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Tyrosine-phosphorylated SOCS3 negatively regulates cellular transformation mediated by the myeloproliferative neoplasm-associated JAK2 V617F mutant

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ABSTRACT

In the majority of myeloproliferative neoplasms (MPNs) patients, a point mutation, V617F has been found in Janus kinase 2 (JAK2) gene, and this JAK2 mutant provoked aberrant signaling pathway. In the current study, we found that suppressor of cytokine signaling proteins 3 (SOCS3) possessed the tumor suppressive activity against the JAK2 V617F mutant-provoked cellular transformation. The knockdown of SOCS3 increased the expression level of the JAK2 V617F mutant, which enhanced the activation of signaling mediators, including signal transducer and activator of transcription 3 and 5 (STAT3, STAT5) and extracellular signal-regulated kinase (ERK), and also increased of the proliferation rate and tumorigenesis activity of Ba/F3 cells expressing the JAK2 V617F mutant and erythropoietin receptor (EpoR). In contrast, the enforced expression of SOCS3 significantly inhibited the JAK2 V617F mutant-induced activation of downstream signaling molecules, cell proliferation, and tumorigenesis by down-regulating the expression level of the JAK2 V617F mutant. SOCS3 interacted with the JAK2V617F mutant through its SH2 domain and was phosphorylated at Tyr-204 and Tyr-221 in its SOCS box by the JAK2V617F mutant. SOCS3 mutants carrying a mutation in the SH2 domain (R71E) and a substitution at Tyr-221 (Y221F) failed to exert inhibitory effects on JAK2V617F mutant-induced cellular transformation and tumorigenesis. Collectively, these results imply that SOCS3 plays a negative role in the JAK2 V617F mutant-induced oncogenic signaling pathway through its SH2 domain and the phosphorylation of Tyr-221 in its SOCS box.

1. Introduction

The somatic mutation at position 617 (valine-to-phenylalanine; V617F) in the tyrosine kinase, janus kinase 2 (JAK2), has been implicated in the development of a number of myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) [1,2]. The JAK2 V617F mutant was shown to be constitutively active when homodimeric type I cytokine receptors such as erythropoietin receptor (EpoR) and thrombopoietin receptor (TpoR) were expressed [3]. The co-expression of the JAK2 V617F mutant and EpoR conferred growth factor independence to the hematopoietic cell line Ba/F3 [4,5]. It is well suggested that the JAK2 V617F mutant possesses the oncogenicity under the condition which co-expresses EpoR, since transplantation of Ba/F3 cells which ectopically expressed JAK2 V617F mutant and EpoR caused tumor formation in nude mice [6].

As a signaling platform, EpoR is phosphorylated by JAK2 V617F mutant at eight tyrosine residues: Tyr-343, Tyr-401, Tyr-429, Tyr-431, Tyr-443, Tyr-460, Tyr-464, and Tyr-479 [7,8]. The phosphorylation of EpoR at tyrosine residues results in the recruitment and activation of

Abbreviations: CHX, cycloheximide; CIS, cytokine-inducible SH2-containing protein; CML, chronic myeloid leukemia; EpoR, erythropoietin receptor; ERK, extracellular signal-regulated kinase; ET, essential thrombocythemia; FBS, fetal bovine serum; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; ires, internal ribosomal entry site; JAK2, janus kinase 2; MPNs, myeloproliferative neoplasms; MSCV, murine stem cell virus; PI3K, phosphoinositide 3-kinase; PMF, primary myelofibrosis; PV, polycythemia vera; s.c., subcutaneous; SH2, Src homology 2; SOCS, suppressor of cytokine signaling proteins; STAT, signaling mediators including signal transducer and activator of transcription; TpoR, thrombopoietin receptor

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downstream signaling molecules such as Grb2, phosphoinositide 3-kinase (PI3K), and signal transducer and activator of transcription 5 (STAT5) through the interaction with their Src homology 2 (SH2) domain [9,10].

We recently reported the presence of essential three tyrosine residues, Tyr-343, 460 and 464 in EpoR for the tumorigenesis and the activation of STAT5 provoked by JAK2 V617F mutant [7–10]. Furthermore, we reported the phosphorylation of Tyr-479 in EpoR is required for the JAK2 V617F mutant-stimulated activation of the PI3K-Akt pathway, which exhibits anti-apoptotic activity [11].

So far, several studies revealed the importance of suppressor of cytokine signaling proteins (SOCS)/cytokine-inducible SH2-containing protein (CIS) family as negative regulator for the cytokine signaling. SOCS/CIS family consists of eight members: SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, SOCS7, and CIS, because of their structural conservation such as a central SH2 domain and a carboxy-terminal 40-amino-acid module known as the SOCS box [12,13]. In amino terminal portion, their structures seem to be diversified, and the uniqueness for their function may be due to these structural differences. Through their SOCS box, SOCS/CIS family members interact with elongin B/C and cullin 5, which possess E3 ubiquitin ligase activity. Substrates for SOCS/CIS-mediated ubiquitination associate with amino-terminal portion of each SOCS/CIS member, and then ubiquitinated proteins are degraded by proteasome [14–16].

Our previous research revealed that the expression of CIS and SOCS1 was significantly enhanced by JAK2 V617F mutant-STAT5 signaling axis [17]. While the role of SOCS1 against JAK2 V617F mutant has not yet been clarified, we clarified that CIS suppressed the JAK2 V617F mutant-provoked tumorigenicity and STAT5 activation through the interaction with EpoR at phosphorylated Tyr-401. Varghee *et al.* performed *in vitro* pulldown experiments and showed physical interaction between SOCS3 and JH1-JH2 fragments containing kinase domain and pseudokinase domain derived from wild type and V617F mutant of JAK2 [18]. They also showed the tumor suppressive effect of SOCS3 on JAK2 V617F mutant-induced oncogenicity. The expression of SOCS3 was shown to be induced by the JAK2 V617F mutant in HEK cells, and SOCS3 colocalized with the JAK2 V617F mutant at the membrane and decreased the protein expression of the JAK2 V617F mutant [19]. On the other hand, there is the conflicting evidence showing that the activity of the JAK2 V617F mutant was enhanced in the presence of SOCS3 by increasing the stability of the JAK2 V617F mutant [20].

In the present study, we re-examined the role of SOCS3 in JAK2 V617F mutant-mediated cellular transformation to clarify its involvement. We found that SOCS3 negatively modulated JAK2 V617F mutant-mediated signaling by interacting with the JAK2 V617F mutant through its SH2 domain and demonstrated that the phosphorylation of Tyr-221 in the SOCS box may be necessary for this inhibitory effect of SOCS3.

