Abstract

Fusion oncogenes in acute myeloid leukemia (AML) promote self-renewal from committed progenitors, thereby linking transformation and self-renewal pathways. Like most cancers, AML is a genetically and biologically heterogeneous disease, but it is unclear whether transformation results from common or overlapping genetic programs acting downstream of multiple mutations or by the engagement of unique genetic programs acting cooperatively downstream of individual mutations. This distinction is important, because the involvement of common programs would imply the existence of common molecular targets to treat AML, no matter which oncogenes are involved. Here we show that the ability to promote self-renewal is a generalized property of leukemia-associated oncogenes. Disparate oncogenes initiated overlapping transformation and self-renewal gene expression programs, the common elements of which were defined in established leukemic stem cells from an animal model as well as from a large cohort of patients with differing AML subtypes, where they strongly predicted pathobiological character. Notably, individual genes commonly activated in these programs could partially phenocopy the self-renewal function of leukemia-associated oncogenes in committed murine progenitors. Furthermore, they could generate AML following expression in murine bone marrow.

In summary, our findings reveal the operation of common programs of self-renewal and transformation downstream of leukemia-associated oncogenes, suggesting that mechanistically common therapeutic approaches to AML are likely to be possible, regardless of the identity of the driver oncogene involved. Cancer Res; 71(12); 4117–29. ©2011 AACR.

Introduction

Acute myeloid leukemia (AML) is characterized by a block in terminal myeloid differentiation, accompanied by uncontrolled proliferation of immature myeloid progenitor cells. However, although there are unifying cellular characteristics, it remains a very heterogeneous disease morphologically, molecularly, and biologically (1). Hundreds of separate genetic lesions have been described in AML, and recent next-generation sequencing analysis of AML genomes has shown the occurrence of multiple lesions within individual leukemias (2). Great improvements have been made in the identification of prognostic factors for AML, such as age, white blood cell (WBC) count, cytogenetics, and mutational analysis of important genes (e.g., FLT3 and NPM1); however, these have reinforced the heterogeneity of AML cases. In addition, gene expression studies have defined signatures downstream of many of these genetic lesions, allowing further molecular characterization (3, 4).

Knowledge of the molecular lesions associated with specific subtypes of AML has led to the introduction of specifically targeted therapeutics, such as all-trans retinoic acid in PML-RARA (retinoic acid receptor α-positive cases, and selective FLT3 inhibitors in cases that harbor an internal tandem duplication of the FLT3 gene (FLT3-ITD). However, AML remains a significant clinical problem with more than 70% of patients succumbing to the disease, accounting for more than 9,000 deaths each year alone in the United States (5), and novel therapeutics with efficacy in the majority of AML patients are urgently required. To this end, it is not known whether transformation is mediated by common or overlapping genetic programs downstream of multiple mutations or through the engagement of unique programs downstream of individual mutations. This distinction is important, as the demonstration of common pathways may identify common
critical molecular targets for the treatment of AML, in all cases or at least in significant subgroups.

The development of cancer is associated with the acquisition of a number of cellular characteristics, including limitless self-renewal, evasion of apoptosis, and self-sufficiency in growth signals (6), and it is possible that these characteristics are governed by a limited number of master regulatory pathways. To use the example of malignant self-renewal, there would appear to be a significant overlap between self-renewal programs in both normal and malignant hematopoietic stem cells. Many genes and pathways implicated in normal stem cell self-renewal, such as the clustered HOX genes, the Wnt/β-catenin pathway, the PTEN/Akt/FOXO axis, and the Notch pathway, are also frequently dysregulated in cancer (reviewed in refs. 7, 8). However, the finding that these pathways are aberrantly activated in both normal and malignant hematopoietic stem cells. Many genes and pathways implicated in normal stem cell self-renewal, may be exploited for therapeutic benefit.

The development of cancer is associated with the acquisition of a number of cellular characteristics, including limitless self-renewal, evasion of apoptosis, and self-sufficiency in growth signals (6), and it is possible that these characteristics are governed by a limited number of master regulatory pathways. To use the example of malignant self-renewal, there would appear to be a significant overlap between self-renewal programs in both normal and malignant hematopoietic stem cells. Many genes and pathways implicated in normal stem cell self-renewal, such as the clustered HOX genes, the Wnt/β-catenin pathway, the PTEN/Akt/FOXO axis, and the Notch pathway, are also frequently dysregulated in cancer (reviewed in refs. 7, 8). However, the finding that these pathways are aberrantly activated or genes in them frequently mutated, along with the demonstration that normal stem cells and cancer stem cells may have different molecular requirements (9), suggests that there are differences between the self-renewal programs in normal and malignant stem cells that may be exploited for therapeutic benefit.

