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RESEARCH ARTICLE

Novel germline *JAK2*^{R715T} mutation causing PV-like erythrocytosis in 3 generations. Amelioration by Ropeg-Interferon

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Abstract

Polycythemia vera (PV) is a clonal disorder arising from the acquired somatic mutations of the *JAK2* gene, including *JAK2*^{V617F} or several others in exon 12. A 38-year-old female had a stroke at age 32 and found to have elevated hemoglobin, normal leukocytes, normal platelets, and tested negative for *JAK2*^{V617F} and exon 12 mutations. Next generation sequencing revealed a novel mutation: *JAK2*^{R715T} in the pseudokinase domain (JH2) at 47.5%. Its presence in her nail DNA confirmed a germline origin. Her mother and her son similarly had erythrocytosis and a *JAK2*^{R715T} mutation. Computer modeling indicated gain-of-function JAK2 activity. The proposita and her mother had polyclonal myelopoiesis, ruling out another somatic mutation-derived clonal hematopoiesis. Some erythroid progenitors of all three generations grew without erythropoietin, a hallmark of PV. The *in vitro* reporter assay confirmed increased activity of the *JAK2*^{R715T} kinase. Similar to PV, the *JAK2*^{R715T} native cells have increased *STAT5* phosphorylation, augmented transcripts of prothrombotic and inflammatory genes, and decreased *KLF2* transcripts. The proposita was not controlled by hydroxyurea, and JAK2 inhibitors were not tolerated; however, Ropeginterferon-alfa-2b (Ropeg-IFN-α) induced a remission. Ropeg-IFN-α treatment also reduced JAK2 activity in the proposita, her mother and *JAK2*^{V617F} PV subjects. We report dominantly inherited erythrocytosis secondary to a novel germline

JAK2^{R715T} gain-of-function mutation with many but not all comparable molecular features to *JAK2*^{V617F} PV. We also document a previously unreported inhibitory mechanism of JAK2 signaling by Ropeg-IFN-α.

1 | INTRODUCTION

There are two types of erythrocytosis: primary and secondary. Primary erythrocytosis is characterized by intrinsic defects in erythroid progenitors, which can either grow independently of erythropoietin (EPO) or exhibit hypersensitivity to it. Subjects with primary erythrocytosis exhibit inappropriately low levels of EPO caused by congenital mutations in the *EPOR* gene (encodes the EPO receptor) or somatic mutations in the *JAK2* gene, resulting in polycythemia vera (PV). Secondary erythrocytosis is caused by high levels of erythropoiesis-stimulating factors such as EPO, cobalt, manganese, IGF1, and testosterone. It can also result from an appropriate response to hypoxia in environments of high altitude or from an increase in hemoglobin oxygen affinity caused by mutated hemoglobin and low 2,3-DPG levels. Inappropriate responses to hypoxia due to mutations in hypoxia-sensing and signaling genes, including *VHL*, *EGLN1*, *EPAS1*, and *EPO*, can also lead to secondary erythrocytosis.[1]

PV is a clonal disorder originating from a pluripotent hematopoietic stem cell. By way of gain-of-function somatic mutations of *JAK2*, primarily *JAK2*^{V617F}, and less so of *JAK2* exon 12, there is an overproduction of myeloid blood cells. The *JAK2* gene encodes a tyrosine kinase enzyme that is involved in the erythropoietin (EPO) signaling pathway as well as various other cytokine signaling[2] and growth factor pathways. *JAK2*^{V617F} includes those that stimulate the production of neutrophils and platelets, whereas *JAK2* exon 12 mutations are not associated with thrombocytosis or neutrophilia.[3] Murine models of *JAK2* exon 12 mutations also showed an increase in red blood cells but not neutrophils and platelets.[4] These mice had decreased hepcidin but increased erythroferrone, further facilitating already increased erythropoiesis[5] from augmented JAK2 signaling. The *JAK2* mutations found in PV result in a constitutively active *JAK2* protein. This leads to augmented STAT phosphorylation, uncontrolled cell growth, and some erythroid colonies (BFU-Es) growing without extrinsic erythropoietin (EPO).[6]

The *JAK2*^{V617F} mutation is the most common *JAK2* mutation. It is present in virtually all patients with PV,[7] ~55% of patients with essential thrombocythemia (ET), and in ~60% of patients with primary myelofibrosis.[8] The *JAK2* exon 12 mutations are found only in a small subset of PV patients. Previous knowledge regarding the development of myeloproliferative neoplasms tells us that these disorders are not caused by germline mutations but rather by acquired somatic mutations that occur and progress over a period of many years across a person's lifetime, possibly occurring as early as fetal life.

Compound heterozygous mutations (p.E846D, p.R1063H) of *JAK2*, causing both hereditary thrombocythemia and erythrocytosis without EPO-independent BFU-Es,[9] and the co-existence of two heterozygous germline mutations (p.T108A, p.L393V) with a somatic *JAK2*^{V617F} mutation[10] have been reported in PV. However, there is currently no evidence to suggest that a single *JAK2* germline mutation causes the full PV phenotype.

We report a novel gain-of-function germline mutation: *JAK2*^{R715T}, which results in PV-like erythrocytosis in three generations of a family. Treatment with Ropeg-IFN-α induced a hematological remission, revealing previously unreported interferon inhibition of JAK2 signaling.

2 | MATERIALS AND METHODS

2.1 | Patient information

A 38-year-old female had a stroke at age 32. She had an elevated Hb of 18 g/dL, normal leukocytes and platelets, and borderline low EPO. Her thrombophilia work-up was negative, and neither *JAK2*^{V617F} nor *JAK2* exon 12 mutations were detected. She had aquagenic pruritus and night sweats, and her bone marrow was interpreted as compatible with PV. Her 68-year-old mother and 11-year-old son were not previously known to have erythrocytosis nor any symptoms nor complications compatible with PV, but were both found to have elevated hemoglobin.

