Cancer Therapy: Preclinical

Clinical Cancer Research

Intrinsic Resistance to JAK2 Inhibition in Myelofibrosis

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Abstract

Purpose: Recent results have shown that myeloproliferative neoplasms (MPN) are strongly associated with constitutive activation of the Janus-activated kinase (JAK)2 tyrosine kinase. However, JAK2 inhibitors currently approved or under development for treating myeloproliferative neoplasms do not selectively deplete the malignant clone, and the inhibition of activity of the drug target (JAK2) has not been rigorously evaluated in the clinical studies. Therefore, in this study we developed an *in vitro* assay to gain insight into how effectively JAK2 activity is inhibited in the samples of patients.

Experimental Design: We treated primary cells from normal donors and patients with MPN with JAK2 inhibitors and measured phosphorylation of downstream targets STAT5 and STAT3 by flow cytometry. Obtained results were next correlated with JAK2 V617F allele burden and plasma cytokine level.

Results: We observed a dose-dependent decrease in pSTAT5 and pSTAT3 in *ex vivo* treated granulocytes. However, phosphorylation of STAT3 and STAT5 in cells from patients with myelofibrosis was significantly less inhibited when compared with cells from patients with polycythemia vera, essential thrombocythemia, and normal donors. Sensitivity to inhibition did not correlate with JAK2 V617F clonal burden. Mixing studies using plasma from patients with myelofibrosis did not transfer resistance to sensitive cells. Likewise, no single cytokine measured seemed to account for the observed pattern of resistance.

Conclusions: Taken together, these observations suggest that there are cell intrinsic mechanisms that define *a priori* resistance to JAK2 inhibition in myelofibrosis, and the lesion is localized upstream of STAT3 and STAT5. *Clin Cancer Res; 19(7); 1729–39. ©2013 AACR.*

Introduction

Polycythemia vera, essential thrombocythemia, and myelofibrosis are myeloproliferative neoplasms (MPN) unified by the constitutive activation of the Janus-activated kinase (JAK)2 pathway, conferred most commonly by a point mutation in the pseudokinase domain of JAK2 ($JAK2^{V617F}$; refs. 1–4). The identification of this mutation has prompted wide interest in targeting JAK2 for therapeutic benefit. Myelofibrosis, whether primary or evolved from polycythemia vera or essential thrombocythemia, has a variable but overall poor prognosis. Clinical investigation of JAK2 inhibitors has focused on myelofibrosis with some unexpected results. Although reduction in spleen size and amelioration of constitutional symptoms are frequent and can be dramatic, only modest changes in mutant clonal burden have been observed and complete responses by any criteria are exceedingly rare (5–7).

Notably, validation of inhibition of activity of the drug target (JAK2) has never been rigorously evaluated in clinical studies. JAK family proteins are nonreceptor tyrosine kinases that are associated with cytokine receptors. Cytokine engagement with receptor leads to the phosphorylation of JAK2 and the recruitment and phosphorylation of cytoplasmic signal transducers and activators of transcription (STAT3 and 5). Upon activation, phosphorylated STAT3 and STAT5 (pSTAT3 and pSTAT5) form homodimers and translocate to the nucleus to activate transcription. In MPN, JAK2 with a V617F substitution is constitutively active and confers cytokine independence/hypersensitivity to hematopoietic tissues. Small-molecule tyrosine kinase inhibitors have been shown to be active in both preclinical and late-phase clinical testing, with analyses of cytokine arrays and measurement of $JAK2^{V617F}$ clonal burden used as potential biomarkers for response (6, 5). To study the efficacy of JAK2 inhibitors to inhibit JAK signaling in primary patient cells, we used phospho-specific flow cytometry on whole peripheral blood from patients with MPN and normal controls to measure phosphorylation of STAT3 and STAT5, the canonical targets of JAK2. We hypothesized that this measurement would be a feasible and informative pharmacodynamic assay for measuring JAK2 signaling in patients on clinical trials and that differences in signaling might be observed across the spectrum of disease.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The identification of Janus-activated kinase (JAK)2 mutations in classical, Philadelphia-chromosome negative myeloproliferative neoplasms (MPN) suggested a target for small-molecule therapy for MPN, with the hope of responses akin to those seen in chronic myeloid leukemia treated with imatinib. Although subsequent studies of JAK2 inhibitors have proven that they are therapeutic, responses have been unexpected, and do not in fact meaningfully deplete the clonal burden of disease. The mechanism(s) through which JAK2 inhibitors exert activity are incompletely understood including how effectively they inhibit JAK2 kinase activity in blood cells. We directly measured downstream inhibition of STAT3 and STAT5 phosphorylation in whole blood and found that across disease subtypes, the response to inhibition varied with myelofibrosis overall resistant. Our findings identify a pattern of resistance that is not in the classical "escape mutation" paradigm and may begin to explain the attenuated clinical responses that have been observed in the clinic.

Materials and Methods

Peripheral blood samples

Peripheral blood samples from patients and normal donors were collected, annotated, and stored through an Institutional review board–approved hematologic diseases tissue bank. Previously collected, anonymous samples were obtained from the Stem Cell and Xenograft Core facility (University of Pennsylvania, Philadelphia, PA).

Reagents

JAK2 inhibitors: CEP701 (lestaurtinib) was provided by Cephalon Oncology or purchased from LC laboratories. CYT387 and INCB018424 were synthesized by ChemieTek and kindly provided by Dr. Ross Levine (Memorial Sloan-Kettering Cancer Center, New York, NY). Antibodies were purchased from BD Bioscience and R&D Systems.