Leukemia-associated fusion genes such as AML1-ETO, NUP98-HOXA9, and MOZ-TIF2 generate aberrant transcriptional programs mediated by their ability to modify chromatin and can confer leukemic stem cell (LSC) properties when retrovirally overexpressed in committed myeloid progenitors (10–12). We assessed the ability of other AML-associated mutations to alter self-renewal properties and chose 2 further transcription factor fusion genes, AML1-ETO (RUNX1-RUNX1T1) and NUP98-HOXA9, and the internal tandem duplication (ITD) mutation of the FLT3 receptor tyrosine kinase (RTK) gene (FLT3-ITD). FLT3-ITD mutations are representative of constitutively active RTK signaling, whereas the AML1-ETO and NUP98-HOXA9 fusion genes, which contain consensus DNA binding sequences, are representative of mutations thought to alter transcriptional programs mainly through transcriptional repression and activation, respectively. In addition, they also represent a prognostic spectrum, with the AML1-ETO rearrangement characteristically associated with a good prognosis (13) and the FLT3-ITD and NUP98-HOXA9 lesions associated with poor prognosis (14, 15).

In this report, we show that the ability to alter self-renewal is a more generalized effect of leukemia-associated transcription factor fusion oncogenes. In addition, taking advantage of this restoration of self-renewal properties in vitro, we use this platform to show that 3 disparate oncogenes initiate early, common, and overlapping transformation and self-renewal signatures involved in leukemia induction. Moreover, elements of these signatures can be detected in established LSCs from an animal model of AML as well as in expression data from a large cohort of patients with differing AML subtypes. Furthermore, these gene sets strongly predict disease biology and correlate with existing prognostic factors. Finally, we show that individual genes from within the signature, such as Sox4 and Bmi1, can at least partially phenocopy the leukemia-associated oncogenes and alter self-renewal in committed murine progenitors and generate AML when expressed in murine bone marrow. This suggests that common transformation and self-renewal pathways downstream of a variety of leukemia-associated oncogenes contribute to the induction and maintenance of AML, a finding with important clinical implications.

Material and Methods

Generation of retroviral constructs

The MOZ-TIF2, NUP98-HOXA9, and FLT3-ITD MSCV vectors were generated as previously described (16–18). The AML1-ETO vectors were a kind gift from Michael Tomasson (Washington University School of Medicine, St. Louis, MO). The cDNA coding regions of murine Sox4 (NM_009238; Gene-service clone BC052736.1), Tcf4 (Geneservice clone BC043050), and murine Bmi1 (Geneservice clone BC053708) were amplified and subcloned into MSCV-ires-GFP and MSCV-pgk-Neo and vectors reverified by sequencing.

Staining and sorting of progenitor populations

Bone marrow mononuclear cells were isolated as previously described (11) and LSK (lineage−, Sca−1−, c-kit+) common myeloid progenitor (CMP), and granulocyte monocyte progenitor (GMP) populations stained as described (19) with minor changes (see Supplementary Methods).

Retroviral transductions, bone marrow, progenitor, and limiting dilution transplant assays

