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Differential Biological Activity of Disease-Associated JAK2 Mutants

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Abstract

The JAK2V617F mutation has been identified in most patients with myeloproliferative neoplasms (MPNs), including polycythemia vera, essential thrombocythemia and primary myelofibrosis. Although JAK2V617F is the predominant allele associated with MPNs, other activating JAK2 alleles (such as K539L, T875N) also have been identified in distinct MPNs. The basis for the differences in the *in vivo* effects of different JAK2 alleles remains unclear. We have characterized three different classes of disease-associated JAK2 mutants (JAK2V617F, JAK2K539L and JAK2T875N) and found significant differences in biochemical, signaling and transforming properties among these different classes of JAK2 mutants.

1. Introduction

Janus Kinase 2 (JAK2) is a member of the Janus family of non-receptor protein tyrosine kinases, which also includes JAK1, JAK3 and TYK2. JAK2 serves as a key mediator of cytokine receptor signaling and plays an important role in hematopoiesis [1,2]. JAK2 is the predominant JAK kinase activated in response to erythropoietin (Epo), thrombopoietin (Tpo), interleukin (IL)-3, granulocyte/macrophage colony stimulating factor (GM-CSF), IL-5, growth hormone and prolactin [3–10]. It is also activated along with other JAKs in responses to a wide variety of cytokines including interferon γ (IFN γ), stem cell factor (SCF), granulocyte colony stimulating factor (G-CSF), IL-6, IL-11, IL-12, IL-19, IL-20, IL-23, IL-24, IL-27, IL-31, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and oncostatin M (OSM) [11–16]. JAK2 contains seven JAK homology domains (JH1–JH7), which include a catalytically active kinase domain (JH1) and a catalytically inactive pseudo-kinase domain (JH2). The JH2 domain is thought to play a negative auto-regulatory role in that deletion of the JH2 domain results in constitutive activation of the JAK2 kinase [17]. JAK2 also contains a FERM (band 4.1, ezrin, radixin, moesin) domain in the N-terminal region (JH4–JH7), which is required for association with cytokine receptors [18].

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A somatic point mutation (V617F) in the pseudokinase (JH2) domain of JAK2 has been found in most patients with polycythemia vera (PV) and 50–60% patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) [19–23]. JAK2V617F is a constitutively active tyrosine kinase, which can transform hematopoietic cell lines and progenitors [19,20,24]. Expression of JAK2V617F mutant activates multiple downstream signaling pathways, such as, Stat5, Stat3, Erk/MAP kinase and PI3 kinase/Akt pathways [19,24]. Expression of JAK2V617F also confers hypersensitivity to Epo [19,20,24], which is a characteristic feature of PV. It has been shown that co-expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation of hematopoietic cells [25]. Transgenic or knock-in mice models of JAK2V617F showed that JAK2V617F is directly responsible and sufficient to cause PV [24,26–30].

Additional mutations in JAK2 also have been observed in hematologic diseases with a much lesser frequency than JAK2V617F. Several mutations in the JAK2 exon 12 including JAK2K539L have been identified in a small number of patients with PV and idiopathic erythrocytosis [31,32]. Unlike JAK2V617F, which occurs in a wide spectrum of myeloid malignancies, JAK2 exon 12 mutations are only observed in JAK2V617F-negative PV with isolated erythrocytosis without concomitant leukocytosis or thrombocytosis, suggesting that JAK2 exon 12 mutations are associated with a distinct clinical phenotype. Another activating mutation in the kinase domain (JH1) of JAK2 (JAK2T875N) has been identified in a cell line derived from a patient with acute megakaryoblastic leukemia (AMKL) [33]. Although frequency of this mutation in AMKL is yet to be determined, expression of JAK2T875N induced a myeloproliferative neoplasm (MPN) with features of AMKL in a murine bone marrow transplantation (BMT) assay [33]. In addition, IREED deletion or substitutions in the conserved R683 residue within the JH2 domain of JAK2 have been identified in Down's syndrome-associated acute lymphoblastic leukemia [34,35]. Thus, different activating JAK2 alleles are associated with different phenotypes. However, the basis for the differences in the *in vivo* effects of different JAK2 alleles remains unclear.

