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# *POU4F1* is associated with t(8;21) acute myeloid leukemia and contributes directly to its unique transcriptional signature

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## Abstract

The t(8;21)(q22;q22) translocation, present in ~5% of adult acute myeloid leukemia (AML) cases, produces the AML1/ETO fusion protein. Dysregulation of the POU domain-containing transcription factor *POU4F1* is a recurring abnormality in t(8;21) AML. Here, we show that *POU4F1* over-expression is highly correlated with, but not caused by AML1/ETO. AML1/ETO markedly increases the self-renewal capacity of myeloid progenitors from murine bone marrow or fetal liver and drives expansion of these cells in liquid culture. *POU4F1* is neither necessary nor sufficient for these AML1/ETO-dependent properties, suggesting that it contributes to leukemia through novel mechanisms. To identify targets of *POU4F1*, we performed gene expression profiling in primary mouse cells with genetically defined levels of *POU4F1* and identified 140 differentially expressed genes. This expression signature was significantly enriched in human t(8;21) AML samples and was sufficient to cluster t(8;21) AML samples in an unsupervised hierarchical analysis. Among the most highly differentially expressed genes, half are known *AML1/ETO* targets, implying that the unique transcriptional signature of t(8;21) AML is, in part, attributable to *POU4F1* and not *AML1/ETO* itself. These genes provide novel candidates for understanding the biology and developing therapeutic approaches for t(8;21) AML.

### Keywords

POU4F1; AML1/ETO; acute myeloid leukemia; gene expression profiling



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**conflict of interest**

The authors declare no conflicts of interest.

**Supplementary Information**

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu).

## introduction

The t(8;21)(q22;q22) translocation produces an in-frame fusion of the first five exons of *AML1* (*RUNX1*) to all but the first exon of *ETO* (*MTG8*) (1). This translocation is detectable in ~10% of *de novo* acute myeloid leukemia (AML) cases of the French-American-British M2 subtype and ~5% of all AML cases (2). AML1 is the DNA binding subunit of core binding factor (CBF), a multimeric transcription factor complex that includes CBFβ and additional transcriptional cofactors. The chimeric AML1/ETO protein has dominant negative effects on genes typically regulated by CBF (3). ETO, also a transcription factor, contains four *nervy* homology regions that contribute directly to the negative regulation of CBF-responsive genes (4). Despite these effects on gene regulation, AML1/ETO is not sufficient to cause AML (5-8), implying that additional genetic events are required.

Genome-wide expression profiling of primary human AML samples, performed by several groups, has identified a robust gene expression profile that distinguishes t(8;21) from other AML subtypes (9, 10). ETO is part of the t(8;21) expression signature. This is not unexpected, since most of the *ETO* coding sequence is contained within the *AML1*/*ETO* fusion transcript. These studies have also demonstrated that the *POU4F1* gene is consistently dysregulated in t(8;21) human patient samples (9, 10).

POU4F1 is a transcription factor, originally identified in rat brain (11). The mouse and human orthologs are highly homologous (95% nucleic acid identity, 99% amino acid identity). POU4F1 contains a homeodomain and a POU-specific domain, both of which are required for DNA binding (11). Pou4f1 is important for embryonic brain development and is expressed beginning at E11.0 in mice (12), but has no reported role in normal or leukemic hematopoiesis. *Pou4f1* null mice die postnatally with developmental anomalies in both the central and peripheral nervous system (13, 14).

The striking correlation between *AML1/ETO* and *POU4F1* expression in human AML led us to hypothesize that *POU4F1* might be a transcriptional target of *AML1/ETO*. Surprisingly, we found that *POU4F1* dysregulation is not caused by *AML1/ETO* and that *POU4F1* is dispensable for AML1/ETO function *in vitro*, but it is an important driver of the unique transcriptional profile associated with t(8;21) AML.

## MATERIALS AND METHODS

### Plasmids

Recombinant murine stem cell proviral plasmids MSCV2.2-*ires-GFP* (MIG) and MSCV2.2-*AML1/ETO-ires-GFP* (MAIG) were provided by Michael Tomasson (Washington University, St. Louis, MO). MSCV2.2-*ires-YFP* (MIY) was created by removing the *GFP* cDNA from MIG and replacing it with *YFP* from pEYFP-N1 (Clontech, Mountain View, CA). MSCV 2.2-*Pou4f1-ires-YFP* (MPIY) and MSCV2.2-*Pou4f1-ires-GFP* (MPIG) were generated by subcloning the mouse *Pou4f1* cDNA (provided by Eric Turner, University of CA, San Diego) into MIY or MIG, respectively.

