF β -SMMHC in leukemia development.¹⁰ The study identified retroviral insertions at the *Plag1* locus in as many as 40% of the samples and at the *Plagl2* locus in 10% of the samples. Interestingly, the proviral insertions mapped upstream of the translation-start site, thus suggesting that up-regulation of these genes may contribute to AML.

The PLAG family is composed of 3 members (*PLAG1*, *PLAGL1/LOT1/ZAC1*, and *PLAGL2*) with highly conserved structure and function.¹¹ These proteins are transcription factors that include a C-terminal *trans*-activation domain preceded by 7 C2H2 zinc fingers with DNA binding function. Genetic studies have associated *PLAG1* with benign tumors harboring 8q12 translocations, including pleomorphic adenomas of the salivary gland, lipoblastomas, and hepatoblastomas.¹²⁻¹⁴ *PLAGL2* has not been previously associated with human cancer, but it has been reported to have similar DNA-binding affinity to the *Plag1* consensus sequence and to induce proliferation in NIH-3T3 cells.¹⁵ Conversely, *PLAGL1* seems to function as a tumor suppressor protein that is found mutated in breast and pituitary tumors^{16,17} and that regulates apoptosis and G₁ cell cycle arrest.¹⁸

In the present study we tested whether *Plag1* and *PLAGL2* are oncogenes in AML using a combination of in vivo and in vitro

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assays. We found that these transcription factors are specifically up-regulated in RIM-induced leukemic samples with retroviral insertions within these loci. To functionally validate their role in leukemogenesis we used a bone marrow transduction assay followed by transplantation (tBMT). Coexpression of either *Plag1* or *PLAGL2* and *Cbfb-MYH11* efficiently induced AML in 100% of recipient mice with a latency of 3 to 12 weeks. Furthermore, *Plag1* or *PLAGL2* induced expansion of hematopoietic progenitors and increased proliferation by inducing G₁ to S transition in vitro. Importantly, *PLAG1* and *PLAGL2* are overexpressed in 20% of human AML. Among these, *PLAGL2* is preferentially overexpressed in samples carrying inv(16). These results are in concordance with our in vivo studies in the mouse and highlight *PLAG1* and *PLAGL2* oncogenic function in human leukemia.

Materials and methods

Reverse transcriptase and quantitative PCR (qPCR) analyses

RT and qPCR of murine samples. RNA was extracted with guanidium thiocyanate followed by centrifugation in cesium chloride solution or with Trizol (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. First-stand cDNA was generated by using 2 μg RNA, 1 U Superscript-2 reverse transcriptase (RT; Invitrogen), and 0.1 μg oligo dT primer in a 20-μL reaction. Each polymerase chain reaction (PCR) included 1 μL cDNA, 1 μL 10 mM primers, 1 μL 10 mM deoxyribonucleoside triphosphates (dNTPs), 5 μL 10 × buffer, 1 U Taq Polymerase (Fisher Scientific, Pittsburgh PA). PCR conditions included a 2-minute step at 95°C followed by a 30-cycle amplification (each with a 30-second denaturing step at 95°C, a 30-second annealing step at 56°C, and a 30-second extension step at 72°C). SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for qPCR according to the manufacturer's instructions. *Plag1* primers were *mP1* × 1 (GGTTCACCTCTCTCA-CACG) and *mP1* × 2 (TGAGTAGCCATGTGCCTTTGTA). *Plag2* primers were *mPL2* × 1 (TATAGGCACATGGCCACCCACT) and *mPL2* × 2 (TGAC-GACGGTATCCCAGCTTT). The long terminal repeat (LTR) primer was LTR1 (CTCTTGCTGTGCATCGACT). *Igf2* primers were *mIgf2-1* (AGTC-CGAGAGGGACGTGTCTAC) and *mIgf2-2* (CGTCCCGCGGACTGTCTC). *Actb* (β-actin) primers were *b-actinFI* (CGAGGCCAGCAAGAGAG) and *b-actinRI* (CGGTTGGCCTTAGGGTTCAG). QPCR was performed in an ABI PRISM 7000 sequence detection system (Applied Biosystems). Samples were normalized to *beta-actin* expression levels, and relative values were determined by the standard curve method.

qPCR of human samples. Bone marrow samples from 162 patients with AML at diagnosis (classified according to the French-American-British nomenclature; Table 1) and 6 healthy volunteers were obtained after informed consent. Approval was obtained from the Erasmus Medical Center institutional review board for these studies. Blasts from patients with AML and mononucleated fractions from normal bone marrow specimens were isolated by Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation and then cryopreserved. After thawing, cells were washed with Hanks Balanced Salt Solution (HBSS) and further processed for RNA isolation. AML samples treated according to this procedure usually contain more than 90% blasts after thawing. Total RNA was extracted with guanidium thiocyanate followed by centrifugation in cesium chloride solution. RNA (1 μg) was transcribed into cDNA by using Superscript (Life Technologies, Merelbeke, Belgium) and random hexamers in a 40-μL reaction, under standard conditions. The qPCR amplification was performed in an ABI PRISM 7900 HT Sequence Detector, using 12.5 μL SYBR Green PCR Master Mix (PE Biosystems, Nieuwekerk a/d IJssel, The Netherlands), 2 μL (1/20th aliquot) cDNA, 2.5 pmol primer mix (Life Technologies), and 10 μL water. The PCR conditions included 2 minutes at 50°C and 10 minutes at 95°C followed by 45 cycles of amplification (each with a 15-second denaturation step at 95°C and a 1-minute annealing/extension step at 60°C). The *PLAG1* primers were *hP1Taq1* (ACACAGGAGAGAG-GCCCTACA) and *hP1Taq2* (ATGAGTAGCCATGTGC TTTGT); the *PLAGL2* primers were *hPL2Taq1* (CACTGTGGCAAGGCTTTTGC) and

