

# The possible role of mutated endothelial cells in myeloproliferative neoplasms



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## ABSTRACT

Myeloproliferative neoplasms (MPN) are chronic, clonal hematologic malignancies characterized by myeloproliferation and a high incidence of vascular complications (thrombotic and bleeding). Although MPN-specific driver mutations have been identified, the underlying events that culminate in these clinical manifestations require further clarification. We reviewed the numerous studies performed during the last decade identifying endothelial cell (EC) dysregulation as a factor contributing to MPN disease development. The *JAK2V617F* MPN mutation and other myeloid-associated mutations have been detected not only in hematopoietic cells but also in EC and their precursors in MPN patients, suggesting a link between mutated EC and the high incidence of vascular events. To date, however, the role of EC in MPN continues to be questioned by some investigators. In order to further clarify the role of EC in MPN, we first describe the experimental strategies used to study EC biology and then analyze the available evidence generated using these assays which implicate mutated EC in MPN-associated abnormalities. Mutated EC have been reported to possess a pro-adhesive phenotype as a result of increased endothelial P-selectin exposure, secondary to degranulation of Weibel-Palade bodies, which is further accentuated by exposure to pro-inflammatory cytokines. Additional evidence indicates that MPN myeloproliferation requires *JAK2V617F* expression by both hematopoietic stem cells and EC. Furthermore, the reports of *JAK2V617F* and other myeloid malignancy-associated mutations in both hematopoietic cells and EC in MPN patients support the hypothesis that MPN driver mutations may first appear in a common precursor cell for both EC and hematopoietic cells.

## Introduction

The Philadelphia chromosome-negative myeloproliferative neoplasms (MPN) include polycythemia vera, essential thrombocythemia and primary myelofibrosis.<sup>1</sup> These clonal hematopoietic stem cell (HSC) disorders are characterized by an increased rate of vascular complications including thrombotic and bleeding episodes.<sup>2,3</sup> However, the mechanisms underlying these vascular events remains uncertain and have been the subject of considerable speculation and debate for decades.<sup>4,5</sup> Recently, new insights into factors contributing to the development of thrombotic events in MPN patients have become available,<sup>6</sup> including the role of endothelial cells (EC) that contain MPN driver mutations. Physiologically, EC participate in the maintenance of vascular integrity, and generate an anti-thrombotic surface.<sup>7</sup> During the last decade, the *JAK2V617F* MPN driver mutation has been shown to be present in EC<sup>8,9</sup> and their progenitors<sup>10-12</sup> in some MPN patients, suggesting a link between mutated EC and the high incidence of vascular events. This concept and its implications remain controversial and its significance has been questioned by some investigators.<sup>9,11,13</sup> The aim of this review is to analyze this evidence in a critical fashion and assess the validity of the link between EC and MPN pathobiology.

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## Myeloproliferative neoplasms and vascular complications

Vascular complications are the most common clinical sequelae and a major cause of morbidity and mortality in MPN patients.<sup>2,3</sup> The incidence and the characteristic clinical presentations of vascular events in MPN patients are summarized in Table 1.

Thrombotic events are often the initial manifestation of an MPN or may precede the diagnosis of the MPN. Thrombosis appears to be more common among patients with polycythemia vera than in those with essential thrombocythemia or primary myelofibrosis both at diagnosis<sup>3</sup> and during follow up<sup>2</sup> (Table 1). Bleeding episodes are less frequent than thrombotic events in MPN patients; and, contrary to thrombosis, occur primarily after the diagnosis of the MPN has been established<sup>14</sup> (Table 1).

### Factors predisposing to thrombosis in patients with myeloproliferative neoplasms

Many features of a patient's demographics are predictive of MPN-associated thrombotic complications<sup>15-17</sup> including age, prior thrombotic events, an inflammatory state, and MPN-associated risk factors, such as degree of erythrocytosis, leukocytosis, and the presence of *JAK2V617F*. By contrast, individuals with *calreticulin* mutations have a lower risk of thrombosis than those with *JAK2V617F*.<sup>2</sup> Notably, the frequency of the *JAK2V617F* variant allele influences the degree of thrombotic risk<sup>18</sup> in patients with

essential thrombocythemia, while contradictory results were found in patients with polycythemia vera. Conventional cardiovascular risk factor (e.g., hypertension, hyperlipidemia, diabetes and smoking) are additional variables associated with an increased rate of thrombosis. Among factors predisposing to thrombosis, only age greater than 60 years and a prior history of a thrombotic event were validated as thrombotic risk factors in MPN patients, while conflicting results have been reported for other proposed predisposing factors.<sup>2,15,16</sup> However, the presence of *JAK2V617F* as an MPN driver mutation has been confirmed as a predictor of additional thrombotic events in patients with essential thrombocythemia.<sup>15,16</sup>

The history of thrombotic events prior to a diagnosis of MPN may also be attributed to the presence in these patients of a clonal hematopoiesis of indeterminate potential (CHIP), involving *JAK2V617F* or *calreticulin* mutations prior to the development of a full blown MPN. Indeed, CHIP has been associated with an increased risk of coronary artery disease and stroke.<sup>19</sup> In particular, *JAK2V617F*<sup>+</sup> CHIP has been most frequently associated with an increased risk of developing cardiovascular diseases, thrombosis and coronary heart disease.<sup>19</sup> Furthermore, Cordua *et al.*<sup>20</sup> have shown that subjects with *JAK2V617F* or *calreticulin* CHIP frequently eventually develop a full-blown MPN.

The underlying events that lead to thromboses in MPN patients remain the subject of investigation. Historically, the thrombotic tendency may be influenced, as outlined

**Table 1. Incidence and main clinical characteristics of vascular events in patients with myeloproliferative neoplasms.**

