



PML-RARA can increase hematopoietic self-renewal without causing a myeloproliferative disease in mice

John S. Welch, Wenlin Yuan, and Timothy J. Ley

Section of Stem Cell Biology, Division of Oncology, Departments of Medicine and Genetics, Washington University School of Medicine, St. Louis, Missouri, USA.

Acute promyelocytic leukemia (APL) is characterized by the t(15;17) translocation that generates the fusion protein promyelocytic leukemia-retinoic acid receptor α (PML-RARA) in nearly all cases. Multiple prior mouse models of APL constitutively express PML-RARA from a variety of non-*Pml* loci. Typically, all animals develop a myeloproliferative disease, followed by leukemia in a subset of animals after a long latent period. In contrast, human APL is not associated with an antecedent stage of myeloproliferation. To address this discrepancy, we have generated a system whereby PML-RARA expression is somatically acquired from the mouse *Pml* locus in the context of *Pml* haploinsufficiency. We found that physiologic PML-RARA expression was sufficient to direct a hematopoietic progenitor self-renewal program in vitro and in vivo. However, this expansion was not associated with evidence of myeloproliferation, more accurately reflecting the clinical presentation of human APL. Thus, at physiologic doses, PML-RARA primarily acts to increase hematopoietic progenitor self-renewal, expanding a population of cells that are susceptible to acquiring secondary mutations that cause progression to leukemia. This mouse model provides a platform for more accurately dissecting the early events in APL pathogenesis.

Introduction

More than 95% of acute promyelocytic leukemia (APL; FAB-M3) cases are associated with the recurrent t(15;17)(q22;q11) translocation, which results in the expression of a fusion transcript promyelocytic leukemia-retinoic acid receptor α (PML-RARA), inconsistent expression of a reciprocal fusion product (RARA-PML), and haploinsufficiency for both PML and RARA (1, 2). Although APL has served as an important model disease for understanding leukemogenic pathways directed by a specific translocation, the precise mechanisms by which t(15;17) initiates leukemia remain unknown.

Several mouse models have been used to study the mechanisms by which PML-RARA causes transformation (reviewed in refs. 3, 4). These models have used viral transduction, transgenic, and knockin strategies; regulatory sequences from the *MRP8* gene (also known as S100 calcium binding protein A8 [*S100A8*]) and human and murine cathepsin G (*CTSG* and *Ctsg*, respectively) genes have been used to direct the expression of PML-RARA to the myeloid compartment (5–8), where it can cause a myeloproliferative disease and APL after a long latent period.

However, there are many issues with existing mouse models of APL that must be addressed to fully understand how PML-RARA works. (a) The dose of PML-RARA is known to be important for AML penetrance, but no model to our knowledge has yet expressed PML-RARA from the endogenous *Pml* locus. This has not previously been attempted, since *Pml* is ubiquitously expressed, and since widespread expression of PML-RARA during development causes embryonic lethality (9, 10). (b) t(15;17) is a somatically acquired event, but all prior models have used constitutive PML-RARA expression, raising the possibility that hematopoietic cells may have adapted to tolerate PML-RARA expression during embry-

genesis and/or early adult life. (c) Although *Pml* haploinsufficiency has been shown to contribute to APL pathogenesis, this has not been routinely integrated into mouse models (11).

To better define the initial effects of PML-RARA on hematopoiesis, we have developed a murine model (mPML-PRflox mice). We used homologous recombination to express PML-RARA under the control of the murine *Pml* locus and used Cre-Lox technology to prevent PML-RARA expression prior to Cre recombination (to prevent embryonic lethality). This model recapitulates multiple elements of the pathologic t(15;17) translocation that have been absent in prior murine models of APL (i.e., *Pml* locus-appropriate expression of PML-RARA, *Pml* haploinsufficiency, and somatic acquisition of oncoprotein expression in adult animals).

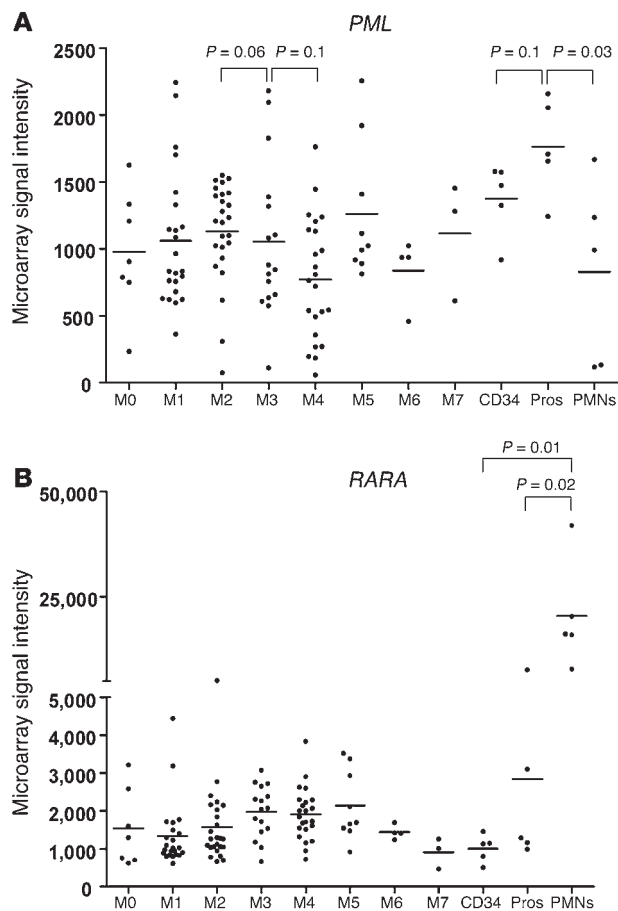
In this report, we show that this model differs substantially from all previous ones and allows interrogation of initial PML-RARA effects on hematopoiesis after somatic acquisition of expression. When an ERT2-Cre cassette (driven by a chicken β -actin/CMV promoter/enhancer) is activated by tamoxifen in adult mice, mPML-PRflox is floxed and induces an inappropriate hematopoietic self-renewal program: floxed mPML-PRflox (designated mPML-fPR) bone marrow CFUs could be serially replated for 6 or more weeks ex vivo and a progressive expansion of mPML-fPR-positive hematopoietic cells occurred in vivo. Remarkably, this expansion occurred in the absence of concurrent myeloproliferation and was observed in both myeloid and lymphoid compartments after competitive transplantation. These studies show that PML-RARA alters self-renewal programs but does not alter homeostatic feedback mechanisms that control the size of the preleukemic myeloid compartment itself.

Results

PML and RARA expression in normal and leukemic human bone marrow cells. Because many translocations result in aberrant expression of at least one nearby gene (12), we evaluated the expression of both

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PML and *RARA* across a wide range of AML patients with various FAB subtypes and in 5 normal volunteers, after flow sorting for CD34⁺, promyelocyte, and neutrophil (PMN) populations (13). We found no significant alteration in the expression of *PML* or *RARA* in patients with t(15;17) compared with those with non-FAB-M3 AML (Figure 1). This suggests that t(15;17) does not influence hematopoiesis by grossly dysregulating the expression of either *PML* or *RARA*. These data also confirmed the prior observation that *RARA* expression is markedly upregulated during terminal myeloid maturation (Figure 1B and ref. 14).

Generation of mice with the *mPML-PRflox* allele. To more closely recapitulate the effects of dose-appropriate expression and somatic acquisition of *PML-RARA* on hematopoiesis, we used homologous recombination to insert a loxP-PGKneo-stop-loxP-*PML-RARA* cDNA into the murine *Pml* 5' untranslated region (designated *mPML-PRflox*) of a C57BL/6-derived (B6-derived) ES cell line (B6-Blu), which was then used to generate B6 mice with this mutant allele (Figure 2A). Five stop codons positioned across all 3 reading frames were inserted downstream from the PGK-neo cassette to prevent expression of *PML-RARA* prior to Cre-mediated recombination. *mPML-PRflox* mice are viable and display normal Mendelian inheritance and normal peripheral blood cell counts (data not shown). The Cre-recombined allele expresses *PML-RARA* mRNA and protein and is referred to hereafter as *mPML-fPR*.