Table 1. Clinical characteristics of the AML samples

Characteristics	Number
Gender	
Male	77
Female	85
Age, median (range), y	42.3 (15.2-60.8)
Age groups	
Younger than 35	51
35-50	62
50 and older	49
FAB	
M0	4
M1	39
M2	33
M3	13
M4	32
M5	36
M6	2
Unclassified	3
Cytogenetic risk group	
Favorable	34
inv(16)	12
t(8;21)	11
t(15;17)	11
Intermediate	111
Unfavorable	17
WBC count, median (range), 10 ⁹ /L	29.8 (0.3-263)
Blast count, median (range), %	66 (0-98)

FAB indicates French-American-British; WBC, white blood count.

hPL2Taq2 (GATGGTCCTTGCGGTGAAACAT). qPCR for normalization control was carried as described previously.¹⁹

To determine *PLAG1* and *PLAGL2* relative expression levels, the average Ct (threshold cycle) values from duplicate readings were normalized for endogenous reference (dCt = Ct target – Ct PBGD) and compared with a calibrator using the “delta-delta Ct method” (δδCt = δCt Sample – δCt Calibrator). As calibrator we used the average Ct value of *PLAG1* or *PLAGL2* in the 6 CD34⁺ healthy volunteer samples. Using the δδCt value, relative expression was calculated (2^{-δδCt}).

Retroviral production

The *Plag1* cDNA was cloned from testis RNA by using PFU polymerase (Stratagene, La Jolla, CA) and primers *mP1FIF* (GATAATCTCGAGAC-CATGGATT ACAAGGATGACGACGACGATAAGGCCACTGTGCAT-TCCTGGTGAT) and *mP1FIR* (GAATGAGAAGCGGCCGACTA-CTGAAAAGCTTGATGGA). The *PLAGL2* cDNA was cloned by using primers *hPL2FF* (GATAATCTCGAGACCATGGA TTACAAGGATGAC-GACGACGATAAGACCACATTTTTTCACCGCGT) and *hPL2FR* (GAATGAGAAGCGGCCGACTACTGGAATGCTTGGTGGA). The PCR amplicons were cut with *Xho1* and *Not1* and cloned into *pMSCViresEGFP(MIG)*.²⁰ Phoenix packaging cells (generously provided by Gary Nolan, Stanford University, CA) were cotransfected with 2 μg retroviral constructs, 2 μg ψ-Eco packaging plasmid with Effectene reagent (Quiagen, Valencia CA) according to manufacturer's protocol. Retrovirus supernatants were taken at 24, 48, and 72 hours and titered in 3T3 cells by fluorescence-activated cell sorting (FACS) analysis for green fluorescent protein (GFP).

Bone marrow transduction and transplantation (tBMT)

Cbfb-MYH11 expression was induced in a *Cbfb*^{56M/+} conditional knock-in mouse model recently generated in our laboratory with the use of the Cre-Lox system. Its characterization will be published elsewhere. *Cbfb-MYH11* was induced by using the *Mx1Cre* transgenic mouse.²¹ Briefly, *Cbfb*^{56M/+}/*Mx1Cre* double transgenics or wild-type control mice were injected with 3 doses of polyinosinic-polycytidylic acid (pI-pC; Sigma, St

Louis, MO) at 3 weeks of age, and cells were harvested 6 days after treatment with 150 mg/kg 5-fluorouracil. Double transgenic bone marrow cells consistently rendered more than 90% of Cre-mediated *Cbfb-MYH11* induction. Cells were spin-infected twice with retrovirus supernatant. Following infection, 5×10^5 to 1×10^6 bone marrow cells were transplanted into 4- to 6-week-old sublethally irradiated (650 rads [6.5 Gy]) 129SvEv wild-type mice intravenously. Mice were under daily observation for early signs of leukemia. These signs included limited motility, pale paws, and dehydration. At first signs of illness, peripheral blood was analyzed for cell number and morphology for the presence of immature cells. FACS analysis of peripheral blood was performed by using antibodies to cell-surface markers Gr-1, CD11b, B220, CD3, Ter119, and c-kit (Pharmingen, San Diego CA). Leukemic cells were harvested from the bone marrow or spleen of affected mice in RPMI 1640 (Invitrogen, Carlsbad, CA) and 20% fetal bovine serum (FBS; Invitrogen), and single-cell suspension aliquots of 1×10^6 cells were transplanted intravenously in sublethally irradiated 4- to 6-week-old 129SvEv recipients.

Southern blot analysis

Southern blot analysis was performed following standard protocols. Briefly, spleen DNA from leukemic and control mice was cut with *HindIII* (to identify viral insertions in sample) separated in a 0.8% agarose gel and transferred onto Hybond-XL nylon membrane (Amersham-Pharmacia Biotech, Buckinghamshire, England). The 800-base pair (bp) GFP probe was an *EcoRI/NcoI* fragment from *MIG*. Hybridizations were performed overnight at 45°C in formamide solution (Hybrisol-I; Serologicals, Norcross, GA).

Methylcellulose colony-forming assay and cell-cycle analysis

129SvEv bone marrow cells expressing *Cbfb-SMMHC* were harvested 6 days after treatment with 150 mg/kg 5-fluorouracil, spin-infected with 2 rounds of retrovirus supernatant (either *MIG*, *MIG-Plag1*, or *MIG-PLAGL2*), and sorted for GFP expression. GFP-positive cells (1×10^4) were plated (in triplicate) in methylcellulose supplemented with interleukin 3 (IL3), IL6, and stem cell factor (SCF; Methocult 3534). Colonies were counted 7 days after plating and analyzed by FACS and cytopsin. Remaining cells were recovered and 1×10^4 cells were serially replated for 3 rounds. For cell-cycle analysis, sorted cells were fixed in 70% ice-cold ethanol, stained with 50 μ g/mL propidium iodide (Sigma), and analyzed by FACS.

Cytology and histology analysis

Morphology analysis of peripheral blood cells was carried out on modified Wright-Giemsa-stained smears and cytopsin. Histology analysis was conducted on sections from paraffin-embedded blocks and stained with

Hematoxylin and Eosin (Histoserv, Gaithersburg, MD). Images were taken using a Zeiss Axioskop 40 microscope (Carl Zeiss, Jena, Germany) with 40 \times or 100 \times (under Zeiss Immersol 518N oil) lenses, or a Zeiss Stemi 2000-C stereoscope at 6.5 \times magnification. The images were acquired with a Zeiss AxioCam MRc camera and MR-Green acquisition software.