Here, we hypothesized that different JAK2 mutants have distinct intrinsic transforming ability, and their biochemical and signaling properties may differ. To test this, we have analyzed the biochemical, signaling and biological properties of three classes/types of disease-associated JAK2 mutants, JAK2V617F (located in the JH2 pseudokinase domain), JAK2K539L (located in the linker region between JH2 and JH3 domains) and JAK2T875N (located in the JH1 kinase domain). We observed significant differences in biochemical, signaling and transforming properties within these three classes of JAK2 mutants.

2. Materials and methods

2.1. Plasmids and reagents

The cDNA encoding murine JAK2 was obtained from Dr. James Ihle (St. Jude Children's Hospital, TN). JAK2V617F, JAK2K539L and JAK2T875N mutants were generated using the Quick Change site-directed mutagenesis kit (Stratagene) and sub-cloned into MSCV-IRES-GFP vector. The monoclonal anti-phosphotyrosine antibody (4G10) was purchased from Millipore. The rabbit antibodies directed against phospho-JAK2 (Y1007/Y1008), phospho-Stat5 (Y694), phospho-Stat3 (Y705), phospho-Akt (S473), phospho-Erk1/2, phospho-Shp2 and phospho-Gab2 were purchased from Cell Signaling Technologies, and the rabbit antibodies against JAK2, Stat5, Stat3, Akt, Erk2, Shp2, Pim-1, Pim-2 and EpoR were obtained from Santa Cruz biotechnology.

2.2 Cell culture and proliferation assay

Ba/F3-EpoR cells were maintained in RPMI medium containing 10% FBS and IL-3 (1 ng/mL). To generate Ba/F3-EpoR cells stably expressing different JAK2 mutants, Ba/F3-EpoR cells were transduced with MSCV-IRES-GFP retroviruses expressing JAK2WT, JAK2V617F, JAK2K539L or JAK2T875N, and sorted for GFP positive cells. For proliferation assays, cells were washed three times in RPMI medium containing 10% FBS (without any cytokine) and seeded in a 96-well plate (2.5×10^3 per well). Cell proliferation was measured at 24 hr, 48 hr and 72 hr after plating using the WST assay.

2.3. Retroviral transduction and colony forming assay

High-titer retroviral stocks of WT or mutant JAK2 were prepared by transient transfection of 293T cells as described previously [36]. Bone marrow cells from wild type C57/BL6 mouse were transduced with retroviruses expressing WT or mutant JAK2 by two rounds of spininfection [36] and plated (1×10^4 per dish) in duplicates in methylcellulose medium without any cytokine (M3234, Stem Cell Technologies). CFU-E colonies were counted after 2 days by morphology and staining with benzidine solution (Sigma). In some experiments, Ba/F3-EpoR cells expressing different JAK2 mutants were assayed for colony formation in methylcellulose medium without any cytokine.

2.4. Immunoprecipitation and immunoblotting

Ba/F3-EpoR cells expressing different JAK2 mutants were deprived of IL-3 for 24 hr and then starved for 6 hr in RPMI medium containing 0.5% BSA at 37°C. Cells were washed in PBS and lysed in 1% Triton X-100 buffer containing 50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM Na_3VO_4 , 5 mM NaF supplemented with protease inhibitor cocktail (Sigma). Cell lysates were subjected to immunoprecipitation with anti-EpoR affinity resin and probed with anti-JAK2 or anti-EpoR antibodies. To detect phosphorylated signaling proteins, cells lysates were separated by SDS-PAGE. Immunoblotting was performed using phospho-specific antibodies as indicated. To control for loading, blots were re-probed with antibodies against total proteins.

