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Roles of p15^{Ink4b} and p16^{Ink4a} in myeloid differentiation and RUNX1-ETO-associated acute myeloid leukemia

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Abstract

Inactivation of p15^{Ink4b} expression by promoter hypermethylation occurs in up to 80% of acute myeloid leukemia (AML) cases and is particularly common in the FAB-M2 subtype of AML, which is characterized by the presence of the *RUNX1-ETO* translocation in 40% of cases. To establish whether the loss of p15^{Ink4b} contributes to AML progression in association with *RUNX1-ETO*, we have expressed the RUNX1-ETO fusion protein from a retroviral vector in hematopoietic progenitor cells isolated from wild-type, p15^{Ink4b} or p16^{Ink4a} knockout bone marrow. Analysis of lethally irradiated recipient mice reconstituted with RUNX1-ETO-expressing cells showed that neither p15^{Ink4b} or p16^{Ink4a} loss significantly accelerated disease progression over the time period of one year post-transplantation. Loss of p15^{Ink4b} alone resulted in increased myeloid progenitor cell frequencies in bone marrow by 10 months post-transplant and a 19-fold increase in the frequency of Lin^c-Kit⁺Sca-1⁺ (LKS) cells that was not associated with expansion of long-term reconstituting HSC. These results strongly suggest that p15^{Ink4b} loss must be accompanied by additional oncogenic changes for RUNX1-ETO-associated AML to develop.

Keywords

RUNX1-ETO; acute myeloid leukemia; p15^{Ink4b}; p16^{Ink4a}; hematopoiesis

1. Introduction

The development of acute myeloid leukemia (AML), like all cancer, is a process that requires multiple genetic and epigenetic changes that result in the clonal outgrowth of malignant myeloid cells [1]. For a number of AML subtypes defined by the French-American-British (FAB) classification system, specific mutations are closely associated with clinically defined categories of disease. One of the most common recurrent mutations associated with approximately 40 percent of the FAB-M2 subtype of AML involves the translocation of chromosome 8 to chromosome 21 in the t(8;21) AML1-ETO (RUNX1-ETO) translocation, which is present in about 8-12 percent of adult AML cases [2-4]. The t(8;21) generates a fusion protein that contains the N-terminal 177 amino acids of the AML1/RUNX1 transcription factor,

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which includes the DNA-binding Runt-homology domain, and almost the entirety of the eight-twenty-one (*ETO/MTG8*) gene, which is a non-DNA-binding factor that is normally involved in recruitment of nuclear co-repressor complexes [5-9].

Animal model studies where a conditional allele of *RUNX1-ETO* expressed from the native *Runx1* locus was activated in adult mice showed that RUNX1-ETO enhanced the self-renewal of myeloid progenitor cells in serial replating assays but animals did not progress to AML in the absence of secondary mutagenic events induced by N-ethyl-N-nitrosourea (ENU) [10]. Similar observations were made in transgenic animals where RUNX1-ETO expression was induced by removal of tetracycline [11] or expressed under the control of the *MRP8* promoter [12]. Similarly, studies where RUNX1-ETO was expressed from a retroviral vector in hematopoietic stem cells (HSC) used to reconstitute lethally irradiated mice did not result in AML but caused significant delays in HSC differentiation (as determined by low-levels of initial donor-cell chimerism), early blocks in both B and T lymphocyte development, higher percentages of myeloid progenitor cells in bone marrow and increased *in vitro* colony-forming progenitors [13,14]. The delayed differentiation of RUNX1-ETO-expressing HSC may have been due to RUNX1-ETO inhibition or downregulation of key myeloid differentiation factors like C/EBP α [13,15-17] and/or to RUNX1-ETO inhibition of cell-cycle progression, which would make RUNX1-ETO-expressing HSC less competitive in a transplant assay.

Previous studies in myeloid cell line models and in primary myeloid bone marrow cells have shown that RUNX1-ETO expression results in a partial G1/S phase arrest [17-19], while Runx1 expression promotes the G1/S phase transition [20,21]. Inhibition of cell-cycle progression by RUNX1-ETO was correlated with downregulation of Cdk4 and c-myc [17] and cyclin D3 expression [22]. RUNX1-ETO has also been shown to induce transcription of the tumor suppressor protein p21/Waf1/Cip [23,24]. Conversely, RUNX1-ETO can directly bind and repress expression of other tumor suppressor genes including *p19^{ARF}* and neurofibromatosis-1 (*NF-1*) [25,26]. Collectively, these observations indicate that the mechanism for RUNX1-ETO inhibition of cell-cycle progression is complex and may involve deregulated expression of multiple cell-cycle-associated genes.

