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Selective Inhibition of JAK2-Driven Erythroid Differentiation of Polycythemia Vera Progenitors

Ifat Geron,[1,6] Annelie E. Abrahamsson,[1,6] Charlene F. Barroga,[1] Edward Kavalerchik,[1] Jason Gotlib,[2] John D. Hood,[3] Jeffrey Durocher,[4] Chi Ching Mak,[3] Glenn Noronha,[3] Richard M. Soll,[3] Ayalew Tefferi,[5] Ken Kaushansky,[1] and Catriona H.M. Jamieson[1,*]

[1]Department of Medicine, University of California, San Diego, La Jolla, CA 92093, USA
[2]Department of Medicine, Stanford University, Stanford, CA 94305, USA
[3]TargeGen Inc., San Diego, CA 92121, USA
[4]Transgenomic Inc., Gaithersberg, MD 20878, USA
[5]Department of Medicine, Mayo Clinic, Rochester, MN 55905, USA
[6]These authors contributed equally to this work.
*Correspondence: cjamieson@ucsd.edu
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SUMMARY

Polycythemia Vera (PV) is a myeloproliferative disorder (MPD) that is commonly characterized by mutant JAK2 (JAK2V617F) signaling, erythrocyte overproduction, and a propensity for thrombosis, progression to myelofibrosis, or acute leukemia. In this study, JAK2V617F expression by human hematopoietic progenitors promoted erythroid colony formation and erythroid engraftment in a bioluminescent xenogeneic immunocompromised mouse transplantation model. A selective JAK2 inhibitor, TG101348 (300 nM), significantly inhibited JAK2V617F$^+$ progenitor-derived colony formation as well as engraftment (120 mg/kg) in xenogeneic transplantation studies. TG101348 treatment decreased GATA-1 expression, which is associated with erythroid-skewing of JAK2V617F$^+$ progenitor differentiation, and inhibited STAT5 as well as GATA S310 phosphorylation. Thus, TG101348 may be an effective inhibitor of JAK2V617F$^+$ MPDs in clinical trials.

INTRODUCTION

Myeloproliferative disorders, such as PV, essential thrombocythemia (ET), and myelofibrosis (MF), are clonal hematopoietic disorders typified by overproduction of terminally differentiated cells in part as a result of hypersensitivity of marrow progenitor cells to hematopoietic growth factors (Adamson et al., 1976; Cashman et al., 1988; Dai et al., 1992; Fialkow et al., 1981; Gilliland et al., 1991; Jamieson et al., 2006; Prchal and Axelrad, 1974; Ugo et al., 2004). Clonal derivation of MPDs from a primitive hematopoietic progenitor was first suggested by studies demonstrating the same glucose-6-phosphate dehydrogenase (G6PD) allele in PV erythrocytes, granulocytes, and platelets (Fialkow et al., 1981) and later confirmed by phosphoglycerate kinase gene PCR that revealed clonal involvement of the myeloid and erythroid lineages in female PV patients (Gilliland et al., 1991). Several recent reports provided critical insight into the molecular events involved in the development of PV by identifying a mutation (V617F) that constitutively activates the JAK2 tyrosine kinase in the majority of patients with PV and approximately 50% of patients with ET and idiopathic myelofibrosis (MF) (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). *JAK2V617F* gene expression in the Ba/F3 growth factor-dependent cell line resulted in erythropoietin (EPO) hypersensitivity and growth factor-independent survival (James et al., 2005). Moreover, transplantation of mouse marrow cells transduced with the *JAK2V617F* mutant allele into lethally irradiated recipients resulted in erythrocytosis typical of PV (James et al., 2005). In addition, mice transplanted with JAK2V617F-expressing cells developed a PV-like disease that

SIGNIFICANCE

Increased morbidity and mortality rates associated with JAK2$^+$ MPDs have provided the impetus for developing small-molecule JAK2 inhibitors. Moreover, JAK2$^+$ MPDs represent an important paradigm for understanding the molecular underpinnings of hematopoietic progenitor cell-fate decisions. We identified an orally bioavailable, selective JAK2 inhibitor, TG101348, which inhibits the aberrant erythroid differentiation potential of PV progenitors in vitro and in a preclinical bioluminescent xenogeneic transplantation model of human PV. This research provides direct in vivo evidence suggesting that JAK2V617F is both necessary and sufficient to drive aberrant human PV progenitor erythroid differentiation through enhanced GATA-1 transcription. This process is potently reversed by TG101348, thereby providing the impetus for its use in clinical trials for JAK2$^+$ MPDs.

progressed to myelofibrosis in a manner analogous to human PV (Wernig et al., 2006). These studies support a critical role for JAK2V617F in the pathogenesis of a large proportion of MPDs (Kaushansky, 2005).

Although JAK2V617F-driven MPDs such as PV, ET, and MF have a combined incidence that is 5-fold higher than chronic myeloid leukemia (CML) (Tefferi and Gilliland, 2007), the first leukemia to be associated with a pathognomic molecular abnormality at the hematopoietic stem cell level (Daley et al., 1990; Fialkow et al., 1977; Holyoake et al., 1999; Jamieson et al., 2004) and the first cancer to be treated with molecularly targeted therapy (Druker et al., 2006; Kantarjian et al., 2006; Talpaz et al., 2006), there has been no treatment developed to date that selectively inhibits JAK2 kinase activity (Tefferi and Elliott, 2007). While JAK2V617F$^+$ PV and ET have a lower rate of progression to acute leukemia than CML, both quality and quantity of life are detrimentally affected by a high prevalence of major thrombotic events (Tefferi and Elliott, 2007). In addition, primary myelofibrosis and acute myelogenous leukemia (AML) or myelofibrosis that develops following sustained myeloproliferation in JAK2V617F$^+$ PV or ET are relatively recalcitrant to current forms of treatment, thereby providing the impetus for developing selective JAK2 inhibitors (Harrison et al., 2005; Kiladjian et al., 2006; Tefferi et al., 2006).

