

Transcriptional Profiling of Polycythemia Vera Identifies Gene Expression Patterns Both Dependent and Independent from the Action of JAK2V617F

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Abstract

Purpose: To understand the changes in gene expression in polycythemia vera (PV) progenitor cells and their relationship to JAK2V617F.

Experimental Design: Messenger RNA isolated from CD34⁺ cells from nine PV patients and normal controls was profiled using Affymetrix arrays. Gene expression change mediated by JAK2V617F was determined by profiling CD34⁺ cells transduced with the kinase and by analysis of leukemia cell lines harboring JAK2V617F, treated with an inhibitor.

Results: A PV expression signature was enriched for genes involved in hematopoietic development, inflammatory responses, and cell proliferation. By quantitative reverse transcription-PCR, 23 genes were consistently deregulated in all patient samples. Several of these genes such as *WT1* and *KLF4* were regulated by JAK2, whereas others such as *NFIB* and *EVI1* seemed to be deregulated in PV by a JAK2-independent mechanism. Using cell line models and comparing gene expression profiles of cell lines and PV CD34⁺ PV specimens, we have identified panels of 14 JAK2-dependent genes and 12 JAK2-independent genes. These two 14- and 12-gene sets could separate not only PV from normal CD34⁺ specimens, but also other MPN such as essential thrombocytosis and primary myelofibrosis from their normal counterparts.

Conclusions: A subset of the aberrant gene expression in PV progenitor cells can be attributed to the action of the mutant kinase, but there remain a significant number of genes characteristic of the disease but deregulated by as yet unknown mechanisms. Genes deregulated in PV as a result of the action of JAK2V617F or independent of the kinase may represent other targets for therapy. *Clin Cancer Res*; 16(17); 4339–52. ©2010 AACR.

Philadelphia chromosome-negative myeloproliferative neoplasms (MPN) are clonally derived hematopoietic malignancies that include polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (MF). The unregulated expansion of mature hematopoietic cell in MPN suggests that the hematopoietic stem progenitor cell has lost key homeostatic factors. The presence of the acquired somatic mutation JAK2V617F is found in ~95% of cases of PV and 50% of ET and MF (1), and helps explain uncontrolled hematopoiesis in MPN. However, the mechanism by which a single mutation contributes to the

pathogenesis of three clinically distinct disorders is still unclear. Retroviral transduction of JAK2V617F into murine bone marrow cells, followed by transplantation of these cells into irradiated recipients, yields erythrocytosis but not thrombocytosis (2). Acquired uniparental disomy of chromosome 9p24, which includes the JAK2 locus, is common in PV and MF, but only a rare event in ET (3, 4), suggesting a role of allele burden in the phenotypic manifestation of MPN. This notion is supported by a transgenic mouse model for MPN, in which mice expressing

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Translational Relevance

To understand the molecular basis of polycythemia vera (PV), we profiled the gene expression in CD34⁺ cells from a set of patients with PV and contrasted this with the gene expression mediated by the inhibition of JAK2V617F activity in cell lines or overexpression of JAK2V617F in normal CD34⁺ cells. We found that a significant amount of gene deregulation in PV and other myeloproliferative neoplasm can be attributed to the activity of JAK2, but there remains a set of gene deregulated and a characteristic of PV that appears independent of JAK2 action. Genes deregulated in PV as a result of the action of JAK2V617F or independent of the kinase may represent potential therapeutic targets.

JAK2V617 at a level lower than wild-type (WT) JAK2 develop an ET-like phenotype, whereas JAK2V617F expressed at levels similar to WT JAK2 results in a PV phenotype (5).

The recapitulation of MPN in animal models by enforced expression of JAK2V617F clearly implicates this mutant kinase in the pathogenesis of disease. However, emerging evidence suggests that there may be other underlying factors at play in the genesis of MPN. The presence of a subpopulation of clonal cells in patients with MPN that lack JAK2 mutation (6) suggests that other molecular lesions in addition to JAK2V617F may contribute to the MPN phenotype and may precede acquisition of JAK2V617F. *TET2*, recently identified as mutated in MPN, may represent one such lesion (7–9). It has been long known that certain chromosomal anomalies such as deletion of 20q can be associated with MPN, and recent data suggest that this is a genetic event independent from the acquisition of JAK2V617F (10). Finally, genome-wide association studies showed that inheritance of a polymorphism outside of the coding region of JAK2 was associated with an increased incidence of MPN, suggesting that altered JAK2 gene expression or regulation in hematopoietic precursor cells could contribute to the acquisition of the JAK2V617F mutation and disease development (11, 12).

To better understand the molecular events underlying PV, we compared the gene expression profiles of CD34⁺ cells purified from the marrow of PV patients with normal CD34⁺ cells and defined a set of genes characteristic of PV. Using cell line models of the action of JAK2V617F by either overexpression or inhibition, we identified sets of genes regulated by JAK2 that could be found within the PV signature set. Comparing with the PV data set, we defined JAK2-dependent and JAK2-independent gene sets to predict other forms of MPN by class prediction analysis. Both sets accurately distinguished MPN from normal controls. These data indicate that the expression profile of PV can be explained in part by the action of JAK2V617F.

However, a component of gene expression aberrant in PV but independent of JAK2 action may also play a role in PV pathogenesis.

Materials and Methods

Specimen isolation and processing

Bone marrow specimens from nine PV patients were obtained after informed consent and approval from the Institutional Review Board of the Mount Sinai School of Medicine. Six of the PV patients had normal karyotype, whereas one patient possessed del5q31, one presented with trisomy 8, and one had several anomalies of chromosome 9. Information on time from diagnosis to sampling and therapies used before sampling are presented in Supplementary Data S1. The JAK2V617F mutation was detected in seven of nine specimens by reverse transcription-PCR (AmpliTaQ Gold) and directly sequencing JAK2 sequences of 1,849 nucleotides amplified. Normal bone marrow CD34⁺ cells were purchased from Allcells. To isolate CD34⁺ cells, bone marrow samples were diluted 1:1 in PBS and layered onto Histopaque 1077 (Sigma; 1.077 discontinuous gradient) to a final concentration of 36% histopaque. CD34⁺ cells were positively selected by two rounds of CD34⁺ affinity purification using MiniMACS MS columns (Miltenyi Biotec). Flow cytometry showed the cells to be 98% CD34⁺ (CD34-PE, BD Biosciences) after the first purification, and $\sim 2 \times 10^5$ CD34⁺ cells were obtained from each specimen. RNA was harvested from CD34⁺ cells (RNeasy MiniKit), subjected to two rounds of linear amplification with 3 μ g of total RNA being used in the first round, and 2 μ g of first round product labeled with biotinylated CTP and UTP (Biotin-11-CTP, Biotin-16-UTP) in the second amplification (MessageAmp aRNA kit, Ambion). Following fragmentation, the final RNA probe was hybridized to HG-U133A GeneChip (Affymetrix; Genomics Core, Mount Sinai School of Medicine).

Cell culture

HEL (American Type Culture Collection; ref. 13) and UKE-1 (gift of Dr. Gabi Vohwinkel, University Hospital Eppendorf, Hamburg, Germany; ref. 14) cells were previously described. HEL cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. UKE-1 cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal bovine serum, heat-inactivated 10% horse serum, 1 μ mol/L hydrocortisone (SIGMA), 100 U/mL penicillin, and 100 μ g/mL streptomycin in the presence or absence of 1 to 2 μ mol/L JAK2 Inhibitor I (Calbiochem). Cell viability was determined by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Total RNA was extracted from HEL and UKE-1 cells in biological triplicate using the RNeasy MiniKit (Qiagen) and profiled using the HumanWG-6_V3 bead chip (Illumina) at the genomics core of the Cleveland Clinic Cancer Center.

