STIMULUS REPORT

blood advances

Downregulated *KLF2* in polycythemia vera and essential thrombocythemia induces prothrombotic gene expression

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Key Points

- *KLF2* is downregulated by augmented JAK-STAT signaling in PV and ET, leading to upregulated thrombotic gene expression.
- *KLF2* transcript levels in PV and ET are inversely correlated with neutrophil and platelet counts but not hemoglobin and hematocrit.

Thromboses are major causes of morbidity and mortality in polycythemia vera (PV) and essential thrombocythemia (ET) diseases associated with $JAK2^{V617F}$ mutation. However, the molecular mechanism(s) of increased thrombosis in PV and ET remain unknown. Kruppel-like factor 2 (KLF2) is a transcription factor that regulates expression of genes associated with inflammation and thrombosis; the absence of KLF2 in neutrophils causes thrombosis by inducing tissue factor. We studied the role of KLF2 in regulating prothrombotic gene expression in PV and ET. Neutrophils and platelets *KLF2* expression in PV and ET was lower than the controls. Furthermore, in patients with thromboses, *KLF2* transcripts were lower in platelets than those without thromboses. $JAK2^{V617F}$ allelic burden was inversely correlated with *KLF2* transcript levels, suggesting JAK-STAT pathway may downregulate *KLF2* expression. Whole transcriptome analyses of neutrophils and platelets showed that a lower *KLF2* expression was associated with an upregulation of KLF2-regulated thrombotic genes. In addition, low *KLF2* expression in platelets positively correlated with thrombotic events. In patients with PV and ET, *KLF2* expression was induced by pegylated interferon alfa (PegINF-α) but not by hydroxyurea treatments. These data suggest that KLF2 may be a regulator of PV and ET thrombosis and a novel therapeutic target to prevent thrombosis.

Introduction

Thrombosis is the most common complication of polycythemia vera (PV) and essential thrombocythemia (ET).[1] The risk factors of thrombosis include high leukocyte and neutrophil counts;[2] however, the molecular mechanisms of thrombosis are not fully known. We previously reported that increased hypoxia inducible factor transcriptional activity in PV and ET induces transcription of proinflammatory and prothrombotic genes.[3]

Kruppel-like factors (KLFs) are zinc finger transcription factors.[4] KLF2 is highly expressed in endothelium and hematopoietic cells and induces myeloid quiescence by inhibiting the recruitment of coactivators of nuclear factor kappa B (NF-κB).[5] The knockdown of *KLF2* in endothelial cells increases prothrombotic gene expression including *F3* (encoding tissue factor [TF]) and *SERPINE1* (encoding plasminogen activator inhibitor 1 [PAI-1]),[6] and low neutrophil KLF2 activates neutrophils and induces TF.[7]

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Any data or resources in the manuscript will be shared upon request: josef.prchal@hsc.utah.edu.

The full-text version of this article contains a data supplement.

