Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling

CHEN, Edwin, et al.

Abstract

The JAK2V617F mutation is associated with distinct myeloproliferative neoplasms, including polycythemia vera (PV) and essential thrombocythemia (ET), but it remains unclear how it generates disparate disorders. By comparing clonally-derived mutant and wild-type cells from individual patients, we demonstrate that the transcriptional consequences of JAK2V617F are subtle, and that JAK2V617F-heterozygous erythroid cells from ET and PV patients exhibit differential interferon signaling and STAT1 phosphorylation. Increased STAT1 activity in normal CD34-positive progenitors produces an ET-like phenotype, whereas downregulation of STAT1 activity in JAK2V617F-heterozygous ET progenitors produces a PV-like phenotype. Our results illustrate the power of clonal analysis, indicate that the consequences of JAK2V617F reflect a balance between STAT5 and STAT1 activation and are relevant for other neoplasms associated with signaling pathway mutations.

Reference


DOI : 10.1016/j.ccr.2010.10.013
PMID : 21074499
Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling

Edwin Chen1, Philip A Beer1,2, Anna L Godfrey1,2, Christina A Ortmann1,2, Juan Li1, Ana P. Costa-Pereira3, Catherine E Ingle4, Emmanouil T Dermitzakis4, Peter J Campbell4, and Anthony R. Green1,2,§

1Cambridge Institute for Medical Research and Department of Haematology, University of Cambridge, Hills Road, Cambridge, CB2 0XY, United Kingdom
2Department of Haematology, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 0XY
3Imperial College London, Department of Surgery and Cancer, Hammersmith Hospital, London W12 0NN
4The Wellcome Trust Sanger Institute, Hinxton, Cambridge, CB10 1SA

SUMMARY

The JAK2V617F mutation is associated with distinct myeloproliferative neoplasms, including polycythemia vera (PV) and essential thrombocythemia (ET), but it remains unclear how it generates disparate disorders. By comparing clonally-derived mutant and wild-type cells from individual patients, we demonstrate that the transcriptional consequences of JAK2V617F are subtle, and that JAK2V617F-heterozygous erythroid cells from ET and PV patients exhibit differential interferon signaling and STAT1 phosphorylation. Increased STAT1 activity in normal CD34-positive progenitors produces an ET-like phenotype, whereas down-regulation of STAT1 activity in JAK2V617F-heterozygous ET progenitors produces a PV-like phenotype. Our results illustrate the power of clonal analysis, indicate that the consequences of JAK2V617F reflect a balance between STAT5 and STAT1 activation and are relevant for other neoplasms associated with signaling pathway mutations.

INTRODUCTION

The myeloproliferative neoplasms (MPNs) are hematological malignancies characterized by a chronic clinical course and a risk of thrombosis and transformation to acute leukemia. These disorders are an attractive model for understanding the earliest stages of cancer development since many patients present at an early stage with an incidental abnormal blood count, the landscape of causative somatic mutations underpinning the disorders is increasingly well understood and tumor cells can be grown and differentiated in vitro from single progenitor cells. The two most common MPNs are polycythemia vera (PV), in which patients present with a raised red cell mass sometimes associated with increased platelet and white cell counts, and essential thrombocythemia (ET), which is defined by an elevated platelet count but normal red cell mass.

The MPNs result from transformation of a multipotent hematopoietic progenitor (Adamson et al., 1976; Delhommeau et al., 2007; Fialkow et al., 1981; Jamieson et al., 2006). In 95%

§Corresponding author: Professor A.R. Green, Cambridge Institute for Medical Research, Hills Road, Cambridge, CB2 0XY, United Kingdom. Telephone: (+44) 1223 336820, Fax: (+44) 1223 762670, arg1000@cam.ac.uk.
of patients with PV and 60% of those with ET, an identical somatically acquired mutation is found in the tyrosine kinase, JAK2 (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005a; Levine et al., 2005). This V617F mutation results in dysregulated kinase activity of JAK2 and produces ligand-independent activation of receptor signaling in cytokine-dependent cell lines. It has been suggested that expression of a homodimeric type I cytokine receptor provides a scaffold necessary for optimal signaling by mutant JAK2 (Lu et al., 2008; Lu et al., 2005) and several signaling cascades are activated by mutant JAK2, including the STAT5, MAPK and PI3K pathways (James et al., 2005; Kralovics et al., 2005a; Laubach et al., 2009; Levine et al., 2005; Oku et al., 2010). Of these the STAT5 pathway has been shown to be necessary and sufficient for at least some aspects of the MPN phenotype in vitro and in vivo (Funakoshi-Tago et al., 2010; Garcon et al., 2006; Grebien et al., 2009; Lu et al., 2008; Ilaria et al., 1999). Drosophila studies have also revealed a non-canonical pathway by which JAK2 regulates chromatin (Shi et al., 2006; Shi et al., 2008) and a direct nuclear function for JAK2 as a histone H3 kinase has recently been reported (Dawson et al., 2009). The JAK2V617F mutation is sufficient to cause an MPN phenotype in mouse models (Akada et al., 2010; Lacout et al., 2006; Li et al., 2010; Marty et al., 2010; Mullally et al., 2010; Tiedt et al., 2008; Wernig et al., 2006; Xing et al., 2008; Zaleskas et al., 2006) and, taken together, existing data demonstrate that mutation of JAK2 plays a central and early role in MPN pathogenesis.