## Mice

*Pou4f1(*Brn3a) mutant mice were provided by Eric Turner (University of CA, San Diego) (15). Embryos from timed matings were genotyped by PCR (primer sequences provided in Supplementary Table 1). *Sca*$^{+/GFP}$ and *Sca*$^{+/AE}$ mice were generated, as previously described (8, 16). All mice were backcrossed at least 10 generations to a C57BL/6J background.

## RT-PCR analysis

RNA was made in Trizol LS (Invitrogen, Carlsbad, CA). All samples, excluding RNA from human AML samples, were treated with DNAse (Roche, Palo Alto, CA). cDNA was made from RNA using M-MLV reverse transcriptase (Promega, Madison, Wisconsin). qRT-PCR was performed using TaqMan Universal PCR Master Mix (Roche) (primer and probe sequences provided in Supplementary Table 1). All samples were run in triplicate on a 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) and analyzed using the standard curve method.

## Retroviral transduction

Retroviral supernatants were generated by transient transfection of 293T cells with Ecopac (Cell Genesys, Forster City, CA) and the MSCV-based retroviral constructs. 48 hrs after transfection, retroviral supernatants were harvested and titered on 3T3 cells using flow cytometry. Bone marrow cells were cultured in complete media (RPMI containing 1% L-glutamine, 20% fetal bovine serum) supplemented with recombinant hematopoietic cytokines (100 ng/mL stem cell factor, 6 ng/mL interleukin-3, 50 ng/mL Fms-related tyrosine kinase 3 ligand, and 10 ng/mL thrombopoietin; all from Peprotech, Rocky Hill, NJ) for 48 hours. 3.0-4.5 × $10^6$ cells were infected on two consecutive days by centrifugation at 2500g for 90 minutes in the presence of 10 ug/ml polybrene (Sigma; St. Louis, MO) and 33 uM HEPES with retroviral supernatants using multiplicities of infection (MOIs) ranging from 1.5-3. Two days post-infection, the cells were seeded in cytokine-supplemented media at $10^5$cells/ml in triplicate. In some experiments, transduced cells were selected by sorting on GFP or YFP (MoFlo, Beckman Coulter, Fullerton, CA) prior to initiating the cultures. Cells were counted twice-weekly and replated in fresh media, maintaining a concentration of $10^5$ cell/mL. The proportion of transduced cells was monitored weekly by flow cytometry (FACScan, Becton Dickinson).

## Hematopoietic progenitor analysis

Bone marrow cells from adult mice or 14.5-16.5 dpc fetal liver cells were transduced, as above. 48 hrs after the first round of infection, the cells were washed three times with RPMI, plated (6.7 × $10^3$ cells/mL for bone marrow or 3.3 ×$10^4$ cells/mL for fetal liver) in cytokine-supplemented methylcellulose media (MethoCult M3434,Stem Cell Technologies; Vancouver, BC, Canada), and incubated at 37°C in 5% $CO_2$. Seven days after plating, the colonies were examined under a fluorescence microscope. GFP+ or YFP+ colonies were individually selected, disrupted, and replated at one colony-equivalent per well in 24-well plates containing fresh methylcellulose media. Colonies were scored for survival and serially replated weekly.

### Gene expression profiling

Murine 14.5-16.5dpc fetal liver cells were sorted for GFP or YFP expression 48 hours after retroviral transduction. Three experimental groups were generated: "*Pou4f1* null" (*Pou4f1*$^{-/-}$ cells transduced with MIY), "*Pou4f1* wildtype" (*Pou4f1*$^{+/+}$ cells transduced with MIY), and "*Pou4f1* high" (*Pou4f1*$^{+/+}$ cells transduced with MPIY). Three independent samples were generated for each group (n=9 total samples). RNA was purified using Trizol LS, quantified by UV spectroscopy (Nanodrop Technologies), and qualitatively assessed using a BioAnalyzer 2100 and the RNA NanoChip assay (Agilent Technologies, Palo Alto CA). All samples were linear amplified, labeled, and hybridized to Affymetrix MOE430v2.0 GeneChip microarrays (Affymetrix, Santa Clara, CA) using standard protocols from the Siteman Cancer Center Multiplexed Gene Analysis Core Facility (for protocols, see http://Pathimm.wustl.edu/~mgacore/index.htm). Data are available from the Gene Expression Omnibus (GSE19997).

