Vascular endothelial cell expression of JAK2^{V617F} is sufficient to promote a pro-thrombotic state due to increased P-selectin expression

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Supplemental Methods

Cell culture/Lentivirus transduction

Human umbilical venous endothelial cells (HUVECs, Lonza, Basel, Swiss) were cultured in EGM-2 media (Lonza, CC-3156) supplemented with EGM-2 Single Quots (Lonza, CC-4176). Cells were transduced at a multiplicity of infection of 20 with GFP positive lentiviruses encoding human JAK2V617F, human JAK2WT, or GFP alone as negative control. HUVECs were used between passage 5 and passage 7.

Thrombin generation

Thrombin generation was measured in freshly prepared platelet free plasma, according to the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, Netherlands). Thrombin generation was performed in 96-well plates containing confluent HUVECs washed with HEPES buffer (20 mM Hepes, 140 mM NaCl, 5 mg/ml BSA, pH 7,35). Thrombin generation was triggered by tissue factor (1 or 2.5 pM final concentration).

Protein C activation

Confluent HUVECs in 96-well plates were washed twice with warm sterile PBS and then incubated with thrombin (2 nM thrombin) and CaCl2 (2.5 mM) for 10 min at 37°C. Protein C was then added to each well at a final concentration of 0.2 μM. The plate was further incubated for different times at 37°C. Aliquots were collected and transferred to a clean 96-well plate. Hirudin (100 U/ml) was added to block thrombin. Activated protein C activity was monitored during 30 minutes at 405 nm on a FLUOStar Optima plate reader (BMG Labtech GMBH, Ortenberg, Germany) using a specific substrate (PNAPEP™ 1566, 100 µL at 0.4 mM). Results were plotted as the rate of substrate hydrolysis over time.

Nitrite and 6-keto Prostaglandin F1-alpha quantification

HUVECs were platted in 24 wells plates to reach confluence. Experiments were performed in the absence or presence of TNF-alpha (Merck Millipore). Nitrite quantification was performed following manufacturer's instructions with the Nitrate/Nitrite colorimetric assay (Cayman chemical, ref: 78001). Prostaglandin quantification was performed following manufacturer's instructions using the 6-keto-prostaglandin F1a kit (Cayman Chemical, ref: 515211).

Video analysis and quantification of leukocytes rolling and adhesion

Rolling leukocytes were quantified by counting the number of rhodamin-marked cells passing a given plane perpendicular to the vessel axis in a 30 second interval. Adherent leukocytes were quantified by counting the number of rhodamin-marked cells motionless during a 30 second interval. The results were expressed as number of rolling leukocytes per 30 seconds. Leukocyte adhesion results were expressed as a ratio of adherent cells/vessel surface (cm2). Video analyses were performed using ZEN imaging software. Leukocytes rolling and adhesion quantification were performed in a blinded fashion, by two independent observers.

Study of adhesion molecules on HUVECs

Transduced HUVECs were platted in 6-well plates to reach confluence. Before the experiment, conditioned medium with or without 10 ng/ml TNF-alpha was added for 4 hours. HUVECs were then washed twice with warm sterile PBS and incubated during 10 minutes with accutase. After two washes with PBS, cells were incubated with anti-ICAM-1 antibody (PE mouse anti-human CD54, HA-58 clone, BD Pharmingen), anti-VCAM-1 antibody (APC mouse anti-human CD106, 51-10C9 clone, BD Pharmingen) and anti-E-Selectin antibody (PE mouse anti-human CD62E, 68-5H11 clone, BD Pharmingen) during 30 minutes and flow cytometry was performed.

Generation and characterization of Pdgfb-iCreERT2;JAK2V617F/WT mice

Conditional heterozygous JAK2V617F/WT knock-in (JAK2V617F KI) mice have been previously described (1). Pdgfb-iCreERT2;JAK2V617F/WT mice were generated by crossing JAK2V617F KI mice with Pdgfb-iCreERT2 transgenic mice (2), allowing tamoxifen-inducible expression of JAK2V617F in endothelium. Pdgfb-iCreERT2-negative; JAK2V617F/WT littermate mice were used as controls. Mutant mice were analysed 15-20 days after tamoxifen administration. Adult mice were genotyped by PCR using the P1/P2 primer set for the wild-type allele and for the JAK2 Flex allele: P1: 5'-GTCTGTCCAAAGAGTC-TGTAAGTAC- 3'; P2: 5' : GCTCCAGGGTTACACGAGTC- 3'. For PDGF Cre-recombinase, forward primer: 5'-CCAGCCGCCGTCGCAACT-3', and reverse primer: 5'-GCCGCCGGGATCACTCTCG-3' 24 were used. Haematocrit, hemoglobin level, platelet, and white cells count were determined using an automated counter (scil Vet abc Plus+).