We first assessed the effect of the *mPML-PRflox* allele on *Pml* expression using Western blotting (Supplemental Figure 1A). As expected, we observed a decrease in *Pml* protein in *mPML-*

Figure 1

Expression profiling. (A and B) Expression array profile of (A) human *PML* and (B) human *RARA* in 111 patients with indicated FAB-AML and 5 flow-sorted normal controls. CD34⁺, CD34⁺ bone marrow cells; Pros, promyelocytes. Individual data points represent results from individual patients. Horizontal bars are the median value.

PRflox^{-/-} spleen cells and a complete loss of *Pml* protein in *mPML-PRflox*^{+/+} spleen cells. These data confirm that *mPML-PRflox* results in a null mutation in *Pml* and that heterozygosity creates haploinsufficiency for *Pml*.

We crossed *mPML-PRflox* mice (made in a B6 ES cell line and maintained in the B6 strain) with several different Cre-expressing mouse lines that were also made in or backcrossed to the B6 background. We first crossed *mPML-PRflox* mice to *Mx-Cre* mice but did not observe any doubly positive *Mx-Cre*^{+/+} × *mPML-PRflox*^{+/+} mice. Because *Mx-Cre* is expressed at low levels even without interferon activation and *Pml* is expressed widely during embryogenesis, we suspected that these mice died during embryogenesis because of *Mx-Cre* “leakiness” (9, 15, 16). To confirm this result, we crossed male *CMV-Cre*^{+/+} mice with female *mPML-PRflox*^{+/+} mice. We observed 7 out of 16 *CMV-Cre*^{+/+} × *mPML-PRflox*^{+/+} male pups but 0 out of 13 *CMV-Cre*^{+/+} × *mPML-PRflox*^{+/+} female pups (*CMV-Cre* is X-linked, and the female pups are obligate Cre heterozygotes) (Figure 2B). We further evaluated 51 E13.5 embryos from a total of 6 litters of *CMV-Cre*^{+/+} × *mPML-PRflox*^{+/+} intercrosses. Of these, 26 embryos were *CMV-Cre*^{+/+}, 16 embryos were *mPML-PRflox*^{+/+}, and 0 embryos were *CMV-Cre*^{+/+} × *mPML-PRflox*^{+/+}. No resorbed embryos were observed in any of the 6 litters. This confirms that the floxed *mPML-fPR* allele causes early embryonic lethality when *PML-RARA* is widely expressed (consistent with prior observations that *PML-RARA* can cause cellular toxicity) (17–19) and provides strong evidence that *PML-RARA* is functional when it is activated by Cre-recombinase in this locus.

In contrast, Cre cassettes with more restricted expression patterns could be successfully intercrossed; doubly heterozygous mice containing the *LysM-Cre* or the *ERT2-Cre* allele survived to adulthood with normal blood counts and without gross phenotypes (*LysM-Cre* expression is limited to differentiated myeloid cells, and *ERT2-Cre* is active only after tamoxifen treatment) (20–22). Due to previously described cellular and embryonic toxicity from *PML-RARA* expression, we were surprised that *ERT2-Cre*^{+/+} × *mPML-PRflox*^{+/+} mice tolerated i.p. tamoxifen administration without evidence of gross toxicity (17–19, 22).

PML-RARA mRNA could be detected using qRT-PCR in *LysM-Cre*^{+/+} × *mPML-PRflox*^{+/+} spleen cells and also in *ERT2-Cre*^{+/+} × *mPML-PRflox*^{+/+} bone marrow cells after tamoxifen treatment (Supplemental Figure 1, B and C; supplemental material available online with this article; doi:10.1172/JCI42953DS1). We compared this expression to our previous model of APL, in which the same *PML-RARA* cDNA is expressed from the *Ctsg* locus using a knockin strategy (herein referred to as *mCG-PR* mice), which results in constitutive *PML-RARA* expression in hematopoietic progenitors, promyelocytes, and PMNs (peak expression is in promyelocytes) (7). The *mPML-fPR* allele yielded lower *PML-RARA* mRNA expression, consistent with the fact that the *Pml* gene is expressed at much lower levels than the *Ctsg* gene during normal hematopoiesis (Welch, et al., unpublished observations). However, *PML-RARA* mRNA was much more abundant in the leukemic spleen cells of a *LysM-Cre*^{+/+} × *mPML-PRflox*^{+/+} mouse, perhaps because virtually all cells in the sample contained an active *mPML-fPR* allele (Supple-

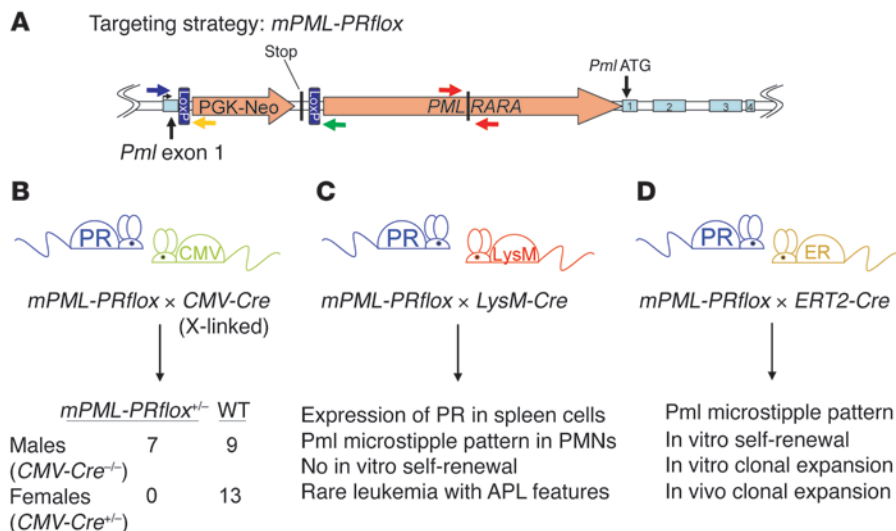


Figure 2

mPML-PRflox targeting strategy and the result of intercrosses with *CMV-Cre*, *LysM-Cre*, and *ERT2-Cre* mice. (A) Targeting strategy. Rectangles represent exons. (B) The results of intercrosses with *mPML-PRflox* × *CMV-Cre*, (C) *mPML-PRflox* × *LysM-Cre*, and (D) *mPML-PRflox* × *ERT2-Cre* mice are shown, with details about resulting pups.

mental Figure 1C). We also detected PML-RARA protein in this leukemia sample, although we were unable to detect PML-RARA protein in bone marrow, spleen, or CFU-granulocyte/CFU-macrophage (CFU-G/CFU-M) cells from healthy 8-week-old *mPML-fPR* or *mCG-PR* mice (Supplemental Figure 1D and data not shown).

PML-RARA activity causes a microstippled pattern of native Pml protein in the nucleus (17). We observed this pattern of Pml nuclear localization in *mCG-PR*^{+/-} and *LysM-Cre*^{+/-} × *mPML-PRflox*^{+/-} splenic PMNs but not in wild-type or *mPML-PRflox*^{+/-} splenic PMNs (Supplemental Figure 1E). After tamoxifen treatment, we also observed microstippling of nuclear Pml in *ERT2-Cre*^{+/-} × *mPML-PRflox*^{+/-} splenic PMNs (Supplemental Figure 1E).

Collectively, these data show that the *mPML-fPR* allele results in *Pml* haploinsufficiency, that *PML-RARA* is expressed after exposure to Cre recombinase, and that *PML-RARA* has the expected effects on cellular functions.

