AML1/ETO Proteins Control POU4F1/BRN3A Expression and Function in t(8;21) Acute Myeloid Leukemia

Jenny Dunne1, Duncan M. Gascoyne2, T. Andrew Lister1, Hugh J.M. Brady2, Olaf Heidenreich3, and Bryan D. Young1

Abstract

A variety of genetic lesions, including chromosomal translocations, internal tandem duplications, and mutations, have been described in acute myeloid leukemia (AML). Expression profiling has shown that chromosomal translocations, in particular, are associated with distinctive patterns of gene expression. AML exhibiting the translocation t(8;21), which fuses the AML1 and ETO genes, has such a characteristic expression profile. One gene whose expression is highly correlated with the presence of the AML1/ETO fusion is POU4F1, which encodes the POU homeodomain transcription factor BRN3A. Here we show using specific siRNA in t(8;21) cells and overexpression studies in progenitor cells that AML1/ETO promotes expression of POU4F1/BRN3A. This effect requires DNA-binding function of AML1/ETO, and accordingly, AML1/ETO is bound to the POU4F1 locus in t(8;21) cells. Functionally, whereas overexpression of Brn3a in murine hematopoietic progenitor cells induces terminal myeloid differentiation, coexpression of AML1/ETO or AML1/ETO9a blocks this effect. Furthermore, Brn3a reduction by shRNA impairs AML1/ETO-induced immortalization of murine progenitors. In summary, we identify POU4F1/BRN3A as a novel potential upregulated AML1/ETO target gene whose dramatically high expression may cooperate with AML1/ETO in t(8;21) cells. Cancer Res 70(10): 3985–95. ©2010 AACR.

Introduction

Gene expression profiling by microarray has identified many genes whose unusually high or low expression shows specificity for a particular cancer type or subtype (1–3). In acute myeloid leukemias (AML), the common chromosome translocations have each been associated with distinctive gene expression patterns. However, the specific relationships between particular gene fusions and many of their associated genes remain obscure.

The t(8;21) translocation fuses the coding sequence of the AML1 gene to that of ETO, producing the chimeric transcription factor AML1/ETO, also called AML1/MTG8 or RUNX1/ETO. In t(8;21) samples, the AML1/ETO fusion is associated with increased expression of genes such as CSF1R and RUNX1T1 (4, 5). Overexpression of AML1/ETO is sufficient to promote self-renewal of both murine and human myeloid progenitor cells in vitro (6–9), and siRNA studies show a continuing requirement for AML1/ETO in maintenance of proliferation and inhibition of terminal differentiation (10–12). Expression of AML1/ETO is not sufficient to generate overt leukemia in vivo (6, 8, 13, 14), suggesting that t(8;21) leukemia requires "second hit" alterations in proliferation-associated pathways such as those of the c-KIT and FLT3 growth factor receptors (5, 15, 16). Additional less-well-characterized splice variants of AML1/ETO such as the oncogenic AML1/ETO9a may also play a role in t(8;21) AML (17).

AML1/ETO retains the AML1 DNA-binding domain and the repressor domain of ETO, and thus has some function by aberrantly repressing transcription (5). Indeed, AML1/ETO binds directly to transcriptional repression machinery and represses expression of genes such as CSF1R (also known as MCSFR or c-FMS) and p14ARF (18, 19). However, mostly as a result of gene expression profiling, it is now becoming clear that whereas AML1/ETO can repress gene expression, it is perhaps also capable of activating gene expression (11, 20–23).

Expression profiling in AML has identified POU4F1 to be highly expressed in t(8;21) samples (1–3). Specificity for t(8;21) is such that high POU4F1 expression can assist positive identification of those carrying this translocation (1). However, functions of the murine Pou4f1 gene product Brn3a have been studied almost exclusively in the nervous system where it promotes neuronal development and survival (24–27). Potential function of Brn3a/BRN3A in hematopoietic cells is presently unknown.

Here we show that expression of POU4F1 is promoted by AML1/ETO proteins, and that DNA binding of AML1/ETO,
which occurs at AML1 binding sites in the POU4F1 locus, is required for this effect. Functionally, overexpression of Brn3a in murine hematopoietic progenitor cells results almost exclusively in terminal macrophage differentiation. In contrast, AML1/ETO proteins inhibit and possibly even reverse this effect, with Brn3a being required for AML1/ETO-dependent growth. Thus, AML1/ETO and BRN3A seem to cooperatively inhibit differentiation and promote growth in t(8;21) AML.

