

# JAK2V617F-Positive Endothelial Cells Induce Apoptosis and Release JAK2V617F-Positive Microparticles

## JAK2V617F-Pozitif Endotel Hücreleri Apoptozu İndükler ve JAK2V617F-Pozitif Mikropartikül Salınımı Yapar

© Hilal Hekimoğlu<sup>1,2</sup>, © Selin Fulya Toprak<sup>1,2</sup>, © Selçuk Sözer<sup>1</sup>

<sup>1</sup>Istanbul University, Aziz Sançar Institute of Experimental Medicine, Department of Genetics, Istanbul, Turkey

<sup>2</sup>Institute of Health Sciences, Istanbul University, Istanbul, Turkey

### Abstract

**Objective:** Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) have a high propensity for thrombosis, which has been attributed to increased blood counts, endothelial cell (EC) dysfunction, and inflammation. The presence of the JAK2V617F mutation in the ECs of MPN patients has been confirmed, but the consequences of EC involvement by JAK2V617F in the pathogenesis of thrombosis are unclear. Endothelial microparticles (EMPs) released from ECs play an important role in endothelial dysfunction and also in the intercellular exchange of biological signals and information. Several studies have revealed that patients with JAK2V617F and a thrombosis history have increased numbers of MPs in their circulation.

**Materials and Methods:** The current study utilized a lentiviral transduction model of JAK2 wild type (JAK2wt) or JAK2V617F encoding green fluorescent protein (GFP) into human umbilical vein ECs to determine the effect of JAK2V617F on ECs. EC infected with JAK2V617F, JAK2WT, and only-GFP were tested after two days of culture.

**Results:** The proteins of ECs that potentially play a role in the development of thrombosis, including endothelial protein C receptor, thrombomodulin, and tissue factor, were detected by flow cytometry analysis with no statistical significance. Increased annexin-V uptake of JAK2V617F and JAK2wt ECs compared to GFP-alone ECs was detected. The EMP production in the supernatants of the EC culture was investigated. Genotyping of the EMPs revealed the presence of genomic DNA and RNA fragments in EMP cargos. JAK2V617F-positive DNA was detected in EMPs released from JAK2V617F-infected ECs and EMPs were shown to carry the genotype of the cell of origin.

**Conclusion:** JAK2V617F-positive EMPs were shown for the first time in the literature. This novel research provides the first evidence that EMPs might regulate neighboring and distant cells via their cargo materials. Thus, the direct effect of JAK2V617F on ECs and their functions suggests that different mechanisms might play a role in the pathogenesis of thrombosis in MPNs.

**Keywords:** JAK2V617F, Endothelial cells, Microparticles, Extracellular genomic materials

### Öz

**Amaç:** Philadelphia kromozom negatif miyeloproliferatif neoplazi'ler (MPN), yüksek tromboz riski taşıyarak inflamasyon, endotel hücre (EH) disfonksiyonu ve artan kan sayıları ile karakterize edilirler. Önceki çalışmalarda, MPN hastalarının EH'de JAK2V617F mutasyonunun varlığı tespit edilmiştir. Ancak tromboz patogeneğinde JAK2V617F mutasyonu taşıyan EH'in rolü belirsizdir. EH'den salınan endotel mikropartikülleri (EMP'ler) biyolojik sinyallerin ve hücreler arası bilginin değişiminde ve endotel disfonksiyonunda önemli rol oynarlar. Birçok çalışmada, JAK2V617F mutasyonu taşıyan ve tromboz öyküsü olan hastaların dolaşımlarında artan sayıda mikropartikül olduğunu ortaya konulmuştur.

**Gereç ve Yöntemler:** Çalışmamızda, JAK2V617F'nin EH üzerindeki etkisini belirlemek için yeşil fluoresan proteini (GFP) ile işaretli JAK2 yabanıl tip (JAK2WT) veya JAK2V617F'li lentiviral transdüksiyon modeli kullanılarak insan göbek kordonundan elde edilmiş endotel hücre hattı (HUVEC) enfekte edildi. JAK2V617F, JAK2WT ve sadece-GFP ile enfekte EH'ler iki gün süren kültür sonrasında test edildi.

**Bulgular:** Akım ölçer analizinde, Endotelial protein C reseptörü, trombomodulin ve doku faktörü dahil olmak üzere tromboz gelişiminde potansiyel rol oynayan EH proteinlerinde istatistiksel anlamlılık olmadığı tespit edildi. JAK2V617F ve JAK2WT enfekte EH'de annexin-V alımının sadece-GFP enfekte EH'ye kıyasla arttığı tespit edildi. EH kültüründen elde edilen süpernatantlarda EMP üretimi araştırıldı. EMP genotiplemesinde EMP kargolarında genomik DNA ve RNA fragmanları varlığı tespit edildi. JAK2V617F enfekte EH'den salınan EMP'lerde JAK2V617F pozitif DNA tespit edildi ve EMP'lerin köken aldıkları hücre genotipini taşıdığı gösterildi.

**Sonuç:** Bu çalışma ile literatürde ilk defa, JAK2V617F pozitif EMP'ler gösterilmiştir. Bu yeni araştırma, EMP'lerin taşıdıkları kargolar aracılığıyla komşu ve uzaktaki hücreleri düzenleme potansiyeli olduğunu göstermektedir. Bu sayede JAK2V617F'nin EH ve işlevleri üzerindeki direkt etkisi ile MPN'lerde tromboz patogeneğinde farklı mekanizmaların rolü olduğunu ileri sürer.

**Anahtar Sözcükler:** JAK2V617F, Endotel hücreler, Mikropartiküller, Ekstraselüler genomik materyaller



## Introduction

Endothelial cells (ECs) are fundamental components of vascular biology, providing hemostasis for the regulation of angiogenesis, coagulation, and inflammation [1]. Endothelial dysfunction usually occurs by apoptosis, leading to increased proinflammatory and prothrombotic properties of the vascular network [2,3,4]. Thrombosis has been shown as the most important cause of mortality among patients with myeloproliferative neoplasms (MPNs) [5,6]. The high incidence of vascular and thrombotic complications in both venous and arterial systems in patients with Philadelphia chromosome-negative MPNs provides evidence for the possible role of the endothelium in MPNs [7].