Analysis of human AML patient samples has shown that up to 80% of AML cases involve transcriptional inactivation of the *p15^{INK4B}* tumor suppressor gene through an epigenetic process involving DNA methylation of the *p15^{INK4B}* promoter region [27,28]. *p15^{INK4B}* inactivation is present at higher frequencies in FAB-M2 leukemia [29,30], suggesting that this event may be AML promoting in FAB-M2 cases. This is further supported by observations of increased susceptibility to leukemia formation in *p15^{Ink4b}*-deficient mice [31]. Concomitant inactivation of *p15^{INK4B}* and the *p16^{INK4A}* tumor suppressor gene, which regulates cyclin-dependent kinase activity and pRb phosphorylation, has been infrequently observed and, when present, is exclusively associated with AML of the FAB-M2 and -M4 subtypes [29,30]. Homozygous deletion of either *p15^{INK4B}* or *p16^{INK4A}*, which are tightly linked genes on human chromosome 9p21, is rare in AML [32,33].

In these studies, we have tested whether the loss of either *p15^{Ink4b}* or *p16^{Ink4a}* would promote AML progression in association with RUNX1-ETO in mice by expressing RUNX1-ETO in the context of wild-type, *p15^{Ink4b}*-deficient or *p16^{Ink4a}* knockout bone marrow cells used to reconstitute lethally irradiated mice.

2. Materials and methods

2.1. Mouse strains and genotyping

Wild-type C57BL/6-Ly-5.1 mice were used as a source of donor cells for retroviral transduction. *p15^{Ink4b}*-deficient animals on a C57BL/6 background were obtained from Linda

Wolff (NCI). p16^{Ink4a}-deficient animals on a FVB/N.129 background were purchased from the Mouse Models of Human Cancers Consortium (NCI-Frederick, strain #01XE4) and were backcrossed onto C57BL/6-Ly-5.1 for 4 generations prior to use. p15^{Ink4b}-deficient animals were genotyped using the following primers: p15KO forward 5'-ATC CGA GTG CCT ACA CCT CCA-3'; p15KO reverse 5'-GCT CCC GAT TCG CAG CGC AT-3'; p15WT forward 5'-GTC ATG ATG ATG GGC AGC G-3'; p15WT reverse 5'-CCG GAA TTC GCG TGC AGA TAC CTC GC-3'. p16^{Ink4a}-deficient animals were genotyped using: C015 5'-GGC AAA TAG CGC CAC CTA T-3'; C016 5'-GAC TCC ATG CTG CTC CAG AT-3'; C017 5'-GCC GCT GGA CCT AAT AAC TTC-3'.

2.2. Retroviral transduction of bone marrow and transplantation

Transduction and transplantation of wild-type C57BL/6, p15^{Ink4b} knockout and p16^{Ink4a}-deficient bone marrow cells was done as previously described [34].

2.3. Bromodeoxyuridine (BrdU) treatment

Two months post-transplant, VEX control and RUNX1-ETO mice were injected IP with a single dose of BrdU (1mg) and then sacrificed at 90 minutes (T1.5), 3 hours (T3), or 10 hours (T10) post-injection. Bone marrow was harvested and stained with antibodies to detect CMP, GMP, and MEP, which were then FACS sorted and fixed for staining with a PE-labeled anti-BrdU antibody and 7-AAD (BrdU Flow Kit, BD Pharmingen). Cells were analyzed on a 4-laser LSRII flow cytometer (BD Biosciences, San Jose, CA).

2.4. Myeloid progenitor cell staining and sorting

Red blood cells were lysed in ACK (8.3g ammonium chloride/1.0g potassium bicarbonate in 1L deionized water) and then bone marrow was stained using biotinylated antibodies to lineage markers (anti-CD3 (KT31.1), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-Gr-1 (RB6-8C5), anti-Ter-119 (TER-119), anti-IgM (R6-60.2) and anti-IL-7R α chain (A7R34)); APC-conjugated anti-c-Kit (2B8); PE-conjugated anti-Sca-1 (E13.141.1); PE-Cy7-conjugated anti-Fc γ RII/III (2.4G2); and FITC-conjugated anti-CD34 (RAM 34). All antibodies were purchased from BD Pharmingen unless otherwise noted. Biotinylated antibodies were visualized using a streptavidin-Pacific Blue secondary antibody (Molecular Probes, Eugene, OR). Cell sorting was performed on a MoFlo (Dako-Cytomation, Fort Collins, CO) and analysis was performed on an LSRII (BD Bioscience).

