A high penetrance mouse model of acute promyelocytic leukemia with very low levels of PML-RARα expression.

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Abstract

Transgenic mice expressing PML-RARα in early myeloid cells under control of human cathepsin G regulatory sequences all develop a myeloproliferative syndrome, but only 15-20% develop acute promyelocytic leukemia (APL) after a latent period of 6-14 months. However, this transgene is expressed at very low levels in the bone marrow cells of transgenic mice. Since the transgene includes only 6 kB of regulatory sequences from the human cathepsin G locus, we hypothesized that sequences required for high-level expression of the transgene might be located elsewhere in the cathepsin G locus, and that a knock-in model might yield much higher expression levels, and higher penetrance of disease. We therefore targeted a human PML-RARα cDNA to the 5’ untranslated region of the murine cathepsin G gene, using homologous recombination in embryonic stem cells. This model produced a high-penetrance APL phenotype, with more than 90% of knock-in mice developing APL between 6-16 months of age. The latent period and phenotype of APL (including a low frequency of an interstitial deletion of chromosome 2) was similar to that of the previous transgenic model. Remarkably, however, the expression level of PML-RARα in bone marrow cells or APL cells was <3% of that measured in the low-penetrance transgenic model. Although the explanation for this result is not yet clear, one hypothesis suggests that very low levels of PML-RARα expression in early myeloid cells may be optimal for the development of APL in mice.
Introduction

Several groups, including our own, have recently modeled acute promyelocytic leukemia (APL) in the mouse \(^1-^5\). When a PML-RAR\(\alpha\) cDNA (PR) derived from a t(15;17) is placed under control of human cathepsin G regulatory sequences, it is expressed in early myeloid cells at low levels \(^1\). Virtually all mice expressing PML-RAR\(\alpha\) in early myeloid cells develop a myeloproliferative syndrome, and 15-20% go on to develop a disease that closely resembles acute promyelocytic leukemia after a latent period of 6-14 months \(^1,^2,^5\). The penetrance of that APL-like disease can be increased nearly 4-fold by co-expressing the reciprocal RAR\(\alpha\)-PML cDNA (RP) in the same early myeloid compartment, but the long latency persists in these doubly transgenic mice \(^6\). These results have suggested that PML-RAR\(\alpha\) is the primary determinant of the phenotype of this disease, and that it is a bona fide leukemia-initiating protein \(^7\).

However, even though this molecule is necessary for the development of APL in the mouse, the previous models suggests that it is not sufficient. The long latency and low penetrance suggest that additional genetic events are required for leukemia progression. Several events associated with progression have been identified cytogenetically, including an interstitial deletion of chromosome 2, gain of chromosome 15, and loss of a sex chromosome \(^8,^9\). In addition, over-expression of bcl-2 in early myeloid cells \(^10\), or the co-expression of an activated FLT3 allele \(^11\), also increase the penetrance of the APL phenotype in transgenic mice expressing PML-RAR\(\alpha\).

The precise mechanism by which PML-RAR\(\alpha\) expression facilitates the development of APL is not yet known. This molecule has been proposed to act in a dominant negative fashion to
suppress the normal function of both RARα and PML. When over-expressed, it interferes with the assembly of RARα-RXR heterodimers, and its ability to be displaced from target sequences by its physiologic ligand is dramatically reduced. Furthermore, when hCG-PML-RARα transgenic mice are intercrossed with mice that are deficient for PML, the penetrance of APL increases, suggesting that enforcement of a dominant negative activity against PML may increase the susceptibility of mice to leukemia.

Since hCG-PML-RARα mice express the transgene at very low levels compared to the endogenous cathepsin G allele, we hypothesized that the penetrance of APL might be greatly increased in these mice if we could increase levels of PML-RARα expression in early myeloid cells. To accomplish this end, we targeted the same bcr-1 derived PML-RARα cDNA used previously in the hCG-PML-RARα transgenic mice to the 5’ untranslated region of the endogenous murine cathepsin G locus, using homologous recombination techniques in embryonic stem cells (we decided not to target PML-RARα into the endogenous mouse PML locus since PML expression is ubiquitous; widespread expression of PML-RARα in transgenic mice may be toxic). We found that the retained PGK-neo cassette in the mutant cathepsin G allele caused transcriptional shutdown of the gene. We therefore removed the PGK-neo cassette from the locus in targeted ES cells using Lox P-Cre-mediated recombination. These ΔPGK-neo mice did express PML-RARα, and more than 90% developed APL with a latency similar to that of the original transgenic model. Surprisingly, however, the expression of PML-RARα in the bone marrow and in APL cells was not higher than that of the transgenic mice; in fact, it was < 3% that of the transgenic model. These results suggest that this high-penetrance model does not arise because of a simple dominant-negative effect, but rather an optimal, low level of PML-
RARα expression that facilitates its gain-of-function effects.
Methods