CD34⁺ cell culture

CD34⁺ cells were cultured at a density of 3×10^3 /mL in six-well plates; in serum-free expansion media (StemCell Technologies) containing bovine serum albumin, recombinant human insulin, iron-saturated human transferrin, 2-mercaptoethanol, and L-glutamine in Iscove's modified Dulbecco's medium, 100 U/mL penicillin, and 100 μ g/mL streptomycin supplemented with one of the following cytokine cocktails: maintenance and expansion media: 100 ng/mL FLT3-Ligand, 100 ng/mL stem cell factor (SCF), and 100 ng/mL thrombopoietin; full myeloid outgrowth media: 50 ng/mL SCF, 20 ng/mL interleukin (IL)-3, 1 U/mL erythropoietin (EPO), 20 ng/mL IL-6, 20 ng/mL IL-11, 10 ng/mL thrombopoietin, 20 ng/mL IL-1 β , and 10 ng/mL granulocyte/macrophage colony stimulating factor (GM-CSF); full erythroid outgrowth media +EPO: 50 ng/mL SCF, 20 ng/mL IL-3, 10 ng/mL GM-CSF, and 1 U/mL EPO; erythroid outgrowth media -EPO: 50 ng/mL SCF, 20 ng/mL IL-3, and 10 ng/mL GM-CSF (all cytokines from Stem Cell Technologies). Normal human CD34⁺ cells, untransduced or nucleofected with JAK2 or JAK2V617F expression vectors, were grown in liquid culture for 3 days in triplicate; RNA was extracted as above and profiled using HG-U133A chips.

Microarray data analysis

PV specimen data were initially analyzed using GeneSpring GX (Stratagene, Agilent Technologies) after quantile normalization using RMA or GC-RMA algorithms. Significance analysis was done using the unpaired T tests, and differentially expressed genes were selected using a 2-fold cutoff and $P < 0.05$. In parallel, feature selection of the PV versus normal was done to ascertain probe sets able to discriminate between patients and healthy donor, by mean of rank product analysis [false-positive predictions (pfp) ≤ 0.05 , 500 permutations; ref. 15].

Normal human CD34⁺ cells nucleofected with JAK2 or JAK2V617F or untransduced were profiled using Affymetrix HG-U133A arrays. The rank product analysis was applied to identify the set of genes differentially regulated in the overexpression of JAK2. A classifier based on shrunken centroids method was applied to identify a JAK2-dependent signature gene set to evaluate the ability of this gene set to discriminate between PV patients and controls (16).

HEL and UKE-1 cells were profiled in triplicate using Illumina Beadchips v3, before and 24 hours after treatment with JAK inhibitor I. Unnormalized raw signal intensities from Illumina Beadchips v3 were generated by BeadStudio software (<http://www.illumina.com>) and loaded on oneChannelGUI (17) Bioconductor (18) package in which values were \log_2 transformed and normalized using the cyclic loess (19). The number of genes evaluated was reduced by applying both an interquartile (IQR < 0.25) and an intensity filter (i.e., removing all probe sets below 0 intensity in $>50\%$ of the arrays) to remove the nonsignificant probe sets (20). Probes in common between the two data sets were selected and analyzed using the integrative correlation coefficient (IC; ref. 21). Using

this method, only the subset characterized by an IC of ≥ 0.3 were kept for further analysis. Feature selection of transcripts differentially expressed was addressed using the so-called rank product nonparametric method (15). This method addresses the multiple comparison problem and performs P value correction by false discovery rate, comparing the true rank product distribution with a random one defined, permutating gene labels in each of the arrays under analysis. Here, we have used 500 permutations and a threshold of percentage of pfp of 0.05.

Real-time expression analyses using Taqman low-density arrays

Customized Taqman low-density arrays (TLDA; Applied Biosystems) representing a subset of 139 differentially expressed genes (at least 2-fold difference, $P < 0.05$ in the gene list from either GC-RMA analysis or Rank product analysis of PV specimens) and 16 controls (ACTB, ACTIN, B2M, GAPDH, GUSB, HMBS, HPRT1, IPO8, PGK1, POLR2A, PPIA, RPLP0, TBP, TFRC, UBC, and YWHAZ) were done to validate microarray analysis using available RNA samples from the same three patients as in microarray experiments and two additional PV patients, and the same four controls with one more control added. Total RNA was converted to cDNA using the Taqman RNA Reverse Transcription kit (Applied Biosystems). cDNA from each sample was mixed with the PCR Master Mix (Applied Biosystems), and the reactions were run in an Applied Biosystems 7900HT Fast Real-Time PCR system at 94.5°C for 10 minutes, followed by 40 cycles at 97°C for 30 seconds and 60°C for 1 minutes. Expression changes were analyzed using the StatMiner software (Integromics), and Δ Ct values from each gene were also normalized against two control genes (*HMBS* and *UBC*) that showed less variability among all samples (chosen by the StatMiner software). Fold changes in gene expression with a P value of <0.05 were calculated by StatMiner using the formula $\text{Log}_{10}\text{RQ} = \text{Log}_{10} 2^{-(\Delta\text{Ct})}$ ($\text{Log}_{10}\text{RQ} = 0$ corresponds to no expression change; $\text{Log}_{10}\text{RQ} = 1$ means that the test sample is expressed at a level 10 times greater than in the calibrator sample; $\text{Log}_{10}\text{RQ} = -1$ means that the test sample is expressed at a level 10 times less than in the calibrator sample). MeV4.1 (MeV: MultiExperiment Viewer; ref. 22) was used to generate unsupervised hierarchical clustering based on Support tree average linkage with Euclidean distance and Pearson correlations.

Retroviral infection and differentiation assays

For virus production, subconfluent 293T cells were transfected using Fugene6 (Roche) with a total of 6 μ g DNA including equal amounts of MIGR1-JAK2-IRES-GFP or MIGR1-V617F-IRES-GFP (2), which express human WT or mutant JAK2 cDNAs, respectively, and GP/ENV (containing the gag/pol genes but lacking an envelope protein) and pMD.G packaging plasmids (vesicular stomatitis virus G protein, viral envelope protein; gift of Luca Gisella, Mount Sinai School of Medicine, New York, NY). Supernatant containing viral particles was collected at 24, 48, and