2.2 | Sanger sequencing

Genomic DNA (gDNA) was isolated from granulocytes and nails. In order to confirm the R715T mutation in granulocytes and nails, exon 17 of the *JAK2* gene was amplified using HotStarTaq Master Mix (Qiagen) and primers including F:5′ CATCCAACCCCTCCAAAATA 3′ and R: 5′ AATGCCACACCAACCTTCTT 3′. The PCR conditions were as follows: initial denaturation at 95°C for 15 min, followed by 33 cycles of 40 s of denaturation at 94°C, 40 s of annealing at 55.1°C, and 35 s of extension at 72°C, followed by a final extension step of 10 min at 72°C. The PCR product was cleaned using EXOSaplt (Thermo Fisher Scientific) to remove PCR primers and dNTPs. Sanger sequencing was performed by the DNA sequencing core at the University of Utah.

2.3 | Clonality assay

The X-chromosome transcription-based clonality assay was performed after testing 5 common exonic X-chromosome polymorphisms[11] and finding that both proposita and her mother were heterozygous, that is, informative for clonality using *BTK* SNP ID: rs1135363 exonic

polymorphism.[11] mRNA was isolated from granulocytes and platelets and reverse-transcribed into cDNA using SuperScript™ VILO cDNA synthesis kit (Thermo Fisher Scientific). Allelic frequencies in granulocytes and platelets were determined by Sanger sequencing. To measure the allelic frequency of rs1135363 exonic polymorphism in granulocytes and platelets, a portion of the *BTK1* transcript was amplified using HotStarTaq Master Mix (Qiagen) and primers including F: 5′ AGACTGCTGAACACATTGCC 3′ and R: 5′ ATTGGGGCTTGTGGAGAAGA 3′. The PCR conditions were as follows: initial denaturation at 95°C for 15 min, followed by 33 cycles of 40 s of denaturation at 94°C, 40 s of annealing at 52.2°C, and 35 s of extension at 72°C, followed by a final extension step of 10 min at 72°C. The PCR product was purified using EXOSaplt (Thermo Fisher Scientific) to remove PCR primers and dNTPs.

2.4 | Erythroid progenitor *BFU-E* colony assay

BFU-E were grown from peripheral blood mononuclear cells in the presence of various EPO concentrations at 0, 15, 30, 60, and 3000 mU/mL.[12] The number of BFU-E colonies was counted after 14 days of incubation.

2.5 | Mutagenesis and vector construction

To introduce the *JAK2*^{R715T} point mutation into a human *JAK2* WT cDNA cloned in pCMV6-AC-IRES-GFP-Puro vector, we amplified a 221-bp segment spanning two unique restriction sites, *XbaI* and *SwaI*, with a reverse primer bearing the mutation (AAATTTTTAGGATTTTCAATGCATT CAGGTGGTACCCATGGTATTgTCTCC). The PCR fragment was then cloned into the pGEM-T Easy vector (Promega). Both plasmids were then digested using *XbaI* and *SwaI* enzymes and ligated to get the final pCMV6-AC-IRES-GFP-Puro *JAK2*^{R715T} vector.

2.6 | Dual luciferase assays for STATs activity assessment

HEK-293 cells were transiently transfected with *JAK2* WT, R715T, and V617F, pGRR5 reporter, STAT5/3/1, p-RLTK, EPOR, TPOR, and GCSFR, at a 3:3:1:1:3 ratio, using Lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours post-transfection, the luciferase production was quantified in cell lysates with the Dual-Luciferase® Reporter Assay kit (Promega) on a Glomax 20/20 luminometer (Promega). Firefly:Renilla luciferase ratios were calculated for each condition in order to normalize the STATs activities for different *JAK2* constructs.

2.7 | BA/F3-EPOR viability assay

The Ba/F3-EPOR cell (1×10^6 cells per sample) were nucleofected with 10 ug pCMV6-AC-IRES-GFP-Puro wild type *JAK2*, *JAK2*^{V617F}, and *JAK2*^{R715T} variants using the NEON device protocol – 1600 V, 10 ms, and 3 pulses (Thermo Fisher Scientific). Proliferation of cells in the absence or presence of EPO (1 U/mL) was quantified 48 hours after transfection by Alamar Blue reagent (Thermo Fisher Scientific) on a Perkin-Elmer Envision analyzer.

2.8 | In vitro erythroid progenitor expansion and p-STAT5 measurement

Erythroid progenitors were expanded *in vitro* from blood mononuclear cells in the presence or absence of JAK1 and JAK2 inhibitor ruxolitinib,[13,14] and collected and counted at various time points. Cell proliferation was assessed by counting the cells and expressed as fold changes relative to the initial count on Day 1. To measure p-STAT5, cells were fixed in 1 mL of cold 0.05% glutaraldehyde for 10 min at room temperature. The cells were washed with staining buffer (BD Bioscience) and permeabilized with 0.1% Triton X-100 for 10 min at room temperature and stained using FITC-CD71, PE-CD235a, and Phosflow™ PE-Cy™7 Mouse anti-Stat5 (pY694) (BD Bioscience). The mean fluorescence of PE-Cy™7 was measured in erythroid progenitors ($CD71^+/CD235a^+$) by the BD LSRFortessa™ Cell Analyzer.

2.9 | Measurement of expression levels of HIF-regulated, inflammatory, and thrombotic genes

Granulocytes and platelets were separated from peripheral blood using Ficoll-Paque/density gradient centrifugation, using the methodology described previously.[15] Total RNA was extracted from granulocytes and platelets using Tri-Reagent per the manufacturer's protocol (Molecular Research Center). cDNA was made from RNA using the SuperScript™ VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Expression levels of selected genes were measured in granulocytes and platelets by qRT-PCR (quantitative reverse transcription polymerase chain reaction) using TaqMan Expression Assays (Thermo Fisher Scientific). Expression levels were normalized against *RPL13A*, calculated by ΔΔCt method, and expressed as fold change as described previously.[16]

3 | RESULTS

3.1 | The *JAK2*^{R715T} mutation is germline

Known *JAK2* mutations previously reported in PV were not detected in the proposita. However, the *Myeloid Malignancies Mutation Panel* by Next Generation Sequencing (NGS) at the University of Utah's ARUP Laboratories revealed a mutation (c.2144G > C, p.Arg715Thr) (Figure 1A) in exon 17 of *JAK2* at a 47.5% allelic frequency. This mutation was confirmed by Sanger sequencing in her granulocytes, and its presence in her nail DNA established a germline origin (Figure 1B). Arg715 is highly conserved across species and across other members of the Janus Kinase (*JAK*) family (Figure 1C). The same *JAK2*^{R715T}

