PML-RARα co-operates with Sox4 in acute myeloid leukemia development in mice

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ABSTRACT

Acute promyelocytic leukemia is characterized by a chromosomal translocation involving the retinoic acid receptor alpha gene. To identify co-operating pathways to leukemogenesis, we crossed *MRP8-PML/RARA* transgenic mice with BXH-2 mice which harbor an endogenous murine leukemia virus that causes acute myeloid leukemia. Approximately half of the leukemias that arose in this cross showed features of acute promyelocytic leukemia. We identified 22 proviral insertion sites in acute promyelocytic-like leukemias and focused our analysis on insertion at *Sox4*, a HMG box transcription factor. Using a transplant model, co-operation between PML-RARα and Sox4 was confirmed with increased penetrance and reduced latency of disease. Interestingly, karyotypic analysis revealed cytogenetic changes suggesting that the factors combined to initiate but not complete leukemic transformation. The co-operation between these transcription factors is consistent with the paradigm of multiple routes to the disease and reinforces the concept that transcription factor networks are important therapeutic targets in myeloid leukemias.

Introduction

While acute myeloid leukemia (AML) presents with a heterogeneous phenotype and genotype, somatic chromosomal aberrations are a common molecular abnormality. The t(15;17) PML-RARα fusion product is characteristic of a subtype of AML, namely acute promyelocytic leukemia (APL), which is recapitulated by the *MRP8-PML/RARA* transgenic model.[1] This has modeled the common, secondary chromosomal aberrations in human APL[2] as well as co-operating pathways during leukemic transformation.[3,4] Current data support the idea that PML-RARα serves as the initiating event in APL. The conceptual model of co-operation between receptor tyrosine kinase mutations (Class-I) and mutations targeting transcription factors (Class-II) has led to significant advances in our knowledge of myeloid leukemogenesis.[5] However, other routes clearly exist, such as co-operative transformation mediated by alterations in more than one transcription factor. Replication-competent murine leukemia viruses (that lack oncogenic sequences) and murine strains with high levels of endogenous retroviral activity[6] have been invaluable tools for the identification of co-operating events.

The Sry-related high mobility group (HMG) box 4 (Sox4) transcription factor binds directly to the minor groove of DNA within a conserved region.[7,8] On the one hand, ablation of Sox4 leads to embryogenic lethality due to cardiac defects,[9] whereas there is mounting evidence that overexpression of Sox4 contributes to tumorigenesis. Directly relevant to myeloid leukemia, the *Sox4* locus has been previously identified as a viral integration target in co-operation with the *Evi1* transcription factor.[10] We describe here the novel co-operation of PML-RARα with Sox4 during myeloid leukemogenesis, highlighting collaborative changes in transcription factors as one route to myeloid leukemia, and emphasizing the importance of disrupting transcription factor abnormalities for treatment of hematopoietic neoplasia.[11,12]

Design and Methods

Mice

Mice were bred and maintained at the University of California in San Francisco in accordance with institutional Animal Care and Use Committee guidelines. FVB/n *MRP8-PML/RARA* mice have been previously described.[1] Animals were sacrificed when moribund or when physical examination and blood cell counts indicated likely rapid progression of illness. Daily inspection of mice included assessment for general signs of illness. Blood was obtained via retro-orbital bleeding of animals presenting with raised criteria for malaise or moribund.

Retroviral transduction and transplantation

The ecotropic BOSC23 packaging line was transfected with retroviral constructs and virus harvested as previously described[3] using the MSCV control plasmid[13] or one carrying the mouse Sox4 cDNA. Transduced cells were washed, counted with trypan blue and injected into the tail vein of lethally (9 Gy) irradiated recipient FVB/n mice.

Quantitative-PCR

Oligo-dTprimed cDNA samples were prepared from total RNA iso-

lated by means of Trizol extraction (Invitrogen, Carlsbad, CA, USA) using Superscript (Invitrogen,Carlsbad, CA, USA). PCR was conducted in triplicate with 20 μL reaction volumes of 1X Taqman buffer (1X Applied Biosystems PCR buffer, 20% glycerol, 2.5% gelatin, 60nM Rox as a passive reference), 5.5 mM $MgCl_2$, 0.5 mM each primer, 0.2 μM each deoxynucleotide triphosphate (dNTP), 200 nM probe, and 0.025 unit/μL AmpliTaq Gold (Applied Biosystems) with 5 ng cDNA. PCR was conducted on the ABI 7900HT (Applied Biosystems) using the following cycle parameters: 1 cycle at 95° for 10 min and 40 cycles at 95° for 15 s, and at 60° for 1 min. Analysis was carried out using the SDS v.2.3 software supplied with the ABI 7900HT to determine the Ct values of each reaction. GapDH (Rodent): Forward TGC ACC ACC AAC TGC TTA G; Reverse GGA TGC AGG GAT GAT GTT C; Probe 5'-FAM- CAG AAG ACT GTG GAT GGC CCC TC-BHQ1-3'. Sfpi1 (Mouse): Forward CGT CCT CGA TAC TCC CAT GGT; Reverse AAG GTT TGA TAA GGG AAG CAC ATC; Probe 5'-FAM-TCA GTC ACC AGG TTT CCT ACA TGC CCC-BHQ1-3'. Further information is available in the *Online Supplementary Appendix.*