Retroviral supernatants were generated in 293T cells as previously described (16). Bone marrow transplants were conducted as described (16), with the exception of culture with 10 ng/mL recombinant SCF (PeproTech) and centrifugation at 2,500 revolutions per minute. For whole bone marrow experiments, 1 × 106 CD45.2+ cells were injected into lethally irradiated (2 × 550 rads) CD45.2+ recipients (Charles River). Double fluorescence-activated cell-sorted cell-sorted CD45.2+ progenitors were supplemented with recombinant murine IL11 (30 ng/mL; R&D Systems), recombinant murine SCF (150 ng/mL; PeproTech), recombinant murine or human IL6 (30 ng/mL; PeproTech), recombinant murine TPO (60 ng/mL; PeproTech), 2% fibronectin solution (StemCell Technologies), 5% FBS, and 1% penicillin/streptomycin (Invitrogen). Cells were spin-inoculated with retroviral supernatants and 4 μg/mL polybrene at 1,800 revolutions per minute for 60 minutes and incubated overnight at 32°C. The next day, progenitors were washed, resuspended in HBSS (Invitrogen), and injected [transplant range CMP: (3.2–5.8) × 103; GMP: (4.2–16.5) × 103; MEP: (3.5–6.8) × 103] into the tail vein of 2 lethally irradiated (2 × 550 rads) CD451+ recipients (Charles River) per progenitor population along with 5 × 105 CD451+ bone marrow mononuclear cells. Each experiment was repeated 3 times. In the adoptive transfer of MOZ-TIF2-associated leukemia cells to enrich for LSCs, leukemic GMPs, leukemic GFP+, and leukemic GFP+ Macl1+Gr1+ were transplanted at limiting dilution doses of 1 × 101, 5 × 101, 1 × 102, 5 × 102, 1 × 103, 1 × 104, and 10 cells into 3 to 5 sublethally irradiated CD45.2+ recipients (1 × 650 rads) as detailed in the text.

Serial replating and growth in liquid culture

Serial replating assays were conducted as previously described (11).
RNA amplification and gene expression analysis

Thirty-six hours after spin-inoculation of oncogene or empty vector-transduced GMP, GFP-positive cells were homogenized with TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA isolated from these populations along with sorted normal LSK, GMP, and MOZ-TIF2 leukemic GMP was quantified with the RiboGreen RNA Quantitation Reagent and Kit (Invitrogen) according to manufacturer's specifications and used for RNA amplification and hybridization as previously published (20), with the modification of biotinylated CTP and UTP (Enzo Diagnostics) in a 2.5:1 proportion to nonbiotinylated CTP and UTP. All RNA populations were simultaneously amplified.

Gene-expression levels were measured using Affymetrix Mouse Genome 430 2.0 GeneChip arrays (45,101 probe sets), with hybridization and washes as per the manufacturer's
A

**GFP**

**GMP**

GFP alone
MOZ-TIF2
NUP98-HOXA9
AML1-ETO

36 h

**Sca-1**

RNA extraction and amplification

B

Oncogene vs. GFP

1,119 probes/1,082 genes

Filter

LSK vs. GMP

182 probes/167 genes

C

**MOZ-TIF2**

NUP98-HOXA9

AML1-ETO

10

2

1

14

9

66

25

D

**MOZ-TIF2**

NUP98-HOXA9

AML1-ETO

459 probes/413 genes

Filter

LSK vs. GMP

100 probes/91 genes

"Leukemia initiation" signature

"Leukemia self-renewal" signature
specifications. The starting point for all analyses was the ".CEL" files from the MAS5 software. Data were analyzed using the R statistical package bioconductor. Data quality was assessed using functions in the affy- and affyPLM packages, and outlier arrays were removed from subsequent analysis. The GCRMA algorithm (version 2.4.1) was used to obtain normalized expression estimates. Genes were selected for further analysis on the following basis: genes that had probe sets for which the expression value was greater than 60 (which in our study constitute the average background reading of all probe sets) and that had a present flag call in at least 2 of 3 samples. To detect significant changes in the expression levels, 2-sample Welch t tests (parametric; assuming unequal variances; Benjamini and Hochberg stepup multiple testing correction at a false discovery rate (FDR) of 0.05) was applied to the resulting genes. Heat map images were developed using GenePattern 3.0 (23) with row-normalized color scheme.

Results

Alteration of self-renewal properties is a common finding with AML-associated transcriptional oncogenes

