ARTICLE

Multilevel defects in the hematopoietic niche in essential thrombocythemia

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ABSTRACT

The role of the bone marrow niche in essential thrombocythemia (ET) remains unclear. Here, we observed multilevel defects in the hematopoietic niche of patients with *JAK2V617F*-positive ET, including functional deficiency in mesenchymal stromal cells (MSC), immune imbalance, and sympathetic-nerve damage. Mesenchymal stromal cells from patients with *JAK2V617F*-positive essential thrombocythemia had a transformed transcriptome. In parallel, they showed enhanced proliferation, decreased apoptosis and senescence, attenuated ability to differentiate into adipocytes and osteocytes, and insufficient support for normal hematopoiesis. Additionally, they were inefficient in suppressing immune responses. For instance, they poorly inhibited proliferation and activation of CD4-positive T cells and the secretion of the inflammatory factor soluble CD40-ligand. They also poorly induced formation of mostly immunosuppressive T-helper 2 cells (Th2) and the secretion of the anti-inflammatory factor interleukin-4 (IL-4). Furthermore, we identified WDR4 as a potent protein with low expression and which was correlated with increased proliferation, reduced senescence and differentiation, and insufficient support for normal hematopoiesis in MSC from patients with *JAK2V617F*-positive ET. We also observed that loss of WDR4 in MSC cells downregulated the interleukin-6 (IL-6) level through the ERK–GSK3β–CREB signaling based on our *in vitro* studies. Altogether, our results show that multilevel changes occur in the bone marrow niche of patients with *JAK2V617F*-positive ET, and low expression of WDR4 in MSC may be critical for inducing hematopoietic related changes.

Introduction

In ET with the acquired *JAK2V617F* mutation, neoplastic clones take over the BM niche, and consequently normal hematopoiesis fails.[1] Multiple mechanisms may be involved in this process; however, the specific mechanisms leading to the replacement of normal hematopoietic stem/progenitor cells (HSPC) by mutant HSPC remain unclear.

Hematopoiesis is a parenchymal process that takes place in the BM, wherein it is tightly regulated by a complex communication network involving various factors that collectively form the niche for hematopoiesis. All blood cells are derived from HSPC that are primarily present in the perivascular niche, along with mesenchymal stromal cells (MSC) that synthesize various factors promoting HSPC maintenance and/or quiescence.[2,3] Perivascular stromal cells marked by nestin (NES) in the BM are closely associated with HSPC and can regulate the proliferation, differentiation, and long-term hematopoietic capacity of HSPC *via* direct or indirect pathways. Importantly, these NES-positive cells maintain HSPC in the BM, and when ectopi-

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cally transplanted, they help to reconstitute hematopoietic activity in the host region.[4] Although malignant hematopoiesis is mainly caused by genetic abnormalities of the mutant stem cells themselves, increasing evidence has shown that abnormal regulation of the hematopoietic niche in the BM has also significant effects.[5,6] Recently, several studies have shown that genetic and physiological changes in MSC may accompany hematopoietic disorders, such as myelodysplastic syndrome, and leukemia.[7-9] However, little is known about BM-derived MSC (BM-MSC) in patients with ET.

Perivascular NES-positive MSC are extensively innervated by sympathetic nerve fibers that play vital roles in hematopoiesis and cancer progression by targeting HSPC, MSC, and osteoblasts *via* the b3 and b2 adrenergic receptor (B3AR and B2AR) signaling pathways.[4,10,11] The nervous system also regulates immunity and inflammation in the BM, which have long been known to extensively participate in regulation of hematopoiesis.[12] Both T-helper 1 cells (Th1) and Th2 cells produce granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes the differentiation of macrophages from hematopoietic stem cells.[13] Th2 cells also produce lineage-specific cytokines, such as IL-4, which can increase production of granulocytes and monocytes from mature unipotential hematopoietic progenitor cells, while contributing to thrombocytopenia *via* inhibitory effects throughout the process of megakaryopoiesis.[14,15] Emerging reports have shown that T-cell imbalance in the BM and abnormal secretion of inflammatory factors can impair normal hematopoiesis while accelerating the proliferation of malignant clones that carry mutations.[16,17] Moreover, the inflammatory microenvironment can impair sympathetic nerve maintenance and regeneration.[18] Recently, neuropathy and inflammation have been reported in a mouse model of *JAK2V617F*-positive myeloproliferative neoplasms (MPN), which tends to exhibit abnormal hematopoiesis.[19] Nonetheless, the sympathetic and inflammatory environment in the BM of patients with ET has not been widely examined.

IL-6 is a multifunctional and pleiotropic cytokine that plays critical roles in the immune system and in a variety of biological processes including hematopoiesis. It is secreted by numerous cell types, including monocytes, dendritic cells, macrophages, T cells, B cells, fibroblasts, osteoblasts, endothelial cells, and particularly MSC.[20-23] IL-6 has both pro- and anti-inflammatory properties and is involved in the pathogenesis of nearly all inflammatory diseases. It has been reported that mice homozygous for a mutation in the IL-6 receptor signaling subunit glycoprotein 130 (gp130Y757F/Y757F) develop a wide range of hematopoietic abnormalities, including splenomegaly, lymphadenopathy, neutrophilia, and thrombocytopenia in addition to elevated myelopoiesis and megakaryopoiesis in the BM.[24,25] These mice show glycoprotein 130-dependent signal transduction and hyperactivation of the transcriptional activator STAT3. IL-6 also participates in the pathogenesis of various blood disorders by increasing the number of early pluripotent precursor cells and committed myeloid precursors in the BM.[22,26,27] The ERK–GSK3β–CREB signaling pathway has been demonstrated to be involved in regulating IL-6; however, to date, its role in BM-MSC remains unclear.[28-30]

WDR4, located in human chromosomal region 21q22.3, codes for a member of the WD repeat protein family, which has been shown to participate in various cellular processes, such as differentiation, apoptosis, cell cycle progression, and stem cell self-renewal by regulating a wide range of signaling pathways *via* epigenetic regulation of gene expression, or ubiquitin-mediated degradation of proteins.[31,32] *WDR4* is involved in the regulation of an prometastatic and immunosuppressive microenvironment in lung cancer.[33] Downregulation of *WDR4* has been detected in the megakaryocytes and platelets of patients with ET, as shown in the GEO Profiles (GEO numbers: GSE2006 and GSE567). However, the role of WDR4 in ET has not been evaluated.

In the present study, we compared the normal BM niche with that of *JAK2V617F*-positive ET patients to have a comprehensive insight into the changes that occur in the BM microenvironment, and to identify the factors correlated with them.

Methods

Patients and samples

Ninety-one untreated ET patients and fifty healthy donors (HD) were included in this study. The characteristics of the subjects are detailed in the *Online Supplementary Table S1*. This study was approved by the hospital-based ethics committee.

Isolation, expansion, and characterization of MSC

BM-MSC were isolated and expanded *in vitro*, and their cell morphology, immunophenotype, proliferation, cell cycle, apoptosis, differentiation, and senescence were evaluated. Additional information on the experimental design is provided in the *Online Supplementary Material and Methods.*

Transcriptomics and quantitative real-time PCR (qPCR) analyses

Total RNA was isolated from MSC at passage four and used for transcriptomic and qPCR analyses. Detailed information is provided in the *Online Supplementary Material and Methods*.

Measurement of cytokine levels

Luminex assay (R&D Systems, Minneapolis, MN, USA) was performed on the supernatants obtained from the BM extracts to assess for inflammatory factors according to the manufacturer's instructions. Enzyme-linked immunosorbent assay (ELISA) was performed on the supernatants obtained from the BM extracts or from the MSC cultures. A list of the ELISA kits is provided in the *Online Supplementary Table S3*.

Colony-forming unit (CFU) assay

To evaluate the capacity of MSC to sustain normal hematopoiesis, a CFU assay was performed according to the manufacturer's instructions. Detailed information is provided in the *Online Supplementary Material and Methods*.