The discovery of the JAK2V617F mutation in ECs [8,9] led many researchers to examine the factors that have roles in EC structure and function and are associated with the pathobiology of MPN thrombosis [10,11,12,13]. Although the presence of the JAK2V617F mutation in ECs was not revealed to disrupt the integrity of ECs in MPNs, several studies have documented that vessel wall endothelium, which works as an anticoagulant in healthy cells, becomes procoagulant in MPNs [14], increasing the propensity for thrombosis [15]. Thus, the effect of the JAK2V617F mutation on the functional properties of ECs becomes a critical indicator of their involvement in thrombosis and detrimental aspects in MPN prognosis [7,16,17]. Many studies have been conducted to reveal possible factors that cause the increased propensity to thrombosis in MPNs, but the causes of morbidity and mortality in this group of diseases remain elusive.

Extracellular genomic materials (EGMs) are a group of molecules characterized by DNA-, RNA-, and DNA/RNA-related molecules released from all types of cells into the extracellular region [18]. Microparticles (MPs) are a type of EGM released into the extracellular region from varying types of cells in response to cellular activation elicited by a variety of stimuli, such as inflammation, oxidative stress, apoptosis, and mechanical/hemodynamic fluctuations contingent upon the host cell [19,20,21,22]. During stimulation, the host cell's intracellular concentration of calcium increases, which affects the activation of calcium concentration-dependent enzymes, such as gelsolin, aminophospholipid translocase, floppase, calpain, and scramblase [21,23]. MPs are small vesicles ranging in size from 0.1 to 1  $\mu\text{m}$  and they do not have a nucleus [22,24]. The cell of origin may transfer some of its components and contents to MPs, including genetic materials and proteins [25]. For example, ECs release MPs to the extracellular region that contain endothelial proteins, such as platelet EC adhesion molecule 1 (PECAM-1), intercellular adhesion molecule 1,  $\alpha\text{v}\beta\text{3}$  integrin, and VE-cadherin, as surface markers that are usually related to ECs [26,27]. These MPs are referred to as endothelial MPs (EMPs) [27].

This process mediates cell-to-cell communications, particularly the activation, phenotypic modification, and reprogramming of selected target cells [2]. In this case, EMPs affect neighboring and distant cells through the transfer of membrane-associated receptors, releasing directly active proteins, exchanging genetic information, or inducing the adaptive immune response [28].

The role of MPs in disorders of hemostasis and thrombosis has been widely revealed [29]. In addition, the presence of phosphatidylserine (PS) and tissue factor (TF) in the EMP membrane might be a reason for their procoagulant properties [30]. EMPs that carry TF bind to monocytes, causing further TF expression and resulting in enhanced transmigration of monocytes through the endothelial junction [31,32]. Elevated numbers of MPs in plasma have also been reported in numerous prothrombotic conditions [29,33] and patients with MPN and thrombosis history [34,35,36,37,38,39,40,41]. Patients with essential thrombocythemia had higher numbers of circulating MPs with platelet and endothelial markers, suggesting an ongoing platelet and endothelial activation [35]. A recent study also demonstrated that erythrocyte-derived microvesicles induce arterial spasms in JAK2V617F MPNs [42]. To date, however, no study has revealed the presence of JAK2V617F in EMPs as part of their cargo or the direct effect of JAK2V617F-mutated ECs on EMPs.

In this study, we utilized a lentivirus-mediated induction of EC with JAK2 wild type (JAK2wt) and JAK2V617F plasmids and analyzed JAK2wt-positive and JAK2V617F-positive ECs. We demonstrated not only that JAK2V617F ECs produce EMPs but also that these EMPs contain RNA and genomic DNA with the JAK2V617F mutation in their cargo. These findings might provide some insights into the propensity to thrombosis in cases of MPNs.

## Materials and Methods

### Cell Lines

The derivative of the human embryonic kidney 293 cell line containing the SV40 T-antigen (HEK 293T) and human umbilical vein ECs (HUVECs) were purchased from the ATCC (Manassas, VA, USA). HEK 293T and HUVECs were cultured in 10% fetal bovine serum and 1% penicillin/streptomycin with Dulbecco's modified Eagle medium.

### Lentiviral Transduction of JAK2wt and JAK2V617F in ECs

Detailed information about the method for and confirmation of the in vitro model for genetically modified ECs with lentiviral plasmids pCDF1-GFP, pCDF1-JAK2wt-GFP, and pCDF1-JAK2V617F-GFP and helper vectors pCI-VSVG and pCPRDenv has been reported elsewhere [43]. The lentiviral plasmids, which were feline immunodeficiency virus-based

(System Biosciences, Palo Alto, CA, USA), were a generous gift from Dr. Camelia Iancu-Rubin from the Icahn School of Medicine at Mount Sinai (New York City, NY, USA). The ECs that were genetically modified with the expression vectors pCDF1-GFP, pCDF1-JAK2wt-GFP, and pCDF1-JAK2V617F-GFP are referred to here as GFP-alone, JAK2wt, and JAK2V617F, respectively.

### Cell Surface Analysis and Sorting of Genetically Modified ECs

After 72 h of incubation at 37 °C in 5% CO<sub>2</sub> with ≥95% humidity with the lentiviruses, ECs were detached with 0.25% trypsin-EDTA (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Following centrifugation, the collected cells were resuspended in 500 µL of buffer containing phosphate-buffered saline (PBS) with 7.5% bovine serum albumin + 0.5 M EDTA and labeled with endothelial protein C receptor (EPCR), also known as activated protein C receptor (CD201-PE, BD Pharmingen, San Diego, CA, USA), and thrombomodulin (TM) (CD141-PE, BD Pharmingen) and tissue factor (TF) (CD142-APC, BioLegend, San Diego, CA, USA). From each antibody, 5 µL was added separately to each genetically modified EC sample. After 30 min of incubation with the antibodies at room temperature, the ECs were washed twice with buffer and acquired on a FACSCalibur instrument (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). Analysis was performed with a GFP-positive gate if the percentage of GFP-positive cells was ≥50%.

### Cell Cycle Assay

Immunofluorescent staining of incorporated 5-bromo-2'-deoxyuridine (BrdU) with newly synthesized genomic DNA during the S-phase of mitosis and flow cytometric analysis of genetically modified ECs were performed for proliferation, cell cycle, and apoptosis. The stain, coupled with 7-amino-actinomycin D (7-AAD), binds to the total DNA to help with the identification and characterization of ECs. A BrdU Flow Kit (BD Pharmingen) was used according to the manufacturer's instructions. Briefly, 31 µL of BrdU was dissolved in 1 mL of EC culture medium, and 6 µL of this solution was added to each sample of genetically modified ECs per well in a 24-well plate for 2 h.