2.5. Quantitative RealTime PCR analysis

RNA was extracted from sorted cell populations using the MicroRNAeasy Kit (Qiagen, Valencia, CA). cDNA was transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Gene-specific primers were generated using Primer Express Software (Applied Biosystems) and were as follows: p16^{Ink4a} forward 5'-CCC AAC GCC CCG AAC T-3'; p16^{Ink4a} reverse 5'-GAG CAG AAG AGC TGC TAC GTG AA-3'; p15^{Ink4b} forward 5'-CAG AGA CCA GGC TGT AGC AAT CT-3'; p15^{Ink4b} reverse 5'-GCC CAA CAT GCC CTT GTC-3'; p19^{Arf} forward 5'-GTT GAG GCT AGA GAG GAT CTT GAG A-3'; p19^{Arf} reverse 5'-AAC GTT GCC CAT CAT CAT CA-3'; p21^{Cip1} forward 5'-GCA GAT CCA CAG CGA TAT CCA-3'; p21^{Cip1} reverse 5'-CGG ACA TCA CCA GGA TTG G-3'; p53 forward 5'-TGAACCGCCGACCTCTCCTTA-3'; p53 reverse 5'-GGC ACA AAC ACG AAC CTC AAA-3'; HPRT forward 5'-CTG GTG AAA AGG ACC TCT CG-3'; HPRT reverse 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'. Primer sets for p18^{Ink4c} were previously published [35]. RT-PCR was performed using SYBR[®] Green PCR Master Mix (Applied Biosystems) and manufacturer recommended conditions in a 20 μ l reaction volume. Reactions for each sample were performed in quadruplicate. Data was collected with a 7900HT Sequence Detection System (Applied Biosystems).

2.6. Myeloid colony-forming cell assay

FACS-sorted VEX⁺ myeloid scatter-gated bone marrow cells isolated from VEX control and RUNX1-ETO mice were plated in MethoCult M3434 methylcellulose media (StemCell Technologies, Vancouver). Colonies of greater than 50 cells were counted, typed and cytopun for Wright-Giemsa staining at 12 days.

2.7. Microscopy and software

All images were captured using a Zeiss Axiovert A1 compound microscope and 60X fluoromat oil immersion lens. Images were acquired using AxioVision rel. 4.5 software.

2.8. Competitive Repopulating Unit Assay

Whole bone marrow cells from 10-month old C57BL/6-Ly-5.1 p15KO mice or control C57BL/6-Ly-5.1 mice were retro-orbitally transplanted into lethally irradiated congenic C57BL/6-Ly-5.2 recipient mice at various doses (8×10^3 , 4×10^4 , 2×10^5 , and 1×10^6) along with a competitive dose of 2×10^5 C57BL/6-Ly-5.2 whole bone marrow cells. Four-month post-transplant animals were bled and blood cells were stained for Ly5.1 expression to determine donor reconstitution. All mice that exhibited >1.5% donor reconstitution in both the lymphoid and myeloid compartment were considered to be positive. Based on Poisson distribution of the negatively reconstituted mice, CRU values were calculated using L-Calc software (Stem Cell Technologies, Vancouver).

3. Results

3.1. RUNX1-ETO slows G1 to S phase progression in myeloid progenitor cells

Previous studies have shown that RUNX1-ETO slows G1-S phase progression when expressed in myeloid cell lines. To determine the effect of RUNX1-ETO on cell-cycle progression of myeloid progenitor cells *in vivo*, we transplanted lethally irradiated, C57BL/6-Ly-5.1 congenic mice with hematopoietic progenitor cells expressing RUNX1-ETO from a murine stem cell virus (MSCV) retroviral vector that co-expressed a violet-excited green fluorescent protein variant (VEX) from an internal ribosome entry sequence (Figure 1A, [13, 36]). At two months post-transplant, animals reconstituted with cells expressing RUNX1-ETO (RUNX1-ETO mice) or the VEX control virus (VEX mice) were intraperitoneally injected with bromodeoxyuridine (BrdU) and sacrificed at either 1.5, 3.0, or 10.0 hours after injection (n=3 for RUNX1-ETO and VEX control mice for each time point). Bone marrow cells were stained for markers identifying common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP), and megakaryocyte/erythrocyte progenitor (MEP) subsets, which were then FACS-sorted for VEX expression and each of the myeloid progenitor cell phenotypes (Figure 1B). Sorted myeloid progenitor cell subsets (collectively referred to as MPC hereafter) from individual animals were then pooled to have enough cells for analysis and subsequently fixed and stained for intracellular BrdU expression and 7-amino-actinomycin D (7-AAD), which intercalates into double-stranded DNA and is a measure of DNA content. As shown in Figures 1C and 1D, RUNX1-ETO slowed G1-S phase progression of MPC at all time points analyzed. G0/G1 and S-phase frequencies for VEX control or RUNX1-ETO-expressing MPC were highly reproducible, with statistically significant increases in the frequency of MPC in S phase from VEX control versus RUNX1-ETO mice (T=1.5, $p < 0.0002$; T=3.0, $p < 0.01$; T=10.0, $p < 0.01$; Student's t-test).