Materials and Methods

Patient samples and cell culture. Leukemia samples at presentation were obtained with informed consent from adult patients at St Bartholomew’s Hospital, and mononuclear cells purified by standard techniques. Primary AML blasts and t(8;21) positive Kasumi-1 cells (DSMZ ACC 220, ref 28) were cultured as described previously (11).

Plasmid construction. To generate pMSCV-3a-neo, murine Brn3a(L) cDNA was inserted into pMSCVneo (Clontech) between EcoRI and BgIII sites. Brn3a(L) was inserted between EcoRI and NoI sites of a modified pMSC-hCD2tailless vector (29) to generate pMSCV-3a-hCD2. To generate Brn3ASHRNA plasmids, double-stranded oligonucleotides were cloned into pMSCV-hCD2tailless-miR30 vector pM2miR (30), generating a fused hCD2-miR transcript. Targeting sequence for sh24 is 5'-CGCATTGAAACTGAGCACTAAA-3, and for sh43, 5'-AGCCGAGACCGTGCATTTAATG-3. pMiG-AML1/ETO, pMiG-AML1/ETO9a, and derived mutant plasmids (17, 31) were a kind gift of Dong-Er Zhang (Department of Pathology, University of California San Diego, La Jolla, CA).

Retroviral infection and culture of hematopoietic progenitors. Progenitor cells were purified from day E12.5 or E13.5 murine fetal livers by Ter119 depletion and c-Kit positive selection as per manufacturer’s instructions (Miltenyi). Cells were cultured in DMEM containing 10% FCS (Stem Cell Technologies; with 10 ng/mL granulocyte macrophage colony-stimulating factor) 72 hours after isolation according to the manufacturer’s protocols, and replatings performed at 6- to 8-day intervals. Primary AML blasts and Kasumi-1 cells were cultured as described previously (11).

siRNA transfection. Cells were electroporated with 100 nmol/L siRNA oligonucleotides as described previously (10, 11). Transfection efficiency was >95% as assessed by electroporation of a Cy3-labeled siLaminA oligonucleotide pair into control cells. Oligonucleotide pairs or commercial reagents used for targeting are described in Supplementary Table S1.

RNA and protein isolation. Total RNA was isolated from cultured cells using RNeasy columns (Qiagen). Protein was isolated from flow-through fractions as previously (11). RNA, DNA, and protein were extracted from patient samples using TRIzol (Invitrogen) as per manufacturer’s instructions.

Protein samples were dissolved in 9 mol/L urea, 1% (w/w) DTT.

Real-time mRNA analysis. cDNA synthesized from 33 ng of RNA using M-MLV Reverse Transcriptase RNase H minus point mutant (Promega) was used per reaction with either Universal or SYBR Green master mix (Applied Biosystems). Oligonucleotide sequences (Sigma-Genosys) or commercial products used are described in Supplementary Table S1. Normalized mean gene expression values ± SD were determined from triplicate cycle threshold (Ct) values for each gene and the housekeeping gene 18S or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative transcript levels were determined by the 2-ΔΔCt method.

Immunoblot analysis. Cell lysates (20–50 μg) were separated by SDS-PAGE, transferred onto membranes, blocked, and subjected to overnight primary antibody incubation with anti-ETO C-20 (Santa Cruz), anti-Brn3a MAB1585 (Chemicon), anti-β-actin (Sigma), anti-GAPDH, anti-β-tubulin, or anti-hemagglutinin (anti-α; Cell Signaling). Expression was quantitated using a GS800 densitometer and QuantityOne software (Bio-Rad).

Electrophoretic mobility shift assay analysis. Whole-cell lysates from Kasumi-1 or 293T cells were incubated with either cold oligonucleotide or specific antisera recognizing AML1 (Ab-1, Calbiochem) or ETO (C-20) before a 20-minute incubation with [32P]dCTP radiolabeled double-stranded oligonucleotides. Complexes were resolved in 5% acrylamide nondenaturing gels. Oligonucleotides are detailed in Supplementary Table S1.

Fluorescence-activated cell sorting analysis. Cells were incubated with either cold oligonucleotide or specific antisera recognizing AML1 (Ab-1, Calbiochem) or ETO (C-20) before a 20-minute incubation with [32P]dCTP radiolabeled double-stranded oligonucleotides. Complexes were resolved in 5% acrylamide nondenaturing gels. Oligonucleotides are detailed in Supplementary Table S1.