Previously, FACS analysis of early phase PV patient samples revealed an expansion in the number of cells with a HSC phenotype (CD34$^+$CD38$^-$CD90$^+$Lin$^-$) suggesting that PV HSC had enhanced proliferative potential (Jamieson et al., 2006). In hematopoietic progenitor assays, PV HSC gave rise to a preponderance of large erythroid colonies that expressed the JAK2V617F mutation (Jamieson et al., 2006). These results suggested that the JAK2V617F mutation had an inherent capacity to skew differentiation of PV HSC toward an erythroid fate (Jamieson et al., 2006). However, the molecular mechanisms mediating JAK2V617F progenitor cell-fate decisions were not elucidated (Jamieson et al., 2006). Moreover, the capacity of a selective JAK2 inhibitor to prevent JAK2-driven engraftment in xenogeneic transplantation models of human PV had not been investigated.

In this study, we assessed the capacity of JAK2V617F to alter human hematopoietic progenitor cell-fate decisions both in vitro and in a bioluminescent xenogeneic transplantation model of human PV. We also examined erythroid differentiation potential in the presence or absence of a selective small-molecule ATP-competitive JAK2 inhibitor, TG101348, as a means of preclinically assessing its potential efficacy as a molecular-targeted therapy for JAK2-driven MPDs. To investigate possible mechanisms of action, we examined the capacity of TG101348 to inhibit JAK2 driven STAT5-phosphorylation and AKT-mediated GATA-1 S310 phosphorylation as well as GATA-1 and PU.1 transcription factor ratios known to regulate hematopoietic progenitor cell-fate decisions (Zhao et al., 2006).

RESULTS

In Vitro Inhibition of PV Progenitor Erythroid Differentiation by TG101348

TG101348 (Figure 1A) was designed and synthesized at TargeGen using structure based drug design methods to inhibit JAK2 and JAK2V617F kinase (IC_{50} = 3 nM for both; data not shown). In contrast to other currently available inhibitors, TG101348 does not inhibit other closely related kinases such as JAK3 (IC_{50} = 1040 nM; data not shown). In five independent experiments, hematopoietic stem cells (HSC; CD34$^+$CD38$^-$CD90$^+$Lin$^-$) and common myeloid progenitor (CMP; CD34$^+$CD38$^+$CD123$^+$CD45RA$^-$Lin$^-$) cells from three JAK2V617F$^+$ PV patients (Table 1) were FACS-sorted (Jamieson et al., 2006; Manz et al., 2002) into methylcellulose supplemented with human cytokines and increasing concentrations of TG101348. Differential colony counts were performed on day 14. These experiments demonstrated that the propensity of PV progenitors to differentiate along the erythroid lineage was significantly inhibited by 300 nM of TG101348 (BFU-E; $p = 0.02$), as was the formation of mixed colonies (CFU-Mix; $p = 0.05$) (Figure 1B). No significant inhibition of other colony types was observed at this dose although there was a trend toward inhibition of CFU-GM ($p = 0.17$), which did not reach statistical significance. Three experiments revealed dose-dependent sensitivity of erythroid colonies (Figure 1C) to the inhibitory effects of TG101348 relative to other colony types (Figures S1A and S1B available online). Colonies were analyzed for JAK2V617F$^+$ expression by a direct semiquantitative sequencing method and revealed a reduction in mutant allele frequency, although individual variability in sensitivity to TG101348 was detected (Figure 1C).

In Vitro Inhibition of JAK2V617F-Driven Erythroid Differentiation with TG101348

The role of JAK2V617F in skewing differentiation potential was investigated by lentivirally-enforced expression (Naldini et al., 1996) of JAK2V617F or wild-type JAK2 in normal cord blood progenitors in hematopoietic progenitor assays. JAK2V617F-expressing cord blood progenitors gave rise to a preponderance of erythroid (BFU-E) colonies, while wild-type JAK2 induced more mixed (CFU-Mix) colony formation over that of backbone vector controls (Figure 2A; n = 4 experiments). PCR performed with primers specific for lentivirally introduced JAK2 (mJAK2) followed by sequencing was used to verify transduction of colonies with the lentiviral vectors (Figure 2B). In subsequent in vitro experiments (n = 4), lentiviral backbone-, JAK2V617F-, or wild-type JAK2 (WT JAK2)-transduced human cord blood HSC were treated with or without 300 nM of TG101348 and plated onto methylcellulose supplemented with human cytokines. These experiments demonstrated selective inhibition of JAK2V617F skewed erythroid colony formation with TG101348 (Figure 2C).

Inhibition of Human PV Progenitor Erythroid Engraftment by TG101348

The capacity of PV stem and progenitor cells to give rise to human erythroid engraftment compared with their normal counterparts was assessed in a bioluminescent xenogeneic transplantation model involving intrahepatic transplantation of neonatal highly immunocompromised (RAG2$^{-/-}\gamma_c^{-/-}$) mice (Traggiai et al., 2004) with luciferase-transduced (Breckpot et al., 2003) human progenitor cells (Figure 3A). While bioluminescent imaging demonstrated comparable rates of engraftment between normal and PV progenitors (Figure 3B), FACS

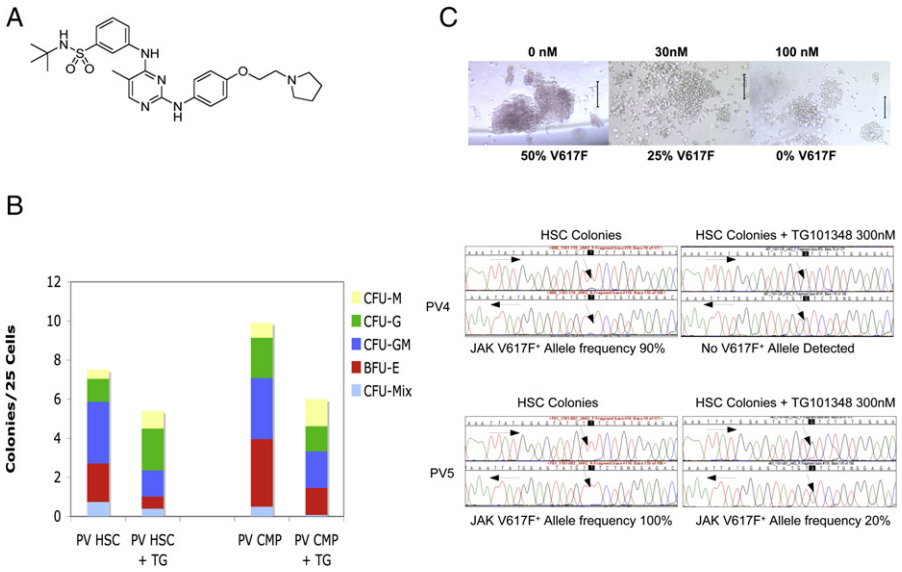

Figure 1. In Vitro Inhibition of PV Progenitor Erythroid Differentiation by TG101348

(A) Structure of TG101348.