One fundamental, unresolved question is how the same mutation in JAK2 can give rise to phenotypically distinct disorders such as ET and PV. Homozygosity for the mutation is common in JAK2V617F-positive PV but rare in JAK2V617F-positive ET (Scott et al., 2006), thus raising the possibility that increased signaling through JAK2V617F may be responsible for the PV phenotype. However attempts to identify different signaling consequences in ET and PV have not yielded consistent results using Western blotting (Heller et al., 2006), immunohistochemistry (Grimwade et al., 2009; Teofili et al., 2007) or expression profiling (Berkofsky-Fessler et al., 2010; Goerttler et al., 2005; Kralovics et al., 2005b; Laubach et al., 2009; Pellagatti et al., 2003; Puigdecarnet et al., 2008; Sleazak et al., 2009; Tenedini et al., 2004). This is likely to reflect several issues that complicate inter-individual comparisons including constitutional variation in transcript levels among normal individuals (Stranger et al., 2005; Stranger et al., 2007), the widely varying proportions of wild-type and mutant clones present in samples obtained from different patients (Dupont et al., 2007; Scott et al., 2006) and treatment differences across patient cohorts. Such difficulties undoubtedly confound similar laboratory studies of other cancers as well, but are generally difficult to identify or quantify and have therefore often not been formally evaluated.

To circumvent these issues, we took advantage of the tractable clinical and laboratory features of MPNs to devise a strategy based on analysis of clonally-derived cells genotyped for JAK2 mutation status. This approach allows comparison of pure populations of phenotypically equivalent mutant and wild-type cells grown from the same patient under identical conditions, and has allowed us to address the mechanisms responsible for the different phenotypic consequences of the JAK2 mutation in ET and PV.

RESULTS

JAK2V617F-associated transcriptome changes are less marked than those due to inter-individual differences and can be identified by clonal analysis

In patients with MPNs, erythroid colonies containing thousands of cells can be grown by in vitro culture in semi-solid media. Each colony represents the in situ expansion of a single erythroid progenitor cell and so reflects the genetic landscape of the initiating cell. Since JAK2 mutant and wild-type progenitor cells coexist in the peripheral blood of patients with ET and PV, colonies from both genotypes can be grown in the same experiment,
individually picked and typed for presence or absence of the V617F mutation, pooled by genotype and studied for phenotypic differences (Figure 1). This strategy enables direct comparison of mutant and wild-type cells within a patient, thereby controlling for differences in age, sex, treatment, germline genetic background, experimental factors and other confounding variables. Laboratory assays that can be applied to phenotype such colonies are flexible and wide-ranging: in this manuscript, we present results of transcriptional profiling, conventional and intracellular flow cytometry, Western immunoblotting, as well as immunohistochemistry.

A total of 5,302 BFU-E colonies from 36 MPN patients (20 ET and 16 PV; Table S1) were grown, collected, genotyped and pooled based on presence or absence of the JAK2V617F mutation. For each patient a pool of JAK2V617F-heterozygous erythroid colonies was compared to a pool of JAK2V617F-negative colonies using gene expression microarrays. We chose to study erythroid cells since it is the presence or absence of a raised red cell mass that is the key distinction between PV and ET. The high erythropoietin concentrations normally used in erythroid colony assays may mask transcriptional consequences of the JAK2 mutation, and so colonies were grown in 0.01 U/ml erythropoietin, a concentration shown to maximize the difference in expression of a known JAK/STAT target (PIM1) between mutant and wild-type colonies (Figure S1A). Comparison of colonies with and without the JAK2V617F mutation revealed no overt differences in colony morphology or size (Figure S1B), and no detectable differences in the degree of erythroblast differentiation (assessed by GPA and CD71 expression and morphological analysis of cytospins) (Figure S1C-D), demonstrating that colonies from the two genotypes represented comparable stages of erythroid development. Gene set enrichment analysis did not show any enrichment of erythroid differentiation genes amongst genes up-regulated in mutant colonies compared to wildtype colonies (p=0.2) or amongst mutant PV colonies compared to mutant ET colonies (p=0.19).

Unsupervised clustering analysis of expression profiles from the two classes of colony demonstrated that JAK2 mutant colonies were more closely related to wild-type colonies from the same patient than to mutant colonies from other patients (Figure 2A). There was no clustering based on other parameters, such as diagnosis, therapy, age or gender. To ensure that this pattern of clustering did not reflect simultaneous processing of paired samples from a given patient, repeat blood samples were drawn from 4 individuals and subjected to the entire process ab initio. The resulting expression profiles still revealed close clustering between samples derived from the same patient, with samples of the same genotype from the same patient clustering closer yet (Figure S2A). Moreover, the ratio of gene expression between the V617F-heterozygous and wild-type colonies for all genes revealed strong concordance in independently performed experiments (Figure S2B). These results demonstrate firstly that variability attributable to inter-assay differences was minimal, and secondly that the transcriptional consequences of the JAK2 mutation are less marked than normal inter-individual variation.