Probe raw signal intensities were summarized into probeset values using RMA (17, 18), and samples were quantile normalized. Only probesets called 'Present' by MAS5 software in all triplicates of at least one experimental group were retained for cluster and differential gene expression analysis (n=17,568 probesets). The overall expression profiles were highly similar for all samples (average Pearson correlation = 0.959) except for one from the wildtype group (average Pearson coefficient = 0.923 compared to the remaining 8 samples). This sample was retained, since the results of downstream analysis were not affected. We performed one-way analysis of variance (ANOVA) to identify probesets exhibiting differential expression between experimental groups. We performed 17,568 statistical tests, and applied *q-value* to the resulting p-values to estimate the genome-wide false discovery rate (19). Gene Ontology enrichment analysis was performed using DAVID (20).

Total RNA from 111 de novo M0-M7 human AML samples was profiled on Affymetrix U133+2 arrays, as previously described (21). Data are available from the Gene Expression Omnibus (GSE10358). Human orthologs of the dysregulated murine genes were identified (n=285 probesets) using BioMart (22). Testing for the enrichment of the *Pou4f1* gene set in human AML samples was performed using Gene Set Enrichment Analysis (23, 24). Samples with or without the t(8;21) were compared, and the genes ranked based on the correlation between their expression and the class distinction using both signal2noise and ratio-of-classes gene ranking metrics (24). Wards hierarchical clustering was performed using Spotfire DecisionSite 8.2 (TIBCO Software Inc, Somerville, Mass). The P-value of the t(8;21) clustering was assessed by determining the number of times that a random selection of 285 probesets would result in the t(8;21) samples being nearest neighbors (distance metric = 1-Pearson correlation), divided by the number of random samplings (n=10,000). The *POU4F1*-independent gene expression profile was identified by removing the probesets for *POU4F1* and its targets from the human AML data. Probesets with fewer than 25% present calls or a CV less than 0.5 were also removed. The remaining 13,700 probesets were used to cluster the AML samples with or without t(8;21). Significant differences in expression were identified by SAM using an FDR threshold <0.05 (25).

## results

### *POU4F1* is associated with t(8;21) AML

We and others have noted that *POU4F1* expression is dysregulated in t(8;21) AML (9, 26-30). We performed gene expression profiling using primary human samples and found that *POU4F1* is not expressed in normal human CD34+ bone marrow cells or in AML samples from most FAB subtypes (Figure 1). High *POU4F1* expression is restricted to M2 samples, with the highest levels noted in t(8;21) positive AML. To confirm these results, we performed quantitative RT-PCR using three samples with t(8;21) and nine randomly selected M2 samples without t(8;21). The t(8;21) samples tested had significantly higher levels of *POU4F1* expression compared to M2 samples lacking t(8;21) ($P<0.001$) (Figure 1).

### *POU4F1* over-expression is AML1/ETO independent

We hypothesized that high *POU4F1* expression in t(8;21) AML is caused by altered transcriptional activity mediated by AML1/ETO (AE). Transient transfection of K562 cells with a construct containing AE (MAIG) or a control (MIG) plasmid did not increase *POU4F1* mRNA above baseline levels (Figure 2). Next, we infected primary mouse bone marrow cells with the MAIG or MIG retroviruses. The cells were placed in culture and serial samples were taken for qRT-PCR analysis to measure AE and *Pou4f1* expression. AE mRNA was readily detectable in cells infected with MAIG, but *Pou4f1* levels were not increased in cells expressing AE (Figure 2). Similar results were obtained when bone marrow cells were first enriched for stem/progenitors by cell sorting (lineage-Kit+Sca+) prior to retroviral transduction (not shown). Finally, *Pou4f1* levels were similar in bone marrow cells from mice heterozygous for an AE allele targeted to the *Sca1* locus, compared to control $Sca^{+/GFP}$ mice (Figure 2). Taken together, these data suggest that high *POU4F1* expression, though correlated with t(8;21), is not caused by AE.

### Role of *POU4F1* in AE-dependent hematopoietic cell growth

To test the impact of AE and *Pou4f1* on the survival and proliferation of hematopoietic cells in vitro, we performed retroviral transduction of primary murine bone marrow cells. Transduced cells were enriched by flow sorting for GFP and/or YFP expression, and cultured in cytokine-supplemented media. Cells transduced with *Pou4f1* (MPIY) or empty vector (MIY) died within two weeks, whereas cells transduced with AE (with or without *Pou4f1*) expanded rapidly beyond three weeks (Figure 3). Coexpression of *Pou4f1* with AE provided no consistent growth advantage compared to AE alone.