(B) In five experiments, PV hematopoietic stem cells (HSC) and common myeloid progenitors (CMP) from JAK2V617F$^+$ PV patients (n = 3 patients) were FACS Aria sorted directly into 96-well plates containing methylcellulose supplemented with human cytokines and treated with 0 or 300 nM of TG101348 (+TG). Differential colony counts were performed on day 14.

(C) Upper panel: representative photomicrographs of PV progenitor colonies (scale bar, 100 μm) treated with 0, 30, or 100 nM of TG101348 and the corresponding *JAK2V617F* allele frequency (% V617F) determined by sequencing on pooled colonies.

Lower panel: in three experiments, sequencing analysis was used to determine *JAK2V617F* allele frequency in pooled HSC colonies treated with 0 or 300 nM of TG101348.

analysis of engrafted hematopoietic organs revealed a propensity for in vivo erythroid differentiation by PV progenitors in hematopoietic organs of transplanted mice (Figure 3C). In four separate experiments, oral gavage administration of TG101348 (120 mg/kg) significantly ($p = 0.02$) inhibited PV progenitor erythroid differentiation in vivo (Figure 3D). Moreover, sequencing analysis of hematopoietic tissues derived from PV progenitor-engrafted mice revealed a corresponding diminution of JAK2V617F expression following TG101348 treatment (Figure S2).

Selective Inhibition of JAK2V617F-Driven Erythroid Engraftment

We investigated whether enhanced PV progenitor erythroid engraftment was dependent on JAK2V617F or wild-type JAK2 expression and whether this engraftment was susceptible to inhibition with TG101348. In these experiments, normal cord blood progenitors were transduced with backbone, JAK2V617F, or wild-type JAK2 and transplanted intrahepatically into neonatal RAG2$^{-/-}\gamma_c{}^{-/-}$ (Traggiai et al., 2004) recipients (Figure S3). Following 12 days of oral gavage treatment with TG101348 (120 mg/kg), quantitative bioluminescence imaging analysis revealed reduced engraftment ($p = 0.08$) by JAK2V617F-expressing progenitors compared with backbone ($p = 0.61$) and wild-type JAK2 ($p = 0.67$) progenitor-transplanted mice (Figure 4A). FACS analysis revealed a significant inhibition of JAK2V617F-driven erythroid engraftment in TG101348-treated transplant recipients ($p = 0.037$) while wild-type JAK2 ($p = 0.077$) and backbone ($p = 0.27$) derived human erythroid engraftment were not significantly reduced (Figure 4B). These in vivo studies suggested that the enhanced erythroid engraftment potential of JAK2V617F-transduced cord blood progenitors was more sensitive to inhibition by TG101348 than wild-type JAK2-transduced progenitors. While TG101348 reduced human engraftment in bone marrow, it did not inhibit thymic T cell engraftment by JAK2V617F-expressing progenitors (Figures S4A and S4B). Since JAK3 is required for T cell development, these observations further emphasize the selectivity of TG101348 toward JAK2.

JAK2-Driven Erythroid Signal Transduction Pathways Are Inhibited by TG101348

The mechanism of JAK2V617F-enhanced erythroid differentiation was investigated by Q-PCR to detect changes in erythroid (GATA-1), and myeloid (PU.1) transcription factor transcripts in PV progenitors (Figure 5A) (Galloway et al., 2005; Hsu et al., 2004). While there were no appreciable differences in PU.1 transcript levels between PV and normal progenitors ($p = 0.44$), there was a significant increase ($p = 0.049$) in GATA-1 expression by PV progenitors in keeping with their enhanced erythroid differentiation potential (Figure 5A). Similarly, lentiviral transduction of JAK2V617F enhanced expression of GATA-1 but suppressed expression of FOG-1, a megakaryocytic transcription factor, further skewing the transcriptome profile toward enhanced erythroid differentiation (data not shown) (Deconinck et al., 2000; Galloway et al., 2005; Hsu et al., 2004). JAK2V617F enhancement of GATA-1 in relation to PU.1 transcripts and inhibition of FOG-1 expression was reversed

Table 1. Patient Characteristics

Patient Number	Sex/Age	Date	WBC	H/H	Plts (mm^3)	Spleen Size (cm below LCM)	Treatment at Time of Sample Acquisition
01	M/68	8/17/05	13	18.7/57.6	461	3	ASA
02[a]	M/62	7/11/05	70.8	12.4/41.7	903	9	hydroxyurea 1 gm + phlebotomy + coumadin
03	F/55	5/23/05	15	15.5/49.5	666	0	ASA + phlebotomy
04	F/60	5/26/06	17.6	12.3/39	198	2	ASA + phlebotomy + Plavix
05	F/42	6/28/06	16.9	20.3/62	574	7	ASA + phlebotomy
06	M/60	6/09/06	8.6	16/51.8	132	0	PV postinduction for AML
07	M/50	5/4/06	13.5	13.5/43.3	724	15	ASA + phlebotomy
		5/15/06	12.1	13.4/43.0	702	15	ASA + phlebotomy
		5/25/06	13.3	12.5/39.5	679	15	ASA + phlebotomy

Characteristics of seven patients with $JAK2V617F^+$ polycythemia vera (PV) who donated peripheral blood including age, sex, date of sample, hemoglobin (Hb), white blood cell count, platelet count, spleen size, and treatment at the time of sample acquisition. WBC, white blood cell count at time of study; H/H, hemoglobin/hematocrit at time of study; Plts, platelet count at time of study; LCM, left costal margin; ASA, aspirin; gm, gram; AML, acute myelogenous leukemia.

[a] Developed acute myelogenous leukemia with 24% marrow blasts on 11/06.

by TG101348 treatment (Figure 5B) The GATA-1/PU.1 transcript ratio decreased significantly to 25% ($p = 0.017$) (Figure 5B, left panel) in the TG101348-treated JAK2V617F-transduced cells, but not in the backbone-transduced cells ($p = 0.47$). Similarly, in TG101348-treated JAK2V617F-transduced cells, FOG-1 transcript levels increased by 30% (data not shown), and the ratio of GATA-1/FOG-1 transcript levels decreased significantly by 52% ($p = 0.05$) (Figure 5B, right panel) (InStat analysis, two-tailed t test), reversing enhanced erythroid differentiation.

