STAT5 Activation Is Critical for the Transformation Mediated by Myeloproliferative Disorder-associated JAK2 V617F Mutant

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It has been well established that disruption of JAK2 signaling regulation is involved in various hematopoietic disorders; however, the detailed mechanism by which abnormal activation of JAK2 exhibits transforming activity remains to be elucidated. Here, to clarify the functional role of the erythropoietin receptor (EpoR) and its downstream transcription factor STAT5 in the abnormal activation of JAK2-induced hematopoietic diseases, we generated a stable transfectant of Ba/F3 cells expressing EpoR and analyzed the molecular mechanism of how JAK2 mutation induces cell growth disorder. JAK2 V617F mutant exhibited transforming activity when EpoR was coexpressed. According to a study utilizing several truncated mutants of EpoR, the ability of EpoR to facilitate the transforming activity of JAK2 V617F mutant required the intracellular domain to interact with STAT5. Strikingly, once the truncated EpoR (EpoR-H) was mutated on Tyr-343, the phosphorylation of which is known to be important for interaction with STAT5, JAK2 V617F mutant failed to exhibit transforming activity, suggesting that STAT5 is critical for JAK2 mutant-induced hematopoietic disorder. Furthermore, the expression of the constitutively active STAT5 mutant exhibited transforming activity in Ba/F3 cells, and short hairpin RNA-mediated knockdown of STAT5 significantly inhibited the transforming activity of JAK2 V617F mutant. Taking these observations together, STAT5 plays an essential role in EpoR-JAK2 V617F mutant-induced hematopoietic disorder. Although it remains unclear why the presence of EpoR is required to activate oncogenic signaling via the JAK2 mutant and STAT5, its interacting ability is a target for the treatment of these hematopoietic diseases.

The nonreceptor tyrosine kinase JAK2 is an essential signal transducer for various cytokine signaling (1). Markedly, JAK2-deficient embryos are profoundly anemic and die at around 12.5 days post-coitum, because JAK2-deficient erythroid progenitor cells exhibit a defective response to erythropoietin (Epo) stimulation and lack the ability to proliferate in response to Epo (2, 3). Once Epo binds to the erythropoietin receptor (EpoR), multiple tyrosine residues of EpoR are immediately phosphorylated by activated JAK2. Tyrosine phosphorylation can trigger the association of EpoR with various signal transducers, including phosphatidylinositol 3-kinase, SHP-1/2, and STAT5, through specific and direct interactions with the Src homology (SH2) domain or the phosphotyrosine-binding domain of signaling molecules (4–8). Some of these signaling molecules are known to be involved in cell proliferation mediated by the activation of Akt through phosphatidylinositol 3-kinase and the Ras/ERK pathway through the association of SHP-2 with the Grb2-mSos complex (4–8). Activation of these cell proliferative signals is triggered by JAK2-induced tyrosine phosphorylation of EpoR, thereby suggesting that JAK2 has a critical role in Epo-induced proliferative signaling.

STAT5 (signal transducers and activators of transcription 5) is also known to bind to the phosphorylated tyrosine residue of EpoR (8). It has been well clarified that STAT5 binds to phosphorylated tyrosine at 343 of EpoR, and the truncated mutant of receptor harboring this tyrosine residue (EpoR-H) is sufficient to activate STAT5. When tyrosine 343 is mutated to phenylalanine, this mutant fails to activate STAT5 in response to Epo stimulation, indicating that EpoR is required to be associated with and phosphorylated by JAK2 for STAT5 activation (9–12). Although the mechanism of STAT5 activation by EpoR/JAK2 has been well characterized, the physiological role of STAT5 in the proliferative signaling of EpoR remains to be elucidated.

Zang et al. (13) have created two strains of knock-in mice, termed H and HM, and analyzed the role of STAT5 in vivo. H strain contains a truncation of the distal half of the cytoplasmic domain, whereas HM strain contains the same truncation as well as the point mutation of Y343F; however, both strains of mice were viable, with only slight alternations in constitutive erythropoiesis or in IL-2-induced T-cell proliferation but few defects in hematopoiesis in vivo (15–18). Combining all reports, the phys-
Role of STAT5 in Transformation Induced by JAK2 Mutant

Experimental Procedures

Reagents—Recombinant human erythropoetin (Epo) (ESPO® 3000) and recombinant murine IL-3 were purchased from Kirin Brewery Co. (Tokyo, Japan) and PeproTech (Rocky Hill, NJ), respectively. Anti-phospho-JAK2 antibody (Tyr-1007/1008), anti-phospho-STAT5 antibody (Tyr-694), anti-STAT5 antibody, anti-phospho-ERK antibody (Thr-202/Tyr-1007/1008), anti-phospho-STAT5 antibody (Tyr-694), anti-HA antibody (3F10) were purchased from Santa Cruz Biotechnology, and anti-Akt antibody were purchased from Cell Signaling. STAT5 antibody, anti-phospho-ERK antibody (Thr-202/Tyr-1007/1008), anti-phospho-STAT5 antibody (Tyr-694), anti-HA antibody (3F10) were purchased from Santa Cruz Biotechnology, and anti-Akt antibody were purchased from Cell Signaling. STAT5 antibody, anti-phospho-ERK antibody (Thr-202/Tyr-1007/1008), anti-phospho-STAT5 antibody (Tyr-694), anti-HA antibody (3F10) were purchased from Santa Cruz Biotechnology, and anti-Akt antibody were purchased from Cell Signaling.

Plasmids—Murine JAK2-HA and murine EpoR c-FLAG were subcloned into retroviral plasmids, murine stem cell virus (MSCV)-Hygro, and MSCV-Puro (Clontech), respectively. Murine EpoR (H) mutant was produced by truncation of the C-terminal 108 amino acids, and the murine EpoR (HM) mutant was produced by the same truncation and a point mutation of Y343F, as described previously (13, 22). The deletion mutants of EpoR were subcloned into MSCV-Puro. MSCV-internal ribosome entry site-green fluorescent protein (GFP)-murine wild-type STAT5 and MSCV-internal ribosome entry site-GFP-murine constitutively active STAT5 mutant were donated by Dr. Ihle (St. Jude Children’s Hospital). A constitutively active form of murine STAT5 cDNA harbors substitutions at amino acid residue 711 from serine to phenylalanine (S711F) and at 299 from histidine to arginine (H299R), as reported previously (25). Mutagenesis of an amino acid residue, V617F in JAK2 was performed using a site-directed mutagenesis kit, according to the manufacturer’s instruction.