*Generation of knock-in mice.* A targeting vector for inserting a bcr-1 PML-RARα cDNA into the 5’ untranslated region of the murine cathepsin G locus (mCG) was generated using the polymerase chain reaction (PCR) to generate 5’ and 3’ targeting arms flanking the insertion site. Oligonucleotide primers were used to generate a 1.9 kb 5’ targeting arm flanked by Sac-I and Bam HI sites at its 5’ and 3’ ends, respectively, and extending to within 1 bp of the translation initiation site within exon 1. Oligonucleotide primers were used to generate a 2.0 kb 3’ targeting arm flanked by Sal I and Hind III sites at its 5’ and 3’ ends, respectively, and extending from the translational initiation site in exon 1 to within exon 4 (nucleotides 461-2441)\(^{19}\). The targeting vector was assembled within a pUC19 backbone, together with a 1.6 kb PGK-neo selectable marker cassette flanked by LoxP1 sequences. The RW-4 embryonic stem (ES) cell line\(^{20}\) was transfected with the targeting vector by electroporation and G418-resistant clones were isolated. To screen for homologous recombination at the mCG locus, DNA samples isolated from resistant clones were digested with Hind III, Southern blotted, and probed using a PCR-generated 512 bp random primer-labeled DNA probe that spanned exons 3-4 and intron 3 of the mCG locus (nucleotide 2415-2926\(^{19}\)), located downstream of the 3’ targeting arm (Figure 1A). To remove the PGK-neo selectable marker cassette, a correctly targeted ES cell clone (# 100) was transfected with the pTurbo-CRE expression cassette and grown in the absence of G418 as described\(^{20}\). Loss of PGK-neo was determined by Southern blotting of Hind III-digested ES cell DNA, which was hybridized with the 3’CG probe described above.
Mutant mice were generated by injection of C57Bl/6 blastocysts with correctly targeted ES cells, that were implanted into pseudopregnant Swiss Webster females. Chimeric male offspring were identified on the basis of coat color and bred to C57Bl/6 female mice. Germline transmission of the mutant CG locus was assessed by Southern blotting of Hind III digested tail DNA as described above. To generate animals homozygous for the targeted mutation, heterozygous animals were intercrossed, and homozygous male offspring were subsequently bred with heterozygous females to generate a colony of PML-RARα heterozygous and homozygous littermates. To generate knock-in heterozygotes with or without a functional CG gene on the residual allele, mCG\textsuperscript{PR/PR} homozygotes were bred with mCG\textsuperscript{+/-} mice.\textsuperscript{21}

\textit{Leukemia development.} To determine the incidence of leukemia among PML-RARα animals, cohorts of each genotype were generated and followed over time. To screen for leukemia development, peripheral blood was obtained for automated complete blood count analysis by retroorbital plexus bleeding at 1-2 month intervals. Animals that became moribund were euthanized and blood and spleen samples were analyzed for evidence of acute leukemia, using Coulter analysis, morphology, flow cytometry, and histopathologic analysis.

\textit{Cryopreservation of tumor cells.} Splenocytes from euthanised leukemic animals were harvested under sterile conditions and cryopreserved in 10% DMSO media in liquid nitrogen as previously described.\textsuperscript{6}

\textit{Flow cytometry.} Cryopreserved tumor samples were thawed, washed with phosphate buffered saline, and samples were prepared for flow cytometric analysis by red cell lysis and incubation
with antibodies to Gr-1, Mac-1, Sca-1, CD34, Ter119, B220, CD3, CD34, or isotype controls (Becton Dickenson). Flow cytometry was performed using a BD FACscan and data was analyzed using CellQuest software.

**Real Time Quantitative RT-PCR.** Bone marrow was collected from the tibias and femurs of 3-6 month old C57Bl/6 wild type, mCG+/PR (+PGK-neo), mCG+/PR (ΔPGK-neo), and transgenic hCG-PML-RARα non-leukemic animals. Whole marrow was washed in PBS and red cells were removed by incubation in red cell lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Cryopreserved splenic tumor cells obtained from overtly leukemic mCG+/PR (ΔPGK-neo) and transgenic hCG-PML-RARα animals were thawed and RNA was purified from 5x10⁶ cells using an RNEasy kit (Qiagen) with on-column DNAseI treatment, per the manufacturer’s protocols.

RNA was subjected to real time quantitative one-step RT-PCR. Briefly, PCR assays were performed using 500 ng of total RNA per reaction in TaqMan One-Step RT-PCR Master Mix (Applied Biosystems), 400 nM each oligonucleotide primer, and 250 nM TaqMan probe (Applied Biosystems), with or without 1.25 U/µL MultiScribe reverse transcriptase. Primers for human PML-RARα were: forward (5’-CCCAGGAGCCCCGTCATAGG-3’), and reverse (5’-CTTGTAGATGCGGGGTAGAGG-3’). Primers for mouse neutrophil elastase were: forward (5’-CCTTCTCTGTACGGGATCTTC-3’), and reverse (5’-ACATGGAGTTCTGTCACCCAC-3’). Primers for mouse MMP9 were: forward (5’-CAGGGAGATGCCATTTTGC-3’), and reverse (5’-GGGCACCATTGGAGTTTCCA-3’). Fluorescent probes were synthesized by Applied Biosystems for human PML-RARα (5’-VIC-TCTGCCCCAACAGCAACCACGT-
TAMRA-3'), for mouse neutrophil elastase (5’-VIC-CCAACGTGCGTGGCCG-CAG-TAMRA-3’), and for mouse MMP9 (5’-VIC-TCGCTGGGCAAAGCGTCG-TAMRA-3’). GAPDH amplification was performed on the same cDNA using the ABI protocol and ABI reagents. RT-PCR was performed on a GeneAmp 5700 (Applied Biosystems) as follows: 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes, then 40 cycles of 95°C for 15 seconds and 62°C for one minute. Fluorescence ΔCₜ values were used to calculate mRNA levels of PML-RARα relative to neutrophil elastase, or of MMP9 relative to GAPDH. Data represent samples obtained from three animals or one tumor, each assayed in duplicate in three independent experiments.