Disease	Molecular features	Main Phenotype	THROMBOSIS			BLEEDINGS			
			Incidence	Type	Clinical characteristics	Ref.	Incidence	Clinical characteristics	Ref.
PV	<i>JAK2 V617F</i> (95%) <i>JAK2</i> exon 12 (5%) Sub-clonal mutations in myeloid genes	<b>Erythrocytosis</b> , which can be associated with leukocytosis and thrombocytosis.	- At diagnosis: 28.6% - During follow up: 3.8 x 100 person/year (1.5 deaths per 100 person/year)	Both arterial and venous	<b>Mild microcirculatory disturbances</b> (headache, itching, buzzing)	2, 3, 17	3–8% (usually after the diagnosis)	- <b>Minor bleeding</b> (e.g. ecchymoses, gingival hemorrhage, menorrhagia and epistaxis)	14
ET	<i>JAK2 V617F</i> (60%) <i>MPL</i> exon 10 (5%) <i>CALR</i> exon 9 (20%) Triple negative (5–10%) Sub-clonal mutations in myeloid genes	<b>Thrombocytosis</b> . Sometimes patients presented with normal white blood cell counts. A reduced red blood cell count can also be observed	- At diagnosis: 20.7% - During follow up: 2.4 x 100 person/year	Mainly arterial	<b>Major arterial and venous thrombotic events</b> (ischemic stroke, peripheral artery disease, splanchnic vein thromboses, cerebral sinus thromboses, myocardial infarction, and deep vein thromboses)	2, 3, 17, 22	3 - 18% (usually after the diagnosis)	- <b>Major bleeding</b> (e.g. intracranial hemorrhage, gastrointestinal bleeding, retroperitoneal bleeding) - Extreme thrombocytosis may cause bleeding due to development of an acquired Von Willebrand syndrome	14, 29
MF	<i>JAK2 V617F</i> (60%) <i>MPL</i> exon 10 (5%) <i>CALR</i> exon 9 (20%) Triple negative (5–10%) Sub-clonal mutations in myeloid genes. ( <i>ASXL1</i> , <i>DMT3A</i> , <i>EZH2</i> , <i>IDH1/IDH2</i> , <i>SRSF2</i> , or <i>TP53</i> are associated with a worse outcome)	<b>Splenomegaly (85%); Cytopenia:</b> - 2/3 of patients had anemia at diagnosis; - 40 to 50% have leukocytosis - 13–32% have thrombocytosis	- At diagnosis: 9.5% - During follow up: 2.2 x 100 person/year	Both arterial and venous	<b>Over-representation of thrombosis in unusual sites</b> (portal system, Budd-Chiari syndrome, cerebral venous thrombosis)	2, 3, 4, 5	19 - 56% (~12% in patients with pre-fibrotic myelofibrosis)	- <b>Main cause of bleedings</b> are Portal hypertension with esophageal varices, the use of anti-platelet and/or anti-coagulant therapy	4, 14, 29

PV: polycythemia; ET: essential thrombocythemia; MF: myelofibrosis; ref.: references.

below, by a combination of increased numbers of abnormal myeloid cells and the co-existence of a chronic inflammatory state.<sup>2</sup> Recently, new evidence has shown a role for endothelial cells, which is the subject of this review.

### **Blood cell alterations and thrombotic tendency in myeloproliferative neoplasms**

The elevated number of red cells and the resultant increased hematocrit levels are well established to have pro-thrombotic effects.<sup>2</sup> Under low shear rates an elevated hematocrit leads to increased blood viscosity, while at high shear rates, the increased red cell numbers disperse platelets toward the vessel walls, resulting in platelet activation. Finally, biochemical changes have been observed in red cell membranes both in patients with polycythemia vera and in those with essential thrombocythemia, causing red blood cell aggregation.<sup>2</sup> In contrast to red blood cells, there are few studies on platelets directly correlating the degree of thrombocytosis with the rate of thrombosis in MPN patients.<sup>21</sup> The impact of leukocytosis on thrombosis has been evaluated in numerous retrospective studies, but with discordant results. Several studies suggest that the adhesion of leukocytes to EC contributes to the development of thrombosis, especially the formation of venous thrombi.<sup>22</sup> By contrast, it has been recently documented that persistent leukocytosis in polycythemia vera was associated with disease progression, rather than thrombosis.<sup>23</sup> In general, neutrophils play a central role in generating the inflammatory response and in activation of the blood coagulation system through the release of proteolytic enzymes and reactive oxygen species and the increased expression of CD11b which activates or damages platelets, EC and coagulation proteins.<sup>2</sup> Moreover, granulocytes in MPN patients produced an increased amount of neutrophil extracellular traps that initiate and propagate arterial and venous thrombosis.<sup>24,25</sup> Mouse models have demonstrated that neutrophil extracellular traps are crucial in the development of thrombosis.<sup>32</sup>

Moreover, MPN blood cells are also qualitatively abnormal due to their procoagulant and proteolytic properties, secretion of inflammatory cytokines, and expression of cell adhesion molecules.<sup>2</sup> In particular, activated platelets in MPN patients express P-selectin and tissue factor and secrete an increased number of platelet activation products.<sup>26</sup>

### **Inflammation and thrombosis**

In concert, inflammatory cytokines secreted by MPN cells and leukocyte-derived proteases damage the integrity of the normal vascular endothelium, leading to the acquisition of a pro-thrombotic phenotype in MPN patients. Specifically, EC overexpress adhesion receptors favoring the attachment of platelets, erythrocytes, and leukocytes to the vascular wall. In addition, MPN patients have increased levels of circulating procoagulant microparticles which are associated with activation of protein C.<sup>2</sup>

### **Endothelial cells and thrombosis**

In general, numerous insults occur in MPN patients, which perturb the integrity of the endothelium, resulting in a pro-adhesive and pro-coagulant EC surface. Over the last decade, increasing evidence has been provided indicating that *JAK2*-mutated MPN EC might also contribute to the MPN pro-thrombotic state.<sup>27,28</sup> This evidence will be reviewed here.

### **Bleeding risk factors in patients with myeloproliferative neoplasms**

Risk factors for developing hemorrhagic events are less well understood. The *JAK2V617F* mutation has not only been related to the rate of thrombosis, but also to the rate of bleeding events.<sup>4</sup> Furthermore, thrombocytopenia due to hypersplenism and/or progressive myelofibrosis may enhance the risk of bleeding.<sup>14</sup> Paradoxically, extreme thrombocytosis is associated with bleeding due to the development of acquired von Willebrand syndrome.<sup>21</sup> The type of MPN also appears to influence the hemorrhagic risk, with an increased incidence being associated with prefibrotic primary myelofibrosis as compared to essential thrombocythemia.<sup>29</sup> In general, the effect of the administration of antiplatelet aggregating agents on bleeding events in MPN patients is debatable. These agents should however be used with caution in patients with extreme thrombocytosis and acquired von Willebrand syndrome, severe thrombocytopenia, or in those receiving oral anticoagulants.

There are several possible factors that contribute to bleeding in MPN patients, including both disease-related factors (e.g., MPN subtypes, thrombocytopenia or extreme thrombocytosis, platelet storage pool defects with a downregulation of glycoproteins (GP)Ib and GPIIb/IIIa and therapy-related factors (e.g., use of antiplatelet and anticoagulant therapies,<sup>27</sup> drug-induced thrombocytopenia due to ruxolitinib, fedratinib, interferon, busulfan or hydroxyurea).