2. Materials and methods

2.1. Reagents

Cycloheximide (CHX) and ruxolitinib were purchased from Nacalai Tesque (Tokyo, Japan) and SYN kinase (San Diego, CA, USA), respectively. Anti-β-actin, anti-EpoR, anti-Pim1, and anti-c-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-Flag antibody (M2) and anti-HA antibody (3F10) were purchased from Sigma-Aldrich (St. Louis, MO) and Roche Applied Science (Penzberg, Upper Bavaria, Germany), respectively. Other first antibodies utilized in this study were obtained from Cell Signaling Technology (Danvers, MA).

2.2. Cell culture

Parental Ba/F3 cells were cultured in RPMI1640 (Nacalai tesque, Japan) containing 10% Fetal bovine serum and 10 ng/ml murine IL-3 (Peprotech, NJ, USA). In the case of Ba/F3 expressing EpoR, cells were maintained by the culture with completed RPMI1640 containing 5U/ml human Epo (ESPO 3000). HEK293T cells were cultured in Dulbecco's Modified Eagle Medium supplied with 10% FBS, penicillin and streptomycin.

2.3. Plasmids

The cDNAs of murine JAK2 C-HA, murine EpoR C-Flag, and murine N-Flag-SOCS3 were inserted into retroviral plasmids, MSCV-Hygro, MSCV-ires-GFP, and MSCV-Puro (Clontech, Mountain View, CA), respectively. The mutagenesis of amino acid residues, V617F in JAK2 and Y204F and Y221F in SOCS3, was performed using a site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instruction.

2.4. RNA interference

An annealed oligonucleotide including the sequences of shRNAs for murine SCOS3 was ligated into *Bgl*II and *Hin*dIII sites of a pSUPER-retropuro (Oligoengine, Seattle, WA). The sequences of oligonucleotides used to construct shRNA retroviral vectors were as follows: sh-SOCS3, 5′-GATCCCCGGGAAGGATGGAAGAGAAATTCAAGAGATTTCTCTTCCATCCTTCCCTTTTTA-3′ and 5′-AGCTTAAAAAGGGAAGGATGGAAGAGAAATCTCTTGAATTTCTCTTCCATCCTTCCCGGG-3′ (underlined sequences correspond to the sequence of murine SOCS3).

2.5. Retroviral infections and cell cultures

The plasmids including retroviral RNA genome and the helper plasmids encoding *gag*, *pol* and *env* were transfected into HEK293T cells using FuGENE 6 (Promega, Madison, WI). Thirty-six hours after transfection, the conditioned culture media including secreted retroviruses was collected. Ba/F3 cells were infected with an empty virus (–), retroviruses harboring wild-type murine JAK2 c-HA, or JAK2 V617F mutant c-HA with murine EpoR using RetroNectin (Takara, Tokyo, Japan) as described previously [11]. Ba/F3 cells expressing the JAK2 V617F mutant and EpoR, named VF cells, were sequentially infected with a retrovirus including the cDNA of Flag-SOCS3 or its mutants, or the sequence of shRNA against SOCS3 using RetroNectin. To concentrate the cells infected with retroviruses, infected cells were cultured in the culture media including 2 mg/ml puromycin or 2 mg/ml hygromycin (InVivoGen, San Diego, CA, USA).

2.6. Ba/F3 cell growth assay

After retroviral infection and selection with antibiotics, the exponentially growing Ba/F3 cells (1×10^5) were washed twice with PBS to remove the growth factors. And then, cells were resuspended with RPMI 1640 medium supplemented with 1% FBS, and cultured for 3 days. The number of living cells and cell viability were measured using a Beckman Coulter VI-Cell (Beckman Coulter, Fullerton, CA) as described previously [11].

2.7. Cell cycle analysis

Transduced Ba/F3 cells were washed twice with PBS and incubated with RPMI 1640 medium supplemented with 1% FBS for 24 h. After that the cells were fixed with 70% (v/v) cold ethanol at −20 °C overnight, cells were collected by centrifuging (5000 rpm, 2 min). Cells were treated with 10 μg/ml RNase A (Wako, Tokyo, Japan), and then stained with 100 μg/ml propidium iodide (Sigma). DNA content and cell cycle distribution were examined by a flow cytometric analysis using FACSCalibur and analyzed using CellQuest software as described previously [11].

Table 1
Cell strains analyzed in the present study and their characteristics.

Cell Strains		JAK2	SOCS3	Degradation of JAK2	Activation of[a] STAT3, STAT5, and ERK	Cell[b] Proliferation	Tumorigenesis in nude mice
1	(–)	None	None	ND[c]	(STAT5: −)	ND	ND
2	WT	Wild-type	None	ND	(STAT5: −)	ND	ND
3	VF	V617F	None	ND	(STAT5: +)	ND	ND
4	Sh-Luc	V617F	None	+	+	+	+
5	Sh-SCOS3	V617F	None	−	+ + +	+ +	+ + +
6	VF(–)	V617F	None	+	+	+	+
7	SOCS3	V617F	Wild-type	+ + +	−	−	−
8	R71E	V617F	R71E	−/+	+	+	+
9	Y204F	V617F	Y204F	+ + +	−	−	−
10	Y221F	V617F	Y221F	−/+	+	+	+
11	Y204/221F	V617F	Y204/221F	−/+	+	+	+

[a] The activation of STAT3, STAT5, ERK, and Akt was tested in the absence of a cytokine stimulation.
[b] Cell proliferation was tested in the absence of a cytokine stimulation.
[c] ND means not detected.