**Western Blotting.** Bone marrow and splenic tumor cells were washed with PBS and subjected to red cell lysis. 2x10⁶ cells were dissolved in 100 µL RIPA buffer, protein was quantitated by a BCA assay (Pierce), and 50 µg total protein was electrophoresed on 8% SDS-PAGE and transferred to PVDF according to standard protocols. Blotting was performed using polyclonal rabbit antisera against human PML, two monoclonal anti-mouse PML antibodies (a generous gift from Scott Lowe), a rabbit polyclonal antibody against RARα (Santa Cruz, C-20), or a goat anti-actin polyclonal antibody (Santa Cruz, C-11). After staining with the appropriate secondary HRP-conjugated antibodies (Amersham) diluted 1:10,000 in TBST, protein was visualized using Maximum Sensitivity Femto ECL (Pierce).

**In vitro ATRA differentiation.** Spleen cells from banked APL tumors were incubated in vitro (1 x 10⁶ cells per ml) with 1 µM ATRA dissolved in EtOH or with EtOH alone (total 0.1% EtOH by
volume) for 72 hours at 37°C in RPM1-1640 with 10% FCS. Total RNA was then prepared and subjected to real time quantitative RT-PCR analysis as described above.

**Molecular Cytogenetics.** Chromosomes were prepared from APL spleen cells as previously described\(^9\). Chromosome preparations prepared from cells cultured for 4-5 days were stained with Giemsa for chromosome counting, or used for fluorescence in situ hybridization (FISH) with a painting probe for mouse chromosome 2. The conditions of hybridization, the detection of hybridization signals, digital-image acquisition and processing, were performed as previously described\(^22\).
RESULTS

*Generation of mCG*<sup>+/PR</sup> *knock-in mice by homologous recombination*

To generate a transgenic mouse line in which a single copy of PML-RARα cDNA was inserted into the murine cathepsin G locus, ES cells were transfected with the targeting vector shown in Figure 1. The PML-RARα cDNA (the same bcr-1 fusion cDNA used in our previous studies)<sup>1,6</sup>, in tandem with a PGK-neo selection cassette, was inserted by homologous recombination into the 5’ untranslated region of the murine cathepsin G locus. Based on evidence that a transcriptionally active selectable marker cassette might alter the expression of nearby genes<sup>20</sup>, we designed the targeting vector with loxP sites flanking the PGK-neo cassette to facilitate its subsequent removal, which was achieved via transient expression of CRE recombinase in the targeted ES clone (Figure 1A). After CRE transfection, a subclone was identified that retained the PML-RARα cDNA in the 5’ untranslated region of the mCG locus, but from which the PGK-neo cassette was excised, leaving only a single loxP site in its place (Figure 1B). Blastocyst injection of both unmanipulated (+PGK-neo) and pTurboCRE-transfected (ΔPGK-neo) clones was performed to generate chimeric male founder animals, and germline transmission of the targeted loci was observed with predicted Mendelian frequency in both +PGK-neo and ΔPGK-neo lines.
Southern blot analysis of genomic DNA from mCG^{+/-PR} animals revealed that the pTurboCRE plasmid had stably integrated into the genome of the ES cell at the time of transfection. The integrated CRE gene was inherited independently from the mCG^{+/-PR} locus, and its presence was not associated with development of APL (data not shown).

**High penetrance acute myeloid leukemia in mCG^{+/-PR} (ΔPGK-neo) mice**

Transgenic mCG^{+/-PR} (+PGK neo) animals were phenotypically normal, with peripheral blood and bone marrow counts that were indistinguishable from wild type littermates (data not shown). None of these animals developed acute leukemia despite observation for up to two years (Figure 2A). In contrast, all tested mCG^{+/-PR} (ΔPGK-neo) animals showed evidence of a myeloid expansion in the bone marrow similar to that described in our previously characterized hCG-PML-RARα model, with progressive splenomegaly and extramedullary splenic hematopoiesis. Peripheral blood counts were not significantly different from wild type littermates during the “pre-leukemic” phase (Figure 3B, Table 1). Between 6-19 months of age, however, these animals exhibited a high probability of developing an abrupt onset, rapidly fatal acute leukemia, characterized by pronounced leukocytosis with increased myeloblasts and promyelocytes, anemia, thrombocytopenia, and massive hepatosplenomegaly with leukemic cell infiltration (Figure 2A, Table 1). Leukemic cells from the spleens of moribund animals displayed an APL phenotype that was similar to that of PR transgenic mice, with markedly increased numbers of immature myeloid cells, including promyelocytes (Figure 3A). The median age of overt leukemia development was 10 months. Leukemia was readily transferable to genetically compatible wildtype secondary recipients (C57Bl/6 x 129Sv/J F1), which developed similar, rapidly fatal leukemias within 6-8 weeks of tumor inoculation (data not shown).
**Aberrant co-expression of CD34 and myeloid differentiation markers in knock-in PML-RARα tumors**

To characterize the leukemias that developed in the mCG^{+/PR}(ΔPGK neo) knock-in animals, flow cytometric analyses were performed on the spleen cells of affected animals, and compared with tumor spleens from hCG-PML-RARα mice. Analysis of multiple independent tumors demonstrated co-expression of myeloid differentiation markers Gr-1 and Mac-1 in leukemic cells, and the absence of B- or T-lymphoid marker expression, or the erythroid lineage marker Ter-119 (Table 1, Figure 3C, and data not shown). The abnormal co-expression of the primitive hematopoietic marker CD34 with Gr-1 was detected in a large population of cells in leukemic spleens, similar to that previously described in hCG-PML-RARα mice \(^6\)\(^,\)\(^9\) (Figure 3A, Table 1). Furthermore, the analysis of spleens prior to the development of leukemia demonstrated increased numbers of Gr-1 positive cells compared to wild type mice. A modest but statistically significant increase in Gr-1/CD34 co-expressing cells was also detected in the non-leukemic spleens (Figure 3C).