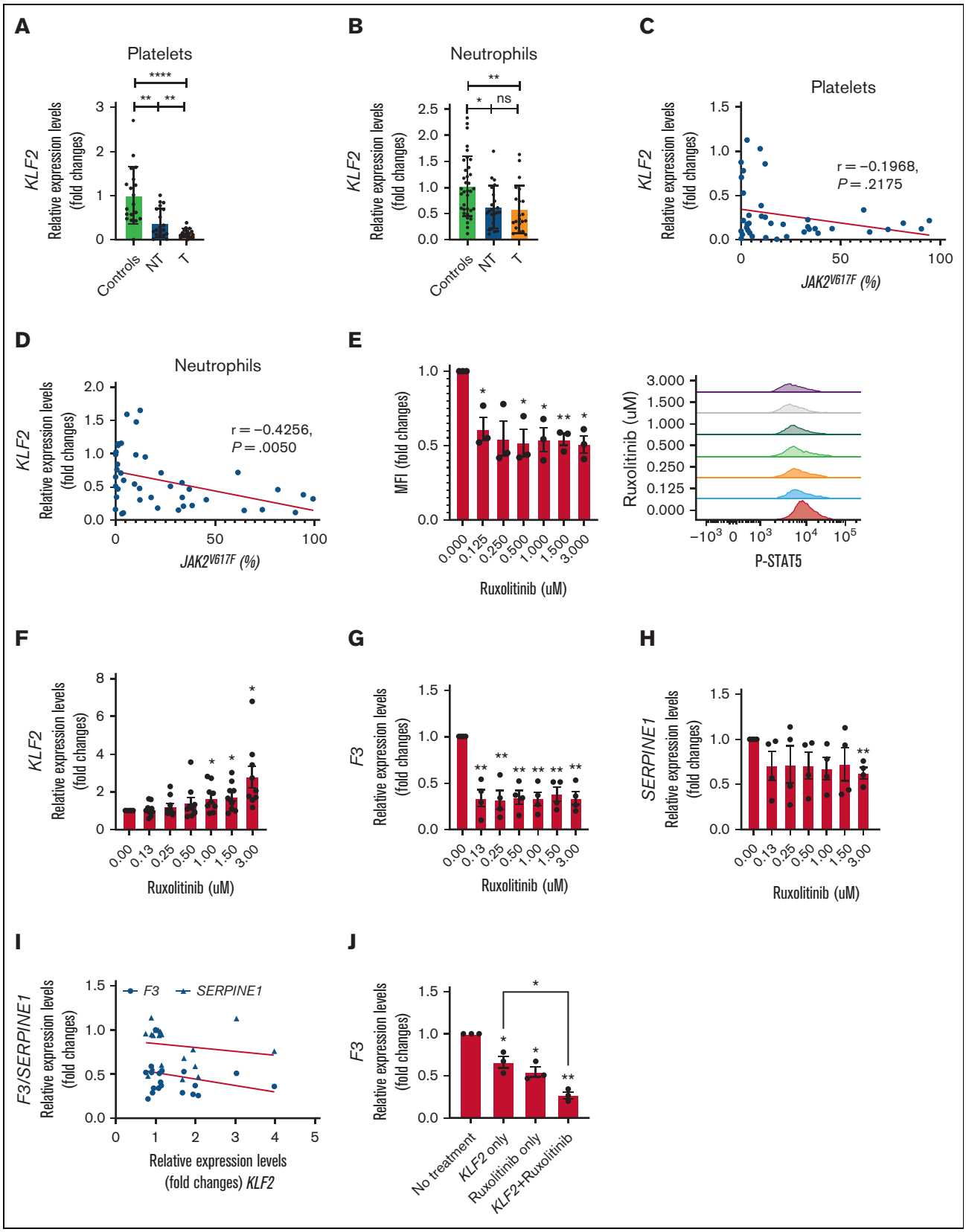

Figure 1. *KLF2* is downregulated in PV and ET platelets and neutrophils by JAK-STAT signaling. *KLF2* transcript levels were measured in (A) platelets and (B) neutrophils and expressed as a fold changes. *KLF2* transcript levels in (C) platelets and (D) neutrophils correlated with *JAK2*^{V617F} allele burden measured in neutrophils.

We investigated possible *KLF2* downregulation in PV and ET and its effect on thromboses and interaction with *JAK2*^{V617F}.

Study design

In this institutional review board–approved study *JAK2*^{V617F} allele burden,[8] *KLF2*, and thrombotic gene transcripts were measured as previously described.[3,9] Ruxolitinib was used for JAK2 inhibition and pCMV6-entry vector containing *KLF2* complementary DNA was used for *KLF2* overexpression; for details, see supplemental Methods.