Following cell detachment with 0.25% trypsin-EDTA, the staining protocol was applied to the cells. The incorporated BrdU was stained with a specific anti-BrdU fluorescent antibody, allophycocyanin (APC), for detection. Two separate sets of experiments were then performed. The DNA histograms of the cells and each phase of the cell-cycle gating strategies have been explained elsewhere [44]. The cell-cycle phases were identified in the DNA histograms and respective contour plots. Analysis was performed based on the percentage of proliferating cells by BrdU-positive cells and the percentage of cells in each cell-cycle phase by 7-AAD fluorescence distribution.

### Apoptosis Analysis

The apoptosis activity of the genetically modified ECs was evaluated by labeling with annexin-V-PE (BD Pharmingen) and 7-AAD (BD Biosciences, Fremont, CA, USA) according to the manufacturer's instructions. After 15 min, the genetically modified ECs were analyzed by flow cytometer.

### Quantitative Real-Time Reverse Transcription PCR Assay

Sorted GFP-positive ECs of GFP-alone, JAK2wt, and JAK2V617F were processed for total RNA extraction using an RNA Purification Kit (Jena Bioscience GmbH, Jena, Germany). First-strand complementary DNA (cDNA) was synthesized from a total of 20 ng of RNA using the SCRIPT cDNA Synthesis-Kit (Jena Bioscience GmbH) according to the manufacturer's instructions. In order to detect the expressional changes of the genes that were relevant in this study, the commercially available PCR Array for the Human JAK/STAT Signaling Pathway (QIAGEN, Dusseldorf, Germany) was applied. Comparative measurements with real-time RT-PCR were performed on a LightCycler 480 instrument (Roche, Basel, Switzerland) using SYBR Green (RT2 SYBR Green qPCR Master Mix, QIAGEN), and reference genes were applied using the housekeeping genes of five different genes in the array. The fluorescence emitted by dye above the baseline signal was detected using the software in real-time, recorded, and represented as the cycle threshold (CT). The arithmetic means of the CT values for *JAK1*, *JAK2*, *JAK3*, and *TYK2* gene expressions, which were measured twice, were calculated for statistical analysis [45]. All gene expressions for each sample were normalized to the control sample, and upregulated/downregulated calculations were performed for comparisons to control samples.

### EMP Analysis

EMPs released from genetically modified ECs were isolated from the collected supernatants of cultured cells using a centrifugation protocol originally optimized for EMP isolation elsewhere [46]. In brief, the collected supernatants of the genetically modified ECs were centrifuged at 500 x g for 10 min to remove the dead cells. The supernatants were collected and given a second centrifugation at 15000 x g for 30 min to condense the EMPs. The pellet was collected and diluted with 1X Dulbecco's PBS (GIBCO, Waltham, MA, USA). The size of the EMPs was determined with a FACSCalibur instrument (Becton Dickinson Biosciences).

The instrument was first rinsed with a particle-free rinse solution for 10 min in order to eliminate background noise. Differently sized latex macrobeads of 0.5, 0.9, 3, and 5 µm (Megamix-Plus FSC, Biocytex, Marseille, France) were applied for the size markers and analysis was performed using a log scale for the forward scatter and side scatter parameters. The flow

cytometry setup introduced for MP dimensions was evaluated and MPs of less than 1  $\mu\text{m}$  in diameter were gated. The EMPs were further labeled with PECAM-1 (CD31-PE, BioLegend) and TF (CD142-APC, BioLegend) antibodies, and the percentages of GFP-positive MPs were detected according to the antibody of interest.

### Detection of JAK2V617F of EMP Genomic DNA by Nested Allele-Specific PCR

Genomic DNA was extracted using a PureLink-Genomic DNA Mini-Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The JAK2V617F mutation was detected by using allele-specific nested PCR as described previously [47].

### Statistical Analysis

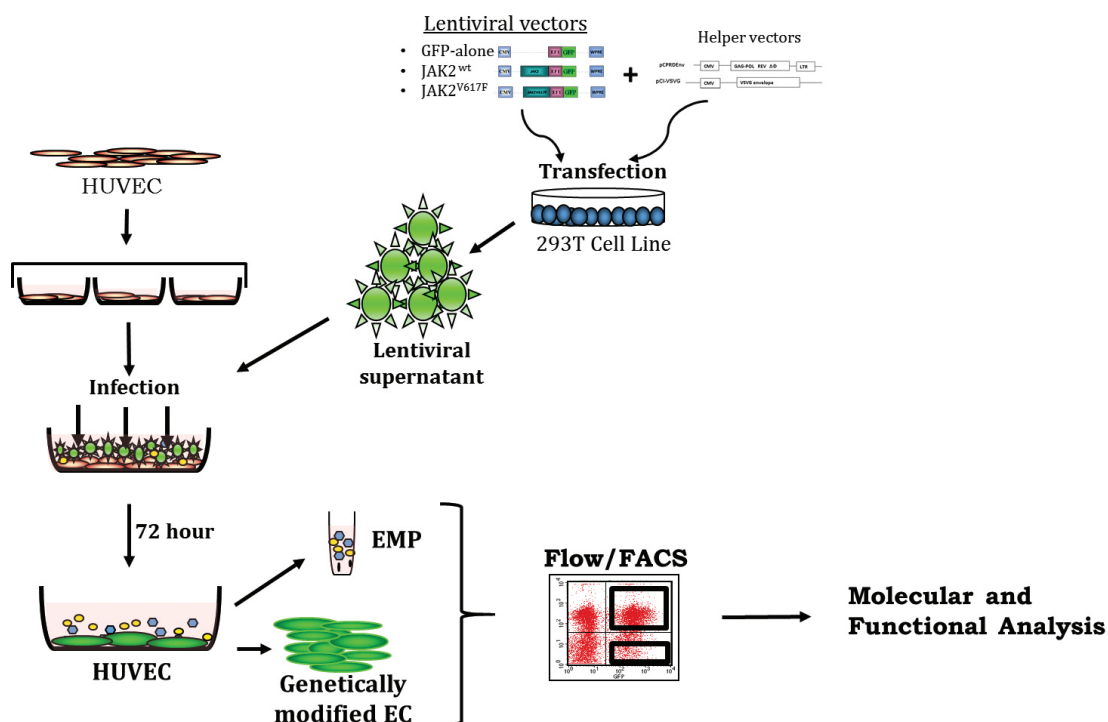
Statistical analyses were performed using one-way ANOVA or a two-tailed Student t-test to determine statistical significance. Values of  $p < 0.05$  and  $p < 0.01$  were considered statistically significant. All values are provided here as mean  $\pm$  standard deviation. A HeatMap map was created by normalizing values to the lowest expression. GraphPad Prism v.8 (GraphPad Prism Inc., San Diego, CA, USA) was used for graphing and statistical analysis.