Next, we performed long term liquid culture of unsorted, retrovirally transduced cells and monitored the proportion of cells expressing GFP (AE reporter) and/or YFP (Pou4f1 reporter). Once again, cells infected with *Pou4f1* alone were rapidly lost, whereas cells transduced with AE (with or without *Pou4f1*) had rapid and indistinguishable growth kinetics (Figure 3). In three independent experiments, the percentage of cells expressing AE only (GFP+YFP−) expanded 8.1-fold (range 5.4-9.6) over 6 weeks, compared to a 0.6-fold increase (range 0.14-6) for AE/*Pou4f1* double positive cells. Although the growth of AE and AE/*Pou4f1* positive cells are similar, the growth is attributable to an increasing population of AE single positive cells. The slight decrease in growth seen in the AE/*Pou4f1* population

therefore, suggests that *Pou4f1* may have a modest inhibitory effect on cell growth stimulated by the presence of AE, although this difference was not statistically significant (p= .22, two-tailed paired t-test).

## Pou4f1 is not required for AE-induced myeloid progenitor self-renewal

To test the importance of these genes for self-renewal of bone marrow myeloid progenitors, we performed serial replating of colonies retrovirally transduced with AE or *Pou4f1* into cytokine-supplemented methocellulose media. In three independent experiments, colonies infected with the AE virus could be replated for at least five weeks (mean =17; range =15-20 per 24 colonies plated), which was significantly longer compared to colonies infected with the *Pou4f1* virus (mean = 2; range = 1-4 per 24 colonies plated; $p<0.001$) or the control virus (mean = 3; range = 0-5;$p<0.001$), indicating that AE, but not *Pou4f1*, is sufficient to enhance progenitor self-renewal (Figure 4). To determine whether cells required *Pou4f1* to self-renew, we serially replated colonies from *Pou4f1*$^{-/-}$, *Pou4f1*$^{+/-}$, or *Pou4f1*$^{+/+}$ fetal liver cells infected with AE or the control virus. Significantly more colonies infected with the AE virus could be replated for 5 weeks (mean = 19; range = 16-24 per 24 colonies), compared to cells infected with the control virus (mean = 4; range = 1-9 per 24 colonies). Similar results were obtained regardless of *Pou4f1* genotype (Figure 4). Therefore, AE promotes self-renewal of myeloid progenitors independent of *Pou4f1*.

## *Pou4f1*-dependent transcriptional profile

Since *POU4F1* dysregulation is not caused by AE, we reasoned that POU4F1 might regulate factors that can cooperate with AE in leukemogenesis. Therefore, we utilized microarray analysis to determine the gene expression profile induced by *POU4F1*. RNA was obtained from flow sorted murine fetal liver cells expressing high, wildtype, or null levels of *Pou4f1*. The global gene expression profiles of all samples were highly similar (>95% correlated); suggesting that over-expression of *Pou4f1* did not dramatically alter the global transcriptional state of murine fetal liver cells, compared to wild-type or *Pou4f1*$^{-/-}$cells. We identified 167 probesets (140 unique genes) that were differentially expressed between at least two experimental groups (Supplementary Table 2). The probeset comparisons were significant between cells expressing high levels of *Pou4f1* compared to cells expressing endogenous levels or no *Pou4f1*. The genes are enriched in several biological pathways relevant for cancer (Supplementary Table 3). *Pou4f1* itself is not part of the gene set, since the *Pou4f1* probesets on the microarray are all 3′ of sequences contained within the transduced *Pou4f1* cDNA.

We next sought to determine whether the set of 140 *Pou4f1*-regulated genes discovered in mouse cells was relevant for human AML. Because *POU4F1* is highly expressed in t(8;21) human AML samples, we hypothesized that the human orthologs of the mouse *Pou4f1* gene set would be enriched in a comparison of AML samples with or without t(8;21). To test this hypothesis, we applied Gene Set Enrichment Analysis (GSEA) to the expression profiles of 111 human AML samples using ratio-of-classes and signal2noise (similar results obtained with both metrics). Significant enrichment of the *Pou4f1* gene set was demonstrated in the t(8;21) positive samples (FDR q-value < 0.001), suggesting that the *Pou4f1* expression signature is robust across species (Figure 5).

Next, we performed unsupervised hierarchical clustering of the human AML samples using the 140 gene (n=285 probeset) *POU4F1* expression signature and found that the t(8;21) samples segregated into a distinct cluster (Figure 5). Because t(8;21) AML samples have a strong, reproducible expression signature (9, 10), these samples might be predicted to cluster using many combinations of 285 probesets. We tested this hypothesis using a random sampling strategy and found that the t(8;21) samples cluster together rarely by chance when gene sets of identical size were used ($P < 0.05$).