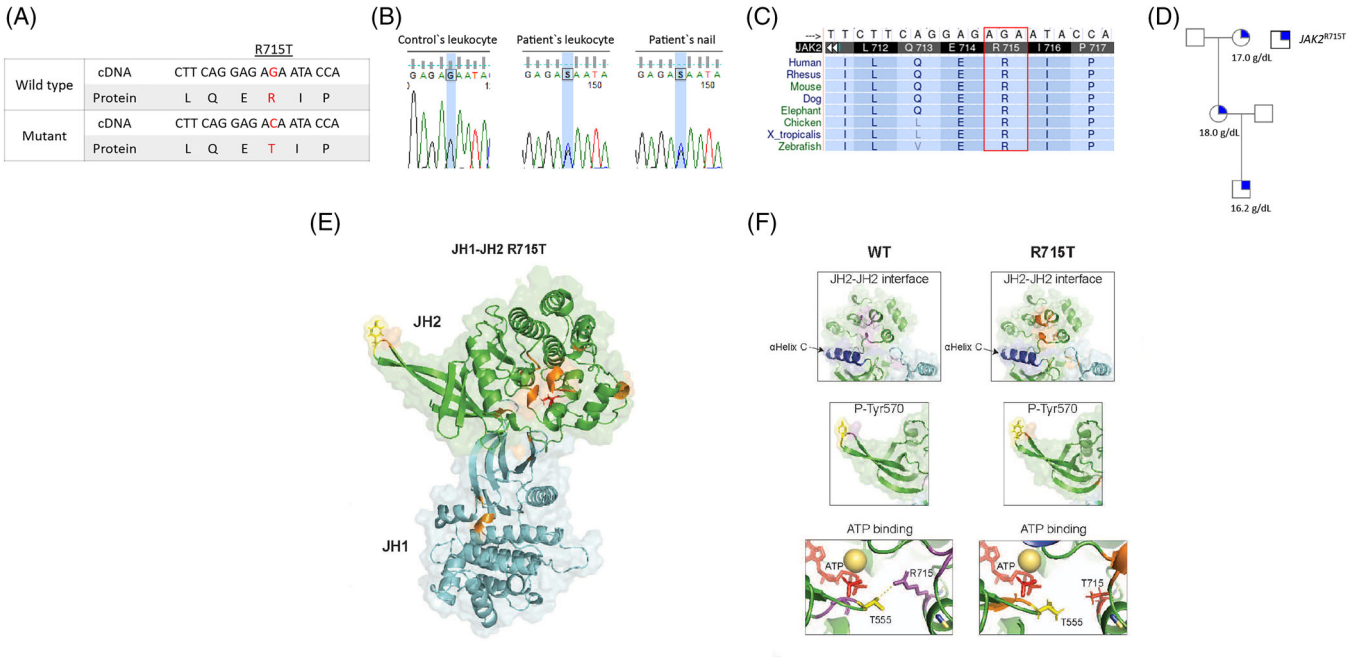

FIGURE 1 *JAK2*^{R715T} mutation detected in PV patients in three generations induced rigid *JAK2* structure. (A) The schematic sequence of *JAK2* with and without the R715T mutation; the mutation site is highlighted in red. (B) Sanger sequencing analysis of the *JAK2*^{R715T} mutation in the patient's nails and leukocytes. (C) Comparison of amino acid sequences of *JAK2* residues from 711 to 717 from different species. R715 residue is in red box. (D) Pedigree of three generations with a heterozygous R715T mutation. Hemoglobin levels were added to the affected individuals below. (E) Protein modeling of the JH1-JH2 domains of *JAK2*^{R715T} and *JAK2*^{WT} using AlphaFold 2.0. JH2 domain is colored green, and JH1 is in cyan. Secondary structure changes compared to *JAK2*^{WT} are highlighted in orange, and Y570 is highlighted in yellow. (F) Comparison of key regions of JH2 between *JAK2*^{R715T} and *JAK2*^{WT}. Regions with different secondary structures are highlighted in purple (*JAK2*^{WT}) and orange (*JAK2*^{R715T}). α-Helix C is shown in dark blue, Y570 and T555 in yellow, and ATP and T715 in red. [Color figure can be viewed at wileyonlinelibrary.com]

mutation was also found in her 11-year-old son and her 68-year-old mother; it was absent in her father, who had normal red cell indices. Her son and mother also had elevated Hb at 16.2 g/dL (normal range: 11.5–15.5 g/dL) and 17.0 g/dL (normal range: 12.6–15.9 g/dL), respectively; repeat testing confirmed persistent elevations (Figure 1D). The deceased brother of the proposita had normal blood counts and his DNA was not available for testing.

3.2 | Proposita and mother have polyclonal hematopoiesis

After testing 5 common exonic X-chromosome polymorphisms[11], we found that both the proposita and her mother were heterozygous for the *BTK* SNP ID: rs1135363 exonic polymorphism. We then measured the transcript levels of the X-chromosome *BTK* rs113536 allele and found that transcripts with both alleles were expressed, indicating that the proposita and her mother had polyclonal granulocytes and platelets. Therefore, erythrocytosis was not caused by clonal hematopoiesis from an additional somatic mutation.