## Results

In this study, we applied a model to investigate ECs and their functional response to JAK2wt and JAK2V617F, as reported elsewhere [43]. In brief, we applied the lentiviral transduction method with vectors carrying JAK2V617F, JAK2wt, or GFP-alone (Figure 1). Successful transductions of JAK2wt and JAK2V617F in ECs were detected via a GFP-positive signal by flow cytometry analysis (Figure 2A). The GFP-gated infection rates for the three vectors were  $54 \pm 21\%$  for JAK2V617F,  $43 \pm 10\%$  for JAK2wt, and  $47 \pm 13\%$  for GFP-alone (Figure 2B). No statistical differences were detected among the groups.

The infected cells were further sorted for GFP-positive signals for each genetically modified EC and processed for RNA isolation. The gene expressions of *JAK1*, *JAK2*, *JAK3*, and *TYK2* were evaluated with real-time RT-PCR analysis. Normalized gene expression analyses revealed them to be overexpressed in ECs genetically modified with JAK2V617F and JAK2wt when compared to GFP-alone. The increased transcript levels of the *JAK1*, *JAK2*, *JAK3*, and *TYK2* genes also provided confidence regarding the induction of JAK2V617F and JAK2wt in ECs (Figure 2C).

In order to understand the role of JAK2V617F in ECs, a functional analysis was performed of genetically modified ECs



**Figure 1.** Illustration of experimental design. Lentiviral plasmids were applied for the genetic modification of endothelial cells (ECs) in green fluorescent protein (GFP)-alone, JAK2 wild type (JAK2wt), and JAK2V617F expression vectors. After infecting human umbilical vein endothelial cells (HUVECs) with virus supernatants, the endothelial microparticles (EMPs) and HUVECs were harvested following molecular and genetic analysis.

for cell surface proteins that play a role in the development of thrombosis, namely EPCR, TM, and TF. After 72 h of infection with lentiviruses, no statistical significance among the genetically modified ECs was found in the cell surface expressions of the EPCR, TM, and TF proteins related to thrombosis (Figure 2D).

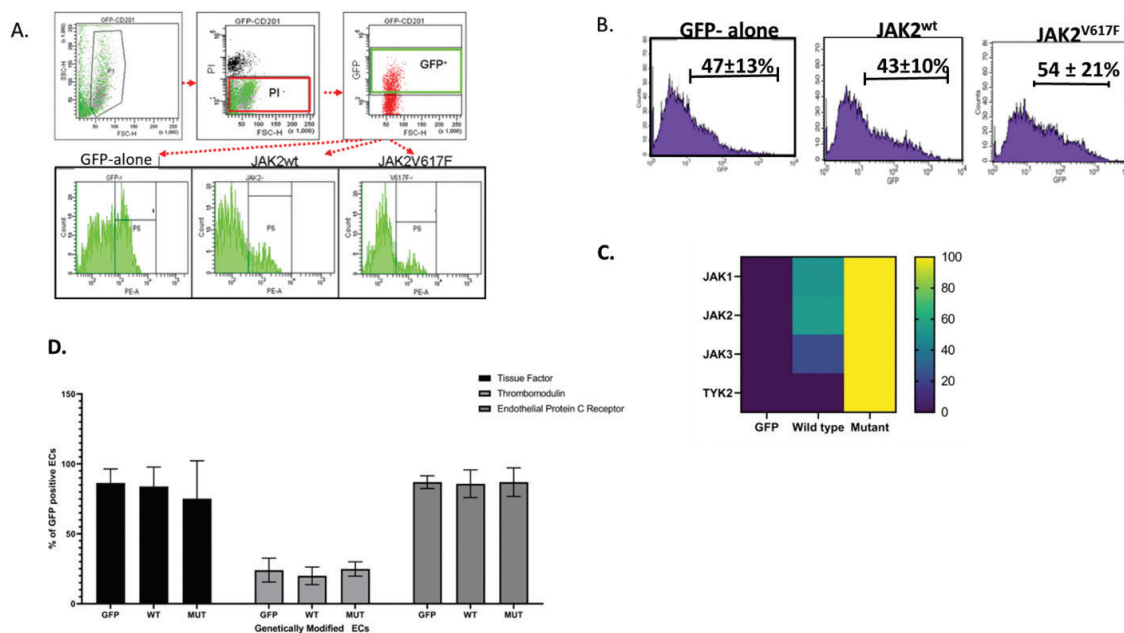
Cell-cycle analysis was performed with DNA histograms and respective contour plots using the BrdU incorporation assay. The analysis was performed based on the percentage of proliferating cells by BrdU-positive cells and the percentage of cells in each cell-cycle phase by 7-AAD fluorescence distribution. The BrdU incorporation analysis for the G0/G1, synthesis (S), and G2/mitosis (M) phases of all genetically modified ECs revealed that a significant number of ECs were in the G0/G1 phase compared to the S and G2/M phases ( $p < 0.01$ ), regardless of the genetically modified EC background. There were no significant differences among the groups within these phases (Figure 3A).

The role of JAK2V617F in the induction of apoptosis in ECs was also evaluated. The apoptosis activity of genetically modified ECs was evaluated by labeling with annexin-V antibody and 7-AAD followed by flow cytometer analysis. Both

JAK2V617F-positive and JAK2wt-positive ECs showed a significant increase in apoptosis compared to GFP-alone ECs ( $p < 0.05$ ). JAK2V617F-positive ECs did not reveal any statistical significance in apoptosis when compared to JAK2wt (Figure 3B).

Since MPs are regulators of EC function, we further investigated the EMPs of genetically modified ECs. Initially, immunophenotype analysis of secreted EMPs collected from supernatants of the genetically modified EC culture according to the established protocols was performed (Figure 4A). We applied PECAM-1 (CD31) to mark the EMPs. The surface expressions of EPCR, TM, and TF in the EMPs of genetically modified ECs revealed no difference in EMP immunophenotypes regarding their thrombotic protein receptors (Figure 4B).

It is well known that MPs carry cargo from their genomic origins. In order to identify the cargo material, genotyping of EMPs was performed by allele-specific nested PCR. This revealed that ECs with JAK2V617F secreted EMPs carrying JAK2V617F DNA fragments (Figure 4C). We also determined the RNA content of the EMP cargo from GFP-alone, JAK2wt, and JAK2V617F ECs to be  $66.6 \pm 5.3$ ,  $126.5 \pm 3.2$ , and  $69.5 \pm 6.8$  ng/ $\mu$ L, respectively.