Flow cytometry

Whole blood samples were fixed and permeabilized using a formaldehyde/triton/methanol method (8, 9) and analyzed by flow cytometry. Briefly, 100 μL of whole blood was exposed to varying concentrations of the inhibitor for 15 minutes at 37°C and then stimulated for 20 minutes at 37°C with 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; for pSTAT5 measurement) or 100 ng/mL granulocyte colony-stimulating factor (G-CSF; for pSTAT3 measurement). After stimulation, the samples were fixed with 4% formaldehyde for 10 minutes at room temperature and permeabilized with 0.1% Triton X-100 for 15 minutes at 37°C. Next, samples were washed twice in cold PBS supplemented with 4% bovine serum albumin (BSA), treated with cold 100% methanol to enhance epitope availability, and stored at −20°C. Before analysis, samples were washed twice in cold PBS supplemented with 4% BSA and then incubated with directly labeled antibodies at room temperature for 30 minutes in the dark. Data were acquired on a BD FACSCalibur using CellQuest Pro software and analyzed using FlowJo version 9.3.1. For sample of each patient, untreated mean fluorescence intensity (MFI) of pSTAT3 and pSTAT5 was subtracted from treated samples and MFI for cytokine-stimulated samples was set at 100%. Persistence of pSTAT3 and pSTAT5 in the presence of inhibitor (X) was calculated as the ratio of mean fluorescence values of gated events relative to cytokine-stimulated sample; inhibition is described as 100−X. Statistical analysis was conducted using GraphPad Prism 5.

JAK2 V617F allele burden

Mononuclear cells and granulocytes were separated by density using Ficoll-Paque (Amersham Bioscience). The genomic DNA from granulocytes was isolated from cells using Gentra Purgene Blood Kit from Qiagen. *JAK2 V617F* allele burden was measured using a quantitative real-time PCR (qRT-PCR) assay adapted from Nussenzveig and colleagues (10) to detect the G→T substitution at position 1849 in exon 14 that uses an allele-specific (wild-type vs. mutant) primer with specificity enhanced by mismatch and a locked nucleic acid modification. Allele frequency was calculated as described by Germer and colleagues (11).

Determination of cytokine level in plasma from patients and normal donors

Plasma samples were obtained by centrifugation of whole peripheral blood at 600 × *g* for 10 minutes and stored at −80°C. Batched frozen samples were analyzed by the Human Immunology Core (University of Pennsylvania) using a Luminex instrument and the Milliplex $_{MAP}$ Human cytokine 9-plex from Millipore.

Statistical analysis

One-way ANOVA and Student *t* test were used to analyze differences between patient and normal donor samples. Correlations were calculated using the Spearman correlation coefficient. All calculations were made using GraphPad Prism 5.

Results

Phosphorylation of STAT5 and STAT3 is detectable with cytokine stimulation and is inhibited in the presence of CEP701

To more precisely validate target and to define biologic response to treatment with JAK2 inhibitors in patients with MPN, we investigated the level of target inhibition in peripheral blood samples collected from 29 patients with MPN (Table 1) and 6 normal donors. We measured phosphorylated STAT3 and 5 (pSTAT3 and pSTAT5) as biomarkers for JAK2 inhibition. In our initial studies we did not observe constitutive basal phosphorylation of STAT3 or STAT5 in any samples evaluated (Fig. 1A and

Table 1. Patient characteristics

ID #	Registry ID	Diagnosis	Age	Sex	Race	WBC (THO/μL)	% Blasts	HGB (g/dL)	PLT (THO/μL)	Cytogenetics	Treatment history	V617F (%)	Years from diagnosis
2211	R1267	Post-PV Myelofibrosis	74	M	W	9.8	0	9.5(t)	49	46,XY [16]	PB, ESA	89	0.9
2212	R0602	Polycythemia vera	56	F	W	4.6	0	14.6	356	46,XX [26]	PB, HU, ASA	67	19.2
2222	R1243	Primary myelofibrosis	68	F	W	5.2	0	8.5 (t)	222	46 XX [22]	THAL/PRED	74	0.7
2223	R1092	Essential thombocythemia	82	F	W	8.7	0	12.8	600	46,XX [20]	HU, ANAG, ASA, CHLR	75	3.4
2273	R0682	Essential thombocythemia	78	M	W	6.5	0	12.6	402	46,XY [8]	HU, ASA	63	4.3
2283	R0633	Primary myelofibrosis	46	F	W	4.1	0	9.1	245	46,XX[20]	ANAG, ASA	0	11.6
2294	R1300	Primary myelofibrosis	65	F	W	5.5	0	8.9	129	46,XX [20]	ESA	ND	0.4
2310	R0973	RARS-T	67	F	AA	12.8	1	8.4	1961	46,XX [20]	HU, ANAG, IFN, ESA	70	0.8
2316	R1231	Primary myelofibrosis	67	M	W	13.7	5	10.7	123	46,XY [20]	ESA	9	1.5
2317	R1312	Polycythemia vera	78	F	W	13.1	0	14.8	346	ND	PB, HU	15	9.4
2327	R1319	Polycythemia vera	41	M	W	4.2	0	14.4	171	ND	PB, ASA,IFN	79	12.7
2328	R1320	Essential thombocythemia	46	M	W	5	0	13.4	320	46,XY [20]	HU	70	15.8
2340	R1231	Primary myelofibrosis	67	M	W	11.6	3	9.3	93	46,XY [20]	ESA	11	1.6
2341	R1325	Primary myelofibrosis	66	M	W	18.1	0	10.1	1183	46,XY [21]	ESA, HU, ASA	73	0.1
2343	R1322	Polycythemia vera	56	M	W	12.1	0	18.2	443	ND	PB, ASA	48	0.1
2359	R1182	Essential thombocythemia	73	F	AA	4.9	0	13.3	721	46,XX [20]	HU, ASA	79	1.1
2369	R0633	Primary myelofibrosis	47	F	W	4.1	2	9.7	217	ND	ANAG, ASA	0	8.4
2370	R1269	Primary myelofibrosis	58	F	W	9.5	0	13.4	89	ND	none	57	0.6
2371	R1332	Essential thombocythemia	40	M	W	13.7	0	17.1	608	46, XY [20]	ASA	91	1.9
2469	R0682	Essential thombocythemia	78	M	W	9.4	0	13.2	550	46, XY [20]	HU, ASA	54	4.8
2470	R1362	Polycythemia vera	45	F	W	4.4	0	12.8	368	46,XX [25]	ANAG, ASA, IFN	ND	17.2
2479	R1308	AML (from post-PV MF)	62	M	AA	121.9	35	ND (t)	91	Complex[a]	PB, HU, ASA, IFN	58	0.8
2487	R0670	Post-PV myelofibrosis	57	F	W	4.7	2	9.6	133	Complex[b]	PB, HU, ASA, IFN, HSCT	52	1.3
2517	R1378	Primary myelofibrosis	63.1	M	W	7.6	0	12.3	110	46,XY [20]	ANAG, HU,ASA	POS	9.0
2521	R1236	Primary myelofibrosis	81.8	F	W	5.1	22	10.6	87	46,XX [20]	HU, ESA	0	5.0
3013	R1550	Polycythemia vera	43	M	W	4.3	0	12.7	227	46,XY [20]	HU, IFN, PB, ASA	POS	1.7
3014	R0639	Essential thombocythemia	60.5	F	W	7	0	11.5	1040	ND	ANAG, HU, ASA	0	13.0
3016	R1589	Polycythemia vera	59	M	AA	6.8	0	15	304	46,XY [20]	ANAG, HU, ASA		6.0
2518	R1319	Polycythemia vera	42	M	W	3.1	0	12.6	122	ND	ASA, PB, IFN	POS	13.0