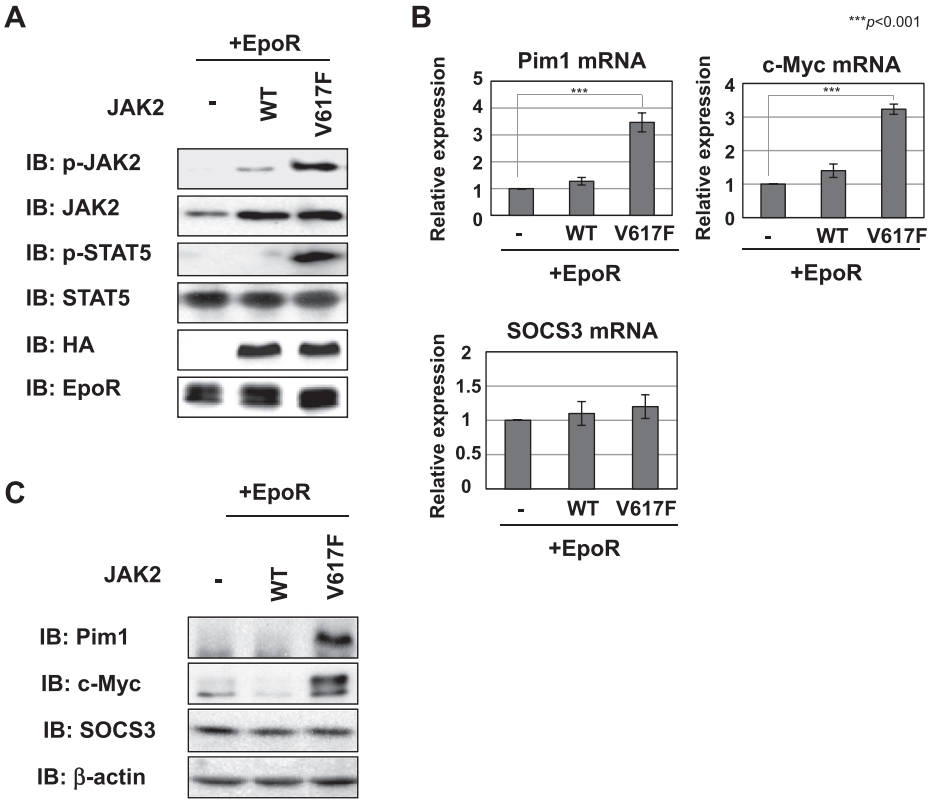

Fig. 1. SOCS3 expression is not affected by JAK2 activity. Using retroviral infectious procedure, (–) cells, WT cells, and VF cells were established, respectively. These sub-cell lines were cultured with DMEM containing 1% FBS for 24 h. (A) Whole cell lysates were prepared and analyzed by immunoblotting with an anti-phospho-JAK2, anti-JAK2, anti-phospho-STAT5, anti-STAT5, anti-HA, or anti-EpoR antibody. (B) Using cDNAs synthesized from total RNAs, the mRNA expression of Pim1, c-Myc, and SOCS3 was assessed by a quantitative RT-PCR analysis. GAPDH mRNA was used as an internal control. Data are shown as the mean ± SD of three independent experiments. ***indicates a significant difference of $p < 0.001$. (C) Whole cell lysates were immunoblotted with an anti-Pim1, anti-c-Myc, anti-SOCS3, or anti-β-actin antibody.

2.8. DNA fragmentation assay

Transduced and exponentially growing Ba/F3 cells were washed twice with PBS. And then, cells were resuspended with RPMI 1640 medium supplemented with 1% FBS, and cultured for 24 h. Preparation of genomic DNA and gel electrophoresis were performed as described previously [11].

2.9. Immunoprecipitation and immunoblot analysis

Cells were harvested in ice-cold PBS and lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM NaF, 0.2 mM Na_3VO_4, 2 mg/ml aprotinin, and 2 mg/ml leupeptin). After centrifuging (15,000 rpm, 15 min), supernatants were incubated with the indicated antibody and the protein complexes were captured with protein G-Sepharose (Zymed Laboratories Inc.) for 4 h. Immunoprecipitates were washed three times with NP-40 lysis buffer, and then eluted with Laemmli's sample buffer for SDS-PAGE. After SDS-PAGE and transfer were performed, each protein was detected using designed antibodies and sites of antibody binding were detected using HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark). To evaluate protein stability, cells were treated with 100 mg/ml CHX for the indicated periods to block new protein synthesis, and cell lysates were then prepared. HA-JAK2 and EpoR remaining in cell lysates were detected by an immunoblot analysis with anti-HA and anti-EpoR antibodies. Protein amounts were quantified by ImageJ, and the results obtained were shown in graphs.

2.10. RT-PCR

cDNAs were synthesized from 2 mg of total RNAs as templates by the reaction with reverse-transcriptase and an oligo $(dT)_{20}$ primer as described previously [11]. The quantitative PCR was performed by using synthesized cDNAs, KAPA SYBR® FAST qPCR Kits (KAPA Biosystems, Wilmington, MA, USA) and an iCycler detection system (Bio-Rad, Berkeley, CA, USA). The primers used to amplify the target cDNAs