Limiting our comparison to the M2 AML patients (n=25), we found that the *POU4F1* gene set again segregated the t(8;21) samples (not shown). When each of the signature genes was tested for differences in expression between samples with or without t(8;21), eight annotated genes were identified (Table 1). Four of these genes show marked (> 2-fold), consistent upregulation in the t(8;21) samples, compared to other AML subtypes (Figure 5).

To ask which genes are have significant differential expression independent of *POU4F1* in t(8;21) AML samples, we used the Significance Analysis of Microarray algorithm after removing the POU4F1 gene set. 115 annotated genes (183 probesets) remained significant in this comparison (Supplementary Table 4).

## discussion

Previous work has demonstrated that expression of the AE fusion gene is not sufficient to induce AML. AE transcripts remain detectable in bone marrow cells from patients with t(8;21) AML in durable remission (5, 31). Expression of an AE cDNA in transgenic mice (6-8) or by retroviral transduction/transplantation (32, 33) does not cause AML, unless additional mutations are induced (6, 7, 32). Gene expression profiling experiments have implicated *POU4F1* as a candidate cooperating factor in t(8;21) AML (9, 26-30). Here, we confirm the observation that *POU4F1* dysregulation is highly correlated with t(8;21) AML and explore the biological consequences of *POU4F1* expression in hematopoietic cells.

Several lines of evidence indicate that *POU4F1* dysregulation is not caused by AE. First, we ectopically expressed the AE cDNA in mouse and human cells by transfection, retroviral transduction, or transgenesis and could not detect differences in *POU4F1* expression under any of these conditions. These results are consistent with previous experiments in cell lines and transgenic zebrafish (34-37). Next, high *POU4F1* expression is detectable in a minority of t(8;21) negative AML cases, suggesting AE is not required for its transcriptional activation. Finally, *Pou4f1* is not expressed in normal hematopoietic or lymphoid cells (38) and is not required for hematopoiesis (11, 13, 14). Taken together, this suggests that AE does not cause *POU4F1* upregulation, nor does it cause *POU4F1* levels to increase by expanding a cellular population that normally expresses *POU4F1*. *POU4F1* and AE, therefore, are concordantly over-expressed in t(8;21) AML, but through independent mechanisms, suggesting that they provide non-redundant signals important for leukemogenesis.

These results support a model in which *POU4F1* upregulation precedes acquisition of the t(8;21). This raises many questions for future investigation, including: what genetic and/or

epigenetic mechanism(s) activate *POU4F1*? Do *POU4F1*-dependent signals facilitate acquisition of the t(8;21) or provide a selective advantage for cells that undergo this translocation? Do *POU4F1* and AE cooperate during induction of leukemia in vivo?

*POU4F1* is a member of the highly conserved family of POU (Pit/Oct/Unc) domain-containing transcription factors. Although we have shown that *Pou4f1* is dispensable for self-renewal of hematopoietic progenitors in vitro, transplantation experiments will be required to address the importance of this factor for pluripotency and self-renewal of hematopoietic stem cells in vivo. The closely related POU family member, *POU5F1* (encoding OCT4), is required to maintain pluripotency in embryonic stem cells (39) and elevated levels drive their differentiation along mesodermal and endodermal lineages (40). *POU5F1* has also been implicated in carcinogenesis. It is over-expressed in breast and germ cell tumors (41, 42). Inducible expression in mice causes dysplasia in the skin and GI tract (43).

Previous studies of POU4F1 have been restricted to neuronal cells, where it has been shown to promote cell survival and inhibit apoptosis, in part by antagonizing p53 and p73 to increase Bcl2 and Bax expression (44, 45). In hematopoietic cells, we observed different effects of Pou4f1 on transcription and cell growth (in fact, Pou4f1 appears to restrain cell growth that was stimulated by AE). These apparently contradictory results may reflect differences in experimental design or cellular context.