Results

Retroviral insertions induce *Plag1* and *Plagl2* overexpression in leukemic samples expressing *Cbfb-MYH11*

Our recent RIM study identified 8 retroviral insertions at the *Plag1* locus and 2 at the *Plagl2* locus in *Cbfb-MYH11*-associated leukemia samples. These results suggested that the transcription factors may play a critical role in inv(16) AML.¹⁰ To determine the precise location of retroviral insertions in the *Plag1* locus, we analyzed the *Plag1* gene structure in the mouse genome. The sequence of the 5' *Plag1* cDNA was cloned from embryo RNA (embryonic day 14.5) by using a combination of RT-PCR and 5'-rapid amplification of cDNA ends (RACE; GenBank accession no. AY574219). Analysis of this sequence using the public mouse genome database (<http://genome.ucsc.edu>) confirmed that the 8 *Plag1*-associated RIM retroviral insertions were located in its introns 1 and 2, upstream of the translation start codon in exon 4, and in the transcriptional orientation¹⁰ (Figure 1A). Notably, these introns are also involved in the breakpoints of 8q21-associated translocations in salivary gland adenomas.²² Considering that the retroviral insertions mapped upstream of *Plag1* and *Plagl2* translational start site and in the same transcriptional orientation, we reasoned that these insertions may up-regulate *Plag1* and *Plagl2* transcription. Therefore, we analyzed *Plag1* and *Plagl2* expression in normal and RIM-AML samples. The *Plag1* transcript was not detected in mouse hematopoietic tissues by RT-PCR (Figure 1B). Furthermore, expression was not detected in a panel of 10 RIM-AML samples with retroviral insertions in other loci (Figure 1B, sample V65; data not shown). Conversely, *Plag1* was expressed in all samples with retroviral insertions in the *Plag1* locus. Finally, since these AML samples were induced in *Cbfb*^{+/MYH11} chimeras, *Cbfb-MYH11* expression was confirmed in leukemia samples by RT-PCR (Figure 1B, middle panel).

In humans, *PLAGL2* is expressed during embryogenesis and not

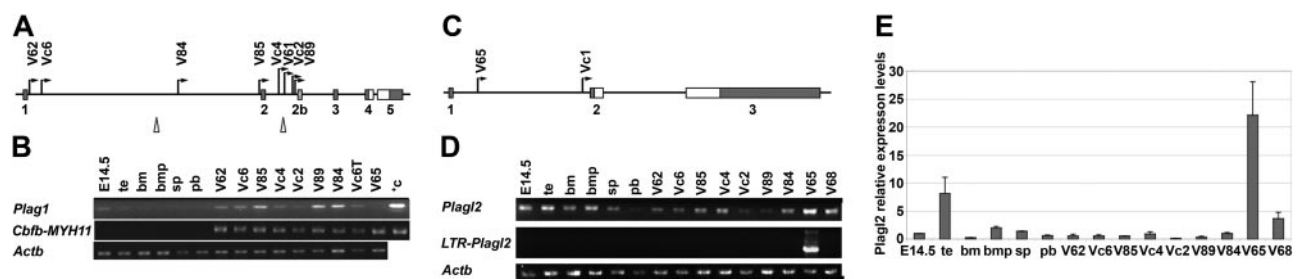


Figure 1. *Plag1* and *PLAGL2* are overexpressed in RIM-AML samples. (A) Representation of *Plag1*-RIM insertions (V62, Vc6, V84, V85, Vc4, Vc2, V89). Thin arrows indicate location of retroviral insertions and direction of LTR-transcription. Triangles indicate regions of chromosomal breakpoints in pleomorphic adenomas.²² Boxes indicate exons, including untranslated (□) and translated (▣) regions, and alternatively spliced exon 2b (▨). (B) RT-PCR analysis of *Plag1* (top panel), *Cbfb-MYH11* (middle panel), and *Actb* (β -actin) (bottom panel), including whole embryo day 14.5 (E14.5), testis (te), bone marrow (bm), hematopoietic progenitor-enriched bone marrow (bmp; bone marrow 6 days after 5-fluorouracil treatment), spleen (sp), peripheral blood white blood cells (pb), *Plag1*-associated RIM-AML samples (V62, Vc6, V85, Vc4, Vc2, V89, and V84), Vc6 transplant (Vc6T), *Plagl2*-associated AML sample (V65), and 0.1 ng *Plag1* plasmid control (+c). (C) Representation of *Plagl2*-RIM insertions (Vc1 and V65). Arrows indicate location of retroviral insertions and direction of LTR-transcription. Boxes indicate exons, including untranslated (□) and translated (▣) regions. (D) RT-PCR analysis of *Plagl2* (top panel), *Plagl2* transcribed from viral LTR (middle panel), and *Actb* (bottom panel); including hematopoietic tissues, *Plag1*-associated RIM-AML samples (V62, Vc6, V85, Vc4, Vc2, V89, and V84), *Plagl2* RIM-AML sample V65, and a representative RIM-AML sample with no viral insertion near *Plag1* or *Plagl2* (V68). (E) Quantitative PCR analysis of *Plagl2*, using *Plagl2* specific primers, in normal and leukemic samples. Expression levels were normalized to *Actb* and shown relative to sample E14.5 (E14.5 = 1). Error bars indicate standard errors from duplicate experiments.

detected in adult tissues.¹¹ In the mouse, however, *Plag2* is detected in several tissues, showing higher expression in lung, spleen, and testes.²³ The RIM-AML samples Vc1 and V65 included retroviral insertions in *Plag2* intron 1, in the same transcription orientation and upstream of its translation start codon (Figure 1C). *Plag2* expression was detected in all normal tissues analyzed (Figure 1D, top panel, lanes 1-6). The RIM-AML samples analyzed also expressed *Plag2* (Figure 1D, top panel, lanes 7-4), with higher levels in the *Plag2*-associated sample V65 (RNA from sample Vc1 was not available). Similar analyses that used LTR-forward and *Plag2*-reverse primers identified a strong band only in sample V65 (Figure 1D, middle panel), confirming that the increase in *Plag2* expression was transcribed from the retroviral LTR. Furthermore, increased *Plag2* relative expression levels (RELS) were confirmed by qPCR using *Plag2*-specific primers (Figure 1E). Therefore,

upstream retroviral insertions up-regulated *Plag1* and *Plag2* transcription in *Plag*-associated leukemic samples.