Results

Gene expression profiles of BM-MSC derived from HD and patients with *JAK2V617F*-positive ET

First, we performed RNA sequencing on MSC of HD or patients with *JAK2V617F*-positive ET. A total of 766 upregulated and 429 downregulated genes were detected in the patient samples (Figure 1A). Gene Ontology analysis revealed changes in the gene sets related to cell cycle, differentiation, proliferation, cell death, and aging (Figure

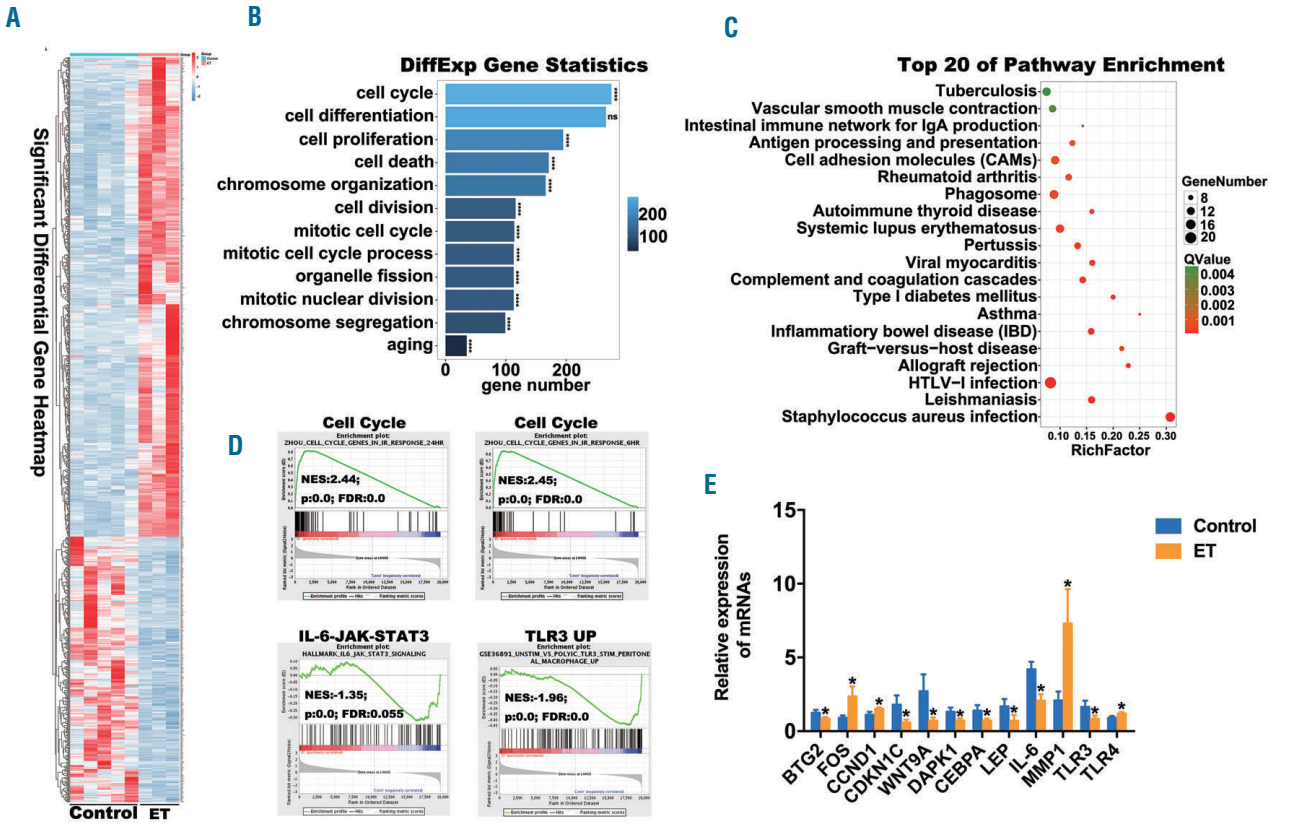

Figure 1. Transcriptomic analysis revealed multiple abnormalities in bone marrow derived mesenchymal stromal cells (BM-MSC) from patients with *JAK2V617F*-positive essential thrombocythemia (ET). A. Heatmap of transcriptomic analysis from eight MSC samples (control, n=5; untreated patients with *JAK2V617F*-positive ET, n=3) demonstrated that MSC from patients with *JAK2V617F*-positive ET differed from those isolated from HD. Briefly, 1,195 genes were identified with a cut-off of greater than 2.0-fold for gene expression change and $P<0.05$. B. GO analysis of genes enriched in terms of different function showed changes in gene sets related to the cell cycle, cell differentiation, proliferation, death, and aging. C. KEGG analysis was carried out to identify differential pathway enrichment between ET and control. Rich factor refers to the ratio of the number of genes differentially expressed in the pathway entry to the total number of genes in the pathway entry. A larger rich factor indicates a higher degree of enrichment. The q-value is the *P*-value after multiple-hypothesis test corrections, ranging from 0 to 1 (a value closer to zero indicates a more significant enrichment). The figure is plotted with the top 20 paths sorted according to the q value from small to large and shows enrichment of genes of inflammation pathways. D. GSEA using MSigDB identified differential gene enrichment between ET and the control. NES, Normal p and FDR q-values for each gene set are shown. The results revealed differential expression of genes involved in cell cycle, inflammatory responses, and hematopoietic support. E. qPCR validation of relevant genes (control, n=16; untreated patients with *JAK2V617F*-positive ET, n=16). MSC used for gene analysis were isolated and expanded *in vitro* and identified according to the minimal criteria for defining multipotent mesenchymal stromal cells stated by the International Society for Cellular Therapy position at passage four.[50] **P*< 0.05; ***P*<0.01, ****P*<0.001; *****P*<0.0001. Data are presented as the mean or mean ± SEM. ET: essential thrombocythemia; HD: healthy donors; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; GSEA: gene set enrichment analysis; MsigDB: Molecular Signatures Database; NES: normalized enrichment score; qPCR: quantitative real-time PCR; n: number of unique donors in each group; ns: not significant; SEM: standard error of mean.

1B). Further analysis showed a prominent abundance of gene signatures associated with inflammation (Figure 1C). Gene Set Enrichment (GSEA) analysis confirmed significant enrichment of dysregulated genes involved in the cell cycle, inflammatory responses, and hematopoietic support (Figure 1D). These changes, confirmed by qPCR (Figure 1E), suggested multiple functional defects in MSC of patients with *JAK2V617F*-positive ET.

BM-MSC from patients with *JAK2V617F*-positive ET show enhanced proliferation and attenuated apoptosis, senescence, and differentiation

To verify the results of the gene expression analyses, we performed Cell Counting Kit 8 (CCK-8) assays, which revealed that the MSC of the patients had higher proliferative capacity than the MSC of the HD (Figure 2A). Furthermore, the apoptosis (Figure 2B) and senescence rates (Figure 2C) of the MSC derived from the patients were lower. The adipogenic and osteogenic differentiation potentials of the ET MSC were also significantly lower (Figure 2D). In line with the above results, the majority of the ET MSC were in S and G2 phases (Figure 2E). Additionally, we detected an increase in the level of NES mRNA (Figure 2F) and the number of NES-positive cells (Figure 2 G-H) in the BM of the patients. No difference in immunophenotype or morphology was detected between the MSC of the HD and those of the patients (*Online Supplementary Figure S1 A-B*).

BM-MSC from patients with *JAK2V617F*-positive ET show insufficient capacity to support normal hematopoiesis

To assess the capacity of MSC to support hematopoiesis, we established a coculture setting involving normal CD34-positive cells and MSC derived from the HD or patients with *JAK2V617F*-positive ET. The recov-

ered CD34-positive cells after seven or 14 days of coculture were plated in a semisolid medium containing methylcellulose or agar in the presence of a cytokine cocktail, and their hematopoietic potential was estimated by determining the number of CFU. We thereby observed that CD34-positive cells had fewer CFU in total (CFU-Total), particularly for granulocytes and macrophages (CFU-GM), when cocultured with the ET MSC relative to those obtained with the HD MSC. There was no significant difference in the number of CFU for megakaryocytes (CFU-MK) between the two co-cultures (Figure 3A-B). These results indicated that BM-MSC from patients with *JAK2V617F*-positive ET were deficient in the maintenance of normal hematopoiesis.

Many cytokines influence hematopoiesis. To identify the effectors, we performed ELISA on the supernatants of