Histology

To quantify thrombus formation in mice, we used Carstairs staining allowing visualization of platelets, red blood cells and fibrin. Slides were hydrated in xylol and ethanol to distilled water, followed by incubation in 5% ferric ammonium sulfate for 5 min, washing, and staining with Mayer hematoxylin for 5 min and Picric Acid-orange G solution for 1 hour. After washing, slides were stained with 1% phosphotungstic acid for 10 min, Ponceau Fuchsin solution for 7 min, washed, and 1% phosphotungstic acid for 10 minutes. After washing, slides were stained with Anilin blue solution for 30 min, and rinsed in distilled water. Slides were dehydrated and covered with a coverslip using mounting medium. Presence of thrombi was assessed by fibrin deposit in thrombi. Thrombus quantification was performed using an optical microscope and the pulmonary area was measured to perform thrombi number/pulmonary area (number of thrombi/cm2) ratio. Immunostaining of platelets and neutrophils was performed using polyclonal anti-PF4 antibody (Peprotech, 500-P05) and anti-Ly6G monoclonal antibody (1A8 clone) respectively. Three slides per mouse were analysed in a blinded fashion, by two independent observers.

Quantification of VWF in HUVECs and supernatant

HUVECs were seeded in 12 well plates (Costar), and at confluence, they were washed with 500 μL of PBS (Gibco). 500 μL of medium was added. 10ng/ml TNF-alpha was added (or not) for 24 hours) (Merck Millipore). Supernatant was then removed, centrifuged at 12000 g for 5 minutes and stored at -80°C for later quantification of secreted vWF. To quantify intracellular vWF, cells were washed with PBS before adding 500 μL PBS and removed with a scrapper. Liquid was removed and 5 cycles of freezing and thawing were performed. Finally, the intracellular lysates were centrifuged at 12000 g for 5 minutes and stored at -80°C. vWF was quantified using a previously published method (3). Ninety-eight well plates (Greiner, Flat Bottom) were coated with anti-vWF antibody (DAKO, A0082) diluted at 1/660 in a coating buffer overnight at 4°C. After washing and blocking, a sample of HUVEC supernatant and intracellular lysate were added to wells. Control wells were treated with platelet poor plasma from a healthy donor. After 2 hours and a second wash, anti-vWF antibody coupled to HRP (DAKO, P0226) diluted at 1/6000 in wash buffer was added to wells.

After 2 hours and a third wash, HRP was revealed with an OPD and H202 solution. After 2 minutes of coloration, the reaction was blocked with a H2S04 3M solution and results were read at 492nnm with an OPTIMA plate reader.

Von Willebrand Factor (vWF) and P-selectin immunostaining

Immunofluorescence analyses were performed on HUVECs, treated with or without TNF-alpha (10ng/ml overnight). For vWF staining, cells were fixed with 2% paraformaldehyde (PFA) for 10 minutes and, after saturation in 5% bovine serum albumin for 1 hour, incubated with rabbit anti-human vWF (Merck Millipore) primary antibody, and resolved with Alexa Fluor 588 conjugated secondary antibody (Invitrogen, Carlsbad, California). Cells were mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, California), imaged with a confocal microscope (Zeiss LSM 700) and analysed by Imaris software (Bitplane). In mice, P-Selectin immunostaining was performed in carotid arteries of PDGFb-iCreERT2;JAK2V617F/WT and control mice.

In vitro and in vivo study of the effects of hydroxyurea on endothelial cells

In vitro, HUVECs were treated with hydroxyurea (HU) (Sigma-Aldrich, H8627) for 24 hours at a concentration of 100 μM, then washed three times with EGM2 medium. Neutrophil static adhesion was performed as previously described. In vivo, Pdgfb-iCreERT2;JAK2V617F/WT and control mice were treated with HU at a dose of 200 mg/kg/day (using oral gavage) for 10 days prior to any experiment.