### **Endothelial cell involvement in myeloproliferative neoplasms**

A significant increase in marrow and splenic microvascular density<sup>30</sup> is a characteristic feature of MPN, particularly polycythemia vera and myelofibrosis. Moreover, neo-angiogenesis represents a hallmark of these diseases.<sup>31</sup> Whether neo-angiogenesis in MPN is an epiphenomenon of the MPN pro-inflammatory milieu or a consequence of EC dysregulation due to the same pathogenic mechanism that leads to the hematopoietic cell proliferation<sup>32</sup> remains controversial. It is important to realize that these two mechanisms are not mutually exclusive and could be operating in concert. In addition, increased serum levels of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), have been reported in MPN patients.<sup>33</sup> It has been suggested that autocrine and paracrine signaling pathways lead to increased levels of VEGF, which may not only contribute to accelerated hematopoietic cell growth but may also contribute to the MPN-associated risk of thrombosis.<sup>34</sup>

The increased marrow and splenic microvessel density and neo-angiogenesis, together with the high incidence of vascular complications, has led some authors to hypothesize direct involvement of EC by the malignant process in MPN. The observation that EC and their precursors may harbor the *JAK2V617F* mutation supports this hypothesis.<sup>8,9,12,39,42,45</sup> However, studying the contribution of EC to human disease development is challenging because endothelium cannot, for ethical reasons, be easily sampled from patients. This limitation has meant that most published papers providing support for the above-mentioned hypothesis are based on *in vitro* studies dealing with circulating endothelial progenitors,<sup>32,34,37</sup> and mature

EC.<sup>39</sup> Moreover, some authors demonstrated that monocytes isolated from MPN patients are capable of generating cells that closely resemble EC, the so-called endothelial-like cells or angiogenic monocytes.<sup>38</sup> Both in animal models and humans, angiogenic monocytes contribute to neo-vessel formation while assuming a mature EC phenotype.<sup>38</sup> However, in humans it is currently thought that endothelial-like cells influence angiogenesis by secreting pro-angiogenic factors (paracrine effects), rather than participating directly in neovascularization.<sup>39</sup>

At present, the true origin of mutated EC in MPN patients remains the subject of debate. Where do these cells originate from? Can we be certain of their true EC nature? In the following sections we review the instruments that are presently used to study EC biology in order for the reader to better appreciate the challenges encountered in understanding the origins and consequences of *JAK2*-mutated EC.

### Assays for endothelial cells and endothelial progenitor cells

A growing number of assays have been utilized to study the origins of EC in MPN. It is impossible to evaluate the validity of such data without first understanding the nature of each of these assays as well as their strengths and limitations. We will describe each of the currently used assays below.

Circulating endothelial progenitor cells (EPC) (Table 2) have the capacity to proliferate, migrate, and differentiate into cells belonging to the endothelial lineage, but do not acquire the characteristic features of mature EC. EPC are very rare peripheral blood cells (0.0001% of circulating nucleated cells).<sup>40</sup> In both animal models and humans they have been reported to play a role in vascular repair and neo-angiogenesis.<sup>40</sup> Asahara *et al.*<sup>40</sup> initially reported the isolation of a putative EPC from human peripheral blood, on the basis of cell surface expression of CD34 (expressed by EC, as well as HSC) and Flk-1 (a receptor for VEGF2). These cells were capable of *de novo* blood vessel tube formation. Subsequently, Urbich and Dimmeler<sup>41</sup> defined EPC as progenitors of EC that were capable of

clonal expansion with stem cell-like characteristics and had the capacity to differentiate into EC. Since these initial observations, there has been a great deal of debate concerning the definition and characterization of these progenitor cells. In addition, a variety of methods have been used to detect and characterize EPC, which has led to disparate results.<sup>49</sup> Three main approaches have been used to identify and isolate EPC.

One approach is to identify EPC using surface antigen expression with cytofluorimetry of circulating cells (Table 3). Unfortunately, the presently used cell surface markers, CD34, VEGFR2 (human KDR and mouse Flk-1) and CD133 do not unequivocally identify EPC.<sup>37</sup> This approach allows EPC to be distinguished from mature circulating endothelial cells (CEC), since CD133 is a stem cell marker expressed by EPC but not by mature EC.<sup>45</sup>

A second method of assaying for EPC consists of plating human peripheral blood or cord blood low-density mononuclear cells in culture dishes coated with fibronectin in a commercially available culture medium rich in EC growth factors and fetal calf serum.<sup>44</sup> After 4-5 days the non-adherent cells are removed and the adherent cells are examined for their ability to bind acetylated low-density lipoprotein and *Ulex europaeus* agglutinin 1 (a plant lectin). The putative EPC identified are called circulating angiogenic cells. These markers, however, lack specificity<sup>45</sup> (numerous blood cells express the integrin receptors for fibronectin) and these cells typically do not form EC colonies *in vitro*.<sup>46</sup> EPC identified in this manner are thought to contribute to neo-angiogenesis by secreting angiogenic factors (paracrine route).<sup>46</sup>

The third method to quantitate the numbers of EPC is based on the *in vitro* colony-forming capacity of cultured CD34<sup>+</sup> cells. Two classes of EPC have been described, which are termed colony-forming unit-endothelial cells (CFU-EC) and endothelial colony-forming cells (ECFC). CFU-EC are assayed by plating CD34<sup>+</sup> cells for 48 h in fibronectin-coated dishes and then replating the non-adherent cells and monitoring for the emergence of the EPC-derived colonies. These CFU-EC, however, fail to display any postnatal vasculogenic activity and are thought ultimately to be the cellular progeny of myeloid cells.<sup>45</sup> Since this assay includes the adhesion of mononu-

**Table 2. Main abbreviations referring to endothelial progenitor cells and mature endothelial cells, and brief definitions of the types of cells.**

Abbreviation	Definition
EPC = endothelial progenitor cell	Endothelial progenitors that differentiate into endothelial cells and may become part of the newly formed vessel wall or favor angiogenesis by secretion of pro-angiogenic factors (paracrine effect). There are several <i>ex vivo</i> assays for EPC.
ECFC = endothelial colony-forming cells	Among the EPC, ECFC originate from peripheral blood mononuclear cells and are able to form large colonies of human CD45 <sup>-</sup> cells after 1–3 weeks of incubation (once called late outgrowth endothelial cells, OEC), which have phenotypic and functional properties of endothelial cells. Indeed, they are able to generate new vessels <i>in vivo</i> and to generate endothelial colonies <i>ex vivo</i> , and are now considered the true precursor cells of endothelial cells.
CFU-EC = colony-forming unit-endothelial cells	These are assayed by plating CD34 <sup>+</sup> cells for 48 h in fibronectin-coated dishes and then replating the non-adherent cells and monitoring for the emergence of the EPC-derived colonies. Because of the brief period of incubation <i>ex vivo</i> they were once called early outgrowth endothelial cells, (EOC). They were initially included as endothelial precursors, but they do not possess any postnatal vasculogenic activity and, therefore, are no longer considered true EPC.
CAC = circulating angiogenic cells	Bone marrow-derived immune cell populations (T cells and certain subsets of monocytes) that stimulate vascular regeneration and angiogenesis through a paracrine mechanism.
CEC = circulating endothelial cells	Mature endothelial cells circulating in the peripheral blood, which are shed from vessel walls as a result of pathophysiological conditions that affect the endothelium.
ELC = endothelial-like cells	Monocytes that closely resemble endothelial cells and acquire endothelial cell surface markers.