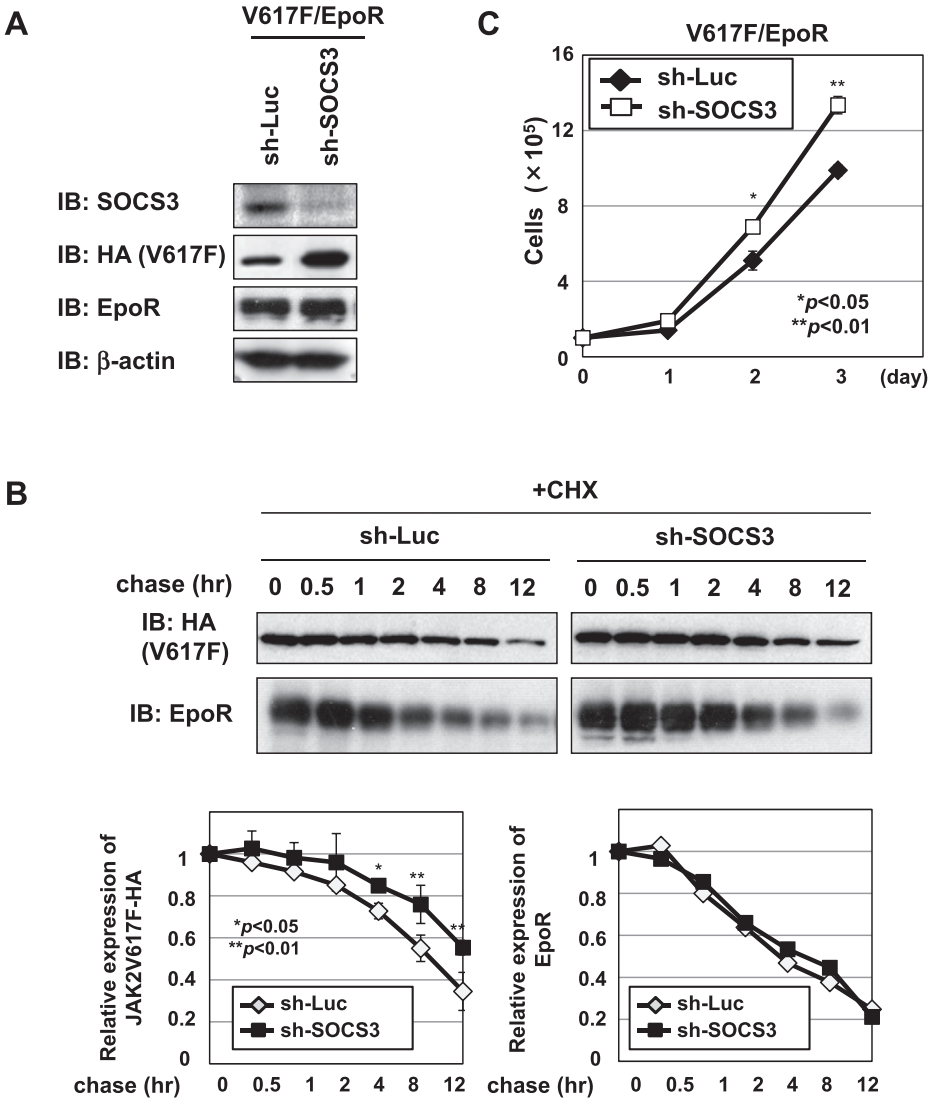

Fig. 2. The knockdown of SOCS3 enhances the proliferation rate of cells transformed by the JAK2 V617F mutant. VF cells were sequentially infected with a retrovirus including control shRNA against luciferase (sh-Luc) or shRNA against SOCS3 and named sh-Luc cells and sh-SOCS3 cells, respectively. (A) Whole cell lysates were prepared from indicated cells, and then analyzed by immunoblot analysis with an anti-SOCS3, anti-HA, anti-EpoR, or anti-β-actin antibody. (B) sh-Luc cells and sh-SOCS3 cells were cultured in the medium including cycloheximide (100 mg/ml) for the indicated periods. Whole cell lysates were immunoblotted with an anti-HA or anti-EpoR antibody. The relative amount of JAK2 V617F mutant c-HA and EpoR was graphed. Data are shown as the mean ± SD of three independent experiments. * and ** indicate significant differences of $p < 0.05$ and $p < 0.01$, respectively. (C) sh-Luc cells and sh-SOCS3 cells were incubated with DMEM containing 1% FBS for 3 days. Viable cells were counted by the trypan blue exclusion method. Results represent the mean ± SD of three independent experiments. (D–F) sh-Luc cells and sh-SOCS3 cells were incubated with DMEM containing 1% FBS for 24 h. (D) Whole cell lysates were analyzed by immunoblotting with an anti-phospho-STAT5, anti-STAT5, anti-phospho-STAT3, anti-STAT3, anti-phospho-ERK1/2, or anti-ERK1/2 antibody. (E) The mRNA expression of Pim1 and c-Myc was assessed by a quantitative RT-PCR analysis. GAPDH mRNA was used as an internal control. Data are shown as the mean ± SD of three independent experiments. **indicates a significant difference of $p < 0.01$. (F) Whole cell lysates were immunoblotted with an anti-Pim1, anti-c-Myc, or anti-β-actin antibody.

were: c-Myc 5′-tgcgacgaggaagagaattt-3′ (forward) and 5′-aaccgctccacatacagtcc-3′ (reverse); Pim1 5′-cttcggctcggtctactctg-3′(forward) and 5′-ccgagctcaccttcttcaac-3′ (reverse); SOCS3 5′-gttgagcgtcaagacccagt-3′ (forward) and 5′-cgttgacagtcttccgacaa-3′ (reverse); GAPDH, 5′-actccactcacggcaaattc-3′ (forward) and 5′-ccttccacaatgccaaagtt-3′ (reverse)

2.11. Animal tumorigenesis

To evaluate the JAK2 V617F mutant-provoked oncogenisity *in vivo*, transduced Ba/F3 cells (1×10^7) were seeded by subcutaneous transplantation into female BALB/c nude mice aged 4 weeks (n = 8). And then mice were sacrificed 16 to 18 days post-inoculation, and the weights of the tumor, liver, spleen and lymph nodes were recorded. All experimental protocols were approved by the Animal Usage Committee of Keio University (Approval number, 15029-(0)). Methods were performed in accordance with the approved guidelines.

2.12. Statistical analysis

Data are presented as the mean ± SD. All experiments were repeated at least three times. The significant differences between mean values for the treatment groups were calculated by the Student's t-test and a one- or two-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered to be significant for values of $p < 0.05$.

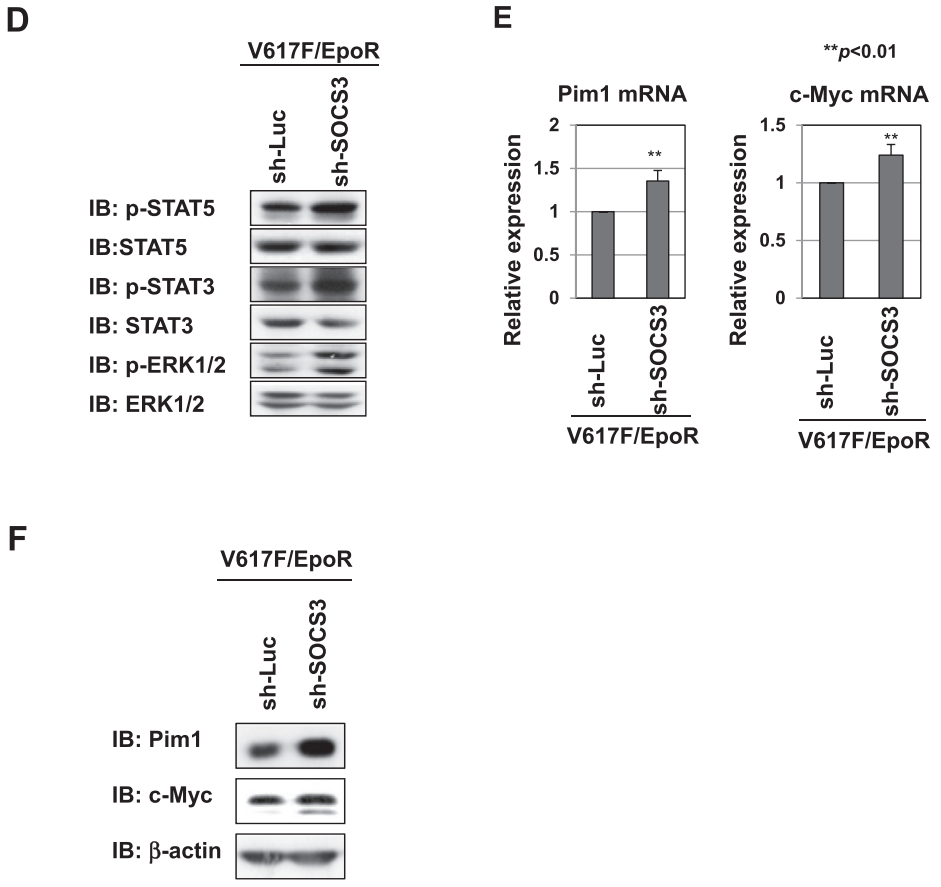

Fig. 2. (*continued*)