Immunofluorescence. Cells were cytospun, paraformaldehyde-fixed, rinsed twice in PBS, and then permeabilized with 0.1% (v/v) Triton X-100 in PBS before incubation in PBS containing 2% FCS, 0.1% bovine serum albumin (BSA), 0.1% sodium azide with isotype controls or specific antibodies (antihuman CD117-PE 555714 (BD), anticyCD11b, CD117, F4/80, CD61, and/or antihumanCD2 (all eBioscience). Data were acquired using LSR (Becton Dickinson) or CyAnADP (DakoCytomation) analyzers. Cell sorting was done using a MoFloXDP (Beckman Coulter).

Chromatin immunoprecipitation. This was performed by a modification of the method of Ivins and colleagues (34). Cells (5 × 10^6) were incubated with 1% formaldehyde in growth medium for 10 minutes at 37°C, washed, and then lysed in 0.5 mL of lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris (pH 8.0), 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin 1 μg/mL leupeptin] for 10 minutes.
on ice. After sonication, 10% lysate was removed as input and the remainder diluted in immunoprecipitation dilution buffer [0.01% SDS, 0.01% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris (pH 8.0), plus protease inhibitors]. Lysate was divided equally and incubated with 1 μg of test or control antibodies: normal goat IgG, α-ETO C-20, α-AML1 (Ab-1), or α-AML1 (Ab-2; Calbiochem); the latter does not recognize AML1 when DNA bound and is a negative control. Precipitated complexes were cross-link reversed and proteinase K digested, and purified DNA was subjected to real-time PCR using oligonucleotide primers and probes (Supplementary Table S1). Quantitation by the 2^−ΔΔCT method was done as per manufacturer’s protocol (Applied Biosystems) comparing input to test samples, with results displayed as ratio of test to control antibody signals for each cell type.

Statistical analyses. Comparisons to determine statistical significance of differences were done using two-sided Student’s t test, either paired or unpaired as appropriate.
Results

Expression of BRN3A is extremely high and nuclear in t(8;21) patient cells. Global gene expression profiling of AML has identified high expression of POU4F1/BRN3A to be correlated closely with presence of t(8;21) translocation (1–3). To confirm high BRN3A expression in primary t(8;21) cells, we performed real-time PCR and immunoblot and immunofluorescence analyses (Fig. 1A–C). Real-time PCR verified that both bone marrow and peripheral blood t(8;21) samples exhibit very high BRN3A expression in comparison with other AML samples (Fig. 1A). Immunoblot and immunofluorescence analyses using two independent antisera confirmed that BRN3A protein is expressed abundantly in t(8;21) primary cells (Fig. 1B and C), with the long isoform BRN3A(L) being predominant. Importantly, expression of BRN3A is nuclear and exhibited by cells also expressing AML1/ETO as detected by α-ETO immunofluorescence (Fig. 1C). In the t(8;21) cell lines Kasumi-1 and SKNO-1, there is significant BRN3A expression; however, this is 50- to 100-fold reduced in comparison with primary t(8;21) samples and similar to that in other cell lines (Fig. 1D).

AML1/ETO proteins promote BRN3A expression in t(8;21) AML cells. All primary adult t(8;21) samples studied thus far exhibit high BRN3A expression (1), and this correlation may result from direct regulation of BRN3A by AML1/ETO. To test this hypothesis, expression of AML1/ETO in primary t(8;21) patient cells was reduced by siRNA, and the effect on endogenous BRN3A transcript levels determined. As observed previously (10, 11), AML1/ETO expression was reduced efficiently by this method (Fig. 2A). Loss of AML1/ETO significantly reduced BRN3A expression (Fig. 2A and B), indicating
BRN3A as a potential AML1/ETO target. Similar AML1/ETO targeting in t(8;21) Kasumi-1 and SKNO-1 cells also decreased low basal BRN3A expression, but generally less than 2-fold (data not shown), explaining why BRN3A was not identified as an AML1/ETO target in previous cell line microarray screens whose cutoff requirement was 2-fold regulation (11).