Abbreviations: ANAG, anagrelide; ASA, aspirin; CHLR, chlorambucil; ESA, erythropoietin/darbopoietin; HSCT, hematopoietic stem cell transplantation; HU, hydroxyurea; ND, no data; PB, phlebotomy; POS, positive but unknown percentage.

[a]47,XY, +der(9)t(1;9)(q21;q21)[1]/47, idem,del(5)(q21q34)[19]. [b]46,XX+der(9)t(1;9)(q12;q21)[20].

Supplementary Fig. S1A; $P = 0.48$). Therefore, we incorporated cytokine activation into the assay. Unmanipulated whole blood from normal donors or patients was stimulated with GM-CSF or G-CSF known to activate phosphorylation of STAT5 or STAT3, respectively, in human myeloid cells (12, 13). Activation was measured in granulocytes by gating on CD15+ cells. There were no differences in cytokine responsiveness across disease and normal samples. The total level of intracellular STAT5 was measured to ensure adequate permeabilization of cells and did not change with stimulation or inhibition (Supplementary Fig. S1B and S1C).

We next used this assay to study the efficacy of JAK2 inhibitors (Fig. 1B and C). For these studies, blood from normal donors, patients with essential thrombocythemia, polycythemia vera, and patients with the more advanced disease, myelofibrosis (either primary or evolved from polycythemia vera or essential thrombocythemia) was first exposed to inhibitor, primarily lestaurtinib (CEP701) at a concentration of 10 to 50 μmol/L and then stimulated with GM-CSF or G-CSF to activate phosphorylation of STAT5 (Fig. 1B) or STAT3 (Fig. 1C), respectively. The range of inhibitor concentration was based on mean steady-state plasma concentrations of 7.7 to 12 μmol/L (1–40 μmol/L) previously described in treated patients for CEP701 (14), and from published pharmacokinetic information on achievable concentrations, when available, for INCB018424 and CYT387 (15). We observed a dose-dependent decrease in cytokine-activated pSTAT3 and pSTAT5 in CD15+ granulocytes from normal donors when treated *ex vivo* with CEP701 (Fig. 1B and C).

Neutrophils from patients with myelofibrosis are intrinsically resistant to JAK2 inhibitors

Next, we sought to measure response to treatment across MPN phenotypes. We found that the response to JAK2 inhibitor differs among patients with MPN. Although phosphorylation of STAT5 (Fig. 2A) and STAT3 (Fig. 2B) in both essential thrombocythemia and polycythemia vera was similar and nearly completely abrogated in the presence of 20 μmol/L CEP701, pSTAT3 and pSTAT5 in myelofibrosis samples were minimally inhibited (Fig. 2A and B). In aggregate across samples, the mean inhibition at 20 μmol/L in essential thrombocythemia/polycythemia vera samples as measured by pSTAT5 (69%), was significantly more than that in myelofibrosis samples (36%, $P = 0.015$; Fig. 3A and Supplementary Table S1). The response to inhibition in normal donors (76% inhibited) was comparable with that observed in essential thrombocythemia and polycythemia vera samples (pSTAT5, 68.9% inhibited; $P = 0.90$). For pSTAT3, polycythemia vera/essential thrombocythemia samples were more sensitive (73% inhibited) when compared with myelofibrosis samples (60% inhibited) although this was not significant ($P = 0.43$). This suggests that either downstream signaling through STAT3 in myelofibrosis samples is truly more responsive to inhibition than STAT5 or may reflect a more subtle difference not captured in this number of samples. We also noted a narrower dynamic range for pSTAT3 measurements (Supplementary Fig. S1B). Thus, we observed that pSTAT5 and pSTAT3 can be measured in whole blood by phospho-flow cytometry in the presence of exogenous cytokine and that pharmacologic inhibition is both dose- and MPN-subtype dependent. To extend this observation using 2 additional tyrosine kinase inhibitors, we repeated these studies with CYT387 and INCB018424, agents currently being investigated/approved for the treatment of myelofibrosis. We found the samples of myelofibrosis to be similarly less sensitive to inhibition with these compounds when compared with polycythemia vera samples (Fig. 3B). The samples of myelofibrosis exposed to 10 and 20 μmol/L CYT387 were significantly more resistant (28% and 36.4% inhibition of pSTAT5, respectively) than polycythemia vera samples (74% and 93% inhibition, $P = 0.003$ for 10 μmol/L; $P = 0.001$ for 20 μmol/L). Differences in response observed to INCB018424 were also significant (10 μmol/L; myelofibrosis samples were 42% inhibited, whereas polycythemia vera samples were 72% inhibited, $P = 0.025$). Taken together, these results suggest that peripheral blood neutrophils from patients with myelofibrosis were intrinsically resistant to JAK2 inhibitors.