3.2. p15^{Ink4b} and p16^{Ink4a} expression is increased in RUNX1-ETO-expressing MPC

To account for the delay in G1-S phase progression in RUNX1-ETO-expressing MPC, RealTime PCR was used to analyze the expression of a panel of tumor suppressor genes that regulate both the p53 and pRb pathways (Figure 2A). RNA was isolated from sorted MPC

from RUNX1-ETO and VEX control mice at 2 months post-transplant (n=5-9 independent FACS sorts on independent days for each of VEX control and RUNX1-ETO mice). After normalization of all samples to an HPRT internal control, fold-difference in expression between RUNX1-ETO and VEX control MPC was determined (reactions for all samples were assayed in quadruplicate). The results showed that p15^{Ink4b} and p16^{Ink4a} mRNA levels were increased approximately 1.7- and 2.2-fold, respectively, in RUNX1-ETO MPC compared to VEX control MPC (for p15^{Ink4b}, p18^{Ink4c}, and p19^{Arf} reactions, n=5 independently sorted MPC samples from each of VEX control and RUNX1-ETO mice, for p16^{Ink4a} and p21, n=7 MPC samples each, and for p53, n=9 samples each). Expression levels of p18^{Ink4c} were not statistically different from control samples. Consistent with previous observations in myeloid cell lines and in primary Lin⁻ bone marrow cells [23], the levels of p21^{Cip1/waf1} were elevated in RUNX1-ETO-expressing MPC and this correlated with a 1.4-fold increase in p53 mRNA levels. Expression levels of p19^{Arf} were significantly reduced in RUNX1-ETO MPC, which is in agreement with studies showing that RUNX1-ETO can directly bind and repress transcription driven by the p19^{Arf} promoter in multiple cell types [25]. Together, these results show that RUNX1-ETO delays G1-S phase progression in MPC and that a number of tumor suppressor genes (particularly p15^{Ink4a}, p16^{Ink4b}, and p21^{Cip1}) are expressed at higher levels in RUNX1-ETO-expressing MPC than in control MPC. These results could be explained by direct or indirect regulation of tumor suppressor gene mRNA expression levels by RUNX1-ETO or simply by the increase in G0/G1 cell frequency within RUNX1-ETO-expressing MPC.

3.3. Lack of p15^{Ink4a} or p16^{Ink4b} does not promote leukemic progression in RUNX1-ETO-expressing mice

Since p15^{Ink4b} and p16^{Ink4a} were both significantly increased in RUNX1-ETO-expressing MPC, we tested whether loss of either tumor suppressor would functionally cooperate with RUNX1-ETO to promote leukemic progression. An enriched population of hematopoietic progenitor cells was isolated from the bone marrow of either wild-type, p15^{Ink4b}- or p16^{Ink4a}-deficient mice four days post-injection of a single dose of 5-fluorouracil (See Figure 2B for verification of knockout genotypes). Cells were then transduced with RUNX1-ETO or VEX control virus and transplanted into lethally irradiated, wild-type C57BL/6 congenic recipient mice and analyzed at various times post-transplantation. FACS analysis of peripheral blood isolated from representative animals at 1 month post-transplant showed that RUNX1-ETO caused a partial terminal myeloid differentiation arrest, as determined by increased Mac-1⁺Gr-1^{int} cells in all genetic backgrounds (wt, p15^{Ink4b}, and p16^{Ink4a}, [13]), as well as an overall increase in the frequency of more differentiated Mac-1⁺Gr-1⁺ myeloid cells among the VEX⁺ cells compared to genotype-matched controls (Figure 3A). In the absence of RUNX1-ETO, the loss of p15^{Ink4b} alone resulted in statistically significant increases in the frequency of Mac-1⁺Gr-1⁺ cells in peripheral blood as compared with animals reconstituted with wild-type bone marrow cells expressing the VEX control retrovirus (Mac-1⁺Gr-1⁺ frequency of 16.2% +/- 0.4% for VEX WT, n=14; 22.4% +/- 8.4% for VEX p15KO, n=14, p=0.04). There were not statistically significant differences in Mac-1⁺Gr-1⁺ frequencies on the p16^{Ink4a} knockout background (n=17). Analysis of B220⁺ B-lineage cells in peripheral blood showed severe reductions in the frequencies of RUNX1-ETO-expressing B cells in all genetic backgrounds (Figure 3B, 4-7-fold reductions in RUNX1-ETO mice compared with genotype-matched controls; VEX, n=14; RE VEX, n=15; p15KO VEX, n=14; p15KO RE VEX, n=17; p16KO VEX, n=17; p16KO RE VEX, n=21). Analysis of peripheral blood cell counts in 10-month post-transplant RUNX1-ETO or VEX control mice (n=3-5 animals for all genotypes) indicated that RUNX1-ETO mice of all genotypes had a 30% reduction in red blood cell counts compared with genotype-matched controls as well as significant reductions in platelets, hematocrit, and white blood cell counts (Table 1). Genetic background did not seem to modify the peripheral blood phenotype of any developmental lineage that was analyzed in RUNX1-