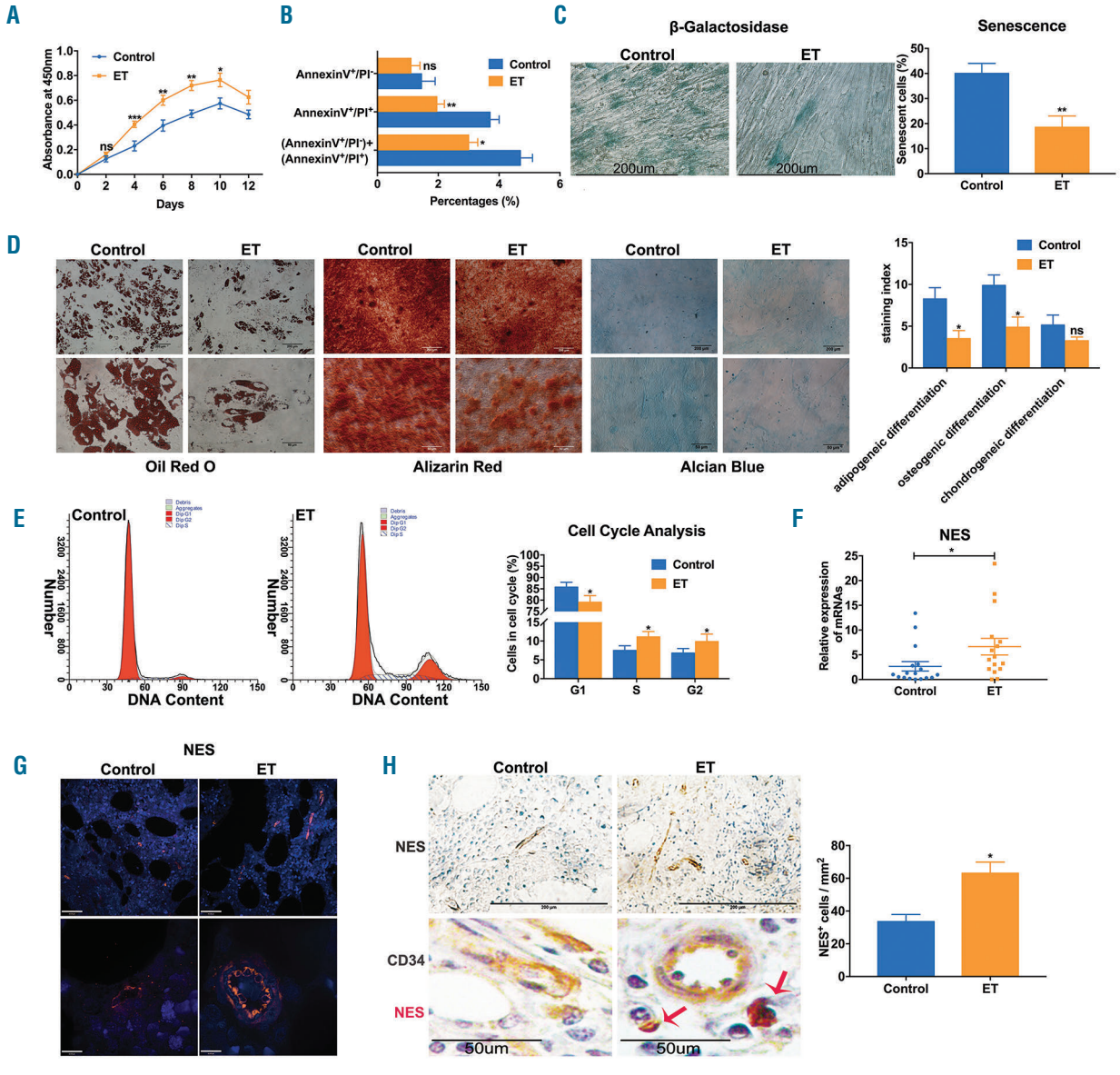

Figure 2. Bone marrow derived mesenchymal stromal cells (BM-MSC) from patients with *JAK2V617F*-positive essential thrombocythemia (ET) show enhanced proliferation and attenuated apoptosis, senescence, and differentiation. A. Growth curves of BM-MSC isolated from HD (n=12) and patients with *JAK2V617F*-positive ET (n=12). The ET MSC grew progressively faster than controls. B. Decreased apoptosis of BM-MSC derived from patients with *JAK2V617F*-positive ET as determined by flow cytometry (control, n=16; ET, n=16). C. The number of β-galactosidase–positive cells were lower in BM-MSC derived from patients with *JAK2V617F*-positive ET compared to those from control patients (control, n=16; ET, n=16). D. Differentiation potentials of MSC toward adipocytes, osteocytes, and chondrocytes were assessed by Oil Red O, Alizarin Red, and Alcian Blue staining, respectively, after induction for 14–21 days. Representative micrographs of BM-MSC derived from HD, and patients with *JAK2V617F*-positive ET are shown. Variations in the differentiation between HD (n=12) and ET samples (n=16) were quantified by the staining index described in the Methods section. E. Cell cycle status was determined by flow cytometry. Patients with *JAK2V617F*-positive ET had less MSC in the G1 phase and more in the S and G2 phases relative to those in the HD controls (control, n=12; ET, n=12). F. NES mRNA expression in BM cells of the controls (n=17) and patients with *JAK2V617F*-positive ET (n=16). G. NES-positive cells in the bone marrow of an HD control (n =1) and patient with *JAK2V617F*-positive ET (n=1) shown by immunofluorescence. H. BM sections of the controls and patients with *JAK2V617F*-positive ET immunostained with NES (brown). (control, n=8; ET, n=37; upper panels); BM sections of the controls and patients with *JAK2V617F*-positive ET immunostained with NES (red) and CD34 (brown). (control, n=8; ET, n=37; lower panels). MSC used in each assay were at passage four (except for those immunostained with NES or NES and CD34). *P<0.05; **P<0.01, ***P<0.001; Data are presented as the mean ± SEM. ET: essential thrombocythemia; HD: healthy donors; n: number of unique donors in each group; IF: immunofluorescence; ns: not significant; SEM: standard error of mean.

the MSC cultures. We thereby observed that ET MSC secreted less IL-6, leptin, and GM-CSF than HD MSC (Figure 3C).

BM-MSC from patients with *JAK2V617F*-positive ET show an impaired immunomodulatory capacity

CD4-positive T cells from patients with *JAK2V617F*-positive ET showed enhanced proliferation and activation compared to normal controls (Figure 4A-B). No changes were observed in the levels of GATA3, T-bet, ROR-γt, or FOXP3 (Figure 4C). Notably, Th2 cell counts were significantly decreased in patients with *JAK2V617F*-positive ET (Figure 4D). The level of the anti-inflammatory factor IL-4 was lower in the patient samples, while pro-inflammatory factors, such as IL-1β, and sCD40L, were upregulated. No difference was observed in IL-6 levels between the patient and healthy samples (Figure 4E).

We next evaluated whether MSC play a role in the immune disorder mentioned above. We observed that CD4-positive T-cell proliferation and activation were remarkably enhanced, and Th2 cell counts were significantly lower when cocultured with ET MSC relative to those observed with HD MSC (Figure 4 F-H). Additionally, IL-4 was downregulated and sCD40L was upregulated with the ET MSC coculture (Figure 4I).

Downregulation of WDR4 is correlated with enhanced proliferation, decreased senescence, and impaired differentiation in BM-MSC of patients with *JAK2V617F*-positive ET

WDR4 was one of the differentially expressed genes according to our RNA sequencing results and was recently reported to regulate the immunosuppressive microenvironment of solid tumors.[25] Downregulation of *WDR4*