Similarly to MLL fusions or MOZ-TIF2, AML1-ETO and NUP98-HOXA9 could alter properties of self-renewal in committed myeloid progenitor cells (CMP and GMP) in vitro, when assessed based on their ability to serially replate in cytokine-supplemented methylcellulose and to grow in short-term liquid culture (Fig. 1B and C). In contrast, transduction of committed progenitors with FLT3-ITD did not alter their self-renewal properties in in vitro or in vivo assays (Fig. 1A), in keeping with our previous findings with another activated tyrosine kinase, BCR-ABL (11). However, differences were present between the effects of the transcription factor fusion genes in vivo. NUP98-HOXA9 was able to generate AML when transduced committed progenitors (CMP, GMP, and MEP) were transplanted into recipient mice (Fig. 1C). Previous studies have shown that expression of AML1-ETO in unfractionated murine bone marrow does not generate AML without additional mutations but does lead to stem cell expansion (24); however, we could not detect the presence of any transplanted cells in recipients of AML1-ETO-transduced committed myeloid progenitors after 4 months (Fig. 1B). These data show that alteration of self-renewal potential is a more generalized property of AML-associated transcription factor fusion genes and is not solely associated with mutations harboring intrinsic chromatin modification properties. However, the extent of this property seems to be variable between different fusions with some, such as MLL fusions, MOZ-TIF2, and NUP98-HOXA9, able to confer self-renewal properties in vivo and generate AML and others, such as AML1-ETO, only able to alter self-renewal in vitro.

Common and overlapping pathways promote transformation and alter self-renewal downstream of AML-associated oncogenes

The aforementioned findings provided us with a platform to investigate whether common, overlapping, or separate self-renewal and transformation pathways are activated downstream of individual oncogenes. The MOZ-TIF2, NUP98-HOXA9, and AML1-ETO oncogenes or a control empty vector were retrovirally expressed in the GMP compartment by using MSCV-ires-GFP retroviral vectors. GFP-positive transduced cells were sorted and RNA populations from these cells were simultaneously amplified and then hybridized to Affymetrix 430A 2.0 arrays (Fig. 2A).

The data were analyzed using 2 complementary methods. Initially, we compared the gene expression profiles of multiple replicates of oncogene-transduced GMP together in a single 2-class comparison with empty vector-transduced GMP (Fig. 2B). In addition, we also compared replicates of each oncogene in 3 separate 2-class comparisons with empty vector–transduced GMP at lower stringency (Fig. 2C). In the first analysis, we identified 1,082 genes/1,119 probe sets with expression levels that significantly differed [false discovery rate (FDR) level = 0.05; Fig. 2B]. There is a loss of self-renewal potential in the transition from the LSK to GMP populations, and to further enrich for potential self-renewal genes, we compared the expression of these probes in sorted normal LSK versus GMP populations. Genes that were not significantly (P < 0.05) and similarly differentially expressed between LSK and GMP were excluded, allowing us to prioritize an immediate "leukemia initiation" program of 167 genes (182 probe sets, Fig. 2B and Supplementary Table S5). This gene signature included genes associated with either normal stem cell self-renewal, such as Foxo3a (25), with malignant transformation, such as Meis1 (26) and Mef2c (12), or with both processes, such as Bmi1 (27).

Figure 2. Identification of immediate leukemia-associated signatures downstream of multiple AML fusion genes. A, schema of the experimental strategy used to identify the leukemia-associated signatures. B, identification and prioritization of genes to generate the 167-gene (182 probe) immediate "leukemia initiation" signature. Initially, the expression profiles for multiple replicates of MOZ-TIF2-, AML1-ETO-, and NUP98-HOXA9-transduced GMPs were compared in a single 2-class comparison to GFP-transduced GMPs. A total of 1,119 genes were commonly dysregulated to an FDR significance level of 0.05. A filter comparing expression of the 1,119 genes between normal hematopoietic stem cells and GMPs was then applied, with genes that were significantly and coordinately differentially expressed (P < 0.05) retained. This prioritized 167 genes to generate our signature. Expression patterns of 20 representative genes are presented in the left part of the panel (all genes are listed in Supplementary Table S1). C, 3 single 2-class comparisons were also made between each of MOZ-TIF2-, AML1-ETO-, and NUP98-HOXA9-transduced GMPs, and GFP-transduced GMPs and genes differentially expressed (P < 0.05) were overlapped with our leukemia initiation signature. The Venn diagram details the number of genes dysregulated by each oncogene and shows significant common and overlapping pathways downstream of the 3 oncogenes. D, identification and prioritization of genes to generate the 91-gene (100 probe) immediate "leukemia self-renewal" signature. The expression profiles for multiple replicates of MOZ-TIF2- and NUP98-HOXA9-transduced GMPs were compared in a single 2-class comparison with AML1-ETO–transduced GMPs. A total of 413 genes were commonly dysregulated (P < 0.01). This gene set was then similarly filtered to generate our immediate leukemia self-renewal signature. Expression patterns of 10 representative probes (9 genes) are presented in the left part of the panel (all genes are listed in Supplementary Table S2).
A