The relatively rapid reduction in BRN3A expression following AML1/ETO siRNA treatment suggested a direct relationship between the fusion protein and BRN3A. To determine whether AML1/ETO is sufficient to increase Brn3a expression, AML1/ETO or its shorter variant AML1/ETO9a (17) was introduced into primary murine hematopoietic progenitor cells. Significant downregulation of myeloid differentiation transcripts C/ebpα and Pu.1 verified correct function of AML1/ETO (P ≤ 0.001; Fig. 2C). In contrast, Brn3a transcript levels significantly increased approximately 3-fold 48 hours after either AML1/ETO or AML1/ETO9a transduction (P ≤ 0.001; Fig. 2C). To investigate the mechanism by which AML1/ETO upregulates Brn3a, the effects of specific AML1/ETO9a mutants and wild-type AML on Brn3a expression were examined (Fig. 2D). AML1/ETO9a mutants R174Q, delNHR2, and del(350–428), which disrupt DNA binding, oligomerization, and NCoR interaction, respectively (31), do not upregulate Brn3a expression (P = 0.238, P = 0.403, and P = 0.188; Fig. 2D). In contrast, AML1/ETO9a with delNHR1 mutation, which disrupts E-protein interaction, remains competent to upregulate Brn3a significantly (P = 0.007). Thus, the pattern of Brn3a regulation by AML1/ETO9a mutants parallels their ability to induce rapid leukemia in vivo (31). In an opposite manner, AML1b significantly represses Brn3a expression in this assay (P = 0.036), further supporting direct regulation of Brn3a by AML1-containing proteins.

The AML1 DNA-binding domain is intact in both AML1/ETO and AML1/ETO9a (17); thus, both are likely to bind DNA in a similar, if not identical, manner to AML1. MatInspector screening (35) of the entire BRN3A locus identified three potential sites with varying homology to the AML1 binding consensus (4) in both upstream and downstream regulatory regions (Fig. 3A and B). Although these AML1 binding sites are not globally conserved in the mouse, all are proximal to validated (36) and predicted BRN3A binding sites. Initially, electrophoretic mobility shift assay (EMSA) analyses showed that all three sites can bind to a specific factor present in 293T cells transfected with AML1/ETO but not green fluorescent protein (GFP; Fig. 3C, top). Mutation of AML1 sites in competing unlabeled oligonucleotides prevented competition for binding, demonstrating interaction specificity. The presence of AML1/ETO in these complexes and in similar endogenous complexes in Kasumi-1 cells was confirmed by supershift analysis using both anti-ETO and anti-AML antisera (Fig. 3C, bottom). An additional complex present in all extracts bound specifically to site 2 in a manner dependent on an intact AML1 site but was not supershifted by either antisera.

To examine AML1/ETO binding to the endogenous Pou4f1/BRN3A gene, chromatin immunoprecipitation (ChIP) assays were done using both t(8;21) patient and Kasumi-1 cells. Importantly, both anti-ETO and anti-AML antisera coprecipitated two of the predicted AML1 binding sites in BRN3A, sites 2 and 3, from patient t(8;21) cells (Fig. 3D, left). Binding to the BRN3A locus occurs also in Kasumi-1 cells, although only significantly at sites 1 and 2 (Fig. 3D, right). This altered binding correlates with reduced BRN3A expression (Fig. 1D) and its reduced dependency on AML1/ETO in Kasumi-1 compared with primary t(8;21) AML. In summary, siRNA, EMSA, and ChIP studies of AML1/ETO in t(8;21) cells alongside AML1/ETO overexpression studies in primary cells suggest that BRN3A is a novel direct target of AML1/ETO.

High Brn3a expression in murine progenitor cells promotes myeloid differentiation. AML1/ETO increases Brn3a expression in murine progenitors in vitro; however, resulting expression levels are modest when compared with those in patient samples. Therefore, to investigate the function of high BRN3A expression in hematopoietic cells, we overexpressed Brn3a in multilineage progenitors and monitored lineage phenotypes and methylcellulose replating capacity. Progenitors were transduced with retroviruses encoding Brn3a cDNA under the control of MSCV promoter (Fig. 4A), and infection was monitored using either selection for neomycin resistance or electronic gating for humanCD2tailess (hCD2) expression. Background expression of Brn3a was low in purified progenitor populations (Fig. 2C and D and data not shown), and expression of Brn3a protein from the viruses was confirmed in the packaging cell line LinXE (Fig. 4A).