3.3 | Impact of 715 on protein structure

The *JAK2*^{R715T} is a gain-of-function mutation, as predicted by DynaMut,[17] to cause a change in fold stability (ΔΔG) of 1.390 kcal/mol of the R715T mutation. To explore the impact of the R715T mutation on kinase activation in detail, AlphaFold 2.0[18] was used to model the JH1-JH2 domains of *JAK2*^{R715T} and *JAK2*^{WT} (Figure 1E,F). In *JAK2*^{WT}, R715 lies next to the JH2 ATP-binding pocket and promotes a closed loop conformation through interaction with T555 of the nucleotide-binding loop (Figure 1F), aligning with the structure of the *JAK2* JH2 domain.[19] In *JAK2*^{R715T}, loss of this interaction results in a more opened-lobe conformation and disturbs ATP binding to JH2 (Figure 1F), known to regulate *JAK2* kinase activity. In addition, the overall structure of the *JAK2*^{R715T} JH2 domain exhibited increased rigidity compared to *JAK2*^{WT}. A detailed comparison of *JAK2*^{R715T} with *JAK2*^{WT} JH2 indicated that the rigidification of the structure involved the JH2-JH2 *trans*-dimerization domain centered around α-helix C[20] (Figure 1F) and a lengthening of the ß-sheet surrounding the auto-inhibitory phosphorylation site Y570.[21]

3.4 | *JAK2*^{R715T} mutation activates JAK–STAT signaling

The signal transduction of *JAK2* variants, measured by STATs activation, was evaluated using dual luciferase assays with STATs responsive element reporter plasmid in HEK-293 cells. In the presence of the EPOR, *JAK2*^{R715T} induced a high constitutive activity of STAT5 and STAT3, but not STAT1, comparable to

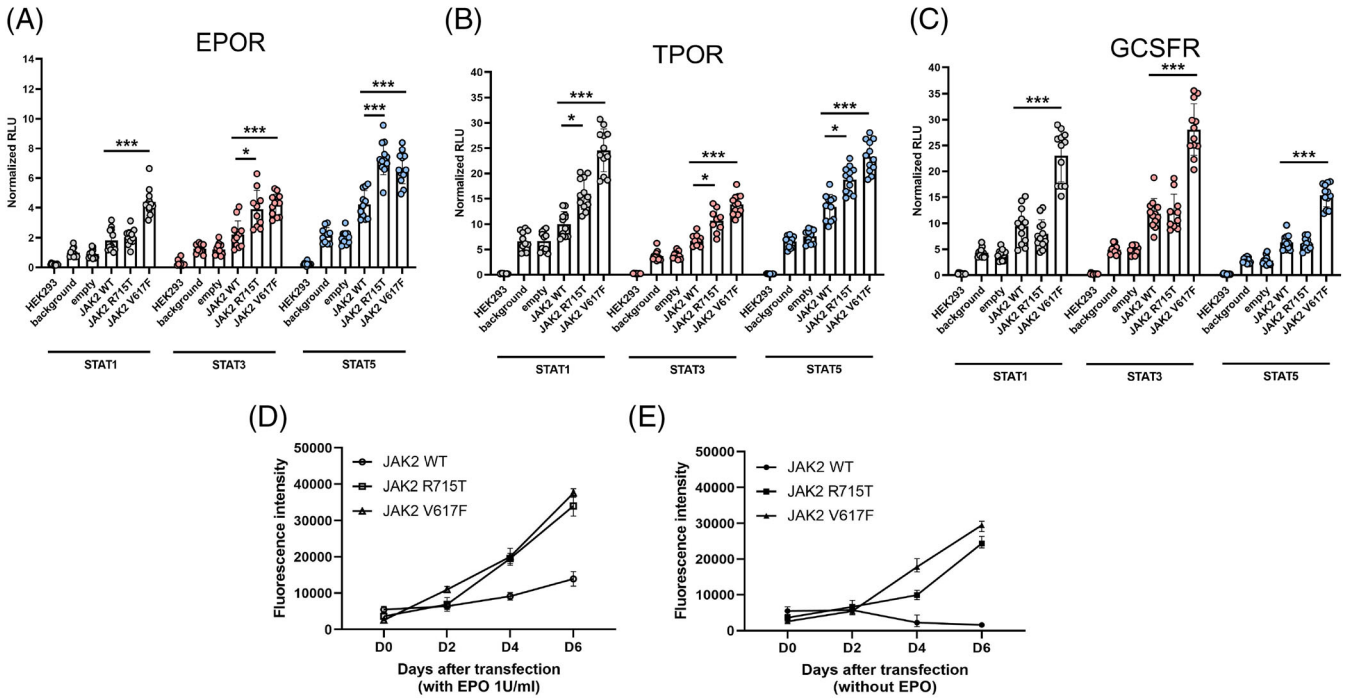

FIGURE 2 *JAK2*^{R715T} activates JAK-STAT signaling. Constitutive STAT5/3/1 activity was assessed by a dual-luciferase assay in HEK-293 cells transfected with *JAK2*^{WT}, *JAK2*^{R715T}, and *JAK2*^{V617F} in the presence of (A) EPOR, (B) TPOR, and (C) GCSFR. Shown are the averages of 12 replicates (± SD) from three independent experiments. Statistical analysis was performed using GraphPad Prism 8.4.3. *p < .001, ***p < .00001, Student's unpaired t test with unequal variance. Proliferation of BAF-3/EPOR cells in the (D) presence/(E) absence of EPO (1 U/mL). Data are shown as fluorescence intensity after 4 h of incubation with Alamar blue reagents. Results are shown as the mean (± SD) of two independent experiments performed in triplicate. [Color figure can be viewed at wileyonlinelibrary.com]

JAK2^{V617F} (Figure 2A). With thrombopoietin receptor (*TPOR*) expression, STAT1, STAT3, and STAT5 activities were induced by *JAK2*^{R715T} more compared to *JAK2*^{WT} but less compared to *JAK2*^{V617F} (Figure 2B). However, *JAK2*^{R715T} did not affect the activities of these three STATs in the presence of the granulocyte colony-stimulating factor receptor (GCSFR) (Figure 2C). Transfected Ba/F3-EPOR cells by *JAK2*^{R715T} and *JAK2*^{V617F} had similar proliferation rates in the presence or absence of EPO (Figure 2D,E).

3.5 | *JAK2*^{R715T} augments *JAK2* kinase activity and erythroid hyperproliferation

STAT5 phosphorylation was assessed in the blood erythroid progenitors (CD71+/CD235a+) of the patient, her mother, her son, 23 patients with *JAK2*^{V617F}-mutated PV, and 13 healthy controls. We found that the proposita and her mother had increased *JAK2* kinase activity at levels higher than those observed in *JAK2*^{V617F} positive PV patients (Figure 3A). Additionally, the proposita's erythroid progenitors had increased proliferation (Figure 3B) that exceeded that of patients with PV. *JAK2* inhibitor ruxolitinib decreased the proliferation of progenitors in the proposita and PV controls (Figure 3C).