ETO or VEX control mice at both early (2 months) and late (10 months) time-points post-transplantation.

3.4. Loss of p15^{Ink4a} results in increased frequencies of myeloblasts/promyelocytes in bone marrow

FACS analysis of RUNX1-ETO bone marrow myeloid subsets showed a similar accumulation of Mac-1^{hi}Gr-1^{int} metamyelocytes/band neutrophils as observed in peripheral blood of all RUNX1-ETO animals irrespective of genotype and time post-transplant (Figure 4A, see arrow in WT RE VEX plot). Also noted was an increase in a Mac-1⁺Gr-1^{hi} myeloblast subset in animals that expressed RUNX1-ETO (see red-gated subset in WT RE VEX plot and cyto-spin preparation, Figure 4A). Even though there were changes in the distributions of the various Mac-1⁺Gr-1⁺ subsets, there was not a significant difference in the total frequency of Mac-1⁺Gr-1⁺ cells between animals. Further FACS analysis of myeloid progenitor cell frequencies (CMP, GMP, and MEP) in bone marrow of RUNX1-ETO and VEX mice at 10 months post-transplant showed that RUNX1-ETO expression resulted in significant fold increases in all myeloid progenitor subsets compared with genotype-matched VEX control mice (CMP, 2-fold, GMP, 3-fold, MEP, 5-fold; Figure 4B). There was also a significant increase ($p < 0.05$) in CMP in p15^{Ink4b}^{-/-} bone marrow in the absence of RUNX1-ETO as well as a 19-fold increase in the frequency of LKS(FLT3⁻) cells (Figure 4B, see bar graph with alternative scale, $n=3$, VEX; $n=3$, p15^{Ink4b}^{-/-}; $n=4$, p16^{Ink4a}^{-/-}). The presence of RUNX1-ETO increased the frequencies of LKS(FLT3⁻) cells 100-200-fold irrespective of the genetic background (Figure 4B), which was noted previously [13]. The increase in LKS cells in the presence of RUNX1-ETO does not represent a significant expansion of true LT-HSC but rather an expansion of mast cells of the LKS phenotype in bone marrow (data not shown, C.S. Swindle and C.A. Klug, manuscript in preparation). Since CMP, GMP, and MEP frequencies are often not an accurate measure of immature myeloid progenitor expansion due to altered cell-surface marker expression in the context of oncogenic mutations (for instance, Gr-1 is normally included in the lineage cocktail for CMP, GMP, and MEP staining, which would exclude the Mac-1⁺Gr-1^{hi} myeloblast subset from analysis in RUNX1-ETO mice, Figure 4A), we performed differential cell counts using bone marrow representing the various genotypes at 10 months post-transplant ($n=3$ for all genotypes). As shown in Table 2, blast/promyelocyte frequencies among the total VEX⁺ myeloid cells in bone marrow were significantly increased in all RUNX1-ETO animals compared with genotype-matched controls, with the exception of p15^{Ink4b}^{-/-} animals, where similar increased frequencies of blasts/promyelocytes were observed in the presence or absence of RUNX1-ETO (also see Figure 4C). Significant myeloid progenitor expansion was not noted in p16^{Ink4a}^{-/-} knockout animals, although p16^{Ink4a} loss may have contributed to enhanced myeloid progenitor expansion in association with RUNX1-ETO (Table 2 and Figure 4B). Further characterization of myeloid progenitor cell frequencies using *in vitro* colony-forming cell (CFC) assays showed no statistical difference in the frequency of myeloid CFC when equivalent numbers of VEX⁺ bone marrow cells were plated from animals that lacked RUNX1-ETO expression (Figure 4D), although we did note that colonies plated from p15^{Ink4b}^{-/-} bone marrow tended to consist of both immature and more differentiated phenotypes while cells from colonies isolated from VEX⁺ control platings were entirely differentiated (data not shown). RUNX1-ETO increased myeloid CFU frequencies 25-50-fold by 10 months post-transplantation independent of genetic background (Figure 4D).