2.5. In vitro JAK2 kinase assay

The cells were lysed in 1% Triton X-100 lysis buffer supplemented with protease inhibitors. JAK2 was immunoprecipitated from the cell lysates using anti-JAK2 antibody. Immunoprecipitates were washed 3 times with lysis buffer and twice with kinase buffer (10 mM HEPES [pH 7.4], 50 mM NaCl, 5 mM MnCl_2 , 5 mM MgCl_2 , 1 mM Na_3VO_4 , 5 mM NaF, 1 mM dithiothreitol plus protease inhibitors). The immune complex was resuspended in kinase buffer supplemented with 10 μM ATP, 2 μCi [γ - ^{32}P] ATP (Perkin Elmer). A peptide derived from Stat5 (AKAADGY⁶⁹⁴VKPQIKQVV) containing the JAK2 phosphorylation site was used as a substrate. The reaction mixture was incubated with 0.5 mM Stat5 peptide substrate at 30°C for 30 minutes. Reactions were terminated by addition of an equal volume of 20 mM EDTA. An aliquot of the reaction mixture was transferred onto P81 phosphocellulose paper (Whatman). After washing the P81 paper three times with 0.75% phosphoric acid, bound radioactivity was measured using a Scintillation Counter. The counts detected in the absence of peptide were used as a background.

2.6. Statistical analysis

Results are expressed as mean plus or minus SEM, and data were analyzed by the two-tailed Student's *t* test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Kinase activity of disease-associated JAK2 mutants

We analyzed three disease-associated JAK2 mutants, JAK2V617F, JAK2K539L and JAK2T875N located in the JH2 pseudokinase domain, the linker region between JH2 and JH3 domains, and the JH1 kinase domain respectively (as depicted in Fig. 1A). We established Ba/F3-EpoR cell lines stably expressing wild type JAK2 (JAK2WT), JAK2V617F, JAK2K539L and JAK2T875N mutants. To assess the kinase activity of these JAK2 mutants, cell lysates were immunoprecipitated with anti-JAK2 antibody and the immunoprecipitated JAK2 kinases were incubated with the Stat5 peptide containing the JAK2 phosphorylation site. As expected, very little kinase activity was seen with vector transfected Ba/F3-EpoR cells (Fig. 1B). All three disease-associated JAK2 mutants were more active than the JAK2WT (Fig. 1B). JAK2T875N exhibited the highest levels of kinase activity among these three classes of JAK2 mutants. JAK2V617F was more active than the JAK2K539L but less active than the JAK2T875N mutant (Fig. 1B). However, the levels of expression of JAK2 were comparable in cells expressing different JAK2 mutants (Fig. 1B, lower panel). Thus, different classes of disease-associated JAK2 mutants may have different levels of catalytic activity.

3.2. Biological effects of JAK2 mutants

To assess the biological effects of JAK2 mutants, we first measured the cytokine-independent proliferation of cells expressing different classes of JAK2 mutants. As expected, Ba/F3-EpoR cells expressing vector (GFP) or JAK2WT failed to proliferate in absence of cytokine (Fig. 2A). Ba/F3-EpoR cells expressing JAK2V617F, JAK2K539L and JAK2T875N mutants continued to proliferate in absence of cytokine. However, expression of JAK2T875N resulted in significantly increased proliferation of Ba/F3-EpoR cells compared with JAK2V617F or JAK2K539L expression (Fig. 2A).

Next we assessed the effects of different JAK2 mutants on clonogenic growth of Ba/F3-EpoR cells. For this purpose, Ba/F3-EpoR cells expressing different JAK2 mutants were plated in methylcellulose medium without any cytokine, and colonies were counted after 5–6 days. Expression of the vector (GFP) or JAK2WT failed to produce any colony in the absence of cytokine, whereas expression of JAK2 mutants resulted in a large number of cytokine-independent colonies (Fig. 2B). Ba/F3-EpoR cells expressing JAK2T875N gave rise to the highest number of cytokine-independent colonies among the three different classes of JAK2 mutants (Fig. 2B), consistent with increased proliferation of cells expressing JAK2T875N compared to other JAK2 mutants. Together, these results suggest that different classes of JAK2 mutants have different levels of biological activity.