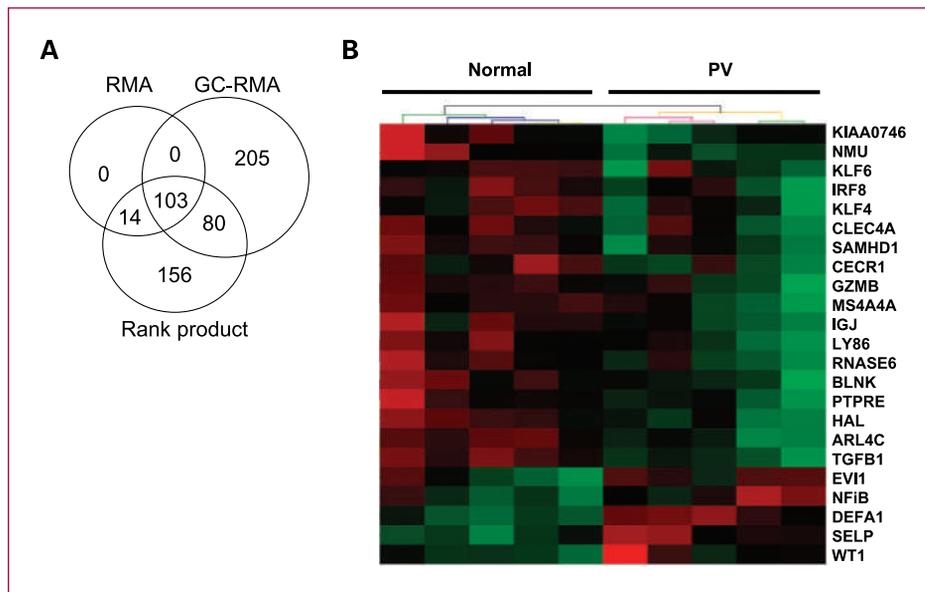


Fig. 1. A, venn diagram showing probe sets identified by GeneSpring GX analysis with a cut off of $FC \geq 2$ ($P < 0.05$) after either RMA or GC-RMA normalization and by rank product analysis. B, supervised hierarchical clustering using Euclidian distance for 23 genes differentially expressed ($P < 0.05$) in PV CD34⁺ cells compared with controls cells as identified by oligonucleotide microarray and verified by TLDA between normal and PV samples. Red, upregulated genes; green, downregulated genes.

72 hours posttransfection and filtered through a low-protein-binding 0.45- μ m filter. Virus was concentrated, resuspended in 200 μ L of PBS, and stored at -80°C . Virus with green fluorescent protein (GFP) visible at a 1×10^{-2} dilution in 293T was used in each experiment. For infections, 1×10^6 CD34⁺ cells in 100 μ L were incubated with 50 μ L of viral stock in serum-free expansion media containing polybrene (5 μ g/mL) and incubated for 48 hours. Transduction efficiency was $\sim 60\%$ as determined by GFP expression. For differentiation liquid culture experiments, cells were cultured either in full myeloid outgrowth media, full erythroid outgrowth media +EPO, or erythroid outgrowth media -EPO media. Differentiation was assessed by flow cytometry for CD71 and glycophorin A (GpA) after 10 days (BD Biosciences). For myeloid and erythroid colony forming assays, cells were plated in triplicate at a density of 1×10^4 cells/mL in methylcellulose (methylcellulose in Iscove's modified Dulbecco's medium, fetal bovine serum, bovine serum albumin, 2-mercaptoethanol, L-glutamine, rhSCF, rhGM-CSF, rhIL-3, and rhEPO; Methocult Complete, StemCell Technologies). Myeloid and erythroid colonies were scored on day 14.

Nucleofection and differentiation assays

Nucleofection of human CD34⁺ cells was done using the Amaxa Human Progenitor kit according to the manufacturer's specifications. MIGR1, MIGR1-JAK2WT, MIGR1-JAK2V617F, and MIGR1-Tel-JAK2 plasmids (18 μ g) were added to cells and nucleofected using Program U-008, and cells were cultured in maintenance and expansion media for 48 hours. Transduction efficiency as determined by GFP expression was 60% to 80%. For differentiation liquid culture experiments, cells were cultured in either full myeloid outgrowth media, full erythroid outgrowth media +EPO, or erythroid outgrowth media -EPO media.

Differentiation was assessed by flow cytometry for CD71 and GpA after 5 days. For analysis of gene expression, cells were collected after 4 days, and total RNA was harvested and hybridized in biological triplicate to HG-U133A gene-chips (Affymetrix).

Results

Gene expression profiling of PV

As indicated in Supplementary Data S1, two patients did not have JAK2V617F mutations. However, when we performed unsupervised hierarchical clustering of all patient samples, the expression profiles of these two patients were similar to those of the other patients and distinct from normal controls, suggesting that these patients may have harbored exon 12 mutations of JAK2 (data not shown). Given the relatively uniform gene expression pattern among all of the PV specimens, we grouped the patients together in further analysis. To identify PV signature genes, we applied both RMA and GC-RMA normalization algorithms before significant analysis. Significant analysis of the RMA-normalized gene expression profiles showed 117 probe sets (104 genes) differentially expressed (≥ 2 -fold, $P < 0.05$) in PV versus normal specimens, whereas the GC-RMA normalization produced 388 probe sets with the same cut-off (Supplementary Data S2 and S3). In both cases, the vast majority of these genes were downregulated in the PV specimens compared with the controls.

Because the different methods of significant analysis produce a slightly different set of differentially expressed genes, we have also analyzed these PV patients array data set with mean of rank product analysis ($p_{\text{pf}} \leq 0.05$, 500 permutations; Supplementary Data S4), which is useful to analyze and compare different platforms of array

data in later studies in our model cell lines. This analysis yielded a total of 353 probe sets (PV signature). Comparing this set with RMA and GC-RMA analysis lists, all 117 probe sets from RMA analysis were identified in the list from the rank product analysis and 183 probe sets were shared between GC-RMA and rank product analysis (Fig. 1A). One hundred three probe sets were selected by all three methods. We used a custom TLDA platform (Applied Biosystem) to validate randomly selected genes (139 genes) among genes identified by either the GeneSpring data set or rank product analysis, and did real-time quantitative reverse transcription-PCR with three of the original nine PV, two additional PV specimens, four original donor specimens, and one additional normal donor for which additional RNA was available. Most of genes tested were validated in this subset of patients, and the TLDA results generally paralleled those of the microarray analysis (data not shown). Among the genes validated by TLDA, we identified 23 that were consistently and strongly deregulated among the PV samples ($P < 0.05$; Fig. 1B).

To identify pathways most significantly dysregulated in PV specimens, we analyzed the 103 probe sets identified by all three methods (Fig. 1A) using the Ingenuity Pathways Analysis. Genes involved in inflammatory response (27 genes), cellular growth and proliferation (33 genes), and hematologic system development and functions (30 genes) were most significantly affected (Supplementary Data S5). Genes in pathways implicated in B-cell development (DNMT and HLA-DRA), antigen presentation pathway (HLA-DPA1 and HLA-DRA), and B-cell receptor signaling (BCL6, BLNK, EGR1, and NFATC3) were all repressed. This suggests that the fate of the hematopoietic progenitors of the patients had indeed been altered, consistent with the myeloproliferative phenotype.

The overexpression of JAK2V617F induced erythroid expansion dependent of EPO in a human hematopoietic progenitor model

To determine the biological and genetic activity of mutant JAK2 in human hematopoiesis, human JAK2V617F was ectopically expressed in normal human CD34⁺ cells, with 60% to 80% efficiency as determined by flow cytometry for GFP expression. These cells were allowed to differentiate in liquid culture or in methylcellulose in media with or without EPO. WT JAK2 was transduced as a negative control, and TEL-JAK2, an oncogenic fusion protein (23), was inserted into these cells as positive control. After 10 days, the percentage of cells expressing GpA and the proliferative marker CD71 was determined by flow cytometry. When grown in media containing EPO, cultures transduced with JAK2V617F or TEL-JAK2 yielded more mature GpA⁺, CD71⁻ cells (63%) than cultures expressing WT JAK2 (19%; Fig. 2A). Similar results were obtained when human bone marrow CD34⁺ cells were transiently transfected with the same constructs used in the viral infections. At day 5 postnucleofection, 75% of mutant JAK2- nucleofected cells were more mature GpA⁺ CD71⁻ compared with 43% for the WT cells. The cell cycle profile

of cells stably expressing JAK2V617F and WT JAK2 was identical (data not shown). These data suggest that cells progress more quickly through the differentiation program in the presence of JAK2V617F than in the presence of WT JAK2. The activity of JAK2V617F was also scored in colony forming assays. In the presence of EPO, CD34⁺ cells transduced with JAK2V617F yielded significantly more erythroid and myeloid colonies than those containing WT JAK2 (Fig. 2B). In the absence of EPO, colony formation was low regardless of which construct was transduced into the cells. Collectively, these data indicate that JAK2V617F can cause erythroid expansion, but this requires the continued presence of EPO.