3.6 | Erythroid progenitors grew without extrinsic EPO

EPO-independent BFU-E is a hallmark of PV.[22] To evaluate the effect of *JAK2*^{R715T} in response to EPO in erythroid cells, erythroid progenitors from the proposita, her mother, and her son were grown for 14 days with various concentrations of EPO together with healthy controls and 2 PV patients. Some erythroid progenitors (BFU-E) of the proposita, her mother, and her son grew without extrinsic EPO (Figure 3D). The percentage of EPO-independent erythroid cells was comparable between *JAK2*^{R715T} and *JAK2*^{V617F} erythroid cells in both of the PV patients.

3.7 | Hypoxia-inducible factor (HIF) transcriptional activity

In PV and ET, the transcriptional activity of HIFs is augmented in neutrophils and platelets.[23] We thus measured transcript levels of *SLC2A1* and *VEGFA*, which are HIF-targeted genes, to assess the transcriptional activity of HIFs in *JAK2*^{R715T} granulocytes and platelets. These two HIF target genes were expressed more in patients with PV and ET compared to controls in both granulocytes and platelets (Figure 4A). In our three affected individuals we evaluated the following: we assessed 4 different time points of the proposita and her mother, both treated with fedratinib

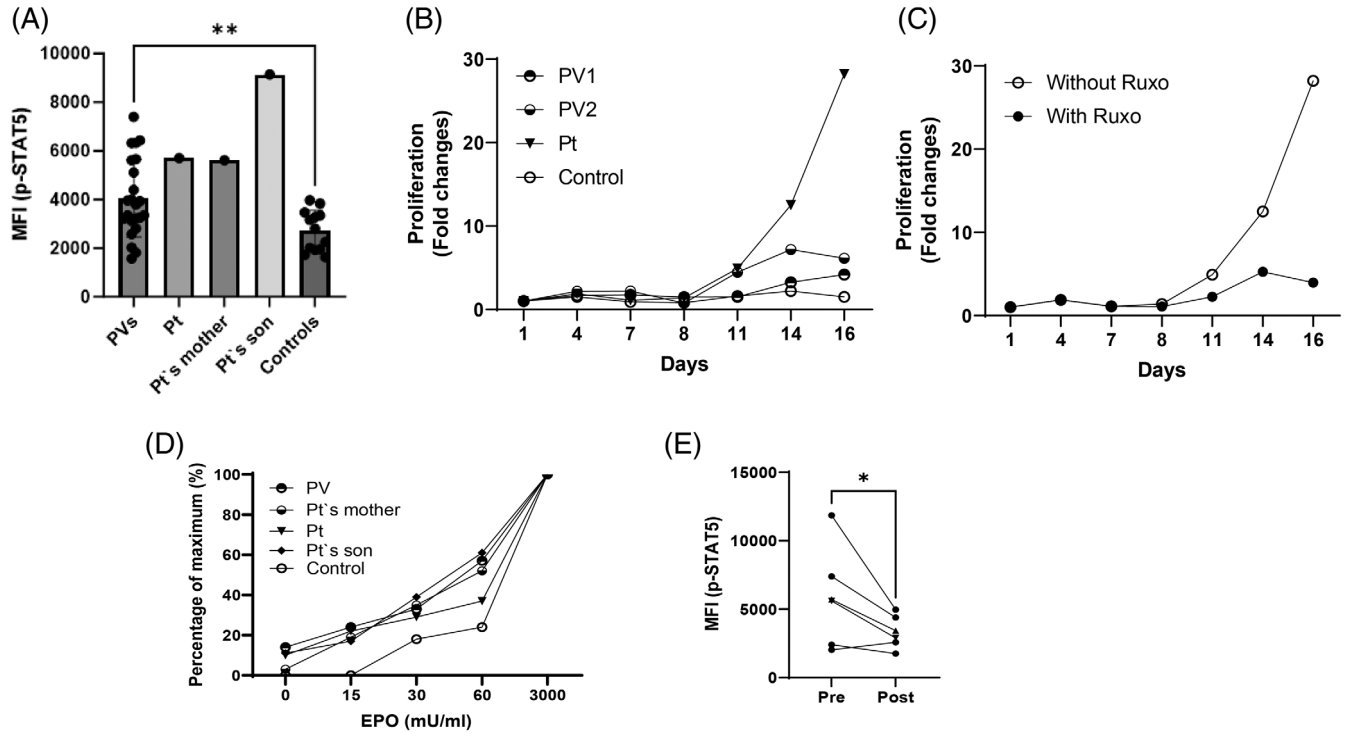

FIGURE 3 *JAK2*^{R715T} augmented *JAK2* kinase activity, leading to increasing erythroid cell proliferation. (A) The mean fluorescence intensity (MFI) of phosphorylated STAT5 in erythroid progenitors (CD71+/CD235a+) was measured by FACS analysis. (B) The proliferation of erythroid progenitors expanded *in vitro*. Cell proliferation was calculated as fold changes from day 1. (C) Ruxolitinib (Ruxo) treatment decreased cell proliferation of erythroid progenitors expanded *in vitro*. (D) Epo-dependent and independent growth of BFU-E colonies. (E) MFI of phosphorylated STAT5 in erythroid progenitors was measured before (pre) and after (post) Ropeg-IFN-a in *JAK2*^{V617F} mutated PV and ET patients. Proposita and her mother were marked as an up-pointing triangle and down-pointing triangle, respectively.

and hydroxyurea, respectively, and 2 time points of her son, who was without treatment. Their granulocytes and platelets did not overexpress the aforementioned HIF-targeted genes. (Figure 4A).