To begin to understand what, if any, coherence might exist between terminally differentiated neutrophils and more primitive progenitor compartments with respect to signaling response, we measured pSTAT5 in CD15+ and CD34+ cells exposed to CYT387 and INCB018424 from a patient with acute myeloid leukemia that had evolved from post-polycythemia vera myelofibrosis (Fig. 4). We found that CD34+ stimulation with GM-CSF results in a more heterogeneous pSTAT5 signal, likely reflecting differences in GM-CSF receptor expression in this compartment (16), and that the response to inhibition in CD34+ cells generally mirrors that in CD15+ cells.

The pattern of resistance is not correlated with *JAK2 V617F* allele burden in myelofibrosis

To gain insight into a mechanism by which STAT3 and STAT5 remain phosphorylated even in the presence of upstream JAK2 inhibition, we investigated whether we could detect a correlation between the frequency of *JAK2 V617F*-mutant allele burden in samples from patients with the mutation and response to drug. Overall, we found no relationship between allele frequency and level of target inhibition in tested samples (Table 2 and Supplementary Fig. S2). Likewise, a similar (and near complete) pattern of resistance was observed in a patient with myelofibrosis who was negative for the *JAK2 V617F* mutation. Thus, the mutant to wild-type ratio of JAK2 does not seem to directly account for the observed pattern of resistance. We did note that in cells from patients without myelofibrosis, there indeed seemed to be a significant direct correlation with higher levels of *JAK2 V617F* and persistent signaling ($P = 0.04$); although we interpret this cautiously, given the numbers of samples ($n = 2$) from patients with lower allele burdens.

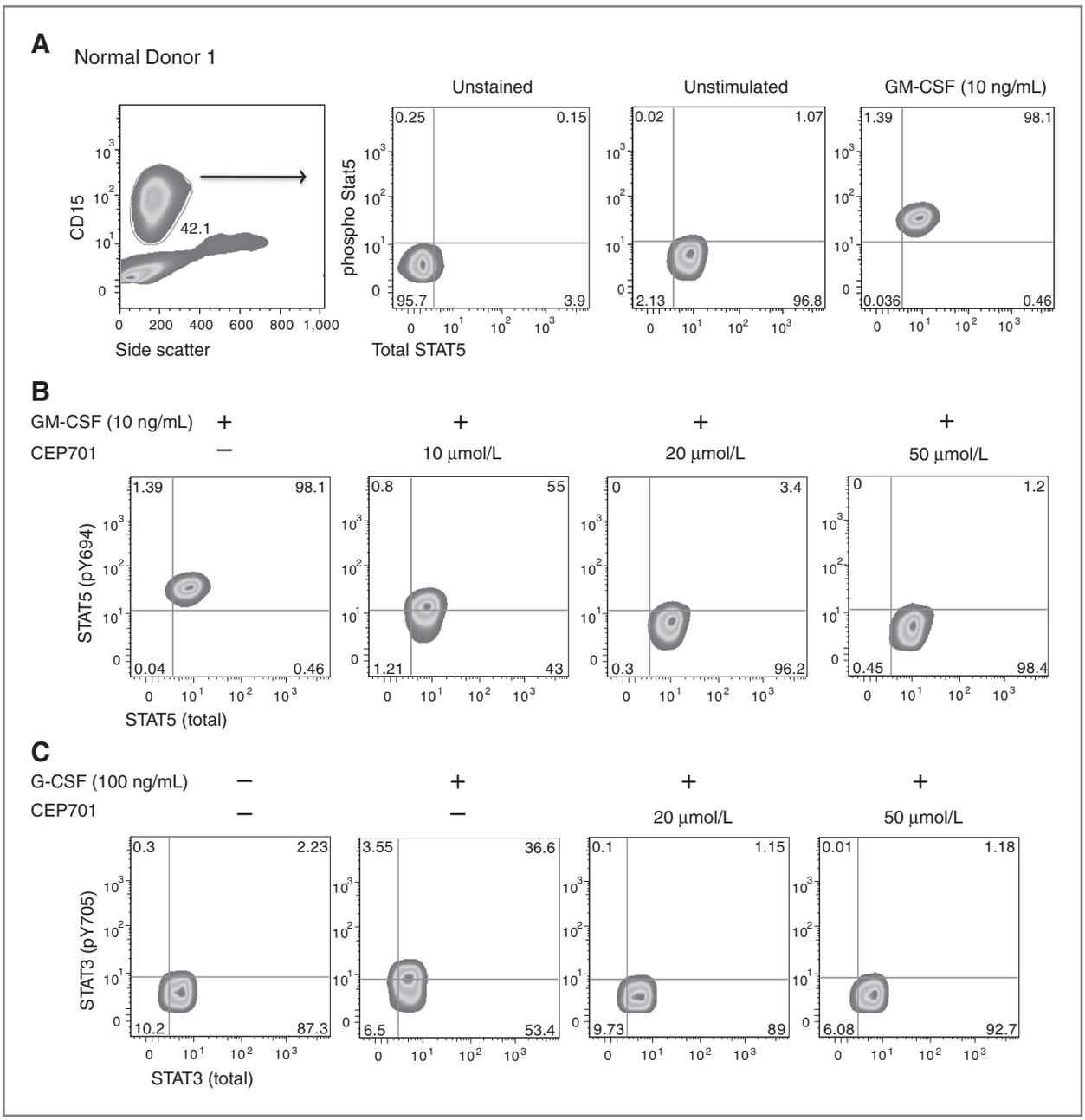

Figure 1. Measurement of STAT5 and STAT3 phosphorylation in normal peripheral blood granulocytes. A, the gating strategy for isolating granulocytes based on forward/side scatter properties and expression of CD15 antigen. Total (*x*-axis) and phospho- (*y*-axis) STAT5 (B) and STAT3 (C); detection of total STAT3 or STAT5 served as an internal control for permeabilization. B and C, target inhibition in response to exposure to CEP701 at 10, 20, and 50 μmol/L doses.