Titration floxing in vivo. Consistent with prior studies by Hayashi et al., *ERT2-Cre* activity resulted in dose-dependent floxing of the LoxP-flanked Neo cassette (22). After 5 doses of tamoxifen at 4 mg per dose i.p., we observed approximately 20% *mPML-fPR* alleles in a wide range of tissues by Southern blotting (data not shown). Because large amounts of DNA are required for this technique, we developed a qPCR-based assay, using primers that span the LoxP Neo cassette (“Floxx” primer pair amplified the floxed locus only) or that span the first LoxP site (“Neo” primer pair amplified the native, unfloxed locus only) (Figure 2A and Supplemental Figure 2A). Amplification of flox and neo primer pairs was normalized to amplification across the *PML-RARA* junction (“PR” primer pair amplified the *mPML-PRflox* allele). By titrating genomic DNA from *mPML-fPR* and *mPML-PRflox*^{+/-} spleen cells we could calculate the percentage of floxed alleles within a population (Supplemental Figure 2A). Amplification of a genomic DNA mixture containing a population of 10% or 90% *mPML-fPR* alleles yielded consistent results across multiple experiments (Supplemental Figure 2B). These samples were amplified during each of the subsequent experiments and were used to calibrate floxing efficiency. Using this approach, we confirmed a dose-dependent increase in the *mPML-fPR* allele in bone marrow from *ERT2-Cre*^{+/-} × *mPML-PRflox*^{+/-} mice: after 1, 5, and 10 doses of 4 mg i.p. tamoxifen, we observed approximately 10%, 30%, and 95% floxed alleles, respectively (Supplemental Figure 2C).

Early effects of PML-RARA on hematopoiesis. Despite the significant embryonic toxicity observed with the *mPML-PRflox* transgene when crossed with *Mx-Cre* or *CMV-Cre*, we observed very little acute hematopoietic toxicity in *ERT2-Cre*^{+/-} × *mPML-PRflox*^{+/-} mice after tamoxifen treatment. After 10 doses of i.p. tamoxifen (4 mg each, administered in twice weekly doses), we evaluated peripheral blood counts and bone marrow progenitor compartments in 4 *ERT2-Cre*^{+/-} × *mPML-PRflox*^{+/-} mice (these mice all achieved more than 95% floxing in their bone marrow cells) and their littermate controls. Tamoxifen treatment did not acutely alter total peripheral blood white blood cell numbers, hemoglobin levels, platelets, or the percentage of lymphocytes, granulocytes, or monocytes (data not shown). Evaluation of bone marrow cells revealed a small decrease in Kit⁺Lin⁻Sca⁺ (KLS) cells in *mPML-fPR* mice (1.3% vs. 2.5% of lineage-negative cells; *P* = 0.09) but no other significant changes in progenitor populations (Figure 3B). Bone marrow cells were cultured in methylcellulose containing IL-3, IL-6, and SCF, and CFUs were assessed after 1 week. *mPML-fPR* did not significantly alter total CFUs or the immunophenotype of these cells after myeloid differentiation (Figure 3, C and D). Short pulses of 6 mg tamoxifen (given i.p. every other day for 10 days) resulted in 75% floxing in bone marrow and spleen cells and a nonsignificant trend toward decreased CFUs and CFU-Ms (Supplemental Figure 3, A and B). Exposure to 4-hydroxytamoxifen in vitro caused a modest decrease in total CFUs and CFU-Ms, but there was no change in total CFUs arising in methylcellulose with G-CSF alone (Figure 3E and Supplemental Figure 3C). Surprisingly, these data suggest that acute exposure of hematopoietic cells to *PML-RARA* (at a dose governed by the *Pml* locus) is minimally toxic to early progenitor populations, leaving the hematopoietic compartments largely unperturbed.

However, Cre-dependent activation of *mPML-PRflox* resulted in dysregulation of hematopoietic self-renewal. After 10 doses of tamoxifen (4 mg i.p. twice weekly), bone marrow cells from *ERT2-Cre*^{+/-} × *mPML-PRflox*^{+/-} mice could be serially passaged for more than 6 weeks (Figure 4B). The self-renewal phenotype was completely dependent on floxing, since the floxed allele frequency increased during replating and represented 100% of the alleles after 4 weeks of culture (Figure 4C). Outgrowth of the *mPML-fPR* allele occurred whether the starting bone mar-