3.8 | Inflammatory and thrombotic gene expression

PV and ET patients have higher expression of inflammatory and thrombotic genes in their granulocytes and platelets.[23] We thus measured the expression of inflammatory genes *CXCL8* and *TNF* and thrombotic genes *SERPINE1*, *F3*, *THBS1*, and *KLF2* in granulocytes and platelets. Like PV and ET, *CXCL8* expression was upregulated in the granulocytes and platelets of *JAK2*^{R715T}-mutated individuals. Upregulated *TNF* was observed only in granulocytes but not in platelets, while *TNF* was upregulated in both granulocytes and platelets of PV and ET (Figure 4B). *SERPINE1* was upregulated in PV and ET granulocytes and platelets but was comparable to controls in granulocytes and was downregulated in the platelets of *JAK2*^{R715T}-mutated individuals. *F3* was upregulated in both granulocytes and platelets in PV and ET and was upregulated only in granulocytes but not in platelets in *JAK2*^{R715T} subjects. While *THBS1* was upregulated in both PV and ET granulocytes and platelets, *THBS1* was upregulated in granulocytes but downregulated in platelets. *KLF2*, an anti-thrombotic gene, is downregulated (Figure 4B,C) in PV and ET granulocytes and platelets. However, *KLF2* expression was comparable to controls in both cell types of these individuals (Figure 4C). These data demonstrate that some, but not all, inflammatory and thrombotic genes are similarly dysregulated in *JAK2*^{R715T} when compared to PV and ET subjects.

3.9 | Proposita's therapy response

After failure to achieve a remission on hydroxyurea, treatment with the *JAK2* inhibitor fedratinib (as ruxolitinib was not approved by insurance) normalized the proposita's hemoglobin to 15.2 g/dL. However, fedratinib was discontinued due to side-effects. The proposita was then started on a low dose of Ropeg-IFN-α: 50 mcg injected subcutaneously every 14 days. Unexpectedly, she achieved complete hematological remission. Her mother later started on Ropeg-IFN-α with similar results. Due to a delay in the resupply of Ropeg-IFN-α, both patients relapsed. Upon restarting Ropeg-IFN-α, they both responded again and have continued to maintain a complete hematological remission. The proposita's aquagenic pruritus and night sweats have fully resolved. Her now 12-year-old son is asymptomatic and has not yet initiated therapy.

In regard to the anti-thrombotic gene *KLF2*, which is decreased in untreated PV and ET patients, we found that, unlike with