3.5. Loss of p15^{Ink4b} results in a significant expansion of LKS(FLT3⁻) cells but no increase in competitive repopulating unit (CRU) frequency

To determine whether the 5-fold (2 months) and 19-fold (10 months) increases in LKS (FLT3⁻) cells were occurring in functional long-term self-renewing HSC (LT-HSC), differing doses of bone marrow from 10-month-old wild-type or p15^{Ink4b}^{-/-} mice were transferred along with 2×10^5 recipient-type bone marrow cells into lethally irradiated

congenic recipient animals. LT-HSC normally comprise approximately 10-15% of LKS (FLT3⁻) cells and represent about 0.005-0.007% of total C57BL/6 bone marrow. Donor cell reconstitution of B, T, and myeloid-lineage cells was assessed by FACS analysis of peripheral blood at 4 months post-transplant. As shown in Figure 5, the CRU frequency for p15^{Ink4b}-deficient bone marrow cells was not significantly different from wild-type C57BL/6 bone marrow (1/65,061 cells versus 1/76,742 cells, respectively). This indicates that the increase in LKS(FLT3⁻) frequency in p15^{Ink4b}^{-/-} animals is not due to expansion of the LT-HSC subset.

4. Discussion

As much as 80 percent of human AML patient samples show epigenetic silencing of the *p15^{INK4B}* gene, which strongly suggests that loss of p15^{INK4B} expression may be a significant event in AML progression in humans. In addition, neonatal *p15^{Ink4b}* heterozygous mice inoculated with the MOL4070LTR virus are susceptible to myeloid leukemia as are animals with a myeloid-specific deletion of the *p15^{Ink4b}* locus ([31] and L. Wolff, unpublished data). However, the long latency and incomplete penetrance associated with leukemia development in these mice suggest that multiple cooperating events are required to promote AML in conjunction with loss of p15^{Ink4b}. In addition, analysis of *p15^{Ink4b}*-deficient mice showed no evidence of myeloid malignancies [37], which further supports the conclusion that p15^{Ink4b} is a rather weak tumor suppressor in the myeloid lineage in C57BL/6 mice as a single mutation. These suggestions are further supported by the results of the present study, where retroviral expression of RUNX1-ETO on a *p15^{Ink4b}*-deficient background did not result in death of any animal due to AML that was analyzed or survived beyond 10 months post-transplantation (n=21). Since there is such a high correlation of *p15^{INK4B}* hypermethylation and locus silencing in human AML associated with RUNX1-ETO, these results strongly suggest that additional genetic events are required to promote leukemic progression. Alternatively, AML development in mice may be less sensitive to loss of p15^{Ink4b} expression than in humans. Further work will be necessary to clarify this issue.

In a similar manner, the loss of p16^{Ink4a} did not promote progression to AML in association with RUNX1-ETO (n=29 mice that survived at least 10 months post-transplant) nor was their any significant consequence of p16^{Ink4a} loss on expansion of myeloid progenitor cells in older animals (Table 2). These results are consistent with a number of studies showing that *p16^{INK4A}* is rarely lost or epigenetically silenced in human AML cases. Due to the tight linkage of the *p16^{Ink4a}* and *p15^{Ink4b}* genes, we were unable to obtain animals with homozygous deletion of both tumor suppressors even though we genotyped over 100 F1 progeny of a cross between *p16^{Ink4a}* and *p15^{Ink4b}* mice for evidence of a crossover event between these loci. It may well be that inactivation of both *p16^{Ink4a}* and *p15^{Ink4b}* genes may contribute to AML development in the context of RUNX1-ETO since both factors are induced in RUNX1-ETO-expressing myeloid progenitor cells (Figure 2A). However, human AML patient studies would suggest that p16^{INK4A} loss is much more highly correlated with lymphoid than with myeloid malignancy.

Even though the loss of p15^{Ink4b} did not promote AML in the context of the RUNX1-ETO mutation in the time frame of our experiments, we did observe a significant increase in the frequencies of more differentiated Mac-1⁺Gr-1⁺ cells in peripheral blood of *p15^{Ink4b}*-knockout mice and a reproducible expansion of myeloid progenitor cells in the bone marrow of older *p15^{Ink4b}*-deficient animals based on differential cell counts (Table 2) and flow cytometric analysis of CMP frequencies (Figure 4B). The increase in Mac-1⁺Gr-1⁺ frequencies was recently observed in another study that also showed increased CMP/GMP frequencies in bone marrow of *p15^{Ink4b}* knockout animals [38]. We did not observe increased GMP frequencies in our experiments, although this difference could be explained by subtle differences in myeloid progenitor subset gating or differences between mouse strains in the independent studies.