To identify V617F-associated genes common to both PV and ET, expression profiles derived from all 36 patients were examined to identify genes consistently dysregulated in V617F-heterozygous colonies relative to autologous wild-type colonies. Following adjustment for age, gender and therapy, and controlling the false discovery rate (FDR) at 10% for multiple hypothesis testing, expression levels of 201 genes were increased in V617F-heterozygous cells and those of 22 genes were reduced (minimum fold change, 1.3; p-values ≤ 0.0034; Figure S2C-D). Gene set enrichment analysis (GSEA) was applied to a pre-ranked gene list ordered according to the significance with which they were differentially expressed in mutant and wild-type colonies. Components of the erythropoietin signaling pathway (net enrichment score (NES) = 1.51; q = 0.02) and previously reported
targets of STAT5A signaling (NES = 1.40; q = 0.09) (Olthof et al., 2008) were enriched amongst genes up-regulated in mutant colonies (Figure 2B). Array results from individual patients for known JAK/STAT targets (PIM1 and CISH) showed up-regulation associated with presence of the JAK2 mutation in most (but not all) patients and were confirmed by quantitative RT-PCR in at least 5 PV and 5 ET patients (Figure 2C). Consistent with these data, nuclear pSTAT5 was increased in JAK2V617F-heterozygous but not wild-type erythroblasts from both PV and ET patients (Figure 2D).

Together these data demonstrate that the effect of the V617F mutation on the transcriptome is surprisingly subtle, and less than the influence of inter-individual variation. However comparison of clonally-derived mutant and wild-type cells from each patient permits identification of mutation-associated expression changes.

**JAK2V617F-heterozygous erythroid cells from ET and PV patients exhibit differential interferon signaling and STAT1 phosphorylation**

We next investigated whether gene expression changes associated with JAK2V617F-heterozygous erythroid colonies were the same in patients with ET and PV. Following adjustment for potential confounders including age, gender and therapy, a linear mixed effects (LME) modeling algorithm was applied to the dataset to identify genes for which a significant interaction existed between JAK2 mutation status and MPN subtype (ie ET or PV). This type of analysis has the advantage of allowing for correction of inter-individual variability without the need to explicitly model it (Li et al., 2004). Controlling the FDR at 10%, a total of 171 genes exhibited significant interaction, all of which fell into one of four basic patterns of gene behavior (Figure S3A): (i) genes up-regulated in JAK2-mutant erythroblasts specifically in ET but not PV (n=83), (ii) genes down-regulated in JAK2-mutant erythroblasts specifically in ET but not PV (n=21), (iii) genes up-regulated in JAK2-mutant erythroblasts specifically in PV but not ET (n=40), and (iv) genes down-regulated in JAK2-mutant erythroblasts specifically in PV but not ET (n=24) (p ≤ 0.0028). A hierarchical clustering showing these combined 171 genes and their ability to subdivide the PV patients from the ET patients is shown in a heat map (Figure 3A). Linear discriminant analysis and leave-one-out cross-validation using the 12 most statistically significant genes resulted in correct classification of 33/36 (92%) of the patients; an identical classification rate was determined using the Stanford pamr software. Taken together, these data demonstrate cell-intrinsic differences in JAK2V617F-heterozygous erythroblasts from PV and ET.

To investigate pathways associated with JAK2V617F expression in ET and PV, GSEA was applied to the entire gene list ranked in order from those “most significantly up-regulated in association with JAK2V617F in ET, compared to PV” to those “most significantly up-regulated in association with JAK2V617F in PV, compared to ET”. At a FDR cut-off of 20%, 23 gene sets were enriched for genes up-regulated in ET, of which 7 were related to interferon signaling whereas 0/25 gene sets enriched for genes up-regulated in PV were interferon related (Figure 3B, Table S2). We then looked for interferon-regulated genes amongst the genes significantly up-regulated by JAK2V617F in an ET-selective or in a PV-selective manner. Interferon regulated genes were defined by their presence in the Interferome database (Samarajiwa et al., 2009), a group of manually curated gene sets comprising known IFNα and IFNγ target genes. Of the 83 genes up-regulated in an ET-selective manner, 26 were IFN targets (8 IFNγ targets, 2 IFNα targets and 16 targets of both). By contrast, only one of 40 genes up-regulated in a PV-selective manner were targets of either interferon (Table S4). Real time qPCR confirmed increased transcript levels of 5 known IFNγ target genes (IFI44L, GBP2, IRF1, HLA-F, IFITM3) in V617F-heterozygous relative to autologous wild-type erythroblasts in 10 ET patients but not in 9 PV patients (Figure 3C). These results demonstrate that JAK2V617F is associated with enhanced IFN
signaling, predominantly involving the IFNγ pathway, in erythroblasts from patients with ET but not in those from patients with PV.