3.3. Differential transforming potentials of disease-associated JAK2 mutants

We and other groups have shown that expression of JAK2V617F in the bone marrow or spleen results in transformation of erythroid progenitors [24,26–30]. So, we asked if there is any difference in the transforming ability of erythroid progenitors among these three classes of JAK2 mutants. We transduced wild type bone marrow cells with retroviruses expressing JAK2WT, JAK2V617F, JAK2K539L and JAK2T875N, and plated in methylcellulose medium in absence of cytokine. Colony forming unit-erythroid (CFU-E) was counted after 2 days. For this purpose, all retroviral titers were determined in Ba/F3 cells based on their GFP expression (Supplementary Fig. 1A), and equivalent titer retroviruses were used for BM transduction. We observed comparable infection efficiency with different JAK2 mutant retroviruses on BM cells (Supplementary Fig. 1B). As expected, expression of retroviral vector alone failed to transform erythroid progenitors (Fig. 3). Overexpression of JAK2WT in the BM gave rise to a few cytokine-independent CFU-E colonies. Expression of

JAK2V617F and JAK2K539L resulted in a large number of cytokine-independent CFU-E colonies (Fig. 3). However, JAK2T875N expression caused significantly less erythroid transformation compared to JAK2V617F and JAK2K539L mutants (Fig. 3) even though JAK2T875N mutant exhibited the highest kinase activity (Fig. 1B). We also observed significantly larger number of CFU-E colonies in the presence of Epo in JAK2V617F and JAK2K539L expressing BM cells compared with JAK2T875N (Fig. 3). These results suggest that different classes of disease-associated JAK2 mutants have distinct intrinsic transforming ability.

3.4. Signaling properties of disease-associated JAK2 mutants

To further study the disease-associated JAK2 mutants, we examined their effects on cell signaling. First, we analyzed their effects on overall protein tyrosyl phosphorylation by immunoblotting of cell lysates from the growth factor starved cells using anti-phosphotyrosyl antibody. Overall tyrosine phosphorylation of proteins was significantly enhanced in cells expressing JAK2 mutants compared with JAK2WT (Fig. 4A). Several proteins (sizes 135, 125, 120, 90, 88, 71, 68, 55 kDa) exhibited increased tyrosyl phosphorylation in cells expressing JAK2T875N compared with JAK2V617F and JAK2K539L (Fig. 4A). Autophosphorylation of JAK2T875N, as monitored by immunoblotting with phospho-specific JAK2 antibody, was significantly higher than JAK2V617F and JAK2K539L mutants (Fig. 4B). However, JAK2V617F was slightly more autophosphorylated than JAK2K539L mutant (Fig. 4B), consistent with the results of the kinase assays. Phosphorylation/activation of the downstream targets, such as Stat5 and Stat3, was also significantly higher in cells expressing JAK2T875N compared to JAK2V617F and JAK2K539L mutants (Fig. 4B). Moreover, expression of Pim-1 and Pim-2, which are known targets of the JAK/Stat pathway [37], was significantly more induced in JAK2T875N-expressing cells compared to cells expressing JAK2V617F and JAK2K539L mutants (Fig. 4B). Expression of JAK2V617F, however, resulted in higher level of activation of Stat5 and Stat3 than JAK2K539L expression (Fig. 4B).

We also studied the effects of disease-associated JAK2 mutants on other downstream signaling molecules and pathways. We observed constitutive phosphorylation of Shp2 and Gab2 in cells expressing activated JAK2 mutants (Fig. 4C). Phosphorylation of Shp2 was comparable in cells expressing JAK2V617F and JAK2T875N mutants. However, phosphorylation of Gab2 was significantly higher in JAK2T875N-expressing cells compared to cells expressing JAK2V617F and JAK2K539L mutants (Fig. 4C). Similarly, activation of Akt and Erk1/2 were enhanced in cells expressing JAK2T875N compared to JAK2V617F and JAK2K539L (Fig. 4C). Thus, different classes of disease-associated JAK2 mutants give rise to different levels of activation of downstream signaling pathways.

Next, we assessed the interaction of different JAK2 mutants with the EpoR. To investigate the interaction between different JAK2 mutants and EpoR, we performed immunoprecipitation experiments. Compared with the wild type JAK2, activating JAK2 mutants exhibited increased interaction with the EpoR (Fig. 4D). Notably, JAK2V617F and JAK2K539L mutants showed significantly more interaction with the EpoR compared with the JAK2T875N mutant (Fig. 4D). Thus, different JAK2 mutants differentially interact with the EpoR. The reduced ability of the JAK2T875N mutant to interact with the EpoR may possibly explain the reduced transforming potential of erythroid progenitors by the JAK2T875N mutant.