3. Results

3.1. SOCS3 expression was not affected by the activity of JAK2

First, we utilized the retroviral infectious technique, and established the Ba/F3 cells were expressing wild-type JAK2 c-HA (WT) or the JAK2 V617F mutant c-HA (V617F) with the co-expression of EpoR, designated as (–) cells, WT cells, and VF cells, respectively. The characteristics of each cell sub-strain were shown in Table 1. As shown in Fig. 1A, the JAK2 V617F mutant was constitutively activated, and caused the phosphorylation of STAT5 without the cytokine stimulation in VF cells, while wild type JAK2 failed to do so. Furthermore, we performed the quantitative RT-PCR to evaluate the expression of STAT5 target genes including Pim1 and c-Myc [21–23]. As shown in Fig. 1B, the mRNA expression of Pim1 and c-Myc was drastically enhanced in VF cells. On the other hand, the expression level of SOCS3 mRNA was equivalent in all cells, such as (–) cells, WT cells, and VF cells (Fig. 1B). Consistent with mRNA expression levels, the protein expression of Pim1 and c-Myc was induced in VF cells only, while the expression of the SOCS3 protein was detected equivalently in all cells, suggesting that the expression of SOCS3 was not affected by the activity of JAK2 (Fig. 1C).

3.2. Knockdown of SOCS3 enhanced the JAK2 V617F mutant-induced signaling pathway

Although the expression level of SOCS3 was not altered by the JAK2 V617F mutant, we investigated whether SOCS3 functions in the transformation induced by the JAK2 V617F mutant by knocking down endogenous SOCS3 in VF cells. To test the effects of silencing the expression of SOCS3, VF cells were infected with retroviruses harboring shRNA against luciferase as the control and SOCS3 and named sh-Luc cells and sh-SOCS3 cells, respectively. The characteristics of each cell strain are shown in Table 1. The knockdown of SOCS3 significantly increased the expression level of the JAK2 V617F mutant, but not EpoR (Fig. 2A). To investigate whether SOCS3 attenuates the protein stability of the JAK2 V617F mutant, new protein synthesis was blocked by the treatment with CHX, and decrease of JAK2 protein was chased by immunoblot analysis. Decrease of protein expression of JAK2 V617F over time was significantly delayed when the expression of SOCS3 was silenced. On the other hand, the knockdown of SOCS3 failed to affect the degradation of EpoR (Fig. 2B). We also analyzed the proliferation of VF cells, and their proliferation was significantly accelerated when the expression of SOCS3 was knocked down (Fig. 2C). Furthermore, the shRNA-caused silencing the expression of SOCS3 augmented the phosphorylation of STAT5, STAT3, and ERK1/2 in VF cells (Fig. 2D). The expression of Pim1 and c-Myc in VF cells at both the mRNA and protein levels also increased when SOCS3 was knocked down (Fig. 2E and F). These observations strongly suggest that SOCS3 negatively regulated the JAK2 V617F mutant-derived signaling pathway.

3.3. SOCS3 functions as tumor suppressor against JAK2 V617F mutant

To elucidate the role of SOCS3 in tumorigenesis caused by the JAK2 V617F mutant, nude mice were inoculated subcutaneously (s.c.) with empty virus-infected Ba/F3 cells (control cells), sh-Luc cells, and sh-SOCS3 cells. While nude mice inoculated with control cells did not possessed any tumors, prominent formation of tumor was observed in nude mice at the injected site of sh-Luc cells and sh-SOCS3 cells. Comparing to the case of sh-Luc cells, the mice inoculated with sh-SOCS3 cells possessed apparent larger tumors (Fig. 3A and B). In addition, the liver, spleen, and lymph nodes were markedly swollen in mice inoculated with sh-Luc cells, and these organs were significantly larger in the case of mice transplanted with sh-SOCS3 cells, while mice inoculated with control cells exhibited no alteration (Fig. 3C and D).