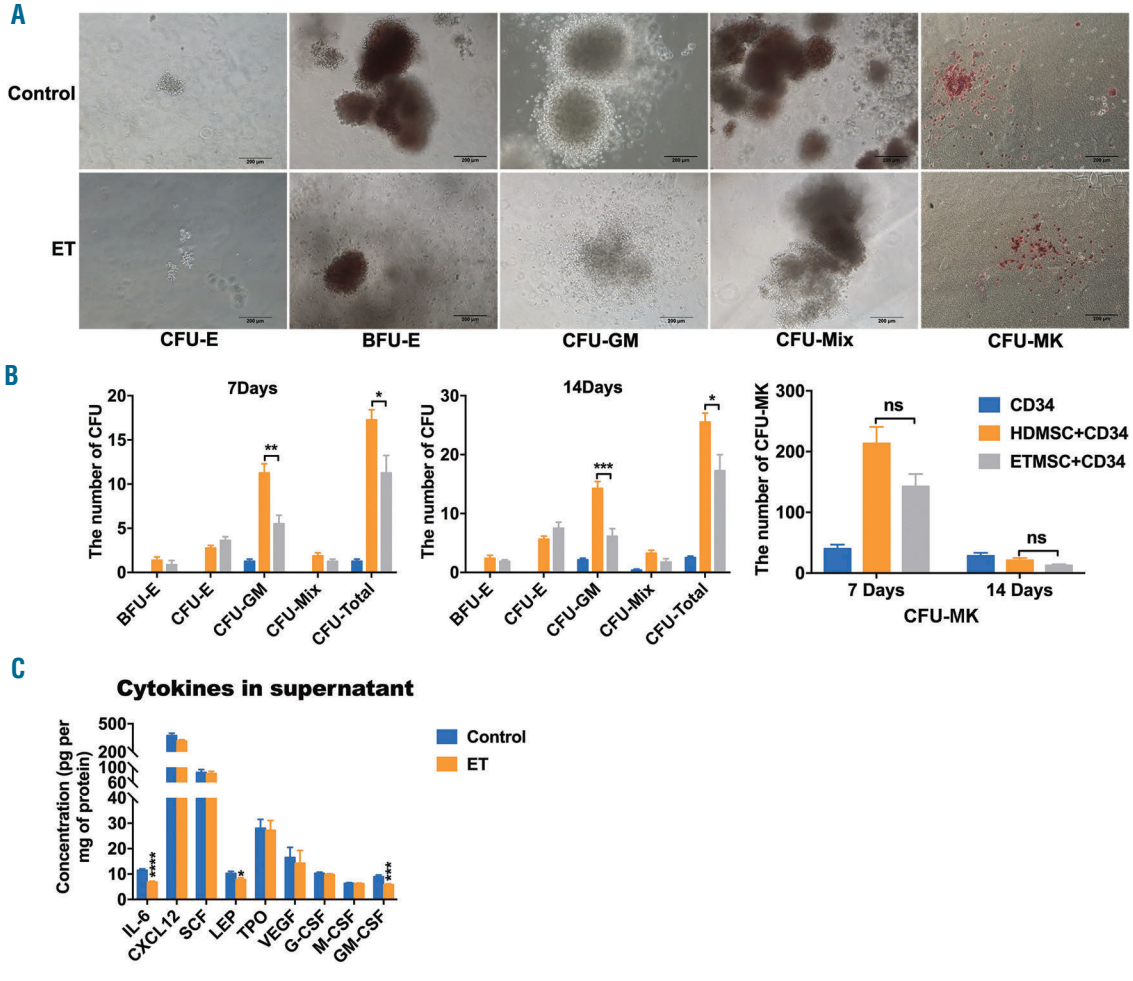

Figure 3. Bone marrow derived mesenchymal stromal cells (BM-MSC) from patients with *JAK2V617F*-positive essential thrombocythemia (ET) show insufficient ability to support normal hematopoiesis. A. Representative micrographs of CFU formed by purified normal CD34-positive cells in the presence of BM-MSC from HD (n=12) and patients with *JAK2V617F*-positive ET (n=16). B. Numbers of BFU-E, CFU-E, CFU-GM, CFU-Mix, CFU-Total, and CFU-MK formed by purified normal CD34-positive cells after coculture with BM-MSC from HD (n=12) or from patients with *JAK2V617F*-positive ET (n=16) for 7 or 14 days. After 7 or 14 days of coculture of purified normal CD34-positive cells and MSC from patients with *JAK2V617F*-positive ET, the numbers of CFU-GM and CFU-Total were significantly lower, with no significant changes in the numbers of BFU-E, CFU-E, CFU-Mix, and CFU-MK. C. Cytokines secreted by BM-MSC into the culture medium analyzed by ELISA (control, n=16; *JAK2V617F*-positive ET, n=16). MSC used in each assay were at passage four. *P<0.05; **P<0.01, ***P<0.001, ****P<0.0001. Data are presented as the mean ± SEM. ET: essential thrombocythemia; HD: healthy donors; ELISA: enzyme-linked immunosorbent assay; n: number of unique donors in each group; ns: not significant; BFU-E: burst-forming unit-erythroid; CFU-E: colony-forming unit-erythroid; CFU-GM: colony-forming unit-granulocyte and macrophage; CFU-Mix: colony-forming units mixed; CFU-Total: total colony-forming units; CFU-MK: colony-forming unit-megakaryocyte; SEM: standard error of mean.

expression was confirmed by qPCR and Western blotting in MSC derived from patients with *JAK2V617F*-positive ET (Figure 5 A-B). To further evaluate the function of WDR4 in BM-MSC, we used lentiviruses carrying the WDR4 cDNA (LV-WDR4) or a specific shRNA targeting WDR4 (LV-shWDR4). LV-shWDR4–infected MSC had significantly lower WDR4 mRNA and protein levels than those in the control groups, and LV-WDR4 effectively increased the WDR4 level in BM-MSC (Figure 5 C-D). We next established coculture systems between normal mononuclear or CD4-positive T cells from the BM, and WDR4 knockdown or overexpressing MSC. Among the subgroups that had different levels of *WDR4* expression, no changes were observed in terms of CD4-positive T-cell proliferation and activation, inflammatory cytokine secretion, or Th2-cell subtype counts (*Online Supplementary Figure S2*). We next examined the role of *WDR4* in BM-MSC regarding functions besides immunoregulation. In HD MSC infected with LV-shWDR4, we observed biological characteristics similar to those in ET MSC, including enhanced proliferation (Figure 5E), decreased senescence (Figure 5F), and an impaired ability to differentiate into adipocytes, osteocytes, and chondrocytes (Figure 5G). Conversely, when ET MSC were infected with LV-WDR4, the previously detected abnormal biological properties were partially reversed (Figure 5 E–G) except for apoptosis (*Online Supplementary Figure S3*).

Insufficient action of the WDR4–IL-6 axis decreases hematopoiesis-supporting activities of BM-MSC from *JAK2V617F*-positive ET patients

We next investigated whether WDR4 affected the hematopoiesis-supporting function of BM-MSC. Hematopoiesis was studied by analyzing CFU. After 14 days of coculture of normal CD34-positive cells with HD