4. Discussion

Different JAK2 mutations are associated with different hematologic disorders, but the basis for various pathologies associated with different JAK2 mutants has remained unclear.

Previous studies suggested that all disease-associated JAK2 mutants have increased basal catalytic activity. We have found that disease-associated JAK2 mutants differ in their catalytic activity, and their effects on downstream signaling also significantly vary. However, catalytic activity alone cannot explain all mutant JAK2-associated pathology. We observed that different disease-associated JAK2 mutants have differential ability to transform erythroid progenitors, which could be due to their differential interaction with the erythropoietin receptor.

Murine models of JAK2V617F, JAK2K539L and JAK2T875N have been reported. We and other investigators have shown that physiologic expression of JAK2V617F in murine hematopoietic progenitors resulted in a PV-like disease characterized by marked increase in hemoglobin and hematocrit, increased RBC, leukocytosis, neutrophilia, thrombocytosis and splenomegaly [24,29,30]. Expression of JAK2 exon 12 mutant JAK2K539L resulted in erythrocytosis (increased RBC, hemoglobin and hematocrit [31,38], whereas expression of JAK2T875N in a murine bone marrow transplant (BMT) model induced a MPN with a megakaryocyte phenotype, including megakaryocytic hyperplasia, impaired megakaryocyte polyploidization and increased reticulin fibrosis in the bone marrow and spleen [33]. JAK2T875N transplanted mice also exhibited an increase in hematocrit without any significant increase in platelets [33]. Previously, it has been observed that BMT approach has inherent deficiency in modeling the thrombocytosis phenotype in mice. Several BMT models for JAK2V617F have been reported; although these mice models showed polycythemia phenotype but they did not show any increase in platelets [39–41]. In contrast, transgenic or knock-in mice models of Jak2V617F reproducibly produced all the features of PV including erythrocytosis, leukocytosis and thrombocytosis [24,26–29]. Thus, the failure to observe thrombocytosis in JAK2T875N transplanted mice does not mean that the mutant is not capable of inducing AMKL. It is also possible that JAK2T875N mutation is one of several genetic events required for development of AMKL. Better mouse models, such as transgenic or knock-in mouse models, of JAK2T875N would determine if JAK2T875N is sufficient to cause AMKL. However, Studies by Mercher and colleagues have suggested that JAK2T875N may have a more prominent effect on megakaryocytic lineage compared with JAK2V617F [33]. In addition, compared with JAK2V617F, JAK2K539L and JAK2T875N mutants did not induce a striking leukocytosis [31,33]. The results from these mice models indicate that different JAK2 mutants perturb hematopoietic signaling in a different manner.

We used Ba/F3-EpoR cells expressing JAK2V617F, JAK2K539L and JAK2T875N mutants to compare the biochemical and signaling properties of JAK2 mutants. In addition, we expressed these JAK2 mutants in murine BM and compared their ability to transform erythroid progenitors. We observed that JAK2 kinase domain (JH1) mutant JAK2T875N has higher basal kinase activity than the JH2 domain mutant JAK2V617F or the JH2–JH3 linker domain mutant JAK2K539L (Fig. 1B). Autophosphorylation of JAK2 mutants as determined by immunoblotting also matched with the results of *in vitro* kinase assays (Figs. 1B, 4B). Several signaling molecules/pathways, such as, Stat5, Stat3, Pim-1, Pim-2, Shp2, Gab2, Akt and Erk, which are inducibly regulated by growth factors or cytokines in hematopoietic cells, are constitutively activated in cells expressing JAK2 mutants. JAK2T875N expression resulted in significantly more activation/induction of these signaling molecules/pathways compared with the JAK2V617F or JAK2K539L mutant (Fig. 4B,C). Haan et al., and Elliott et al., also have compared the effects of these JAK2 mutants by expressing in HEK293 cells [42,43]. However, they did not observe any significant difference in activation of the JAK2 mutants and their downstream signaling. It is possible that the effects of these JAK2 mutants on 293 cells and hematopoietic cells could be different. Moreover, excessive overexpression of the JAK2 mutants (as usually occurs in 293 cells) might not allow us to see the differences in their catalytic activity and downstream