Plasma components do not confer resistance to JAK2 inhibition

We next investigated the possibility that plasma components might contribute to or confer resistance of myelofibrosis samples to JAK2 inhibitors. We mixed cells from a patient with polycythemia vera (sample#: 2518) with plasma from patients with myelofibrosis (samples#: 2517 and 2521) and evaluated inhibition of pSTAT5 (Fig. 5A–F). For a given patient, response to CYT387 or INCB018424 as measured by mean pSTAT5 fluorescence followed a similar trend regardless of plasma source, which suggested that neither resistance to inhibition nor sensitivity to treatment was conferred by plasma components. We also analyzed basal cytokine levels in plasma samples from MPN samples and normal donors. Plasma cytokines that signal through JAK2 and/or are known to be altered in myelofibrosis were measured. No meaningful differences that could directly explain the differences in response were observed. For most

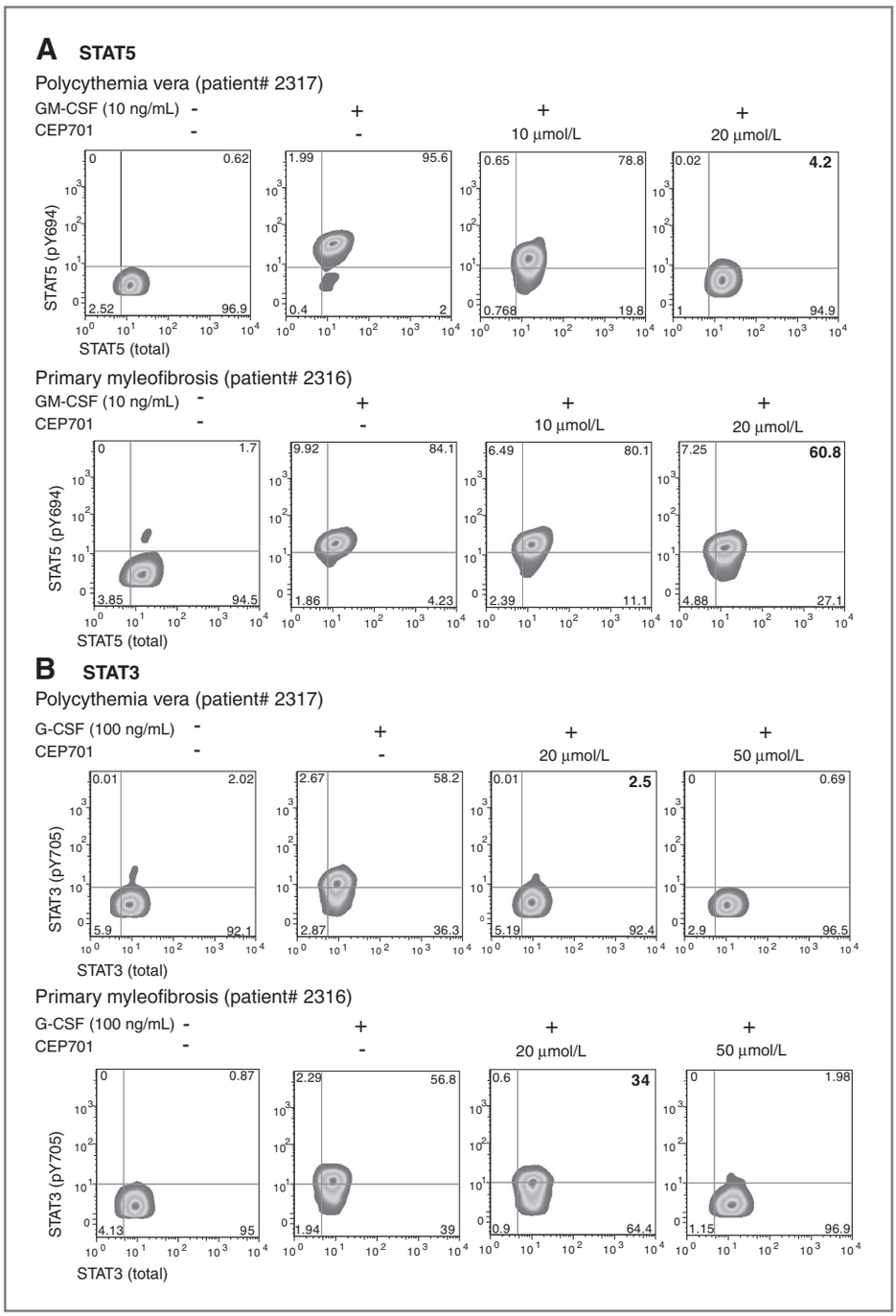

Figure 2. Relative resistance to inhibition of STAT5 and STAT3 phosphorylation in myelofibrosis. Representative flow cytometry plots show level of inhibition of STAT5 (A) and STAT3 (B) phosphorylation after exposure to CEP701 for patients respectively diagnosed with polycythemia vera (top) and primary myelofibrosis (bottom).

of the 9 evaluated cytokines (MIP-1β, TNF-α, IL-6, GM-CSF, IFN-γ, G-CSF, IFN-α, and Rantes), the observed concentration in patients with MPN was higher than that in normal donors, consistent with other reports (refs. 6, 17; Supplementary Fig. S3). In particular, the basal plasma levels of the 2 cytokines also used exogenously in our experiments (G- and GM-CSF) were plotted against response to inhibitor, and there was no significant correlation between basal GM-CSF levels and response to inhibitor ($P = 0.18$, Supplementary Fig. S3B–S3D). These observations thus fail to show a soluble cell extrinsic factor that explains JAK2 inhibitor resistance in granulocytes from patients with myelofibrosis.