Results and discussion

KLF2 transcript levels were downregulated in neutrophils and platelets of PV and ET (n = 45) compared with controls (n = 36, granulocytes; n = 18, platelets) (Figure 1A-B). In those with thrombosis (n = 23), platelet *KLF2* transcript levels were lower than those without thrombosis (n = 22). In neutrophils, *KLF2* transcript levels were comparable in both groups (Figure 1A-B). To determine if downregulation of *KLF2* alters the expression of its target genes, we analyzed whole transcriptome data of neutrophils (22 PV and 10 controls) and platelets (24 PV and 4 controls)[3] available from our prior work. In neutrophils, 149 KLF2-targeted genes were expressed, 30 were upregulated, and 5 were downregulated. In platelets, among 169 KLF2-regulated genes, 26 genes were upregulated, and 2 genes were downregulated (adjusted *P* value <.05, Log2 fold changes >1) (supplemental Figure 1A,B). The transcript levels of these dysregulated genes correlated with *KLF2* transcript levels (supplemental Tables 1 and 2). We quantified transcript levels of some of the *KLF2* target genes including prothrombotic genes of *F3*, *CD36* (platelet glycoprotein 4), *VWF* (von Willebrand factor), *SERPINE1,* and *THBS1* (Thrombospondin 1) and antithrombotic genes of *COL4A1* (Collagen Type IV Alpha 1) and *CD40LG* (CD40 Ligand). Then, these transcript levels were correlated with *KLF2* transcript levels (supplemental Figure 2). In general, prothrombotic genes were upregulated in PV and ET neutrophils and platelets (supplemental Figure 2A-E), whereas antithrombotic genes were downregulated (supplemental Figure 2F,G). In platelets, *F3*, *CD36*, and *VWF* transcript levels were higher in those with thrombosis than in those without thrombosis (supplemental Figure 2A-C). In neutrophils, *CD36* levels were higher in patients with thrombosis (supplemental Figure2B). *KLF2* transcript levels in platelets but not in neutrophils inversely correlated with prothrombotic gene expression (supplemental Figure 2A-E). In contrast, antithrombotic gene expression both in platelets and neutrophils positively correlated with *KLF2* transcript levels (supplemental Figure 2F-G). Although the absence of *KLF2* in neutrophils alone induces thrombosis in murine models,[7] we found an even stronger correlation of KLF2 and its target gene expression in platelets. Thus, downregulated *KLF2* in PV and ET in platelets induces prothrombotic gene expression.

Given the variable *JAK2*^{V617F} allelic burden is a thrombosis risk in PV and ET,[10,11] we measured it in PV and ET clonal neutrophils. *JAK2*^{V617F} allelic burden inversely correlated with *KLF2* transcript levels in neutrophils (r = −0.4160, *P* = .0068) only, but not in platelets (Figure1C,D), suggesting that augmented JAK2-STAT5 signaling in PV and ET[12] because of *JAK2*^{V617F} may downregulate neutrophil *KLF2* expression. To test this hypothesis, transcript levels of *KLF2* and its target genes (*SERPINE1*, and *F3*) were measured in myeloid leukemia cell lines human erythroleukemia (HEL) (*JAK2*^{V617F} positive) incubated with ruxolitinib (JAK1 and JAK2 inhibitor[13]). We measured the JAK2 activity to confirm ruxolitinib effect by measuring phosphorylated STAT5 by flow cytometry.[14] Phosphorylated STAT5 decreased with ruxolitinib treatment, whereas *KLF2* transcripts increased in dose dependent manner (Figure 1E), indicating that *KLF2* expression is downregulated by augmented JAK2-STAT5 activity in PV and ET (Figure 1F). We show that ruxolitinib treatment decreased *F3* and *SERPINE1* transcript levels (Figure 1G,H), however, it did not correlate with *KLF2* transcript levels (Figure 1I).

To test if *F3* transcript is regulated by KLF2 and/or JAK2-STAT5, we overexpressed *KLF2* in HEL cells. *KLF2* overexpression led to decreased *F3* transcript levels. Ruxolitinib treatment further decreased the *F3* transcript levels (Figure 1J). These data demonstrate that low *KLF2* transcript levels in PV and ET are mediated by augmented JAK2-STAT5 signaling due to *JAK2*^{V617F} and *F3* expression. Augmented JAK2-STAT5 signaling also increases *F3* expression, independently. These data are consistent with higher TF activity in PV and ET with *JAK2*^{V617F} mutation than in ET with *CALR* mutation or controls.[15]

KLF2 transcript level in platelets inversely correlated with neutrophil (r = −0.3061, *P* = .0226) and platelet numbers (r = −0.2949, *P* = .032) (Figure 2A,B) but not with hemoglobin or hematocrit (Figure 2C,D). Multivariate analyses showed that only leukocytosis/neutrophilia associated with higher thrombotic risk generally require myelosuppressive therapy.[2] Neutrophils make up 50% to 70% of leukocytes.[16] Neutrophil counts correlated positively with leukocyte counts (r = 0.9210, *P* < .0001). High platelet counts increase 1.8-fold risk of arterial thrombosis in brain[17] whereas lower platelet counts are associated with venous thromboses in ET,[18] suggesting that high leukocyte and platelet numbers increase the risk of thrombosis because of low expression levels of *KLF2* and further support that normalization of leukocyte and platelet numbers reduces the risk of thrombosis.