To better understand the mechanism of TG101348 reversal of enhanced erythroid differentiation, we analyzed the effect of TG101348 on JAK2-mediated signaling in a human erythropoietin responsive cell line UT7/EPO (Figure 5C). Notably, TG101348 (at 300 and 600 nM) inhibited STAT5 phosphorylation more effectively than AG490, a JAK2 and JAK3 inhibitor. Inhibition of STAT5 phosphorylation was more pronounced with 600 nM TG101348 than with 50 μM or even 100 μM of AG490 (data not shown). While AG490 did not affect AKT phosphorylation in UT7/EPOcells, TG101348 potently inhibited AKT phosphorylation in a manner similar to a PI-3 kinase inhibitor, LY294002 (20 μM). Analysis of GATA-1 revealed that erythropoietin enhanced GATA-1 serine 310 (S310) phosphorylation while LY294002 inhibited this slightly. In addition, TG101348 reduced GATA-1 S310 phosphorylation, consistent with potent inhibition of AKT phosphorylation (Figure 5C). Notably, GATA-1 S310 phosphorylation activates GATA-1-mediated transcription of genes involved in erythroid proliferation and differentiation (Zhao et al., 2006). Thus, inhibition of AKT-regulated GATA-1 S310 phosphorylation and reduced STAT5 phosphorylation provides a potent mechanism of inhibition of JAK2-mediated signaling pathways and JAK2V617F-driven erythroid differentiation by TG101348 (Figure 5D).

DISCUSSION

Recently, we combined multicolor FACS analysis and hematopoietic progenitor assays with targeted sequencing analysis of purified PV HSC and progenitors in order to identify the stage of hematopoiesis at which the *JAK2V617F* mutation occurs. Our data revealed an increase in HSC in PV peripheral blood, as well as an expansion of the common myeloid progenitor (CMP) pool and emergence of an IL-3 receptor alpha-high population during disease progression (Jamieson et al., 2006). In addition, we detected a qualitative alteration in HSC differentiation potential. Moreover, *JAK2V617F* mutation expression by PV HSC was clonally transmitted to committed progenitors. Finally, greater inhibition of HSC erythroid differentiation was observed with a JAK inhibitor, AG490, in PV than in normal HSC (Jamieson et al., 2006). These findings suggested that, in addition to enhanced proliferative capacity, one of the main defects in PV may be altered differentiation potential at the stem cell level. However, a direct role for JAK2V617F in altering primitive hematopoietic progenitor cell-fate decisions was not established.

In this study, the role of JAK2V617F in altering hematopoietic differentiation was demonstrated by lentiviral transduction of cord blood progenitors with JAK2V617F. As seen with $JAK2V617F^+$ PV progenitors, only JAK2V617F overexpression resulted in enhanced erythroid colony formation, while wild-type JAK2 increased the number of mixed rather than erythroid colonies compared with backbone vector controls. These data suggested a direct link between JAK2V617F expression and enhanced erythropoiesis in vitro. Furthermore, we demonstrated that the propensity of $JAK2V617F^+$ PV HSC and progenitors to differentiate into erythrocytes was selectively blocked by a potent JAK2 inhibitor, TG101348 (TargeGen Inc.). TG101348 is a small-molecule ATP-competitive inhibitor that occupies the ATP-binding pocket of JAK2 (J. Cao, C. Chow, E. Dneprovskaia, L. Hwang, D. Lohse, C.C.M., M. Martin, A. McPherson, G.N., M.S.S. Palanki, V.P. Pathak, J. Renick, R.M.S., B.Tam, B. Zeng, J.D.H., unpublished data). In addition, TG101348 selectively inhibited erythroid colony formation by JAK2V617F-transduced cord blood progenitors indicative of an innate sensitivity of JAK2V617F-driven erythroid signal transduction pathways to TG101348 inhibition.

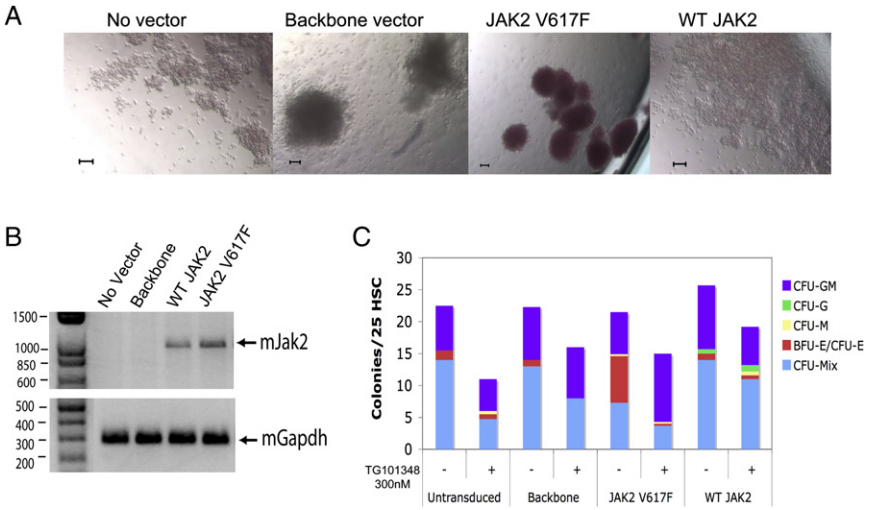

Figure 2. In Vitro Inhibition of JAK2V617F-Driven Erythroid Differentiation with TG101348

(A) Representative photomicrographs (scale bar 100 μm) of day 14 colonies derived from normal cord blood HSC transduced with no vector, backbone vector, JAK2V617F or wild-type JAK2 (WT JAK2) vector (n = 4 experiments).

(B) Human cord blood HSC-derived colonies were collected after 14 days in methylcellulose culture, and lentiviral JAK2 (mJAK2) PCR was used to verify transduction. In addition, bands were extracted and sequenced to verify presence of WT JAK2 and JAK2V617F.

(C) Human cord blood HSC transduced with lentiviral backbone, JAK2V617F, or wild-type JAK2 (WT JAK2) vectors, (25 cells/well with human cytokine supplemented methylcellulose) were treated with (+) or without (–) 300 nM of TG101348, and colonies were scored on day 14. Untransduced HSC served as a control. Representative of n = 4 experiments.