**Induction of myeloid differentiation following ATRA treatment**

To determine whether leukemia cells derived from the knock-in mice respond to ATRA treatment, we devised an assay to measure the induction of MMP9, a gene that is expressed at its highest levels during the late stages of myeloid development. RNA isolated from hCG-PML-RARα or mCG^{PR/PR} tumor spleens was reverse transcribed into DNA and subjected to quantitative real time PCR amplification. We observed 1.5 to 12-fold increases in MMP9 mRNA abundance following treatment of tumor cells from either model with 1 μM ATRA for 72 hours.
over treatment with vehicle alone, after normalization against GAPDH expression levels (Figure 3D). Increases in MMP9 mRNA levels correlated with an increase in the proportion of mature myeloid cells in the cultures over time (data not shown). ATRA sensitivity was similar in the transgenic and knock-in tumor cells. Spleen cells from one knock-in tumor did not exhibit an increase in MMP9 expression level or in myeloid differentiation over time.

A small decrease in leukemia latency with a two-fold increase in PML-RARα gene dosage

To determine the effect of gene dosage on leukemia development, heterozygous mCG+/PR animals (one PML-RARα copy) were bred together to generate offspring homozygous at the CG locus for the PML-RARα knock-in mutation (two PML-RARα copies). Although the high lifetime probability of leukemia development was the same for heterozygous and homozygous animals, the median age at leukemia development was decreased by ~6 weeks among homozygous animals (Figure 2B). Flow cytometric analysis demonstrated no differences in expression of differentiation markers between PML-RARα heterozygous and homozygous tumors (Figure 3, Table 1). Moreover, tumor inoculation into secondary recipients showed no difference in the ability of heterozygous versus homozygous tumors to transfer leukemia to wild type recipients (data not shown).

Endogenous Cathepsin G loss-of-function does not contribute to leukemia development

Because the PML-RARα knock-in mutation results in disruption of one or both mCG loci, it was formally possible that the difference in leukemia incidence between the transgenic and knock-in models resulted from loss of cathepsin G function. We considered this possibility to be unlikely, since both CG+/− and CG−/− mice lack a detectable hematopoietic phenotype 21, and since AML
has never been observed in any CG<sup>+/−</sup> or CG<sup>−/−</sup> animal (C. Pham and T.J. Ley, unpublished). To formally test the effect of mCG loss-of-function in the setting of PML-RARα expression, we bred mCG<sup>PR/PR</sup> homozygous animals with CG<sup>+/−</sup> animals. The offspring of these animals all carried a single PML-RARα cDNA inserted into one mCG allele, and either a wildtype mCG gene or a null mutation at the other allele. These mice were therefore either haploinsufficient or null for Cathepsin G. The probability of leukemia development among these animals was identical, indicating that homozygous loss-of-function for cathepsin G played no detectable role in the development of leukemia in this system (Figure 2C).

**Expression of PML-RARα mRNA in transgenic vs. knock-in mice**

We designed a quantitative RT-PCR assay to determine the relative levels of PML-RARα mRNA expression in the knock-in model and the hCG-PML-RARα transgenic model previously described. Total RNA was harvested from the bone marrow of 3 month old C57Bl/6 wild type mice and from the non-leukemic bone marrow of 3 month old knock-in and transgenic animals. Additionally, RNA was harvested from cryopreserved splenic tumors arising from both APL models. Primers and probes specific for the PML-RARα cDNA, mouse neutrophil elastase, and mouse GAPDH were designed to quantitate PML-RARα abundance with respect to whole cell mRNA abundance (GAPDH) and promyelocyte-specific mRNA abundance (neutrophil elastase). Neutrophil elastase (NE) is coordinately regulated with cathepsin G, both of which demonstrate maximal expression in the promyelocyte compartment<sup>24,25</sup>. Normalizing PML-RARα mRNA abundance to NE mRNA abundance controls for variation in the number of early myeloid cells in each sample (i.e. expression of PML-RARα will not be overestimated in samples with
increased numbers of early myeloid cells). Levels of PML-RARα mRNA normalized to GAPDH mRNA yielded similar relative expression values in all samples tested (data not shown).

Since the transgenic model contains ~50 concatamerized copies of the PML-RARα cDNA \(^1\) (versus only one copy in the knock-in model), the following steps were taken to avoid DNA contamination in the PCR reaction: 1) RNA samples were treated with DNAseI on a purification column, 2) samples were then digested with HaeIII, which cleaves the PML-RARα cDNA within the 200 base pair PCR amplicon, and 3) lack of contamination was verified by requiring at least a five cycle difference in \(\Delta C_T\) between reactions with and without reverse transcriptase.