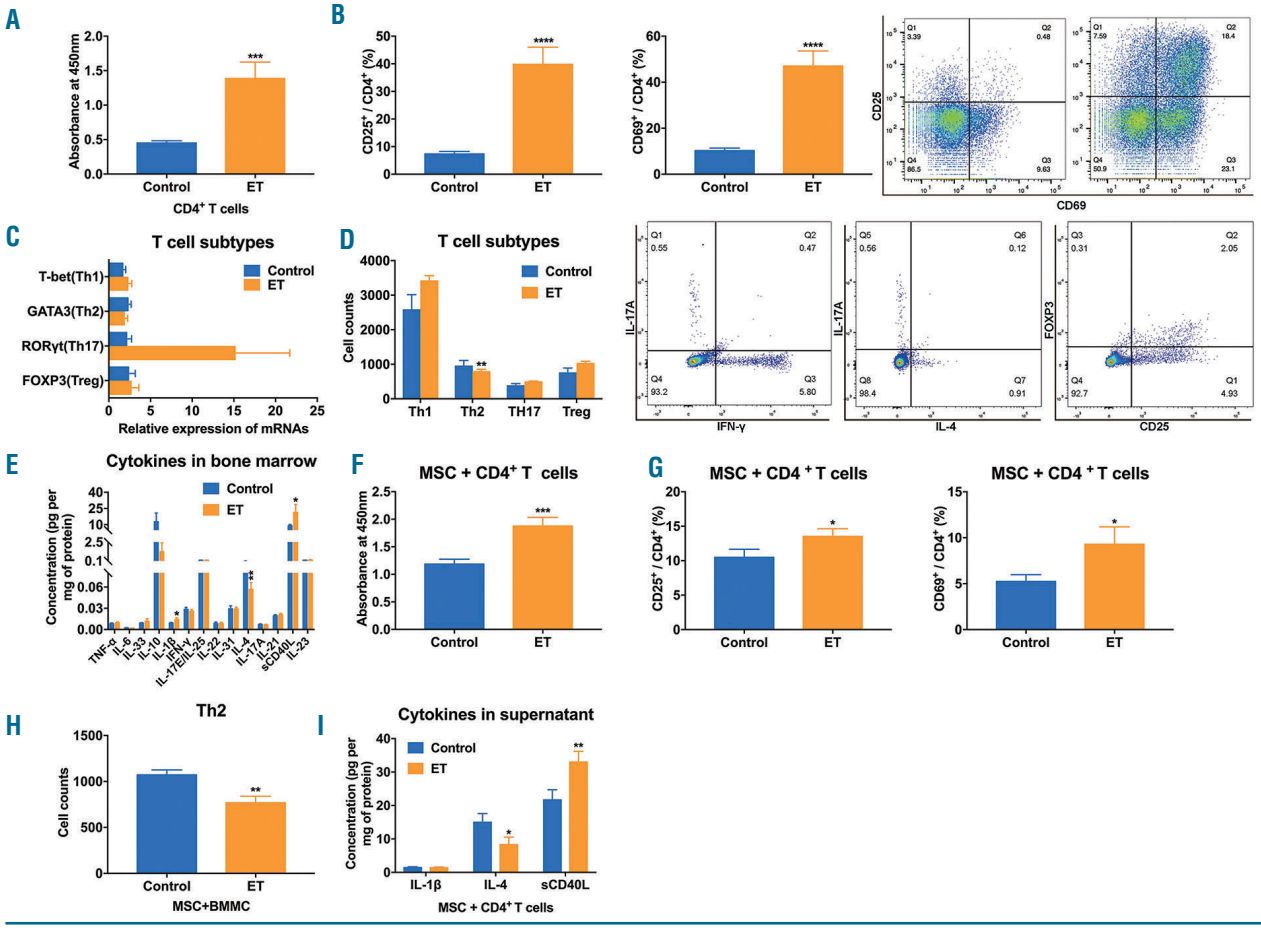

Figure 4. Bone marrow derived mesenchymal stromal cells (BM-MSC) from patients with *JAK2V617F*-positive essential thrombocythemia (ET) have an impaired immunomodulatory capacity. A–B. Proliferation (A) and activation (B) of CD4-positive T cells from HD (n=12) and patients with *JAK2V617F*-positive ET (n=12). C. Expression of T-cell subset transcription factors in bone marrow mononuclear cells (BMMC) derived from HD (n=12) and patients with *JAK2V617F*-positive ET (n=12). T-cell–expressed transcription factors T-bet, GATA-3, RORγt, and FOXP3, representing Th1, Th2, Th17, and Treg cells, respectively. D. Flow-cytometric analysis of the T-cell subset in the bone marrow of HD (n=16) and patients with *JAK2V617F*-positive ET (n=16). CD4-positive cells were sorted into Th1, Th2, Th17, and Treg subsets according to the expression of IFN-γ, IL-4, IL-17 and FOXP3 with CD25. The number of the Th2 subset in patients with *JAK2V617F*-positive ET was lower relative to that in the control group. E. A Luminex assay performed using the supernatant of bone marrow extract from HD (n=20) and from patients with *JAK2V617F*-positive ET (n=24), revealed a decreased level of IL-4 and elevated IL-1β and sCD40L levels in ET. F–G. Proliferation (F) and activation (G) of normal CD4-positive T cells were higher after coculture with BM-MSC from patients with *JAK2V617F*-positive ET (n=8) relative to those observed with HD MSC (n=8). H. Flow-cytometric analysis of T-cell subsets showed lower number of Th2 cells formed from normal BMMC after coculture with BM-MSC from patients with *JAK2V617F*-positive ET (n = 12) relative to those with HD MSC (n=12). I. Decreased level of IL-4 and increased level of sCD40L were found in the supernatant of cell coculture medium of normal CD4-positive T cells and BM-MSC isolated from patients with *JAK2V617F*-positive ET, as determined by ELISA (control, n=12; ET, n=12). MSC used in each assay were at passage four. **P*<0.05; ***P*<0.01, ****P*<0.001, *****P*<0.0001. Data are presented as the mean ± SEM. ET: essential thrombocythemia; HD: healthy donors; BMMC: bone marrow mononuclear cells; ELISA: enzyme-linked immunosorbent assay; n: number of unique donors in each group; SEM: standard error of mean.

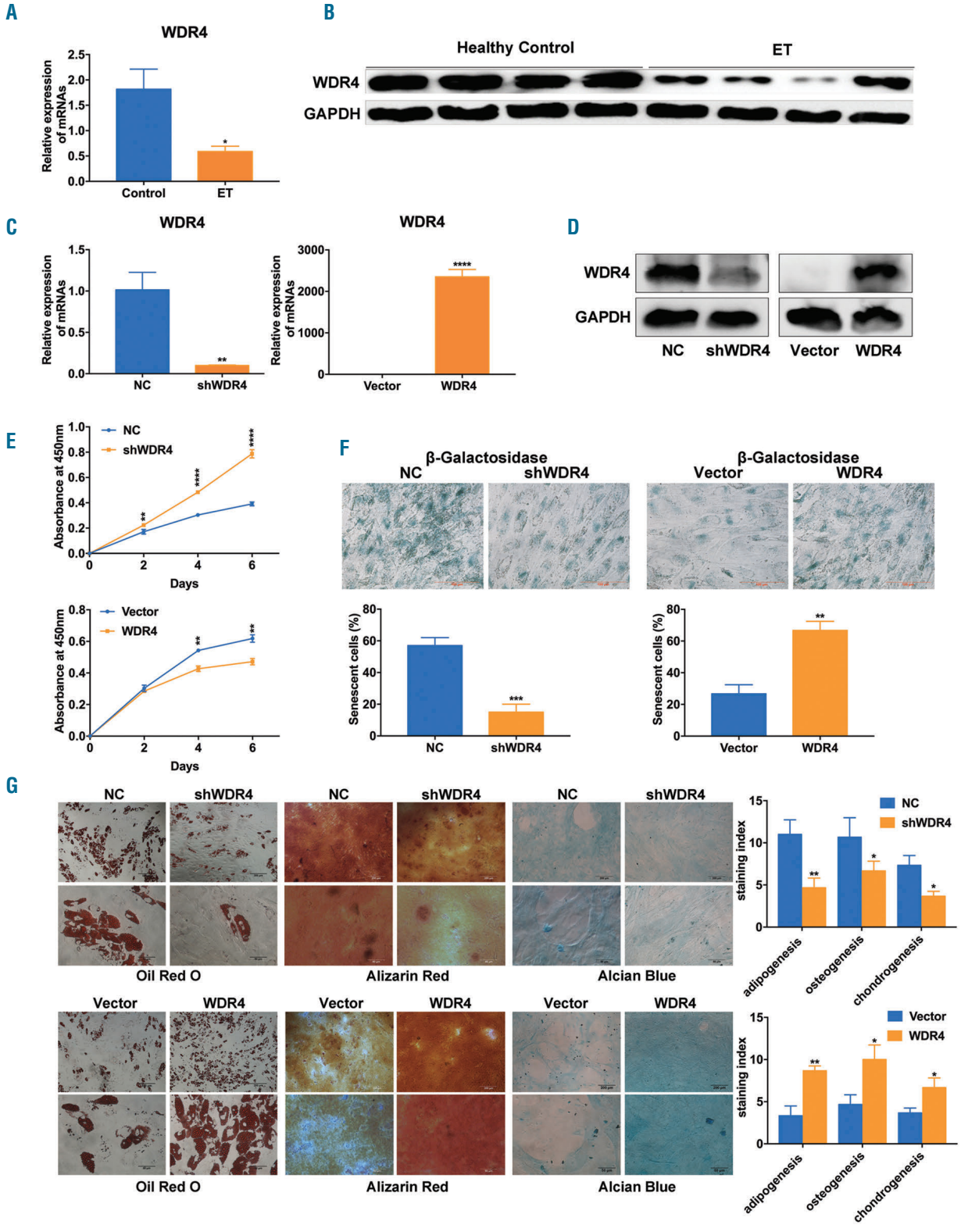

Figure 5. Low expression of WDR4 is correlated with enhanced proliferation, decreased senescence, and impaired differentiation of bone marrow derived mesenchymal stromal cells (BM-MSC) isolated from patients with *JAK2V617F*-positive essential thrombocythemia (ET). A. WDR4 mRNA expression in BM-MSC isolated from the controls (n=20) and patients with *JAK2V617F*-positive ET (n=15).B. WDR4 protein expression in BM-MSC isolated from the controls (n=4) and patients with *JAK2V617F*-positive ET (n= 4). C–D. WDR4 shRNA and cDNA decreased or increased WDR4 expression in BM-MSC efficiently, as determined by qPCR (C) and Western blotting (D). E. WDR4 cDNA treatment decreased the proliferative capacity of BM-MSC, while WDR4 shRNA treatment increased the proliferation of BM-MSC, as measured by the CCK-8 assay. F. WDR4 cDNA increased senescence of BM-MSC as measured by β-galactosidase staining while WDR4 shRNA had the opposite effect. G. WDR4 increased the differentiation potential of BM-MSC into adipocytes, osteocytes, and chondrocytes as indicated by Oil Red O, Alizarin Red, and Alcian Blue staining, respectively. MSC used in each assay were at passage four. All the experiments (except for the quantitation of WDR4 mRNA and WDR4 protein expression in clinical samples) were repeated at least three times. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as the mean ± SD (except for mRNA expression of WDR4 in clinical samples, mean ± SEM). ET: essential thrombocythemia; CCK-8: Cell Counting Kit 8; n: number of unique donors in each group; NC: normal control; SD: standard deviation; SEM: standard error of mean.

