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Extra Views

Dominant Negative Effects of the AML1/ETO Fusion Oncoprotein

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KEY WORDS

AML1/ETO, Runx, Sca1, hematopoietic stem cell

ABBREVIATIONS

AE	AML1/ETO
AML	acute myeloid leukemia
CBF	core binding factor
GFP	green fluorescent protein
HSC	hematopoietic stem cell

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ABSTRACT

The t(8;21)(q22;q22) translocation, present in 10–15% of acute myeloid leukemia (AML) cases results in the production of the AML1/ETO fusion protein. Expression of AML1/ETO in patients or mouse models is not sufficient to induce AML. Despite convincing evidence that AML1/ETO is directly involved in the pathogenesis of AML, the underlying mechanism is not well understood. Genetic and biochemical experiments suggest that AML1/ETO is a dominant inhibitor of the core binding factor (CBF) transcription complex that includes AML1 (RUNX1), the N-terminal fusion partner in the t(8;21). We generated and recently characterized a novel strain of transgenic mice in which the AML1/ETO cDNA was inserted into the *Ly-6A* gene that encodes Sca1, a well-characterized marker of murine hematopoietic stem cells. Unexpectedly, transgene expression assessed by flow cytometry was significantly lower than predicted in lymphocytes from these mice. We have confirmed this finding at the mRNA level and suggest that this phenotype is a consequence of dominant inhibition of transgene expression by AML1/ETO. The dominant negative characteristics of AML1/ETO may be important for AML pathogenesis and may provide a molecular target for therapeutic intervention.

TARGETING THE SCA1 LOCUS TO MODEL t(8;21) AML IN MICE

Acute myelogenous leukemia (AML) accounts for 35% of adult leukemia cases.[1] A distinct subset of these leukemias is characterized by the (8;21) translocation.[2] The t(8;21) fuses the N-terminal 177 amino acids of the transcription factor AML1 (RUNX1) in frame with all but the first 30 amino acids of ETO.[3] AML1 is the DNA-binding subunit of core binding factor (CBF), a multimeric transcription factor complex that includes CBFβ and additional transcriptional coactivators. The AML1/ETO fusion protein retains the Runt homology domain of AML1 (responsible for DNA binding and interaction with CBFβ) and lacks the C-terminal transactivation domain.[4] The role of AML1/ETO in the pathogenesis of AML is not yet well understood.

The t(8;21) is detectable years prior to the onset of AML and can persist for years in remission bone marrow samples.[5-7] Similarly, several independently generated murine transgenic strains in which AML1/ETO is expressed in adult bone marrow cells fail to develop spontaneous leukemias.[8-12] Taken together, these observations suggest that AML1/ETO is not sufficient to induce AML. The t(8;21) is likely an early event in the pathogenesis of AML. AML1/ETO appears to act on primitive stem/progenitor cells, promoting self-renewal over differentiation.[10,13] This may contribute to leukemogenesis by increasing the pool of cells available to acquire additional mutations.

The leukemia-initiating cell in t(8;21) AML has a primitive stem/progenitor phenotype.[14,15] We, therefore, hypothesized that targeting AML1/ETO expression to HSC might be important for experimental models of this disease. We chose to utilize the *Ly-6A* (*Sca1*) locus because we demonstrated previously that this strategy yields high-level transgene expression in the hematopoietic stem/progenitor compartment in adult mice.[16]

In contrast to germline $Aml1^{+/AE}$ mice, $Sca^{+/AE}$ mice are viable and develop normally.[17] Hematopoiesis is normal at baseline, apart from a modest elevation of the white blood cell count and a decrease in the frequency of myeloid progenitors. After a long latency, more than 80% of the mice develop a myeloproliferative disorder characterized by elevation of the neutrophil count, bone marrow myeloid hyperplasia, splenomegaly, and extramedullary hematopoiesis.[17] As noted with previous AML1/ETO mouse models, these animals do not develop AML spontaneously. Nevertheless, this model should provide a useful platform to study the biology of AML1/ETO and to evaluate the potential of additional mutations to cooperate with AML1/ ETO in the induction of AML.