**Figure 2.** Analysis of genetically modified green fluorescent protein (GFP)-positive endothelial cells (ECs) by flow cytometer. **A)** Representative flow cytometry analysis of genetically modified ECs gated for living cells after excluding propidium iodide (PI)-positive cells. The second gating was performed on GFP-positive signals for GFP-alone, JAK2 wild type (JAK2wt), and JAK2V617F ECs. **B)** Infection rates of the genetically modified ECs as detected by flow cytometry analysis; values are mean  $\pm$  standard deviation (SD). **C)** Quantitative real-time reverse transcription-PCR analysis of sorted GFP-positive ECs for GFP-alone, JAK2wt, and JAK2V617F. The fold change in the gene expressions of *JAK1*, *JAK2*, *JAK3*, and *TYK2* were investigated for all genetically modified ECs. Analysis of gene expressions was performed with housekeeping genes. Heat maps were generated using normalized gene expressions in GraphPad Prism v.8. **D)** Analysis of cell surface receptor expression against endothelial protein C receptor (EPCR), also known as activated protein C receptor (CD201-PE), and thrombomodulin (TM) (CD141-PE) and tissue factor (TF) (CD142-APC) was performed for genetically modified ECs. All bar graphs reflect mean  $\pm$  SD. Results were calculated with unpaired, two-tailed Student t-tests using Microsoft Excel with  $p < 0.05$  considered statistically significant.

Surprisingly JAK2wt ECs released significantly more RNA in their EMP cargo than the other genetically modified ECs.

### Discussion

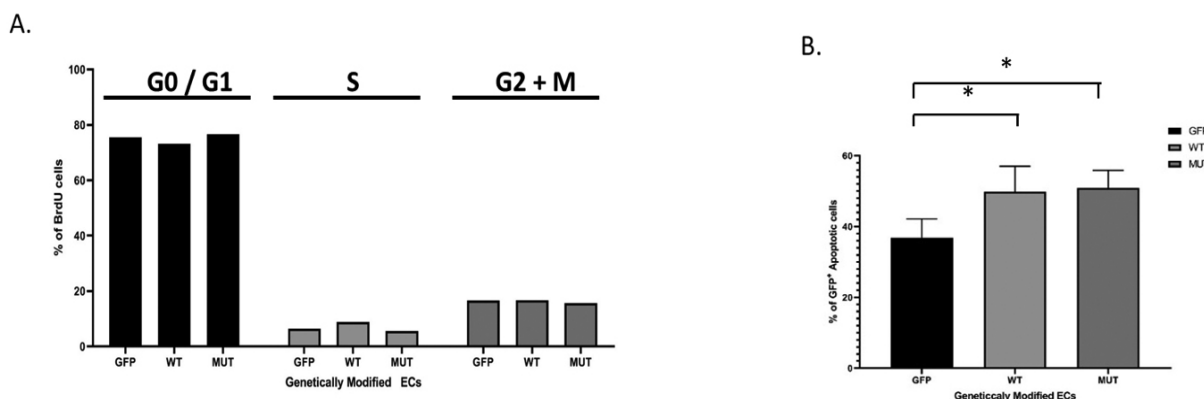
Thrombotic events are frequently observed in patients with MPNs. Since the identification of the JAK2V617F mutation in the ECs of PV patients with Budd-Chiari syndrome [8], the main research focus has been on the effect of mutation on EC functions, the rationale here being that an acquired genetic mutation can affect the prothrombotic nature of ECs, leading to a hypercoagulative state. To test this hypothesis, we applied a lentiviral transduction of the JAK2V617F mutation in an EC line and created an in vitro platform where genetically modified ECs could be functionally and genetically studied in a model environment. Although the model inevitably has many limitations when compared to an in vivo study, it nevertheless provided a controlled setting for understanding and comparing the effects of mutation in a single-sided approach.

The study began with an analysis of the activity of Janus kinases *JAK1*, *JAK2*, *JAK3*, and *TYK*, which were all determined to have upregulated gene expressions in ECs with JAK2wt and JAK2V617F induction when normalized to GFP-alone. The upregulation in gene expressions revealed that lentiviral vectors induce JAK/STAT pathway activation in ECs. We then investigated the EC markers on EC cell surfaces related to hemostasis and blood coagulation. There was no statistically significant difference among the genetically modified ECs. A recent study investigating the JAK2V617F mutation in an in vivo murine model reported similar results with a pro-adhesive phenotype of JAK2V617F mutant ECs [15].

Annexin-V labeling was used to test the induction of apoptosis in ECs modified with JAK2wt and JAK2V617F. Again, similar results to those found here were reported in a study in which a murine model of MPN was used. In that study, the JAK2V617F mutation was expressed in specific cell lineages, including blood cells and vascular ECs, and an increased apoptosis rate in ECs was revealed [48]. These results might lead to speculation that the induction of apoptosis in ECs induces endothelial dysfunction, leading to hypercoagulability and vascular complications in patients with MPNs.

A surprising result was obtained with the study of EMPs in this setup. Although we did not detect any difference in EMP immunophenotypes among the genetically modified ECs, genotyping revealed profound results. It has been proposed that EMP cargos carry genomic DNA (gDNA) fragments of their cells of origin; in this case, the gDNA fragment of JAK2V617F and many ribonucleic acids were detected in the EMP cargo of JAK2V617F ECs. These results demonstrate the direct effect of JAK2V617F in the biology of ECs by inducing signals and accumulating the secretion of EMPs with JAK2V617F-positive DNA, thus elucidating the involvement of ECs in MPNs. It is well known that host inflammatory responses as a consequence of cytokines and other mediators via malignant cells initiate a thrombotic state of acquired thrombosis in cases of MPNs; EMPs with JAK2V617F-positive DNA might also be involved as players within this mechanism.

TF-positive MPs have been considered the initiators of thrombotic events in many cases [49]. In some studies, patients with thrombosis events and JAK2V617F mutation showed elevated levels of TF-positive MPs [39]. EMPs are also found



**Figure 3.** Cell cycle analysis of genetically modified green fluorescent protein (GFP)-positive endothelial cells (ECs) by flow cytometer. **A)** Cell cycle status after BrdU treatment followed by 7-amino-actinomycin D (7-AAD) labeling analyzed with flow cytometry. The G0/G1, S, and G2/M phases are shown on the GFP-positive gate of the genetically modified ECs. **B)** Apoptosis analysis by flow cytometer for GFP-positive ECs. The GFP, JAK2 wild type (JAK2wt), and JAK2V617F ECs were analyzed for their annexin-V uptake. 7-AAD labeling is also included in this analysis. Error bars represent mean ± SD (\*: p<0.05). **B)** Apoptosis analysis by flow cytometer for GFP-positive ECs. The GFP, JAK2 wild type (JAK2wt), and JAK2V617F ECs were analyzed for their annexin-V uptake. 7-AAD labeling is also included in this analysis. Error bars represent mean ± SD (\*: p<0.05).

to have procoagulant and pro-adhesive properties [50], which promote coagulation and induce vascular inflammation [28].