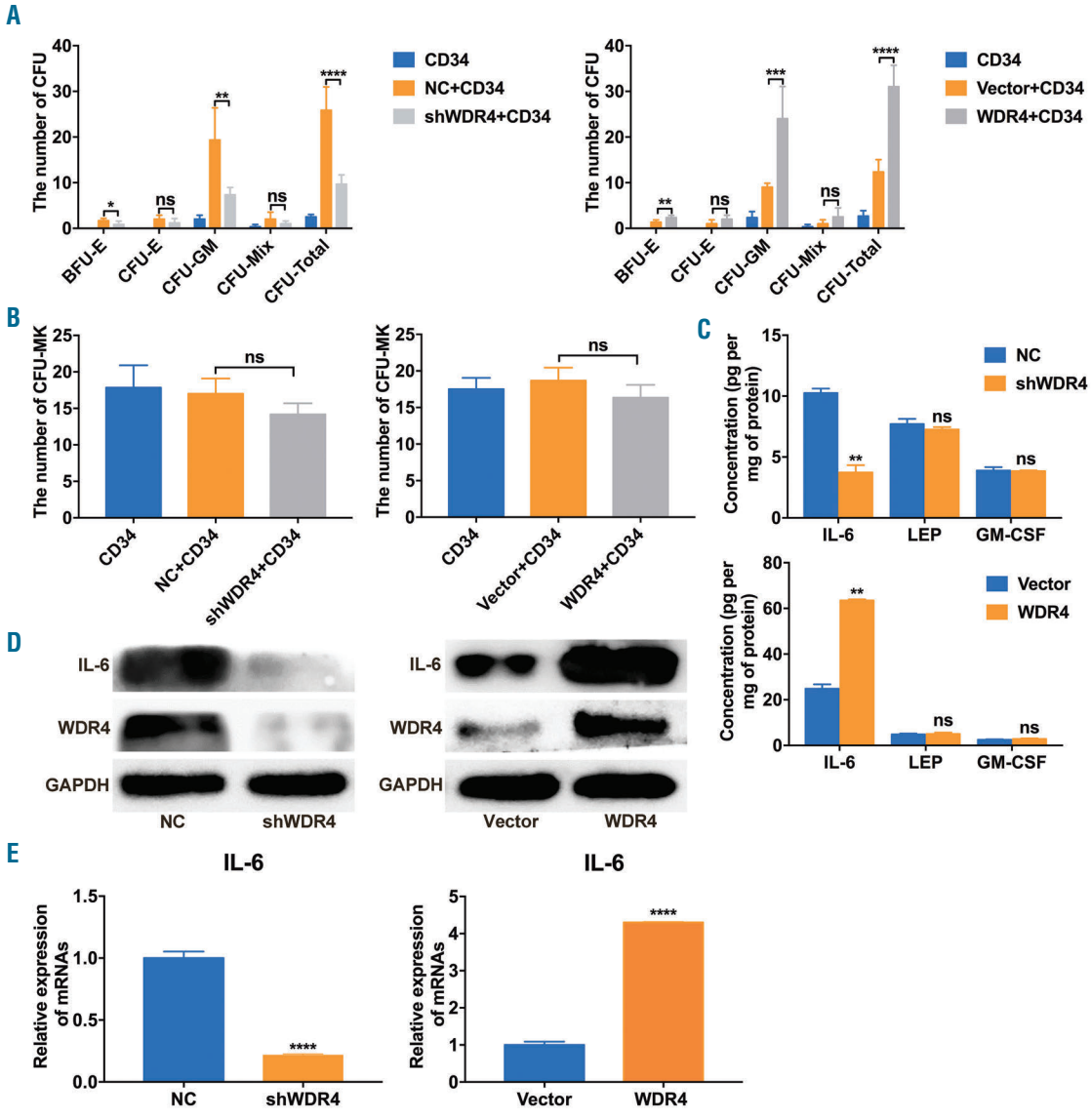

Figure 6. Insufficient action of the WDR4–IL-6 axis decreases hematopoiesis-supportive activities of bone marrow derived mesenchymal stromal cells (BM-MSC) from patients with *JAK2V617F*-positive ET. A–B. Numbers of BFU-E, CFU-E, CFU-GM, CFU-Mix, and CFU-MK formed by purified normal CD34-positive cells after coculture with BM-MSC infected with LV-shWDR4, or LV-WDR4. WDR4 increased the number of BFU-E, CFU-GM, and CFU-Total formed by normal CD34-positive cells (A). No changes were observed in the number of CFU-MK (B). C. WDR4 increased the secretion of IL-6 from BM-MSC as determined by ELISA on the supernatant obtained from the MSC cultures. D–E. WDR4 increased the intracellular expression of IL-6 in BM-MSC as determined by Western blotting (D) and qPCR (E). MSC used in each assay were at passage four. All the experiments were repeated at least three times. *P 0.05; **P<0.01; ***P<0.001; ****P<0.0001. Data are presented as the mean ± SD. qPCR: quantitative real-time polymerase chain reaction; CFU-E: colony-forming unit-erythroid; CFU-GM: colony-forming unit-granulocyte and macrophage; CFU-Total: total colony-forming units; CFU-MK: colony-forming unit-megakaryocyte; n: number of unique donors in each group; ns: not significant; NC: normal control; SD: standard deviation.

MSC infected with LV-shWDR4, the numbers of erythroid burst-forming units (BFU-E), CFU-GM, and CFU-Total were significantly lower than those in the control groups (Figure 6A). In contrast, increasing WDR4 expression corrected the ET MSC-mediated defects, as evidenced by higher numbers of BFU-E, CFU-GM, and CFU-Total relative to those in the control groups (Figure 6A). No changes were observed in the CFU-MK number (Figure 6B).

We next examined whether WDR4 regulated the hematopoietic cytokines mentioned above that were differentially produced between the two MSC samples. The results revealed a link between IL-6 and WDR4 (Figure 6C). We next performed qPCR and Western blotting to assess whether IL-6 expression was affected by WDR4. In WDR4 knock-down HD MSC, intracellular IL-6 protein (Figure 6D) and mRNA levels (Figure 6E) were decreased. Furthermore, in the ET MSC, restoration of WDR4 expression alleviated the decrease in IL-6 levels (Figure 6 D–E). Taken together, these results indicate that WDR4 promotes the intracellular expression and secretion of IL-6 by BM-MSC.

WDR4 acts through the ERK–GSK3β–CREB pathway to increase IL-6 expression and secretion by BM-MSC

To identify candidate kinases through which WDR4 may act on IL-6, we evaluated the levels of 43 phosphorylated kinases in HD MSC infected with LV-shWDR4 rela-

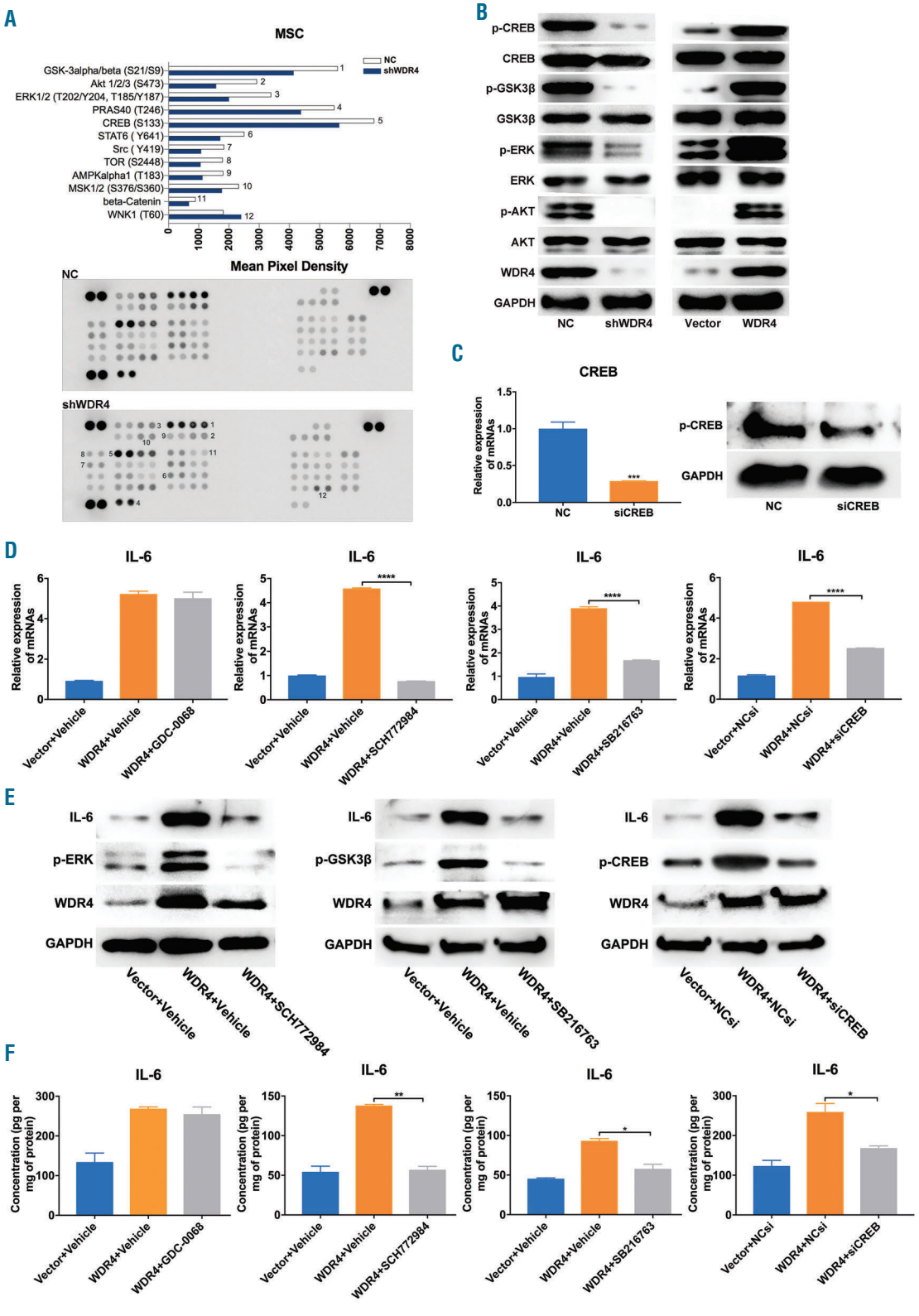

Figure 7. WDR4 acts through the ERK–GSK3β–CREB pathway to enhance IL-6 expression and secretion by bone marrow derived mesenchymal stromal cells (BM-MSC). A. Graphic representation of the quantification of 12 proteins with the most significant difference in phosphorylation status between MSC infected with LV-shWDR4 and MSC in the control group, as measured by a phospho-kinase array of 43 phosphorylated kinases. B. Western blot analysis of phosphorylation levels of GSK3β (S9), AKT1/2/3 (S472/S473/S474), ERK1/2 (T202/Y204, T185/Y187), and CREB (S133) in MSC infected with LV-shWDR4 or LV-WDR4 and their respective controls. C. CREB-specific siRNA decreased CREB expression in BM-MSC efficiently, as determined by qPCR and Western blotting. D–F. IL-6 induction by WDR4 over-expression was at least partially suppressed by an ERK1/2 inhibitor (SCH772984), GSK3 inhibitor (SB216763), or CREB-specific siRNA as determined by qPCR (D), Western blotting (E), and ELISA (F). MSC used in each assay were at passage four. All the experiments were repeated at least three times. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as the mean ± SD. qPCR: quantitative real-time polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; NC: normal control; SD: standard deviation.

tive to those in the controls. Among the kinases that were affected by LV-shWDR4 infection, GSK3β, AKT, ERK, and CREB are known to be upstream of IL-6 (Figure 7A). We next validated the results by Western blotting. Phosphorylation levels of GSK3β (S9), AKT1/2/3 (S472/S473/S474), ERK1/2 (T202/Y204, T185/Y187), and CREB (S133) significantly decreased with WDR4 knockdown in HD MSC and increased with WDR4-overexpression in ET MSC (Figure 7B).