Inclusion of an *ires* EGFP reporter cassette in the targeting vector allowed us to monitor transgene expression in $Sca^{+/AE}$ mutant mice by flow cytometry using GFP as a surrogate for AML1/ETO. Identically targeted $Sca^{+/GFP}$ mice controlled for GFP expression and integration effects at the *Sca1* locus.[16] The transgene is highly expressed in mature myeloid cells from both strains.[17] Unexpectedly, transgene expression is significantly lower in B cells, immature thymocytes, and peripheral CD4+ T cells in $Sca^{+/AE}$ mice compared to $Sca^{+/GFP}$ mice.[17]

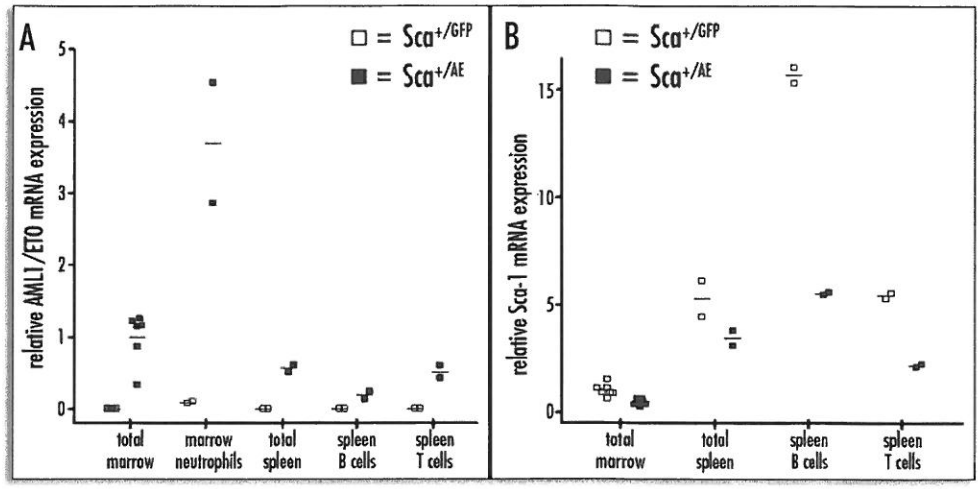

Figure 1. Real-time PCR analysis of gene expression. (A) *AML1/ETO* mRNA expression in cells from $Sca^{+/AE}$ mice (filled symbols) is shown. Expression is significantly higher in neutrophils compared to T and B cells. No background signal is detectable using mRNA from $Sca^{+/GFP}$ cells (open symbols). Each point represents mRNA expression in a sample from an individual animal measured in triplicate and averaged. (B) *Sca1* expression is shown for the indicated cellular populations. *Sca1* expression is significantly lower in bone marrow, B cells, and T cells from $Sca^{+/AE}$ mice compared to $Sca^{+/GFP}$ mice ($p<0.005$).

ROLE OF AML1 IN HEMATOPOIETIC DEVELOPMENT

Aml1 is expressed in functional HSCs[18] and is required for specification of adult definitive hematopoiesis.[19,20] In contrast to this early embryonic requirement, Aml1 is not necessary for maintenance of HSC in the adult mouse.[21] Interestingly, *Aml1* haploinsufficiency leads to a 50% reduction in long-term repopulating HSCs (LTR-HSCs), with a concomitant increase in multilineage committed progenitors, suggesting that this compartment is highly sensitive to *Aml1* gene dosage.[22] Conditional *Aml1* deletion revealed an absolute requirement for CBF function in normal lymphocyte and platelet development.[21] In contrast, Aml1 is apparently dispensable for myelomonocytic differentiation[21] despite high level expression in these lineages.[23] Overexpression of dominant Runx inhibitors can impair granulocytic differentiation,[24-26] raising the possibility that other Runx family members (Aml2 or Aml3) may compensate for the absence of Aml1 in the myeloid lineage.

Given the body of evidence indicating an important role for Aml1 in lymphocyte development, we hypothesized that the unexpectedly low level transgene expression we detected in lymphocytes from $Sca^{+/AE}$ mice might result from deleterious effects of AML1/ETO on hematopoietic lineages in which Runx factors are essential. Since our previous analysis relied on a reporter gene, we decided to measure AML1/ETO mRNA expression directly in purified cells from $Sca^{+/AE}$ mice using real-time quantitative RT-PCR. We found that AML1/ETO mRNA is expressed at relatively high levels in neutrophils, and at reduced levels in lymphocytes (Fig. 1A). This reduction in AML1/ETO mRNA is particularly apparent in B cells. These data mirror our flow cytometric analysis.[17] Since Sca1 protein expression assessed by flow cytometry is not proportionately reduced in these cells (not shown), these results suggested that the *AML1/ETO* mutant allele might be selectively downregulated. To test this hypothesis, we analyzed *Sca1* mRNA expression by quantitative RT-PCR. In contrast to the results from flow cytometric analysis, we found that *Sca1* mRNA expression is significantly decreased in whole bone marrow, B cells, and T cells in $Sca^{+/AE}$ mice compared to $Sca^{+/GFP}$ mice (Fig. 1B). These results suggest that AML1/ETO is acting in *trans* to reduce expression of both the wildtype and targeted *Sca1* alleles.