Recent studies have provided evidence for the functioning of MPs as vectors for the intercellular exchange of biological signals and cellular information by the transcellular exchange of proteins and genetic materials [18]. This delivery system establishes a communication network in which the transcriptomes and proteomes of recipient cells might be influenced and shared by the host and signaling transducers not only in their local environments but also at remarkable distances from their sites of origin. Moreover, we detected RNA in their cargos. It is also possible that in some events, such as inflammation and cancerogenesis, the transferred cargo is selective in sorting (or "packaging") the host cell components and contents that induce finely tuned or exacerbated effects on target cells [28,51]. Since MPs freely circulate in the bloodstream, they may serve as shuttle modules.

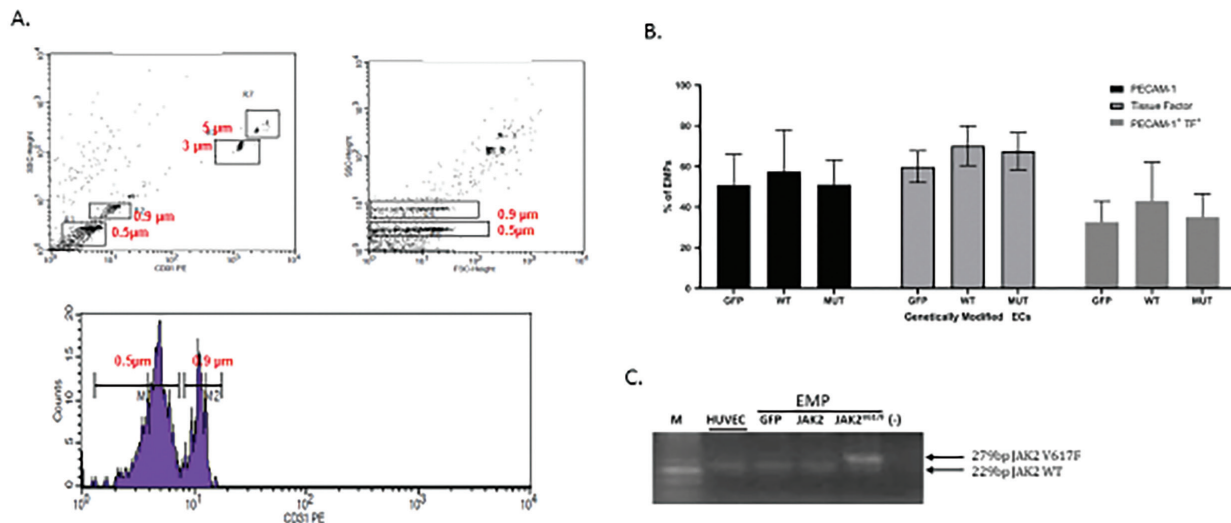
The characterization and quantification of MPs is challenging due to their submicron sizes. Flow cytometry remains the most frequently utilized strategy for MP detection. We applied 1-µm beads gated for MPs smaller than 1 µm and further stained them with PECAM-1 to mark EMPs analyzed for TF. In our results, no significant changes were detected for the percentages of TF-positive MPs in JAK2V617F ECs. Further studies are needed

to identify the direct role of JAK2V617F-positive EMPs in inflammation and thrombosis in cases of MPNs. It is also possible that EMPs as well as other EGMs may be diagnostic and prognostic markers for many cancer types with mutations [52].

It is known that the expression construct of model systems incorporating a lentiviral-based vector will transduce in target cells and integrate into gDNA, providing stable, long-term expression of the target gene. The EMP protocol applied in this study requires several washing and centrifugations steps. Therefore, the likelihood of gDNA contamination from any other source appears to be minimal.

### Conclusion

ECs are a crucial element in hematopoiesis and the hematopoietic microenvironment as well as in circulation. The data in the literature to date reveal features of ECs with JAK2V617F that have often been associated with dysfunctional endothelium, such as sustained inflammation or permeability, limited cell growth, and senescence [53]. The present study is the first to provide data revealing the importance of EMPs in JAK2V617F ECs as players in cellular crosstalk and suggesting that JAK2V617F expression in ECs induces a response in this cell type. Furthermore, the concerning induction of not only the inflammation and/or thrombosis observed in MPNs but also of microenvironmental changes of the mutated clone must be



**Figure 4.** Endothelial microparticle (EMP) analysis of genetically modified endothelial cells (ECs) by flow cytometer. **A)** Flow cytometry analysis strategy for EMP identification. Varying-size latex macrobeads (0.5, 0.9, 3, and 5 µm) were introduced to flow cytometry to identify the microparticle (MP) dimensions based on known macrobeads. MPs of less than 1 µm in diameter were gated, and the quantities of green fluorescent protein (GFP)-positive MPs were detected according to the antibody of interest. **B)** Flow cytometry analysis of percentage of EMPs released from genetically engineered ECs and labeled with PECAM-1 and TF antibodies. Error bars represent mean±SD. **C)** JAK2V617F allele status of EMPs from genetically modified ECs by allele-specific nested PCR. The final PCR products were analyzed on 2.5% agarose gels. The 279-bp product is the JAK2V617F-positive allele, whereas the 229-bp product is the wild-type allele. The presence of two bands represents JAK2V617F heterozygous alleles; only the wild type product represents JAK2 wild type (JAK2wt) alleles. PCR analyses were performed with the following order: Lane 1, marker (50 bp); Lane 2, human umbilical vein ECs (HUVECs); Lane 3, GFP EMPs; Lane 4, JAK2wt EMPs; Lane 5, JAK2V617F EMPs; and Lane 6, negative control.

considered [18]. One thing should be stated: the production of EMPs is not a dysfunction of ECs but rather a part of their cellular activation that might be triggered by JAK2V617F. This study may be pioneering in the field of MPN research for illuminating both the thrombotic complications of MPNs and treatment modalities of various cancers.

## Ethics

**Ethics Committee Approval:** No ethics approval was required for this study.

**Informed Consent:** Obtained.

## Authorship Contributions

Concept: S.S.; Supervision: S.S.; Materials: H.H., S.F.T., S.S.; Data Collection and/or Processing: H.H., S.F.T., S.S.; Analysis and/or Interpretation: H.H., S.S.; Literature Search: H.H., S.F.T.; Writing: H.H., S.S.; Critical Review: S.S.