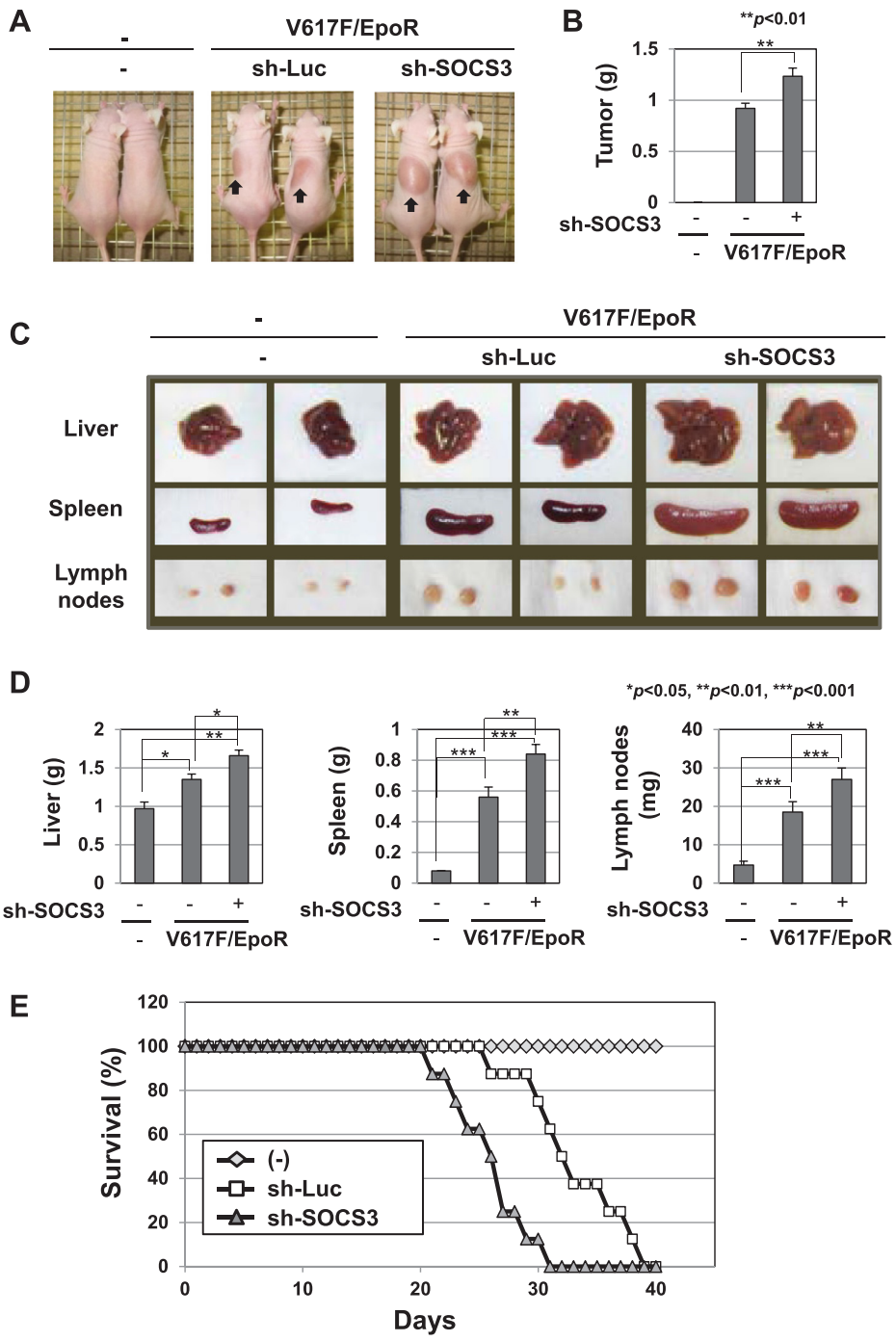

Fig. 3. The knockdown of SOCS3 enhances the tumorigenesis activity of cells transformed by the JAK2 V617F mutant. VF cells infected with an empty virus (–), sh-Luc cells, and sh-SOCS3 cells (1×10^7 cells) were subcutaneously injected into nude mice (n = 8). (A) Nude mice inoculated with transduced cells were photographed 16 days post-inoculation. Arrowheads indicate tumors in nude mice. (B) Tumors obtained from each transplanted mice shown in (A) were weighed and plotted. ** indicates a significant difference of $p < 0.01$. (C) and (D) Morphological changes in the liver, spleen, and lymph nodes were photographed, and their weights were measured and shown in the graph in (D). *, **, and *** indicate significant differences of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. (E) The survival of eight nude mice inoculated with transduced cells was monitored daily until 40 days post-inoculation.

Whereas the inoculation of control cells exhibited no lethality until 40 days after transplantation, the inoculation of sh-Luc cells let all mice die within 40 days. Significantly, the life span of nude mice receiving sh-SOCS3 cells was markedly shorter than mice inoculated with sh-Luc cells (Fig. 3E). These results indicate that SOCS3 negatively regulated tumorigenesis induced by the JAK2 V617F mutant.

3.4. Enforced expression of SOCS3 inhibited the JAK2 V617F mutant-induced signaling pathway through the SH2 domain

To elucidate the mechanisms by which SOCS3 functions in JAK2V617F mutant-induced cellular transformation, VF cells were infected with empty virus (–) or retroviruses encoding SOCS3 and its mutant carrying a mutation in the SH2 domain (R71E). As shown in Fig. 4A, these Flag-tagged proteins were detectable by immunoblotting, and the total amounts of SOCS3 was slightly increased. The enforced expression of SOCS3 markedly reduced the expression level of the JAK2 V617F mutant, while the R71E mutant did not. On the other hand, the expression level of EpoR was not affected by the enforced expression of SOCS3 or its R71E mutant (Fig. 4A). JAK2V617F mutant was detected in the immunoprecipitates of SOCS3 but not in the immunoprecipitates of its R71E mutant (Fig. 4B). Kershaw et al. solved the crystal structure of protein complexes including an intracellular fragment of interleukin-6 (IL-6) receptor β-chain, JAK2 and SOCS3 [24]. In the crystal structure, SOCS3 exhibited binding activity to both the kinase domain of JAK2 and IL-6 receptor β-chain. Therefore, the SH2 domain of SOCS3 is most likely critical for the interaction with JAK2-EpoR complexes. Although the expression of SOCS3 increased the degradation of the JAK2