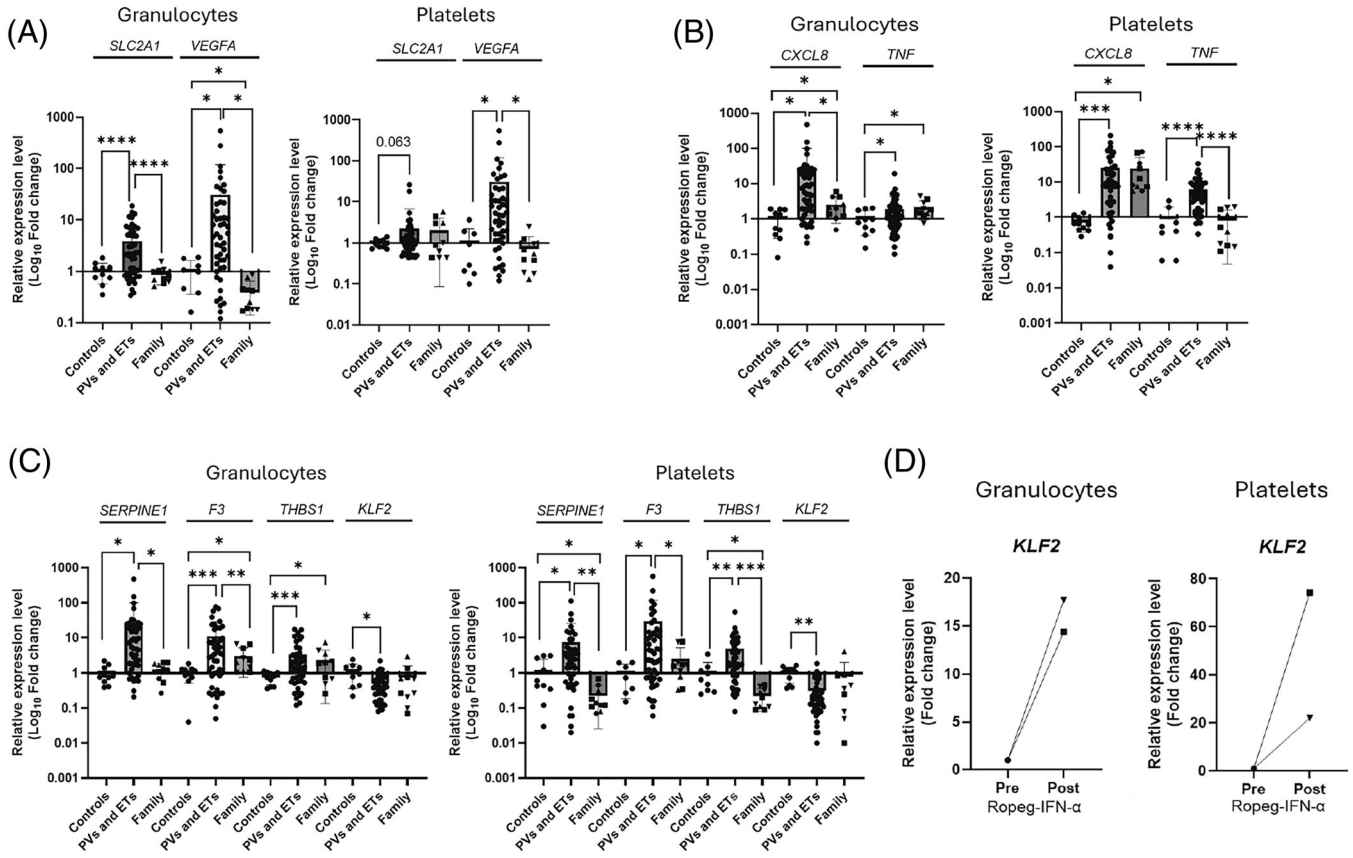

FIGURE 4 Expression levels of HIF-regulated inflammatory, and thrombotic genes in granulocytes and platelets. (A) HIF-regulated genes (*SLC2A1* and *VEGFA*), (B) inflammatory genes (*CXCL8* and *TNF*), and (C) thrombotic genes (*SERPINE1*, *F3*, *THBS1*, and *KLF2*) expression levels were measured in granulocytes and platelets of controls, patients with polycythemia vera (PV) and essential thrombocythemia (ET), and individuals with the *JAK2*^{R715T} mutation (Family). The expression levels were calculated against controls set as 1 and expressed as Log10 fold changes. P value was calculated by a paired *t*-test using GraphPad v10. 1. 0. ****$p < .0001$, ***$p < .001$, **$p < .01$, and *$p < .05$. (D) *KLF2* expression levels in granulocytes and platelets of the proposita (square) and her mother (upside-down triangle) before and after Ropeg-IFN-α treatment. The expression levels were calculated against the pre-treatment set as 1 and expressed as fold changes.

hydroxyurea, this decrease was partially ameliorated by treatment with Ropeg-IFN-α.[24] We also observed that Ropeg-IFN-α treatment increased *KLF2* expression in granulocytes and platelets of both the proposita and her mother (Figure 4D).

3.10 | Ropeg-IFN-α ameliorates *JAK2* activity

Treatment with Ropeg-IFN-α in PV specifically targets clonal *JAK2*-mutated cells, as evidenced by normalization of blood cell counts[25] and reduction of the *JAK2*^{V617F} variant allele frequency,[26] demonstrating its specific targeting of *JAK2*^{V617F}-mutated cells.[27] Given this known mechanism, we did not expect a complete response in polyclonal germline-mutated *JAK2*^{R715T} cells. However, to our surprise, complete hematological remission was achieved not only in the proposita but also in her mother. This previously unreported interferon suppression of *JAK2* signaling (Figure 3E, proposita and her mother marked in up-or down-pointing triangles, respectively) was then also verified in *JAK2*^{V617F}-mutated PV and ET patients treated by Ropeg-IFN-α (Figure 3E).

4 | DISCUSSION

The proposita, with a history of thrombosis, was referred by her hematologist to one of us (JTP) for consultation due to high hemoglobin levels. Neither *JAK2*^{V617F} nor *JAK2* exon 12 mutations were detected. The Myeloid Malignancies Mutation Panel by NGS identified a mutation (c.2144G > C, p.Arg715Thr) with a 47.5% allelic frequency, suggesting the possibility of a germline mutation. We then also found this mutation in her nail DNA and in her mother and her son, who were not previously known to have abnormal blood counts. However, on repeated testing, they both had consistent erythrocytosis. This data confirms that this dominantly inherited germline mutation is associated with erythrocytosis.

Somatically mutated hematopoietic stem cells (HSCs) of PV render the normal HSCs dormant, resulting in clonal hematopoiesis.[26–28] In female embryos, X-chromosome inactivation occurs randomly. Therefore, normal female tissue is a mixture of cells with either active paternal or maternal X-chromosomes, but not both, leading to genetic mosaicism. In our experience, all PV females, unless treated with interferon, express a single, identical active

X-chromosome in myeloid cells (clonality).[28,29] Thus, clonal hematopoiesis caused by acquired somatic *JAK2* mutations is one of the cardinal features of PV. To determine if *JAK2*^{R715T} may be associated with clonal hematopoiesis by an unrelated concomitant somatic mutation, we tested for the presence of clonality and observed polyclonal granulocytes and platelets in the proposita and her mother, excluding this possibility.

The proposita and her affected mother had low to normal levels of EPO (13 and 14 mU/mL, respectively), but their erythroid progenitors grew without EPO. The ability of erythroid progenitors to grow without EPO was further confirmed by Ba/F3-EPOR cells transfected with *JAK2*^{R715T} and *JAK2*^{V617F}. The similar proliferation rate of Ba/F3-EPOR *JAK2*^{R715T} and *JAK2*^{V617} transfected cells further confirms that their erythrocytosis is primary erythrocytosis and that the *JAK2*^{R715T} mutation is its cause.

The erythrocytosis phenotype was observed in all three *JAK2*^{R715T}-affected individuals without the thrombocytosis and leukocytosis commonly observed in *JAK2*^{V617F}-mutated patients. The absence of thrombocytosis and leukocytosis is also seen in *JAK2* exon 12 mutations,[4] which suggests that the *JAK2*^{R715T} mutation might exhibit limited gain-of-function characteristics in other myeloid cells. Thus, we evaluated the kinase activity of *JAK2*^{R715T} and its interactions with EPOR, TPOR, or GCSFR through the measurement of STATs activation using dual luciferase assays with a reporter plasmid responsive to STATs activation. *JAK2*^{R715T} was found to activate STAT3 and STAT5 but not STAT1 in the presence of EPOR, whereas *JAK2*^{V617F} activated all three STAT proteins. Patients with PV show elevated phosphorylation of both STAT5 and STAT3, whereas patients with ET display high STAT3 phosphorylation but low STAT5 phosphorylation,[30] further confirming that the *JAK2*^{R715T} induces an erythrocytosis-like phenotype. The high *JAK2* activity of *JAK2*^{R715T} was also confirmed by measuring phosphorylated STAT5 in reticulocytes in peripheral blood. Treatment with ruxolitinib (a JAK1 and *JAK2* inhibitor) decreased the proliferation of erythroid progenitors. These data suggest that upregulated STAT5 activity and *JAK2* activity by *JAK2*^{R715T} accounted for the high hemoglobin levels in the proposita and her *JAK2*^{R715T} affected relatives. The downregulation of STAT1 activity in CD34+ cells from *JAK2*^{V617F}-heterozygous ET progenitors resulted in a PV-like phenotype, while overexpression of STAT1 increased megakaryocyte production.[31] The elevated levels of phosphorylated STAT1 were reported in *JAK2*^{V617F} heterozygous ET BFU-Es compared to wild-type ET BFU-Es, but in PV patients, phosphorylated STAT1 levels showed no difference, indicating a specific association of STAT1 with megakaryopoiesis.[32] Interaction between *JAK2*^{R715T} and TPOR induced activation of all STATs; however, the affected individuals do not have thrombocytosis, possibly because the activation of STATs by *JAK2*^{R715T} was less pronounced than that by *JAK2*^{V617F} (Figure 2B). Erythroid progenitors respond to thrombopoietin (TPO), and its synergistic effect with EPO increases erythrocyte production.[33,34] This implies that enhanced TPOR-*JAK2*^{R715T}-interaction activated STAT signaling may further stimulate erythropoiesis. A basal level of STAT3 phosphorylation is sufficient for the differentiation of myeloid progenitor cells into neutrophils, a process enhanced by granulocyte-colony stimulating factor (GCSF) treatment or the overexpression of STAT3.[35] The activation of STAT3 is also associated with erythropoiesis.[36] High STAT3 activity, induced by *JAK2*^{R715T} and its interaction with EPOR, corresponds with high hemoglobin in these affected individuals. The absence of STAT activation by *JAK2*^{R715T} in the GCSFR reporter assay likely explains the normal white blood cell counts in all 3 generations (Figure 2C).