To determine which kinases are involved in WDR4-mediated IL-6 upregulation, we used kinase inhibitors specific for AKT1/2/3 (GDC-0068), ERK1/2 (SCH772984), and GSK3β (SB216763), and an siRNA specific for CREB in ET MSC infected with LV-WDR4. We thereby found that inhibition of ERK1/2, GSK3β, or CREB could at least partially suppress WDR4-induced IL-6 upregulation, while inhibition of AKT1, -2, or -3 caused no significant effect as assessed with qPCR (Figure 7D), Western blotting (Figure 7E), and ELISA (Figure 7F). These findings indicate that WDR4 promotes IL-6 expression and secretion *via* the ERK–GSK3β–CREB signaling pathway in BM-MSC.

Neuropathy and aberrant expression of IL-1β in the BM of patients with *JAK2V617F*-positive ET

Markedly lower numbers of sympathetic nerve fibers and insheathing Schwann cells were found in the BM of patients with *JAK2V617F*-positive ET (Figure 8A). Norepinephrine, which is mainly secreted by sympathetic nerve fibers, was also significantly downregulated in ET BM (Figure 8C). Additionally, B3AR, a norepinephrine receptor, and IL-1β levels were upregulated in the BM of patients with *JAK2V617F*-positive ET (Figure 8B-D).

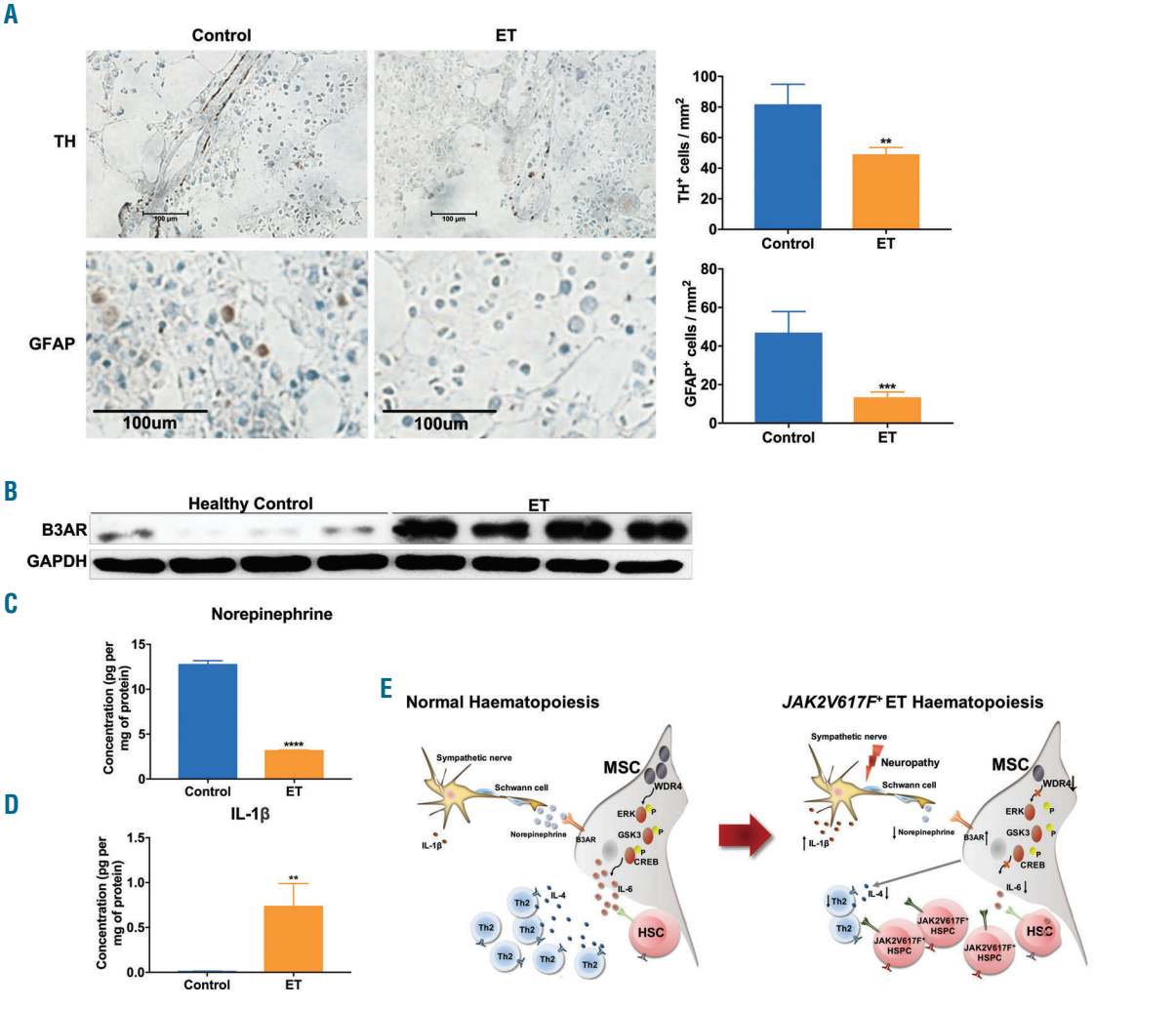

Figure 8. Neuropathy and aberrant expression of IL-1β in the bone marrow (BM) of patients with *JAK2V617F*-positive essential thrombocythemia (ET). A. Sympathetic nerve fibers quantitated with the help of an anti-TH antibody (control, n=9; ET, n=42), and Schwann cells visualized using an anti-GFAP antibody (control, n=9; ET, n=40) decreased in the BM of patients with *JAK2V617F*-positive ET relative to those in the HD, as determined by immunohistochemistry. B. Increased expression of B3AR in the BM of patients with *JAK2V617F*-positive ET (n=4) relative to the HD (n=4) as determined by Western blotting. C–D. Lower NE levels (C) (control, n=20; ET, n=20) and higher IL-1β levels (D) (control, n=20; ET, n=20) in the BM of patients with *JAK2V617F*-positive ET relative to the control, as measured by ELISA on the supernatant of the BM aspirates. E. A model illustrating BM hematopoietic dysfunction in *JAK2V617F*-positive ET. **P*<0.05; ***P*<0.01, ****P*<0.001; *****P*<0.0001. Data are presented as the mean ± SEM. ET: essential thrombocythemia; HD: healthy donors; n: number of unique donors in each group; TH: tyrosine hydroxylase; GFAP: glial fibrillary acidic protein; NE: norepinephrine; B3AR: β3 adrenoceptor; SEM: standard error of mean.