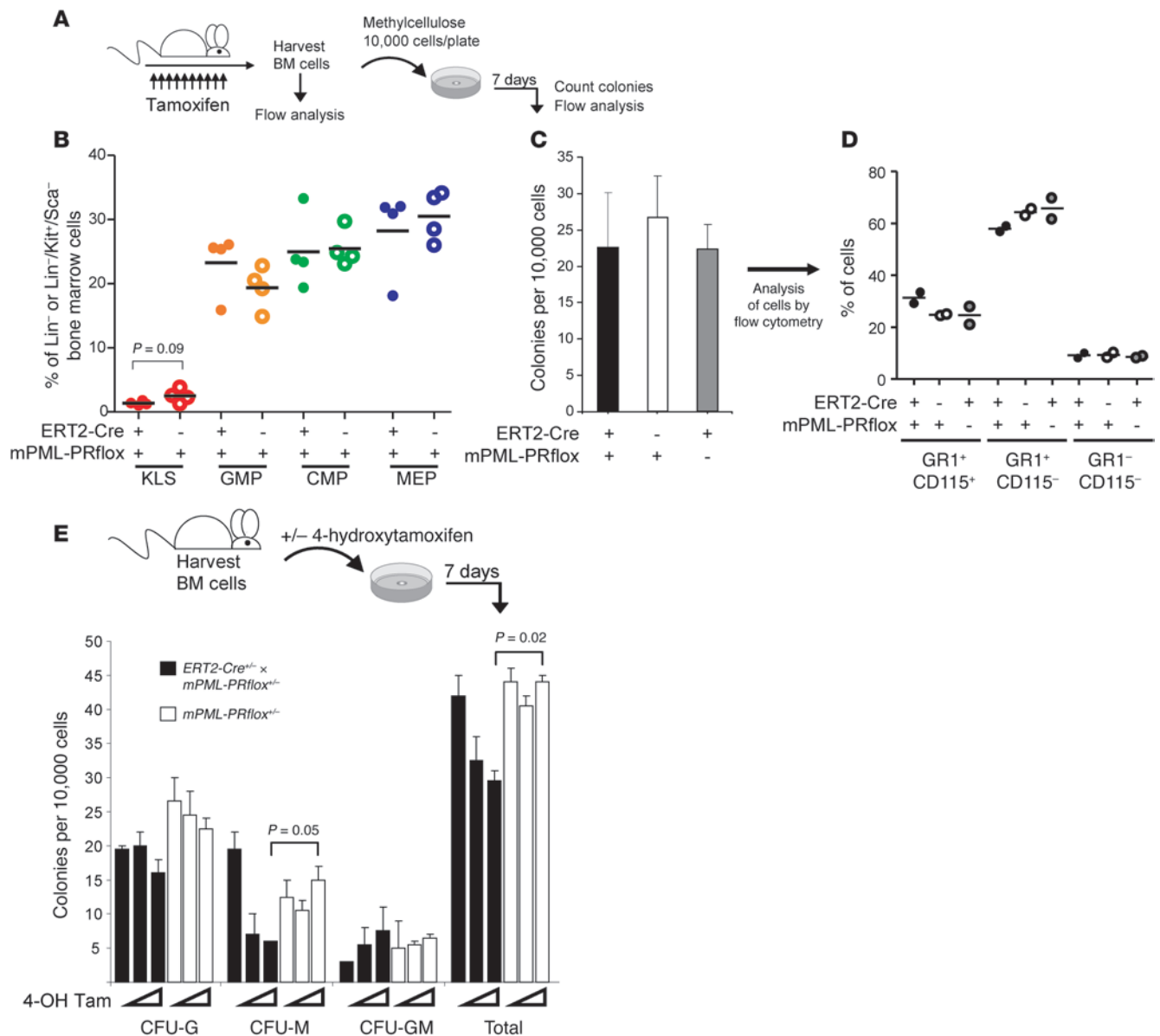


Figure 3

mPML-fPR does not grossly alter bone marrow progenitor populations, CFUs, or ex vivo myeloid differentiation. **(A)** Schema showing that mice were treated with 10 doses of tamoxifen (4 mg i.p. twice weekly for 5 weeks) and analyzed 2 weeks later. **(B)** mPML-fPR modestly decreased the percentage of Lin⁻cKit⁺Sca⁺ (KLS) cells but not the percentage of Lin⁻cKit⁺Sca⁻CD16/32⁺CD34⁺ (GMP), Lin⁻cKit⁺Sca⁻CD16/32⁻CD34⁻ (CMP), or Lin⁻cKit⁺Sca⁻CD16/32⁻CD34⁻ (MEP) cells. KLS is expressed as the percentage of Lin⁻ cells; the others are expressed as the percentage of Lin⁻cKit⁺Sca⁻ cells. Results are from 4 independent mice in each genotype. Horizontal bars are the median value. **(C)** mPML-fPR did not alter the number of CFUs in the bone marrow cells when grown for 7 days in methylcellulose containing IL-3, IL-6, and SCF (average ± SD). **(D)** mPML-fPR did not alter the immunophenotype of cells maturing during the methylcellulose culture in **C**. After 1 week in culture, cells were collected from methylcellulose, washed, and stained as indicated. Representative results are from 2 experiments (average ± SD). Horizontal bars are the median value. **(E)** Acute exposure of ERT2-Cre^{+/+} × mPML-PRflox^{+/+} bone marrow cells to 4-hydroxytamoxifen (4-OH tam) (0.1 M or 1 M) modestly decreased the number of CFU-Ms and total CFUs. Bone marrow cells from mice without prior tamoxifen exposure were harvested and plated in methylcellulose, as above, containing increasing doses of 4-hydroxytamoxifen (average ± SD).

row contained 40% or 95% floxed alleles (Figure 4C). LysM-Cre did not recapitulate the replating phenotype, nor did it result in an expansion of the mPML-fPR allele during serial passaging (Figure 4, B and C). The differences observed between floxing induced by ERT2-Cre (active in diverse tissues during tamoxifen therapy and in early hematopoietic stem cells) (23) versus

that induced by LysM-Cre (active predominantly in late myeloid cells) (21) suggest that PML-RARA expression in late myeloid cells cannot reprogram these cells to self-renew.

In vivo effects of PML-RARA on hematopoiesis. To define the effects of mPML-fPR on hematopoiesis, we generated a tumor watch consisting of LysM-Cre^{+/+} × mPML-PRflox^{+/+} mice (n = 54) from 2

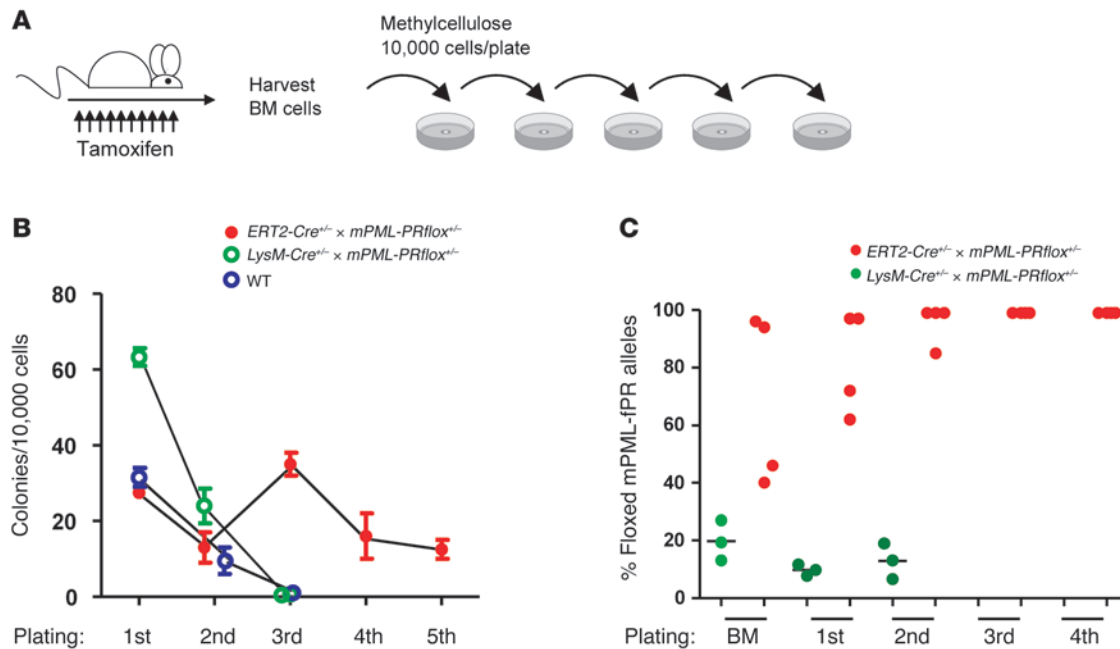


Figure 4 mPML-fPR leads to inappropriate ex vivo self-renewal of bone marrow CFUs. (A) Experiment schema showing that *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* mice and littermate controls were treated with tamoxifen (4 mg i.p. twice weekly for 10 doses). Bone marrow cells were plated in methylcellulose containing IL-3, IL-6, and SCF. At 7-day intervals, colony numbers were counted and cells were harvested and replated as indicated. (B) mPML-PRflox leads to inappropriate self-renewal when intercrossed with ERT2-Cre but not LysM-Cre. Representative results are from 2 experiments. Data points represent average and SD from 4 individual mice. (C) The mPML-fPR allele was associated with continued CFU activity in ERT2-Cre, but not LysM-Cre, bone marrow. The percentage of mPML-fPR alleles was assessed at each time point in B using qPCR. Data points represent results from individual mice. Horizontal bars are the median value.

founder mPML-PRflox lines and their littermate controls. After 18 months of observation, we observed leukemia in only 1 *LysM-Cre^{+/-} × mPML-PRflox^{+/-}* mouse (on day 481; Figure 5, A and B). Leukemic spleen cells expressed a primitive myeloid phenotype: cKit⁺Sca⁺CD34⁺CD16⁺Gr1^{dim}. Analysis of this mouse identified 90% and 95% mPML-fPR alleles in the peripheral blood and spleen cells, respectively. Interestingly, this mouse presented with a white blood cell count of 3,600 cells/ul and only modest splenomegaly (460 mg spleen weight). These spleen cells reconstituted leukemia in 5 adoptively transferred B6 mice, whose leukemic spleens contained 100% floxed alleles (Figure 5, C–E).

Unexpectedly, the peripheral blood and spleen cells at 18 months in nonleukemic mice demonstrated no consistent expansion of a floxed population or evidence of myeloproliferation (Figure 5, A, F, and G). Analysis of 5 *mPML-PRflox^{+/-}* mice at 18 months showed no evidence of the floxed allele in the absence of Cre recombinase (data not shown). Regardless, these data confirm that the mPML-fPR transgene, activated in the LysM cell population, is capable of causing myeloid leukemia, albeit rarely.

In contrast, after limited exposure to tamoxifen in *ERT2-Cre^{+/-}* mice, we observed that mPML-fPR caused a competitive advantage within hematopoietic cells in vivo. We treated 2 cohorts of mice (comprised of mice from 2 different founder mPML-PRflox lines and their littermate controls) with 1 (*n* = 6) or 5 (*n* = 4) doses of tamoxifen (4 mg i.p. twice weekly) and serially assessed the percentage of mPML-fPR alleles within the peripheral blood. Acquisition of the mPML-fPR allele did not alter peripheral blood counts during 6 months of evaluation (Figure 6B and data not shown). How-

ever, we observed a marked increase in the percentage of peripheral blood mPML-fPR alleles over this same time period, regardless of the initial efficacy of floxing (Figure 6, C and D). A third cohort of *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* mice were followed without tamoxifen treatment. Minimal floxing was detected in the peripheral blood of these mice, showing that leakiness of ERT2-Cre during adult life could not account for the expansion of mPML-fPR-positive cells after tamoxifen treatment (Figure 6D).