RNA extraction and real-time qPCR

RNA were prepared using Trizol reagent as recommended by the manufacturer. RNA were then treated with RNAse-free DNAse (Promega, reference: M610A). For *P-selectin* mRNA quantification, complementary DNA (cDNAs) were synthetized from 1 µg of RNA, using the M-MLV reverse transcriptase (Promega, reference: M1708) according to manufacturer instructions. The real time quantitative PCR (qRT-PCR) was performed using One-Step SYBR® Green qRT-PCR Kit for iQ (Quantabio) on a AriaMx thermocycler (Agilent). Data were analyzed by the 2-ΔΔCt method (4) with the reference gene GAPDH. The sequence of the primers used were: P-selectin : 5'-CTGCCAAGCAGGACCATTGA and 5'-AGGACTCGGGTCAAATGCAG, GAPDH: 5'-AGGTGAAGGTCGGAGTCAACG and 5'-GCTCCTGGAAGATGGTGATGG.

Supplemental references

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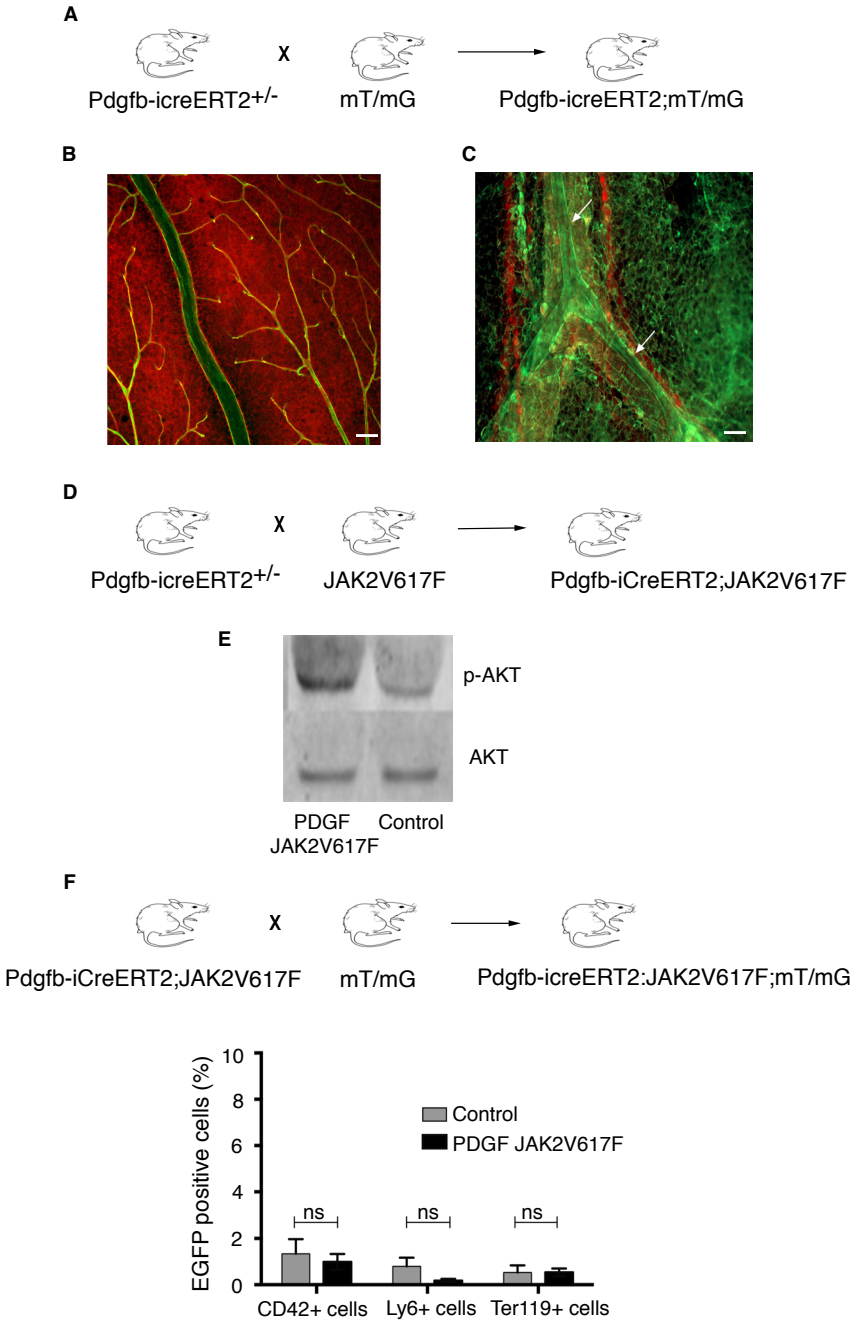
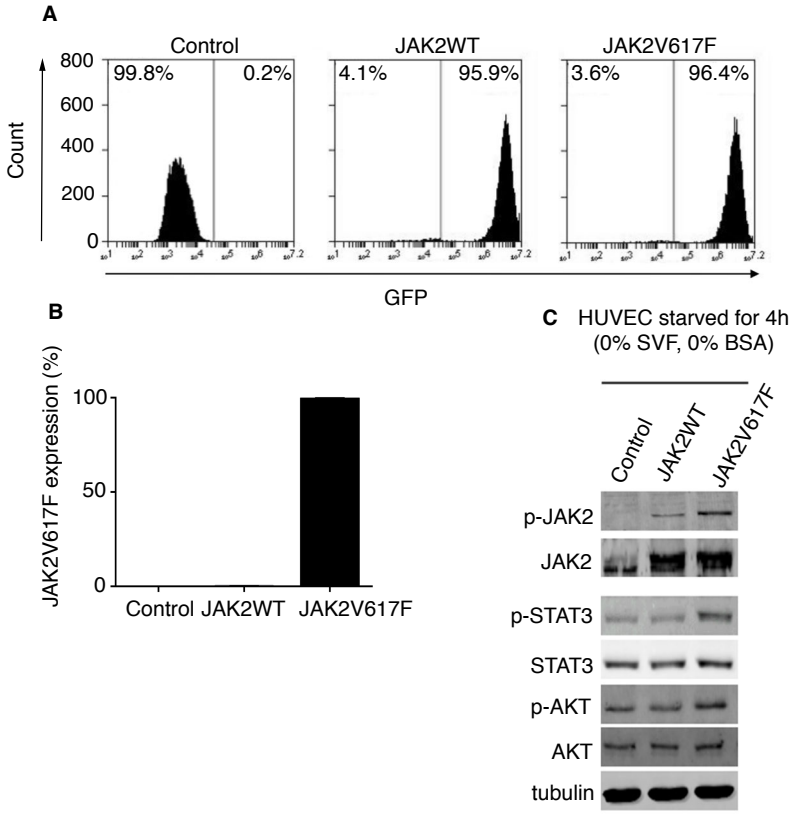
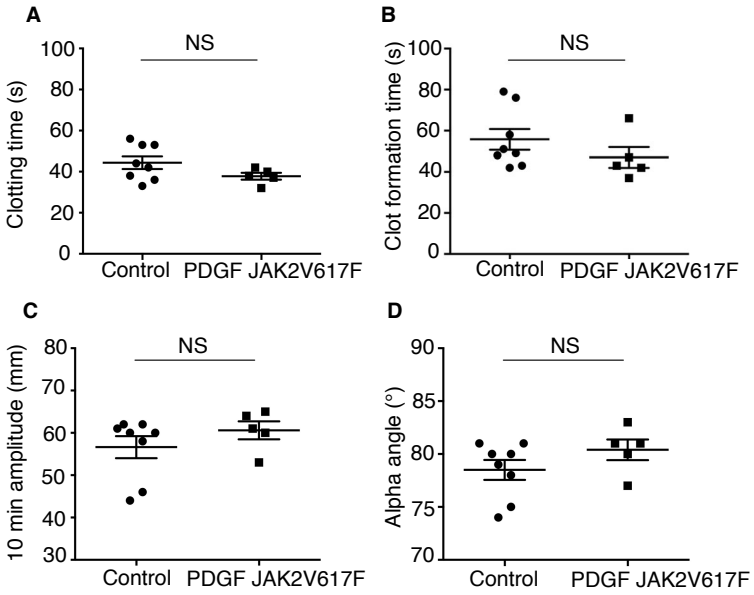
Supplemental Figures