Discussion

In the present study, we observed transcriptional and functional abnormalities in the hematopoietic niche of patients with *JAK2V617F*-positive ET, including functional deficiency in MSC, immune imbalance, and sympathetic neuropathy, relative to those in HD controls. BM-MSC from patients with *JAK2V617F*-positive ET showed an altered transcriptome, faster proliferation, attenuated apoptosis and senescence, decreased potential to differentiate into adipocytes and osteocytes, and insufficient support for normal hematopoiesis. These findings partially agree with those found in previous studies of the myelodysplastic syndrome, leukemia, and MPN by other groups, but differ from the conclusions obtained from an MPN mouse model and patients.[7-9,19,34] NES-positive cells have been reported to be heterogeneous populations comprising mesenchymal cells and endothelial cells in the BM.[4] We co-stained bone marrow samples with NES and CD34 (endothelial cell marker), and found that patients with *JAK2V617F*-positive ET showed higher numbers of NES-positive mesenchymal cells in the BM relative to those in the HD controls. This observation contradicts the results obtained from the MPN mouse model and patients mentioned above but is consistent with the results obtained in patients with ET, polycythemia vera (PV), and primary myelofibrosis (PMF) by other group.[34] NES^-/leptin $receptor^-$/$CXCL12^+$ MSC subpopulations have been reported to be essential for the maintenance of HSC in mouse models.[35] Furthermore, in BM sections from patients with myelodysplastic syndrome, HSC are mostly in close contact with $CD271^+$/NES^- MSC.[36] Hock *et al*. confirmed that HSC with elevated proliferation rates were functionally compromised.[37] Therefore, given that MSC of patients with *JAK2V617F*-positive ET showed enhanced proliferation, we hypothesize that functional deficits are also present in these MSC. These studies, as well as our data, may explain the poor ability of the expanded MSC isolated from patients with *JAK2V617F*-positive ET to support normal hematopoiesis. Regarding the paradox with the mouse models of MPN, it is possible that the heterogeneity of the patients with MPN contributed to the observed discrepancy since our study involved only patients with *JAK2V617F*-positive ET as subjects, but not those with PV or PMF. Because of the low number of the primitive non-passaged BM-MSC (0.08% of BM mononuclear cells), MSC used for functional analysis were expanded *ex vivo* in the present work. It is possible that this process caused changes that were not completely consistent with the most primitive state *in vivo*.[38] Additionally, although mouse models are powerful tools for studying MSC *in vivo*, animals may not fully recapitulate the medical conditions in humans, and inter-species differences in structure, function, and immunophenotype may have contributed to the contradictory results. Additionally, *JAK2V617F* mutation is detected in approximately 95% of patients with PV and 50% of patients with ET and PMF, and mouse models of *JAK2V617F* show a tendency to develop a PV phenotype more often than ET.[39-40] Furthermore, the hematopoietic niches of different MPN phenotypes may differ to some extent. Therefore, both animal models and clinical specimens help us to better understand the intrinsic state of MSC under medical conditions in humans. The ratio of mutant to wild-type *JAK2* has been proved to be critical for the phenotypic manifestation.[41] Nonetheless, a successful model that accurately recapitulates the human manifestations of *JAK2*-positive ET is currently not available for us. Collectively, the present *in vitro* findings revealed a perturbed transcriptome and aberrant biological characteristics of BM-MSC of patients with *JAK2V617F*-positive ET.

It has been reported that expansion of BM CD4-positive T cells can lead to exhaustion of hematopoietic cells.[42] In this study, we found that MSC of patients with *JAK2V617F*-positive ET showed reduced inhibition of CD4-positive T-cell proliferation and activation, and secretion of the inflammatory cytokine sCD40L. In addition, they showed decreased induction of mostly immunosuppressive and antineoplastic Th2 cell formation, and secretion of the anti-inflammatory cytokine IL-4. Th2 formation is highly dependent on the activation of signal transducers and activators of transcription 6 by IL-4.[43] Thus, low secretion of IL-4 may be both the cause and consequence of blockage of the Th2 response. These results are mostly compatible with the results of previous studies.[14,15,42] Attenuated senescence of MSC from patients with *JAK2V617F*-positive ET was observed in the present study. Thus, one paradox arises, given that decreased senescence of MSC is typically linked to their anti-inflammatory status. The concept that MSC are highly plastic and the local inflammatory environment thus can shape the immunomodulatory effects of MSC may help to improve the understanding of the state of MSC in pathological processes. Specific inflammatory signals prompt MSC to switch between the proinflammatory and anti-inflammatory phenotypes.[44] One possible explanation for the contradiction between aging and their inflammatory phenotype is that aging-related changes may be compensated to some extent by the local inflammatory milieu. Additionally, MSC can be polarized by downstream Toll-like receptor (TLR) signaling into two homogenous phenotypes. TLR4-primed MSC mostly produce pro-inflammatory cytokines, while TLR3-primed MSC express mostly immunosuppressive cytokines.[45] TLR4 has been confirmed to inhibit senescence via epigenetic silencing of senescence-related genes.[46] Thus, another possible explanation is that upregulation of TLR4 and downregulation of TLR3 polarize the MSC from patients with *JAK2V617F*-positive ET to a proinflammatory phenotype with an anti-senescence effect. Collectively, these results indicate that MSC contribute at least partially to the immune imbalance in the BM of ET patients.

The changes mentioned above provide a possible link between the alterations in hematopoietic niches to the pathophysiology of *JAK2V617F*-positive ET in humans. Nonetheless, the underlying mechanisms remain unclear. In this study, we determined a mechanism whereby WDR4 deficiency impairs the ability of BM-MSC to support normal differentiation of hematopoietic progenitors in patients with *JAK2V617F*-positive ET. This effect occurs due to decreased IL-6 expression and secretion through suppression of the ERK–GSK3β–CREB pathway. Overall patients with MPN have been described to have higher IL-6 levels in the BM and there is published data on the oncogene-dependent mechanisms of fibroblasts expansion and IL-6 upregulation in fibroblasts in patients with *JAK2V617F*-positive MPN.[47] Nonetheless, in the present study, a prominent reduction in IL-6 levels was found in the supernatants of the culture medium of BM-MSC from patients with *JAK2V617F*-positive ET, with no obvi-

ous changes in IL-6 levels in the BM extract. This may be the case because the myelofibrosis grade of the patients enrolled in this study was 0-1. The significance of the hematopoiesis-supporting role of IL-6 has been well-documented.[24-27] Most HSPC are in contact with MSC in the BM, forming a highly sophisticated interaction network through direct contact and paracrine effects.[2] Thus, one possible explanation is that IL-6 levels in MSC contribute to the regulation of hematopoietic progenitors through local signals. Interestingly, our CFU assays showed that although some types of colonies depended on WDR4-IL6 axis, CFU-MK did not. *JAK2V617F* leads to increased HSC survival and biased differentiation to the lineages that signal through JAK2, including MK-platelets.[41] Therefore, it is reasonable to hypothesize that these *JAK2V617F* CFU-MK would outgrow other lineages that are more dependent on the WDR4-IL6 axis in the BM of ET patients. Further work is required. In the present study, CD34-positive cells cultured with MSC infected with LV-shWDR4 showed a different phenotype to those with ET MSC in terms of BFU-E. We observed a trend towards BFU-E reduction in patients with ET, but the difference was not significant. The difference is likely related to the diversity between these groups. Both groups treated with WDR4 shRNA or control shRNA showed similar characteristics in terms of clinical or laboratory features, while an overlap existed between BFU-E levels in patients and control samples, possibly because of the heterogeneity of clinical specimens and variable disease progression of the patients. Together these findings indicate that dysregulation of the WDR4-IL6 axis is involved in most dysfunctions in ET MSC.

Neuropathy was detected in the present study in BM sections of patients with *JAK2V617F*-positive ET. Although 10 μm sections are not optimal for accurate identification and quantification of BM fibers, no thicker sections were approved due to the scarcity of the clinical specimens. Aberrant expression of NE and B3AR were also detected in the BM of the patients. IL-1β, secreted by hematopoietic progenitors and many types of stromal cells, has been shown to mediate neuropathy in the BM of *JAK2V617F*-positive MPN mice and patients.[19] We also found elevated IL-1β levels in the BM extract of patients with *JAK2V617F*-positive ET, which may explain why neuropathy was detected. Neuropathy has been linked to the loss of NES-positive cells in MPN mice and patients,[19] while denervation of the BM in HD or patients with acute myeloid leukemia can lead to increased numbers of NES-positive cells.[9] A higher number of NES-positive cells was estimated in this study when thinner sections (5 μm) were used. Given that combining data derived from sections with different thickness may introduce significant bias, further studies are required to understand the relevance between neuropathy and NES-positive cells. IL-1β has previously been reported to upregulate NES,[48] suggesting that immune cues are involved in altering both the sympathetic nervous system and MSC. NE inhibits nuclear factor-κB activity and pro-inflammatory cytokine release by binding to B2AR and B3AR on MSC and other immune cells.[12,18] The decreased production of NE, possibly caused by increased neurotoxic inflammatory cytokines, in ET can facilitate the formation of an inflammatory environment. Further studies are required to elucidate the connection between MSC, immunity, and sympathetic nerves.

In summary, this study revealed multilevel defects in the hematopoietic microenvironment of patients with *JAK2V617F*-positive ET and demonstrated that one of the differentially expressed genes, WDR4, underlies most dysfunctions in ET MSC *via* downregulation of IL-6 expression and secretion through suppression of the ERK–GSK3β–CREB pathway. Activation of the JAK-STAT3 pathway through IL-6 binding and its receptor is essential for maintaining normal hematopoiesis. A study recently revealed that IL-6 stimulated JAK2-STAT3 signaling in PV and PMF, but suppressed this signaling in ET.[49] Our results, alongside with those of previous studies, provide a compelling rationale for exploring the in vivo effect of the WDR4-IL-6 axis against ET in the future. Due to technical limitations, we also suggested a number of hypotheses and potential explanations for the paradoxes with other groups and future work is required for their validation.

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