signaling pathways. Also, previous studies did not compare the effects of these JAK2 mutants on hematopoietic progenitors. Our studies suggest that different JAK2 mutants may have differential ability to transform hematopoietic progenitors. Although JAK2T875N exhibited significantly more basal catalytic activity than the JAK2V617F or JAK2K539L mutant (Fig. 1), it was less potent in transforming erythroid progenitors in the BM (Fig. 3). Thus, our data show that transforming ability is not directly proportional to the catalytic (kinase) activity of the JAK2 mutants. Similar results were observed previously with the Noonan syndrome- and leukemia-associated PTPN11 mutants, in which catalytic (phosphatase) activity of PTPN11 mutants did not correlate with their transforming ability and associated pathology [44].

Although all disease-associated JAK2 mutants have increased basal kinase activity, the mechanisms underlying the constitutive activation of these different JAK2 mutants remain elusive. Based on the computer modeling, it was suggested that V617 residue in the JH2 domain directly interacts with the JH1 kinase activation loop to keep in an inactive conformation [45,46]. The V617F mutation disrupts this critical interaction between JH2 and JH1 domains and results in constitutive activation of the JAK2 kinase [45,46]. The K539 residue lies within a region linking JH2 and JH3 domains. Several residues within this linker region are believed to be important to support the local conformation near V617 [46]. The K539L mutation distorts the local conformation surrounding V617 and causes significant changes in the JH1/JH2 interface [46]. It has been found that residues within the linker region between JH2 and JH3 domains are essential for interaction with EpoR and JAK2 activity [38]. The T875 residue lies within the $\beta 2$ - $\beta 3$ loop of the JH1 kinase domain [33]. It was proposed that T875N mutation could potentially disrupt the JH1/JH2 interface [33]. Computer based models only helped to make some predictions on the structural effects of JAK2 mutants. However, crystal structure of full-length JAK2 would be required to better understand the mechanism of constitutive activation of the JAK2 mutants and why some mutants are more active than others.

Previous studies have demonstrated the requirement of homodimeric type I cytokine receptor expression in JAK2V617F-mediated transformation of hematopoietic cells [25,47]. Hematopoietic cell transformation by other JAK2 mutants (JAK2K539L, JAK2T875N) also requires the presence of homodimeric type I cytokine receptor [31,33]. This raises the possibility that differential expression of homodimeric type I cytokine receptors in different hematopoietic lineages and different levels of interaction of the JAK2 mutants with the type I cytokine receptor may modulate the disease phenotype. Indeed, we observed that PV and idiopathic erythrocytosis associated JAK2 mutants, JAK2V617F and JAK2K539L, were more potent at transforming erythroid progenitors and exhibited significantly more interaction with the EpoR compared with the AMKL-associated JAK2T875N mutant (Figs. 3, 4D). We also have tried to analyze the interaction of different JAK2 mutants with the EpoR in murine bone marrow cells. However, available EpoR antibodies failed to efficiently pull down endogenous EpoR from the primary bone marrow cells (data not shown). Nevertheless, we have provided evidence that different JAK2 mutants have differences in their ability to interact with the EpoR.

In conclusion, our studies show that disease-associated JAK2 mutants have differences in the kinase activity, and their biological and signaling properties may also differ. However, the extent of activation of the JAK2 kinase alone cannot predict the disease phenotype of a JAK2 mutant. We have found evidence that disease-associated JAK2 mutants have distinct intrinsic transforming properties, which could be due to differential activation of downstream signaling pathways and their specific interaction with certain cytokine receptor. High-resolution crystal structure of full-length JAK2 is yet to be resolved. Structural insights

into the effects of different JAK2 mutations would shed light in further understanding the relationship between JAK2 activation, intracellular signaling and pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

JAK	Janus kinase
Epo	erythropoietin
EpoR	erythropoietin receptor
MPNs	myeloproliferative neoplasms
PV	polycythemia vera
ET	essential thrombocythemia
PMF	primary myelofibrosis

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Fig. 1.