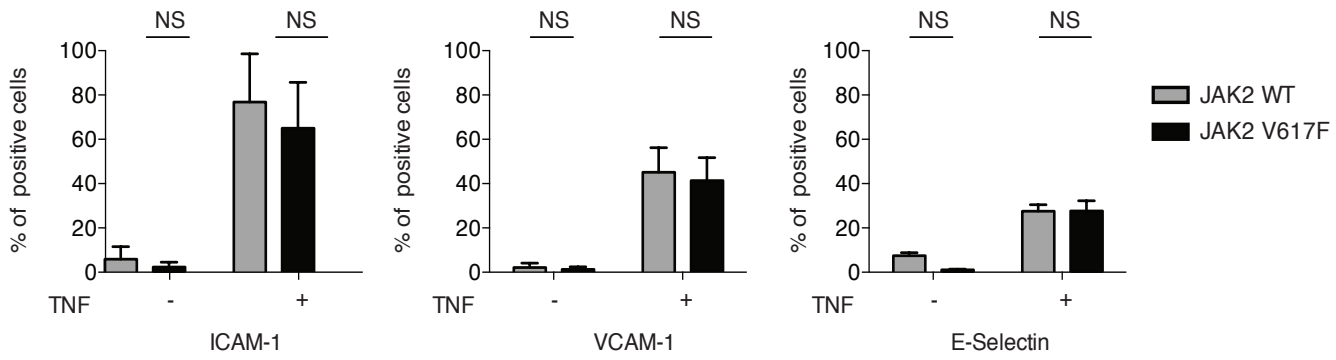
Supplemental Figure 1. Characterization of *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$* mice. **(A)** *Pdgfb-iCreERT2* were crossed with *mT/mG* mice to obtain *Pdgfb-iCreERT2;mT/mG* mice. Efficient endothelial recombination after oral administration of tamoxifen in the retina **(B)** and mesentery **(C)** of *Pdgfb-iCreERT2;mT/mG* mice. White arrows indicate GFP positive endothelium. **(D)** Conditional JAK2V617F$^{WT/Flex}$ mice were crossed with *Pdgfb-creERT2* mice for tamoxifen-inducible adult expression of JAK2V617F in endothelial cells. **(E)** AKT phosphorylation is increased in lung endothelial cells from *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$* mice. **(F)** *Pdgfb-icreERT2;JAK2$^{V617F/WT}$* mice were crossed with *mT/mG* mice to obtain *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$; mT/mG* mice. Flow cytometry analysis did not reveal any expression of the Cre in granulocytes (Ly6+), platelets (CD42+) and red blood cells (Ter119+) in *Pdgfb-iCreERT2;mT/mG;JAK2$^{V617F/WT}$* mice 20 days after oral tamoxifen administration. Results are mean value +/- s.e.m from 5 mice per genotype. Statistical significance by Mann-Whitney-test.



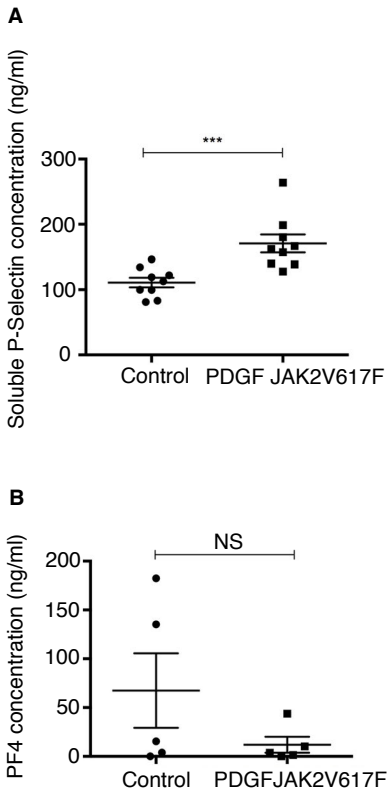
Supplemental Figure 2. **(A)** Expression of GFP up to 95% in JAK2WT and JAK2V617F HUVECs. **(B)** Measurement of JAK2V617F allelic burden in HUVEC transduced either with control, JAK2WT or JAK2V617F lentiviruses. **(C)** Studies of JAK2, STAT3 and AKT phosphorylation by western blotting in HUVECs overexpressing JAK2WT or JAK2V617F.