Discussion

Our findings show that in myelofibrosis, cell intrinsic properties bestow resistance to JAK2 inhibition, although the mechanism remains unclear. We found that overall

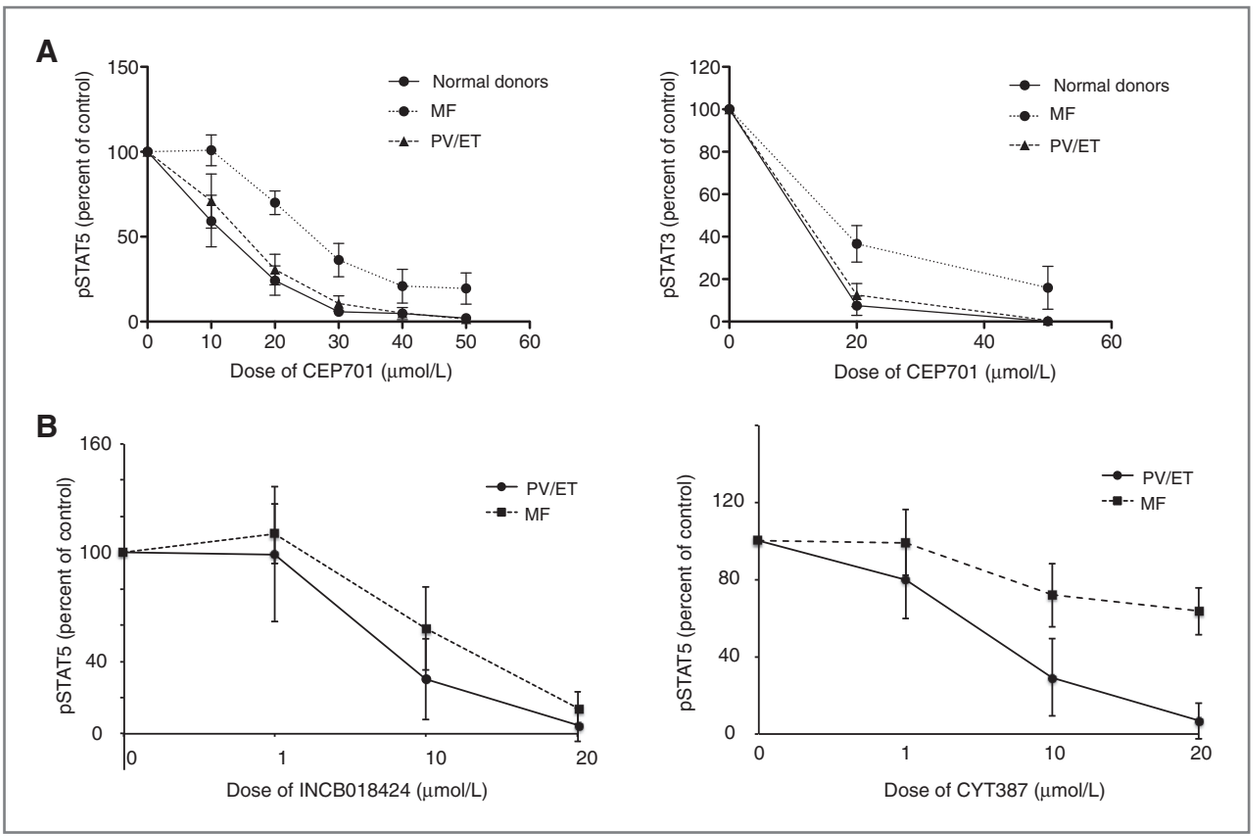

Figure 3. A, CD15+ cells from patients with myelofibrosis are significantly more resistant to JAK2 inhibition than cells from patients with polycythemia vera (PV), essential thrombocythemia (ET), and normal controls. Graphs show molecular response to various doses of JAK2 inhibitor, CEP701, in normal donors when compared with samples from patients with polycythemia vera/essential thrombocythemia and myelofibrosis (MF). Molecular response to the drug is presented as a percentage of STAT5 (left) and STAT3 (right) phosphorylation remaining after exposure to CEP701. Mean fluorescence from cells stimulated with GM-CSF for pSTAT5 or G-CSF for pSTAT3 minus background (mean fluorescence of unstimulated cells) was set as a 100%. B, resistance to inhibition can be generalized to additional JAK2 inhibitors under clinical development. Response to CYT387 (right) and INCB018424 (left) are plotted as the percentage STAT5 phosphorylation after exposure to JAK2 inhibitors. MFI from cells stimulated with GM-CSF for pSTAT5 minus background (mean fluorescence of unstimulated cells) was set as a 100%.

polycythemia vera and essential thrombocythemia samples are sensitive to inhibition by multiple investigational inhibitors, with responses overall comparable with those of normal donors. In contrast, in cells from patients with treatment-naïve myelofibrosis, STAT5 remained phosphorylated despite treatment with inhibitors, suggesting either incomplete upstream inhibition of JAK2 and/or alternative inputs to STAT5 (and possibly STAT3) that are specific to myelofibrosis, typically a more advanced and genetically complex disease. Our studies did not support a role for specific cell extrinsic soluble factors in plasma as major mediators of resistance, nor could we correlate the clonal burden of JAK2 V617F with the resistance phenotype, suggesting that additional acquired features specific to myelofibrosis mediated the attenuated response.

Our signaling studies focused largely on mature, terminally differentiated neutrophils from patients, an experimental design conceived with the intent of developing a feasible peripheral blood-based pharmacodynamic assay for measuring response to treatment. But whether our observation in neutrophils extends to signaling in more primitive, disease-initiating compartments is also of great interest. Our exploratory studies of CD34+ blasts and neutrophils from a patient with acute leukemia evolved from post-polycythemia vera myelofibrosis showed a response that mirrors the response in granulocytes, supporting the possibility that a signaling phenotype carries through to terminal differentiation, but additional studies are needed to draw major conclusions about signaling responses throughout differentiation. Of note, other studies have confirmed that granulocytes from patients with myelofibrosis are derived largely from the malignant clone (18). Although our studies cannot directly distinguish signaling within clonally derived granulocytes versus those derived from the premalignant stem cell pool, the signaling patterns in myelofibrosis tended to be relatively uniform, and would be expected to represent, at least proportionally, clonally derived cells. In contrast, clonal dominance is not the predominant pattern in polycythemia vera and essential thrombocythemia, and this may explain the overlapping signaling phenotype among polycythemia vera, essential thrombocythemia, and normal donors (18). Although we