clear cells *in vitro*, this approach may select for monocytes, expressing “endothelial-specific” markers.<sup>53</sup>

Another assay system identifies outgrowth EC. This assay identifies clonal ECFC capacity of EPC, which form large colonies of human CD45<sup>-</sup> cells after 1-3 weeks of incubation.<sup>45</sup> The cells within these colonies are thought to be of EC origin because of their: EC morphology, expression of EPC/EC-related markers (CD31, CD105, CD144, CD146, VWF, and KDR)<sup>36</sup> and spontaneous formation of human blood vessel tubes *in vitro*<sup>47</sup> and *in vivo* (postnatal vasculogenesis).<sup>48</sup> The ability of ECFC to display spontaneous vasculogenic properties and to remodel into arteries and veins *in vivo* distinguishes ECFC from all other EC precursor or progenitor cell types previously described.<sup>45</sup> ECFC are likely the cell population that represents a true lineage-restricted EC progenitor cell.

### Circulating endothelial cells

CEC are mature differentiated EC that are shed from vessel walls as a result of pathophysiological conditions that affect the endothelium.<sup>49</sup> CEC were first identified in the 1970s although more user-friendly techniques to isolate CEC have only recently become available.<sup>50</sup> Prolonged or exaggerated activation by environmental stress leads to dysfunction and to irreversible loss of EC integrity with cell detachment, apoptosis and necrosis, which results in greater EC turnover and increased CEC levels in peripheral blood.<sup>50</sup>

CEC were initially identified using morphological criteria. Subsequently, objective methods to identify CEC with the application of immunofluorescence, and the use of antibodies against various EC markers, were introduced although these efforts have been hampered by the lack of reliable cell-specific markers.<sup>51</sup> Recently a consensus definition of CEC has been reached,<sup>52</sup> according to which CEC are large (>10 µm in length) CD146<sup>+</sup> cells. CD146 (MUC18) is expressed by CEC but not by monocytes, granulocytes, platelets, megakaryocytes, T or B lympho-

cytes.<sup>55</sup> A battery of markers is now used to identify cells of endothelial origin, including CD31, CD105, and CD141.<sup>54</sup> Notably, the absence of CD133 may also be used to distinguish CEC from EPC.<sup>55</sup> Currently, CEC can also be isolated by immunomagnetic selection (CD146<sup>+</sup> cells) or by flow cytometry. Notably, in 2008, Widemann *et al.*<sup>49</sup> reported a hybrid assay that incorporated an algorithm combining immunomagnetic selection of CD146<sup>+</sup> cells with flow cytometric quantification. In parallel, Terstappen's group<sup>56</sup> developed a semi-automatic method for the detection of CEC, also using a combination of iron microbeads and monoclonal antibodies. These assays overcome the lack of standardization and the variability in CEC detection associated with the methods previously described. Moreover, the true endothelial nature of the CEC obtained using this technology was confirmed by gene expression profiling studies.<sup>57</sup>

In healthy individuals, the endothelial layer lining blood vessels is continuously being renewed at a low replication rate of 0-1% per day since normal laminar flow suppresses EC apoptosis. CEC are rare cells, with as few as 0-10 CEC/mL being observed in healthy donors.<sup>58</sup> By contrast, elevated levels of CEC have been reported in patients with various types of diseases, including cardiovascular,<sup>59</sup> infectious,<sup>60</sup> and immune disorders,<sup>61</sup> diabetes, chronic kidney disease,<sup>62</sup> after hematopoietic stem cell transplantation,<sup>63</sup> and cancer.<sup>64</sup> Several pioneering studies have shown that raised CEC levels are also associated with specific tumor types, stage and prognosis,<sup>65</sup> and can be used to monitor responses to chemotherapy.<sup>66</sup> In addition, CEC have been proposed as a non-invasive marker of angiogenesis.<sup>67</sup> In contrast to EPC, which are a proposed marker of regeneration and vessel proliferation, CEC serve as a marker of endothelial damage/dysfunction and reflect a pro-thrombotic tendency.<sup>68</sup> Notably, the numbers of CEC are increased in MPN patients, regardless of their driver mutational status,<sup>69</sup> highlighting the involvement of endothelium in these chronic hematologic neoplasms.

CEC may provide a means to study mature EC that avoids laser microdissection or the limitations associated with performing the tedious and time-consuming EPC assays. However, a consensus on CEC phenotype and the origin of these cells is lacking and the possibility that EC or endothelial-like cells originate from monocytes remains.

**Table 3. Biological characteristics and immunophenotype of endothelial progenitor cells and circulating endothelial cells.**

	CAC	EPC CFU-EC	ECFC	CEC
Immunophenotype	CD34 <sup>+</sup> / <sup>-</sup> * VEGFR2 <sup>+</sup> * CD133 <sup>+</sup> CD31 <sup>+</sup> CD146 <sup>-</sup> CD45 <sup>+/-</sup>	CD34 <sup>+</sup> * VEGFR2 <sup>+</sup> * CD133 <sup>+</sup> CD31 <sup>+</sup> CD146 <sup>-</sup> CD45 <sup>+/-</sup>	CD34 <sup>+</sup> * VEGFR2 <sup>+</sup> * CD133 <sup>+</sup> CD31 <sup>+</sup> CD146 <sup>-</sup> CD45 <sup>-</sup>	CD34 <sup>+</sup> * VEGFR2 <sup>+</sup> * CD133 <sup>-</sup> CD31 <sup>+</sup> CD146 <sup>+</sup> CD45 <sup>-</sup>
Origin	BM	BM	EC?/BM?	EC
Proliferative capacity	-	-	+	-/+
Replating ability	-	-	+	-/+
<i>In vitro</i> tube formation	+/-	+/-	+	-
<i>In vivo de novo</i> formation	-	-	+	-
Paracrine augmentation of angiogenesis	+	+	+/-	NA
Phagocytosis of bacteria	+	+	-	-

\*In common with hematopoietic stem cells. The main differences between CEC and EPC are shown in red. EPC: endothelial progenitors cells; CEC: circulating endothelial cells; CAC: circulating angiogenic cells; CFU-EC: colony-forming unit-endothelial cells; ECFC: endothelial colony-forming cells; VEGFR2: vascular endothelial growth factor receptor 2; BM: bone marrow; EC: endothelial cells; NA: not applicable.