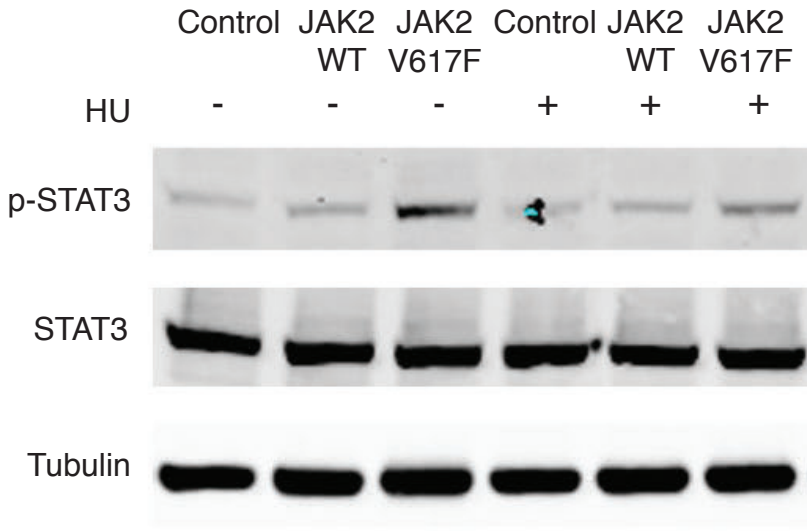
Supplemental Figure 3. Coagulation parameters of control and *Pdgfb-iCreERT2;JAK2*$^{V617F/WT}$ mice evaluated by thromboelastometry. (A) Clotting time is not different between *Pdgfb-iCreERT2;JAK2*$^{V617F/WT}$ mice and control mice. **(B)** Clot formation time is not different between *Pdgfb-iCreERT2;JAK2*$^{V617F/WT}$ mice and control mice. **(C)** Ten minute amplitude is not different between *Pdgfb-iCreERT2;JAK2*$^{V617F/WT}$ mice and control mice. **(D)** Alpha angle is not different between *Pdgfb-iCreERT2;JAK2*$^{V617F/WT}$ mice and control mice. Results are expressed as mean value ± s.e.m. Statistical significance determined by Mann-Whitney test.



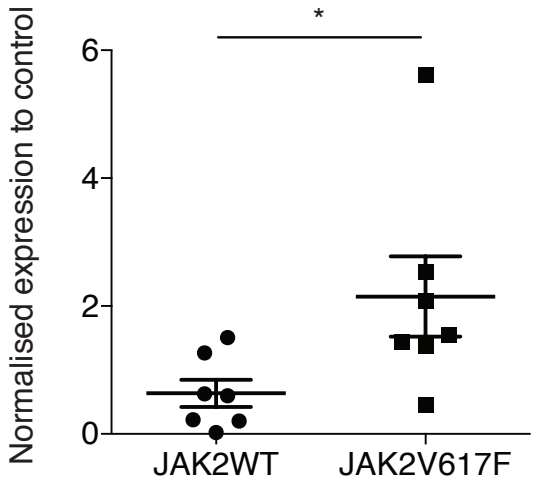
Supplemental Figure 4. No difference of ICAM-1, VCAM-1 and E-Selectin expression between JAK2WT and JAK2 V617F HUVEC. Results expressed as percentage of positive cells, mean and SEM. Statistical significance by Mann-Whitney test.



Supplemental Figure 5. (A) Soluble P-Selectin concentration is higher in *Pdgfb-iCreERT2;JAK2*$^{V617F/WT}$ mice. **(B)** PF4 concentration is not different in *Pdgfb-iCreERT2;JAK2*$^{V617F/WT}$ mice and control mice. Results are mean value +/- s.e.m. Statistical significance by Mann-Whitney test. *** $P < 0.005$.



Supplemental Figure 6. Effect of hydroxyurea (HU) administration on STAT3 and p-STAT3 expression in control, JAK2 WT and JAK2 V617F HUVEC. Administration of Hydroxyurea at the concentation of 100μM during 24 hours lead to a decrease in p-STAT3 expression.



Supplemental Figure 7. P-Selectin RNA expression is increased in JAK2V617F HUVEC. HUVECs were transduced with lentivirus vectors carrying the cDNA of JAK2 WT or V617F or an empty vector. Six days after transduction, RNAs were extracted for evaluation of P-selectin expression. Results are expressed as the expression of P-selectin on GAPDH expression and normalised on the empty-vector control condition according to the 2-ΔΔCt method (n= 3). Statistical significance by Mann-Whitney test. * $P < 0.05$.

Supplemental Table 1

	Control	PDGF JAK2V617F	
Platelets (10^3/mm^3)	1074 +/- 52.94	1106 +/- 64.92	*p = 0.72*
Leukocytes (10^3/mm^3)	4.94 +/- 0.68	4.37 +/-0.60	*p = 0.49*
Hemoglobin (g/dl)	11.68 +/-0.25	11.44 +/- 0.23	*p = 0.49*
Hematocrit (%)	37.96 +/- 0.90	36.37 +/- 0.79	*p = 0.20*