Transplant into secondary recipients at limiting dilution:

1 x 10^6
5 x 10^5
1 x 10^5
1 x 10^4
5 x 10^3
1 x 10^3
1 x 10^2
10 Cells

GFP+ Mac1+ Gr1+
LSC frequency
1/1,007,149

GFP+
1/50,335

GFP+ L-GMP
1/304

LSC potential

B

GMP vs. Leukemic GMP

CyclinD2
CyclinD2
Smoophesec
TGFβR1
Sug5
Tgfβ1
Grf1
Sno9
Smad7
Rab
Meis1
Bmi1
Hoxa10
Cfrtr
Ctnnz1
Lmo2
Runx1
Hoxa9
N-Myc
Our second analysis showed differential expression of 5,750, 1,161, and 5,109 genes, when MOZ-TIF2–, NUP98-HOXA9–, and AML1-ETO–transduced GMP were respectively compared with empty vector–transduced GMP (P < 0.05). When our leukemia initiation signature was compared with the single comparisons, 127 of 167 (76%) genes overlapped. Fourteen genes were differentially expressed downstream of all 3 oncogenes, 77 genes were differentially expressed downstream of 2 oncogenes, and the remaining 36 genes were only differentially expressed downstream of a single oncogene (Fig. 2C and Supplementary Table S5). Taken together, these data show that both common and overlapping genes are transcriptionally dysregulated downstream of disparate AML-associated oncogenes.

Because there was a distinction between the effects of MOZ-TIF2, NUP98-HOXA9, and AML1-ETO in their ability to alter self-renewal in vivo and generate AML from committed progenitors, we next assessed differences between the gene expression signatures of MOZ-TIF2– and NUP98-HOXA9–transduced GMP in comparison with AML1-ETO–transduced GMP. We detected 413 genes (459 probe sets) that were differentially expressed, and we identified, using a similar filtering strategy, an immediate leukemia self-renewal program of 91 genes/100 probe sets (Fig. 2D and Supplementary Table S6). This gene program was not only mutually exclusive from the leukemia initiation program but also included genes previously implicated in self-renewal and transformation, such as Hoxa9 (28), C-Myc (29), and Cbx5 (Hpi10; ref. 29).

Established LSCs exhibit induction of similar oncogenic pathways

To assess the requirement for self-renewal and transformation pathways during leukemic evolution, we next wanted to compare our immediate “preleukemic” signatures with the gene expression pattern in established murine LSCs. We have previously shown that the GMP compartment is the earliest phenotypic population present in MOZ-TIF2–associated leukemias (11). In addition, this same phenotypic population was highly enriched for LSC activity in an experimental schema used to establish an LSC hierarchy (Fig. 3). Mice developed phenotypically identical leukemias (data not shown) but at incidences that varied dramatically according to the transplanted population. Our results showed a hierarchy in MOZ-TIF2–associated leukemias, and consistent with normal ontogeny we found the L-GMP population to be at the apex of leukemic differentiation and to be highly enriched for LSC potential (approximately 166- and 3,300-fold enrichment over bulk leukemic and mature leukemic cells, respectively, with an LSC frequency of 1/300 cells, Fig. 3A).

To assess the evolutionary nature of transcriptional programs critical for leukemia induction and maintenance, the gene expression profiles of replicate L-GMP were then compared with their normal phenotypic counterpart. A total of 2,715 genes were differentially expressed between normal and leukemic GMPs (P < 0.05). Similarities were seen between our immediate preleukemic signatures and the gene expression profiles in the overt leukemias. The overlap with the leukemia initiation and leukemia self-renewal signatures was highly significant at 59 of 167 genes (35%) and 29 of 91 genes (32%), respectively (both P < 0.0001). Overlapping genes included Bmi1, Mes1, Sox4, Tcf7, HoxA9, and Smad7. However, new genes, not present in our preleukemic signatures but widely implicated in leukemogenesis, were also significantly upregulated, including Hoxa10, Hoxa7, Runx1, Lmo2, and Ctnna1 (Fig. 3B). Therefore, although there are similarities with the genetic programs that establish LSCs, additional programs, possibly the result of later cooperating mutations, may be required for the further evolution and maintenance of LSCs during leukemogenesis.