Cell Cultures—Ba/F3 cells were infected with empty virus (−), wild-type murine JAK2 c-HA, or a mutant of murine JAK2 c-HA (V617F) with full-length murine EpoR c-FLAG or deletion mutants of murine EpoR c-FLAG (H or HM) (1–375 amino acids) and established as described previously (22). Ba/F3 cells transfected with empty virus (−) were further infected with wild-type murine STAT5 or a constitutively active mutant of murine STAT5. Empty virus corresponding to the retrovirus expression vector of each molecule was used for all experiments. These cells were cultured in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum (BioWest, France), 2 mm 1-glutamine (Nacalai Tesque, Tokyo, Japan), 100 units/ml penicillin (Nacalai Tesque), 100 μg/ml streptomycin (Nacalai Tesque), and 2 ng/ml IL-3 or 5 units/ml Epo (Kirin Brewery Co.). To select infected cells, 5 μg/ml puromycin and 200 μg/ml hygromycin were used. To detect apoptosis, cells were incubated with RPMI 1640 medium supplemented with 1% fetal bovine serum.

RNA Interference—Annealed oligonucleotide coding shRNA for murine STAT5 was inserted into pSUPER-retropropu retroviral plasmid (Oligoengine, Seattle, WA). The retroviral plasmid and the plasmid encoding retroviral helper were transfected into HEK293T cells using FuGENE 6. Thirty six hours after transfection, the retrovirus secreted into culture supernatant was collected. Ba/F3 cells expressing wild-type JAK2 or JAK2 V617F mutant and EpoR were infected with retroviruses harboring shRNA against STAT5 using RetroNectin (Takara, Tokyo, Japan). Three days later, the efficiency of knockdown was checked by immunoblot analysis for STAT5. The sequences of oligonucleotides used for constructing shRNA retroviral vectors were as follows: shRNA-1, 5′-GATCCCGGGGAAAGCGGAGATC-TCGTTTCGTTTTA-3′ and 5′-AGCTTAAAAACGGA-GAACAGCTGAAGATTTACAGAGATTTACAGCTCGTTCGTTTCTTCTTTTTTTT-3′ and 5′-AGCTTAAAAACGGA-GAACAGCTGAAGATTTACAGAGATTTACAGCTCGTTCGTTTCTTCTTTTTTTT-3′ and 5′-AGCTTAAAAACGGA-GAACAGCTGAAGATTTACAGAGATTTACAGCTCGTTCGTTTCTTCTTTTTTTT-3′ and 5′-AGCTTAAAAACGGA-GAACAGCTGAAGATTTACAGAGATTTACAGCTCGTTCGTTTCTTCTTTTTTTT-3′ and 5′-AGCTTAAAAACGGA-GAACAGCTGAAGATTTACAGAGATTTACAGCTCGTTCGTTTCTTCTTTTTTTT-3′ (underlined sequences correspond to the sequence of murine STAT5a and STAT5b).
Role of STAT5 in Transformation Induced by JAK2 Mutant

**FIGURE 1.** EpoR is required for cytokine-independent cell survival induced by JAK2 V617F mutant. Ba/F3 cell lines were infected with empty virus (−) and retrovirus encoding wild-type JAK2 c-HA or JAK2 mutant c-HA (V617F) and EpoR c-FLAG (FL). Wild-type JAK2, JAK2 V617F mutant, and full-length EpoR are shown as WT, V617F, and FL, respectively. Each cell strain was named −/−, −/FL, WT/−, WT/FL/V617F/−, and V617F/FL cells. A, cell lysates of transduced Ba/F3 cells were blotted with anti-HA antibody (Ser-473), anti-Akt antibody, or anti-β-actin antibody. B, transduced Ba/F3 cells were washed twice with PBS and left untreated or stimulated with Epo (5 units/ml) for 24 h. Whole cell lysates were immunoblotted (IB) with anti-phospho-STAT5 antibody (Tyr-694), anti-STAT5 antibody, anti-phospho-ERK antibody (Thr-202/Tyr-204), anti-ERK antibody, anti-phospho-Akt antibody (Ser-473), anti-Akt antibody, or anti-β-actin antibody. C, transduced Ba/F3 cells were washed twice with PBS and left untreated or stimulated with Epo (5 units/ml) for 48 h. The viability of these cells was determined by the trypan blue exclusion method. Results represent the mean ± S.D. of three independent experiments. D and E, transduced Ba/F3 cells were washed twice with PBS and left untreated or stimulated with Epo (5 units/ml) for 24 h. D, cells were fixed, treated with propidium iodide, and subjected to FACS analysis, as described under “Experimental Procedures.” E, DNA was isolated from cells and subjected to agarose gel electrophoresis.

serum, 2 mM L-glutamine in the presence or absence of Epo (5 units/ml) for 48 h. Living cells were counted using a Beckman Coulter VI-Cell (Beckman Coulter, Fullerton, CA). Cell viability was checked by the trypan blue exclusion method (23).

**Cell Cycle Analysis—**After treatment, cells were fixed with 70% (v/v) ethanol at −20 °C overnight. Cells were then centrifuged at 5,000 rpm for 2 min and resuspended in PBS containing 10 μg/ml RNase A (Wako, Tokyo, Japan) and 100 μg/ml propidium iodide (PI) (Sigma). Following a 30-min incubation, cell cycle parameters were determined by flow cytometry analysis using FACSCalibur (23). All data were recorded and analyzed using CellQuest software.

**DNA Fragmentation Assay—**Genomic DNA was prepared for gel electrophoresis as described previously (23). Electrophoresis was performed on a 1% (w/v) agarose gel in Tris/boric acid buffer. Fragmented DNA was visualized by staining with ethidium bromide after electrophoresis (23).

**Immunoprecipitation and Western Blotting—**Cells were harvested in ice-cold PBS and lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 20 mM NaF, 0.2 mM Na3VO4) supplemented with protease inhibitors. Cell lysates were centrifuged at 15,000 rpm for 15 min to remove debris, and the supernatants were incubated with the indicated antibody for 4 h. Immune complexes were precipitated with protein G-Sepharose (Zymed Laboratories Inc.), washed three times with lysis buffer, and then eluted with sample buffer for SDS-PAGE. Eluted proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were probed using the designated antibodies and visualized with the ECL detection system (GE Healthcare) (23).