When the in vivo engraftment and differentiation capacity of human PV progenitors was compared with that of normal cord blood progenitors in a bioluminescent xenogenic transplantation model, PV progenitors had similar rates of engraftment but gave rise to increased numbers of human erythroid (glycophorin A^+) cells in hematopoietic organs of transplanted mice. Similarly, JAK2V617F-expressing progenitors promoted erythroid engraftment, as had been reported previously with TEL-JAK2 (Kennedy et al., 2006), in $RAG2^{-/-}\gamma_c^{-/-}$ mice, demonstrating a direct correlation between JAK2V617F and in vivo human erythroid cell-fate decisions. Targeted inhibition of JAK2 with TG101348 by oral gavage-mediated administration resulted in a significant reduction in erythroid engraftment by both $JAK2V617F^+$ PV- and JAK2V617F-transduced cord blood progenitors, further underscoring the sensitivity of JAK2V617F-driven erythroid differentiation to TG101348.

Investigation of the mechanisms linking JAK2V617F expression to enhanced erythroid differentiation in vitro and in vivo by quantitative PCR (Q-PCR) demonstrated that FACS-purified PV stem and progenitor cells harbored higher transcript levels of GATA-1, an erythroid transcription factor, than PU.1, a myeloid transcription factor. This Q-PCR analysis indicated that, in PV primitive progenitors, cell-fate decisions are irrevocably altered by an imbalance in erythroid, GATA-1, versus myeloid, PU.1, transcription factors. To determine whether this imbalance was a consequence of JAK2V617F expression, we transduced normal cord blood progenitors with JAK2V617F and analyzed transcript levels of GATA-1, PU.1, and FOG-1, a megakaryocyte transcription factor that has been shown to inhibit erythroid formation (Deconinck et al., 2000). Q-PCR analysis following treatment with vehicle or TG101348 in vitro (Galloway et al., 2005; Hsu et al., 2004) revealed that JAK2V617F-enhanced GATA-1 expression was significantly reduced relative to PU.1 ($p = 0.017$) and that the ratio of GATA-1 to FOG-1 transcripts also decreased significantly ($p = 0.05$), thereby reestablishing the balance of transcription factors required for normal primitive progenitor differentiation. Furthermore, western blot analysis of a human erythropoietin (EPO) responsive cell line, UT7/EPO, demonstrated that TG101348-inhibited activation of GATA-1 regulated transcription by blocking AKT-mediated GATA-1 S310 phosphorylation, potentially providing another mechanism for restoring a balance in the effects of lineage skewing transcription factors (Zhao et al., 2006). In addition, TG101348 treatment resulted in decreased STAT5 phosphorylation, further explaining the potency of TG101348 in blocking JAK2-mediated signaling. Because previous studies involving targeted inhibition of BCR-ABL in CML demonstrated that hematopoietic stem and progenitor cells represent a reservoir for relapse, the capacity of TG101348 to potently inhibit signaling through JAK2V617F at the primitive progenitor level may be particularly relevant in determining the successful outcome of MPD clinical trials with JAK2 inhibitors (reviewed in Kaushansky, 2005) (Holyoake et al., 1999; Jamieson et al., 2004; Kaushansky, 2005; Weissman, 2005).

Recent reports revealed that some MPDs harbor mutations outside the JAK2V617F region, for example in exon 12 of the JAK2 gene or in the MPL gene (Pardanani et al., 2007; Pikman et al., 2006; Scott et al., 2007). However, over 97% of patients with PV and approximately 50% of patients with ET and myelofibrosis harbor the JAK2V617F mutation, underscoring its importance as a therapeutic target (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). In this study, inhibition of JAK2 with TG101348 decreased the aberrant erythroid differentiation potential of JAK2V617F-positive progenitors both in vitro and in a bioluminescent xenogeneic transplantation model. This occurred, in part, because of decreased GATA-1 transcription as well as inhibition of both STAT5 and GATA-1 phosphorylation (Zhao et al., 2006). These data suggest that TG101348, a JAK2 selective tyrosine kinase inhibitor, may be an excellent candidate for targeted therapy of JAK2 driven MPDs.

EXPERIMENTAL PROCEDURES

Samples

Peripheral blood (n = 9) samples, including phlebotomies, were donated on multiple occasions by patients with PV. Normal cord blood (n = 8) and peripheral blood samples (n = 4) were provided by healthy volunteers. Samples were

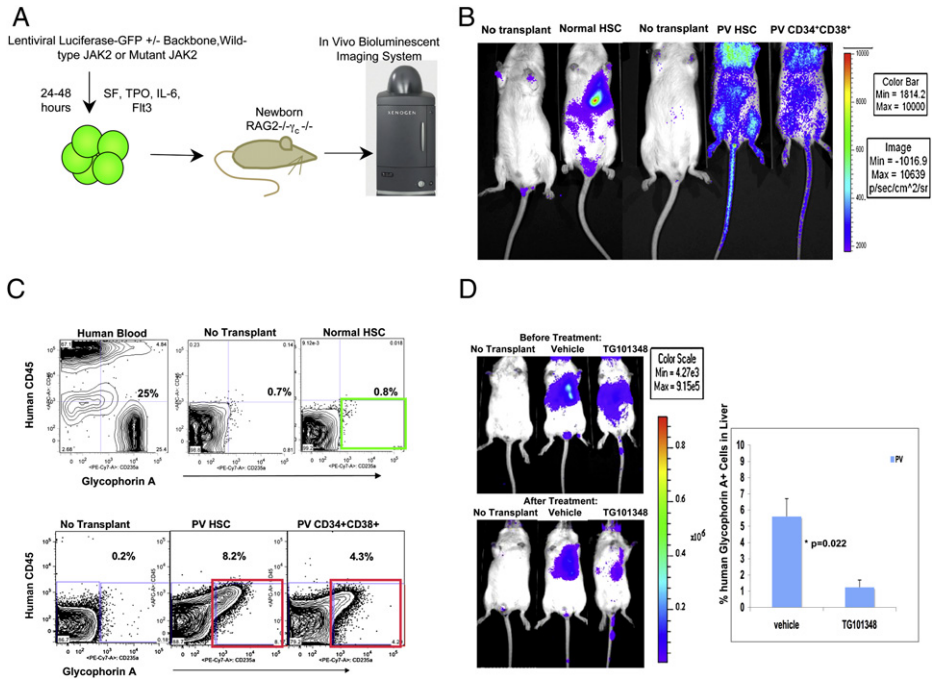

Figure 3. Human PV Progenitor Erythroid Engraftment Is Inhibited by TG101348

(A) Schema of Bioluminescent Engraftment Analysis of Human Progenitors in RAG2$^{-/-}\gamma_c^{-/-}$ Mice. Normal cord blood or PV progenitor cells were cotransduced with lentiviral luciferase GFP and backbone, wild-type, or mutant (V617F) JAK2 lentiviral vectors followed by intrahepatic transplantation into neonatal RAG2$^{-/-}\gamma_c^{-/-}$ mice. Bioluminescence imaging was performed weekly with the aid of a Caliper IVIS Imaging System.