Genes regulated by JAK2 and JAK2V617F in normal CD34⁺ cells

To identify the downstream effects of aberrant JAK2V617F signaling, we profiled mRNA harvested from cultures of normal human CD34⁺ cells (control)

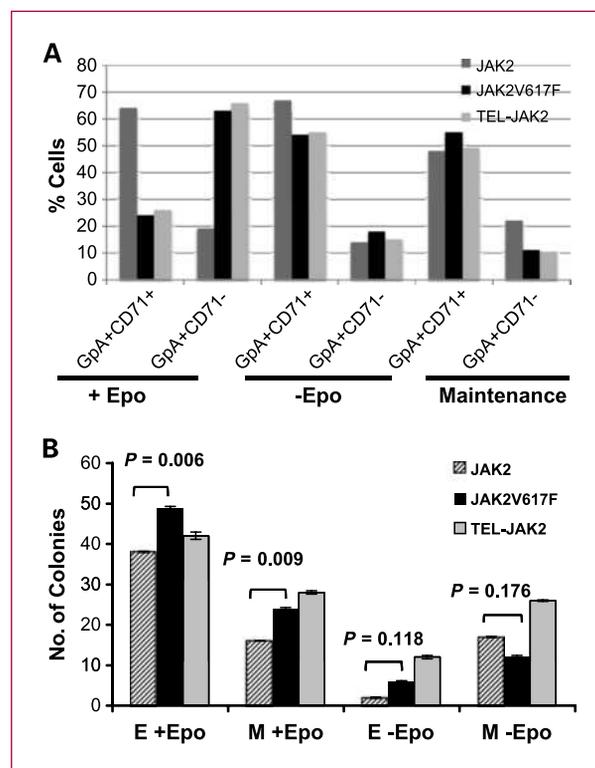


Fig. 2. A, erythroid differentiation of CD34⁺ cells transduced with JAK2. Fluorescence-activated cell sorting analysis for GpA and CD71 cell surface antigens was done at 10 d posttransduction with WT JAK2, JAK2V617F, or Tel-JAK2. Cells were grown in full erythroid media +/- EPO or maintenance media. The experiment was repeated thrice with similar results obtained. B, cells were grown in methylcellulose containing or lacking EPO for 12 d after transduction with the indicated JAK2 allele. The average (\pm SEM) of erythroid or myeloid colonies was determined from counts of eight replicate plates of cells. Statistical significance was calculated (Student's *t* test) comparing JAK2V617F and JAK2wt. The experiment was repeated thrice with similar results obtained.

or triplicate cultures of CD34⁺ cells nucleofected with WT or mutant JAK2 (V617F), in the presence of EPO. Differential expression was estimated with the rank product method ($p_{fp} \leq 0.05$, 500 permutations) comparing WT versus control, and V617F versus control. Three hundred five probe sets (Supplementary Data S6) and 168 probe sets (Supplementary Data S7) were identified as differentially expressed between JAK2 and control, and between JAK2V617F and control, respectively. Comparing the gene lists generated by the enforced expression of JAK2 versus control and JAK2V617F versus control in CD34⁺ cells, 83% of the genes differentially expressed in response to JAK2V617F were in common with genes regulated in response to JAK2 (Fig. 3A). Hence, despite the increased biological activity of JAK2V617F in stimulating erythroid expansion, JAK2V617F produced a response very similar to that of CD34⁺ cells overexpressing the WT JAK2. Overexpression of the WT JAK2 in CD34⁺ cells modulated ~50% more genes than JAK2V617F. Unsupervised hierarchical clustering of the three groups of genes showed that genes regulated in common in response to JAK2V617F and JAK2 segregated normal from WT and mutant JAK2-expressing cells (Fig. 3B, left). Genes specifically regulated in response to WT JAK2 did not distinguish between WT JAK2 and

mutant JAK2-expressing cells, revealing that these genes were actually regulated in a similar manner in these samples (Fig. 3B, middle). By contrast, those genes specifically regulated in JAK2V617F-transduced cells were not homogeneously expressed in cells harboring WT JAK2 (Fig. 3B, right). The smaller set of genes identified in JAK2V617F-expressing cells may be due in part to the limited statistical power of the JAK2V617F data set, consisting of two arrays, whereas the JAK2 data set contained three arrays. Nevertheless, it is clear that there were some genes regulated by JAK2V617F that were not affected by WT JAK2 (Supplementary Data S8). Among these were Cyclin A2, the heat-shock 70-kDa protein 4 and 9 (*HSPA4* and *HSPA9*), members of the heat-shock protein family overexpressed in a wide range of human cancers, the Rh blood group-associated glycoprotein (*RHAG*); and genes involved in mitochondrial metabolism such as NADH:ubiquinone oxidoreductase (*NDUFS1*) and mitochondrial intermediate peptidase (*MIPEP*). This genetic activity is consistent with an expanded biological activity of the V617F allele of JAK2. However, the majority of the transcriptional activity of JAK2V617F in CD34⁺ cells was similar to the action of WT JAK2 (Fig. 3A). Although there are 26 genes exclusively regulated by JAK2V617F in our system (Supplementary Data S8, second column),