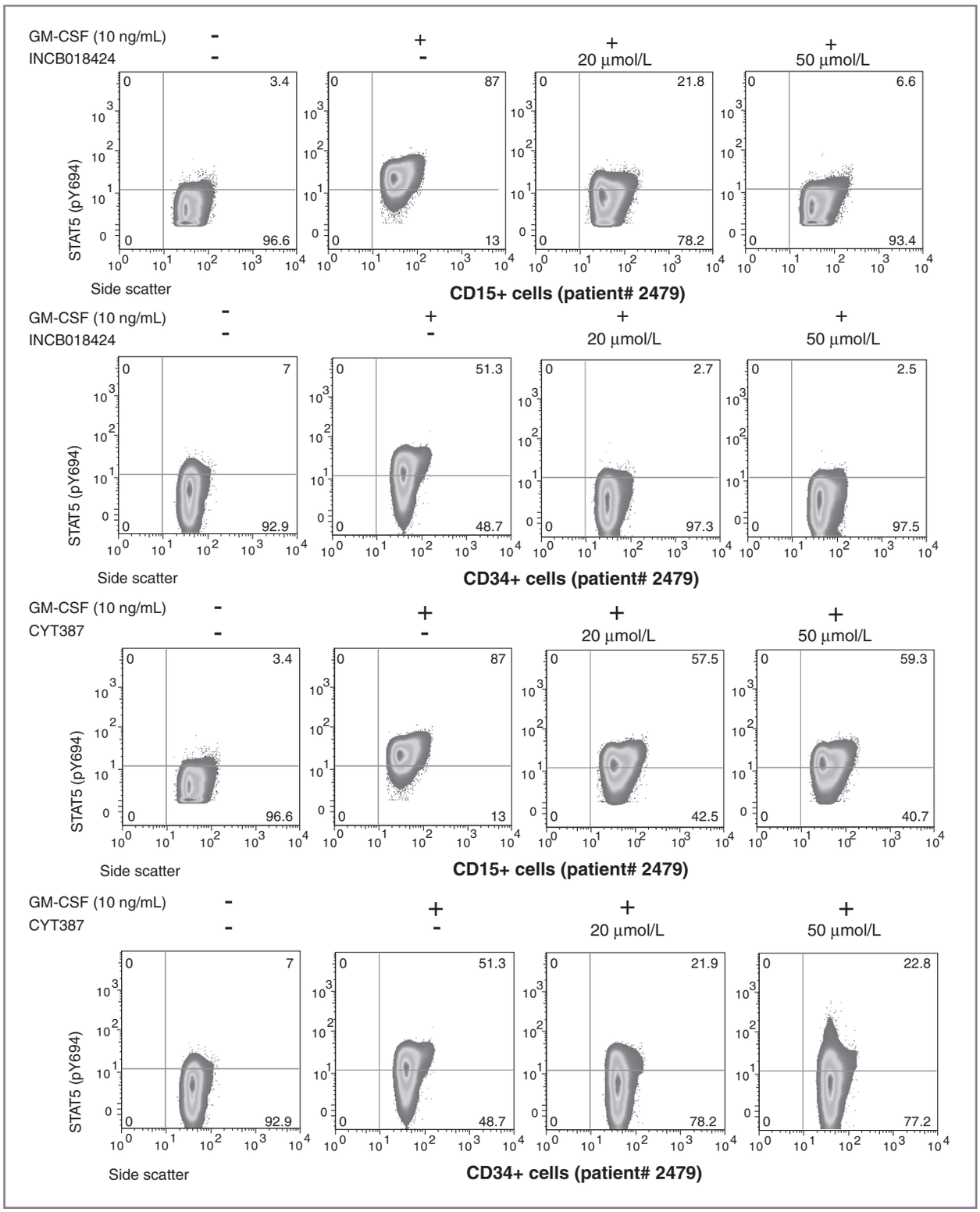

Figure 4. Coherence between terminally differentiated and progenitor signaling response. Measurement of pSTAT3 and pSTAT5 in CD15+ and CD34+ cells exposed to INCB018424 (top) and CYT387 (bottom) from a patient with acute myeloid leukemia evolved from post-polycythemia vera myelofibrosis.

Table 2. Level of mutant *JAK2 V617F* allele frequency in granulocytes does not correlate with response to inhibition of phosphoSTAT5

Stratified by *JAK2 V617F* allele freq	Mean allele freq (%)	Mean % pSTAT5 at 20 μmol/L	*n*
All patients with *JAK2 V617F* mutation			
<50%	17	49.6	5
>50%	74	48.6	11
Polycythemia vera/essential thrombocythemia			
<50%	31	4.05	2
>50%	75	35.74	7
Myelofibrosis			
<50%	8	80.03	3
>50%	73	71.02	4

found no direct correlation between *JAK2 V617F* allele burden and resistance in myelofibrosis, but the samples from patients with essential thrombocythemia or polycythemia vera with higher *JAK2 V617F* seemed to be less sensitive to treatment than samples with lower allele burdens. Taken together, one wonders whether clonal dominance in a bulk population might influence probability of individual cell signaling.