(B) Representative bioluminescence images of mice transplanted with normal HSC, PV HSC, or PV progenitors compared with no transplant controls (n = 4 experiments).

(C) FACS analysis of human erythroid (FSc^{lo}human glycophorin A$^+$CD45$^{-/lo}$) engraftment in spleens of mice transplanted with normal HSC (upper panel), PV HSC, and progenitors (lower panel) compared with their untransplanted controls.

(D) Left panel: representative bioluminescent images of mice transplanted with lentiviral luciferase GFP-transduced PV (n = 4 separate patients) CD34$^+$ progenitor cells before (4 weeks posttransplant) and after 12 days of treatment with vehicle or 120 mg/kg of TG101348. Right panel: comparative graph of human erythroid engraftment (% human Glycophorin A+ Cells in Liver ± SEM) determined by FACS analysis of mice transplanted with PV progenitors and treated with TG101348 compared with vehicle-treated controls.

obtained with informed consent according to Stanford University and UCSD IRB-approved protocols. Normal bone marrow and cord blood samples were also purchased from All Cells.

Human Hematopoietic Stem Cell and Myeloid Progenitor Flow-Cytometric Analysis and Cell Sorting

Mononuclear fractions were extracted from peripheral blood or bone marrow following Ficoll density centrifugation according to standard methods. Samples were analyzed fresh or subsequent to rapid thawing of samples previously frozen in 90% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO) in liquid nitrogen. In some cases, CD34$^+$ cells were enriched from mononuclear fractions with the aid of immunomagnetic beads (CD34$^+$ Progenitor Isolation Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany) (Jamieson et al., 2004, 2006; Manz et al., 2002) (See Supplemental Experimental Procedures for details regarding fluorescence-activated cell sorting analysis and sorting of HSC and myeloid progenitor populations).

Human Hematopoietic Progenitor Assays

Normal and PV HSC (CD34$^+$CD38$^-$CD90$^+$Lin$^-$), common myeloid progenitors (CMP; CD34$^+$CD38$^+$IL-3Ralpha$^+$CD45RA$^-$Lin$^-$), granulocyte-macrophage progenitors (GMP; CD34$^+$CD38$^+$IL-3Ralpha$^+$CD45RA$^+$Lin$^-$), and megakaryocyte-erythroid progenitors (MEP; CD34$^+$CD38$^+$IL-3Ralpha$^-$CD45RA$^-$Lin$^-$) were sorted with the aid of a FACS Aria directly into 12 well plates containing complete methylcellulose (GF$^+$H4435, StemCell Technologies Inc., Vancouver, Canada) according to the manufacturer's specifications (Jamieson et al., 2004, 2006) with or without 0, 30, 100, 300, or 600 nM of a selective JAK2 inhibitor, TG101348 (TargeGen Inc., San Diego, CA). Colonies were incubated in a 37°C 7% CO_2 humidified incubator and scored on day 14 as colony-forming unit mix (CFU-Mix), burst-forming unit erythroid or colony-forming unit erythroid (BFU-E/CFU-E), CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-megakaryocyte (CFU-Mega), or CFU-granulocyte-macrophage (CFU-GM) (Jamieson et al., 2004, 2006). Phase contrast photomicrographs of colonies were obtained on day 14 with a Zeiss Axiovert phase-contrast inverted microscope with the aid of SPOT software.

JAK2 Mutation Screening

JAK2V617F mutation genotyping was performed on peripheral blood mononuclear cells derived from patients with PV, as well as normal peripheral blood, bone marrow, and cord blood. Red blood cells were lysed, and DNA was extracted with the QiaAmp DNA Blood Mini kit according to the manufacturer's directions (QIAGEN, Valencia, CA) and then stored at −80°C until amplification-based testing. Extracted DNA was prepared for *JAK2V617F* mutation analysis by LightCycler methodology as previously described (Jamieson et al., 2006).

PV Progenitor Colony JAK2 Mutation Analysis

Sequencing analysis of *JAK2V617F* expression was performed on pooled PV progenitor colonies treated with vehicle or TG101348. Colonies were plucked and resuspended in 200 μl of RLT buffer supplemented with β-mercaptoethanol (QIAGEN RNeasy) and frozen immediately at −80°C. Samples were thawed and RNA extracted followed by cDNA preparation and PCR amplification with JAK2 specific primers (Jamieson et al., 2006). Mutation analysis of the