We took an unbiased approach to identify transcriptional targets of *Pou4f1* in primary murine hematopoietic cells and then cross-validated this expression signature in primary human AML samples. The *Pou4f1* expression signature is sufficient to cluster t(8;21) AML samples in an unsupervised analysis and the *Pou4f1* gene set is enriched in t(8;21) AML, suggesting that expression of these target genes discovered in mouse is relevant in human cells and preserved in fully transformed leukemias. Four of these genes (*SOX*, *CAV1*, *ROBO1*, *PPADC1B*) are also regulated independently by AE (35-37). The nerve growth factor receptor TrkA has also been shown to be a target of both *Pou4f1* and AE (46, 47). The other four genes in the *POU4F1* signature (*PELI2*, *FAM69B*, *H2AFV*, *PLXDC2*) are *POU4F1*-specific (i.e., they are over-expressed in t(8;21) AML samples, but not activated by AE). These results imply that the gene expression profile of t(8;21) AML is, in part, attributable to *POU4F1* and not solely AE itself.

Several members of the *Pou4f1* gene set have been previously implicated in cancer. SOX4 is a transcription factor important for regulation of embryonic development and determination of cell fate (48-50). Depending on context, SOX4 has both oncogenic (51-53) and tumor suppressor properties (54). *SOX4* is activated by retroviral integration in mice, and cooperates with *Evi1* in the induction of AML (55). *CAV1* is a lipid raft protein that binds to the G-protein coupled receptor, GLP-1, and is involved in subcellular localization, trafficking, and signaling (56). In AML samples, CAV1 and MDR1 colocalize and their expression is highly correlated (57). *ROBO1* is a receptor for *SLIT* and interacts with Rho GTPase activating proteins important for cell motility and angiogenesis (58, 59). Downregulation of *ROBO1* in breast cancer may initiate a metastatic phenotype (60).

*PPADC1B* encodes a transmembrane protein phosphatase that can induce anchorage-independent growth in 3T3 fibroblasts (61).

Although additional work is needed to elucidate the mechanism by which *POU4F1* contributes to t(8;21) AML, it is clear that this transcription factor is a critical driver of disordered gene expression in this disease. Further research on factors both upstream and downstream of *POU4F1* should shed new light on the pathogenesis of t(8;21) positive AML.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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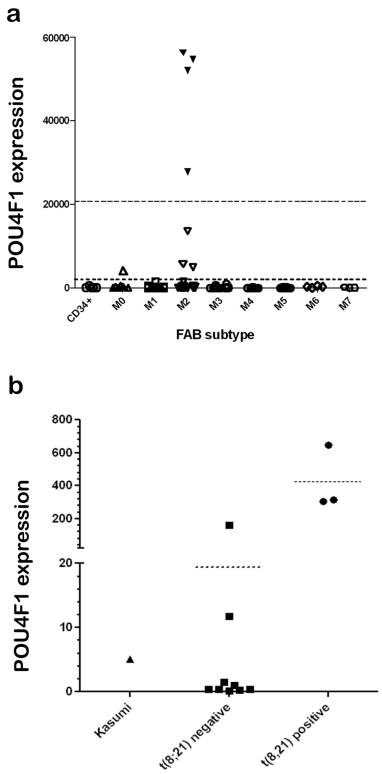


**Figure 1. *POU4F1* is upregulated in t(8;21) AML**

(**a**) Normal human CD34+ bone marrow cells (n=5) and FAB M0-M7 AML patient samples (n=111) were analyzed on the Affymetrix U133 Plus 2.0 array. High *POU4F1* expression is restricted to the M2 samples and is highest in the subset with t(8;21) (filled triangles). The mean across all arrays (dotted line) and 2 standard deviations above (dashed line) for *POU4F1* (probeset 211341_at) are shown. (**b**) qRT-PCR validation demonstrates significantly higher *POU4F1* RNA expression levels in t(8;21) (●) vs. non t(8;21) (■) M2 samples (P<0.0001). The mean expression in each group (dotted line) is shown in comparison to the t(8;21) positive Kasumi cell line.