Preleukemic signatures predict the biology of human AML

Significant differences have been reported in mechanisms of transformation between mice and humans, and our signatures have been generated following retroviral overexpression of oncogenes. Therefore, to validate our genetic programs in human AML, we compared our leukemia initiation and leukemia self-renewal gene sets across bulk gene expression profiles from 253 unselected cases of AML. These cases represented a wide variety of cytogenetic and genetic mutations and were treated uniformly in the Austrian–German AML studies (Supplementary Table S7). In comparing the gene expression from this data set to our own signatures, probes/genes that were not represented on both the human and mouse arrays or that were not of sufficient quality in the human analyses were excluded. This allowed a direct...
comparison of 84 of 167 genes in the leukemia initiation signature and 61 of 91 genes in the leukemia self-renewal signature. For the leukemia initiation signature 67 of 84 genes (80%, represented by 109 probe sets) were differentially expressed, and for the leukemia self-renewal signature 31 of 61 genes (51%, represented by 37 probe sets) were differentially expressed in the human AML cohort.

Using the leukemia initiation gene set, we next conducted unsupervised hierarchical clustering of the human AML samples. This classified the cohort into 5 different patient groups (Fig. 4A), with this association shown to be stable following iterative consensus clustering. These patient groups differed significantly in known prognostic characteristics (karyotype, P < 0.0001; mutational status for molecular lesions such as FLT3-ITD, CEBPA, and NPM1c, all P < 0.0001; age, P = 0.005; and WBC count at diagnosis, P = 0.0035; Fig. 4C and D and Supplementary Fig. S1) and in survival (both event-free survival P < 0.0018 and overall survival P < 0.0001; Fig. 4B). Although genes from our signature were differentially expressed across all patient groups, the leukemia initiation signature was representative of patient group 1 (containing 71 patients), within which it tightly associated when included in the clustering analysis (Fig. 4A). It was noted that group 1 had the worst prognosis both for overall survival and for event-free survival (Fig. 4B). In this respect, the poor prognosis for group 1 patients may be explained by a direct correlation with known poor prognostic determinants (an increased incidence of complex karyotype and the presence of FLT3-ITD mutations) and an inverse correlation with good prognostic determinants (a decreased incidence of rearrangements of the core-binding factor subunits, RARA and CEBPA4 mutations; Fig. 4C and D and Supplementary Fig. S1). In addition, when our gene set was used to classify an independently published data set of 283 patients (4), similar associations between the leukemia initiation gene set and cytogenetic, molecular genetic, and survival findings were obtained (Supplementary Fig. S2). Moreover, an association was shown between our gene set and expression of ETV1, a transcriptional repressor whose expression associates with a poor prognosis in AML patients within this data set (4).

We found similar associations on using our leukemia self-renewal gene set to interrogate the initial AML patient cohort. The self-renewal gene set, using unsupervised clustering, again grouped the cohort into 5 groups (Supplementary Fig. S3). Similarly, these patient groups differed significantly in survival and known prognostic characteristics. On this occasion, our signature tightly clustered with group E (comprising 68 patients). This group again was enriched for poor prognosis patients and correlated with an increased incidence of poor risk cytogenetics, although not on this occasion with FLT3-ITD status (Supplementary Fig. S3).

**Candidate genes alter self-renewal properties in vitro and generate AML in vivo**

Finally, to prove proof of principle that the genes identified in our signatures could alter self-renewal and mediate transformation, 3 genes from the leukemia initiation signature that were present in mouse and human comparisons were chosen: Tcf4, Bmi1, and Sox4. These genes were assessed for their ability to phenocopy the leukemia-associated fusions in serial replating and transplantation assays. Transcription factor A, Tcf4, is an E box–binding helix–loop–helix transcription factor associated with the mental retardation Pitt–Hopkins syndrome (31); Bmi1 is a component of the polycomb PRC1 complex that has been implicated in normal hematopoietic, leukemic, and neural stem cell functions (27); and Sox4 is an HMG box transcription factor involved in cardiac and lymphoid development and has been described as a retroviral integration site in murine leukemias (32, 33).