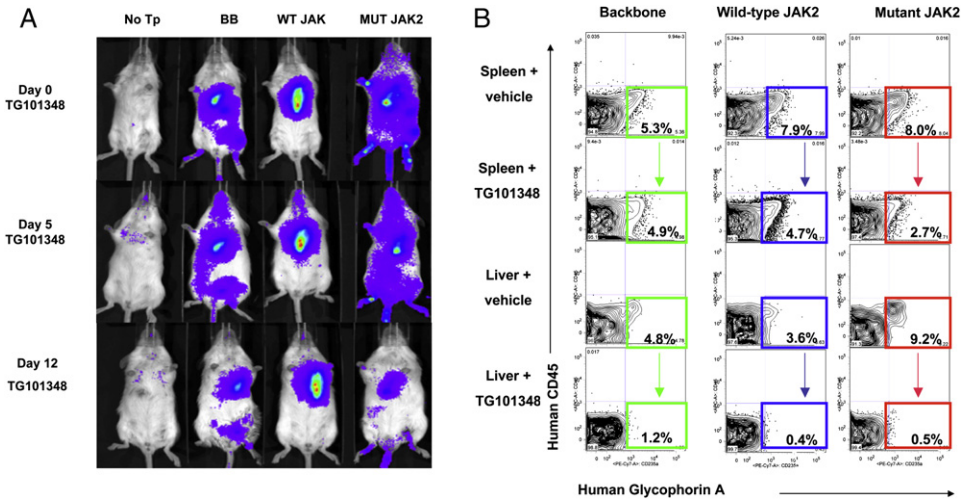

Figure 4. Selective Inhibition of JAK2V617F-Driven Erythroid Engraftment

In four experiments, neonatal RAG2$^{-/-}\gamma_c^{-/-}$ mice (n = 24; 6 mice/group) were transplanted intrahepatically with normal cord blood progenitors that were cotransduced with lentiviral luciferase GFP and a lentiviral backbone vector (BB), wild-type *JAK2* (*WT JAK2*), or *JAK2V617F* (*MUT JAK2*) and treated by twice daily oral gavage for 12 days with vehicle (DMSO) or TG101348 (120 mg/kg). Nontransplanted littermates (no Tp) served as negative controls.

(A) Representative bioluminescence images of transplanted mice before (4 weeks posttransplant), during (day 5 TG101348), and after (day 12 TG101348) treatment with vehicle or TG101348.

(B) FACS analysis of human erythroid (CD45$^-$GlycophorinA$^+$) engraftment following treatment with vehicle or TG101348.

JAK2 cDNA PCR product was conducted using fluorescent denaturing high performance liquid chromatography (DHPLC) technology and SURVEYOR mismatch cleavage analysis both with the WAVE-HS System (Transgenomic, Gaithersberg, MD). Aliquots of PCR product (3–15 μl) from all samples were scanned for mutations by DHPLC, confirmed by Surveyor mismatch cleavage, and identified with bidirectional sequence analysis on an ABI 3100 sequencer using BigDye V3.1 terminator chemistry (Applied Biosystems, Inc., Foster City, CA). In addition, for semiquantitative determination of mutant and normal allele frequencies, relative peak areas of DHPLC elution profiles and Surveyor mismatch cleavage products were determined after normalization and comparison to reference controls using the WAVE Navigator software (Supplemental Experimental Procedures) (Jamieson et al., 2006).

Lentiviral JAK2 Vector Construction

Wild-type *JAK2* and *JAK2V617F* (~3.4 kb) were excised from Jak2-mus-MSCV-neo at the Not I and Age I sites, blunt-ended, and cloned into the Smal site of the self-inactivating lentiviral vector, pLV CMV IRES2 GFP. The pLV CMV IRES2 GFP was modified from pLV CMV GFP Nhe (Naldini et al., 1996) by replacing GFP with IRES2GFP (Clontech) (C. Barroga, unpublished data). Both *WT JAK2* and *Jak2V617F* sequences were verified using murine Jak2 specific primers flanking the mutation. Lentiviral vectors were simultaneously prepared by cotransfection of *WT JAK2*, *JAK2V617F* or backbone vector, together with pCMV-Δ8.9 and pCMV-VSV-G using Lipofectamine 2000 into 293TFT (Invitrogen, Carlsbad, CA). Viral supernatants were collected after 48 hr and concentrated by centrifugation. Viral titers of the lentiviral backbone varied in the range of 4×10^7 to 1×10^9 iu/ml.

Progenitor Transduction with Lentiviral Vectors

Wild-type JAK2, JAK2V617F, and backbone lentiviral vectors were used to transduce normal cord blood hematopoietic stem cells (HSC) and progenitor cells, which were grown in methocult, with (+) or without (−) TG101348. Colonies were counted at day 14, and RNA was extracted, reversed transcribed, and amplified with murine JAK2 specific primers. Sequencing of PCR products confirmed the presence of WT *JAK2* and *JAK2V617F*, respectively.

Bioluminescent Xenogeneic Transplantation Model of Human PV

Immunocompromised RAG2$^{-/-}\gamma_c^{-/-}$ mice were a kind gift from Dr. Irving Weissman. Mice were bred and maintained on sulfamethoxazole water in the animal care facility at UCSD Moores Cancer Center. All mouse experiments were performed in accordance with national guidelines and an animal care protocol (no. S06015) approved by the UCSD Institutional Animal Care and Use Committee.

To assess engraftment potential and in vivo differentiation capacity, JAK2V617F$^+$ PV CD34-enriched cells, HSC or progenitors (CD34$^+$CD38$^+$Lin$^-$) were transduced with lentiviral luciferase GFP (Breckpot et al., 2003) for 48 hr and transplanted intrahepatically into neonatal nonirradiated RAG2$^{-/-}\gamma_c^{-/-}$ mice (Traggiai et al., 2004). Engraftment was analyzed by noninvasive bioluminescent imaging (IVIS 200, Caliper Inc.) and by FACS analysis of hematopoietic tissues. In separate experiments, normal cord blood progenitors were transduced with lentiviral luciferase GFP together with JAK2 WT-, MT-, and backbone lentiviral vectors, followed by intrahepatic transplantation into RAG2$^{-/-}\gamma_c^{-/-}$ mice according to previously published methodology, and analyzed for human engraftment by noninvasive bioluminescent imaging and FACS (Traggiai et al., 2004). Transplanted RAG2$^{-/-}\gamma_c^{-/-}$ mice were also treated with a selective JAK2 inhibitor (TG101348, 120 mg/kg) or vehicle (DMSO) by oral gavage twice daily for 12 days, and the effect on engraftment was analyzed. In another series of experiments, HSC were transduced with the *JAK2V617F* or backbone lentiviral vector with (+) or without (−) TG101348 (IN) or the vehicle (DMSO) and grown for 7 days in myelocult media (Stem Cell Technologies, Inc.), and transcript levels of erythroid transcription factors were quantified by Q-PCR (Jamieson et al., 2006).