AML1/ETO IS A DOMINANT INHIBITOR OF RUNX FUNCTION

Mice heterozygous for AML1/ETO targeted to the *Aml1* locus are phenotypically identical to Aml1 and Cbfβ deficient mice,[19,20,27,28] providing genetic evidence that AML1/ETO is a dominant inhibitor of CBF function.[29,30] Direct biochemical evidence for a dominant negative effect of AML1/ETO came from cotransfection experiments showing that AML1/ETO can block AML1-dependent transcriptional activation,[4] and that this property of AML1/ETO is dependent on the C-terminus of ETO.[31] AML1/ETO recruits histone deacetylases and the corepressors mSin3A and N-CoR, providing the basis for the prevailing model of dominant inhibition in which the CBF transcriptional activator is converted into a repressor.[32-35] Tetramerization of AML1/ETO increases its affinity for DNA, thereby conferring a competitive advantage over the wildtype AML1 protein.[36] In an alternate model, changes in the pattern of transcription caused by AML1/ETO result from misdirection of the fusion protein to transcriptionally inactive subnuclear domains.[37]

We identified three consensus Runx sites in the 5' flanking region of the *Ly-6A* (*Sca1*) gene, located 0.8, 1.0 and 1.5 kb upstream of exon 1. We propose that *Sca1* transcriptional activity may be regulated by CBF, and that this provides a potential mechanism to explain the observed decrease in *Sca1* mRNA in $Sca^{+/AE}$ mice compared with $Sca^{+/GFP}$ mice. In this model, AML1/ETO represses transcription from both the wildtype and mutant *Sca1* alleles in $Sca^{+/AE}$ mice (Fig. 2).

CONCLUSIONS AND IMPLICATIONS

Sca1 transcriptional activity is strongly activated by types I and II interferon.[38] This may be an adaptive response to cytokines produced during an immune response, since Sca1 is required for normal regulation of antigen-induced T cell proliferation.[39] We have shown that the targeted allele remains interferon-responsive in *Sca1* mutant mice.[16] The data presented here imply that *Sca1* may also be a Runx-regulated

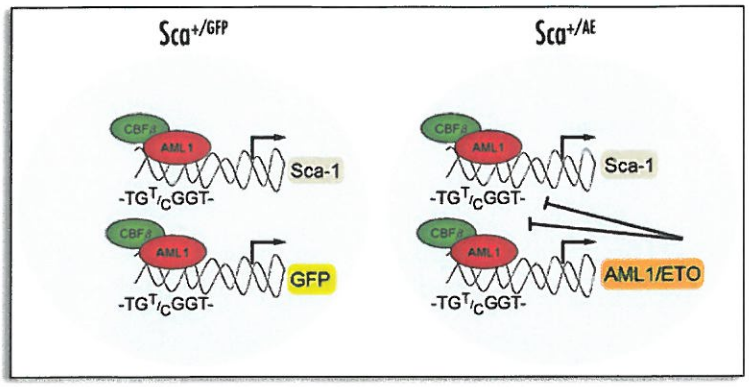

Figure 2. Model of dominant inhibition of *Sca1* by AML1/ETO. The wildtype and targeted *Sca1* alleles in heterozygous $Sca^{+/GFP}$ (left) and $Sca^{+/AE}$ (right) cells are depicted. There are three consensus Runx binding sites in the proximal *Sca1* promoter. Expression of GFP in $Sca^{+/GFP}$ mice does not affect transcription of either the wildtype or mutant alleles. In the proposed model, AML1/ETO expression in $Sca^{+/AE}$ mice represses transcription from both the wildtype and mutant alleles.

gene. Formal proof would require direct demonstration that Runx factors bind the *Sca1* promoter (e.g., by chromatin immunoprecipitation) and alter transcriptional activity (e.g., by promoter assays). An additional implication of these data is that the $Sca^{+/AE}$ mutation amounts to a Sca1 knockdown (through the combined effects of haploinsufficiency and dominant inhibition of the remaining wildtype allele). This introduces a potential caveat in the interpretation of hematopoietic phenotypes observed using the $Sca^{+/AE}$ mice. We and others have found that resting hematopoiesis is intact in Sca1 null mice (Ito et al.[40] and T.A.G., unpublished results). However, serial transplantation revealed a potential role for Sca1 in hematopoietic stem cell self-renewal,[40] raising the possibility that AML1/ETO could have pleiotropic effects on this compartment in $Sca^{+/AE}$ mice.

The dominant negative effects of AML1/ETO are most evident in lymphocytes because Runx factors are specifically required for the development of these lineages. In conditional $Aml1^{+/AE}$ mice, AML1/ETO-expressing lymphocytes are selectively lost.[8] Since $Sca^{+/AE}$ mice are not mosaic, all lymphocytes contain the transgene and compensatory mechanisms must select for low AML1/ETO-expressing cells. Although transgene expression is lower in lymphocytes from $Sca^{+/AE}$ mice, we have shown that their T and B subsets are present in normal numbers.[17] None of the AML1/ETO mouse models are overtly immune deficient. It remains formally possible that subtle immune defect exist in patients and mice expressing AML1/ETO, although this has not been reported.