Cytoreductive treatment hydroxyurea (HU) and Pegylated interferon alfa (PegINF-α) is used for patients with high risk of thrombosis.[19] We evaluated if these therapies modulate *KLF2* expression in

Figure 1 (continued) (E) Phosphorylated STAT5 (P-STAT5) were measured in HEL cells treated with various concentrations of ruxolitinib by fluorescence activated cell sorting analysis and mean fluorescence intensity was calculated against samples without treatment which is taken as 1 and expressed as fold changes. (F) *KLF2*, (G) *F3*, and (H) *SERPINE1* transcript levels were measured in HEL cells treated with various concentration of ruxolitinib. (I) *KLF2* transcript levels were not correlated with *F3* and *SERPINE1* transcript levels in ruxolitinib treated HEL cells. (J) *F3* transcript levels were measured in HEL cells with only *KLF2* overexpression, only ruxolitinib treatment, and both *KLF2* overexpression and ruxolitinib treatment. Expression levels were expressed as fold changes and no treatment control was taken as 1. *P* value was calculated by unpaired t test or paired t test. Correlation analysis was performed by Spearman correlation using GraphPad prism. **** *P* < .0001, ** *P* < .01, * *P* < .05. P-STAT5, Phosphorylated STAT5; NT, no history of thrombosis; T, history of thrombosis.