This competitive advantage continued through 18 months of age, when the mice were sacrificed. In bone marrow and spleen cells, the mPML-fPR allele comprised more than 95% of mPML-PRflox alleles in nearly all mice treated with 1 or 5 doses of tamoxifen (Figure 6E and data not shown). The relative percentage of bone marrow floxed alleles at 18 months correlated with prior observations in peripheral blood cells; the 2 mice that did not achieve more than 95% floxed alleles in the bone marrow had the lowest percentage of peripheral blood floxed alleles from the beginning of the experiment (mouse 11941 and 11933). Again, this expansion of mPML-fPR-positive cells was not associated with myeloproliferation; the mice did not develop splenomegaly or exhibit increased myeloid cells in the bone marrow or spleen (Figure 6F and Supplemental Figure 4). One mouse (mouse 11401) developed a cutaneous infiltrate reminiscent of the cutaneous *MRP8-PML-RARA* phenotype and was sacrificed on day 249 (data not shown) (6). One mouse (11930) developed splenomegaly. This was associated with a transplantable leukemia that appeared unrelated to mPML-fPR; bone marrow and spleen cells contained only 10% floxed alleles and did not contain detectable amounts of PML-RARA protein (Figure 6E,

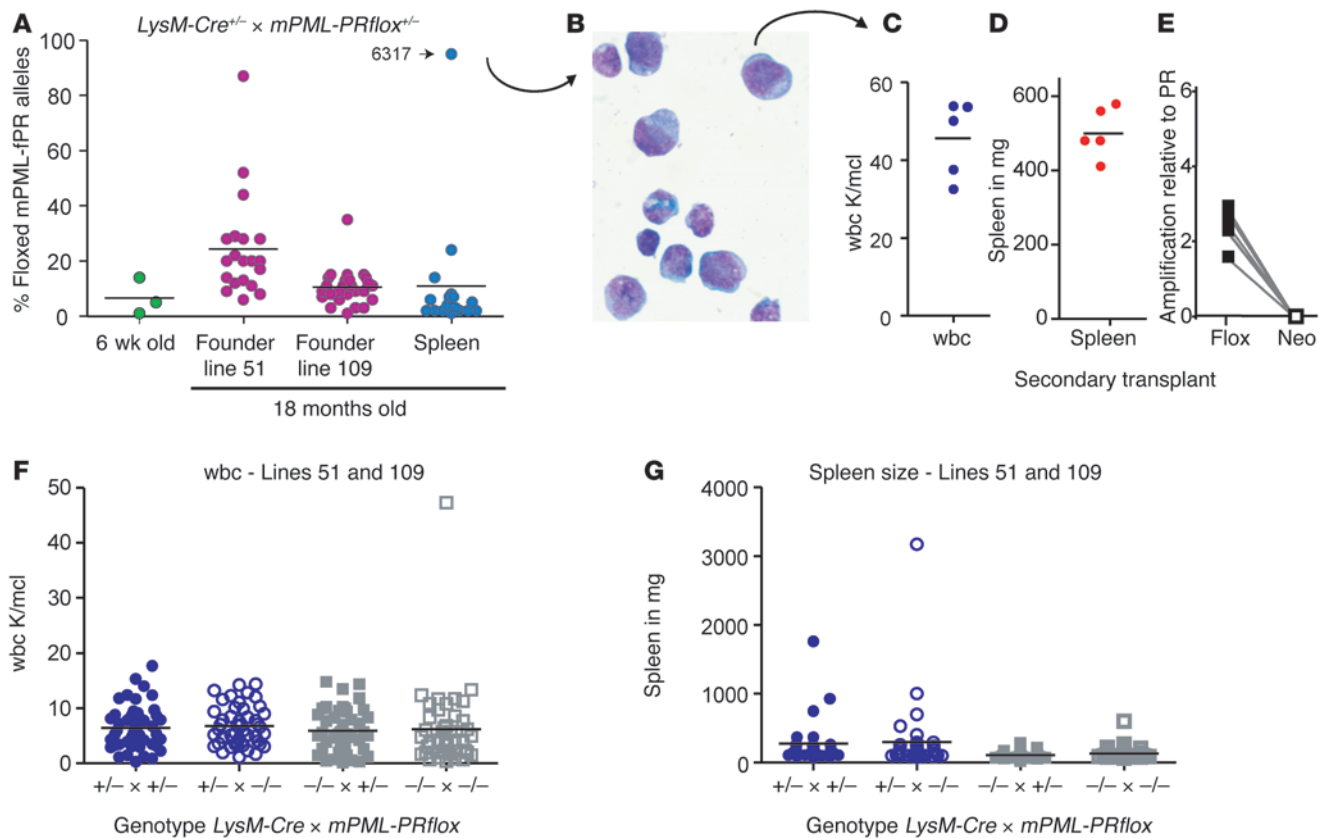


Figure 5

Rare leukemia in *LysM-Cre* × *mPML-PRflox* mice. (A) A tumor watch of *LysM-Cre*^{+/+} × *mPML-PRflox*^{+/+} mice (*n* = 54) resulted in leukemic mouse (mouse 6317, founder line 51) with 90% and 95% peripheral blood and spleen *mPML-fPR* alleles, respectively. Nonleukemic mice were evaluated at 18 months and showed little evidence of expanded *mPML-fPR* alleles in either peripheral blood or spleen. (B) Spleen cell cytomorphology from mouse 6317, stained with Wright-Giemsa (original magnification, ×1,000). (C–E) Spleen cells from mouse 6317 were transplanted into non-irradiated B6 mice (*n* = 5). In 6 weeks all mice were moribund, with (C) elevated white blood cell counts and (D) splenomegaly. (E) qPCR evaluation of the spleen cells in moribund secondary recipients revealed 100% *mPML-fPR*. (F and G) Analysis of *LysM-Cre*^{+/+} × *mPML-PRflox*^{+/+} mice and littermate controls at 18 months revealed normal (F) white blood cell and (G) spleen sizes, with rare evidence of splenomegaly in *LysM-Cre*^{+/+} mice, regardless of *mPML-PRflox* genotype. Data points represent results from individual mice. Horizontal bars are the median value.

Supplemental Figure 1D, and data not shown). These data confirm that *mPML-fPR* provides a competitive advantage in hematopoietic cells *in vivo* as well as *in vitro*. Unlike prior models of APL (but similar to human APL), the competitive advantage of *mPML-fPR* was not associated with myeloproliferation, and homeostatic feedback regulation of myeloid compartment size was preserved.

PML-RARA-dependent leukemia has not yet been observed in *ERT2-Cre*^{+/+} × *mPML-PRflox*^{+/+} mice. An additional 12 mice were treated with 5 doses of tamoxifen and were followed for 15 months. One mouse became ill with myelodysplastic-like disease characterized by pancytopenia, a normal spleen (150 mg), and an infiltrate of Gr1⁺cKit⁺CD11b⁺ cells (79%) in the bone marrow, consisting predominantly of differentiated PMNs with few blasts (data not shown). CD45.2⁺ cells engrafted 2 sublethally irradiated CD45.1 recipients but did not recapitulate the proband’s disease (data not shown).

To determine whether the self-renewal phenotype was related to alterations in cell cycle or apoptosis, we examined BrdU uptake in *ERT2-Cre*^{+/+} × *mPML-PRflox*^{+/+} cells, both *in vitro* and *in vivo*. We observed no difference in BrdU uptake after *in vivo* tamoxifen or after acute exposure to 4-hydroxytamoxifen *in vitro*. We examined unmanipulated total bone marrow cells and bone marrow

cells grown *ex vivo* in liquid culture containing IL-3, IL-6, and SCF, liquid culture containing G-CSF and SCF, or methylcellulose plating experiments containing the IL-3, IL-6, and SCF (data not shown). We examined annexin V/propidium iodine staining under similar conditions and identified no differences in the frequency of apoptotic cells based on genotype or tamoxifen exposure (data not shown). This suggests that the self-renewal phenotype may occur either through non-cell cycle/apoptotic pathways or that only a small subset of cells may be affected.