Bone marrow cells derived from mice with the retained PGK-neo cassette lacked PML-RARα expression that was detectable above the background levels observed in wild type littermates (Figure 4, inset). Removal of the PGK-neo cassette, however, led to consistently detectable PML-RARα expression (Figure 4). Surprisingly, PML-RARα mRNA levels from pre-leukemic knock-in bone marrow cells were significantly lower than those of similar hCG-PML-RARα transgenic mice (48 ± 5.8 fold, \(p<0.005\)). These data were highly reproducible and represent the average expression for three mice of each genotype assayed in duplicate in three separate experiments. This expression relationship was also true for independent tumors that arose in both models (Figure 4). In these samples, where the cells were nearly all leukemic, the levels of PML-RARα mRNA were again much higher in the tumors derived from the transgenic mice. These data represent average data from three tumors in each model, assayed in duplicate in two separate experiments.
Undetectable expression of PML-RARα protein in transgenic and knock-in APL cells

Total cellular protein was extracted from wild type C57Bl/6 bone marrow cells, 3 month old non-leukemic transgenic, and knock-in bone marrow cells, and from several independent tumors arising in each mouse model. Western blotting was performed using several different antibodies directed against RARα or PML. In Figure 5, lane 3 represents U937 cells transiently expressing the human bcr-1 PML-RARα cDNA that was used to create the knock-in and transgenic mouse models. Full length PML-RARα protein is detected at ~120 kd. Despite using highly sensitive chemiluminescence reagents and long exposure times, full length PML-RARα protein was not detected in non-leukemic bone marrow cells from either model (data not shown). Furthermore, in the tumor cells, which represent a more homogeneous cell population, endogenous mouse RARα protein was detectable at ~55 kD, but again, full length PML-RARα was undetectable (Figure 5, lanes 5-9). Similarly, if anti-PML blots were highly overexposed, endogenous mouse PML was visible at ~70 kD, but full length PML-RARα remained below the limit of detection (data not shown). These sensitive Western blots were repeated with a second rabbit polyclonal antibody to PML and with two different monoclonal antibodies against mouse PML. In no case was full length PML-RARα detectable, nor was it detectable in several other tumors from each mouse model (data not shown). Further, lysis by boiling in loading buffer containing 2% SDS did not change these results (data not shown). Together, these data indicate that the abundance of PML-RARα protein is less than that of endogenous PML and RARα in both non-leukemic marrow, and in splenic tumors derived from both mouse models.
An interstitial deletion of Chromosome 2 is found in a small fraction of knock-in tumor cells

We analyzed 9 cryopreserved tumor samples with FISH using a chromosome 2-specific painting probe, and by post-hybridization DAPI-produced G-like banding (Table 2). Six tumors from mCG+/PR mice (13437, 13441, 13487, 13644, 13646 and 13659) had a homogenous, near diploid cell population with a chromosome number ranging from 38 to 46 chromosomes; most of the cells from these 6 tumors contained 40 chromosomes. Among these, tumor 13437 had fewer cells with the normal chromosome number (2n=40). Tumors 13498 and 13499 showed wider variations in chromosome number, which ranged from 39-50 and 36-62, respectively. Only one tumor (13843) consisted of two subpopulations with near-diploid and near-tetraploid chromosome numbers (ranging from 40 to 47, and 71-81 chromosomes, respectively). All lines were examined for alterations of chromosome 2, which is a recurrent alteration in tumors derived from hCG-PML-RARα transgenic animals. An interstitial deletion of chromosome 2, similar to the deletion previously described for transgenic hCG-PML-RARα x hCG-RARα-PML tumors, was detected in tumors 13843 and 13499. The deleted material from chromosome 2 was not translocated to any other chromosome. No other gross chromosomal abnormalities were identified.
Discussion

In this report, we describe mice that express PML-RARα under control of the endogenous murine cathepsin G locus. Mice heterozygous or homozygous for this mutation (which also creates a null cathepsin G allele) are viable and fertile, but all display a myeloproliferative syndrome early in life. After a long latent period, mice bearing this mutation develop a fatal APL-like syndrome that is characterized by the accumulation of early myeloid cells in the bone marrow, spleen, and liver, as well as sensitivity to ATRA in vitro. In contrast to the low penetrance of APL (15-20%) observed in transgenic mice expressing PML-RARα under control of a human cathepsin G targeting cassette, the penetrance of APL in the knock-in mice was greater than 90%. The latency of the two models was similar, however, suggesting that both require additional genetic events for APL progression. Although we predicted that the knock-in model would express PML-RARα at high levels in early myeloid cells, expression was actually much lower than that observed in the previous transgenic model. These results suggest that there may be an optimal level of PML-RARα expression that facilitates APL development, and that this level is lower than that previously predicted.

The most striking feature of the knock-in model was the very high penetrance of APL. In previously reported experiments, we and others have demonstrated that an identical PML-RARα cDNA, when expressed in transgenic mice under the control of human cathepsin G regulatory sequences, results in a myeloproliferative syndrome in all of the mice, but APL development in only 15-20%, and only after a latent period of 6-14 months\textsuperscript{1,2,4,5}. Similarly, mice that express PML-RARα under control of the MRP8 promoter (which is expressed in both early and late myeloid cells) have a low frequency of APL development also characterized by long latency\textsuperscript{3}.  