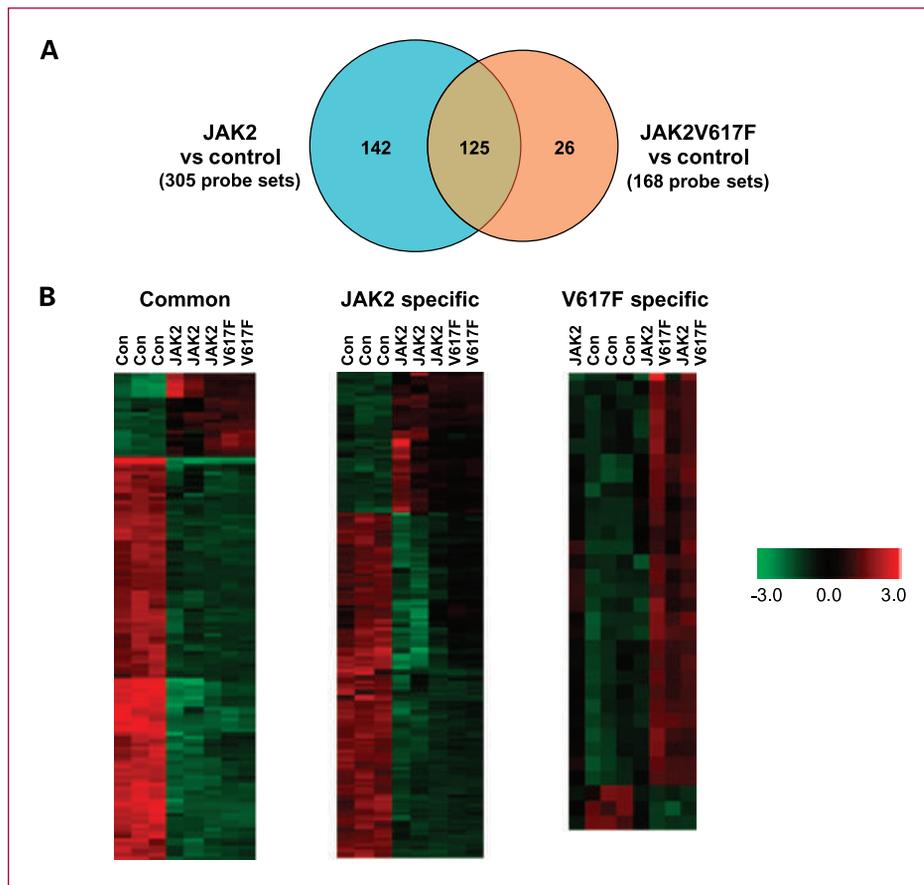


Fig. 3. A, venn diagram showing the overlap between the set of genes found differentially expressed in CD34⁺ cells transduced with WT JAK2 when compared with control cells and in cells transduced with JAK2V617F when compared with controls. B, hierarchical clustering of the genes differentially expressed in cells transduced with either JAK2 or JAK2V617F when compared with untransduced cells: genes found in common (left), genes specific to JAK2 (middle), genes specific to JAK2V617F (right).

we found that several of these genes were previously identified as genes regulated by WT JAK2 such as *HGF* (24), *TFAM* (25), and *TXN* (26).

To complement the gain of function experiments identified the transcriptional consequences of the JAK2V617F mutation, we documented the transcriptional changes in response to an inhibitor of JAK2 in cell lines harboring JAK2V617F. Treatment of HEL or UKE cells with JAK2 Inhibitor I induced a dose-dependent inhibition of cell growth in both these JAK2V617F-positive leukemia cell lines but not in the BCR-ABL-transformed K562 cells (data not shown). Probes behaving similarly in terms of activation or repression in the two different cell lines were detected using IC (27), which quantifies cross-study reproducibility without relying on direct assimilation of expression measurements across experiments. The probe subset characterized by an IC of ≥ 0.3 was kept for further analysis. Principal component analysis (Supplementary Data S9A) on this subset of genes showed that the pattern of gene expression of these cells at baseline is quite different (PC1), but gene expression in the two cell lines shifted in a similar manner in response to the JAK2 inhibitor (PC2). Genes consistently differentially expressed in the two cell lines upon the inhibitor treatment were defined using the Rank Product statistics (15) considering as the "batch effect" the different cell lines used (Supplementary Data S9B and S10). There was very limited overlap between the JAK2 overexpression and JAK2 inhibition expression data sets (Supplementary Data S9C). This could have been due to the different cellular systems (CD34⁺ cells versus leukemia cell lines) and array (Illumina versus Affymetrix) systems used. Nevertheless, Ingenuity Pathways Analysis showed that many of the same pathways were affected upon JAK2 overexpression or inhibition (Supplementary Data S9D).

To ascertain whether the gene characteristics of PV CD34⁺ cells were regulated by JAK2, we measured the expression of a subset of six genes by real-time PCR after JAK2 inhibition in our cell line models (Fig. 4A). *WT1* was overexpressed in PV specimens, and treatment with a JAK2 inhibitor suppressed *WT1* expression in HEL or UKE cells but had no effect on *WT1* in K562 cells (Fig. 4A). *BCL6* and *FLT3*, both showing decreased expression in PV relative to controls, were upregulated upon JAK2 inhibitor treatment of HEL and UKE but not K562 cells. By contrast, three genes deregulated in the PV specimens, *EVI1* (upregulated), *SEPT6*, and *KLF6* (both downregulated), were not affected by the JAK2 inhibitor in HEL or UKE cells (Fig. 4B). This suggests that only part of the gene deregulation found in the PV specimens can be attributed to the action of JAK2V617F.

Predication of MPN using gene sets derived from PV, JAK2 overexpression, and JAK2 inhibition

The set of genes associated with JAK2 inhibition as measured by Illumina arrays (Supplementary Data S10) that had annotated Entrez gene identifiers were remapped onto 195 Affymetrix probe set identifiers. Although the queried

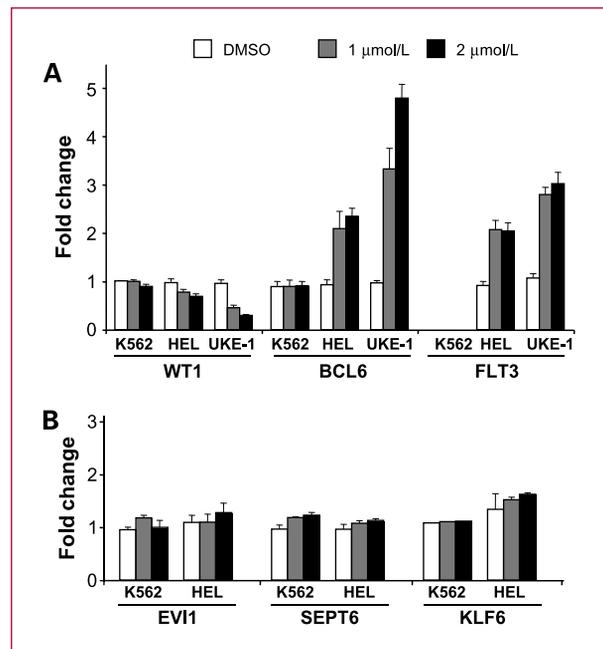


Fig. 4. A, expression of *WT1*, *BCL6*, and *FLT3* mRNA in HEL and UKE-1 cells (mutant JAK2) and K562 (wt JAK2; BCR/ABL positive) as measured by reverse transcription-PCR, at 24 h posttreatment with 1 or 2 $\mu\text{mol/L}$ JAK inhibitor I. B, expression levels of *EVI1*, *SEPT6* after treatment as in A.

data set is small, the 195 probe sets can distinguish between patients and control specimens (Fig. 5A, top). For JAK2-dependent signature genes, we applied a classifier based on shrunken centroids method (16) to the set of genes detected in the JAK2 overexpression (Fig. 3A, 304 probe sets) and identified a set of 14 genes (Table 1A). These JAK2-dependent genes separated disease and normal specimens with high efficiency (Fig. 5A, middle). Furthermore, we defined the JAK2-independent PV signature by subtracting JAK2 inhibition/overexpression signatures from PV signature to test its ability to discriminate between patients and normal donors (Fig. 5B). The shrunken centroids method yielded a total of 12 JAK2-independent genes (Table 1B) as the best group to allow a separation between patients and control group. The high level of discrimination between patient and control samples could be due to overfitting (i.e., the genes most different between sets were chosen to differentiate those same sets; Fig. 5A, bottom left).

To determine if these results could be more generalized to other forms of MPN, we tested whether the JAK2 inhibition probe sets and JAK2-dependent and JAK2-independent signature genes could distinguish between patients with ET from normals (28). Of note, these latter gene expression signatures were obtained from platelets of ET patients and not CD34⁺ cells. Nevertheless, the JAK2 inhibition signature accurately distinguished normal from ET. The JAK2-dependent and JAK2-independent PV signature were generally able to correctly classify the specimen but with less reliability in cross-validation (Fig. 5A).