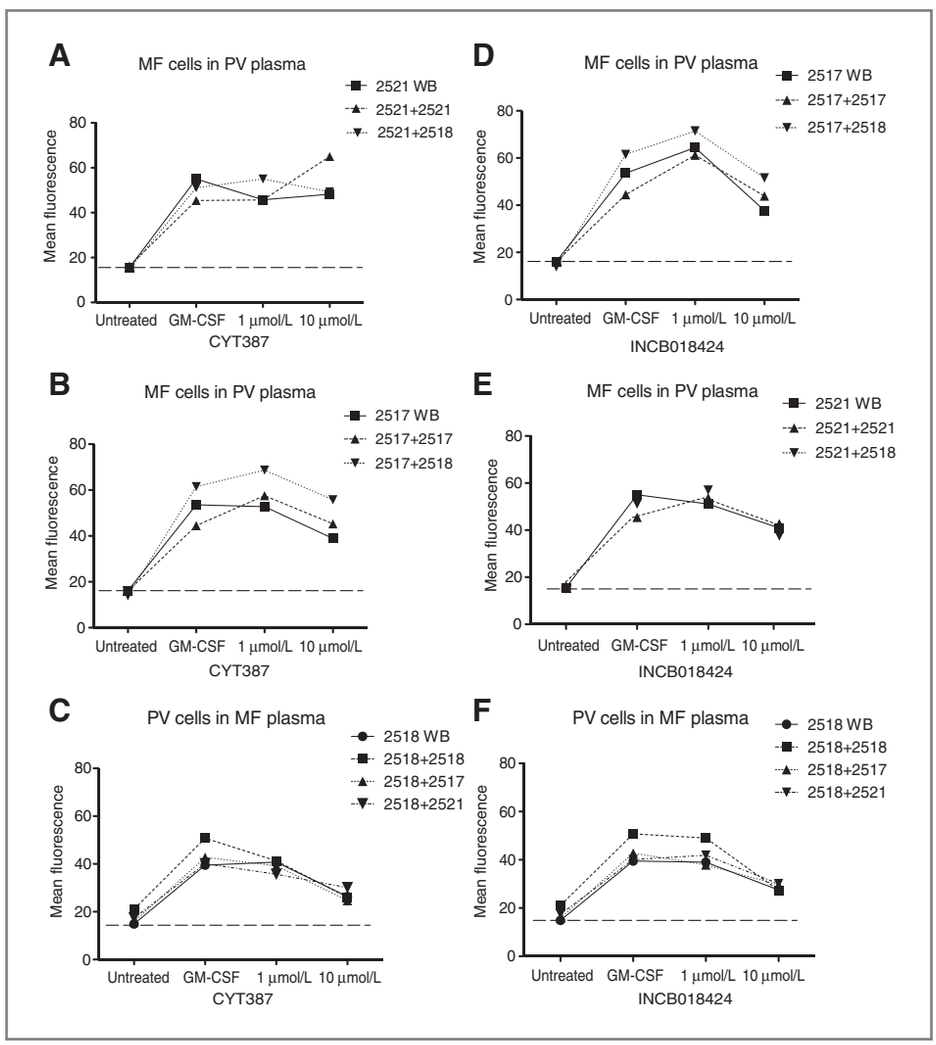

Figure 5. Plasma components do not alter sensitivity to JAK2 inhibitor. Effect of plasma components on response to CYT387 (A–C) or INCB018424 (D–F) as measured by STAT5 phosphorylation and presented as MFI. Peripheral blood cells from 2 patients with myelofibrosis (2521, A and 2517, B) were treated *ex vivo* with GM-CSF in the presence or absence of CYT387, as whole, unperturbed blood (WB), as plasma-depleted blood reconstituted with native plasma (e.g., 2521 + 2521), or with polycythemia vera patient (2518) plasma of equivalent volume (e.g., 2521 + 2518). Likewise, polycythemia vera peripheral blood was exposed to CYT387 (C) or INCB018424 (F) as whole blood, native reconstituted blood, or with plasma from either myelofibrosis patient (2518 + 2517 and 2518 + 2521). Myelofibrosis blood cells (2517, D and 2521, E) were treated with GM-CSF with or without INCB018424 in native plasma or polycythemia vera plasma.

Acquired resistance to targeted therapies has been well described (19), although classical escape mutations do not seem to be a major mechanism of resistance to JAK2 inhibitors. Perhaps unique features of JAK2 as a target lead to less selective pressure for outgrowth of resistant clones, so that classical gatekeeper mutations as seen in chronic myeloid leukemia would not be favored: JAK2 is absolutely required for normal hematopoiesis, and consequently defines a more narrow therapeutic window between diseased, JAK2 dysregulated and normal cells. Elegant experimental engineered *ex vivo* systems of chronic drug exposure have likewise shown that resistance mutations do not account for persistent (or reactivated) downstream signaling in *JAK2 V617F* cells that grow despite the presence of inhibitor (20). Rather, signaling *in trans* of JAK2 by JAK1 (and TYK2) seems to mediate a novel mechanism of drug insensitivity that seems to be epigenetically determined at the JAK2 locus itself. Our data are distinguished from these in that the cells have not been chronically exposed to drug but instead are derived from treatment-naïve patients and yet our observations may have similar underpinnings. Myelofibrosis is increasingly recognized as an entity governed by epigenetic dysregulation that confers competitive advantage to the diseased stem cell compartment, an advantage with which JAK2 V617F itself may conspire (21, 22), but where the mutation alone cannot confer such an advantage (23).

We used pSTAT5 as a surrogate for activation of the JAK2 pathway. One limitation of this strategy is that we cannot exclude the possibility that JAK2 was appropriately inhibited in myelofibrosis samples, whereas STAT5 remained activated in response to other signaling pathways. We were not able to directly detect JAK2/phospho-JAK2 in primary MPN samples. Future studies using other strategies will address whether novel heterodimers can be detected in primary cells, and if present, whether they correlate with persistent activation of STAT3 and STAT5. Our methods also could not specifically address microenvironmental factors known to play an important role in drug response and resistance, which might carry through in progeny of diseased stem/progenitor cells (24). Of particular interest is whether terminally differentiated cell signaling in a clonal population faithfully recapitulates that of more primitive ancestors within the stem/progenitor cell compartment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Kalota, E.O. Hexner
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Kalota, G.R. Jeschke, E.O. Hexner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Kalota, G.R. Jeschke, M. Carroll, E.O. Hexner
Writing, review, and/or revision of the manuscript: A. Kalota, G.R. Jeschke, M. Carroll, E.O. Hexner
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Kalota, E.O. Hexner
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