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Our previously reported hCG transgenic mouse model exhibited relatively low levels of expression of PML-RARα mRNA in the bone marrow, compared with endogenous PML and/or endogenous cathepsin G\(^1\); none of eight transgenic lines expressing hCG-PML-RARα expressed high levels of PML-RARα mRNA\(^1\). In contrast, the unmanipulated 6.0 kB human cathepsin G cassette was expressed at high levels in most transgenic founders, although it did exhibit integration site-specific variegation\(^26\).

These results suggested that high level expression of PML-RARα could not be achieved in the early myeloid cells of transgenic mice. There are at least three possibilities for why this might occur: 1) Insertion of the PML-RARα cDNA into the 5’UT of the cathepsin G gene disrupted a critical regulatory element. 2) A critical regulatory element(s) (e.g., an enhancer or locus control region) that is required for high level expression is missing from the 6.0 kB human cathepsin G targeting cassette. 3) PML-RARα is toxic to the early myeloid cells in which it is expressed.

To address some of these issues, we generated the knock-in model described in this report. The same PML-RARα cDNA was targeted (via homologous recombination) into the identical position of the murine cathepsin G 5’ untranslated region as in the human cathepsin G transgene. With this approach, any missing regulatory element in the cathepsin G locus would be captured. When the PGK-neo cassette was left in the mutant locus, virtually no expression of the cDNA was detected, strongly suggesting that the retained PGK-neo cassette dramatically reduced expression from the mutant locus\(^20\). We therefore removed the PGK-neo cassette by expressing Cre-recombinase in the targeted embryonic stem cell line. This manipulation yielded a functional mutant allele, whose expression could readily be detected, but only with RT-PCR.
The expression level from the mutant allele was dramatically less than that of the unmodified cathepsin G allele in heterozygous animals (data not shown). When expression from the knock-in allele was compared to PML-RARα expression in a transgenic hCG-PML-RARα founder line, we were surprised to find that it was much lower. We and others \(^{14}\) had predicted that the opposite would occur. Furthermore, full length PML-RARα protein could not be detected in the bone marrow of non-leukemic mice, nor in tumors derived from knock-in or transgenic mice, using antibodies that can detect endogenous levels of PML and RARα. This high-penetrance model of APL is therefore associated with an extremely low level of PML-RARα expression in early myeloid cells.

A number of potential explanations could account for these unexpected results: first, the mouse strains used to generate the two models are different. Our transgenic model was made in C3H x C57Bl/6 mice, and the knock-in model was made in 129/SvJ x C57Bl/6 mice. The 129/SvJ component could potentially provide a susceptibility locus for APL development that has not yet been characterized. This possibility seems unlikely to us, since the PML-RARα cDNA has previously been expressed in a number of different genetic backgrounds, and the latency and penetrance of APL development in all strains has been similar \(^{1-5}\). However, this remains a formal possibility, and a backcross to C57Bl/6 mice is therefore in progress; this experiment will require another 2-3 years to complete.

Second, the knock-in mutation actually results in both a gain-of-function and a loss-of-function change in each cathepsin G locus. The gain-of-function mutation is provided by the PML-RARα cDNA placed in the 5’UT of the cathepsin G gene. This mutation also causes a loss-of-function
change: a null mutation of the same cathepsin G gene. However, it does not appear that the loss-
of-function mutation plays a significant role in the phenotype. We did not observe a further
alteration in the latency or penetrance of APL in knock-in mice that had a null mutation of
cathepsin G on the residual allele. Furthermore, since cathepsin G null mice lack any detectable
alterations in myeloid development and do not develop AML, the cathepsin G
haploinsufficiency caused by the targeting event is unlikely to have impacted these results.

Third, it is possible that there is a difference in the early myeloid compartment targeted by the
human cathepsin G transgene vs. the knock-in cathepsin G locus. To explore this possibility, we
purified Sca+Lineage- mononuclear cells from 5-FU treated transgenic and knock-in bone
marrow cells maintained in stem cell factor, Flt3 ligand, IL-3, and TPO for 3 days. Analysis of
these populations does not show any significant difference in PML-RARα mRNA expression
measured by Real Time RT-PCR. Culturing these cells for two days in media containing stem
cell factor and G-CSF induces differentiation of the immature progenitor cells into a
predominantly promyelocytic population; these cells display the same relative levels of PML-
RARα expression as seen in Figure 4 (A Lane and TJ Ley, manuscript in preparation). This
suggests that the knock-in mCG locus does not target expression to a significantly earlier
myeloid progenitor compartment than the hCG transgene.

Fourth, the translatability of the PML-RARα mRNA produced in the knock-in mice may be
greater than that of the transgenic model, where the mRNA is produced from a long concatemer
of transgenes. We cannot directly address this point, since we cannot accurately measure total
PML-RARα protein levels via sensitive western blotting techniques. However, in many previous studies of multi-copy transgenic mice where RNA and protein levels could be measured, direct correlations were observed\textsuperscript{28-30}. For this reason, we feel that this explanation for these results is unlikely.