### JAK2V617F-positive endothelial cells in patients with myeloproliferative neoplasms (Figure 1)

In 2009 Sozer *et al.*<sup>8</sup> reported that mature EC captured by laser microdissection from the lumen of hepatic venules harbored the JAK2V617F mutation in three MPN patients with Budd-Chiari syndrome (Figure 1, on the right). Rosti *et al.* further confirmed the presence of JAK2V617F in micro-laser dissected EC from the splenic vein in MPN patients, but absence of the driver mutation in the ECFC residing in the spleen<sup>9</sup> (Figure 1). Assayable MPN CFU-EC<sup>11,45,70</sup> were first shown to be JAK2V617F<sup>+</sup> while ECFC from these same patients were found to be JAK2V617F<sup>-</sup> (Figure 1). Only 3% of the ECFC colonies analyzed by Yoder *et al.*<sup>45</sup> were JAK2V617F<sup>+</sup>. Interestingly, these mutated-ECFC were derived from the same patient, who presented with a thrombotic event and only later

developed classic hematologic signs of polycythemia vera. Notably, increased numbers of both CFU-EC<sup>32,34,70</sup> and ECFC<sup>71</sup> have been found in the blood of patients with MPN, regardless of their mutational status. The absence of the *JAK2* mutation in ECFC from MPN patients was recently confirmed by Guy and colleagues.<sup>13</sup> Teofili *et al.*<sup>12</sup> however, reported that ECFC from patients with MPN were *JAK2V617F*<sup>+</sup> (Figure 1). Almost half of the MPN patients studied were reported to have MPN-like genetic abnormalities in their ECFC, including either *SOCS* gene hypermethylation or the presence of *JAK2V617F*. Notably, mutated ECFC were detected only in patients with a history of thrombotic events.<sup>12</sup> Moreover, the presence of *JAK2V617F* or other evidence of clonality in ECFC was associated with JAK/STAT pathway activation and significantly greater adhesion of mononuclear cells to mutated EC than normal ECFC.<sup>12</sup> These reports support the hypothesis that EC and HSC may derive from a common progenitor cell, the "hemangioblast",<sup>72</sup> which results in mutated EC and myeloid cells in a subpopulation of patients with MPN. It must be said, however, that conclusive evidence unequivocally demonstrating the existence of the "hemangioblast" *in vivo* in higher vertebrates is lacking. Indeed, most of the published studies have been largely based on experiments that relied on the isolation, culture, and/or manipulation of cells *in vitro*,<sup>72,73</sup> while various fate-mapping studies in the mouse, chick, and zebrafish have led to contradictory conclusions.<sup>74,75</sup> Fate mapping in the zebrafish gastrula has indicated that the "hemangioblasts" are interspersed with hematopoietic and endothelial progenitors in the ventral-lateral mesoderm.<sup>76,77</sup> In contrast, several other studies have suggested

that endothelial and hematopoietic lineages are independently derived from mesodermal cells.<sup>76,79</sup>

The discovery that MPN patients may share the *JAK2V617F* driver mutation has shed new light on this hypothesis. Moreover, some authors recently suggested that *JAK2V617F*, along with other myeloid malignancy-associated gene mutations, may be detected in CEC<sup>80</sup> and HSC in patients with primary myelofibrosis. The concordance between mutations in HSC and CEC may further support the hypothesis of a common progenitor that generates these two subpopulations, but peer-reviewed studies are still required to confirm this hypothesis. Regardless of the presence of a common precursor, each of these observations supports the hypothesis that mutated EC in MPN represent a "neoplastic" vascular niche,<sup>81</sup> which allows blood cell adhesion and tumor cell growth, as demonstrated using *in vitro* and *in vivo* assays.

### Impact of *JAK2V617F* endothelial cells on hematopoiesis and vascular complications in myeloproliferative neoplasms (Figure 2)

#### *In vivo* and *in vitro* models

The observation that EC from some MPN patients were *JAK2V617F*<sup>+</sup> stimulated the performance of additional studies exploring the possible functional consequences of *JAK2*-mutated EC.

Etheridge *et al.*<sup>82</sup> first described the critical role of *JAK2V617F*-mutated EC in the development of bleeding abnormalities using murine models. They used *FF1* transgenic mice to express *JAK2V617F* in different cell lineages. In their model *JAK2V617F* was exclusively present in