To validate the self-renewal phenotype *in vivo* and to define the compartment affected by *mPML-fPR*, we performed both competitive and noncompetitive transplantation experiments. Initial competitive transplantation of bone marrow from *ERT2-Cre*^{+/+} × *mPML-PRflox*^{+/+} mice treated with 10 doses of tamoxifen was confounded by an early engraftment defect and wide variability in subsequent competitive expansion (Supplemental Figure 5A). We observed a similar engraftment defect in bone marrow cells from *ERT2-Cre*^{+/+} mice treated with tamoxifen, suggesting that this phenotype was caused by Cre toxicity to stem cells, which has been observed by others, and may partially explain the decrease in KLS cells seen in Figure 3B (23). We also observed a consistent competitive advantage associated with

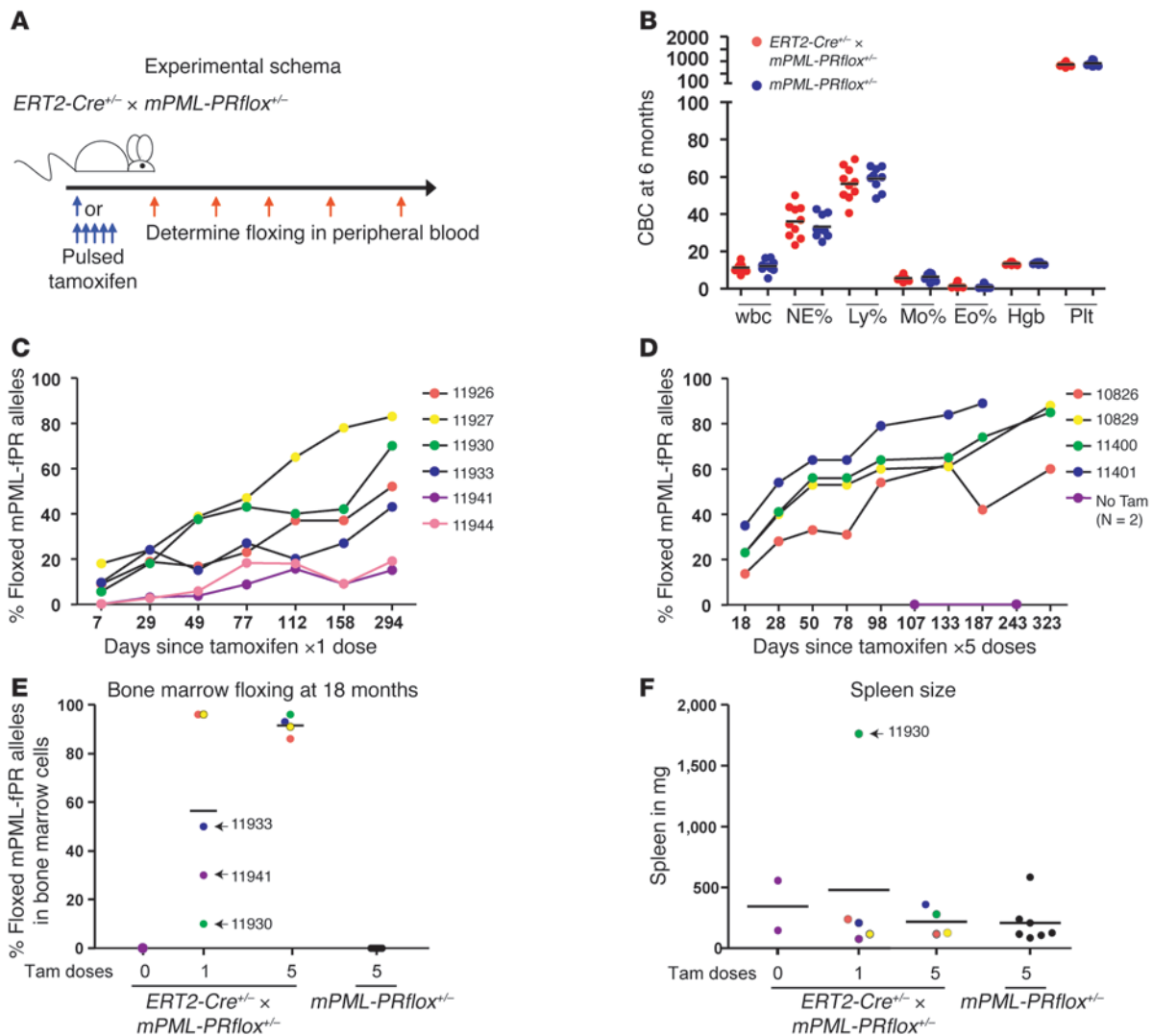


Figure 6

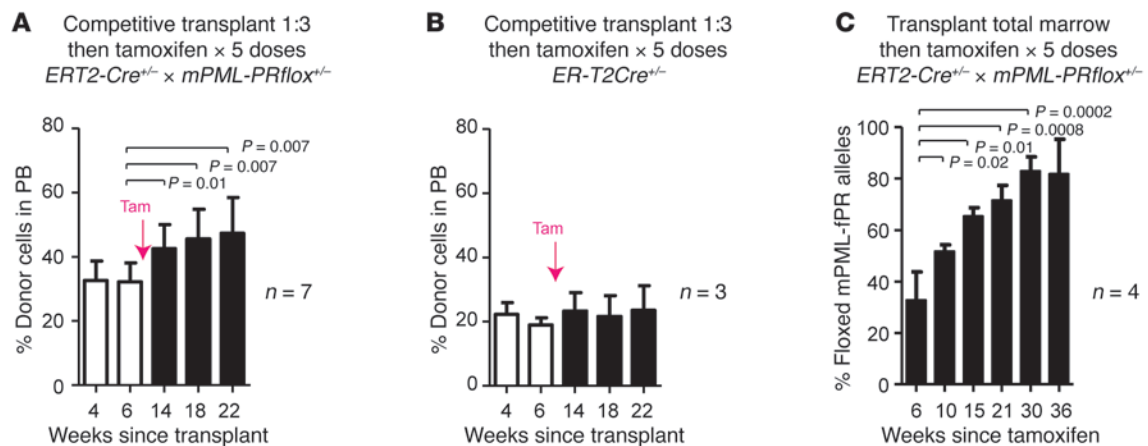
Expansion of mPML-fPR alleles after pulsed tamoxifen. (A) Experimental schema showing *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* mice and *mPML-PRflox^{+/-}* littermate controls were treated with 0, 1, or 5 doses of tamoxifen (4 mg i.p.). (B) mPML-fPR did not alter peripheral blood counts at 6 months. NE, neutrophil; Ly, lymphocytes; Mo, monocytes; Eo, eosinophils; Hgb, hemoglobin; Plt, platelet. (C and D) After the initial pulse of tamoxifen, the percentage of mPML-fPR alleles in peripheral blood increased and was measured using qPCR at indicated time points. Numbers next to colored dots indicate specific mice. (E) At 18 months, the percentage of mPML-fPR alleles was assessed in bone marrow cells. (F) Expansion of mPML-fPR alleles was not associated with splenomegaly. Data represent results from individual mice at indicated time points. Horizontal bars are the median value.

Pml haploinsufficiency in *mPML-PRflox^{+/-}* bone marrow in both the granulocyte and lymphocyte compartments, consistent with prior observations that *Pml* haploinsufficiency shortens mCG-PR leukemic latency (Supplemental Figure 5C, ref. 11, and data not shown).

We observed a competitive expansion of *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* bone marrow cells after competitive and noncompetitive transplantation when donor bone marrow was transplanted prior to tamoxifen exposure (Figure 7). After confirming engraftment of competitor and donor cells, we administered 5 doses of tamoxifen (posttransplant recipients tolerated i.p. tamoxifen poorly, and we administered the drug by gavage). During 16 weeks of follow-up, we observed a consistent expansion of donor CD45.2⁺ peripheral blood cells in *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* recipients but not in *ERT2-Cre^{+/-}* recipients (Figure 7, A and B). Similarly, after noncom-

petitive transplantation of total bone marrow from *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* donors, we observed a consistent expansion of cells with mPML-fPR alleles (Figure 7C). More robust expansion of *PML-RARA* cells in noncompetitive transplants compared with that in competitive transplants is partly explained by the fact that only a portion (~40%) of the CD45.2⁺ donor cells were floxed in the competitive studies (Figure 7, A and C, and data not shown).