No serial replating was seen in LSK cells or whole bone marrow transduced with Tcf4 (Fig. 5A). However, for Bmi1 or Sox4, we showed that retroviral expression could partially phenocopy the effects of fusion oncogene expression and alter the in vitro self-renewal potential of committed progenitors in serial replating assays (Fig. 5B and C). In addition, LSK cells and whole bone marrow transduced with either Sox4 or Bmi1 could grow in cytokine-supplemented liquid culture, although CMPs and GMPs transduced with either gene only grew for a limited period of 4 weeks in culture. Finally, although Bmi1-transduced hematopoietic tissue and Sox4-transduced progenitors did not generate disease, recipient mice transplanted with Sox4-transduced whole bone marrow developed AML and died from AML with a median latency of 28 weeks (Fig. 5D).

**Discussion**

Our data show an alteration of self-renewal in murine stem and progenitor populations following expression of AML1-ETO and NUP98-HOXA9. A reductionist view of the pathogenesis of AML proposes that its development minimally requires cooperation between type I mutations, which alter cellular proliferation and survival (such as FLT3-ITD or c-Kit mutations), and type II mutations, which block myeloid differentiation (of which AML1-ETO and NUP98-HOXA9 would be...
Figure 5. Individual genes in the leukemia initiation signature partially phenocopy AML-associated fusion genes. A, Tcf4 did not alter the properties of bone marrow, LSK, or progenitors in methylcellulose (left) and Tcf4 cells failed to grow in liquid culture (right). B, Left, whole bone marrow, LSK, and myeloid progenitors (CMP and GMP) transduced with Bmi1 serially replate in methylcellulose cultures. Both whole bone marrow and LSK transduced with Bmi1 continue to grow in cytokine-supplemented liquid culture, whereas growth of CMP and GMP populations is limited to 4 weeks in culture before their involution (middle). Right, whole bone marrow or progenitors transduced with Bmi1 did not generate AML following transplantation. C, left and middle, similar findings for stem and progenitor populations transduced with Sox4. In addition, bone marrow transduced with Sox4 generated leukemia with a median latency of 28 weeks (right). D, representative photomicrographs of bone marrow (left) and spleen (middle) from animals with Sox4-associated AML show heavy infiltration with immature blasts. Representative flow of the GFP-positive cells from these leukemias (right) shows a Gr1intermed/Mac1intermed myeloid phenotype.
A similar ability to alter self-renewal in committed progenitors has been shown by others and us for MLL fusions, MOZ-TIF2, and CEBP-α mutants (10–12, 34). Taken together, these data strongly link self-renewal and transformation in AML and suggest that an ability to restore self-renewal or to augment existing self-renewal is a more generalized property of these type II mutations. However, our data also show a differential ability to alter self-renewal in vivo between AML1-ETO and NUP98-HOXA9, with only the latter able to generate AML. As both fusion oncogenes are thought to be initiating events in leukemogenesis (35, 36), our data therefore suggest that the potential cell(s) of origin of the LSCs that propagate the disease may differ according to the properties of the initiating lesion. This may be one explanation for the growing demonstration of heterogeneity within the LSC compartment (37). Furthermore, it also suggests an association between altered self-renewal and aggressive disease, corroborating recent functional findings in murine xenotransplant experiments wherein levels of engraftment from individual patients correlate with their disease outcome (38).

Global gene expression profiles of bulk blasts from AML patients have greatly informed the study of AML biology and have been particularly useful in the classification of specific prognostic groups, detailing that many individual genetic lesions are associated with a characteristic gene expression signature (3–4). However, data showing whether the induction of common, overlapping, or unique pathogenetic pathways occurs downstream of multiple mutations in AML are generally lacking. In this report, we show that 3 disparate but functionally related AML-associated fusion oncogenes cause similar differential expression of common and overlapping gene programs following short-term expression in the murine hematopoietic progenitor compartment. These same pathways were aberrantly regulated in populations enriched for established and functionally validated LSCs, although further genes and pathways associated with leukemogenesis were also perturbed. Taken together, these data show the evolutionary nature of transcriptional programs during leukemogenesis, suggesting that additional programs, possibly the result of later cooperating mutations (39), are required for the further evolution and maintenance of LSCs during leukemogenesis.