Plag1 and Plag2 cooperate with Cbfb-MYH11 to induce leukemia

The *Plag1* and *Plag2* overexpression in mouse RIM-AML samples suggests that these zinc finger transcription factors may participate in leukemogenesis. Therefore, we tested whether *Cbfb-MYH11* and *Plag1* or *Plag2* coexpression is sufficient to trigger AML in the mouse by using a tBMT assay (described in "Materials and methods"). All mice that received transplants with bone marrow cells expressing *Cbfb-MYH11/MIG-Plag1* (n = 22) or *Cbfb-MYH11/MIG-PLAGL2* (n = 19) readily developed leukemia 3 to 12 weeks after transplantation (Figure 2A). Control groups remained healthy

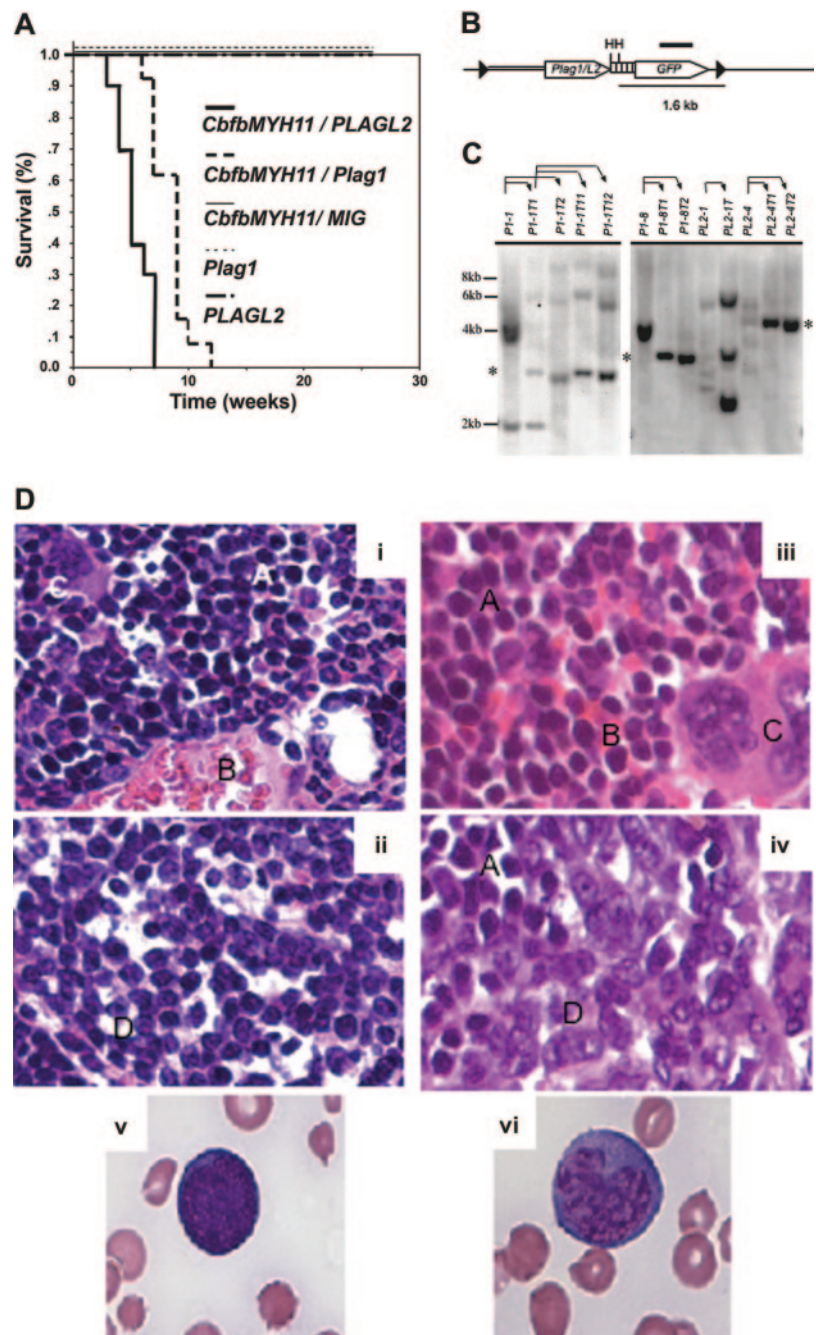


Figure 2. *Plag1* and *PLAGL2* cooperate with *Cbfb-MYH11* to induce AML in mice. (A) Kaplan-Meier survival curve of mice that received transplants with bone marrow cells expressing: *Cbfb-MYH11* and *MIG-Plag1* (thick dashed line), *Cbfb-MYH11* and *MIG-PLAGL2* (thick solid line), *Cbfb-MYH11* and *MIG* (thin solid line), *MIG-Plag1* (thin dashed line), or *MIG-PLAGL2* (dotted-dashed line). (B) Representation of the *MIG-Plag1/L2* provirus. The LTRs (▶), coding sequence for *Plag1/L2* (sequence encoding *Plag1* or *PLAGL2*) and *GFP* (open arrows), internal ribosome entry site (IRES; ▭), probe (thick line), *HindIII* restriction sites (H), genomic (thin line) and viral (double thin line) sequences, are detailed. (C) Representative Southern blot analysis of transplantation of leukemic cells. DNA from *PLAG*-associated primary leukemia (lanes 1, 6, 9, 11), secondary transplanted leukemias (lanes 2, 3, 7, 8, 10, 12, 13), and tertiary transplanted leukemias (lanes 4 and 5) were probed with the retroviral *GFP* sequence. Thin arrows indicate which primary leukemic cells were used for secondary and tertiary transplants. Asterisk shows a band that represents the clonal expansion of a population of leukemic cells that became predominant in mice that receive transplants. The expected molecular weight of expected fragments (in kb) is indicated at the left. (D) Pathologic characteristics of leukemic cells. Wright-Giemsa staining of ($\times 10$ magnification) normal (panel i) and leukemic (panel iii) bone marrow, normal (panel ii) and leukemic spleen (panel iv), and representative blastlike (panel v) and monocytic-like (panel vi) cells ($\times 100$ magnification). Note area with lymphoid (A), red (B), megakaryocytes (C), and leukemic (D) cells.