**Conflict of Interest:** No conflict of interest was declared by the authors.

**Financial Disclosure:** This work was supported by the Scientific Research Projects Coordination Unit of İstanbul University (Project Number: 27570) and by the Scientific and Technological Research Council of Turkey (TÜBİTAK, Grant No. 111S173).

## References

- Kaushansky K, Zhan H. The regulation of normal and neoplastic hematopoiesis is dependent on microenvironmental cells. *Adv Biol Regul* 2018;69:11-15.
- Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. *Circ Res* 2010;107:1047-1057.
- Endemann DH, Schiffrin EL. Endothelial dysfunction. *J Am Soc Nephrol* 2004;15:1983-1992.
- Barbui T, Finazzi G, Falanga A. Myeloproliferative neoplasms and thrombosis. *Blood* 2013;122:2176-2184.
- Barbui T, Finazzi G, Carobbio A, Thiele J, Passamonti F, Rumi E, Ruggeri M, Rodeghiero F, Randi ML, Bertozzi I, Gisslinger H, Buxhofer-Ausch V, De Stefano V, Betti S, Rambaldi A, Vannucchi AM, Tefferi A. Development and validation of an International Prognostic Score of thrombosis in World Health Organization-essential thrombocythemia (IPSET-thrombosis). *Blood* 2012;120:5128-5133.
- Falanga A, Marchetti M. Thrombosis in myeloproliferative neoplasms. *Semin Thromb Hemost* 2014;40:348-358.
- Farina M, Russo D, Hoffman R. The possible role of mutated endothelial cells in myeloproliferative neoplasms. *Haematologica* 2021;106:2813-2823.
- Sozer S, Fiel MI, Schiano T, Xu M, Mascarenhas J, Hoffman R. The presence of JAK2V617F mutation in the liver endothelial cells of patients with Budd-Chiari syndrome. *Blood* 2009;113:5246-5249.
- Sozer S, Ishii T, Fiel MI, Wang J, Wang X, Zhang W, Godbold J, Xu M, Hoffman R. Human CD34+ cells are capable of generating normal and JAK2V617F positive endothelial like cells in vivo. *Blood Cells Mol Dis* 2009;43:304-312.
- Sozer S, Wang X, Zhang W, Fiel MI, Ishii T, Wang J, Wisch N, Xu M, Hoffman R. Circulating angiogenic monocyte progenitor cells are reduced in JAK2V617F high allele burden myeloproliferative disorders. *Blood Cells Mol Dis* 2008;41:284-291.
- Teofili L, Martini M, Iachinoto MG, Capodimonti S, Nuzzolo ER, Torti L, Cenci T, Larocca LM, Leone G. Endothelial progenitor cells are clonal and exhibit the JAK2<sup>V617F</sup> mutation in a subset of thrombotic patients with Ph-negative myeloproliferative neoplasms. *Blood* 2011;117:2700-2707.
- Oppliger Leibundgut E, Horn MP, Brunold C, Pfanner-Meyer B, Marti D, Hirsiger H, Tobler A, Zwicky C. Hematopoietic and endothelial progenitor cell trafficking in patients with myeloproliferative diseases. *Haematologica* 2006;91:1465-1472.
- Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* 2007;109:1801-1809.
- Falanga A, Marchetti M. Thrombotic disease in the myeloproliferative neoplasms. *Hematology Am Soc Hematol Educ Program* 2012;2012:571-581.
- Guy A, Gourdou-Latyszenok V, Le Lay N, Peghaire C, Kilani B, Dias JV, Duplaa C, Renault MA, Denis C, Villeval JL, Boulaftali Y, Jandrot-Perrus M, Couffinhal T, James C. Vascular endothelial cell expression of JAK2<sup>V617F</sup> is sufficient to promote a pro-thrombotic state due to increased P-selectin expression. *Haematologica* 2019;104:70-81.
- Qin Y, Wang X, Zhao C, Wang C, Yang Y. The impact of JAK2V617F mutation on different types of thrombosis risk in patients with essential thrombocythemia: a meta-analysis. *Int J Hematol* 2015;102:170-180.
- Edelmann B, Gupta N, Schnoeder TM, Oelschlegel AM, Shahzad K, Goldschmidt J, Philipsen L, Weinert S, Ghosh A, Saalfeld FC, Nimmagadda SC, Müller P, Braun-Dullaeus R, Mohr J, Wolleschak D, Kliche S, Amthauer H, Heidel FH, Schraven B, Isermann B, Müller AJ, Fischer T. JAK2-V617F promotes venous thrombosis through  $\beta 1/\beta 2$  integrin activation. *J Clin Invest* 2018;128:4359-4371.
- Koçana CÇ, Toprak SF, Sözer S. Extracellular genetic materials and their application in clinical practice. *Cancer Genet* 2021;252-253:48-63.
- Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol* 2011;31:27-33.
- Distler JH, Pisetsky DS, Huber LC, Kalden JR, Gay S, Distler O. Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases. *Arthritis Rheum* 2005;52:3337-3348.
- Krajewska-Włodarczyk M, Owczarczyk-Saczonek A, Żuber Z, Wojtkiewicz M, Wojtkiewicz J. Role of microparticles in the pathogenesis of inflammatory joint diseases. *Int J Mol Sci* 2019;20:5453.
- Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 2009;19:43-51.
- Barteneva NS, Fasler-Kan E, Bernimoulin M, Stern JN, Ponomarev ED, Duckett L, Vorobjev IA. Circulating microparticles: square the circle. *BMC Cell Biol* 2013;14:23.
- Palmisano G, Jensen SS, Le Bihan MC, Lainé J, McGuire JN, Pociot F, Larsen MR. Characterization of membrane-shed microvesicles from cytokine-stimulated beta-cells using proteomics strategies. *Mol Cell Proteomics* 2012;11:230-243.
- Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* 2007;110:2440-2448.
- Peterson DB, Sander T, Kaul S, Wakim BT, Halligan B, Twigger S, Pritchard KA Jr, Oldham KT, Ou JS. Comparative proteomic analysis of PAI-1 and TNF-alpha-derived endothelial microparticles. *Proteomics* 2008;8:2430-2446.