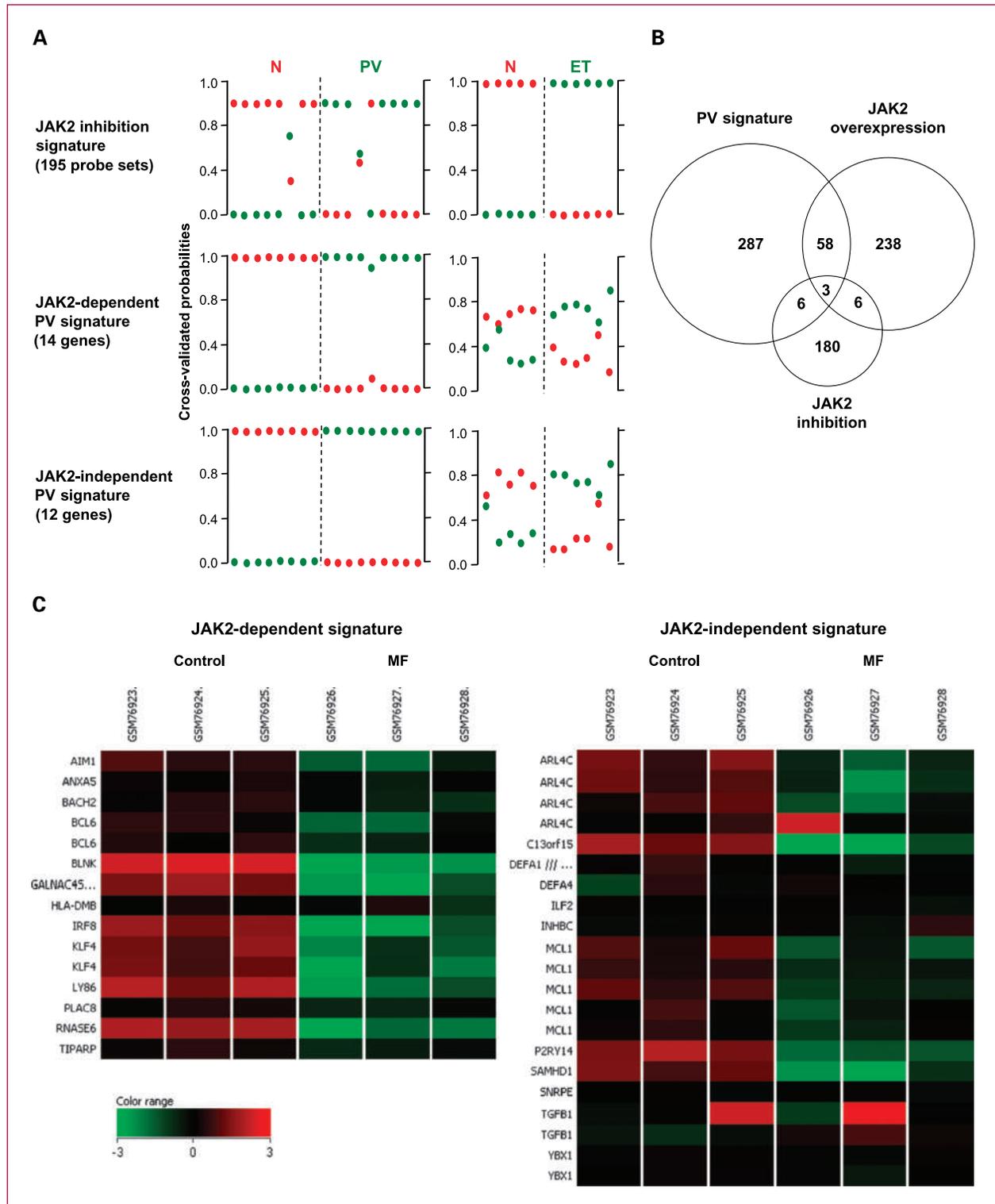


Fig. 5. A, cross-classification plots generated by prediction analysis for microarrays R software (16) for PV versus normal and ET versus normal specimens. Red and green dots, the specimens analyzed with different list of genes: the JAK2 inhibition signature, JAK2 overexpression signature, and PV signature. B, venn diagram showing the relationship between the PV expression signature, genes regulated in response to JAK2 overexpression in CD34⁺ cells, and genes regulated in JAK2V617F harboring cell lines treated with JAK inhibitor. The 287 probe sets remaining from the PV signature can still be used to distinguish PV from normal specimens. C and D, the GSE3410 data set (C) and GSE9827 (D) were imported onto GeneSpring GX11, and both JAK2-dependent and JAK2-independent gene sets were used to classify samples by unsupervised hierarchical clustering.