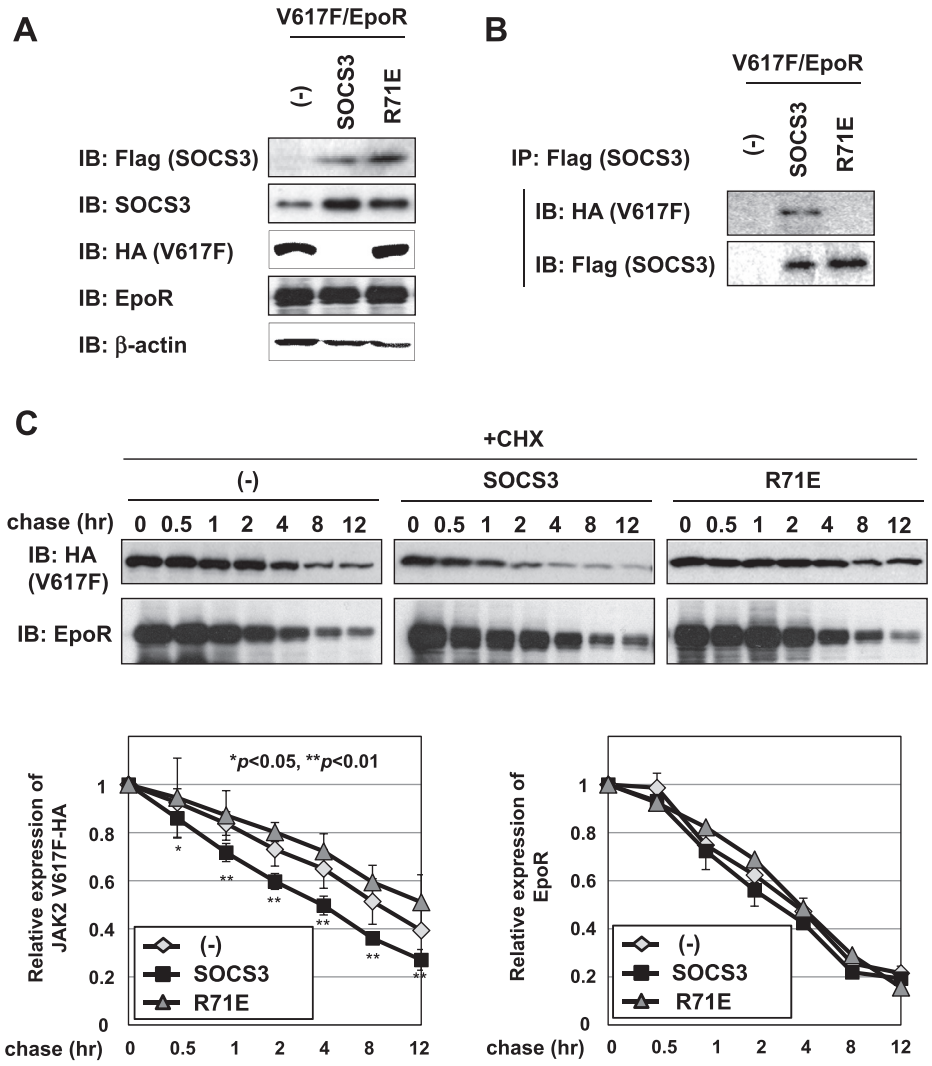

Fig. 4. Enforced expression of SOCS3 decreases the stability of the JAK2 V617F mutant, but not EpoR via its SH2 domain. VF cells were infected with an empty virus (–) and a retrovirus encoding Flag-tagged wild-type SOCS3 or its mutant (R71E) and named VF (–) cells, SOCS3 cells, and R71E cells, respectively. (A) Whole cell lysates were immunoblotted with an anti-Flag, anti-SOCS3, anti-HA, anti-EpoR, or anti-β-actin antibody. (B) Cell lysates were utilized for immunoprecipitation (IP) with an anti-Flag antibody and then, samples were immunoblotted with an anti-HA or anti-Flag antibody. (C) VF (–) cells, SOCS3 cells, and R71E cells were cultured in the medium containing cycloheximide (100 mg/ml) for the indicated periods. Whole cell lysates were immunoblotted with an anti-HA or anti-EpoR antibody. The relative expression of JAK2 V617F mutant c-HA and EpoR was graphed. Data are shown as the mean ± SD of three independent experiments. * and ** indicate significant differences of $p < 0.05$ and $p < 0.01$, respectively.

V617F mutant in the presence of CHX, the R71E mutant only exerted a negligible effect on its degradation. The degradation of EpoR was not affected by the expression of SOCS3 or the R71E mutant (Fig. 4C). Furthermore, the expression of SOCS3, but not the R71E mutant significantly inhibited the proliferation rate of VF cells (Fig. 5A). Next, to investigate whether SOCS3 alters the distribution of cell cycle phases, each infected cell sub-line was cultured without IL-3 for 24 h. The expression of SOCS3, but not the R71E mutant increased the sub-G1 phase, which is consistent with apoptotic cells (Fig. 5B). Furthermore, to confirm whether the enforced expression of SOCS3 causes apoptotic cell death, we observed the fragmentation of genomic DNA in VF cells expressing SOCS3 but not R71E following 24 h of IL-3 deprivation (Fig. 5C). The expression of SOCS3 inhibited the phosphorylation of STAT5, STAT3, and ERK in VF cells, whereas that of the R71E mutant did not (Fig. 5D). Furthermore, the expression of SOCS3, but not the R71E mutant significantly reduced the expression of Pim1 and c-Myc at both the mRNA and protein levels (Fig. 5E and F); therefore, the enforced expression of SOCS3 appears to have inhibited the JAK2 V617F mutant-induced signaling pathway through its SH2 domain.

3.5. The phosphorylation of SOCS3 was induced by the JAK2 V617F mutant

Since a previous study reported that SOCS3 was phosphorylated by JAK2 [25] and we observed an interaction between SOCS3 and the JAK2 V617F mutant, as shown in Fig. 4B, we investigated whether SOCS3 was phosphorylated in VF cells. Although SOCS3 was not phosphorylated in (–) cells or WT cells following 24 h of IL-3 deprivation, the tyrosine phosphorylation of SOCS3 was noted in VF cells (Fig. 6A). As reported previously [26], the JAK2 inhibitor, ruxolitinib paradoxically induced JAK2 activation loop phosphorylation and inhibited its activity, resulting in reductions in STAT5 phosphorylation in VF cells (Fig. 6B). Ruxolitinib significantly inhibited the phosphorylation of SOCS3 in VF cells (Fig. 6B). In addition, the tyrosine phosphorylation of SOCS3 was observed in human erythroleukemia HEL cells, which possess the V617F mutation in JAK2 [27], and the treatment with ruxolitinib diminished the phosphorylation of SOCS3 as well as STAT5 (Fig. 6C). These results indicate that the phosphorylation of SOCS3 was induced in a manner that depended on JAK2 activity.

3.6. The phosphorylation of SOCS3 at Tyr-204 and Tyr-221 was induced by the JAK2 V617F mutant

The principal phosphorylation sites on SOCS3 were previously reported to be tyrosine residues at 204 and 221 in the carboxyl terminus [28]. To identify the phosphorylation sites of SOCS3 in VF cells, we generated SOCS3 mutants in which Tyr-204 and/or Tyr-221 were substituted with phenylalanine (Fig. 7A). VF cells were infected with an empty virus (–) and a retrovirus encoding SOCS3, the Y204F mutant, Y221F mutant, and Y204/221F mutant and named VF (–) cells, SOCS3 cells, Y204F cells, Y221F cells, and Y204/221F cells, respectively. The characteristics of each cell strain are shown in Table 1. The expression