Wild-type JAK2 binds the IFNγ1 component of the IFNγ heterodimeric receptor (Ihle, 1994; Parganas et al., 1998; Silvennoinen et al., 1993), and our results suggest that JAK2V617F enhances activity of this pathway in JAK2-mutant erythroblasts from ET patients. Indeed, relative to expression of wild-type JAK2, expression of JAK2V617F in γ2A cells (which lack endogenous JAK2) resulted in increased STAT1 phosphorylation on the tyrosine-701 residue (pSTAT1) in response to IFNγ (Figure S3B). Similar results were obtained using 293T cells (Figure S3C). These data demonstrate that, analogous to its well-documented effects in conferring cytokine hypersensitivity on type I homodimeric cytokine receptors (Lu et al., 2008; Lu et al., 2005), JAK2V617F also enhances signaling from IFNγ receptors.

To investigate the mechanisms responsible for the striking lack of interferon signaling in JAK2V617F-heterozygous erythroblasts from PV patients, we focused on STAT1 since it is essential for IFNγ receptor signaling (Gough et al., 2010). Levels of pSTAT1 were measured in mutant and wild-type erythroblasts from ET and PV patients using intracellular flow cytometry. Relative to autologous erythroblasts, elevated expression of pSTAT1 was seen in JAK2V617F-heterozygous erythroblasts from all 6 ET patients tested but not in any of 5 PV patients (Figure 3D). Consistent with these results, pSTAT1 was detected by immunofluorescence in the nucleus of JAK2-mutant erythroblasts from ET but not PV patients (Figure 3E). Immunoblotting revealed no differences in total STAT1 levels between mutant and wild-type erythroblasts in either disease (Figure S3D).

These data demonstrate that JAK2V617F-heterozygous PV erythroblasts lack detectable pSTAT1 and therefore provide a mechanism for the differential activation of IFN signaling pathways in ET and PV.

**Increased STAT1 activity produces an ET-like phenotype with enhanced megakaryocytic differentiation and restrained erythroid differentiation**

The results described above raised the possibility that differences in the level of STAT1 activity may contribute to the distinct biological features of ET and PV. ET is characterized by increased megakaryopoiesis whereas the dominant feature of PV is enhanced erythropoiesis. We therefore assessed the effect of altering STAT1 activity on megakaryocytic and erythroid differentiation.

Lentiviral constructs were used to express wild-type or constitutively active STAT1 (STAT1C) (Liddle et al., 2006) in the hematopoietic progenitor cell line K562, which was then induced to undergo megakaryocytic or erythroid differentiation by exposure to phorbol 12-myristate 13-acetate (PMA) or hemin respectively. Transduction efficiencies for all constructs were >95% as determined by GFP positivity by FACS, and expression levels of exogenous STAT1 and STAT1C were equivalent but pSTAT1 levels were only increased in cells expressing STAT1C (Figure 4A). Low concentrations of PMA (insufficient to generate detectable megakaryocytic differentiation of unmodified K562 cells) induced substantial megakaryocytic differentiation of K562 cells expressing STAT1C but not those expressing STAT1. K562 cells expressing STAT1C up-regulated the megakaryocytic markers CD41 (Figure 4B), CD61 (Figure S4A) and GPIIX (Figure 4C) and also developed increased DNA ploidy (Figure 4D; Figure S4B). Exposure to hemin induced erythroid differentiation as indicated by increased levels of γ-globin transcripts and hemoglobinisation. However the increases in γ-globin transcripts and hemoglobin levels were both inhibited in K562 cells expressing STAT1C (Figure 4E-G). These results demonstrate that increased STAT1

*Cancer Cell. Author manuscript; available in PMC 2011 May 1.*
activity in K562 cells results in enhanced megakaryocytic and repressed erythroid differentiation.

To confirm our results in primary hematopoietic progenitors, purified CD34+ cord blood cells were infected with lentiviruses expressing STAT1 or STAT1C and then grown in conditions promoting either megakaryocytic or erythroid differentiation. At day 3 post-infection pSTAT1 was readily detected in cells expressing STAT1C but not in those expressing STAT1 (Figure 5A). No differences in growth kinetics were detected in cells expressing either STAT1 isoform when cultured in either megakaryocytic or erythroid conditions (Figure 5B). However expression of STAT1C increased the proportion of cells expressing the megakaryocytic markers CD41 (Figure 5C; Figure S5A), CD61 (Figure 5D; Figures S5B) and GPIX (Figure 5E), and also increased the proportion of polyploid cells (Figure 5F; Figure S5C). Moreover expression of STAT1C resulted in a reduced proportion of GPA+CD71+ erythroid cells (Figure 5G; Figure S5D) and reduced levels of γ-globin transcripts (Figure 5H). These results accord with our K562 data and demonstrate that increased STAT1 activity in primary hematopoietic progenitors is sufficient to produce an ET-like phenotype with enhanced megakaryocytic and reduced erythroid differentiation.