**Animal Tumorigenesis—**To investigate oncogenic potentials in vivo, 1 × 107 transduced Ba/F3 cells were injected subcutaneously into female BALB/c nude mice aged 4 weeks (23, 26). Twelve or 17 days post-inoculation, the animals were sacrificed, and the weights of the tumor, liver, and spleen were recorded. The tumors isolated from nude mice were frozen with the liquid nitrogen, and cell lysates were then prepared. After sacrifice, the liver of each nude mouse was fixed in 4% paraformaldehyde and then dehydrated gradually in alcohol. Tissues were embedded in paraffin and sectioned at a thickness of 4 μm. The sections were stained with hematoxylin–eosin and analyzed for the presence of tumor cell infiltration using an OLYMPUS BX50 microscope (Olympus, Tokyo, Japan) with Olympus Micro DP70 software (Olympus) (26).

**RESULTS**

EpoR Was Required for JAK2 V617F Mutant-induced Transformation of Ba/F3 Cells—A point mutation of JAK2 (V617F) has been found in the majority of myeloproliferative disorders, including PV patients, essential thrombocythemia patients, and PMF patients (19–21). To investigate the function of this V617F mutation, we generated Ba/F3 cells by retroviral infection with empty virus (−) and viruses, including the wild-type JAK2 c-HA or the JAK2 mutant c-HA harboring V617F mutation (V617F) with or without retrovirus, including cDNA of the full length (FL) of EpoR c-FLAG (Fig. 1A). The characteristics of each cell strain are shown in Table 1 and designated as −/−, −/FL, WT/−, WT/FL/V617F/−, and V617F/FL cells. To test whether coexpression of EpoR could affect the function of the JAK2 V617F mutant, the phosphorylation level of its downstream molecules, including STAT5, ERK, and Akt, was examined. In the presence of EpoR, Epo stimulation induced the phosphorylation of these molecules in both −/FL and WT/FL cells. On the other hand, constitutive activation of STAT5, ERK, and Akt was observed in V617F/FL cells; however, in the absence of EpoR, not only wild-type JAK2 but also JAK2 V617F
Role of STAT5 in Transformation Induced by JAK2 Mutant

Phosphorylation at Tyr-343 in EpoR is required for constitutive activation of STAT5 induced by JAK2 V617F mutant. A, schematic diagram of full-length EpoR and deletion mutants of EpoR (H and HM). The relative positions of tyrosine residues are marked. EpoR-HM mutant harbors Y343F substitution. TM indicates the transmembrane region, and aa indicates amino acid. JAK2 interacts with EpoR through Box1 and Box2 regions (upper panel). Ba/F3 cell lines were infected with empty virus (−) and retrovirus encoding wild-type JAK2 c-HA or JAK2 mutant c-HA (V617F) with EpoR c-FLAG (FL), EpoR-H c-FLAG (H), or EpoR-HM c-FLAG (HM). Wild-type JAK2, JAK2 V617F mutant, full-length EpoR, EpoR-H mutant, and EpoR-HM mutant are shown as WT, V617F, FL, H, and HM, respectively. Each cell strain was named −/−, −/FL, −/H, −/HM, WT/−, WT/FL, WT/H, WT/HM, V617F/−, V617F/FL, V617F/H, and V617F/HM cells. Cell lysates of transduced Ba/F3 cells were blotted with anti-HA antibody, anti-FLAG antibody, or anti-β-actin antibody (bottom). B–D, transduced Ba/F3 cells were washed twice with PBS and left untreated or stimulated with Epo (5 units/ml) for 24 h. B, cell lysates were subjected to immunoprecipitation (IP) using anti-FLAG antibody and immunoblotted (IB) with anti-HA antibody or anti-FLAG antibody. C, cell lysates were subjected to immunoprecipitation using anti-HA antibody and immunoblotted with anti-phospho-JAK2 antibody (Tyr-1007/1008) or anti-HA antibody. D, whole cell lysates were immunoblotted with anti-phospho-STAT5 antibody (Tyr-694), anti-STAT5 antibody, anti-phospho-ERK antibody (Thr-202/Tyr-204), anti-ERK antibody, anti-phospho-Akt antibody (Ser-473), anti-Akt antibody, or anti-β-actin antibody.

**TABLE 1**

| Cell strains analyzed in this study and their characters | JAK2 | EpoR | STAT5 activation | Anti-apoptosis without Epo | Tumorigenesis in nude mice |
|--------------------------------------------------------|------|------|------------------|---------------------------|--------------------------|
| 1                                                      | −/−  | None | ND               | ND                        | ND                       |
| 2                                                      | −/−  | FL   | None             | ND                        | ND                       |
| 3                                                      | −/−  | H    | None             | ND                        | ND                       |
| 4                                                      | −/−  | HM   | None             | ND                        | ND                       |
| 5                                                      | WT   | None | None             | ND                        | ND                       |
| 6                                                      | WT   | FL   | Full length      | ND                        | ND                       |
| 7                                                      | WT   | H    | Full length      | ND                        | ND                       |
| 8                                                      | WT   | HM   | EpoR-H           | ND                        | ND                       |
| 9                                                      | V617F| None | None             | ND                        | ND                       |
| 10                                                     | V617F| FL   | Full length      | ++                        | +++                      |
| 11                                                     | V617F| H    | EpoR-H           | ++                        | +++                      |
| 12                                                     | V617F| HM   | EpoR-H           | ND                        | +                        |

a Fig. 2 shows structures of EpoR mutants, EpoR-H and EpoR-HM.
b STAT5 activation was tested under the absence of Epo stimulation.
c ND means not detected.

Expression of JAK2 V617F mutant failed to induce the activation of these molecules (Fig. 1B). In the absence of EpoR expression, all three cells (−/−, WT/−, and V617F/− cells) died regardless of Epo stimulation. Following Epo withdrawal, both −/FL and WT/FL cells died. On the other hand, V617F/FL cells could survive for 48 h after Epo deprivation (Fig. 1C). We next determined the different phases of cell cycle distribution in these cells following 24 h of Epo deprivation. In the absence of EpoR expression, there was a significant increase of the sub-G1 phase, which is consistent with apoptotic cells. Also, in both −/FL and WT/FL cells, cells in the sub-G1 phase were increased after Epo deprivation (Fig. 1D). Furthermore, a ladder pattern of DNA internucleosomal fragmentation clearly appeared in these cells following 24 h of Epo deprivation, confirming that these cells underwent apoptotic cell death (Fig. 1E). On the other hand, there were no marked changes in cell cycle distribution and DNA fragmentation in V617F/FL cells regardless of Epo stimulation (Fig. 1, D and E); therefore, the coexpression of EpoR with JAK2 V617F mutant conferred growth factor independence on Ba/F3 cells, as reported previously (24).