Catalytic activities of disease-associated JAK2 mutants. (A) Schematic structure of JAK2 and the locations of the disease-associated JAK2 mutants used in this study. (B) Mutant JAK2 proteins were immunoprecipitated and subjected to an *in vitro* kinase assay using a Stat5-derived peptide as a substrate. Phosphorylated peptides were measured by a scintillation counter (upper panel). Note that JAK2T875N mutant exhibited significantly more kinase activity than JAK2V617F ($p < 0.005$) or JAK2K539L mutant ($p < 0.005$), whereas JAK2V617F showed higher kinase activity than JAK2K539L ($p < 0.005$). Immunoprecipitated samples were separated by SDS-PAGE and subjected to immunoblotting with anti-JAK2 antibody (bottom panel) to show equivalent expression of mutant JAK2 proteins.

Fig. 2.

Biological effects of disease-associated JAK2 mutants. (A) Ba/F3-EpoR cells stably expressing GFP (vector), JAK2WT, JAK2V617F, JAK2K539L and JAK2T875N mutants were plated in triplicates in a 96-well plate in the absence of cytokine, and cell proliferation was measured using the WST assay. Asterisk indicates significant differences ($p < 0.01$). Note that JAK2T875N-expressing cells exhibited significantly more cytokine-independent proliferation compared with cells expressing JAK2V617F and JAK2K539L. (B) Clonogenic growth of Ba/F3-EpoR cells expressing different JAK2 mutants was measured in the absence of cytokine in a methylcellulose-based colony assay. Results represent the mean $\pm$ SEM of three independent experiments. Asterisk indicates significant differences ($p < 0.005$). Expression of JAK2T875N caused significantly more clonogenic growth of Ba/F3-EpoR cells than cells expressing JAK2V617F ($p < 0.005$) or JAK2K539L ($p < 0.005$).

Fig. 3.

Transformation of erythroid progenitors by JAK2 mutants. BM from wild type C57/BL6 mice were transduced with equal titer retroviruses expressing GFP (vector), JAK2WT, JAK2V617F, JAK2K539L and JAK2T875N mutants and plated (1×10^4 cells per dish) in methycellulose medium in the absence or presence of Epo. CFU-E colonies were counted after 2 days. Results represent the mean $\pm$ SEM of three independent experiments. Note that JAK2V617F and JAK2K539L expression resulted in transformation of erythroid progenitors and gave rise to a large number of CFU-E in the absence or presence of Epo. However, JAK2T875N was less potent in transforming erythroid progenitors and gave rise to significantly less CFU-E in the absence or presence of Epo compared with JAK2V617F and JAK2K539L mutants (JAK2V617F vs JAK2K539L, $p < 0.05$; JAK2V617F vs JAK2T875N, $p < 0.0005$; JAK2K539L vs JAK2T875N, $p < 0.0005$).

Fig. 4.

Effects of JAK2 mutants on cell signaling. (A) Ba/F3-EpoR cells expressing different JAK2 mutants were starved in RPMI plus 0.5% BSA for 6 hrs. Cell lysates were prepared in RIPA buffer and subjected to immunoblotting with anti-phosphotyrosine (4G10) antibody. (B) Activation of the components of the JAK-Stat signaling pathway, which included JAK2, Stat5, Stat3, Pim-1 and Pim-2 were evaluated in Ba/F3-EpoR cells expressing JAK2 mutants by immunoblotting using phospho-specific antibodies followed by re-probing with total antibodies. (C) Effects of activating JAK2 mutants on other downstream signaling molecules, such as Shp2, Gab2, Akt and Erk1/2, were analyzed by immunoblotting using respective phospho-specific antibodies. Immunoblottings using antibodies against respective total proteins were used as loading controls. (D) Lysates from Ba/F3-EpoR cells expressing different JAK2 mutants were immunoprecipitated with anti-EpoR antibody followed by immunoblotting with anti-JAK2 antibody. The membrane was then re-probed with an antibody against EpoR. Cell lysates were also directly immunoblotted with anti-JAK2 antibody to confirm equivalent JAK2 expression in all mutant expressing cells. Note that association of EpoR with JAK2T875N was significantly less than with JAK2V617F or JAK2K539L.