Competitive expansion occurred across multiple hematopoietic lineages, suggesting that mPML-fPR affects a stem cell compartment with multilineage potential (Supplemental Figure 6, A–D). As expected, we observed initial peripheralization of donor Gr1⁺ and Nk1.1⁺ cells from both *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* and *ERT2-Cre^{+/-}* donor cells, while peripheralization of CD19⁺ and CD3⁺ donor cells occurred more slowly (Supplemental Figure 6). After

**Figure 7**

Competitive and noncompetitive transplantation. Bone marrow from indicated donor mice (CD45.2) was transplanted with competitor bone marrow (CD45.1) into lethally irradiated recipients (CD45.1). After engraftment, recipients were treated with the indicated doses of tamoxifen (3 mg by gavage). At the indicated time points, peripheral blood (PB) was assessed by flow cytometry. (A) Assessment of *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* donor bone marrow cells. (B) Assessment of *ERT2-Cre^{+/-}* donor bone marrow cells. (C) Assessment of *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* donor bone marrow cells. Results represent average and SD from indicated numbers of mice in each cohort.

tamoxifen, we observed expansion of *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* donor cells within CD19⁺, Nk1.1⁺, and CD3⁺ lymphoid compartments but no expansion of *ERT2-Cre^{+/-}* donor cells (Supplemental Figure 6, B–D and F–H). By 14 weeks after transplant, the percentage of donor *ERT2-Cre^{+/-}* cells declined in Gr1⁺ myeloid cells and remained constant with respect to the percentage of total donor cells in the peripheral blood at 6 weeks (Supplemental Figure 6E). In contrast, the percentage of Gr1⁺ *ERT2-Cre^{+/-} × mPML-PRflox^{+/-}* donor cells remained significantly higher than the percentage of total donor cells in the peripheral blood at 6 weeks (Supplemental Figure 6A). As before, expansion of mPML-fPR-positive cells was not associated with myeloproliferation; recipient mice maintained normal CBCs and peripheral blood differential counts throughout the experiment (data not shown). These data provide further evidence that *PML-RARA* results in competitive expansion across multiple hematopoietic lineages, without disturbing the homeostatic regulation of myeloid or lymphoid compartments.

Discussion

PML-RARA expression has been shown to lead to leukemia in multiple prior mouse models (reviewed in refs. 3, 4). This leukemia is typically associated with antecedent myeloproliferation (increased promyelocytes and CFUs), requires cooperating progression mutations, and generally presents with high white blood cell counts (7, 8, 24, 25). Because human APL is characterized by low white blood cell counts and an acute onset without antecedent myeloproliferation (1), we sought to more accurately model human APL by placing *PML-RARA* under the regulatory control of the *Pml* locus, in the setting of somatic acquisition of expression and *Pml* haploinsufficiency. Our observations that *PML* and *RARA* are not grossly dysregulated in t(15;17) human leukemic bone marrow samples suggest that the translocation does not dramatically alter the expression of either gene and that accurate, somatic expression of *PML-RARA* is likely to be important.

Our data show that adult acquisition of hematopoietic *PML-RARA* expression leads to a competitive advantage that allows *PML-RARA*-expressing cells to expand more efficiently than their wild-type

counterparts both in vitro and in vivo. However, this expansion is not associated with evidence of myeloproliferation or dysregulation of hematopoietic homeostasis (mice had normal progenitor numbers, CFUs, and normal CBCs and differentials during follow-up). However, *LysM-Cre* (which is predominantly active in mature PMNs and macrophages) (20) did not recapitulate this self-renewal phenotype, raising the possibility that *PML-RARA* cannot reprogram terminally differentiated myeloid cells to self-renew.

The in vivo self-renewal phenotype was recapitulated after competitive and noncompetitive transplantation (i.e., it was cell autonomous), and it was associated with both lymphoid and myeloid expansion but normal CBCs and differential counts. Collectively, these experiments demonstrate that *PML-RARA* affects a multipotent progenitor compartment. These results are supported by our recent findings in mCG-PR mice, in which we have demonstrated that *PML-RARA* is expressed and functional in the KLS compartment (Welch et al., unpublished observations). Both results contradict the current paradigm, which suggests that *PML-RARA* transforms a committed myeloid precursor (i.e., a promyelocyte) by being expressed at that stage of development (26–29). This is clearly not the case. It will be important to determine whether the maturation arrest results from development-specific cooperating factors (i.e., neutrophil elastase [*ELANE*]) or from cooperating mutations that display myeloid selectivity (i.e., mutations that occur in genes with myeloid-restricted expression) (17).

The leukemic penetrance of mPML-PRflox was very low in both *LysM-Cre* and *ERT2-Cre* intercrosses. This represents the sixth mouse model generated using the same *PML-RARA* cDNA. Each of these prior models have resulted in very different leukemic penetrance, ranging from zero (*cFes* and *CD11b*) to nearly complete (mCG-PR and *MRP8-PR*), suggesting that the dose and timing of *PML-RARA* expression do significantly alter the susceptibility of mice to leukemia development (Welch et al., unpublished observations) (7–9, 30–32).

The model described here differs from the prior models in several ways. Importantly, the expression of *PML-RARA* mRNA from the *Pml* locus is even lower than that measured from the mCG-PR



mice, which is in turn much lower than the expression in hCG-PR transgenic animals. Consistent with this, we have recently found that the expression pattern of *PML* is very different from that of *CTSG* and *MRP8* in both mice and humans; it is much lower with no dynamic induction during promyelocyte maturation (Welch et al., unpublished observations). Our group has previously shown that reduced dose levels of *PML-RARA* are associated with less neutrophil elastase-dependent myeloid toxicity (17). However, the dose of *PML-RARA* expression obtained in mCG-PR mice is still associated with myeloid proliferation, reminiscent of “rebound” after recovery from cytotoxic therapy. In contrast, after acute exposure to mPML-fPR, we observed essentially no hematologic toxicity and no evidence for myeloproliferation (normal progenitor numbers, normal differential counts, and normal CFU numbers). This decrease in toxicity and low leukemic penetrance makes this model an attractive system for testing mutational cooperativity. Studies are ongoing to assess the effects of *JAK1* V658F (33) and radiation in these mice (34). Because antecedent myeloproliferation is not observed in APL patients, we suggest that the myeloproliferation and the increased penetrance observed in mCG-PR mice may partially reflect the hematologic toxicity of overexpressed *PML-RARA* and the resultant early hematopoietic expansion that may occur as a consequence. These results suggest that *PML-RARA*, when expressed from the *Pml* locus, is not a “potent” oncogene and raise questions about the true penetrance of APL in people who spontaneously acquire t(15;17) in hematopoietic cells.

Finally, these data suggest a mechanism of initial t(15;17) activity. *PML-RARA* is necessary but not sufficient to cause leukemia in mice (in mouse models there is substantial leukemic latency that can be shortened by radiation, alkylator treatment, or cooperation with other known leukemia progression mutations) (3, 35). However, human APL is characterized by an acute presentation, often in extremis (1). How does t(15;17) lead to an acute disease if it requires additional cooperating mutations? The results presented here and other data suggest that t(15;17) leads to a competitive advantage that allows for expansion of t(15;17)-positive cells (36). This increases the probability of acquiring additional mutations in t(15;17)-positive cells, but it does not lead to myeloproliferation that could cause a chronic hematopoietic disorder. *PML-RARA* increases the number of self-renewing progenitors, but homeostatic feedback is lost only after critical secondary mutations are acquired. Further studies will be required to fully validate this model, but the data presented here have uncovered substantial differences from previous models with less physiologic designs. Once again, this study points out the importance of accurately reproducing every aspect of oncogene activation to mimic the evolution of human cancers.

