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# JAK2$^{V617F}$-mutant vascular niche contributes to JAK2$^{V617F}$ clonal expansion in myeloproliferative neoplasms

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ABSTRACT

The myeloproliferative neoplasms (MPNs) are characterized by hematopoietic stem/progenitor cell (HSPC) expansion and overproduction of blood cells. The acquired mutation *JAK2$^{V617F}$* plays a central role in these disorders. Mechanisms responsible for MPN HSPC expansion is not fully understood, limiting the effectiveness of current treatments. Endothelial cells (ECs) carrying the JAK2$^{V617F}$ mutation can be detected in patients with MPNs, suggesting that ECs are involved in the pathogenesis of MPNs. Here we report that JAK2$^{V617F}$-bearing primary murine ECs have increased cell proliferation and angiogenesis *in vitro* compared to JAK2$^{WT}$ ECs. While there was no difference between JAK2$^{V617F}$ and JAK2$^{WT}$ HSPC proliferation when co-cultured with JAK2$^{WT}$ EC, the JAK2$^{V617F}$ HSPC displayed a relative growth advantage over the JAK2$^{WT}$ HSPC when co-cultured on JAK2$^{V617F}$ EC. In addition, the thrombopoietin (TPO) receptor MPL is up regulated in JAK2$^{V617F}$ ECs and contributes to the maintenance/expansion of the JAK2$^{V617F}$ clone over JAK2$^{WT}$ clone *in vitro*. Considering that ECs are an essential component of the hematopoietic niche and most HSPCs reside in the perivascular niche, our studies suggest that the JAK2$^{V617F}$-bearing ECs form an important component of the MPN vascular niche and contribute to mutant stem/progenitor cell expansion, likely through a critical role of the TPO/MPL signaling axis.

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## 1. Introduction

The marrow consists of the hematopoietic cells and non-hematopoietic stromal cells, including fibroblasts, reticular cells, endothelial cells (ECs), macrophages, adipocytes and osteoblasts. In addition to its role in normal HSPC biology, an altered microenvironment is an important contributor to the development of hematologic malignancies [1–3]. In a reciprocal fashion, myeloid malignancies also affect the function of the marrow microenvironment to impair normal hematopoiesis while favoring malignant stem cell expansion [4,5].

The chronic Philadelphia chromosome (Ph) negative myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), are clonal stem cell disorders characterized by HSPC expansion, overproduction of mature blood cells, a tendency to extramedullary hematopoiesis, and transformation to acute leukemia or myelofibrosis at variable rates. The acquired signaling kinase mutation *JAK2$^{V617F}$* plays a central role in the pathogenesis of MPN, but mechanism(s) responsible for MPN HSPC expansion is not fully understood, limiting the effectiveness of current treatments. Although the etiology of dysregulated hematopoiesis has been mainly attributed to the molecular alterations within the hematopoietic stem/progenitor cells, abnormalities of the marrow microenvironment are beginning to be recognized as an important factor in MPN development [1,5–7]. The diseased niche could impair normal hematopoiesis and favor the competing malignant stem cells, which could contribute to the poor engraftment and treatment-related mortality following allogeneic stem cell transplantation, the only curative treatment for patients with MPNs [1–3,5,8,9].

ECs are an essential component of the hematopoietic niche and most hematopoietic stem/progenitor cells (HSPCs) reside close to a marrow sinusoid (the "perivascular niche") [10–13]. In addition, ECs are an important niche component of the extramedullary (splenic) hematopoiesis, which is almost always present in patients with MPNs and is associated with MPN disease progression [13,14]. Although the existence and cell of origin of endothelial progenitors is still a matter of debate, JAK2$^{V617F}$ mutation can be detected in endothelial progenitors derived from the hematopoietic lineage (the so-called endothelial cell colony-forming units; CFU-ECs) and, in some reports, in the true endothelial colony-forming cells (ECFC) based on *in vitro* assays [15–

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19]. JAK2$^{V617F}$ mutation is also present in ECs isolated by microdissection from liver and spleen samples of patients with MPNs [17,20]. In addition, we and others have shown that JAK2$^{V617F}$ ECs are critical in the development of the bleeding abnormalities in a murine model of JAK2$^{V617F}$-positive MPNs in which JAK2$^{V617F}$ is expressed in all hematopoietic cells and endothelial cells [21]. All of these observations suggest that ECs are involved in the pathogenesis of MPNs.