Other tumor suppressor genes that were increased in RUNX1-ETO-expressing myeloid progenitor cells were p21 and p53 (Figure 2A), which could have also contributed to the slowed cell-cycle of RUNX1-ETO-expressing myeloid progenitor cells (Figures 1C, D). Loss of p21 was shown by a recent study to cooperate with RUNX1-ETO in promoting progression to AML with a mean survival of approximately 30 weeks [23]. All p21-deficient animals that lacked RUNX1-ETO survived beyond 10 months post-transplantation. The lack of cooperation in leukemic progression evidenced by RUNX1-ETO expression on a p15^{Ink4b} or p16^{Ink4a} knockout background may suggest that p21 is the critical tumor suppressor that needs to be inactivated for progression to AML in the context of a RUNX1-ETO translocation. Alternatively, it could be argued based on the relatively slow kinetics of AML development in p21-deficient bone marrow cells expressing RUNX1-ETO that multiple tumor suppressor pathways must be repressed to promote more aggressive tumor formation.

Since cell-cycle progression is slowed by RUNX1-ETO, it is not surprising that patients in long-term remission following induction chemotherapy still show evidence of the RUNX1-ETO transcript in their peripheral blood and in FACS-sorted hematopoietic stem/progenitor cells [39,40]. These observations indicate that the RUNX1-ETO mutation likely occurs in HSC and that secondary mutations leading to AML progression sensitize these cells to standard chemotherapy approaches that target actively cycling cells. Activating mutations in growth stimulatory pathways that counteract the anti-proliferative activity of RUNX1-ETO are therefore attractive candidates as AML- promoting mutations in the context of the RUNX1-ETO translocation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

BrdU incorporation into myeloid progenitor cells (MPC) of VEX control and RUNX1-ETO mice. (A) Schematic diagram of MSCV retroviral construct used to co-express RUNX1-ETO and VEX (IRES=internal ribosome entry site). (B) Representative FACS analysis of MPC subsets analyzed from a VEX control mouse at 2 months post-transplantation. (C) Representative FACS analysis of BrdU and 7-AAD incorporation in myeloid progenitor cells at various times post-IP injection of a single dose (1mg) of BrdU. Numbers indicate the frequency of cells in G0/G1, S, and G2/M phases of the cell cycle (n=3 mice for each time point for both VEX control and RUNX1-ETO mice). (D) Frequencies of VEX-positive myeloid

progenitor cells in G0/G1, S, and G2/M phase at various time points (hours) post-BrdU injection. Error bars represent standard error of the mean (SEM).

Fig. 2.

(A) RealTime RT-PCR analysis of tumor suppressor gene expression in FACS-sorted MPC isolated from RUNX1-ETO or VEX control mice at 2-months post-transplantation. Fold-difference was calculated after normalization of all samples to an internal HPRT control reaction and assuming a mean expression value of 1.0 for VEX control reactions. Error bars represent SEM, which was calculated from values obtained from 5-9 independent reactions performed in quadruplicate for each target gene. The 5-9 independent MPC samples from each of VEX control and RUNX1-ETO mice were isolated on independent days. (B) PCR genotyping of *p15^{Ink4b}* and *p16^{Ink4a}* knockout bone marrow. PCR was done using DNA from FACS-sorted, VEX⁺ bone marrow cells isolated from VEX control mice that were generated using either *p15^{Ink4b}* (p15KO, top panel) or *p16^{Ink4a}* (p16KO, bottom panel) knockout cells. Control reactions using primers that amplified the wild-type (WT control) or deleted (MT control) *p15^{Ink4b}* and *p16^{Ink4a}* alleles were done using DNA isolated from bone marrow cells obtained from either *p15^{Ink4b}* (top panel, +) or *p16^{Ink4a}* (bottom panel, +) heterozygous mice. Lanes designated “-” indicate reactions with no template added.

Fig. 3.

(A) Peripheral blood analysis of representative reconstituted animals at 1 month post-transplant. Numbers represent the frequencies of cells within the indicated gates. (B) Fold-reductions in peripheral blood B220⁺ cells in RUNX1-ETO (RE) mice were 5-fold (VEX versus RE VEX), 7.7-fold (p15KO VEX versus p15KO RE VEX), and 4.0-fold (p16KO VEX versus p16KO RE VEX). Numbers of animals analyzed representing each genotype are given in the Results. Error bars represent 1 standard deviation (1SD) from the mean.