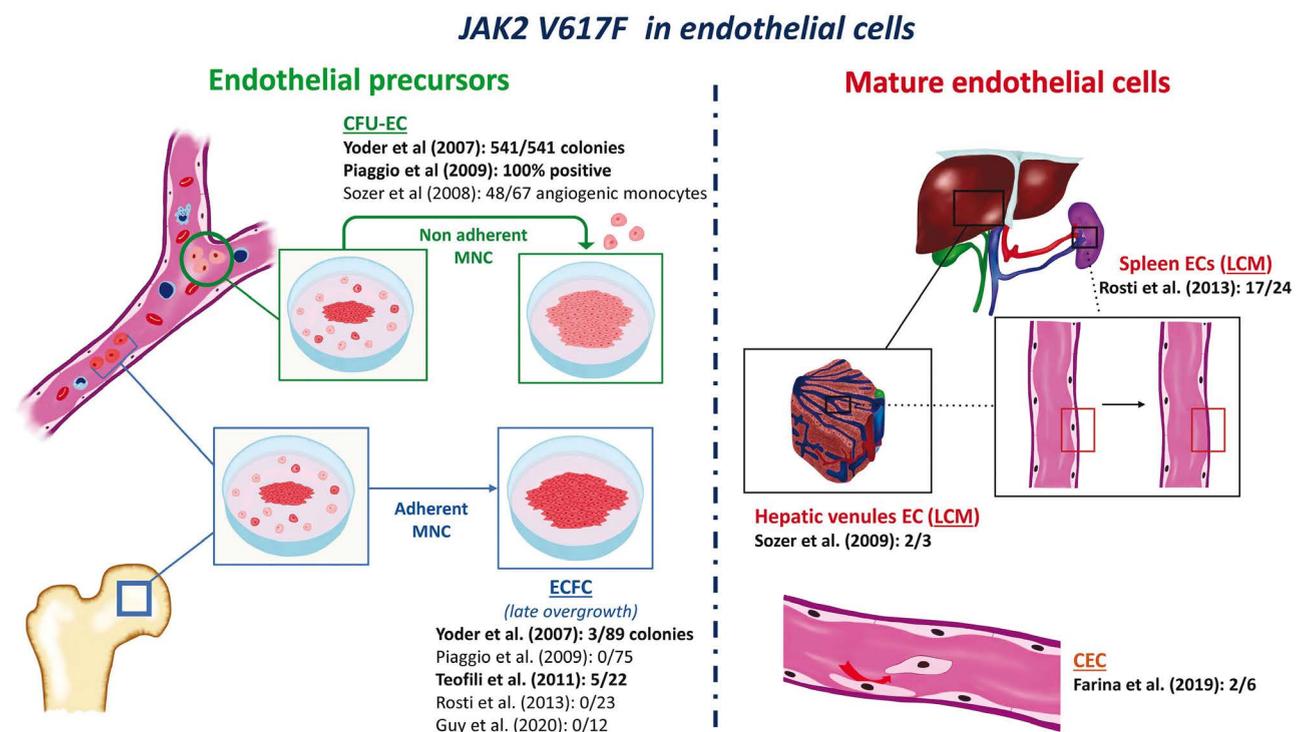
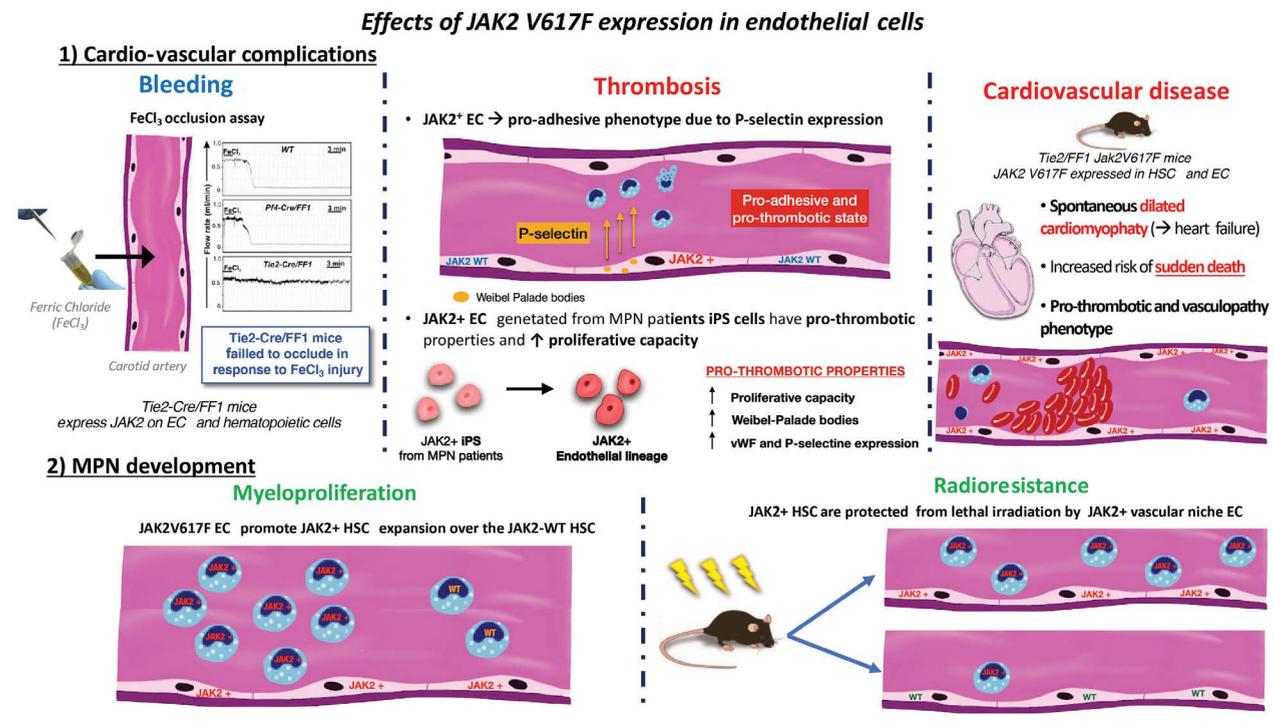


Figure 1. Evidence for *JAK2V617F* mutated endothelial cells in patients with myeloproliferative neoplasms. *JAK2V617F* has been detected in both endothelial progenitors and mature endothelial cells. Studies in which a *JAK2* mutation was detected in endothelial progenitor cells or mature endothelial cells are shown in bold. Positive colonies or patients are expressed on the total number of colonies or patients analyzed. CFU-EC: colony forming unit-endothelial cells, derived from non-adherent mononuclear cell culture (see the text); MNC: mononuclear cells; EC: endothelial cells; ECFC: endothelial colony-forming cells, derived from long-term adherent MNC culture (see the text); LCM: laser-capture microdissection; CEC: circulating endothelial cells; Drawn by C. Luzzani, Medical School, University of Milan

EC, and the mice were characterized by dysfunctional hemostasis in response to injury, resembling the bleeding diathesis observed in MPN patients.<sup>82</sup> One of the potential mechanisms proposed by Etheridge and colleagues was related to von Willebrand factor (VWF) regulation. More recently, using both an *in vitro* model of human *JAK2V617F*-mutated EC and an *in vivo* model of mice with endothelial-specific *JAK2V617F* expression, Guy *et al.*<sup>27</sup> have shown that *JAK2V617F*<sup>+</sup> EC in the absence of similarly mutated hematopoietic cells are associated with a higher rate of thrombosis due to a pro-adhesive phenotype as a result of increased endothelial P-selectin exposure, secondary to degranulation of Weibel-Palade bodies.<sup>27</sup> Interestingly, these mice displayed a higher propensity for thrombosis in spite of having normal blood counts and normal rates of thrombin generation.<sup>27</sup> In contrast, their EC were characterized by increased surface expression of P-selectin and VWF, both of which are contained within Weibel-Palade bodies. Moreover, the thrombotic tendency was accentuated by the creation of a pro-inflammatory milieu due to the administration of low doses of tumor necrosis factor- $\alpha$ .<sup>27</sup> Furthermore, the pro-adhesive properties of the *JAK2V617F*-mutated EC were reversed by treatment with either a P-selectin blocking antibody or hydroxyurea.<sup>27</sup> In addition, Poisson *et al.* showed an increased degree of arterial constriction in response to agents that promote vasoconstriction in mice with *JAK2V617F*<sup>+</sup> HSC and EC.<sup>83</sup>