Methods

Expression array profiling. Human AML and normal hematopoietic cell expression profiling has been previously described (37). In brief, bone marrow aspirates were obtained from patients that gave informed consent. RNA was prepared from the unfractionated snap-frozen cell pellets and profiled using the Affymetrix Human Genome U133 Plus 2.0 Array GeneChip microarrays (Affymetrix) at the Siteman Cancer Center Molecular and Genomic Analysis Core Facility (38).

Mice. The mPML-PRflox transgene was generated as described in Figure 2. *PML-RARA* cDNA was cloned downstream of a loxp-PGK-neo-stop-loxp cassette and then flanked by the 4.5-kb XbaI-EcoRI region upstream of *Pml* exon 1 and by the 4-kb EcoRI-ClaI fragment downstream of *Pml* exon 1.

This construct was electroporated into B6-Blu ES cells made in the Siteman Cancer Center Embryonic Stem Cell Core (<http://escore.im.wustl.edu/>). Two independent targeted clones (numbered 51 and 109) were identified by Southern blotting and injected into B6 blastocysts. Chimeric male offspring were identified by LacZ activity in the peripheral blood, and the targeted locus in both clones was transmitted through the germline of B6 mice. Inter-crosses were performed as described. Mx-Cre (B6.Cg-Tg[Mx1-cre]1Cgn/J), CMV-Cre (B6.C-Tg[CMV-cre]1Cgn/J), LysM-Cre (B6.129-Lyz2^{tm1(cre)Jfo}/J), and ERT2-Cre (B6.Cg-Tg[cre/Esr1]5Amc/J) mice were obtained from The Jackson Laboratory, and all were otherwise on a B6 background. Activation of ERT2-Cre was performed by i.p. injection or gavage of tamoxifen dissolved in sterile corn oil (Sigma-Aldrich) as previously described (22). In wild-type B6 mice, 6 mg i.p. tamoxifen proved to be dose limiting, and we thus typically administered 4 mg tamoxifen. Peripheral blood counts were accessed as indicated (Hemavet 950, Drew Scientific Group).

Competitive repopulation was performed by retro-orbital injection of 1×10^6 total bone marrow cells into lethally irradiated Ly5.1 recipients (CD45.1) that received 1,100 cGy total body irradiation (Mark 1 Cesium¹³⁷ irradiator, JL Shepherd) 24 hours prior to transplantation.

The Washington University Institutional Review Board approved the collection and molecular characterization of bone marrow cells collected from AML patients and from normal subjects. The Washington University Animal Studies Committee approved all animal experiments.

Hematopoietic progenitor assay. After lysis of red blood cells (ACK buffer: 0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA), bone marrow cells were counted using a hemocytometer and plated in duplicate in 1.1 ml of methylcellulose medium containing IL-6, IL-3, and SCF (MethoCult M3534, Stem-Cell Technologies) (36). Cells were plated at 8.3×10^3 cells/ml. Colonies with more than 30 cells were counted on day 7. Cells were collected in warm DMEM, washed, and counted. Cells were then replated at the same density. This process was repeated for 6 weeks or until colony formation failed.

Flow cytometry. After lysis of red blood cells, peripheral blood, bone marrow, or spleen cells were treated with anti-mouse CD16/32 (eBioscience, clone 93) and stained with the indicated combinations of the following antibodies: FITC- α Gr1 (BD Pharmingen, clone RB6-8C5); PE- α CD11b (eBioscience, clone M1/70); PE- α CD115 (eBioscience, clone AFS98); FITC- α CD34 (BD Pharmingen, clone RAM34); FITC-NK1.1 (eBioscience, clone PK136); APC- α Kit (eBioscience, clone 2B8); PE- α CD3 (BD Pharmingen, clone 145-2C11); PE- α CD19 (eBioscience, clone MB19-1); PE- α B220 (BD Pharmingen, clone RA3-6B2); biotin- α CD34 (eBioscience, clone RAM34); PercP-Cy5.5- α Gr1 (eBioscience, clone RB6-8C5); PerCP-Cy5.5- α CD45.2 (eBioscience, clone 104); APC- α CD45.1 (eBioscience, clone A20); APC- α Sca (eBioscience, clone D7); APC-Cy7- α CD117 (eBioscience, clone 2B8); or lineage cocktail (α CD3, CD3e, α CD8 (clone 53-6.7), α CD4 (clone L3T4), α CD19 (clone 1D3), α B220 (clone RA3-6B2), α Gr1 (clone RB6-8C5), or α Terr-119 (clone Ly-76) (all PE-Cy7-conjugated antibodies, eBioscience). Analysis was performed using a FACScan (Beckman Coulter) or Reflection cell sorter (iCyt), and data analyzed using FlowJo, Excel (Microsoft), and Prism 5 (Graphpad).

Western blotting. Whole cell lysates were prepared from spleen cells and NB4 cells in 2 \times sample buffer. Lysates from equal numbers of cells were separated on a 10% NuPAGE Bis-Tris gel (Invitrogen) and transferred onto Hybond C Extra nitrocellulose membrane (Amersham). The membrane was probed with an anti-Pml antibody (1:500; Millipore, clone 16.1-104), a polyclonal anti-RARA antibody (a gift from Scott Kogan, UCSF, San Francisco, California, USA) or anti- α Actin (1:1,000; Millipore). Immune complexes were revealed by a peroxidase-conjugated anti-mouse IgG (1:10,000; GE) or conjugated anti-rabbit IgG (1:2,500; GE) and visualized by chemiluminescence (Pierce).

Immunofluorescence. Spleen cells were fixed in methanol at -20°C and then rehydrated in PBS. Staining was done with α -Pml (1:200; Millipore, clone 16.1-104), goat α -mouse IgG Alexa Fluor 488 (1:200; Invitrogen) in



2% goat serum, and Vectashield (H-1200, Vector Laboratories). Cells were visualized on a Microphot-SA with Digital Sight imaging (Nikon).

qRT-PCR. Bone marrow and spleen cells were prepared using TRIzol (Invitrogen). RNA was treated twice with Turbo DNase (Ambion). cDNA was prepared using Superscript (Invitrogen) and amplified using QuantiTect SYBR Green (Qiagen) and 7300 Real Time PCR (Applied Biosystems) per manufacture specifications. Primers used were as follows: PML-RARA (ENF903 TCTTCTGCCCAACAGCAA; ENR962 GCTTGTAGATGCGGGGTAGAG); Pml (mPML-F CAGCCATTGAGACCCAGAGCAG; mPML-R GCAAGGCTTGTAGATGCGGGGTAG); and *Gapdh* (GAP-F TGCACCACCAACTGCTTAG; GAP-R GGATGCAGGGATGATGTTTC).

qPCR. The percentage of floxed alleles was determined after DNA extraction using a DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA was sonicated at 50% for 30 seconds using a Branson Sonifier 450 (VWR Scientific). Quantitative PCR was performed using QuantiTect SYBR Green (Qiagen) and a 7300 Real Time PCR (Applied Biosystems) per manufacturer specifications. Primers used were as follows: floxed (Floxed2F CCATTTCCCGCCTTACCAGACCTA; Floxed2R GATCGGGATCGAATTCGGCTTG-TACTGA); unfloxed (Floxed2F CCATTTCCCGCCTTACCAGACCTA; Neo7R GTAGCGCCAAGTGCCAGC); and PR control (ENF903 TCTTCTGCCCAACAGCAA; ENR962 GCTTGTAGATGCGGGGTAGAG).

Statistics. Two-sided Student's *t* test was calculated using Excel (Microsoft). *P* values < 0.05 were considered statistically significant. Results with signifi-

cant *P* values are indicated in the text and figures, and the corresponding *P* values are provided. Nonsignificant *P* values generally are not included in figures. Data presented with column graphs and error bars represent the average \pm SD. Other data represents results from individual mice.

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Address correspondence to: Timothy J. Ley, Box 8007, 660 South Euclid Ave., Washington University School of Medicine, St. Louis, Missouri 63119, USA. Phone: 314.362.8831; Fax: 314.362.9333; E-mail: timley@wustl.edu.

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