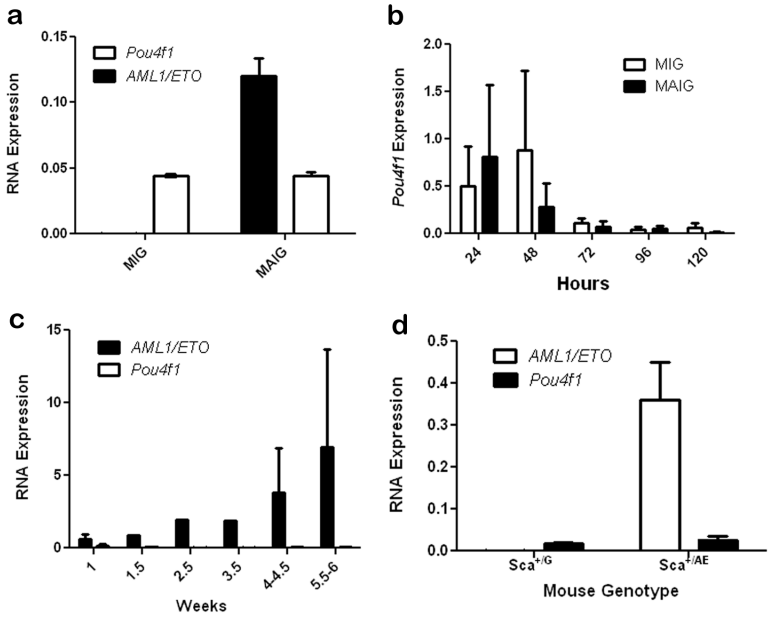


**Figure 2. AML1/ETO does not activate Pou4f1 expression**

(**a**) K562 cells were transfected with *AML1/ETO* (MAIG) or a control vector (MIG) and analyzed by qRT-PCR. *AML1/ETO* is present in MAIG transfected cells, as expected. *Pou4f1* levels are unchanged. (**b**) Mouse bone marrow cells were infected with MAIG vs. MIG and analyzed by qRT-PCR. Mean transduction efficiencies were 19.4% for MIG and 14.9% for MAIG. There is a transient non-specific increase in *Pou4f1* levels after infection, but no significant difference in cells expressing *AML1/ETO* vs. control. (**c**) *AML1/ETO* expression increases over 5 weeks in MAIG-infected bone marrow cells with no detectable expression of *Pou4f1*. (**d**) *Pou4f1* is expressed at similar levels in bone marrow cells from mice heterozygous for either GFP or *AML1/ETO* targeted to the *Sca1* locus.

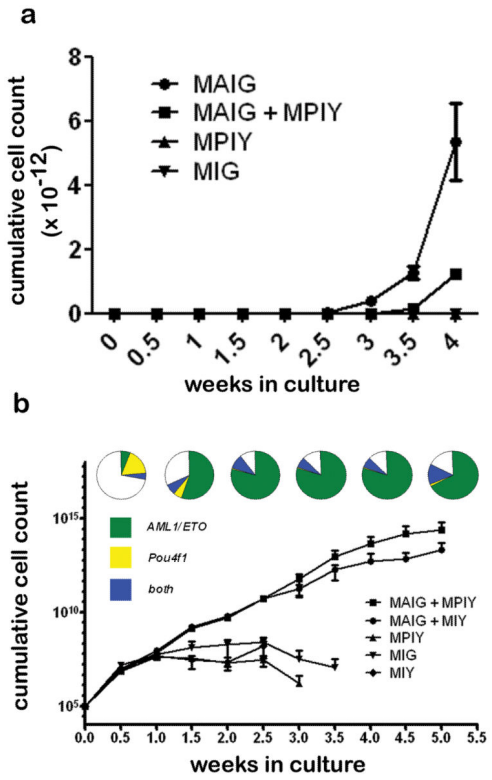


**Figure 3. Pou4f1 does not affect the growth of bone marrow cells**

(**a**) Mouse bone marrow cells were transduced with *AML1/ETO* (MAIG), *POU4F1 (MPIY)*, or a control vector (MIG), positively selected by flow sorting, and cultured for up to 5 weeks, with cell counts and replating performed twice weekly. Cell growth was significantly enhanced by *AML1/ETO*, but not by *Pou4f1*. (**b**) Bone marrow cells were transduced as in (**a**) and cultured directly without sorting. The proportion of cells expressing *AML1/ETO* (GFP+), *Pou4f1* (YFP+), or both (GFP+YFP+) are shown at weekly intervals for cells transduced with both *AML1/ETO* (MAIG) and *Pou4f1* (MPIY). The relative expansion of *AML1/ETO* single positive cells (green) is greater than the expansion of *AML1/ETO*/*Pou4f1* double positive cells (blue).