Statistical analysis

Ct values were determined for three test and three reference reactions in each sample, averaged, and subtracted to obtain the ΔCt [ΔCt = Ct (test locus) – Ct (control locus)]. PCR efficiencies were measured for all custom assays and were greater than or equal to 90%. Therefore, relative fold difference was calculated for each primer/probe combination as $2^{-\Delta Ct}$ x 100.

Results and Discussion

With the aim of identifying co-operating events with PML-RARα during myeloid leukemogenesis, we first back-crossed *MRP8-PML/RARA* transgenic mice with the BXH-2 mice three times and aged mice for leukemia development. BXH-2 strain mice harbor an endogenous retrovirus that can induce AML.[14] Although latency of disease was similar in BXH-2 mice with and without *MRP8-PML/RARA*, leukemias with features of APL were only observed when PML-RARα was present (*data not shown*). Twenty-two insertion sites were identified in eight leukemias with a promyelocytic morphology; with nine being reported as common insertion sites in the Mouse Retroviral Tagged Cancer Gene Database (RTCGD; *http://rtcgd.ncifcrf.gov*) which included Sox4, Meis1, Lck, Sfpi1, Nfil3 and Cbl-b. We focused our analysis on an insertion identified in one animal in the disease relevant HMG box transcription factor *Sox4*. Previously published sample data sets using Affymetrix Human Genome U133 show significantly increased levels of *Sox4* transcript from APL patients *versus* normal human promyelocytes ($P<0.005$)[5] (*Online Supplementary Figure S1*).

To assess the potential co-operation between PML-RARα and Sox4 we utilized a bone marrow transplant assay using retroviral transduction of *Sox4* into control or *MRP8-PML/RARA* cells. Representative pathology at necropsy from PML-RARα animals is consistent with previous observations,[1] where promyelocytes are observed in marrow cytology with immature cells observed by marrow histopathology (Figure 1A). Marrow cytology (Table 1) and histopathology from PML-RARα + Sox4 mice showed more differentiated cells, predominantly with features of metamyelocytes (Figure 1B). Cells appeared most blastic in the leukemias that developed in Control/Sox4 animals (Figure 1C). Leukemic infiltration into other tissues including spleen, liver and lymph nodes was also detected in leukemic animals in all three groups (Figure 1A-C and *data not shown*). Mice that received cells with both PML-RARα
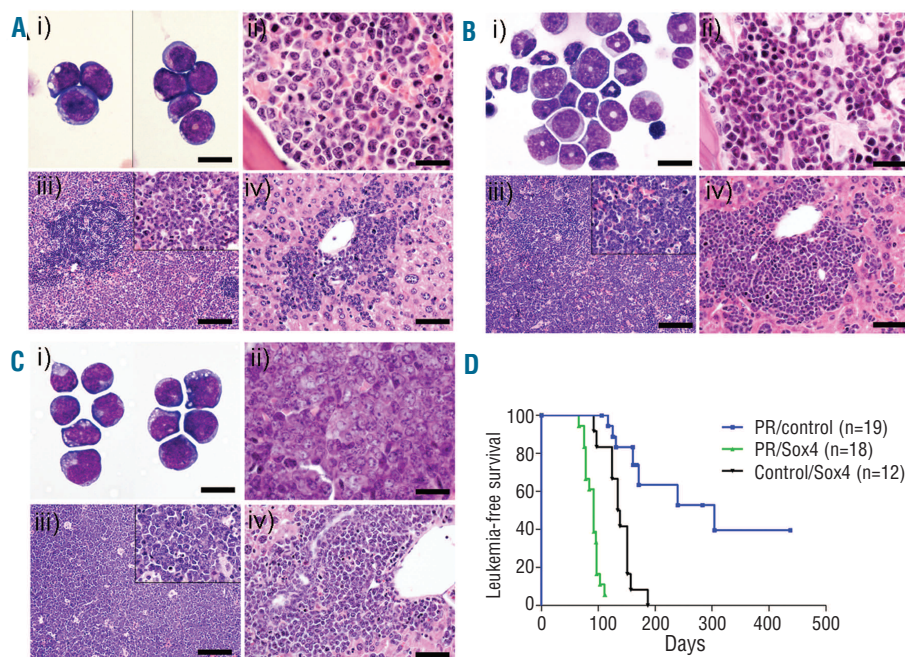