up to 5 months (experimental end point), including mice that received transplants with cells expressing *Cbfb-MYH11*/MIG ($n = 24$), *MIG-Plag1* ($n = 17$), or *MIG-PLAGL2* ($n = 17$).

To confirm that these cells were leukemic, bone marrow cells from affected mice (*Plag1*, $n = 10$; *PLAGL2*, $n = 9$) were transplanted into sublethally irradiated secondary recipients (2 transplants per donor cell population). In all cases, the leukemic cells were readily transplantable with an average disease onset of 4 weeks.

As *Plag1* and *PLAGL2* are expressed from the *MIG-LTR* (Figure 2B), we were able to examine the clonality of disease by Southern blot analysis using primary, secondary, and tertiary leukemic samples. Multiple bands were detected in most primary leukemic samples (Figure 2C, lanes 1, 6, 9, and 11), while secondary (Figure 2C, lanes 2, 3, 7, 8, 10, 12, and 13) and tertiary (Figure 2C, lanes 3-5) transplants frequently displayed enrichment of subclones underrepresented in the primary transplant (note asterisks). These data reveal that *Plag1* and *PLAGL2* efficiently cooperate with *Cbfb-MYH11* to induce AML in mice. In addition, the short latency and oligoclonality of disease is consistent with the hypothesis that leukemia may rise from few genetic alterations.

Leukemic mice exhibited an increase of immature cells and a decrease of red blood cells and megakaryocytes in bone marrow (Figure 2D, panels i and iii). Likewise, spleens were consistently enlarged (with spleen weight of 601.5 ± 234.7 mg for *Plag1*, and 197 ± 106.8 mg for *PLAGL2*) with an altered architecture and evident invasion of leukemic cells (Figure 2D, panels ii and iv). Leukemic cells were progressively predominant in peripheral blood (*Plag1* = 23.5×10^6 , range 7.3×10^6 to 74.4×10^6 cells/mL; *PLAGL2* = 12.5×10^6 , range 0.3×10^6 to 61.7×10^6 cells/mL), including blastlike and monocytic-like cells (Figure 2D, panels v-vi). Occasionally, enlargement of liver resulting from focal infiltrations of leukemic cells was also observed. However, enlargement of the thymus and lymph nodes was not observed (data not shown), indicating a nonlymphoid disease.

We characterized the leukemic cells by FACS analysis. The predominant cells in peripheral blood were *c-kit*⁺ and *Lin*⁻ (*Lin*: CD3, Gr1, B220, Ter119, Mac1; Figure 3). Furthermore, the leukemic cells were consistently GFP⁺, confirming the presence of the provirus (Figure 3). Finally, *Cbfb-MYH11* and either *Plag1* or *PLAGL2* expression in leukemic cells was confirmed by RT-PCR (data not shown).

Taken together, the morphology and FACS analysis data indicate that these leukemic cells are predominantly blastlike and monocytic-like cells and confirm that they express *Plag1* and *Plag2*. Finally, the leukemic cells resemble *Cbfb-MYH11*-associated leukemic cells previously described,^{9,10} supporting the idea that *Cbfb-SMMHC* directs the phenotypic characteristics of leukemic cells.

Plag1 and PLAGL2 expand hematopoietic progenitors in *Cbfb-MYH11*-expressing cells by increasing G₁ to S phase transition of cell cycle

To further understand the role of *Plag1* and *Plag2* in AML development, *in vitro* differentiation assays were performed in GFP-sorted bone marrow progenitors expressing *Cbfb-MYH11* and either *MIG-Plag1*, *MIG-PLAGL2*, or *MIG*. At day 7, the number of *Plag1*- and *PLAGL2*-expressing colonies increased significantly (Figure 4A, P1). In addition, *Plag1* and *PLAGL2* colonies were predominantly compact and large when compared with diffuse colonies in controls, suggesting an increased progenitor proliferation (Figure 4B). Furthermore, cytology analysis revealed that

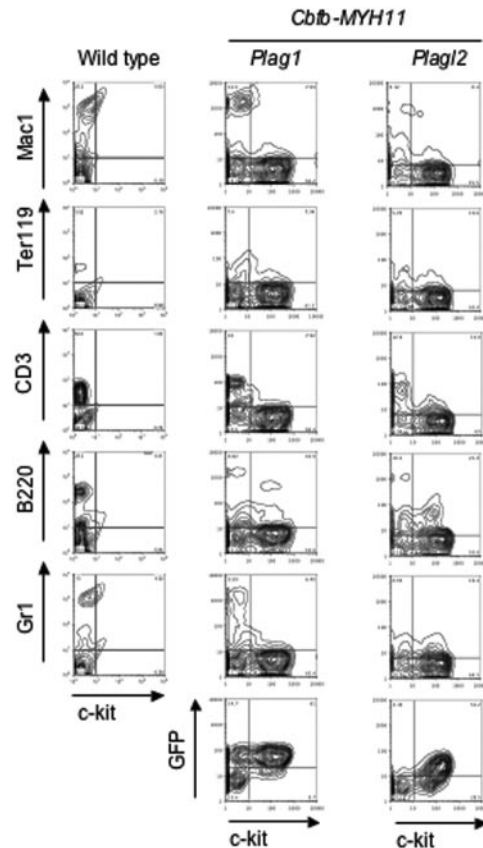


Figure 3. Peripheral blood leukemic cells are *Lin*⁻/*c-kit*⁺ and GFP⁺. Representative examples of peripheral blood leukemic cell FACS analysis. FACS analysis of lineage markers (Gr1, B220, CD3, Ter119, and Mac1) and GFP, versus hematopoietic progenitor marker (*c-kit*) for wild-type (left column) and leukemic cells expressing either *Cbfb-MYH11/Plag1* (middle column) or *Cbfb-MYH11/PLAGL2* (right column).

colonies expressing *Plag1* or *PLAGL2* included predominantly immature blasts when compared with *MIG* control colonies (Figure 4C). This enrichment for immature forms suggests that *Plag1* and *PLAGL2* selectively expanded *Cbfb-MYH11* differentiation-blocked progenitors.