Down-regulation of STAT1 activity in JAK2V617F+ progenitors from ET patients results in a PV-like phenotype with increased erythroid and reduced megakaryocytic differentiation

To test the hypothesis that increased STAT1 activity constrains erythropoiesis in patients with ET, a dominant negative form of STAT1 (STAT1DN) was expressed in CD34+ hematopoietic stem and progenitor cells derived from patients diagnosed with ET. In order to perform these experiments at a clonal level, CD34+ progenitors were isolated from two ET patients, infected with a lentivirus containing an empty vector (VA) or expressing a cDNA encoding a dominant negative form of STAT1 (STAT1DN) in which the Tyr-701 residue was mutated to a phenylalanine (Walter et al., 1997). Infected cells were subsequently sorted at one cell per well into media capable of supporting both erythroid and megakaryocytic differentiation. After 7 days in culture each clone was genotyped to identify JAK2V617F mutant clones, which were subsequently expanded in fresh media for an additional 7-14 days (Figure 6A). Within three weeks, three distinct categories of GFP+ JAK2V617F+ clones were evident: GPA-expressing erythroid clones, CD41-expressing megakaryocytic clones and mixed erythromegakaryocytic clones comprised of cells expressing GPA or CD41 (Figure 6B). In both ET patients examined, the percentage of GFP+ erythroid clones derived from cells infected with the STAT1DN lentivirus was increased and that of the megakaryocytic clones was decreased compared to cells infected with the empty vector (p<0.05, Fisher-Freeman-Halton test) (Figure 6C). These results demonstrate that STAT1 activity is necessary to repress erythropoiesis in JAK2V617F-heterozygous ET progenitors, and that reducing STAT1 activity results in a switch to a PV-like phenotype with increased erythroid and reduced megakaryocytic differentiation.

DISCUSSION

In this paper we address the apparent paradox that an identical JAK2 mutation is associated with different clinical phenotypes. Using a strategy based on analysis of clonally-derived and genotypically-defined cell populations, we have circumvented the problems associated with inter-individual comparison of expression profiles. Our results illuminate the pathogenesis of the MPNs and are also of broad relevance for cancer biology.

We report the surprising finding that, in the vast majority of PV and ET patients, the expression profiles of JAK2V617F-positive erythroid cells are more closely related to wild-type cells from the same individual than to mutant cells from other patients. This
observation demonstrates that normal inter-individual variation in gene expression is greater than the transcriptional consequences of the JAK2 mutation, a finding with relevance for many somatic genetic lesions associated with cancer. Indeed it is increasingly recognized that quantitative differences in transcript levels are under genetic control in both man (Stranger et al., 2005; Stranger et al., 2007) and mouse (Breitling et al., 2008; Gerrits et al., 2009). Our results therefore emphasize the power of comparing clonally-derived, phenotypically equivalent cell populations from the same individual, together with the importance of using physiological levels of cytokines that do not swamp the signaling pathways of interest.

Our results also have implications for the strategy of individualized cancer therapy, an approach based on the concept of identifying genetic lesions in each tumor and using this information to select targeted therapies. Mutant JAK2 activates multiple receptor-associated pathways and we show that some of these, such as STAT5 and STAT1, can have competing consequences. Our results also demonstrate that patients can carry an identical JAK2 mutation but harbor striking differences in the degree to which the mutation activates the STAT1 pathway. The consequences of inhibiting a particular activated kinase may therefore be different for individual tumors and difficult to predict without a detailed knowledge of the signaling environment within a given tumor. Moreover, since some pathways downstream of an activated kinase may, like STAT1 activation, restrain disease evolution, therapeutic kinase inhibition could potentially result in paradoxical tumor-enhancing effects.

The mechanism whereby a single JAK2 mutation can give rise to distinct diseases has been a major lacuna in our understanding of MPN pathogenesis, and distinguishing JAK2V617F-positive ET from PV represents a diagnostic challenge when patients have borderline hemoglobin levels. Patients with JAK2V617F-positive ET exhibit an increased erythroid drive but lack overt erythrocytosis (Campbell et al., 2005), indicating the existence of additional mechanisms that either constrain erythropoiesis in JAK2 mutation-positive ET or enhance it in PV. The data presented here demonstrate that STAT1 is activated in association with JAK2V617F in ET but not PV and that pSTAT1 levels provide a molecular marker that distinguishes these disorders. Importantly, we demonstrate that inhibition of STAT1 signaling in ET progenitors resulted in enhanced erythropoiesis and reduced megakaryopoiesis. Together, our results indicate that the clinical phenotype developed by a given individual reflects the opposing effects of STAT5 and STAT1 signaling. In ET, the intact pSTAT1 response to JAK2V617F constrains erythroid and promotes megakaryocytic differentiation – the reduced pSTAT1 response in PV removes the “brake” on erythropoiesis, thus allowing the development of an overt erythrocytosis, and also reduces megakaryopoiesis (Figure 7). This model is consistent with previous studies of the effects of STAT5 and STAT1 on hematopoietic differentiation (Huang et al., 2007; Olthof et al., 2008; Socolovsky et al., 1999).