Castiglione *et al.*<sup>84</sup> have reported that when *JAK2V617F* was expressed by both hematopoietic cells and EC in a murine model of MPN, the mice developed an MPN phe-

notype and a spontaneous age-related dilated cardiomyopathy with an increased risk of sudden death as well as a pro-thrombotic and vasculopathic phenotype. In contrast, mice expressing solely *JAK2V617F* in blood cells did not demonstrate any evidence of cardiac dysfunction or thrombosis, suggesting that expression of the MPN driver mutation in EC is required for the development of the cardiovascular disease phenotype. Moreover, the authors demonstrated that the *JAK2V617F*<sup>+</sup> EC were associated with the development of a pro-inflammatory milieu. Finally, *JAK2*-mutated EC have been reported to respond to shear flow in a different manner than wild-type EC, leading to upregulation of EC adhesion molecules (platelet endothelial cell adhesion molecule and E-selectin). Guadall *et al.*<sup>28</sup> have provided additional evidence that *JAK2V617F*<sup>+</sup> EC possess pro-thrombotic properties. Using *JAK2V617F*<sup>+</sup> and *JAK2* wild-type induced pluripotent stem cells generated from an MPN patient and redirecting these cells towards the endothelial lineage, the authors observed that *JAK2V617F*<sup>+</sup> EC had a greater proliferative capacity compared with wild-type EC. The numbers and fluorescence intensity of Weibel-Palade bodies as well as the expression of VWF and P-selectin were significantly greater and these effects were accompanied by greater accumulation of P-selectin at the cell surface of *JAK2V617F*<sup>+</sup> EC than wild-type EC. The transcriptomic profile of these mutated cells revealed overexpression of transcripts for genes that are involved in inflammation and cell adhesion, extracellular matrix regulation, the generation of glycoproteins, and a variety of processes that occur in venous stenosis and thrombosis.



**Figure 2.** Effects of *JAK2V617F* expression in endothelial cells. The presence of the *JAK2V617F* mutation in endothelial cells (EC) has an impact on both (1) vascular complications and (2) the development of myeloproliferative neoplasms (MPN). Specifically, it affects bleeding<sup>82</sup> (the carotid arteries of Tie2-Cre/FF1 mice expressing *JAK2* mutations on both EC and hematopoietic stem cells (HSC) failed to occlude in response to ferric chloride, which normally induces occlusive thrombosis in murine carotid arterial); thrombosis (both in a mice model, due to enhanced P-selectin expression,<sup>27</sup> and in an *in vitro* model of induced pluripotent stem cells<sup>28</sup>) and cardiovascular disease (mice expressing *JAK2V617F* had spontaneously dilated cardiomyopathy and an increased risk of sudden death<sup>84</sup>). Finally, *JAK2*-mutated EC affect MPN development, promoting *JAK2* HSC expansion,<sup>85</sup> and radioresistance.<sup>88</sup> FeCl<sub>3</sub>: ferric chloride; EC: endothelial cells; MPN: myeloproliferative neoplasms; iPS: induced pluripotent stem cells; HSC: hematopoietic stem cells; WT: wild-type.

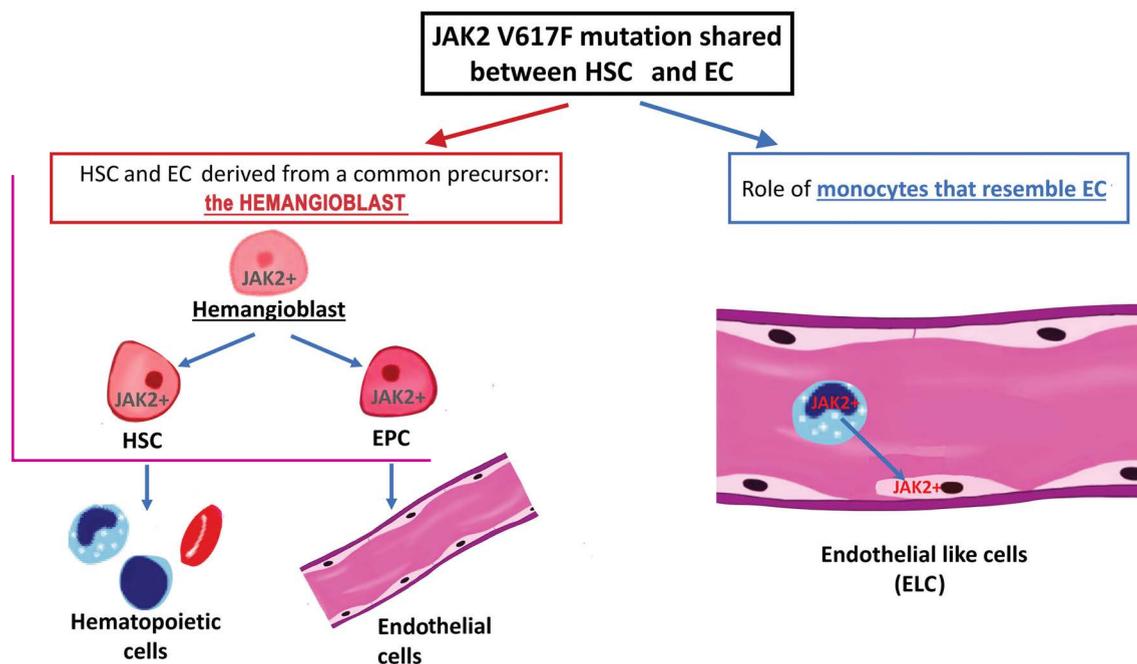


Figure 3. Possible origin of *JAK2*-mutated endothelial cells. The documentation of *JAK2* myeloproliferative neoplasm driver mutations in myeloid cells and endothelial cells (EC) suggests that in some individuals both cell types originate from a “hemangioblast”, which might serve as the cell of origin for myeloproliferative neoplasms during embryogenesis. On the other hand, *JAK2*-positive EC may derive from monocytes that resemble EC (endothelial-like cells) as well. EC: endothelial cells; EPC: endothelial progenitor cells; HSC: hematopoietic stem cells; ELC: endothelial-like cells.