Finally, it is possible that there is a narrow “window” of PML-RARα expression in early myeloid cells that is optimal to cause the changes that ultimately lead to the development of APL. If levels of this protein are too high in these cells, they may die or become disabled, so that they cannot contribute to APL development. A larger pool of cells expressing a smaller amount of protein may make it more likely that the critical “hits” needed for APL progression may occur in a larger fraction of mice. The slightly decreased latency seen with two copies of PML-RARα may reflect a narrow dose response for this protein, perhaps by increasing the proportion of early myeloid cells that are susceptible for secondary transforming events. These observations are supported by observations from Pelicci’s group that have shown that PML-RARα can be toxic to hematopoietic cells when overexpressed\textsuperscript{31,32}. Furthermore, in a recent study from the same group, splenic APL tumors derived from cells transduced with a retrovirus expressing PML-RARα and an IRES-GFP cassette yielded no detectable GFP positive cells\textsuperscript{33}. These results support the hypothesis that cells expressing high levels of PML-RARα may be deleted in vivo.

An interstitial deletion of chromosome 2 was detected in two of nine APL tumors obtained from the knock-in mice. This frequency is similar to that detected in transgenic hCG-PML-RARα mice (1/5) and in MRP8-PML-RARα mice (3/30)\textsuperscript{10}, but is significantly different (p<0.05) from
that seen in transgenic hCG-PML-RARα x hCG-RARα-PML mice, where 11/13 tumors contained del (2)⁹. These results show that a high penetrance model of APL is not necessarily associated with a high frequency of del (2) during progression. The data also support the hypothesis that it is truly the expression of the RARα-PML cDNA that facilitates the acquisition of del (2) in hCG-PML-RARα x hCG-RARα-PML mice ⁹. Collectively, these results suggest that there are many kinds of genetic events that can contribute to APL progression in the mouse model, and that del (2) is simply one of the most obvious and frequently detected at the whole chromosome level.

The results presented in this study indirectly address the hypothesis that PML-RARα contributes to the pathogenesis of APL by acting predominantly as a dominant-negative molecule for PML and RARα. In vitro, PML-RARα can clearly act as a dominant-negative to inhibit both PML and RARα function when overexpressed ¹²⁻¹⁴. Furthermore, PML haploinsufficiency in hCG-PML-RARα mice appeared to increase the likelihood of APL development, suggesting that PML loss-of-function might somehow “cooperate” with PML-RARα, perhaps by enforcing a dominant-negative signal ¹⁸. However, the results presented here suggest that low levels of PML-RARα expression are more efficient at producing APL than higher levels. The data are more consistent with the hypothesis that there is an optimal pathogenetic PML-RARα “dose” that produces disease. Expression levels that are too low result in no disease, while levels that are too high may result in toxicity and select against the expressing cells. Levels that are most appropriate lead to alterations in expressing cells that make it more likely that they will acquire the critical progression mutations that lead to the development of APL. Further experiments designed to rigorously test this hypothesis are in progress.
Acknowledgments

We gratefully acknowledge the efforts of Kelly Schrimpf for blastocyst injections, and Mieke Hoock for excellent mouse colony management. Elaine Ross and Jacque Mudd provided excellent assistance with the ES targeting work. Nancy Reidelberger provided expert editorial assistance.
References


fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell. 1993;74:423-431


Figure legends

Figure 1. Targeting PML-RARα to the murine cathepsin G locus by homologous recombination and generation of transgenic mice.

A). A targeting vector consisting of a PML-RARα and PGK-neo selectable marker cassette, flanked by targeting arms from the murine cathepsin G (mCG) locus were transfected into embryonic stem cells. Coding exons of the mCG gene are represented as solid boxes (not to scale). The PML-RARα cDNA and PGK-neo selectable marker cassette are represented as open boxes. Transcriptional start sites for the mCG gene and PGK-neo selectable marker cassette are indicated by arrows. The mCG 5’ untranslated region is represented by a shaded box. LoxP sites utilized for CRE recombinase-mediated cassette excision are represented as shaded ovals. The position of an external 3’ mCG DNA probe is indicated as a hatched box under mCG exons 3 and 4. The PGK-neo cassette was subsequently removed from a homologously recombinant ES cell clone by transient transfection of targeted ES cells with a Cre recombinase expression vector.

B) The targeted mCG locus was identified in ES cells by a size shift from 3.2 kb (WT) to 7.9 kb (targeted, +PGK-neo) following Hind III digestion of genomic DNA. Removal of the PGK-neo cassette by Cre recombinase resulted in a shift in the size of the recombinant band to 6.4 kb (a loading artifact caused the slight apparent differences in the sizes of the WT mCG bands at 3.2 kb).

C) Tail DNA from the offspring a heterozygous CG^{PR+/- } intercross was screened by Southern blotting to identify wild type (CG^{+/-}), heterozygous (CG^{+PR}), and homozygous (CG^{PR/PR}) animals.

D) Tail DNA from the offspring of CG^{PR/PR} x CG^{+/-} animals was screened by Southern
blotting to identify animals that carried one PML-RARα allele together with either an intact CG gene (CG+/PR) or a CG null mutation (CG-/PR) at the other allele.

**Figure 2. Development of acute myeloid leukemia in transgenic and knock-in animals.**

A). The Kaplan-Mayer probability of leukemia-free survival of wild type (CG+/+), mCG+/PR (+PGK-neo) and mCG+/PR (ΔPGK-neo) animals is plotted against time, and is compared to leukemia free survival of a simultaneous cohort of hCG-PML-RARα animals from line #134 (the same data was used in reference 6). The number of mice in each cohort were: CG+/+= 25; mCG+/PR (+PGK-neo) = 26; mCG+/PR (ΔPGK-neo) = 118; hCG-PR = 27.