Homozygosity for the JAK2 mutation is common in PV but rare in ET (Scott et al., 2006), and has been suggested as a potential mechanism for increased erythropoiesis in PV (Dupont et al., 2007). Our results show that reduced pSTAT1 levels precede homozygosity and enhance erythropoiesis, but do not exclude a subsequent role for JAK2V617F homozygosity. Once the pSTAT1 pathway is repressed, homozygosity of JAK2V617F would be predicted to enhance erythroid differentiation by increasing pSTAT5 signaling. By contrast, in the presence of intact STAT1 signaling, JAK2V617F homozygosity may fail to confer any growth or selective advantage (or even result in a disadvantage). We are collecting JAK2V617F-homozygous colonies in order to investigate the transcriptional consequences of homozygosity.
The process responsible for loss of STAT1 phosphorylation in PV remains unclear. Our data do not distinguish between constitutional or acquired mechanisms. However the pSTAT1 response to IFNγ is normal in T cells from patients with PV (data not shown) indicating that there is no constitutional block to STAT1 activation. Moreover the fact that some patients with high-risk ET develop overt PV transformation (Harrison et al., 2005) argues for an acquired genetic modifier in at least a subset of patients, a concept consistent with the observation that 10% of JAK2V617F knock-in mice also develop PV transformation (Li et al., 2010).

Our results also have implications for interferon therapy, HSC behavior and and clonal evolution in the MPNs. JAK2-mutant erythroblasts from PV patients have markedly reduced pSTAT1 levels (relative to ET patients), and yet both categories of patients respond to therapy with IFNα. This suggests that such responses may reflect non cell-intrinsic mechanisms, that reduced STAT1 phosphorylation in PV can be overcome by pharmacological doses of IFNα, or that IFNα elicits its effects through STAT1-independent pathways (Lu et al., 2010). Normal HSC function is regulated by basal IFN tone (Baldridge et al., 2010; Essers et al., 2009; Zhao et al., 2010) and increased IFNγ or IFNα signaling results in HSC exhaustion (Trumpp et al., 2010). Hence within a JAK2V617F-positive population, a subclone that acquires a defect in STAT1 activation may not only give rise to erythrocytosis but also display a competitive advantage within the HSC compartment, thus providing a potential mechanism for clonal evolution.

**EXPERIMENTAL PROCEDURES**

**Patients and samples**

A total of 36 MPN cases (18 men/18 women) with a median age of 64 (ranging from 12 to 89 years), diagnosed with either essential thrombocythemia or polycythemia vera according to the World Health Organisation (WHO) criteria were recruited for this study from the MPN clinic at the Addenbrooke’s Hospital in Cambridge, UK. All patients were shown to possess the JAK2V617F mutation in granulocyte DNA using allele-specific PCR. Two PV patients and one ET patient also possessed mutations in TET2. None harbored MPL or JAK2 exon 12 mutations. The clinical and biological features of all patients at diagnosis are listed in Table S1. All patients gave informed written consent before participating in the study. The study was approved by the Cambridge and Eastern Region Ethics Committee, and was carried out in accordance with the principles of the Declaration of Helsinki. Venous blood samples (20 ml) were collected from each patient, and peripheral blood mononuclear cells were isolated using Lymphoprep (Axis Shield PLC) according to the manufacturer’s protocols, and plated in Methocult (H4531; Stem Cell Technologies) supplemented with 0.01 U/ml erythropoietin at a density of 3×10⁵ cells/ml. Cultures were incubated at 37°C for 14 days.

**RNA extraction**

For each patient, individual BFU-E colonies were plucked into 100 µl of Buffer RLT (Qiagen). A portion of the sample in Buffer RLT for each colony was used for genomic DNA (gDNA) extraction, followed by genotyping by real-time qPCR to determine its JAK2 mutational status, as described previously (Levine et al., 2006). Colonies with V617F percentages below 10% were designated “wild-type” and those between 40-60% were designated “heterozygous”. Colonies not fulfilling any of the two criteria were discarded. Up to 20 colonies of each JAK2 genotype were pooled, and total cellular RNA from each sample was isolated using the Qiagen RNeasy kit following the manufacturer’s protocols. RNA quality was assessed by nanoelectrophoresis using the Pico lab-on-a-chip assay (Bioanalyzer, Agilent Technologies) and by agarose gel electrophoresis.
Microarray analysis

Total RNA (100 ng) obtained from wild-type and V617F-heterozygous BFU-E colonies from each patient were reverse transcribed into cDNA and amplified by *in vitro* transcription into biotinylated cRNA using the Illumina TotalPrep RNA Amplification Kit (Ambion). For each sample, 1.5 μg of amplified cRNA was used for hybridization onto Illumina WG-6 v2.0 Expression BeadChips, comprising ~48,000 oligonucleotide probes.

Single cell erythroid/megakaryocyte differentiation cultures

Peripheral blood mononuclear cells were obtained from peripheral blood of patients diagnosed with ET over a ficoll gradient, and CD34+ cells were then selected using a magnetic cell sorting system (Miltenyi Biotech), according to manufacturer’s instructions. Purified CD34+ cells were immediately infected with either an empty vector or a dominant negative STAT1-expressing lentiviruses by spinoculation for 2h in the presence of 4 μg/ml polybrene, followed by culturing for 2 days in SFEM medium supplemented with 100 ng/ml Flt3 ligand and 10 ng/ml rhTPO. Subsequently, cells were sorted based on GFP positivity and seeded as single cells in a well of a 96-well plate with SFEM media supplemented with 25 ng/ml rhSCF, 0.5 U/ml rhEPO, 100 ng/ml rhTPO, 30 μg/ml holo-transferrin, 10 nM β-mercaptopethanol and 4 μg/ml dexamethasone, in order to support both erythroid and megakaryocytic differentiation. After 7 additional days in culture, 10% of the cells were removed for JAK2 genotyping as described above. After an additional 7-14 days of culture, V617F-heterozygous clones were analyzed for GPA and CD41 expression.