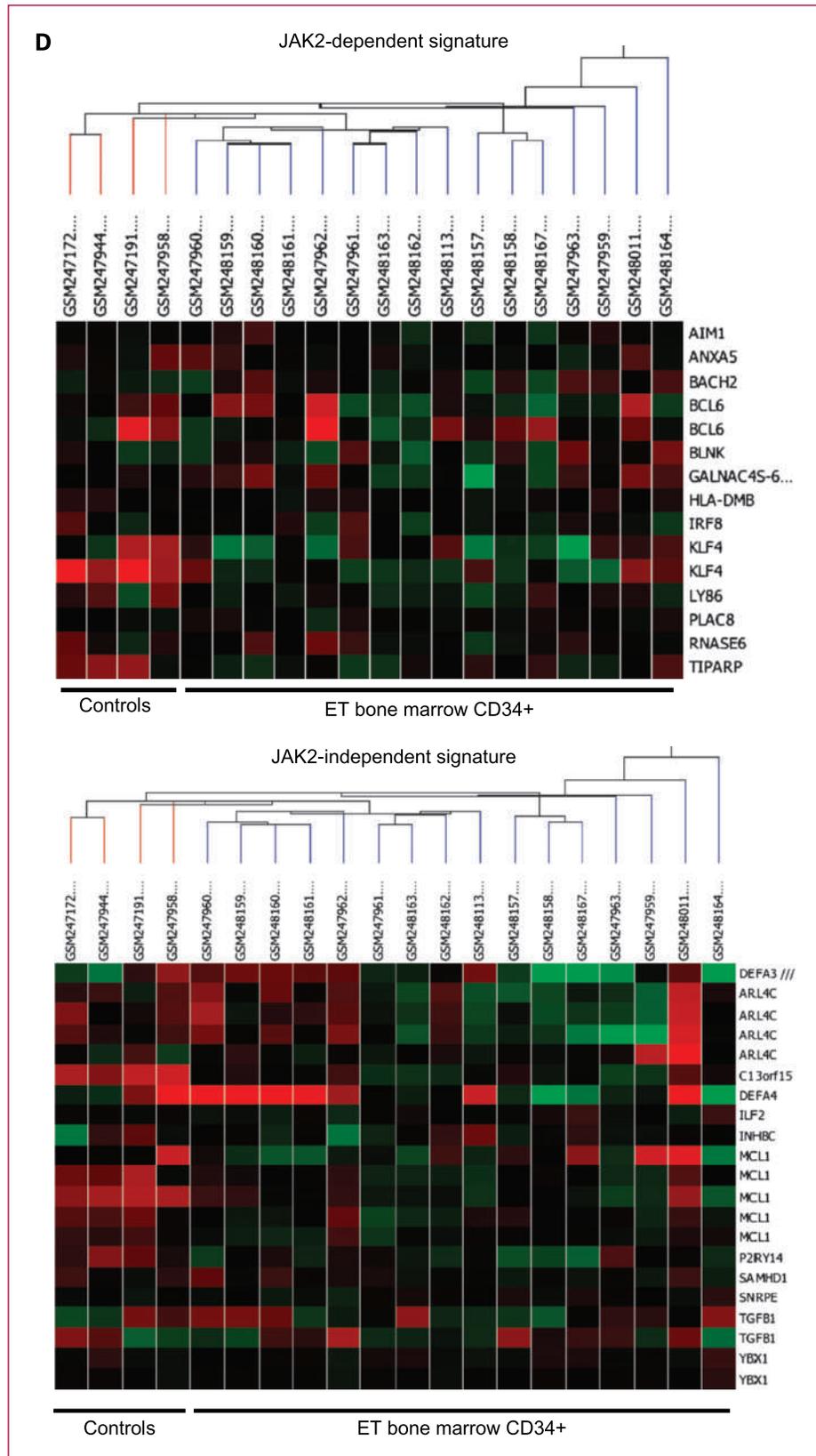


Fig. 5. Continued.

Table 1. JAK2-dependent and JAK2-independent gene sets

Name	Affymetrix ID	Log ratio (normal/PV)	P	Entrez gene ID
A. JAK2-dependent PV signature genes*				
AIM1	212543_at	2.6	0.021	202
ANXA5	200782_at	2.3	0.0424	308
BACH2	221234_s_at	3.7	0.0007	60468
BCL6	203140_at	3.9	0.0003	604
BLNK	207655_s_at	4.3	0	29760
GALNAC4S-6ST	203066_at	3.7	0.0009	51363
HLA-DMB	203932_at	2.5	0.0257	3109
IRF8	204057_at	4.6	0	3394
KLF4	221841_s_at	2.2	0.0485	9314
LOC100133941	266_s_at	3	0.007	100133941
LY86	205859_at	2.4	0.036	9450
PLAC8	219014_at	3.4	0.0023	51316
RNASE6	213566_at	2.5	0.0233	6039
TIPARP	212665_at	2.5	0.0253	25976
B. JAK2-independent PV signature genes†				
DEFA1	205033_s_at	-2.3	0	1667
MCL1	214057_at	1.5	0.0001	4170
P2RY14	206637_at	1.7	0	9934
C13orf15	218723_s_at	2	0	28984
INHBC	207688_s_at	1.6	0.0001	3626
SAMHD1	204502_at	1.4	0.0003	25939
ARL4C	202207_at	1.5	0.0002	10123
TGFB1	201506_at	1.5	0	7045
DEFA4	207269_at	-1.6	0	1669
ILF2	200052_s_at	1.1	0.0164	3608
SNRPE	215450_at	1.4	0.0006	6635
YBX1	208627_s_at	1.2	0.006	4904

*A set of 14 genes common in the PV and cell line data sets, which distinguish normal specimens and PV with high efficiency.

†A set of 12 genes derived from the PV expression signature, from which JAK2 regulated genes were subtracted, which distinguish normal specimens and PV with high efficiency.

Intriguingly, starting from the JAK2-dependent signature and the JAK2-independent PV signature, it is possible to select a subset of genes that discriminate very efficiently between ET and control specimens, but these genes differ from those most efficient in distinguishing PV and controls (data not shown). By contrast, the JAK2 inhibition signature is similarly efficient in predicting ET or PV specimens from controls (Fig. 5A, top).

Using these JAK2-dependent and JAK2-independent signature genes, we analyzed another set of MPN patient gene expression profiles (GSE3410) to cluster gene patterns. Of note, these gene expression profiles were obtained from CD34⁺ cells. Hierarchical clustering by euclidean centroid linkage nicely separated the normal and MF specimens (Fig. 5C). These data suggest both JAK2-dependent and JAK2-independent PV signature genes were predictive of other forms of MPN.

While this article was under revision, a new data set representing gene expression profiles of CD34 cells from ET patients was deposited (GSE9827). Unsupervised hierarchical clustering of specimens based on the expression of the JAK2-dependent and JAK2-independent gene sets separated ET CD34⁺ cells versus CD34⁺ controls, although differential expression was less distinctive than in the case of MF CD34⁺ versus controls (compare Fig. 5C and D). Using these new data sets, we compared and contrasted gene expression among the MPN phenotypes analyzing CD34 cells from PV, ET, and MF, and the data set from ET platelets. Significance analysis (one-way ANOVA with corrected *P* value from Benjamini Hochberg false discovery rate of 0.05 with SNK *post hoc* test) showed that there were minimal differences between the CD34⁺ cells of ET and MF (16 of 16,212 probe sets) and no significant differences between ET and PV. There were only 21 significant differentially expressed probe

sets between PV and MF. When ET CD34⁺ cells and ET platelets were compared, there were 416 probe sets differentially expressed. Shown graphically, when the specimens are clustered based on gene differences, the small number of differences among the PV, ET, and MF CD34⁺ cells leads to these specimens all being intermixed yet distinct from the ET platelets (Supplementary Fig. S11). It is remarkable that despite the differences in gene expression between ET platelets and ET CD34⁺ cells, the PV CD34⁺ cell-derived JAK2-dependent and JAK2-independent gene signature could still distinguish ET platelet samples from controls.

Discussion

The molecular pathology of PV in large part depends on the presence of the JAK2V617F and, in rare cases, activating mutations within exon 12 (29). The essential nature of JAK2V617F to PV was shown by the ability of overexpressed JAK2V617F to increase erythroid colony formation and cell maturation when expressed in CD34⁺ cells. A set of genes derived from the enforced expression of JAK2V617F in normal CD34⁺ cells or inhibition of JAK2V617F in cell lines could be used to distinguish PV, ET, or MF specimens from normal controls, indicating that a subset of the dysregulated gene in PV and other forms of MPN can be attributed to this mutant kinase. The genes deregulated in our series of PV patients tended to cluster in hematopoietic development, inflammation, and proliferation. Among 14 JAK2-dependent gene sets, KLF4, generally downregulated in the PV specimens, is a critical factor maintaining pluripotency of embryonic stem cells (30), and its decreased level in MPN CD34⁺ cells might denote increased commitment of these cells to differentiation. KLF4 in particular is downregulated in response to JAK2 and may represent a JAK2 effector gene whose decline allows for increased proliferation of progenitor cells.

Although many genes of the PV signature could be clearly related to the action of the mutant JAK2 kinase, there remains another set of genes whose expression is not affected by the inhibition of JAK2 in the HEL and UKE cell lines, and is unaffected by the overexpression of JAK2V617F in human CD34⁺ cells. There are several possible explanations for this gene deregulation. Gains or losses in chromosomal segments that we¹¹ and others (31–35) have begun to characterize may affect the expression of specific sets of genes in MPN. Alterations in DNA methylation patterns and the chromatin state of MPN hematopoietic progenitors could also set up new patterns of gene expression. Tet2, which shares structural similarity with Tet1, a protein recently found to have the ability of oxidize methyl cytosine residues (36, 37), could also affect the expression of specific sets of genes.

¹¹ W. Zhang, R. Levine, C. Gilliland, B. Ebert, and J. Licht, unpublished data.