In contrast to AML1/ETO, whose expression is sufficient to immortalize murine progenitor cells in methylcellulose replating assays (6), overexpression of Brn3a alone inhibited replating capacity (Fig. 4B). Whereas control cultures contained multiple myeloid lineages at the first round of replating, Brn3a-hCD2–transduced cultures were composed almost exclusively of macrophages, with occasional neutrophils (Fig. 4B). Similarly, Brn3a-neo–infected cultures failed to replate and exhibited macrophage morphology (data not shown). Furthermore, real-time PCR analyses showed that ectopic Brn3a increases the expression of myeloid genes Pu.1, Cebpα, and macrophage-specific Mmp12 (Fig. 4C), while decreasing the expression of the neutrophil marker gene Lactoferrin. Fluorescence-activated cell sorting (FACS) analysis showed that ectopic Brn3a decreases CD117/c-Kit and CD61 expression and increases CD11b and F4/80 expression on the cell surface (Fig. 4D). Thus, combined data from replating assays and morphology, gene expression, and surface marker analyses indicate that ectopic Brn3a induces monocyte/macrophage differentiation at the expense of neutrophilic and erythroid differentiation.

AML1/ETO proteins alter Brn3a function in myeloid differentiation. High BRN3A levels in AML occur only in the context of t(8;21)-dependent AML1/ETO expression. Therefore, AML1/ETO was coexpressed alongside Brn3a in murine progenitors by dual infection using GFP and hCD2 markers, respectively. As previously described (6, 8), AML1/ETO alone impaired myeloid differentiation, as determined by reduced CD11b expression at the first round of replating, as did the splice variant AML1/ETO9a [Fig. 5A (top) and B]. AML1/ETO reduced cell number immediately after transduction, consistent with the growth arrest observed in previous studies (8), whereas either AML1/ETO or AML1/ETO9a
Figure 3. AML1/ETO can bind to the POU4F1/BRN3A locus via AML1 sites. A, a schematic of AML1 sites identified close to the BRN3A exons by screening the entire human POU4F1/BRN3A locus (accession AL445209) for potential AML1 and BRN3A binding sites using MatInspector software (35). B, detail of potential binding sites in the BRN3A locus. Numbering relative to translation start is indicated on the left; potential binding sites are underlined; dotted arrows indicate sites of primers used for ChIP analysis; end of exon 2 is indicated in bold capital. C, EMSA analysis of AML1/ETO binding to AML1 sites 1, 2, and 3 in the POU4F1/BRN3A locus. Radiolabeled site 1, 2, and 3 probes as indicated were incubated without lysate (−) or with lysate from GFP-transfected 293T cells (G), AML1/ETO-transfected 293T cells (A), or Kasumi-1 cells (K). Top, reactions were subjected to either no competition (−) or competition with a 50-fold excess of either unlabeled same oligonucleotide (S) or same oligonucleotide with mutant AML1 binding site (M). A potential AML1/ETO complex migrated above a nonspecific complex (triangle) and a specific complex formed only with site 2 probe (asterisk). Bottom, identity of the potential AML1/ETO complex was confirmed by supershift with either an anti-ETO antibody (E) or an anti-AML antibody (A). The faster-migrating site 2–specific complex (asterisk) was unaffected by either antibody. D, left, ChIP analysis of AML1/ETO binding to potential BRN3A sites in t(8;21) patient blasts (expt1 M2, expt2 M1) cultured for 24 h. Negative controls were non-t(8;21) blasts (M4 NK, white columns). Specific signals relative to input are expressed as fold enrichment over signal obtained with total IgG (for anti-ETO) or anti-AML-RHD (for anti-AML). AML1/ETO binding determined by anti-ETO and anti-AML precipitation data combined was significant at site 3 \( (P = 0.046) \), borderline significant at site 2 \( (P = 0.058) \), and not significant at site 1 \( (P = 0.361) \). D, right, Kasumi-1 cells were subjected to ChIP analysis as in A to measure binding of AML1/ETO to the BRN3A locus (gray columns). AML1/ETO binding determined by anti-ETO and anti-AML precipitation data combined was significant at sites 1 and 2 \( (P = 0.012 \) and \( P = 0.004, \) respectively) and not significant at site 3 \( (P = 0.103) \).
alone enabled continued long-term replating and immortalization (data not shown). Loss of CD117/c-Kit and gain of CD11b induced by ectopic Brn3a (Fig. 4D) was not affected by cotransduction with GFP control virus [Fig. 5A (left) and B]. Importantly, coexpression of AML1/ETO or AML1/ETO9a respectively reduced and abolished the ability of Brn3a to induce differentiation. In fact, Brn3a overexpression in the presence of AML1/ETO9a seemed to inhibit differentiation as indicated by increased percentage of c-Kit positivity (Fig. 5b). Morphologic analyses of FACS-sorted Brn3a-hCD2+/GFP + and Brn3a-hCD2+/AML1/ETO9a+ infected populations at round 1 of replating (Fig. 5C) confirmed impairment of Brn3a-dependent differentiation by AML1/ETO9a. Furthermore, gene expression analyses 48 hours post-transduction (Fig. 5D) showed that AML1/ETO9a inhibits Brn3a-dependent activation of Pu.1 and C/ebpα but may cooperate with Brn3a to repress Lactoferrin and activate Mmp12.