Figure 1. Sox4 co-operates with PML-RARα to induce AML. Representative pathology of: (i) marrow cytology, (ii) marrow histopathology, (iii) spleen (or lymph node in the case of Sox4) and (iv) and liver for **(A)** PML-RARα, **(B)** PML-RARα + Sox4 and **(C)** Sox4 mice. Although all groups represent similar degrees of organ infiltration of blast cells, PML-RARα + Sox4 group show a more pronounced involvement of metamyelocytes. Scale bar represents 13.3 micrometers for marrow cytology, 20 micrometers for marrow histopathology, 100 micrometers for spleen/lymph node and 40 micrometers for spleen/lymph node inset, and 50 micrometers for liver. Marrow cytology: Wright's Giemsa stain. Histopathology: Hematoxylin & Eosin. **(D)** Kaplan-Meier survival curves of animals in days from transplant censored for non-leukemic deaths with similar incidences between the groups. These represent significantly reduced survival in PML-RARα + Sox4 mice as compared to the PML-RARα or Sox4 alone animals (*P<0.0001*). Non-leukemic deaths were at 116 days (euthanized due to fighting), 161 days (T-cell lymphoma phenotype), 293 days (lung tumor) and 431 days (euthanized due to age without disease).

and Sox4 had consistently increased white blood cell counts (*Online Supplementary Figure S2*) but with similar immunophenotypic profiles as compared to single expressors (using the Mac-1/Gr-1/c-Kit/CD34 markers; *data not shown*) and increased leukemia-associated mortality, with median survival of 92 days as compared to 136 days or 304 days in mice with Sox4 or PML-RARα alone, respectively (Figure 1D). Interestingly, peripheral platelet counts were somewhat higher in PML-RARα + Sox4 leukemic animals as compared to PML-RARα alone leukemic mice, suggesting that thrombocytopenia does not track the course of the leukemic progression in the PML-RARα+ Sox4 model (*Online Supplementary Figure S2*). Additionally, interpretable SKY analysis of leukemia cells from 7 PML-RARα + Sox4 animals revealed 3 with no clonal abnormalities, 2 with loss of a sex chromosome only, and 2 with loss of a sex chromosome together with a gain of chromosome 8. While the cytogenetic pattern of leukemia cells from Sox4-only animals were not obtained, the specific karyotypic abnormalities seen in PML-RARα + Sox4 animals were consistent with that of our previously published observations from the PML-RARα animals with a high frequency of loss of a sex chromosome (57% *vs.* 55%); however the overall frequency of abnormalities appeared reduced in the PML-RARα + Sox4 cohort (57% *vs.* 93%)[3] (*Online Supplementary Table S1*). Our findings indicate that although the combination of Sox4 and PML-RARα is neither sufficient to complete leukemogenesis nor able to fundamentally change the karyotypic pathway to progression, the proteins can work co-operatively to strongly initiate leukemic transformation.

The fact that PML-RARα + Sox4 leads to myeloid leukemia is conceptually consistent with observations of co-operation between transcriptions factors, such as Sox4/PU.1,[16] Sox4/CREB,[17] Sox4/Evi1,[10] Hoxa9/Meis1a[18] and that of PML-RARα/Myc[426]. PML-RARα can promote increased proliferation of hematopoietic progenitors[19] and Sox4 can enhance proliferation and decrease differentiation.[10,20,21] Co-operative expansion of myeloid progenitors by these proteins might provide a platform for the cumulative acquisition of additional genetic changes that complete leukemic transformation. Interestingly, in the context of another myeloid leukemia model, Sox4 has been shown to transactivate the AKV LTR and up-regulate provirally activated *Evi1*.[10] The cumulative impact of transcription factor changes in this model and in our model is the dysregulation of differentiation program and interference with the normal control of proliferation and survival.

Our analyses of published array data concur with the recently described inverse correlation between SOX4 and PU.1 (encoded by the *SPI1* gene) in human AMLs[15,17] (*data not shown*). However, when APL patient samples were specifically analyzed, the relationship is less robust and a range of *SPI1* expression levels was observed even when there was significant expression of *SOX4* (*Online Supplementary Figure S3*). Furthermore, in the context of our *in vivo* mouse model, exogenous expression of Sox4 on the background of PML-RARα was associated with a relative increase in *Sfpi1* levels, the murine homolog of *SPI1* (*Online Supplementary Figure S4*). Hence, although downregulation of *SPI1* levels by SOX4 appears to be one mechanism by which SOX4 contributes to AML, this does not appear predominant in APL: relatively higher rather than lower *SPI1/Sfpi1* levels are observed both in a subset of human APLs and in our murine model. Given the observations that PML-RARα + Sox4 leukemias showed greater neutrophilic

Table 1. Differential cell counts of Wright's Giemsa stained bone marrow cells from PML-RARα[24] and PML-RARα + Sox4 leukemias.