B). The Kaplan-Mayer probability of leukemia-free survival of heterozygous mCG+/PR (ΔPGK neo) vs. homozygous (mCG PR/PR) animals is plotted against time. The difference between the curves is statistically significant (p=0.002). mCG+/PR (ΔPGK-neo) = 118 mice; mCG PR/PR (ΔPGK-neo) = 99 mice.

C). The Kaplan-Mayer probability of leukemia-free survival of animals carrying a single PML-RARα gene copy together with either an intact CG gene (CG PR/+), or a null mutation (CG PR/-) at the other allele is plotted against time. The differences between the curves are not statistically significant (p=0.48). mCG+/PR (ΔPGK-neo) = 60 mice; mCG PR/PR (ΔPGK neo) = 71 mice.

**Figure 3. Characterization of APL Cells**

A). Morphology by May-Grunwald-Giemsa staining and flow cytometric analysis for Gr-1 and CD34 is shown for two independent splenic tumors arising in hCG-PML-RARα animals and in two mCG PR/PR animals. Numbers represent the percentage of cells in each quadrant.
B). Peripheral white blood cell counts (WBC) and absolute neutrophil counts (ANC) determined by automated counting are shown for wild type littermates, non-leukemic mCG+/PR, and leukemic mCG+/PR and mCGPR/PR animals. For panels B and C: wild type = 7 mice; non-leukemic mCG+/PR = 9 mice; leukemic mCG+/PR = 9 mice; leukemic mCGPR/PR = 10 mice.

C). The fraction of Gr-1⁺, CD34⁺, and Gr-1⁺/CD34⁺ cells in the spleens of wild type, non-leukemic, and leukemia cells from mCG+/PR (+/-) and mCGPR/PR (+/+ ) knock-in animals are shown.

D). The relative increase in abundance of MMP9 mRNA in individual APL samples after 3 days of treatment with 1µM ATRA relative to diluent alone is shown. (Left) 5 independent hCG-PML-RARα tumors, (Right) 6 independent mCGPR/PR tumors. MMP9 is normally expressed at its highest levels in terminally differentiated myeloid cells. Expression levels from each sample were normalized by measuring the abundance of GAPDH mRNA, which is expressed at all stages of myeloid development.

Pairwise comparisons that yielded statistical significance of p<0.01 are indicated by asterisks.

Figure 4. Expression of PML-RARα mRNA in non-leukemic and leukemic spleen cells.

Real time quantitative PCR was performed on the bone marrow RNA derived from three non-leukemic animals of each genotype at 3-6 months of age, and on three independent splenic tumors each from leukemic mCG+/PR (ΔPGKneo) or transgenic hCG-PML-RARα animals. Each sample was analysed in duplicate at least twice. Levels of human PML-RARα mRNA were normalized to levels of endogenous mouse neutrophil elastase mRNA, to control for the contribution of early myeloid cells (which express abundant neutrophil elastase mRNA) in each sample. All of the normalized data was pooled and averaged; means and standard errors of the
mean are shown. Statistical significance was determined by a two-tailed Student’s t-test. Inset.

Enlarged view of PML-RARα expression in the non-leukemic bone marrow RNAs of C57Bl/6 wild type, mCG+/PR (+PGK-neo), and mCG+/PR (ΔPGK-neo) animals.

**Figure 5. Expression of PML-RARα protein in leukemic spleen cells.** Western blots were performed for RARα and actin on transiently transfected U937 cells (harvested 4 hours after transfection) and on independent tumors from each model. Lane 1 represents U937 cells electroporated with an empty expression plasmid; Lane 2: U937 cells expressing the RARα portion of human PML-RARα (RARα exons 3-9); Lane 3: U937 cells expressing full length human PML-RARα (importantly, high level expression of PML-RARα in these cells is highly toxic, causing the death of most U937 cells within 24 hours of transfection27). Lanes 5, 6, and 7: hCG-PR tumors; Lanes 8 and 9: mCG+/PR (ΔPGK neo) tumors. The positions of RARα (~55 kD) and full length PML-RARα (~120 kD) are marked. A band of ~50 kD marked “*” represents an unknown protein recognized by the RARα antibody in hCG-PML-RARα tumors. It could potentially represent an altered PML-RARα protein.
Table 1. Peripheral blood counts and splenic flow cytometry on pre-leukemic and leukemic animals. Peripheral blood white cell counts (WBC, x10^6/mL), percent neutrophils (%PMN), platelet counts (PLT, x10^9/mL), and hematocrit (HCT) were determined by automated counting for wild type, non-leukemic mCG+/PR, and leukemic mCG+/PR and mCG-PR/PR animals at the indicated ages. Spleens of the same animals were analyzed by flow cytometry for expression of the surface markers Gr-1 and CD34.

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N=Normal. Del(2) refers to the interstitial deletion of chromosome 2 previously described."
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Figure 2
Figure 3
Figure 4
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![Image of protein gel with bands at 120 kD, 85 kD, 60 kD, and 50 kD](image)

**Figure 5**
A high penetrance mouse model of acute promyelocytic leukemia with very low levels of PML-RARα expression

Peter Westervelt, Andrew A Lane, Jessica L Pollock, Kristie Oldfather, Matthew S Holt, Drazen B Zimonjic, Nicholas C Popescu, John F DiPersio and Timothy J Ley