Figure 4. High Brn3a expression promotes myeloid differentiation. A, left, retroviral plasmids encoding Brn3a(L) cDNAs under the control of MSCV promoter and coexpressing tailless human CD2 (hCD2) or neomycin resistance marker genes were generated as described in Materials and Methods. A, right, Brn3a overexpression from retroviruses in comparison with pMSCV-neo (control) was confirmed by immunoblotting of packaging cells. B, semisolid colony-forming assays of murine hematopoietic progenitor cells from day E13.5 fetal liver transduced with pMSCV-hCD2 (hCD2) or pMSCV-3a-hCD2 (Brn3a-hCD2) viruses; 1 × 10⁴ FACS-sorted hCD2+ cells were plated into each culture 2 d after infection. Colony number per plate and cell number were monitored at rounds 1 and 2, and cell morphology (×40 magnification) at round 1. C, real-time PCR analysis of gene expression as indicated relative to that of 18s in progenitor cells transduced with pMSCV-neo (control) or pMSCV-3a-neo (Brn3a) and cultured for 6 d under G418 selection. D, cell-surface FACS analysis of cells transduced with pMSCV-neo (gray filled histogram) or pMSCV-3a-neo (black line histogram) viruses as in C performed at day 9 postinfection; data are representative of three independent experiments.
with Brn3a having an important role in immortalization driven by AML1/ETO.

Discussion

The most widely described mechanism of action for AML1/ETO is one of DNA-binding dependent transcriptional repression at AML1 binding sites in genes whose expression is activated by normal AML1 (4). Recent gene expression profiling experiments indicate that AML1/ETO may not only repress a specific set of target genes but also increase the expression of others (11, 20–23). However, although mechanisms for AML1/ETO-dependent transcriptional inhibition have been relatively well characterized (4, 18, 19), the means by which AML1/ETO might directly activate transcription are unclear.

We show that BRN3A expression in t(8;21) cells is maintained by AML1/ETO, that AML1/ETO can upregulate Brn3a expression in a DNA-binding dependent manner, and that AML1/ETO can bind to consensus AML1 TG^1/C-GGT sites in the BRN3A gene. These findings therefore add BRN3A to the short list of genes including TRKA whose expression is upregulated by AML1/ETO via potentially direct means (22). It is most likely that BRN3A has not previously been identified as a potential AML1/ETO target gene due to moderate degree of regulation by AML1/ETO in cell lines and potential tissue-specific requirements for this effect. Previous studies have shown negative autoregulation by Brn3a at its own locus (36), and binding of AML1/ETO adjacent to BRN3A may activate BRN3A expression via a de-repressive effect (38, 39). Our results from AML1/ETO9a mutant studies do suggest that corepressor recruitment to AML1/ETO9a

Figure 5. AML1/ETO proteins inhibit Brn3a function in myeloid differentiation. A and B, FACS analysis of CD11b and CD117 expression at day 3 on progenitors infected at day 0 with pMSCV-GFP (GFP), pMiG-AML1/ETO (AML1/ETO), or pMiG-AML1/ETO9a (AML1/ETO9a) and at day 1 with either pMSCV-hCD2 (hCD2 control) or pMSCV-3a-hCD2 (Brn3a). Graph represents mean percentage of total viable population ± SD from three experiments. Differentiated cell number (c-Kit^−/CD11b^+) relative to progenitor number (c-Kit^+/CD11b^−) was increased in Brn3a/GFP cultures (P = 0.013), not significantly different in Brn3a/AML1ETO cultures (P = 0.386), and decreased in Brn3a/AML1ETO9a cultures (P = 0.010). C, morphology of FACS-sorted GFP^+ hCD2^+ cells. D, real-time PCR analysis of gene expression relative to that of 18s in hCD2^+/GFP^+ sorted progenitors 48 h after dual transduction, relative to hCD2/GFP control.
may be important for Brn3a control. Additionally, upregulation of BRN3A by AML1/ETO in t(8;21) AML may be enhanced by either in vivo factors or possible second-hit pathways (5, 16, 40) in this disease.