EpoR-HM Mutant Inhibited STAT5 Activation Induced by JAK2 V617F Mutant—The roles of the distal region of EpoR and receptor tyrosines have been extensively studied in transfected cell lines (4–8). In particular, phosphorylation at Tyr-343 in EpoR is required for the recruitment and activation of STAT5 (8–11). To address the role of STAT5 activation in the function of the JAK2 V617F mutant, we further generated transduced Ba/F3 cells by retroviral infection with the empty virus (−), viruses including the wild-type JAK2 c-HA or the JAK2 V617F mutant c-HA (V617F) with retrovirus, including the full length of EpoR, and the Epo deletion mutants, EpoR-H (H) or EpoR-HM (HM). The EpoR-H mutant deleted the distal 108 amino acids of EpoR. Because the EpoR-H mutant contained one tyrosine (Tyr-343), an Epo-R-HM mutant was created in which the distal region was deleted and Tyr-343 was...
mutated to phenylalanine (Fig. 2A). The characteristics and name of each cell strain are shown in Table 1.

We first investigated whether wild-type JAK2 and JAK2 V617F mutant interacted with EpoR deletion mutants. Immunoprecipitation of EpoR mutants followed by Western blotting for JAK2 mutants demonstrated that not only wild-type JAK2 but also JAK2 V617F mutant associated with all EpoR mutants, even in the absence of Epo stimulation (Fig. 2B). Then, to verify whether JAK2 activation was affected by EpoR mutants, phosphorylation of JAK2 at Tyr-1007/1008 within the activation loop was determined using a specific phospho-JAK2 antibody. Epo induced the phosphorylation of wild-type JAK2 in the presence of EpoR and EpoR mutants, H and HM. Interestingly, the JAK2 V617F mutant was slightly activated only when expressed. Moreover, the phosphorylation of JAK2 V617F mutant was remarkably reinforced by the coexpression of EpoR or EpoR deletion mutants, H and HM (Fig. 2C). Therefore, it was suggested that the coexpression of EpoR was important for full activation of the JAK2 V617F mutant, although its distal region was not necessary for JAK2 activation. The effect of STAT5 activation by the expression of EpoR mutants was examined by Western blotting. Consistent with previous reports (8–11), whereas Epo induced STAT5 activation through the Epo-H mutant, Epo-induced STAT5 activation was not observed in /HM and WT/HM cells. Furthermore, although the EpoR-H mutant as well as EpoR-FL induced the constitutive activation of STAT5 induced by JAK2 V617F mutant, coexpression of the EpoR-HM mutant with JAK2 V617F mutant failed to induce STAT5 activation regardless of Epo stimulation (Fig. 2D). For the activation of ERK and Akt, no difference was observed in the function of not only full-length EpoR but also the EpoR-H mutant (H) and EpoR-HM mutant (HM) in cells expressing wild-type JAK2. Furthermore, Epo-induced activations of ERK and Akt were detected in these cells regardless of the concentration or the treated periods of Epo (supplemental Fig. S1). However, in the absence of Epo, the constitutive activation of ERK and Akt observed by coexpression of the JAK2 V617F mutant and EpoR (FL) was inhibited under the expression of EpoR-H mutant or EpoR-HM mutant. This result indicates that constitutive activation of ERK and Akt induced by the JAK2 V617F mutant requires the distal region of EpoR. On the other hand, in the presence of Epo stimulation, activation of ERK and Akt was observed in all infected cells, suggesting that the distal region of EpoR is not necessary for Epo-induced activation of ERK and Akt (Fig. 2D and supplemental Fig. S1 and Fig. S2). Considering these results, it is suggested that the difference in the function of EpoR-H mutant and EpoR-HM mutant is the ability to induce STAT5 activation; therefore, it was thought that the importance of STAT5 was appreciable by examining the functions of cells expressing these two EpoR deletion mutants, H and HM.

**STAT5 Activation Was Required for JAK2 V617F Mutant-induced Transformation of Ba/F3 Cells**—To investigate the function of STAT5 activation in the anti-apoptotic effect of the JAK2 V617F mutant, we first examined the cell viability of infected Ba/F3 cells after Epo deprivation. As shown in Fig. 3A, in the absence of Epo, Ba/F3 cells survived only when JAK2 V617F mutant was coexpressed with EpoR (FL) or Epo-R-H mutant. On the other hand, Ba/F3 cells expressing JAK2 V617F mutant and EpoR-HM mutant died in the absence of Epo. /FL, /H, /HM, WT/FL, WT/H, and WT/HM cells died after Epo deprivation; however, in the presence of Epo stimulation, all Ba/F3 cells could survive, suggesting that STAT5 is not necessary for Epo-mediated cell survival and also that the STAT5-independent pathway is activated in Epo-stimulated cells expressing the EpoR-HM mutant. In addition, there was a significant increase in the sub-G1 phase in /FL, /H, /HM, WT/FL, WT/H, and WT/HM cells after Epo deprivation (Fig. 3B). Furthermore, a ladder pattern of DNA internucleosomal fragmentation clearly appeared in these cells after Epo deprivation, confirming that these cells underwent apoptotic cell death (Fig. 3C). Although neither marked changes in cell cycle distribution nor DNA fragmentation was detected in V617F/FL and V617F/H cells regardless of Epo stimulation, the deprivation of Epo clearly induced apoptotic responses in V617F/HM cells (Fig. 3, B and C). Therefore, these results indicate that STAT5 activation was required for the anti-apoptotic effect of the JAK2 V617F mutant in the absence of Epo stimulation.