27. Markiewicz M, Richard E, Marks N, Ludwicka-Bradley A. Impact of endothelial microparticles on coagulation, inflammation, and angiogenesis in age-related vascular diseases. *J Aging Res* 2013;2013:734509.
28. Liu Y, Zhang R, Qu H, Wu J, Li L, Tang Y. Endothelial microparticles activate endothelial cells to facilitate the inflammatory response. *Mol Med Rep* 2017;15:1291-1296.
29. Mooberry MJ, Key NS. Microparticle analysis in disorders of hemostasis and thrombosis. *Cytometry A* 2016;89:111-122.
30. Morel O, Toti F, Hugel B, Bakouboula B, Camoin-Jau L, Dignat-George F, Freyssinet JM. Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol* 2006;26:2594-2604.
31. Sabatier F, Roux V, Anfoso F, Camoin L, Sampol J, Dignat-George F. Interaction of endothelial microparticles with monocytic cells in vitro induces tissue factor-dependent procoagulant activity. *Blood* 2002;99:3962-3970.
32. Jy W, Minagar A, Jimenez JJ, Sheremata WA, Mauro LM, Horstman LL, Bidot C, Ahn YS. Endothelial microparticles (EMP) bind and activate monocytes: elevated EMP-monocyte conjugates in multiple sclerosis. *Front Biosci* 2004;9:3137-3144.
33. Diamant M, Tushuizen ME, Sturk A, Nieuwland R. Cellular microparticles: new players in the field of vascular disease? *Eur J Clin Invest* 2004;34:392-401.
34. Zhang W, Qi J, Zhao S, Shen W, Dai L, Han W, Huang M, Wang Z, Ruan C, Wu D, Han Y. Clinical significance of circulating microparticles in Ph(-) myeloproliferative neoplasms. *Oncol Lett* 2017;14:2531-2536.
35. Trappenburg MC, van Schilfgaarde M, Marchetti M, Spronk HM, ten Cate H, Leyte A, Terpstra WE, Falanga A. Elevated procoagulant microparticles expressing endothelial and platelet markers in essential thrombocythemia. *Haematologica* 2009;94:911-918.
36. Aswad MH, Kissová J, Rihova L, Zavrelva J, Ovesná P, Penka M. High level of circulating microparticles in patients with BCR/ABL negative myeloproliferative neoplasm - a pilot study. *Klin Onkol* 2019;32:109-116.
37. Marchetti M, Tartari CJ, Russo L, Panova-Noeva M, Leuzzi A, Rambaldi A, Finazzi G, Woodhams B, Falanga A. Phospholipid-dependent procoagulant activity is highly expressed by circulating microparticles in patients with essential thrombocythemia. *Am J Hematol* 2014;89:68-73.
38. Duchemin J, Ugo V, Iannotto JC, Lecucq L, Mercier B, Abgrall JF. Increased circulating procoagulant activity and thrombin generation in patients with myeloproliferative neoplasms. *Thromb Res* 2010;126:238-242.
39. Holy EW, Akhmedov A, Luscher TF, Tanner FC. Berberine, a natural lipid-lowering drug, exerts prothrombotic effects on vascular cells. *J Mol Cell Cardiol* 2009;46:234-240.
40. Kissova J, Ovesna P, Bulikova A, Zavrelva J, Penka M. Increasing procoagulant activity of circulating microparticles in patients with Philadelphia-negative myeloproliferative neoplasms: a single-centre experience. *Blood Coagul Fibrinolysis* 2015;26:448-453.
41. Charpentier A, Lebreton A, Rauch A, Bauters A, Trillot N, Nibourel O, Tintillier V, Wemeau M, Demory JL, Preudhomme C, Jude B, Lecompte T, Cambier N, Susen S. Microparticle phenotypes are associated with driver mutations and distinct thrombotic risks in essential thrombocythemia. *Haematologica* 2016;101:e365-e368.
42. Poisson J, Tanguy M, Davy H, Camara F, El Mdawar MB, Kheloufi M, Dagher T, Devue C, Lasselin J, Plessier A, Merchant S, Blanc-Brude O, Souyri M, Mougenot N, Dingli F, Loew D, Hatem SN, James C, Villevall JL, Boulanger CM, Rautou PE. Erythrocyte-derived microvesicles induce arterial spasms in JAK2V617F myeloproliferative neoplasm. *J Clin Invest* 2020;130:2630-2643.
43. Göksu A, Hekimoğlu H, Sözer Tokdemir S. The effect of JAK2V617F mutation to the endothelial cells and the expression profiles of SOCS1-4 genes in myeloproliferative neoplasms. *Istanbul University Institute of Health Sciences Journal of Advanced Research in Health Sciences* 2020;3:135-147.
44. Tunç BS, Toprak F, Toprak SF, Sozer S. In vitro investigation of growth factors including MGF and IGF-1 in neural stem cell activation, proliferation, and migration. *Brain Res* 2021;1759:147366.
45. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta C_t}$  method. *Methods* 2001;25:402-408.
46. van Ierssel SH, Van Craenenbroeck EM, Conraads VM, Van Tendeloo VF, Vrints CJ, Jorens PG, Hoymans VY. Flow cytometric detection of endothelial microparticles (EMP): effects of centrifugation and storage alter with the phenotype studied. *Thromb Res* 2010;125:332-339.
47. Ishii T, Zhao Y, Shi J, Sozer S, Hoffman R, Xu M. T cells from patients with polycythemia vera elaborate growth factors which contribute to endogenous erythroid and megakaryocyte colony formation. *Leukemia* 2007:2433-2441.
48. Castiglione M, Jiang YP, Mazzeo C, Lee S, Chen JS, Kaushansky K, Yin W, Lin RZ, Zheng H, Zhan H. Endothelial JAK2V617F mutation leads to thrombosis, vasculopathy, and cardiomyopathy in a murine model of myeloproliferative neoplasm. *J Thromb Haemost* 2020;18:3359-3370.
49. Tesselaar ME, Romijn FP, Van Der Linden IK, Prins FA, Bertina RM, Osanto S. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost* 2007;5:520-527.
50. Combes V, Simon AC, Grau GE, Arnoux D, Camoin L, Sabatier F, Mutin M, Sanmarco M, Sampol J, Dignat-George F. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J Clin Invest* 1999;104:93-102.
51. Mai J, Virtue A, Shen J, Wang H, Yang XF. An evolving new paradigm: endothelial cells-- conditional innate immune cells. *J Hematol Oncol* 2013;6:61.
52. Yokoi A, Villar-Prados A, Oliphant PA, Zhang J, Song X, De Hoff P, Morey R, Liu J, Roszik J, Clise-Dwyer K, Burks JK, O'Halloran TJ, Laurent LC, Sood AK. Mechanisms of nuclear content loading to exosomes. *Sci Adv* 2019;5:eaax8849.
53. Cosgrove ME, Suman R, Harrison HJ, Jackson GE, Howard MR, Hitchcock IS. Endothelial JAK2V617F expression drives inflammation and cellular senescence; new evidence for the roles of endothelial cells in MPN-related clotting abnormalities? *Blood* 2016;128:3134.