Brn3a is sufficient to induce terminal neuronal differentiation when overexpressed in neuronal precursor type cells (24, 27, 41). The present study provides evidence that, in an analogous manner, Brn3a overexpression in hematopoietic progenitors (in the absence of AML1/ETO) promotes terminal monocyte/macrophage differentiation, associated with Pu.1, C/ebpa, and Mmp12 activation and Lactoferrin repression. Candidate Brn3a binding sites in the activated promoters hint at some direct Brn3a-dependent regulation of these important hematopoietic genes, whereas potential inhibition of Brn3a expression by exogenous AML further suggests a role for Brn3a in hematopoiesis.

In the presence of AML1/ETO, Brn3a seems to support immortalization. Previously, the Brn3a long isoform predominant in t(8;21) cells has been described to cooperate with Ras to transform rat fibroblasts (42), and we show here that it may cooperate with AML1/ETO, but not E2A/HLF, to inhibit myeloid differentiation and promote growth of murine progenitor cells. This AML1/ETO-dependent switch of Brn3a function, from inducer to inhibitor of differentiation, provides a parallel with the inhibition of Brn3a neuronal differentiation function by EWS/FLI1 fusion proteins present in Ewing’s sarcoma (41).

Importantly, AML1/ETO prevents Brn3a-induced Pu.1 and C/ebpa activation while potentially coactivating Mmp12. There are several possibilities regarding the mechanisms for these phenomena. First, AML1/ETO could bind Brn3a directly, but despite interaction in pulldown assays, the two proteins do not extensively colocalize in t(8;21) cells. Possibly adjacent AML1 and Brn3a binding sites in Mmp12 facilitate interaction and cooperative target regulation. In contrast, AML1/ETO is likely to control Brn3a function at Pu.1 and C/ebpα promoters (where adjacent binding sites seem to be absent) via altered cofactor recruitment or changes in promoter structure. Inhibitory interaction of Brn3a with HIPK2 (43), which is mutated in

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Figure 6. Brn3a is required for AML1/ETO-dependent immortalization of murine progenitors. A, real-time PCR analysis of Brn3a expression relative to that of 18s in unsorted progenitors 48 h after transduction to ~80% efficiency with nontargeting pM2miR-based retrovirus (Con) or viruses designed to target Brn3a (21, 24, 38, and 43). B, representative FACS analysis of hCD2 and GFP positivity in murine progenitors 48 h (postinfection) after simultaneous transduction with pM2miR (shcon, sh24, or sh43) alongside pMIG-AML1/ETO (GFP) to 60% to 70% double-positive efficiency. Positivity was monitored during subsequent semi-solid methylcellulose culture (round 3) and liquid culture (day 16). C, representative hCD2+ in AML1/ETO and E2A/HLF cultures cotransduced with pM2miR viruses, monitored 48 h after transduction (48R) at rounds 1 to 3 of semi-solid culture replating (r1–r3) and at days 1, 9, and 16 of subsequent liquid culture (d1, d9, d16). D, numbers of viable hCD2+/GFP+ cells during suspension culture.

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* D.M. Gascoyne, unpublished data.
some AML (44), may also modulate Brn3a function in t(8;21) cells. The ability of both AML1/ETO and Brn3a to upregulate TrkA growth factor expression (22, 45) may also be relevant in t(8;21) AML pathogenesis.

In summary, AML1/ETO contributes to both high expression and aberrant function of Brn3a in t(8;21) cells. Identification of specific pathways and target genes regulated by the AML1/ETO and BRN3A combination and additional factors controlling Brn3a expression should provide insight into t(8;21) AML pathogenesis. Reactivation of BRN3A pro-differentiation function by inhibition of AML1/ETO and/or inhibition of BRN3A expression directly may prove therapeutically beneficial in t(8;21) AML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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Jenny Dunne, Duncan M. Gascoyne, T. Andrew Lister, et al.

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