Previously, we and others have shown that the thrombopoietin (TPO) receptor MPL is essential for the development of HSPC expansion in MPNs [22,23]. MPL is expressed in long-term HSPCs and is associated with both HSPC repopulating activity and HSPC quiescence [24,25]. MPL is also expressed on several types of endothelium [26–28]. Whether EC MPL receptor affects the vascular niche function and contributes to MPN development is not known. In this study, we examined the roles of JAK2$^{V617F}$-bearing ECs in MPN hematopoiesis. We found that the MPN vascular niche contributes to the growth advantage of JAK2$^{V617F}$ HSPC over the JAK2$^{WT}$ HSPC. We also found that the EC MPL receptor is important for the JAK2$^{V617F}$ clonal expansion in MPNs.

## 2. Materials and methods

### 2.1. Experimental mice

JAK2$^{V617F}$ Flip-Flop (FF1) mice [29] were kindly provided by Radek Skoda (University Hospital, Basal, Switzerland), Tie2-Cre mice [30] by Mark Ginsberg (University of California, San Diego), and MPL knockout mice (MPL$^{-/-}$) [31] by Warren Alexander (Melbourne, Australia). FF1 mice were crossed with Tie2-Cre to express JAK2$^{V617F}$ specifically in hematopoietic cells and ECs (Tie2/FF1 mice) as we previously did [21]. All mice used were on a C57BL/6 background and were bred in a pathogen-free mouse facility at Stony Brook University. CD45.1$^{+}$ congenic mice (SJL) were purchased from Taconic Inc. (Albany, NY). Animal experiments were performed in accordance with the guidelines provided by the Institutional Animal Care and Use Committee at Stony Brook University.

### 2.2. Isolation of murine hematopoietic stem/progenitor cells (HSPCs)

14–18-week old mice were euthanized and the femurs and tibias removed. A 25-gauge needle was used to flush the marrow with PBS + 2% FBS. Cells were triturated and filtered through 70 μM nylon mesh (BD Biosciences, San Jose, *CA*) to obtain a single cell suspension. For depletion of mature hematopoietic cells, the Lineage Cell Depletion Kit (Miltenyi Biotec, Cat. 130-090-858, San Diego, *CA*) was used. The lineage (CD5, CD45R, CD11b, Ter119, and GR-1) negative cells were collected and then positively selected for CD117$^{+}$ (cKit$^{+}$) cells using CD117 microbead (Miltenyi Biotec, Cat. 130-091-224) to yield Lineage$^{neg}$cKit$^{+}$ (Lin$^{-}$cKit$^{+}$) HSPCs.

### 2.3. Isolation of murine lung endothelial cells

Primary murine lung EC isolation was performed as we previously did [32]. Briefly, 14–18 week old mice were euthanized using 100% $CO_2$ inhalation followed by cervical dislocation. The chest was immediately opened through a midline sternotomy. The left ventricle was identified and the ventricular cavity was entered through the apex with a 27-gauge needle. The right ventricle was identified and an incision was made in the free wall to exsanguinate the animal and to allow the excess perfusate to exit the vascular space. The animal was perfused with 30 mL of cold PBS. The lung tissue was collected and minced finely with scissors. The tissue fragments were digested in DMEM medium containing 1 mg/mL Collagenase D (Roche, Switzerland), 1 mg/mL Collagenase/Dispase (Roche) and 25 U/mL DNase (Sigma, St. Louis, MO) at 37 °C for 2 h with shaking, after which the suspension was homogenized by triturating. The homogenate was filtered through a 70 μm nylon mesh (BD Biosciences, San Jose, *CA*) and pelleted by centrifugation (400*g* for 5 min). Cells were first depleted for CD45$^{+}$ cells (Miltenyi Biotec) and then positively selected for CD31$^{+}$ cells (Miltenyi Biotec) using magnetically labeled microbeads according to the manufacturer's protocol. Isolated ECs (CD45$^{-}$CD31$^{+}$) were cultured in EC culture medium with no medium change for the first 72 h to allow EC attachment followed by medium change every 2–3 days. Cells were re-selected for CD31$^{+}$ cells when they reach >70–80% confluence (usually after 3–4 days of culture).