Fig. 4. Analysis of myelopoiesis in bone marrow. (A) Representative Mac-1⁺Gr-1⁺ staining of bone marrow at 2- and 10-months post-transplant (n=3-7 at 2 months and 3 at 10 months). Red-boxed inset represents gated Mac-1⁺Gr-1^{hi} cells that were FACS-sorted and cytopspun for Wright-Giemsa staining (shown on right). The arrow in the WT RE VEX plot indicates a Mac-1^{hi}Gr-1^{int} metamyelocytes/band neutrophil subset expanded in RUNX1-ETO mice. (B) CMP, GMP, and MEP myeloid progenitor cell and LKS (FLT3⁻) multipotent cell frequencies in bone marrow at 10 months post-transplant. Error bars represent the SEM of 3 independent animals analyzed per genotype except for p16^{Ink4a}, where n=5. (C) Representative cytopsin and Wright-Giemsa staining of VEX⁺ bone marrow cells FACS-sorted from each genotype

(all images at 600X). Differential bone marrow counts are shown in Table 1. (D) Methylcellulose assay indicating the number of myeloid CFC per 1000 cells plated. Platings from each animal were performed in triplicate using bone marrow from a total of 3 animals per genotype. Error bars represent SEM.

Fig. 5.

Analysis of stem cell frequency in *p15^{Ink4b}*-knockout mice. Limiting dilution analysis to determine the number of competitive repopulating units (CRU) in donor bone marrow cells isolated from 2 age- and gender-matched control (C57BL/6-Ly-5.2, open square and black line) and 2 *p15^{Ink4b}* knockout (red square and red line) animals at 10 months of age. A total of 10 secondary recipient mice were transplanted at each cell dose. Transplanted congenic secondary recipient mice (C57BL/6-Ly-5.1) were analyzed for donor-derived lymphoid and myeloid cells at 4 months post-transplant. Points plotted represent the percentage of transplanted animals expressing less than 1.5% of donor-derived lymphoid and myeloid cells in peripheral blood at 4 months post-transplant. CRU frequency was determined to be the point at which 37% of mice are negative for donor-lineage reconstitution.

Table 1

Peripheral blood cell counts in 10-month post-transplant animals

	RBC (M/ul)	Hematocrit (%)	WBC (K/ul)	Plt (K/ul)
Wild type VEX	9.03 ($\pm$ 0.48)	43.68 ($\pm$ 2.87)	17.72 ($\pm$ 5.99)	638 ($\pm$ 257)
Wild type RE	7.26 ($\pm$ 2.21)	40.80 ($\pm$ 5.55)	12.13 ($\pm$ 5.75)	353 ($\pm$ 132)
p15KO VEX	9.31 ($\pm$ 0.54)	43.96 ($\pm$ 2.52)	11.79 ($\pm$ 3.34)	805 ($\pm$ 126)
p15KO RE	6.90 ($\pm$ 0.94) ^{**}	36.79 ($\pm$ 3.68) [*]	7.64 ($\pm$ 2.24) ^{**}	789 ($\pm$ 176)
p16KO VEX	9.22 ($\pm$ 0.48)	45.13 ($\pm$ 1.01)	13.20 ($\pm$ 4.85)	602 ($\pm$ 151)
p16KO RE	7.09 ($\pm$ 1.04) [*]	41.45 ($\pm$ 10.73)	7.76 ($\pm$ 3.05) [*]	480 ($\pm$ 212)

Data shown are mean ($\pm$ SD) values from three to five mice per genotype^{*}
 $p < 0.05$,^{**}
 $p < 0.005$ when compared to wild type VEX

Table 2
Differential counts of sorted VEX (+) myeloid cells from 10-month post-transplant animals

	Blast/Pro (%)	Myelo(%)	Band(%)	Eos (%)	Mono (%)
Wild type VEX	8.9	15.0	73.7	0.4	2.0
	10.7	12.0	75.7	0.8	0.8
Wild type RE	31.5	11.7	51.7	2.3	2.7
	35.4	13.0	46.3	0.4	4.9
	35.0	15.4	46.2	1.1	1.3
p15KO VEX	33.8	12.1	47.0	1.0	6.1
	27.5	35.8	30.0	2.5	4.2
	36.0	24.2	39.3	0	0.6
p15KO RE	14.6	24.5	58.8	0.3	1.9
	25.4	35.7	36.4	0.4	2.1
	32.2	12.4	47.2	1.9	6.2
p16KO VEX	15.0	25.0	57.0	2.3	1.0
	13.4	9.1	67.2	9.1	1.2
	18.4	10.4	62.0	4.4	4.8
p16KO RE	56.8	15.7	15.3	4.7	7.6
	45.7	23.3	25.7	2.7	2.7
	38.1	24.6	34.7	2.1	0.5

Data shown are percentages of >250 cells counted per sample.

Blast/Pro: myeloblast and promyelocytes; Myelo: myelocytes; Band: band-form granulocytes; Eos: eosinophils; Mono: monocytes