### Effects of *JAK2V617F*-positive endothelial cells on hematopoiesis

*JAK2V617F*<sup>+</sup> EC have also been shown to contribute not only to thrombo-hemorrhagic events, but also to MPN-associated myeloproliferation.<sup>85</sup> *JAK2V617F*-bearing EC have been reported to promote the proliferation of *JAK2*-mutated hematopoietic progenitor/stem cells over *JAK2* wild-type ones *in vitro*. This proliferative advantage has been hypothesized to be due to activation of the thrombopoietin/MPL signaling axis.<sup>85</sup> Subsequently, Zhan *et al.* provided *in vivo* evidence that *JAK2V617F*<sup>+</sup> vascular niche cells promote *JAK2V617F*<sup>+</sup> myeloid cell expansion, while inhibiting *JAK2* wild-type hematopoiesis. Zhan *et al.* also reported that *JAK2V617F*<sup>+</sup> HSC transplanted into wild-type recipient mice were incapable of developing an MPN phenotype in the absence of *JAK2V617F*<sup>+</sup> vascular niche EC. Therefore, in this model MPN myeloproliferation requires *JAK2V617F* expression by both HSC and EC.<sup>86</sup> However, there is evidence from mouse models indicating that the presence of the *JAK2* mutation in HSC alone is sufficient to induce an MPN.<sup>87</sup>

In support of this role of mutated EC in MPN hematopoiesis, Lin and colleagues reported that *JAK2V617F*<sup>+</sup> HSC were protected from lethal doses of irradiation by *JAK2V617F*<sup>+</sup> vascular niche EC.<sup>88</sup> These authors hypothesized that the relative resistance of MPN to radiation-based conditioning regimens used prior to allogeneic stem transplantation could be due to the presence of *JAK2V617F*<sup>+</sup> EC within the patient’s bone marrow HSC vascular niche.<sup>88</sup>

### Monocytes can assume the identity of endothelial cells

Notably, some authors have reported that monocytes

isolated from MPN patients resemble endothelial-like cells, accounting for the detection of MPN driver mutations in EC and hematopoietic cells (Figure 3). Leibundgut *et al.*<sup>32</sup> initially reported that CD14<sup>+</sup> monocytes were capable of generating *JAK2V617F*<sup>+</sup> EC *in vitro*. Subsequently, Sozer and colleagues<sup>10</sup> showed that human CD34<sup>+</sup> cells, too, were capable of generating normal and *JAK2V617F*<sup>+</sup> endothelial-like cells *in vivo*. These reports suggest that *JAK2*-mutated CD34<sup>+</sup> cells and CD14<sup>+</sup> monocytes (both elevated in MPN) may both transform to *JAK2V617F*<sup>+</sup> endothelial-like cells. These observations have led to considerable confusion, suggesting to some investigators that monocytes can transition to EPC<sup>46</sup> and then acquire an endothelial-like phenotype. However, a more plausible hypothesis is that monocytes can serve as circulating regulators of the angiogenic response and play a crucial role in neo-angiogenesis during wound healing, tissue ischemia, and tumorigenesis by secreting pro-angiogenic factors rather than by directly participating in neo-vessel formation or endothelial turnover.<sup>39,89</sup>

### Do endothelial cells and hematopoietic stem cells share a common precursor cell in patients with myeloproliferative neoplasms?

HSC and EC are both derived from the mesodermal layer during fetal development. Some authors have speculated that they may be derived from a common precursor cell, termed a “hemangioblast”. The term “hemangioblast” was initially coined by Murray in 1932,<sup>90</sup> referring to a mass of cells derived from the primitive streak mesoderm containing both endothelial and blood cells. This term was meant to complement and contrast with the term “angioblast,” which was thought to be the source of vessels and endothelium.<sup>91</sup> During the late

1990s, the concept of the “hemangioblast” was developed, based on observations that single mesodermal cells isolated from mice had the potential to generate both blood cells and EC.<sup>92</sup>

Interestingly, in many species HSC appear as clusters attached to the endothelium that lines the ventral wall of the abdominal aorta during embryonic development; this observation has long implicated the hemogenic endothelium as the source of developing blood cells. Indeed, when EC isolated from mouse embryos are grown in culture, the hemogenic endothelium possesses the potential to develop into mature blood cells.<sup>93</sup> During development this hemogenic endothelium, gives rise to HSC/hematopoietic progenitor cells that seed the fetal liver and the adult bone marrow.<sup>94</sup> Lineage-tracing markers in mice have identified that definitive HSC arise in the aorta-gonad-mesonephric region of embryos from hemogenic endothelium which gives rise, by asymmetric division, to resident EC and HSC that are released into the blood and then colonize the liver.<sup>95</sup> Peault’s team subsequently described the presence of definitive HSC in the aorta-gonad-mesonephric region of human embryos which were capable of colonizing adult xenografts and reported that definitive HSC were derived from hemogenic endothelium resembling those observed in mouse embryos.<sup>96</sup> The relationship between HSC and hemogenic endothelium has been further clarified,<sup>94</sup> based on continuous single-cell imaging which indicated that freely moving cells expressing blood-specific markers (CD45, CD41, CD11b) were generated from EC expressing vascular endothelial cadherin (VE-cadherin, also known as Cdh5).<sup>97</sup>

The reports discussed above showing that the *JAK2V617F* driver mutation<sup>8,9,11,12,37,98</sup> and other myeloid-associated genes mutations<sup>90</sup> may be present in both hematopoietic cells and EC in MPN patients have reinforced the evidence supporting the existence of a common precursor cell for both EC and hematopoietic cells. In addition, some authors have recently provided evidence that *JAK2V617F* may be acquired *in utero*<sup>99</sup> or during childhood<sup>100</sup> by MPN patients in whom *JAK2V617F* was the only or the first driver mutation. This finding indicates that the acquisition of *JAK2V617F* in MPN patients can occur *in utero* and is at least chronologically consistent with involvement of the “hemangioblast” by MPN driver

mutations (Figure 3). Since the period when EC are hemogenic may be very brief and occurs very early during embryogenesis, the “hemangioblast” may acquire the MPN driver mutation in only a limited group of patients. These assumptions would support the observation that not all *JAK2V617F* MPN patients possess mutated EC.

## Conclusions

The findings summarized here indicate that mutated EC play multiple roles in the development of the clinical phenotype of MPN (Figures 2 and 3). The interaction between EC and MPN HSC creates microenvironmental niches which promote the predominance of the malignant MPN myeloid cells at the expense of the normal HSC. In addition, the documented MPN driver mutations in myeloid cells and EC suggest that in some individuals both cell types originate from a “hemangioblast” present during fetal development or which persists during adult life, and serves as the cell of origin of MPN. Further investigation using single-cell analysis of the putative MPN “hemangioblast” will be required to further confirm this hypothesis. A significant body of evidence indicates that *JAK2V617F*<sup>+</sup> EC contribute to the thrombotic and bleeding tendencies of MPN patients. Additional work will also be required to assess the relative contribution of monocytes that resemble EC and mutated EPC to the prothrombotic MPN milieu. A likely scenario is that the contribution of these two types of EC to the prothrombotic tendency in MPN varies from patient to patient and may be determined in part by the vascular beds in which the thrombotic events occur.

## Disclosures

No conflicts of interest to disclose.

## Contributions

MF and RH conceived and wrote the manuscript. DR wrote the manuscript. All the authors approved the final version.

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