To assess the effect of *Plag1* and *PLAGL2* on the renewal capacity of progenitors, 10^4 cells from day 7 pooled colonies were serially replated. In contrast to *MIG* controls, *MIG-Plag1* and *MIG-PLAGL2* transduced cells exhibited a marked increase in replating potential (Figure 4A, P2-P3). As *Plag1* and *PLAGL2* seemed to expand progenitors, we next assessed their effect on cell-cycle progression. Bone marrow cells expressing *Cbfb-MYH11* were sorted after infection with *MIG-Plag1*, *MIG-PLAGL2*, or *MIG* and analyzed with the use of propidium iodine staining by FACS. Notably, *Plag1* and *PLAGL2* induced entry into S phase and reduced the number of cells in G₀/G₁ phase (Figure 4D). The increase of cells in S phase was also confirmed by bromodeoxyuridine (BRDU) incorporation (data not shown). Overall, these data indicate that *Plag1* and *Plag2* stimulate proliferation and renewal of hematopoietic progenitors expressing *Cbfb-MYH11*.

PLAG1 and *PLAGL2* regulate gene expression by binding to the same DNA consensus site.²⁴ The insulin growth factor 2 gene (*IGF2*) harbors 8 *PLAG* binding sites in its promoter 3 and is up-regulated in cell lines ectopically expressing *PLAG1* as well as in salivary gland tumors with *PLAG1*-associated translocations.^{15,24,25} In addition, AML samples frequently show loss of imprinting at the *IGF2* locus associated with *IGF2* overexpression.²⁶ Therefore, it is possible that *PLAG1* and *PLAGL2* could

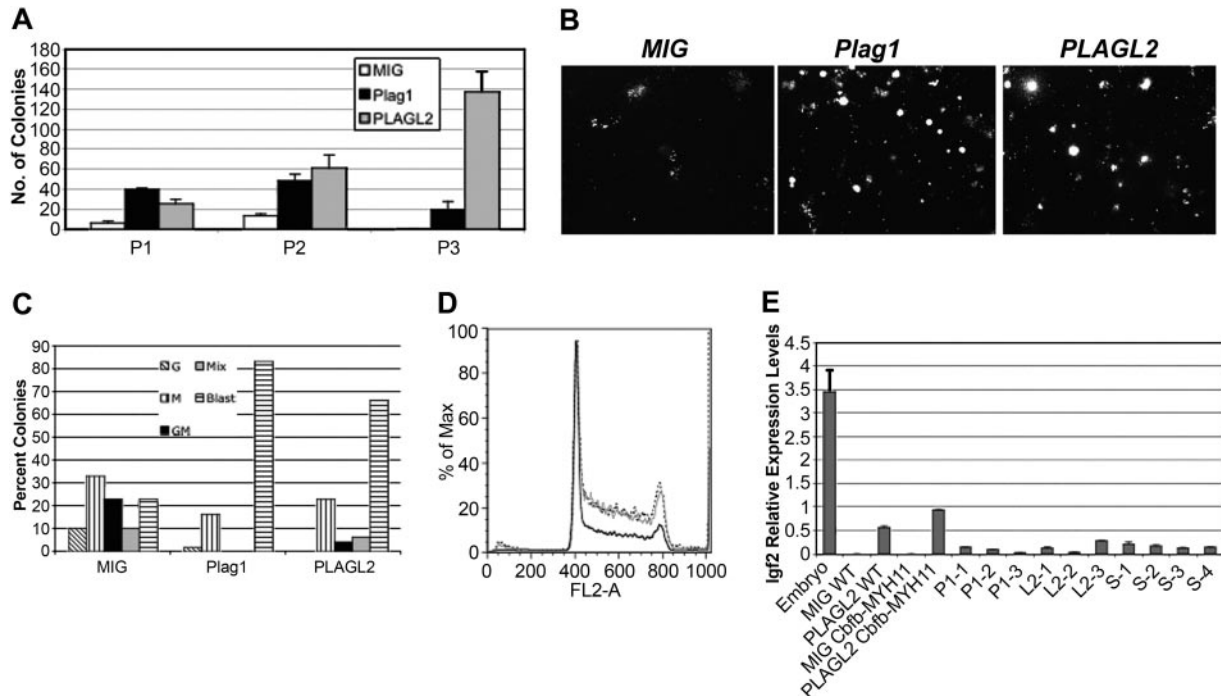


Figure 4. *Plag1* and *PLAGL2* increase proliferation of hematopoietic progenitor in vitro by inducing entry into S phase. (A) Serial replating (P1 to P3) of 10^4 *Cbfb-MYH11*-expressing bone marrow cells infected with *MIG* (□), *MIG-Plag1* (■), or *MIG-PLAGL2* (▨) and cultured in methylcellulose culture for 7 days. (B) Representative images of colony morphology at day 7. (C) Lineage distribution colony-forming units (CFUs) from P1 analyzed by cytospin of individual colonies. Colony types are indicated as granulocytic (G; ▨), monocytic (M; ▩), granulocytic and monocytic (GM; ▧), mix (▤), and blast (▥). (D) Cell-cycle analysis of sorted bone marrow cells expressing *Cbfb-MYH11* and *MIG-Plag1* (gray line), *MIG-PLAGL2* (dotted black line), or *MIG* (solid black line) and stained with propidium iodide. (E) The *Igf2* RELs determined by quantitative PCR in E14.5 total embryo (lane 1), *MIG* wild-type bone marrow (lane 2), *MIG-PLAGL2* wild-type bone marrow (lane 3), *MIG Cbfb-MYH11* bone marrow cells (lane 4), *MIG-PLAGL2 Cbfb-MYH11* bone marrow (lane 5), *Plag1/Cbfb-MYH11* leukemias (lanes 6-8), *PLAGL2/Cbfb-MYH11* leukemias (lanes 9-11), and leukemias not overexpressing *Plag1* or *PLAGL2*.¹²⁻¹⁵ Values were normalized to *Actb* expression levels. Error bars indicate standard error from triplicate experiments.