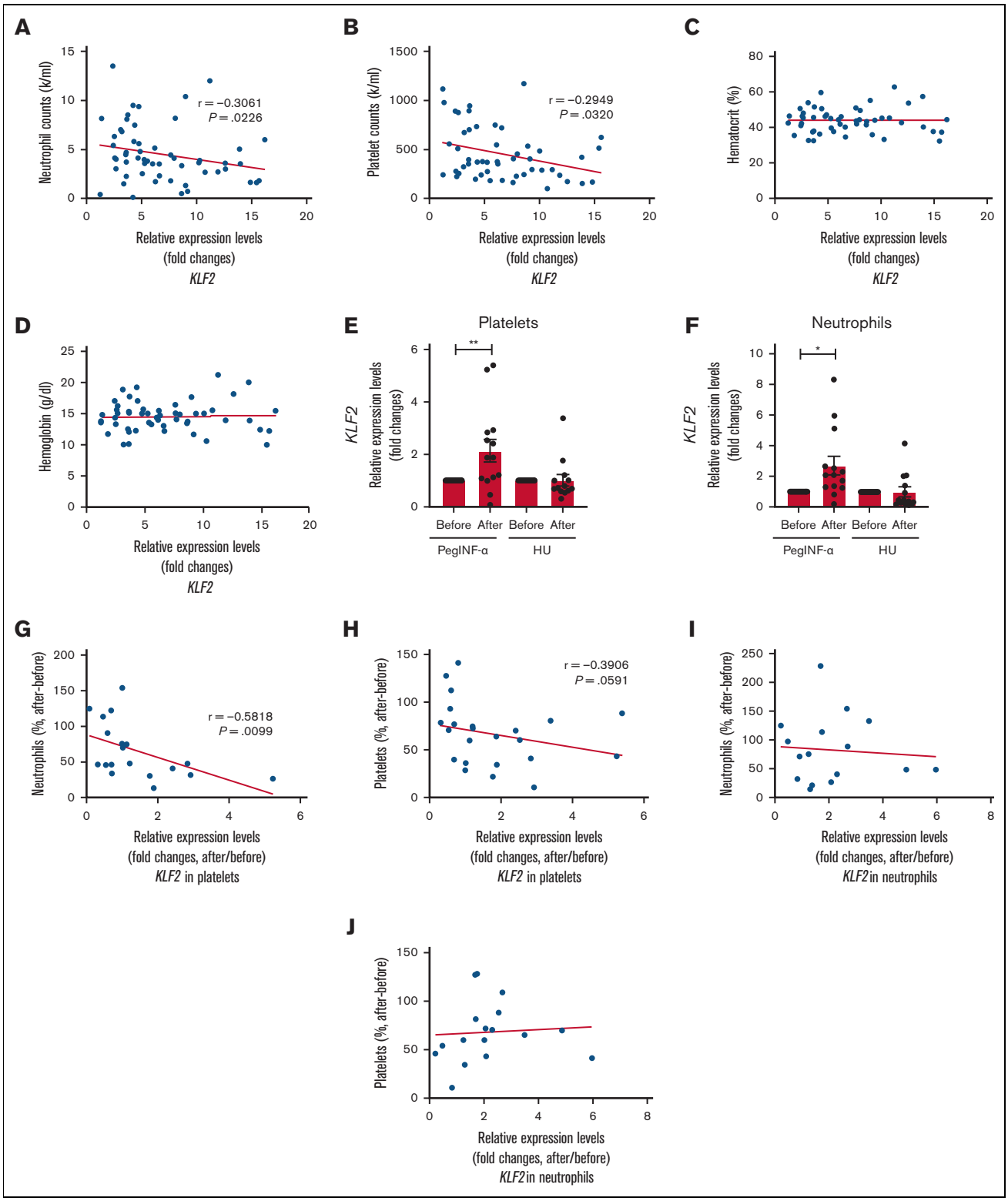

Figure 2. *KLF2* levels in platelets are inversely correlated with neutrophil and platelet counts and increased with PegINF-α treatment. *KLF2* transcript level in platelets was inversely correlated with (A) neutrophil counts and (B) platelet counts but not with (C) hematocrit and (D) hemoglobin concentration. *KLF2* transcript levels were measured in (E) platelets and (F) neutrophils before and after PegINF-α or HU. Changes of (G) neutrophil counts and (H) platelets counts were calculated against before treatment which was taken as 100% and inversely correlated with changes of *KLF2* transcript levels in platelets. Changes of (I) neutrophil counts and (J) platelets counts were calculated against before treatment which was taken as 100% and inversely correlated with changes of *KLF2* transcript levels in neutrophils. *P* value was calculated by paired t test. Correlation analysis was performed by Spearman correlation using GraphPad prism. ** $P < .01$, * $P < .05$.

neutrophils and platelets in 13 patients treated with HU and 14 patients with PegINF-α. Because the patients have different initial *KLF2* transcript levels according to their inflammation or thrombosis status, we used each pretreatment sample as a control for matching post treatment sample. Median duration of treatment with PegINF-α or HU was 165 and 175 days, respectively. PegINF-α treatment increased *KLF2* transcript levels in both neutrophils and platelets, whereas HU treatment did not (Figure 2E-F). *JAK2*^{V617F} allele burden decreased in 53% of patients treated with HU and 30% of the patients treated with PegINF-α. Neutrophil and platelet counts decreased from baseline after HU (83%, 85%) and PegINF-α treatment (85%, 91%), respectively. Increased *KLF2* transcript levels in platelets (Figure 2G-H) but not in neutrophils (Figure 2I-J) inversely correlated with decrease of neutrophils ($r = -0.5818$, $P = .0099$) and platelet counts ($r = -0.3906$, $P = .0591$). However, these levels did not correlate with the decreased hemoglobin and hematocrit (data not shown). Tumor necrosis factor (TNF) treatment reduces *KLF2* transcript and protein levels through NF-kB signaling in human umbilical vein endothelial cells.[20] *TNF* transcript levels in CD34+ cells and in vitro expanded erythroid cells decreased with PegINF-α but not with HU.[21] Increased *KLF2* transcripts in platelets but not in neutrophils after PegINF-α treatment correlated with decreased *TNF* transcript ($r = -0.4682$ $P = .0432$, figure not shown). PegINF-α decreases expression of genes associated with inflammation and immune cell function.[22] Although it remains to be confirmed if PegINF-α treatment increases *KLF2* transcript levels by decreasing inflammation, our observations suggest that increased *KLF2* transcript levels after PegINF-α treatment may be due to PegINF-α–mediated decreased inflammatory factors such as tumor necrosis factor.

We determined that downregulated *KLF2* in PV neutrophils and platelets is mediated by augmented JAK2 activity, leading to increased risk of thrombosis, which is corrected by PegINF-α treatment (supplemental Figure 3).

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Authorship

Contribution: J.G. suggested to P.T., J.T.P., and J.S. to focus on KLF2 in PV and ET; J.S. designed and performed the experiments; P.T. and J.T.P. contributed to the study design; J.S. and S.J.K. performed whole transcriptome analysis; S.J.K. collected and critically analyzed clinical data with J.T.P.; J.S. and J.T.P. wrote the manuscript; and P.T., J.G. and S.J.K. critically revised the manuscript and edited and approved the final version of the manuscript.

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