Comparisons based on differences in the in vitro and in vivo properties conferred by the 3 oncogenes allowed us to define mutually exclusive leukemia initiation and leukemia self-renewal signatures. Similar signatures defining self-renewal and the maintenance of LSCs have been published for MLL fusion proteins (12, 29). When we compared our leukemia initiation and leukemia self-renewal signatures with those of Krivtsov and colleagues (12) and Somervaille and colleagues (29), we found only modest overlap (Supplementary Fig. S4). This corroborates the findings in bulk human AML blasts, in which the global gene expression profiles of MLL rearranged and MLL germ line leukemias were seen to segregate (40). However, these small overlaps were greatly enriched for genes that alter self-renewal and/or are critical for AML pathogenesis and included genes such as Hoxa9 (28), Meis1 (26), Mef2c (12), Myb (41), and Cbx5 (ref. 29; Supplementary Fig. S4A and B). This is a further demonstration that common pathways are critical effectors of leukemogenesis in subsets of AML patients.

The gene sets that comprised our signatures could also classify 2 independent data sets of global gene expression profiles from large cohorts of AML patients. Patients were assigned into groups that significantly differed in existing prognostic characteristics such as patient age, presenting WBC count, karyotype, and mutation status for critical genes such as FLT3-ITD and CEBPA. Although the majority of genes within our signatures were differentially expressed across all groups, our signatures were most representative of specific groups with a particularly poor prognosis. This probably reflects that patients within these groups showed a decreased incidence of good risk characteristics such as rearrangement of core binding factors and RARA and an increased frequency of known poor prognostic factors such as a complex karyotype, FLT3-ITD, and overexpression of EVII. As such, our signatures and genes within them may represent biological effectors downstream of these poor risk mutations. Furthermore, because long-term survival in patients with poor risk factors may be as low as 10% (13), the identification of downstream pathways and potential targets may inform therapeutic design to improve the dismal current outcomes in this group.

Candidates identified in our common signatures are known to alter self-renewal in hematopoietic cells and to critically mediate leukemogenesis. From our leukemia self-renewal signature, Hoxa9 and Myb have been shown to be critical for self-renewal of leukemic and normal stem cells (28, 29, 41, 42), whereas Cbx5 is necessary for MLL fusion LSC maintenance (29). From our leukemia initiation signature, identified genes such as Mef2c, Meis1, and Tal1 have demonstrable roles in LSCs (12, 26). As further proof of principle, we validated another 2 candidates chosen from our leukemia initiation signature to at least partially phenocopy the original leukemia-associated oncogenes. The polycomb group gene Bmi1 has been shown to be a requirement for both leukemic and normal stem cell functions (27) and was upregulated following overexpression of both MOZ-TIF2 and AML1-ETO (Supplementary Table S5). Although its overexpression as a solitary abnormality was insufficient to generate AML in vivo, it did alter self-renewal properties of hematopoietic stem and progenitor cells in vitro. Sox4 was commonly upregulated downstream of MOZ-TIF2, AML1-ETO, and NUP98-HOXA9 (Supplementary Table S5) and is a high-mobility group box transcription factor involved in cardiac and lymphoid development (32). It has previously been implicated in murine leukemogenesis (33) and is a homolog of Sox2, a critical mediator of the cellular reprogramming of induced pluripotent stem cells (43). Importantly, although it may augment their self-renewal properties on overexpression (44), Sox4 seems to be dispensable for normal hematopoietic stem cell function (32). We showed further that Sox4 expression is altered in human AML and that overexpression of Sox4 can alter self-renewal properties in hematopoietic stem and progenitor cells in vitro and can generate AML in vivo. Taken as a whole, these cross-species findings validate common and
overlapping genes and transcriptional programs downstream of oncogenic transcriptional fusion genes to alter self-renewal properties and contribute to the evolution of AML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

B.T. Kvinlaug assisted with study design, conducted experiments, analyzed data, and helped write the manuscript. W.-I. Chan, D. Foster, R. Okabe, B.H. Lee, and I.D. Silva conducted experiments. L. Bullinger, M. Ramaswami, C. Sears, A. Renner, and S.E. Lazi analyzed data. P.J.M. Valk, R. Delwel, and H. Döhner contributed valuable data sets. S.A. Armstrong and D.G. Gilliland helped with initial study design. B.J.P. Huntly designed and guided the study, analyzed data, and wrote the manuscript.

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