**STAT5 Activation Is Required for JAK2 V617F Mutant-induced Tumorigenesis in Nude Mice**—Next, to address the role of STAT5 activation in the function of JAK2 V617F mutant in vivo, we examined whether subcutaneous inoculation of trans-
duced Ba/F3 cells with EpoR mutants into nude mice could induce tumor formation. Although nude mice inoculated with Ba/F3 cells expressing only JAK2 V617F mutant (V617F/FL) appeared to show no change, significant tumor formation was observed in nude mice receiving V617F/FL and V617F/H cells 12 days after inoculation. Interestingly, tumor formation was observed at the injection site in mice inoculated with V617F/HM cells; however, the size and weight were lower than those of mice inoculated with V617F/FL and V617F/H cells (Fig. 4A). To investigate the activation of STAT5, ERK, and Akt in vivo, we performed immunoblot analysis for cell lysates prepared from tumors isolated from nude mice injected with Ba/F3 cells harboring each combination of JAK2 mutants and a series of EpoR or its mutants. As shown in Fig. 4B, phosphorylation of STAT5 was not detected in the tumor from V617F/HM cell-inoculated mice, whereas tumors from other injected nude mice showed activation of STAT5 by JAK2 V617F. On the other hand, activation of ERK and Akt was observed in tumors derived from V617F/FL, V617F/H, and V617F/HM cells. Furthermore, in nude mice receiving V617F/FL and V617F/H cells, the spleen and liver were abnormally enlarged compared with mice receiving V617F/HM, WT/V617F, V617F/FL, V617F/HM, and WT/HM cells. In contrast, 12 days after inoculation, in nude mice injected with V617F/FL and V617F/H cells, the spleen and liver were not enlarged (Fig. 4, C and D). In addition, liver sections were prepared and stained with hematoxylin-eosin (magnification ×400). F, 10 nude mice were injected for Ba/F3 cell lines expressing JAK2 mutant c-HA (V617F) with EpoR c-FLAG (FL), EpoR-H c-FLAG (H), or EpoR-HM c-FLAG (HM). For 35 days post-inoculation, mouse survival was monitored daily.

FIGURE 4. STAT5 activation is required for JAK2 V617F mutant-induced tumor formation in nude mice. Ba/F3 cell lines were infected with retrovirus empty virus (-) and encoding wild-type JAK2-c-HA or JAK2 mutant c-HA (V617F) with EpoR-c-FLAG (FL), EpoR-H c-FLAG (H), or EpoR-HM c-FLAG (HM). Wild-type JAK2, JAK2 V617F mutant, full-length EpoR, EpoR-H mutant, and EpoR-HM mutant are shown as WT, V617F, FL, H, and HM, respectively. 1 × 10⁷ cells of transduced Ba/F3 cells were subcutaneously injected into nude mice. A, nude mice were photographed 12 days post-inoculation. Arrows indicate tumors in nude mice (left). 12 days post-inoculation, tumors at injected sites were weighed and plotted. * indicates significant difference p < 0.01 (right). N.D. indicates not detected. B, 12 days post-inoculation, mice were sacrificed, and cell lysates were prepared from tumors inoculated with V617F/FL, V617F/H, and V617F/HM cells. Whole cell lysates were immunoblotted (IB) with anti-phospho-STAT5 antibody (Tyr-694), anti-STAT5 antibody, anti-phospho-ERK antibody (Thr-202/Tyr-204), anti-ERK antibody, anti-phospho-Akt antibody (Ser-473), or anti-Akt antibody. C, 12 days post-inoculation, mice were sacrificed. In nude mice injected with Ba/F3 cells expressing JAK2 mutant c-HA (V617F) with EpoR c-FLAG (FL), EpoR-H c-FLAG (H), or EpoR-HM c-FLAG (HM), morphological changes of the spleen and liver were photographed. D, 12 days post-inoculation, mice were sacrificed, and the spleen and liver were weighed and plotted. * and ** indicate significant differences p < 0.01 and p < 0.005, respectively. E, 12 days post-inoculation, liver sections were stained with hematoxylin-eosin (magnification ×400).
Role of STAT5 in Transformation Induced by JAK Mutant

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F3 cell lines were infected with empty virus (−) and viruses, including wild-type STAT5 or constitutively active STAT5 (Fig. 5A). Strikingly, following IL-3 withdrawal, although BaF3 cells infected with empty virus (−) and wild-type of STAT5 died, the expression of a constitutively active STAT5 mutant inhibited cytokine deprivation-induced cell death (Fig. 5B). There was a significant increase in the sub-G1 phase in both BaF3 cells infected with empty virus (−) and wild-type of STAT5 (Fig. 5C). In addition, a ladder pattern of DNA internucleosomal fragmentation was clearly observed in these cells following IL-3 deprivation, confirming that these cells underwent apoptotic cell death (Fig. 5D). On the other hand, there were no marked changes in cell cycle distribution and DNA fragmentation in BaF3 cells when expressing constitutively active STAT5 (Fig. 5, C and D).

To further examine the role of STAT5 in the functions of JAK2 V617F mutant in more detail, BaF3 cells were infected with JAK2 V617F mutant and EpoR-HM and then sequentially infected with empty virus (−), wild-type STAT5, or constitutively active STAT5 (Fig. 5E). Interestingly, following Epo withdrawal, whereas V617F/HM cells expressing wild-type STAT5 induced apoptosis and died, the expression of a constitutively active STAT5 mutant completely rescued cytokine deprivation-induced apoptosis and the cell death of V617F/HM cells (Fig. 5, F–H).

Constitutively Active Mutant of STAT5 Induced Tumor Formation of Ba/F3 Cells and Restored Tumor Formation Ability of Ba/F3 Cells Expressing JAK2 V617F Mutant and EpoR-HM Mutant—Next, to examine the role of constitutive STAT5 activation in tumorigenesis, we investigated whether subcutaneous inoculation of transduced Ba/F3 with wild-type STAT5 or constitutively active STAT5 into nude mice could induce tumor formation. 17 days after inoculation, mice inoculated with Ba/F3 cells transduced with empty virus (−) and wild-type STAT5 exhibited no changes in appearance. On the other hand, in mice inoculated with Ba/F3 cells expressing constitutively active STAT5, tumors first appeared at the injection sites after 5 days and then markedly grew to an average of 1.05 ± 0.15 g at 17 days (Fig. 6A). Furthermore, in nude mice injected with Ba/F3 cells

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FIGURE 5. Constitutively active mutant of STAT5 induced cytokine-independent cell survival of Ba/F3 cells and restored cell survival of Ba/F3 cells expressing JAK2 V617F and EpoR HM mutants. A–D, Ba/F3 cells were infected with empty virus (−) and retrovirus encoding wild-type STAT5 or constitutively active mutant of STAT5 (1*6). E–F, Ba/F3 cell lines were infected with retrovirus encoding JAK2 mutant c-HA (V617F) with EpoR-HA-FLAG (FL) and sequentially infected with empty virus (−) and retrovirus encoding wild-type STAT5 or constitutively active mutant of STAT5 (STAT5 1*6). A and C, cell lysates of transduced Ba/F3 cells were blotted with anti-STAT5 antibody, anti-HA antibody, anti-FLAG antibody, or anti-β-actin antibody. B and F, transduced Ba/F3 cells were washed twice with PBS and left untreated or stimulated with Epo (5 units/ml) for 48 h. The viability of these cells was determined by the trypan blue exclusion method. Results represent the mean ± S.D. of three independent experiments. C, D, G, and H, transduced Ba/F3 cells were washed twice with PBS and left untreated or stimulated with Epo (5 units/ml) for 24 h. C and G, cells were fixed, treated with propidium iodide, and subjected to FACS analysis as described under “Experimental Procedures.” D and H, DNA was isolated from cells and subjected to agarose gel electrophoresis. IB, immunoblot.