expand hematopoietic precursors via the IGF2 pathway to induce AML in cooperation with Cbfb-SMMHC. To test this hypothesis, we assessed *Igf2* levels in GFP-sorted bone marrow cells and leukemic samples expressing *Plag1*, *PLAGL2*, or without expression of these genes by qPCR. *Igf2* RELs were increased by *Plag1* and *PLAGL2* in bone marrow (Figure 4E; data not shown). However, RELs were not increased in leukemia samples when compared with non-*Plag1*- or -*PLAGL2*-induced AMLs. These results indicate that *Igf2* transcripts are up-regulated by *Plag1* and *PLAGL2* in hematopoietic progenitors, but that *Plag1* and *PLAGL2* do not maintain increased levels of *Igf2* in leukemic progression.

PLAG1 and PLAGL2 are overexpressed in human AML

AML is a heterogeneous disorder with regard to both morphology and chromosomal aberrations detected in the leukemic cells. Currently, AML is classified according to pretreatment karyotype and treatment outcome. For example, cytogenetic groups with *inv(16)/t(16;16)*, *t(15;17)*, and *t(8;21)* define an AML subset with increased survival (favorable outcome).

To investigate whether *PLAG1* or *PLAGL2* could also play a role in human AML, we asked whether *PLAG1* and *PLAGL2* are up-regulated in human AML samples. The RELs of *PLAG1* and *PLAGL2* were analyzed in a panel of 162 human primary AML samples by qPCR. The REL values were normalized to *PBGD* expression and compared with expression levels in normal CD34⁺ bone marrow cells. The panel was divided into 4 cytogenetic groups: 12 samples with *inv(16)(p13q22)/t(16;16)* expressing *CBFB-MYH11*, 11 samples with *t(15;17)*, 11 samples with *t(8;21)*, and 128 samples without these translocations. The characteristics of the AML samples are detailed in Table 1.

Overall, *PLAGL2* was overexpressed in 15% of the samples. Strikingly, higher RELs were associated with *inv(16)* than with the other cytogenetic subgroups (Figure 5A). The association between *PLAGL2* and *CBFB-MYH11* was highlighted, as no other cytogenetic change was common in *inv(16)* samples (Table 2). In addition, *PLAGL2* expression was significantly higher in *t(8;21)* and *t(15;17)* subgroups (with $P < .01$ and $P < .05$, respectively; 2-tailed paired *t* test) when compared with samples without these 3 chromosomal rearrangements or *inv(16)*.

The RELs of *PLAG1* transcript were also significantly increased 1.4- to 2.4-fold ($P < .0001$; 2-tailed paired *t* test) in 5% (8 of 162) of the samples analyzed (Figure 5B). The overexpression did not correlate with a distinct cytogenetic subgroup, suggesting that *PLAG1* may cooperate with other mutations associated with AML. *PLAG1* overexpression has been associated in tumors with chromosome 8 polyploidy,^{14,29} and trisomy of chromosome 8 is a frequent rearrangement in human AML. However, we found no correlation between trisomy 8 and increased *PLAG1* expression in this AML panel (trisomy 8 REL = 0.14 ± 0.13 , $n = 18$; disomy 8 REL = 0.24 ± 0.44 , $n = 144$). These data reveal that *PLAG1* and *PLAGL2* expression is deregulated in human AML. Furthermore, *PLAGL2* overexpression strongly correlates with *inv(16)* AML.

Discussion

AML arises from the uncontrolled clonal expansion of hematopoietic progenitor cells that have acquired (at least) 2 types of genetic alterations. These cooperating mutations have been classified as

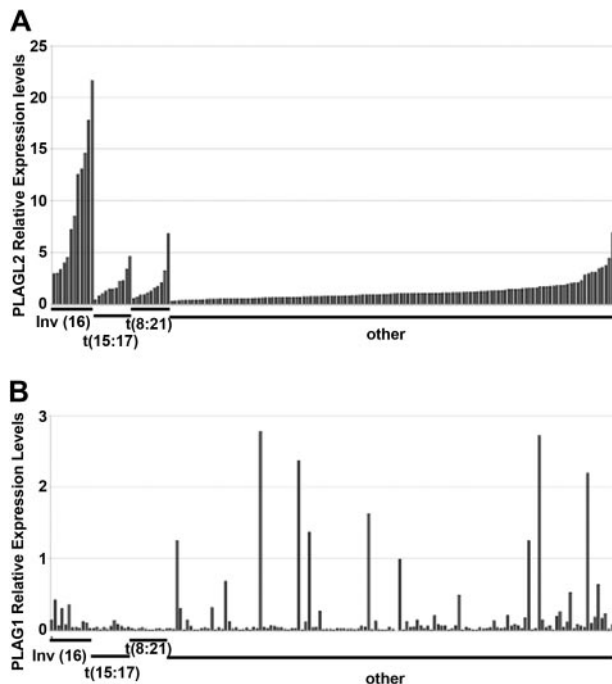


Figure 5. Quantitative PCR analysis of *PLAG1* and *PLAGL2* in a panel of 162 human AML samples. *PLAGL2* (A) and *PLAG1* (B) RELs. Samples are clustered in 4 cytogenetic groups: samples showing inv(16)(p13;q22), t(15;17), t(8;21), and samples without these rearrangements (other). Within each group, samples are ordered by increased *PLAGL2* RELs. RELs represent the average of duplicate values and are compared with the value of 6 healthy CD34⁺ bone marrow samples.