The panel of 12 JAK2-independent genes able to separate normal and disease specimens included DEFA1 (also known as HNP1) and DEFA4, generally upregulated in PV specimens. These are normally expressed later in myeloid development and may represent altered differentiation of the progenitor cells due to mechanisms apart from the action of JAK2V617F (38). A recent study showed that defensin 1, 2, and 3 expression was dramatically increased in chronic myelomonocytic leukemia and could potentially account for aberrant monocyte maturation in this disorder. Intriguingly, defensins stimulate the production of IL-8 (39), which is elevated in PV and other MPNs (40). IL-8 in turn can stimulate the production of EPO-independent erythroid colony growth (41). Hence, deregulated defensin expression could contribute to aberrant hematopoiesis in PV.

Among the JAK2-independent gene set, SAMHD1, recently identified as a gene whose mutation leads to Aicardi-Goutières syndrome and characterized by inappropriate inflammatory responses (42), was downregulated in PV specimens. SAMHD1 seems to prevent the inappropriate activation of IFN and other innate immune responses. Transforming growth factor (TGF) β 1 and INHBC were also downregulated, suggesting that there could be anomalies in the TGF β signaling in the PV progenitor cells. TGF β and related cytokines tend to inhibit hematopoietic proliferation (43). Downregulation of INHBC was also observed in expression profiles from patients with the myelodysplastic syndrome (44). Collectively, these data suggest that CD34⁺ cells derived from PV patients have a disordered state of differentiation and homeostasis.

Several genes previously implicated in leukemia and cancer were also deregulated in PV. For example, WT1 upregulation is also characteristic of acute myelogenous leukemia and has a well-defined effect on myeloid proliferation and differentiation (45, 46). Hematopoietic stem cells from mice devoid of WT1 show decreased competitive repopulation activity after transplantation (45). Accordingly, WT1 upregulation, which we showed was dependent on JAK2 activity, could contribute to the self-renewal potential of the malignant MPN progenitor cell. EVI1, another zinc finger transcription factor, is generally expressed at low levels in normal marrow and was one of the relatively few genes overexpressed in the PV data set. EVI1 upregulation due to the rearrangement of chromosome 3q26.2 in acute myelogenous leukemia contributes to the development of acute myelogenous leukemia by promoting of cell cycle progression (47). KLF6, another transcription factor downregulated in the PV signature but not affected by manipulation of JAK2, has tumor-suppressive activity (48). *NFIB*, a gene encoding a CAAT box binding transcription factor, was upregulated in most of the PV specimens. *NFIB* was previously localized in the region of uniparental disomy, common in MPN on chromosome 9p, which also harbors the JAK2 locus (49). Engineered *NFIB* expression altered the TGF β response of cell lines (49), and whether this

protein plays a contributory role to myeloproliferation remains to be determined. Together, this information suggests that alterations of gene expression both dependent and independent of constitutive JAK2 activity may contribute to the development and/or progression of MPN.

Treatment of JAK2V617F-positive leukemia cell lines with a JAK inhibitor graphically showed that some of the genes consistently deregulated in PV were not likely to be regulated by the mutant kinase, whereas expression of other genes such as FLT3 and BCL6 is likely to be a direct or indirect result of JAK2 expression. Intriguingly, BCL6 was recently reported to be upregulated in chronic myelogenous leukemia cells in response to the kinase inhibitor imatinib mesylate (50). BCL6 was proposed to modulate some of the antiproliferative activity of imatinib through repression of the cyclin D2 promoter. A more recent report suggested that BCL6 upregulation could protect cells from p53-mediated apoptosis, and accordingly, inhibition of BCL6 with a small peptide increased cell killing by imatinib (51). These results suggest a possible therapeutic strategy for the treatment of MPN. It is also notable that inhibition of JAK2 activity was associated with a dramatic increase in FLT3 expression in the HEL and UKE cell lines. If this occurred in patients as with MPN, the result might be continued growth and survival of the malignant clone. In this regard, it should be noted that TG101348 (52) and CEP701 (Lestaurtinib; ref. 53) both in clinical trial for MPN target JAK2 as well as FLT3 and thus might have an advantage over an agent such as INCB018424 (54), which is highly selective for JAK2. Further translational studies correlating response of primary specimens to clinical results will be needed to determine if specific targeting of JAK2 or more broad inhibition of kinases will be a superior approach to the treatment of MPN.

Prior studies of the gene expression profile of MPNs have focused on readily obtained granulocytes. Pellagatti et al. (55) profiled gene expression from granulocytes of PV patients using a custom cDNA array. Unlike our study in which most genes differentially expressed between PV and normal specimens were downregulated, this group identified 147 genes upregulated 3.5× or more and only 20 downregulated genes. Among their set of upregulated genes, we also noted DEFA1 as a characteristic upregulated gene in MPN. Goertler et al. (56) devised a 64-gene signature from the profile of pooled mRNA from PV granulocytes that was able to distinguish PV from secondary erythrocytosis. Only one gene from their predictor, *KLF4*, a downregulated gene, overlapped with our disease predictor set. This may be due to the use of different platforms and the fact that we profiled CD34⁺ cells. CD34⁺ cells from MF patients were expression profiled to determine a class predictor that was useful on a second set of patients (57). There was no overlap between the characteristic gene set of MF in this study and our own set of deregulated genes. This may be due to the distinct nature of MF, which less commonly harbors the JAK2V617F mutation. Kralovics and colleagues identified 280 differentially expressed

genes in granulocytes from PV versus control patients (58). Fifteen of the most highly regulated genes were examined in detail. Among these, *KLF4* was upregulated. Curiously, *KLF4* was upregulated in PV granulocytes, whereas it was downregulated in CD34⁺ cells in our set of PV patients, and *KLF4* was increased in response to inhibition of JAK2V617F. This may represent a differentiation stage-specific action of the mutant JAK2 kinase. Another study of gene expression profiles of granulocytes from ET patients also addressed the issue of JAK2-mediated gene expression (59). JAK2-negative specimens showed no constitutive Stat3 phosphorylation and showed lower expression of known JAK/STAT target genes. Similarly Puigdecane et al. (60) compared gene expression in granulocytes from JAK2V617F-positive and JAK2V617F-negative patients, and identified a group of eight genes, several of which were known to be induced by the JAK/STAT pathway. Although we identified genes regulated by JAK2 that could serve as predictors for PV versus control, our gene set did not overlap with that of Puigdecane et al. (60), perhaps due to the difference between granulocyte and CD34⁺ cell profiles. In fact, another group found that many of the genes differentially expressed in JAK2V617F-positive ET cases were not differentially expressed in CD34⁺ cells from JAK2V617F-positive and JAK2V617F-negative patients (61).

Guglielmelli et al. (62) profiled pooled CD34⁺ cells from MF patients and validated 36 genes as differentially expressed. A subset of eight genes could correctly separate MF from ET, PV, and control granulocytes. These genes did not overlap with our JAK2-dependent and JAK2-independent predictor sets and seem to be unique to MF.

In conclusion, the molecular profiling of PV reveals that many aspects of the aberrant program of these cells can be attributed to the action of JAK2V617F. Genes deregulated in PV and regulated by the action of JAK2 represent potential disease effectors and secondary genetic targets for therapy. In our data set, there remains a set of genes clearly aberrantly expressed relative to normal CD34⁺ cells but apparently not regulated by JAK2V617F. Further studies will be required to determine the etiology of their aberrant expression and their importance in disease pathogenesis.

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No potential conflicts of interest were disclosed.

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