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in the liver of mice inoculated with V617F/− or V617F/HM cells (Fig. 4E). Compared with mice inoculated with V617F/− cells, the life spans of mice inoculated with V617F/FL or V617F/HF cells were greatly reduced, and all mice had died by 21 or 26 days after inoculation, respectively. Importantly, the life span of nude mice receiving V617F/HM cells was effectively extended as compared with these mice (Fig. 4F). Thus, these results indicate that the oncogenic properties of JAK2 V617F mutant are strongly associated with STAT5 activation.

Constitutively Active Mutant of STAT5 Significantly Induced Cellular Transformation of Ba/F3 Cells and Ba/F3 Cells Expressing JAK2 V617F Mutant and EpoR-HM Mutant—To investigate the role of STAT5 in cellular transformation, Ba/F3 cell lines were infected with empty virus (−) and viruses, including wild-type STAT5 or constitutively active STAT5 (Fig. 4A). Importantly, the life span of nude mice receiving V617F/HM cells was effectively extended as compared with these mice (Fig. 4F). Thus, these results indicate that the oncogenic properties of JAK2 V617F mutant are strongly associated with STAT5 activation.

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Role of STAT5 in Transformation Induced by JAK2 Mutant

expressing constitutively active STAT5, the spleen and liver were abnormally enlarged compared with mice receiving control Ba/F3 cells (−) and Ba/F3 cells expressing wild-type STAT5 (Fig. 6, B and C). In addition, marked invasion of tumor cells into the liver was observed only in mice receiving Ba/F3 cells expressing constitutively active STAT5 (Fig. 6D). Compared with nude mice that were inoculated with control Ba/F3 cells (−), there was little change in survival days when mice were inoculated with Ba/F3 cells expressing wild-type STAT5; however, the life span of nude mice inoculated with Ba/F3 cells expressing constitutively active STAT5 was significantly shortened (Fig. 6E).

Furthermore, we examined the effect of STAT5 on tumor formation by Ba/F3 cells expressing JAK2 V617F mutant and EpoR-HM mutant. 17 days after inoculation, compared with mice inoculated with V617F/HM cells, no significant difference in tumor formation was observed when inoculated with V617F/HM cells expressing wild-type STAT5. On the other hand, in mice inoculated with V617F/HM cells expressing constitutively active STAT5, the expression level of STAT5 rather than shRNA-2. In the absence of Epo stimulation, shRNA-1 enhanced the tendency of cell death of −/FL and WT/FL cells. Although control shRNA had no effect on the cell viability of V617F/FL cells, shRNAs against STAT5 significantly decreased cell viability to less than 10%. In contrast, Epo stimulation effectively inhibited the cell death of these cells in which endogenous STAT5 was knocked down, suggesting that STAT5 could be dispensable in the presence of Epo (Fig. 8B). Consistently, in the absence of Epo stimulation, shRNA against STAT5 induced a significant increase of the sub-G1 phase and DNA fragmentation in V617F/FL cells, suggesting that STAT5 is required for the anti-apoptotic effect of V617F/FL cells (Fig. 8, C and D); however, in the presence of Epo stimulation, in −/FL, WT/FL, and V617F/FL cells, apoptosis induced by shRNA against STAT5 was effectively inhibited. Taken together with the results using the EpoR-HM mutant, it is suggested that STAT5 is essential for JAK2 V617F mutant-induced anti-apoptotic signals but not for Epo-induced survival signals.

Knockdown of STAT5 Inhibited Tumor Formation Ability of Ba/F3 Cells Expressing JAK2 V617F Mutant and EpoR-HM Mutant—Next, we tested if STAT5 knockdown could diminish tumor formation induced by JAK2 V617F mutant and EpoR in nude mice. 17 days after inoculation, compared with mice inoculated with V617F/HM cells expressing constitutively active STAT5 but not wild-type STAT5 (Fig. 7D). Compared with nude mice that were inoculated with V617F/HM and V617F/HM cells expressing wild-type STAT5, the life span of nude mice inoculated with V617F/HM cells expressing constitutively active STAT5 was significantly shortened, suggesting that STAT5 activation is essential for JAK2 V617F mutant-induced cellular transformation and tumorigenesis in nude mice (Fig. 7E).

Knockdown of STAT5 Significantly Inhibited Cellular Transformation of Ba/F3 Cells Expressing JAK2 V617F Mutant and EpoR—To gain further insight into the role of STAT5 in the oncogenic functions of JAK2 V617F mutant, we knocked down the expression of endogenous STAT5 using shRNA. Ba/F3 cells expressing wild-type JAK2 or JAK2 V617F mutant with EpoR (FL) were infected with control shRNA or two kinds of shRNA against STAT5 (1, 2). As shown in Fig. 8D, knockdown of STAT5 significantly inhibited the growth of Ba/F3 cells expressing constitutively active JAK2 V617F mutant and EpoR-HM mutant. However, it is not clear whether STAT5 expression is involved in the anti-apoptotic effect of V617F/FL cells (Fig. 8, E and F); however, in the presence of Epo stimulation, in −/FL, WT/FL, and V617F/FL cells, apoptosis induced by shRNA against STAT5 was effectively inhibited. Taken together with the results using the EpoR-HM mutant, it is suggested that STAT5 is essential for JAK2 V617F mutant-induced anti-apoptotic signals but not for Epo-induced survival signals.