“class-I” mutations, conferring hematopoietic progenitor expansion (such as increase in proliferation, cell survival, or self-renewal potential), and “class-II” mutations, inducing impairment of hematopoietic differentiation.³⁰ Previously, we demonstrated that expression of the inv(16)-associated *Cbfb-MYH11* fusion gene (a class-II mutation) impairs hematopoietic differentiation in the mouse, and that this effect is required for leukemogenesis.⁹ Our genetic study identified *Plag1* and *Plag2* as class-I candidate genes for *Cbfb-MYH11*-associated leukemogenesis in the mouse.⁹ The current study demonstrates that up-regulation of *Plag1* and *PLAGL2* is a class-I mutation that induces AML in cooperation with *Cbfb-SMMHC*.

Plag1 and *Plag2* were specifically overexpressed in RIM-AML samples with retroviral insertions at these loci, confirming the genetic data previously published and further implicating these genes in mouse leukemia. Using a tBMT assay, we demonstrate that both transcription factors can independently cooperate with *Cbfb-SMMHC* to induce AML in 3 to 12 weeks with 100% penetrance. Mice consistently display expansion of immature cells and infiltration into peripheral blood, spleen, and liver, whereas lymph nodes and thymus remain unaffected. The leukemic cells in peripheral blood morphologically appear as blastlike and monocytic-like cells and are predominantly Lin⁻/c-kit⁺. Interestingly, although the leukemic cells have a myeloid morphology, their immunophenotype suggests that this population contains blastlike cells stalled at an early stage of differentiation. This phenotype is similar to the AML observed in *Cbfb⁺/MYH11* knock-in mice treated with chemical or retroviral mutagens. Taken together, these studies suggest that *Cbfb-MYH11*, and not the cooperating gene(s), determines the phenotype of the disease.

Consistent with the oligoclonal and aggressive course of disease, coexpression of *Cbfb-MYH11* and *Plag1* or *Plag2* may be

sufficient to trigger AML in the mouse. Alternatively, as the tBMT assays require insertion of the retroviral vector in the hematopoietic progenitor’s genome, it is possible that a third hit could be required for AML development. We show that *Plag1* and *Plag2* oncogenic function is associated with expansion of hematopoietic progenitors expressing *Cbfb-SMMHC*. This is evident in the blastlike colony morphology, the increase of blast cells in these colonies, the increase of cells entering into S and G₂/M phases, and the expanded replating potential. Interestingly, these results are similar to the mitogenic effect that *Plag1* or *Plag2* overexpression provides NIH-3T3 cells.¹⁵

The effect of *Plag1* and *Plag2* in leukemogenesis is similar to that of other class-I genes mutated in human inv(16) AML, including activating (oncogenic) mutations in *KRAS* and *FLT3*.^{31,32} For example, expression of activated *Flt3* or *Kras* alone cannot induce AML in the mouse,³³⁻³⁵ but *Flt3* efficiently induces AML in cooperation with promyelocytic leukemia-retinoic acid receptor α (*PML-RAR α*).³⁶ Conversely, activated *Kras* or *Flt3* alone induces a myeloproliferative-like disease, while *Plag1*- and *Plag2*-expressing mice remain healthy. Also, the in vitro expansion of hematopoietic precursors observed on *Plag1* or *PLAGL2* expression was not observed in oncogenic *Kras* cultures.³⁴ These differences may indicate that distinct pathways are involved.

Previous studies have shown that *PLAG1* and *PLAGL2* up-regulate *IGF2*.^{15,24} In addition, AML samples frequently show loss of imprinting at the *IGF2* locus associated with *IGF2* overexpression.²⁶ Surprisingly, our study shows that *Plag1* and *PLAGL2* induce moderate *Igf2* transcript levels in bone marrow cells, but that this increase is not maintained in leukemic samples. These results suggest that the *Plag* transcription factors may affect an *Igf2*-independent pathway in leukemogenesis or play a role in initiation but not maintenance of leukemia.

We find *PLAGL2* expression preferentially induced in human AML samples with inv(16), supporting our findings using the mouse model. Interestingly, *PLAGL2* was also significantly induced in t(8;21)- and t(15;17)-associated AML samples, suggesting that *PLAGL2* may also induce AML in cooperation with other fusion genes. This is the first study implicating *PLAGL2* in human cancer and demonstrating that both *Plag1* and *PLAGL2* function as oncogenes in vivo. It will be, therefore, interesting to examine *PLAGL2* function in other cancers. Considering the in vivo effect of *Plag1* and *Plag2* in *Cbfb-MYH11*-dependent AML in mice and increased *PLAG1* and *PLAGL2* levels in human AML without inv(16) cytogenetics, it will be important to test whether these transcription factors can also induce AML in cooperation with other class-II fusion genes in vivo. Interesting examples could

Table 2. Cytogenetics of inv(16) AML samples

ID	Cytogenetics
1	46, XY, inv(16)(p13;q22)
2	46, XY, inv(16)(p13;q22)
3	46, XY, inv(16)(p13;q22)[37]/47,id, + 8[2]/47,id, + 21[4]
4	46, XY, inv(16)(p13;q22)
5	46, XY, t(16;16)(p13;q22)
6	46, XX, inv(16)(p13;q22)
7	46, XY, inv(16)(p13;q22)
8	46, XX, inv(16)(15%)/47,XX,inv(16), + 22 (85%)
9	46, XX, inv(16)(p13;q22)
10	46, XX, inv(16)(p13;q22)
11	46, XX, inv(16)(p13;q22)
12	46, XX, inv(16)(p13;q22)[8]/47,idem, + 8[12]

include transgenic mice models for *AML1-ETO* and *PML-RAR α* .³⁷⁻³⁹ Finally, the finding that *PLAG1* and *PLAGL2* zinc finger proteins participate in AML development implicates novel pathways in leukemogenesis and may provide additional targets in the design of improved therapies.

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