Inhibition of EPO Signaling in UT7/EPO Cells with TG101348

The human erythro-megakaryocytic cell line, UT7/Epo cells were starved in 0.1% FBS in IMDM for 24 hr prior to stimulation. Cells were preincubated with various inhibitors including LY294002 (20 μM; Calbiochem, San Diego, CA), AG490 (50 μM; Calbiochem, San Diego, CA), and TG101348 (300 nM and 600 nM; TargeGen, San Diego, CA) 1 hr prior to stimulation with 10 U/ml EPO for 30 min. Cells were lysed with 1% NP40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, complete protease inhibitors and PhosStop; Roche, Indianapolis, IN). Equal amounts of lysate (20 μg) were loaded in 4%–15% Tris-SDS PAGE gels (Biorad, Hercules, CA) and probed with phospho-JAK2 (Tyr1007/1008) (Upstate, Millipore, Billerica, MA), phospho-STAT5 (Tyr694), and phospho-AKT (Ser473) (Cell Signaling, Danvers, MA), and phospho-GATA-1 (S310) (Abcam, Cambridge, MA). Blots were stripped and reprobed with anti-JAK2 (Upstate), anti-pAKT (Cell

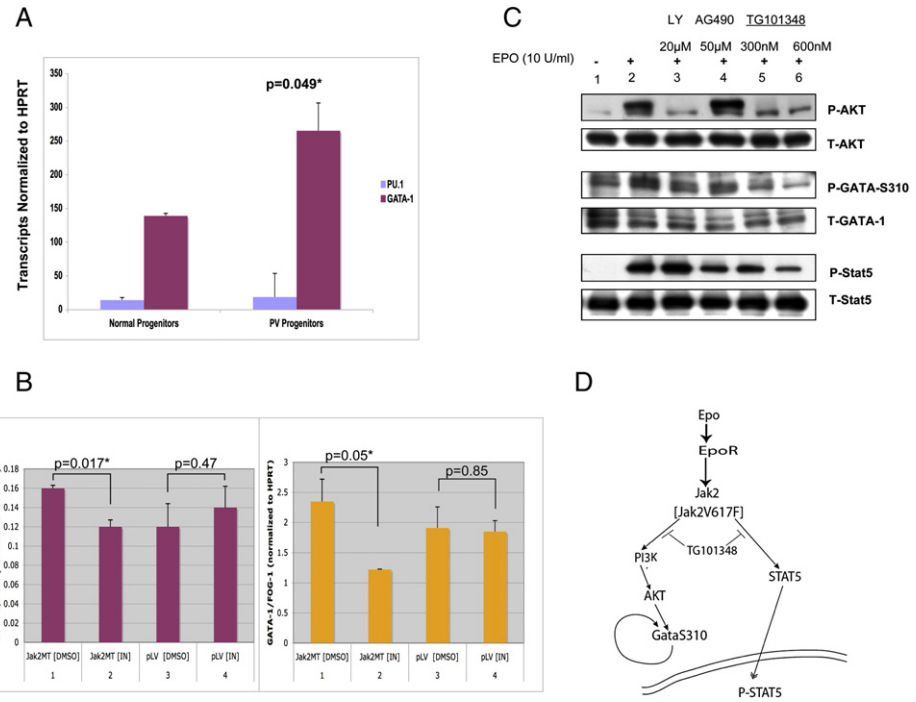

Figure 5. JAK2 Driven Erythroid Signal Transduction Pathways Are Inhibited by TG101348

(A) Quantitative RT-PCR (Q-PCR) analysis (± SEM) of GATA-1 and PU.1 expression was performed on FACS sorted normal and PV $CD34^+$ progenitor cells. Results were normalized to human HPRT expression.

(B) Graph demonstrating the GATA-1/PU.1 (left panel) or GATA-1/FOG-1 (right panel) transcript ratios (transcripts normalized to HPRT ± SEM) determined by Q-PCR following treatment of JAK2V617F (Jak2MT) or backbone (pLV) lentivirally transduced cells with vehicle (DMSO) or TG101348 (300 nM; IN).

(C) Western blot analysis of AKT, GATA-S310, and STAT5 phosphorylation in UT7/EPO cells stimulated with EPO and treated with a PI3 kinase inhibitor, LY294002 (LY; 20 μM); AG490, a nonselective JAK2 inhibitor (50 μM); or TG101348 (300 or 600 nM).

(D) In a proposed model of the mechanism of inhibition of JAK2-driven erythroid differentiation, TG101348 inhibits JAK2-mediated STAT5 as well as AKT-mediated GATA-1 S310 phosphorylation, leading to a potent block in erythroid differentiation following erythropoietin (EPO) binding to the erythropoietin receptor (EpoR) or activation of signaling through JAK2V617F.

Signaling), anti-STAT5 (sc-835) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-GATA-1 (Abcam, Cambridge, MA).

Quantitative RT-PCR Analysis of GATA-1, PU.1, and FOG-1 Transcript Levels

Quantitative RT-PCR (Q-PCR) analysis of GATA-1 and PU.1 transcript levels was performed on FACS-purified PV progenitors as previously described (Jamieson et al., 2006). First, HSC and progenitor (1,000 to 50,000) cells were sorted directly into RLT Buffer and total RNA was isolated using RNeasy Micro Kit (QIAGEN, Valencia, CA, USA), according to manufacturer's protocol (Jamieson et al., 2006). Then, a SYBR Greener two-step Q-RT-PCR Kit for the iCycler (Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA and assess GATA-1 and PU.1 relative transcript quantities according to the manufacturer's protocol. Briefly, 8 μl of 4 to 75 ng/μl of RNA were mixed with RT Reaction Mix and RT Enzyme Mix and incubated at 25°C for 10 min, followed by 50°C for 30 min and finally 85°C for 5 min. The tubes were then chilled and 1 μl of RNase H was added to the reaction followed by a 20 min incubation at 37°C. The quantitative PCR (Q-PCR) reaction was performed in duplicate using 2 μl of the template in 25 μl reaction volume containing SYBR Greener Super Mix and 0.4 μM of each forward and reverse primer. Relative values of transcripts were determined according to a standard curve. GATA-1 and PU.1 values were then normalized to HPRT values (for additional details please see Supplemental Experimental Procedures; Jamieson et al., 2006).

Statistical Analysis

Standard deviation (SD), standard error of the mean (SEM), and numbers of HSC and progenitors per 10^5 mononuclear cells were measured using FlowJo and Excel software. A two-tailed Student's t Test (Excel software) was used to analyze statistical differences in the types of colonies derived from PV patient samples treated with vehicle or TG101348 as well as engraftment rates in $RAG2^{-/-}\gamma_c^{-/-}$ mice. A two-tailed t test (InStat and software) was used to analyze statistical differences in transcript levels of JAK2V617F transduced CD34 treated with vehicle or TG101348.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and four supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/4/321/DC1/.

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