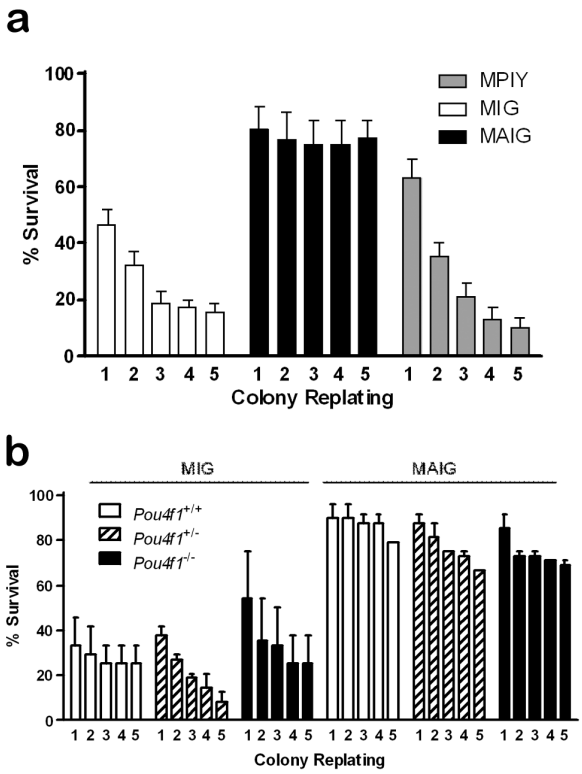


**Figure 4. Effect of *AML1/ETO* and *POU4F1* on self-renewal of hematopoietic progenitors**

(**a**) Bone marrow cells infected with *AML1/ETO* (MAIG), *Pou4f1* (MPIY), or control (MIG) virus were serially replated in methylcellulose. Colonies expressing *AML1/ETO* have increased self-renewal. (**b**) Fetal liver cells from *Pou4f1*$^{-/-}$, *Pou4f1*$^{+/-}$ or *Pou4f1*$^{+/+}$ mice were transduced with AML1/ETO (MAIG) or control (MIG) virus and serially plated in methylcellulose. *Pou4f1* deficiency does not impair colony self-renewal induced by AML1/ETO.

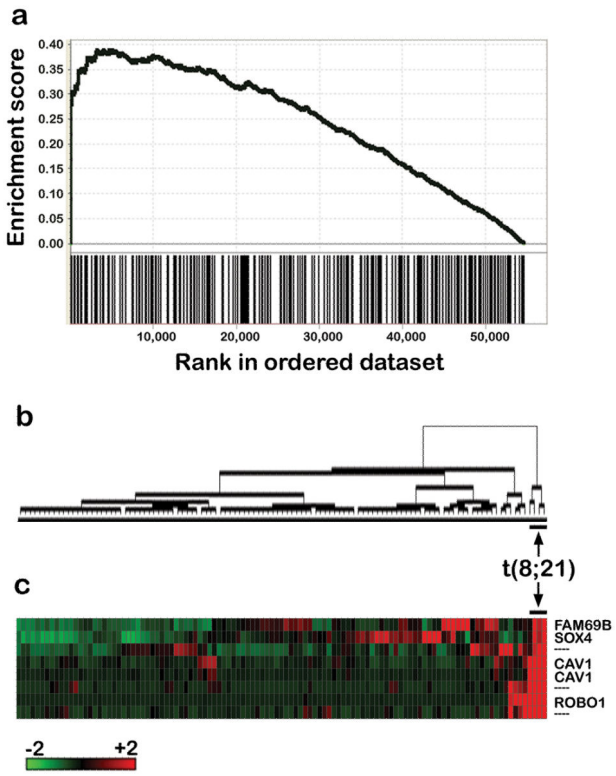


**Figure 5. Contribution of *Pou4f1* target genes to the transcriptional profile of t(8;21) AML**

(**a**) Gene Set Enrichment Analysis was performed on de novo AML samples (n=111) using the human orthologs of the 140 genes differentially regulated by *POU4F1* expression. The *POU4F1* gene set is significantly enriched in human t(8;21) positive AML samples (FDR<0.001). (**b**) Dendrogram of AML samples after unsupervised hierarchal clustering using the *Pou4f1* gene set demonstrates segregation of the t(8;21) patients. (**c**) Four annotated genes (and 3 unannotated probesets) are consistently and significantly ($P < 0.01$) differentially expressed between AML samples with or without t(8;21).

## Table 1

Gene expression differences in FAB M2 AML samples with or without t(8;21).

| Gene | Fold Change* |
|---|---|
| *CAV1* | 34.48 |
| *CAV1* | 11.78 |
| *ROBO1* | 26.01 |
| *FAM69B* | 2.54 |
| *SOX4* | 1.97 |
| *PELI2* | 1.69 |
| *PPAPDC1B* | 1.56 |
| *H2AFV* | 1.49 |
| *PLXDC2* | 0.12 |

* Fold change = mean expression in t(8;21) samples divided by mean expression in AML samples without t(8;21). All values are significant by unpaired two-tailed t-test ($P < 0.01$).