Accession number

All expression profiling data have been deposited into ArrayExpress (accession number E-MTAB-384)

**SIGNIFICANCE**

Our results reveal a central role for STAT1 activation in the pathogenesis of JAK2V617F-positive MPNs and indicate that the phenotypic consequences of JAK2V617F reflect a balance between hematopoietic effects of STAT1 and STAT5 activation. In addition to providing a molecular explanation for this long-standing conundrum, several aspects of our data are of general relevance: 1) the transcriptional consequences of JAK2V617F are less than normal inter-individual variation, a finding with implications for other somatic mutations; 2) tumors harboring an identical mutation can exhibit strikingly different activity of associated signaling pathways, an observation with implications for targeted therapy; 3) our results demonstrate the power of comparing clonally-derived mutant and wild-type cells obtained from the same patient and grown under identical conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the following: patients and staff of the Addenbrookes MPN clinic who have contributed samples to this study; Joanna Baxter, Clare Hodkinson, Jenny Webb and Anthony Bench for sample banking and processing; David Frank (Harvard) for supplying the STAT1C expression construct; Yvonne Silber, Dean Pask, Tina Hamilton, Rachel Pestridge, Rebecca Kelley and Jacinta Carter for technical assistance; Anna Petrunkina for flow cytometry sorting; Maria Ahn, Francesca Pagano and Mark Dawson for constructive comments on the manuscript and Brian Huntly, Bertie Gottgens, Katrin Ottersbach for useful discussions. This work was supported by the Leukemia & Lymphoma Research, the Kay Kendall Leukaemia Fund, the Cambridge NIHR Biomedical

*Cancer Cell*. Author manuscript; available in PMC 2011 May 1.
Research Center, and the Leukemia & Lymphoma Society of America. PJC is a Wellcome Trust senior clinical fellow. ALG is a Kay Kendall junior clinical fellow. The authors declare no competing financial interests.

References


Cancer Cell. Author manuscript; available in PMC 2011 May 1.


Figure 1. Strategy for analysis of paired normal and \textit{JAK2V617F} samples from MPN patients
BFU-E colonies were grown from 20 ET and 16 PV patients in methylcellulose media supplemented with 0.01 U/ml erythropoietin, individually plucked and genotyped for \textit{JAK2V617F} mutation burden. Up to 20 wild-type and V617F-heterozygous colonies were pooled and subjected to further analysis. See also Figure S1.
Figure 2. 

**JAK2V617F-associated transcriptional changes are less than those due to inter-individual differences and include activation of STAT5 signaling**

(A) Dendrogram constructed from unsupervised hierarchical clustering of all 72 datasets (paired wild-type and V617F-heterozygous datasets from 20 ET and 16 PV patients) using Pearson correlation. Datasets from PV patients (listed PV.1 to PV.16) are depicted as light red for expression profiles from wild-type erythroblasts and dark red for expression profiles from V617F-heterozygous erythroblasts, with each patient connected by a line to their two paired expression profiles. Datasets for ET patients (ET.1 to ET.20) are similarly depicted as light blue for expression profiles from wild-type erythroblasts and dark blue for expression profiles from V617F-heterozygous erythroblasts. **(B)** Gene set enrichment analysis for genes significantly associated with V617F mutation across all MPN patients. Enrichment was seen for gene sets comprised of components of the erythropoietin pathway and for STAT5A targets. **(C)** qPCR validation of PIM1 and CISH up-regulation in V617F-heterozygous erythroblasts. Fold increase represents the ratio of gene expression in V617F-heterozygous compared to wild-type erythroblasts, with each data point representing an individual ET (blue) or PV (red) patient. **(D)** Immunocytochemical staining of cytospins of wild-type and V617F-heterozygous BFU-E pools from PV and ET patients (left panel). Histograms show significantly higher numbers of cells positive for pY694-STAT5 in V617F-heterozygous colonies compared to wild-type colonies in both PV and ET (right panel). Scale bars indicate 5 μm. Results represent the mean ± S.D. for 3 PV and 3 ET patients. See also Figure S2.
Figure 3. Activation of interferon signaling and STAT1 phosphorylation in JAK2V617F-heterozygous erythroblasts from patients with ET but not those with PV