	PML-RARα	PML-RARα + Sox4
Blast + Pro	81.3±8.1	39.2±19.6
Imm neut	4.5±2.6	38.6±13.5
Mat neut	0.7±0.7	12.0±5.3
Erythroid	8.7±7.7	7.2±4.9
Lymphocytes	4.5±2.2	1.6±1.2
Eosinophils	0.3±0.3	1.4±2.3

Percentages of nucleated cells were derived from 200-400 cell counts, with data shown as arithmetic means±SD. Blast + pro: blasts and promyelocytes. Imm neut: neutrophilic myelocytes and metamyelocytes; Mat neut: neutrophilic band, mature ring, and polymorphonuclear forms.
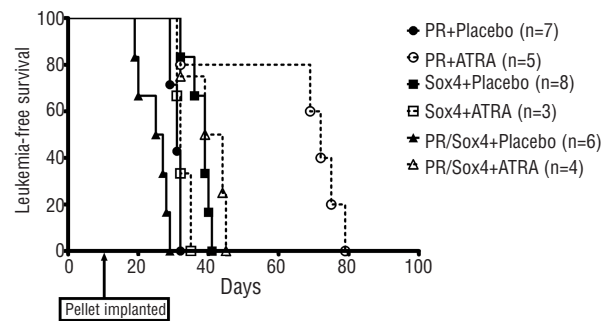

Figure 2. ATRA is able to extend survival of PML-RARα and PML-RARα + Sox4 secondary transplanted mice. Kaplan-Meier survival curves of secondary transplanted animals with placebo or ATRA pellet implantation. Compared to their respective placebo controls, ATRA is able to significantly extend survival in PML-RARα mice (2.3-fold difference in median survival; *P*=0.0052) as well as in PML-RARα + Sox4 mice (1.6-fold difference in median survival; *P*=0.0038).

differentiation than PML-RARα leukemias (Table 1), it was possible that the higher *Sfpi1* levels seen in a subset of these leukemias (*Online Supplementary Figure S4*) reflected this increased maturation. To assess this possibility PML-RARα, Sox4, and PML-RARα + Sox4 leukemias were transplanted into secondary recipients and *Sfpi1* levels were assessed in both leukemic spleens and in sorted promyelocytes. *Sfpi1* levels in all three leukemias were higher than in normal promyelocytes and *Sfpi1* appeared enriched in sorted promyelocytes as compared to unsorted leukemic spleens for both PML-RARα and PML-RARα + Sox4 leukemias (*Online Supplementary Figure S5*). The increased *Sfpi1* in sorted promyelocytes compared to total leukemic cells suggests that the high levels of Sfpi1 in a subset of PML-RARα + Sox4 leukemias do not simply reflect the presence of maturing neutrophilic cells. Hence, the range of *SPI1/Sfpi1* levels measured in human and mouse APL (*Online Supplementary Figures S3 and S4*) appears to reflect true biological variation. Interestingly, for the PML-RARα + Sox4 leukemia studied in *Online Supplementary Figure S5*, *Sfpi1* levels were reduced relative to the PML-RARα alone cells, consistent with previous observations.[16] Altogether, our findings support the idea that inhibition of *SPI1/Sfpi1* expression plays a role in some, but not all, SOX4 expressing leukemias.

Increased expression of SOX4 has been reported in

many cancers including medulloblastoma, as well as cancers of the prostate, bladder, colon and lung (non-small cell).[22] Our models described here provide robust systems in which gene expression profile analyses may reveal additional PML-RARα targets alone or in combination with Sox4 and as comparators to human APL. Furthermore, our finding that PML-RARα + Sox4 co-operate in leukemogenesis is consistent with the observation that therapeutic levels of retinoic acid result in reduced levels of SOX4 in the context of APL.[23] To further examine the therapeutic potential of ATRA, leukemia-free survival of secondary recipients with placebo or ATRA was determined. Interestingly, ATRA is able to significantly increase the leukemia-free survival of PML-RARα + Sox4, as well as the expected PML-RARα cohort, as compared to their respective placebo treated cohorts (P=0.0038 and P=0.0052). Sox4 alone animals were insensitive to the ATRA treatment (Figure 2). Given the leukemic potency of Sox4 we observed, this protein is an attractive target for therapy of malignancies in which it is over-expressed whose efficacy may be exponentially raised in concert with retinoic acid in the presence of the PML-RARα lesion.

Authorship and Disclosures

Information on authorship, contributions, and financial and other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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