The germline $Aml1^{+/AE}$ mutation is embryonic lethal, apparently because Runx activity is lowered below a critical threshold through the combined effects of *Aml1* haploinsufficiency and dominant inhibition by AML1/ETO. Using *Sca1* as a reporter gene, we found that AML1/ETO represses Runx activity between 54% (bone marrow) and 65% (B cells) in $Sca^{+/AE}$ mice (Fig. 1B). Since these mice are wildtype at the *Aml1* locus, this may explain in part why this is not an embryonic lethal mutation. Although our model captures the dominant negative effects of AML1/ETO, to fully model the milieu in t(8;21) leukemic cells, it may be necessary to lower Aml1 (and perhaps Eto) activity further.

Although AML1/ETO expression is not sufficient to induce AML, continued expression may be required for persistence of leukemia. Even in genetically complex tumors, inactivation of a critical oncogene can cause regression by inducing proliferation arrest, differentiation, or apoptosis.[41] Therefore, AML1/ETO may provide a relevant target for novel AML therapeutics. Proposed strategies include redirecting transcriptional repression mediated by AML1/ETO,[42] inhibition of histone deacetylases recruited by the fusion protein,[43] or targeting the AML1/ETO fusion directly via RNA interference.[44]

MATERIALS AND METHODS

Cell sorting. Bone marrow cells and splenocytes were harvested from 6-10 week old $Sca^{+/GFP}$ and $Sca^{+/AE}$ mice. After red cell lysis in hypotonic buffer (150 mM NH_4Cl, 100 mM $KHCO_3$, 0.1 mM EDTA), cells were washed and resuspended in FACS buffer (PBS with 0.2% BSA and 0.01% sodium azide). Cells were counted and viability assessed by trypan blue exclusion. Bone marrow cells and splenocytes were stained with directly conjugated anti-B220, CD3, CD11b, or Gr-1 antibodies (eBioscience). Approximately 2–3 x 10^6 B220 + CD3 - (B cells), 2–3 x 10^6 B220 - CD3+ (T cells), and 4–6 x 10^6 CD11b + Gr - 1 + (neutrophils) were sorted using a MoFlo cell sorter (DAKO Cytomation). Post-sort analysis confirmed high purity (>99%) and viability (>90%).

RNA preparation and cDNA synthesis. Total RNA was isolated from freshly sorted cells using the High Pure RNA Isolation kit (Roche). RNA was quantitated using either the Ribogreen reagent (Molecular Probes), or a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Random hexamer-primed cDNA was synthesized using AMV reverse transcriptase (Promega).

Real-time PCR. Mouse *GAPDH*, human *AML1/ETO*, and *Sca1* cDNAs were amplified and quantitated using dual-labeled fluorescent internal probes. Between 1–10% of the cDNA preparation was added to a 25 uL reaction containing 1X JumpStart Taq ReadyMix (Sigma), 200 nM each primer, 280 nM probe and 1X ROX reference dye (Sigma). Thermal cycling and data collection was performed using a GeneAmp 5700 system (Applied Biosystems). Thermal cycling was as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation (15 seconds) and 60°C annealing/extension (60 seconds). The primer and probe sequences used were as follows: *hAML1/ETO* forward primer 5'-AATCACAGTGGATGGGCCC, *hAML1/ETO* reverse primer 5'-TGCGTCTTCACATCCACAGG, *hAML1/ETO* probe 5'-6FAM- CTGAGAAGCACTCCACAATGCCAGACT-MGBNFQ, *mGAPDH* forward primer 5'-TGCACCACCAACTGCTTAG, *mGAPDH* reverse primer 5'-GGATGCAGGGATGATGTTC, *mGAPDH* probe 5'-HEX-CAGAAGACTGTGGATGGCCCCTC-TAMRA, *Sca1* forward primer 5'-TGGAGATCCTGGGTACTAAGG, *Sca1* reverse primer 5'-AGCTCAGGCTGAACAGAAGCA, and *Sca1* probe 5'-6FAM-CACCTGGACCATGGC-MGBNFQ. The *Sca1* primer set is specific for this gene since no signal was obtained using cDNAs from the other highly homologous Ly-6 family members Ly-6C, Ly-6G or Ly-6M (data not shown). Serial dilutions of calibrator cDNA samples were included in each experiment to allow for quantitation using the relative standard curve method. To correct for variability in mRNA content, mRNA quality, and efficiency of cDNA synthesis, relative GAPDH expression was measured for each sample. The relative mRNA levels for AML1/ETO and Sca1 were then corrected by dividing by the relative GAPDH expression for each sample. Differences between the means were assessed using a 2-tailed t-test.

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