Although INF-α was not our preferred therapy, the proposita has been treated with Ropeg-IFN- α because of her intolerance of hydroxyurea and fedratinib. Ropeg-IFN-α, a mono-pegylated version of IFN-α-2b, has improved tolerability, an extended half-life compared to Peg-IFN-α-2b, and is FDA approved for the treatment of PV. Ropeg-IFN-α treatment normalizes blood cell counts in PV and ET by reducing the *JAK2*^{V617F} variant allele frequency through its ability to selectively target *JAK2*^{V617F}-mutated cells[27] and better targets homozygous *JAK2*^{V617F}-mutated cells. IFN-α treatment also restores dormant normal HSCs, leading to the conversion of clonal to polyclonal myelopoiesis.[29,37] The proposita and her mother have achieved a complete hematological remission with Ropeg-IFN-α. As all the proposita's blood cells were heterozygous for *JAK2*^{R715T}, such a response was unexpected. We then demonstrated that Ropeg-IFN-α reduces *JAK2* activity, resulting in the suppression of augmented myelopoiesis, which we also confirmed in *JAK2*^{V617F}-mutated PV and ET samples studied before and after Ropeg-IFN-α treatment. These findings reveal a novel beneficial molecular mechanism of Ropeg-IFN-α therapy.

Thromboses are a major cause of morbidity and mortality in PV and ET.[38] As the proposita also experienced thrombosis, we examined the expression of genes associated with thrombosis, many of which are regulated by HIFs.[23] Multivariate analysis of blood lineage correlation with thrombosis revealed that only neutrophil count was correlated with thrombosis.[39] We previously reported that elevated levels of HIFs in PV and ET induce the expression of inflammatory and thrombotic genes in neutrophils and platelets,[23] indicating the potential role of increased HIF activity in PV-related thrombosis.[40] *KLF2*, an anti-thrombotic factor, was shown to be downregulated in granulocytes and platelets in PV and ET and is associated with thrombosis. *KLF2* expression is regulated by the JAK-STAT pathway. IFN-α treatment partially augments decreased *KLF2* expression in granulocytes and platelets, suggesting an anti-thrombotic effect from IFN-α treatment.[24] We also observed lower *KLF2* expression in the *JAK2*^{R715T}-affected subjects, although it was not statistically different from controls. This is possibly due to the low number of testable samples. Low *KLF2* expression is due to high *JAK2* activity.[24] Importantly, in both the proposita and her mother, treatment with Ropeg-IFN-α increased *KLF2* expression, suggesting a response mechanism similar to PV and ET. We measured HIF transcriptional activity by assessing HIF target genes *VEGFA* and *SLC2A1* and found no difference compared to controls in the affected individuals. However, inflammatory genes such as *CXCL8* (encodes IL-8) and *TNF*, and some thrombotic genes [*SERPINE1* (encodes plasminogen activator inhibitor type 1), *THBS1* (encodes thrombospondin 1), and *F3* (encodes tissue factor)] were found to be upregulated in affected *JAK2*^{R715T} individuals, but the degree of upregulation was less than those observed in PV and ET. This can be

explained by two possibilities: (1) There was less activation of STATs observed in our *JAK2*^{R715T} individuals when compared to patients with *JAK2*^{V617F} mutations. (2) They had normal white blood cell counts. Since the upregulated gene expression levels observed in PV and ET positively correlate with white blood cell counts, their normal white blood cell counts may not significantly induce these gene expressions.

5 | CONCLUSION

In summary, this study of dominantly inherited PV-like erythrocytosis, caused by a novel germline *JAK2*^{R715T} gain-of-function mutation, presents many but not all comparable molecular and phenotypic features with *JAK2*^{V617F} PV. In these studies, we also uncovered previously unreported IFN-α inhibition of *JAK2* signaling.

AUTHOR CONTRIBUTIONS

JS wrote the manuscript, performed initial computer modeling, evaluated erythropoiesis in liquid cultures and STAT5 phosphorylation, analyzed the gene expression data, edited, and approved the final manuscript. LL designed and performed in vitro analysis of the *JAK2* variants in BA/F3-EPOR and HEK-293 cells and edited the approved final manuscript. SK performed and analyzed BFU-E cultures, in vitro erythroid expansions, genotyped the specimens, performed X-chromosome analysis, measured HIF-target gene, inflammatory, and thrombotic gene expression, and edited and approved the manuscript. NP performed and analyzed computer modeling using AlphaFold 2.0 and edited and approved the manuscript. JM identified and evaluated the affected son of the propositus and edited and approved the manuscript. SNC supervised NP to perform computer modeling to predict *JAK2* activity and edited and approved the manuscript. BP obtained and summarized clinical information and edited and approved the manuscript. JFP wrote manuscript and suggested some experiments and edited and approved the manuscript. JTP diagnosed propositus and her mother, wrote manuscript, supervised, helped interpret the experimental data, reviewed and provided clinical data, and edited and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data will be shared upon request to josef.prchal@hsc.utah.edu.

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