Knockdown of STAT5 Significantly Inhibited Cellular Transformation of Ba/F3 Cells Expressing JAK2 V617F Mutant and EpoR—To gain further insight into the role of STAT5 in the oncogenic functions of JAK2 V617F mutant, we knocked down the expression of endogenous STAT5 using shRNA. Ba/F3 cells expressing wild-type JAK2 or JAK2 V617F mutant with EpoR (FL) were infected with control shRNA or two kinds of shRNA against STAT5 (1, 2). As shown in Fig. 8D, knockdown of STAT5 significantly inhibited the growth of Ba/F3 cells expressing constitutively active JAK2 V617F mutant and EpoR-HM mutant. However, it is not clear whether STAT5 expression is involved in the anti-apoptotic effect of V617F/FL cells (Fig. 8, E and F); however, in the presence of Epo stimulation, in −/FL, WT/FL, and V617F/FL cells, apoptosis induced by shRNA against STAT5 was effectively inhibited. Taken together with the results using the EpoR-HM mutant, it is suggested that STAT5 is essential for JAK2 V617F mutant-induced anti-apoptotic signals but not for Epo-induced survival signals.

FIGURE 6. Constitutively active mutant of STAT5 induced tumor formation in nude mice. Ba/F3 cells were infected with empty virus (−) and retrovirus encoding wild-type STAT5 or constitutively active mutant of STAT5 (1/6). 1 × 10⁵ cells of transduced Ba/F3 cells were subcutaneously injected into nude mice. A, nude mice were photographed 17 days post-inoculation. Arrows indicate tumors in nude mice (left). 17 days post-inoculation, tumors at the injected sites were weighed and plotted (right). * indicates significant difference p < 0.01, n.d. indicates not detected. B, 17 days post-inoculation, mice were sacrificed. Morphological changes of the spleen and liver were documented. C, 17 days post-inoculation, four mice were sacrificed, and the spleen and liver were weighed and plotted. * and ** indicate significant differences of p < 0.01 and p < 0.005, respectively. D, 17 days post-inoculation, liver sections were stained with hematoxylin-eosin (magnification ×400). E, eight nude mice were injected with Ba/F3 cell lines infected with empty virus (−) and retrovirus encoding wild-type STAT5 or constitutively active mutant of STAT5. For 40 days post-inoculation, mouse survival was monitored daily.
mutant-induced tumorigenesis indicate that STAT5 is a critical mediator in JAK2 V617F.

STAT5 was effectively extended (Fig. 9), mice inoculated with V617F/FL cells expressing shRNA against STAT5 but not control shRNA (Fig. 9A). The inhibition of tumor formation exhibited dependence on the knockdown efficiency of STAT5. On the other hand, no significant difference in tumor formation was observed when inoculated with V617F/FL cells infected with control shRNA. Furthermore, shRNA against STAT5 remarkably inhibited the spleen and liver enlargement induced by JAK2 V617F mutant and EpoR (Fig. 9, B and C). Marked invasion of tumor cells into the liver was also inhibited in mice receiving V617F/FL cells expressing shRNA against STAT5 but not control shRNA (Fig. 9D). Compared with nude mice that were inoculated with V617F/FL and V617F/FL cells expressing control shRNA, the life span of nude mice inoculated with V617F/FL cells expressing shRNA against STAT5 was effectively extended (Fig. 9E). These results clearly indicate that STAT5 is a critical mediator in JAK2 V617F mutant-induced tumorigenesis in vivo.

DISCUSSION

The Janus kinase family contains seven highly conserved domains, namely JH domains 1–7. The C-terminal JH1 domain encodes the catalytic domain of tyrosine kinase, and the phosphorylation of tyrosine residues at 1007/1008 in JH1 is well known to be essential for activation following cytokine stimulation. The JH2 pseudokinase domain has been reported to negatively regulate the activity of JAKs through interaction with the JH1 kinase domain (1, 2). Recently, JAK2 V617F mutation in JH2 domain has been found as a common genetic abnormality in patients with myeloproliferative disorders (MPDs), and it was reported that this mutation leads to constitutive activation of JAK2 (19–21). As in previous reports, leukemia-associated L611S mutation in JAK2 also exhibits a similar phenotype to V617F mutation, and these mutations in the JH2 domain of JAK2 may disrupt intramolecular interaction between JH1 and JH2 (26).

However, Lu et al. (24) demonstrated that constitutive activation of the JAK2 V617F mutant requires coexpression of a cognate homodimeric cytokine receptor, suggesting that the association of V617F mutant with the receptor seems to be essential for exhibition of its disordered proliferative activity. Indeed, the N-terminal JH3 to JH7 domains of the JAK family, named the FERM domain (also known as the protein 4.1, ezrin, radixin, moesin domain), mediate their association with membrane proximal regions of cytokine receptors (1). Wernig et al. (27) also reported that constitutive activation of the JAK2 V617F mutant was easily disrupted by introducing a point mutation in the FERM domain (Y114A). It was also reported that the phosphorylation of tyrosine 119 in the FERM domain is important for regulation of the interaction between EpoR and JAK2 (28), and we additionally observed that another point mutation (Y119E) disrupts the constitutively active phenotype of JAK2 V617F (data not shown). As shown in Fig. 2C, whereas the JAK2 V617F mutant was slightly activated only when expressed, the activity of the JAK2 V617F mutant was markedly enhanced by the coexpression of EpoR. Furthermore, it was observed that the JAK2 V617F mutant interacted with EpoR regardless of Epo stimulation (Fig. 2B); therefore, it is expected that the JAK2 V617F mutant could induce the conditions for full activation by interacting with EpoR.

When mice were transplanted with bone marrow cells expressing the JAK2 V617F mutant, the mice developed erythrocytosis and exhibited PV-like features, showing that MPDs are caused by this mutation in vivo (19). Recently, JAK2 V617F transgenic mice were developed and showed granulocytosis, leucocytosis, and thrombocytosis (29). Previously, we reported that the JAK2 V617F mutant is capable of inducing tumor cell...
invasion (23). As shown in Fig. 4, at the subcutaneous injection site, Ba/F3 expressing both JAK2 V617F mutant and EpoR behaved like malignant tumor cells and could actively grow and penetrate various organs, including the liver, demonstrating that the JAK2 V617F mutant showed potent oncogenic activity to stimulate a series of cancer-related processes, not only for cellular transformation and tumorigenesis but also for tumor cell invasion in vivo. In a previous study, we observed that the Ba/F3 cells transformed by JAK2 mutant invaded various organs (23). To detect the transplanted cells, we introduced the GFP expression in the cells transformed by JAK2 mutant and control cells and chased where transplanted cells infiltrate. When Ba/F3 cells transformed by JAK2 mutant were transplanted, GFP-positive cells were detected in hepatocytes and splenocytes, suggesting that the JAK2 mutant-induced transformation of Ba/F3 cells finally tend to result in infiltration of tumor cells into these organs.