(A) Hierarchical clustering of V617F-associated expression profiles of all 36 patients according to the 171 genes which showed significant interaction between the V617F mutation and each disease class (i.e. PV or ET). Each gene is expressed as a ratio of expression in V617F-heterozygous to autologous wild-type sample. Bars at the top of the graph display disease phenotype for each patient (Blue: ET; red: PV). (B) GSEA demonstrates that targets of interferon signaling are enriched amongst genes upregulated by JAK2V617F in ET but not PV. (C) Real-time quantitative RT-PCR analysis of five IFNγ target genes (IFI44L, GBP2, IRF1, HLA-F, IFITM3) in V617F-heterozygous and wild-type colonies from ET patients (n=10) and PV patients (n=9). Fold increase represents the ratio of gene expression in V617F-heterozygous relative to autologous wild-type erythroblasts, with each data point representing an individual ET (blue) or PV (red) patient. Closed points represent patients used in the gene expression profiling; open points represent a patient from an independently derived validation cohort. (D) Intracellular flow cytometry shows elevated expression of pY701-STAT1 in V617F-heterozygous BFU-E colonies relative to autologous wild-type colonies from ET patients (n=6), and not in PV patients (n=5). (E) pY701-STAT1 staining of cytospins of wild-type and V617F-heterozygous BFU-E pools from PV and ET patients (left panel). Histogram depicts increase in percentage of cells positive for pSTAT1 in V617F-heterozygous colonies from ET patients only compared to PV (right panel). Scale bars indicate 5 μm. Results represent the mean ± S.D. for 3 PV and 3 ET patients. See also Figure S3.
Figure 4. STAT1 activation enhances megakaryocytic differentiation and inhibits erythroid differentiation in K562 cells

(A) Western immunoblot shows increased pY701-STAT1 expression in K562 cells transduced with a constitutively active form of STAT1 (STAT1C), compared to cultures transduced with an empty vector (VA) or wild-type STAT1 cDNA (STAT1WT). (B) Representative FACS profiles showing that PMA treatment results in increased numbers of CD41-expressing cells in the STAT1C-infected. Also note concomitant increase in cell size as assessed by forward scatter (FSC), consistent with a megakaryocytic-like phenotype. (C) Real-time quantitative RT-PCR of PMA-treated K562 cells shows increased expression of GpIX transcripts in STAT1C-infected K562 cultures relative to VA and STAT1WT-infected control cultures. (D) PMA treatment results in increased numbers of polyploid (>4n) cells in STAT1C-infected K562 cultures. (E) Real-time quantitative RT-PCR demonstrating lower levels of γ-globin transcripts in STAT1C-infected K562 cultures relative to VA and STAT1WT-infected control cultures following erythroid differentiation with hemin. (F) o-Dianisidine staining showing decreased numbers of hemoglobin-positive cells in STAT1C-expressing K562 cells differentiated with hemin. (G) Increased hemoglobin levels in STAT1C-expressing cells differentiated with hemin. (C-G) Results represent the mean ± S.D. for 3 independent experiments. See also Figure S4.
Figure 5. STAT1 activation enhances megakaryocytic differentiation and inhibits erythroid differentiation in normal human progenitors

(A) Intracellular flow cytometry to detect pSTAT1 in cord blood CD34+ cells following transduction with lentiviruses expressing wild-type STAT1 (STAT1WT) or constitutively active STAT1C (STAT1C). Presence of pY701-STAT1 was detected in GFP+ subpopulation of STAT1C-transduced cultures only. (B) STAT1WT- and STAT1C-infected cells were cultured in megakaryocytic differentiation conditions (blue) or erythroid differentiation conditions (red) (refer to Supplemental Experimental Procedures). Total numbers of GFP+ cells during 12 days of culture under both differentiation conditions are shown. (C-F) Expression of STAT1C in cord blood-derived CD34+ cells grown in conditions supporting megakaryocyte differentiation result in increased numbers of GFP+CD41+ cells (Panel C), increased numbers of GFP+CD61+ cells (Panel D), increased expression of GpIX transcripts (Panel E), and increased numbers of polyploid (>4n) cells (Panel F). (G-H) Expression of STAT1C in CD34+ cells grown in conditions supporting erythroid differentiation resulted in decreased numbers of GFP+GPA+CD71+ cells (Panel G), and decreased expression of γ-globin (Panel H). (B-H) Results represent mean ± S.D. for 3 independent cultures. The data are representative of two independent experiments. See also Figure S5.
Figure 6. Down-regulation of STAT1 activity in JAK2V617F-heterozygous progenitors from ET patients results in a PV-like phenotype with increased erythroid and reduced megakaryocytic differentiation.  

(A) Strategy for assessing erythroid/megakaryocytic differentiation potential of CD34+ cells from ET patients following expression of an empty vector (VA) or a dominant negative STAT1 (DN). (B) Typical FACS profiles illustrating the three clone types generated: megakaryocytic, erythrocytic and mixed. (C) Histogram showing the relative proportions of the 3 different types of GFP+ V617F+ clones derived from cells infected with virus expressing empty vector (VA) or the dominant negative STAT1 (DN). Increased percentages of GFP+ erythroid clones and decreased percentages of GFP+ megakaryocytic clones were derived from cells infected with the DN lentivirus compared to cells infected with the VA. Numbers above bars indicate total number of clones tested. Testing for statistical significance was performed using a Fisher-Freeman-Halton test.
Figure 7. Model for the different effects of JAK2V617F in ET and PV
In ET, JAK2V617F induces simultaneous activation of both STAT5 and STAT1 signaling pathways. Activation of pSTAT1 constrains erythroid and promotes megakaryocytic differentiation. In PV, reduced pSTAT1 response to JAK2V617F removes the “brake” on erythropoiesis, thus allowing the development of an overt erythrocytosis, and also reduces megakaryopoiesis.