### 2.4. Flow cytometry

CD45.1 (A20) antibody or isotype control was used for chimerism studies. All staining steps were performed in ice-cold PBS containing 2% fetal bovine serum. All samples were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Postacquisition data analysis was performed with FlowJo software V9.2.3 (Treestar, *CA*).

### 2.5. Polymerase chain reaction

Human JAK2 cDNA-specific primers (5′-GAAGAACTTCAGCAGTCTT AAAGATC-3′ and 5′-CCATGCCAACTGTTTAGCAACTTC-3′) were used to detect the expression of human JAK2$^{V617}$F in ECs from Tie2/FF1 mice using reverse transcription polymerase chain reaction (RT-PCR). The primers amplify a 573bp fragment that would be detected on 2% agarose gel.

The TaqMan® Gene Expression Assay (Applied Biosystems) was used for real-time quantitative polymerase chain reaction (qPCR) to verify differential expression of MPL (Mm00440306_g1) on an ABI ViiA™ 7 Real-Time PCR machine (Applied Biosystems). The gene expression levels were normalized to Actin beta (Actb) expression and relative fold changes was calculated by the $2^{\Delta\Delta CT}$ method. All assays were performed in triplicate.

### 2.6. Assays to examine endothelial cell in vitro angiogenesis

EC tube formation assay was performed as a measure of angiogenesis *in vitro* [32]. Matrigel® matrix (10 mg/mL, Corning Inc., Corning, NY) were thaw overnight at 4 °C and kept on ice until use. 150 uL Matrigel per well was added to pre-chilled 48-well culture plate. After gelation at 37 °C for 30 min, gels were overlaid with $6 \times 10^4$ JAK2$^{V617F}$ ECs (from Tie2/FF1 mice) or JAK2$^{WT}$ ECs (from control mice) (passage 3–4) in 300 uL of complete EC medium. Tube formation was inspected after a period of 2, 4, 6, and 8 h and images were captured with a phase-contrast microscope (AMEX-1200, AMG, Bothell, WA). The quantification of the capillary tube formation was performed using the ImageJ® software (National Institute of Health, Bethesda, MD) by counting the number of nodes (or branch points), loops, and tubes in 4 non-overlapping areas at ×40 magnification in two duplicate wells.

### 2.7. In vitro cell culture

Lin$^{-}$cKit$^{+}$ HSPCs were cultured in StemSpan® serum-free expansion medium (SFEM) containing 100 ng/mL recombinant mouse SCF, 6 ng/mL recombinant mouse IL3 and 10 ng/mL recombinant human IL-6 (all from Stem Cell Technologies, Vancouver, BC)

ECs were cultured on 1% gelatin coated plates in complete EC medium which is consisted of advanced DMEM/F12 (ThermoFisher, Waltham, MA) medium containing 20% fetal bovine serum, 50 μg/mL endothelial cell growth supplement (Alfa Aesar, Ward Hill, MA), 1% Antibiotic-antimyotic solution (Cat. 15240-062, ThermoFisher), 10 mM HEPES buffer (ThermoFisher), 5 μM SB431542 small molecule (R&D, Minneapolis, MN), 50 μg/mL Heparin (Sigma), 1% Glutamax 100× solution (ThermoFisher), 1% non-essential amino acid (ThermoFisher), recombinant mouse VEGF 10 ng/mL (PeproTech, Rocky Hill, NJ; add fresh when changing medium) and recombinant