Recently, Grimwade et al. (30) performed immunocytochemistry on bone marrow biopsies of MPD patients with JAK2 V617F mutation and examined the phosphorylation level of STAT5. In their study, JAK2 V617F-positive MPDs, including PV, essential thrombocythemia, and PMF, exhibited a 1.2–1.4-fold increase of phospho-STAT5 in megakaryocytes compared with normal controls and JAK2 V617F-negative MPDs. Teofili et al. (31) also analyzed the phosphorylation level of STAT3 and STAT5 in patients with PV and PMF that harbor V617F mutation in the JAK2 locus; however, their results failed to agree with the report by Grimwade et al. (30). Teofili et al. (31) showed that the phosphorylation of STAT3 and STAT5 is uniformly increased in the bone marrow of PV patients and is reduced in the bone marrow of PMF patients. These reports suggest that the role of STAT molecules in oncogenic JAK2-induced hematopoietic disorder remains controversial. This strongly encouraged us to examine the role of STAT5 in JAK2 V617F mutant-mediated cellular transformation and tumorigenesis, which are general experiment systems.

The cellular transformation and tumorigenesis induced by the JAK2 V617F mutant require the expression of EpoR, and this also could be observed in the presence of the EpoR-H mutant, which was able to induce STAT5 activation but not the activation of other signals, including ERK and Akt. On the other hand, the functions of the JAK2 V617F mutant was not exhibited when cells expressed the EpoR-HM mutant, which lacks the ability of STAT5 activation (Figs. 3 and 4). Furthermore, a constitutively active form of STAT5 exhibited sufficient oncogenic activity, and these data seem to indicate the essential and sufficient role of STAT5 in oncogenic JAK2-induced tumorigenicity at a glance; however, in the presence of Epo, STAT5 is dispensable for Epo-induced cell survival. Epo stimulation apparently induced the activation of endogenous JAK2, which should be the wild type, and activated endogenous JAK2 may not require STAT5. Although this is speculative, the

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**FIGURE 8. Knockdown of STAT5 significantly inhibited cytokine-independent cell survival induced by JAK2 V617F mutant and EpoR.** Ba/F3 cell lines were infected with empty virus (−) and retrovirus encoding wild-type JAK2 c-HA or JAK2 mutant c-HA (V617F) with EpoR c-FLAG (FL). Transduced Ba/F3 cells were sequentially infected with retrovirus harboring control shRNA or two kinds of shRNAs against murine STAT5 (panels 1 and 2). After puromycin selection, cells were harvested. A, whole cell lysates were immunoblotted (IB) with anti-STAT5 antibody, anti-HA antibody, anti-FLAG antibody, or anti-β-actin antibody. C, control. B, transduced Ba/F3 cell lines with shRNAs were washed twice with PBS and left untreated or stimulated with Epo (5 units/ml) for 24 h. The viability of these cells was determined by the trypan blue exclusion method. Results represent the mean ± S.D. of three independent experiments. C, cells were fixed, treated with propidium iodide, and subjected to FACS analysis, as described under “Experimental Procedures.” D, transduced Ba/F3 cells were washed twice with PBS and left untreated or stimulated with Epo (5 units/ml) for 24 h. DNA was isolated from cells and subjected to agarose gel electrophoresis.

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**Role of STAT5 in Transformation Induced by JAK2 Mutant**
requirement of STAT5 may be a difference between wild-type JAK2 and the oncogenic JAK2 mutant. Interestingly, in cells expressing EpoR-H or EpoR-HM, Epo stimulation could activate Akt and ERK; however, the V617F mutant failed to activate these signaling pathways (Fig. 2). This result suggests that the required region of EpoR for ERK and Akt activation is quite different when stimulated with Epo and the JAK2 V617F mutant. Of course, it is also possible that JAK2 V617F mutant could activate another signaling pathway, and this also could be sufficient to exhibit JAK2 V617F mutant-induced cell survival and proliferation.

We also utilized shRNA against STAT5 and tested the effect of STAT5 knockdown for JAK2 V617F mutant-induced anti-apoptotic effect and tumorigenicity. STAT5 knockdown had a more severe effect than when EpoR-HM was used. Currently, there is no thorough explanation for the difference between our two experiments. In the course of our in vitro study, all experiments testing apoptosis were performed in the presence of 1% serum with/without Epo. Several reports indicate the requirement of STAT5, not only for the JAK2 pathway (32–34). In our experiments, STAT5 may be also required for serum-induced priming signals emphasizing the effect of Epo, and this could be one reason why our experiments using shRNA against STAT5 had a more severe effect.

Furthermore, to show that the JAK2 V617F mutant could stimulate a series of cancer-related processes in vivo, we injected a series of Ba/F3 cells expressing various combinations of JAK2 V617F and EpoR mutants. The results showed that JAK2 V617F was still able to form tumors when EpoR-HM or shRNA against STAT5 was coexpressed (Figs. 4 and 9), although treatments inactivating STAT5 markedly slowed down tumor progress to malignancy. These data were also controversial; however, this could be due to the difference in the environment around cells between in vitro and in vivo. When tumor cells were injected into nude mice, it should be considered that originally there was cellular interaction between tumor cells and other cells in mice. These cells could affect tumor cells and stimulate their progression, including metastasis. Under these conditions, STAT5 could be dispensable; however, it harbors potent ability to contribute to tumor malignancy.

Previous studies have shown that tumor-related and mitogenic signaling pathways, such as phosphatidylinositol 3-ki-
nase-Akt and MEK-ERK, are predominantly active in tumor cells with invasive and metastatic characteristics (35, 36). As shown in Fig. 4B, the tumors formed in mice inoculated with V617F/FL, V617F/H, and V617F/HM cells showed that the activation of ERK and Akt occurred regardless of EpoR deletion mutants in vivo. Thus, it is easily speculated that the phosphatidylinositol 3-kinase-Akt and MEK-ERK pathways also play certain roles in cancer development caused by the JAK2 V617F mutant and also induced by Epo. When V617F/FL cells were treated with the phosphatidylinositol 3-kinase inhibitor, LY294002, the cells underwent apoptotic cell death (data not shown). On the other hand, treatment with U0126, MEK inhibitor, decreased the S phase but was not able to increase the sub-G1 population in V617F/FL cells (data not shown). These observations indicate that the phosphatidylinositol 3-kinase-Akt pathway contributes to the survival of V617F/FL cells and that the MEK-ERK pathway is important for the proliferation of V617F/FL cells, respectively. Although only the role of STAT5 of wild-type STAT5a and the constitutively active STAT5a mutant.

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