Fig. 5

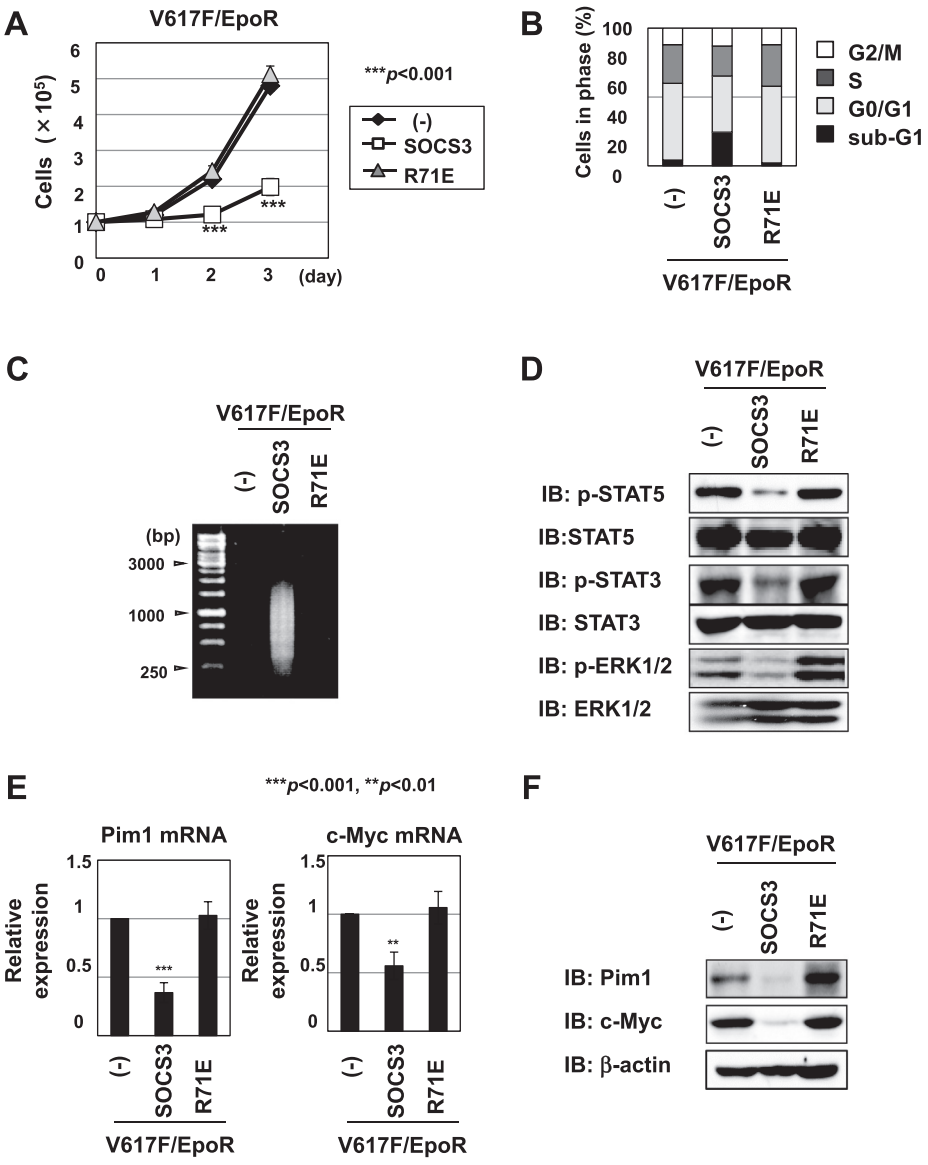

Fig. 5. Enforced expression of SOCS3 inhibited the proliferation of cells transformed by the JAK2 V617F mutant. (A) VF (–) cells, SOCS3 cells, and R71E cells were cultured with DMEM containing 1% FBS for 3 days. The number of viable cells were counted and shown in the graph. Results represent the mean ± SD of three independent experiments. (B-F) VF (–) cells, SOCS3 cells, and R71E cells were cultured with DMEM containing 1% FBS for 24 h. (B) Cells were fixed, treated with propidium iodide, and the cell cycle was analyzed by a flow cytometric analysis. (C) DNA was isolated from cells and subjected to agarose gel electrophoresis. (D) Whole cell lysates were immunoblotted with an anti-phospho-STAT5, anti-STAT5, anti-phospho-STAT3, anti-STAT3, anti-phospho-ERK1/2, or anti-ERK1/2 antibody. (E) Total RNA was prepared and the expression of Pim1 mRNA and c-Myc mRNA was assessed by a quantitative RT-PCR analysis. GAPDH mRNA was used as an internal control. Data are shown as the mean ± SD of three independent experiments. **indicates a significant difference of $p < 0.01$. (F) Whole cell lysates were immunoblotted with an anti-Pim1, anti-c-Myc, or anti-β-actin antibody.

of the JAK2 V617F mutant was decreased in SOCS3 cells and Y204F cells, but not in other cells (Fig. 7B). We then examined the phosphorylation level of each SOCS3 mutant. The phosphorylation of the Y204F mutant and Y221F mutant was markedly weaker than wild-type SOCS3, and the Y204/Y221F mutant was not phosphorylated at all (Fig. 7C). These results revealed that SOCS3 at both Tyr-204 and Tyr-221 was phosphorylated in VF cells. In addition, whereas the expression of SOCS3 and the Y204F mutant increased the degradation of the JAK2 V617F mutant, that of the Y221F mutant and Y204/221F mutant had no effect on the degradation of the JAK2 V617F mutant. On the other hand, the degradation level of EpoR was not changed by the expression of each SOCS3 mutant (Fig. 7D).

3.7. The phosphorylation of SOCS3 at Tyr-221 was required for its inhibitory effects on the JAK2 V617F mutant-induced signaling pathway

To gain further insights into the role of the phosphorylation of SOCS3 in VF cells, the proliferation rates of VF (–) cells, SOCS3 cells, Y204F cells, Y221F cells, and Y204/221F cells were examined. As shown in Fig. 8A, while the proliferation rates of SOCS3 cells and Y204F cells were significantly reduced, those of Y221F cells and Y204/221F cells were equivalent to that of VF (–) cells. No marked changes were detected in the cell cycle distribution under the conditions which Y221F or Y204/221F mutant was forcibly expressed, whereas the enforced expression of SOCS3 and Y204F increased the population of cells in the sub-G1 phase after IL-3 deprivation (Fig. 8B). Furthermore, DNA fragmentation was detected in SOCS3 cells and Y204F cells (Fig. 8C). The phosphorylation of STAT5, STAT3, and ERK was effectively inhibited in Y204F cells and SOCS3 cells (Fig. 8D). The expression of Pim1 and c-Myc at both the mRNA and protein levels was also reduced in Y204F cells and SOCS3 cells (Fig. 8E and F). These results suggest that the phosphorylation of SOCS3 at Tyr-221 in the SOCS box is required for its inhibitory effects on the JAK2 V617F mutant-induced signaling pathway.

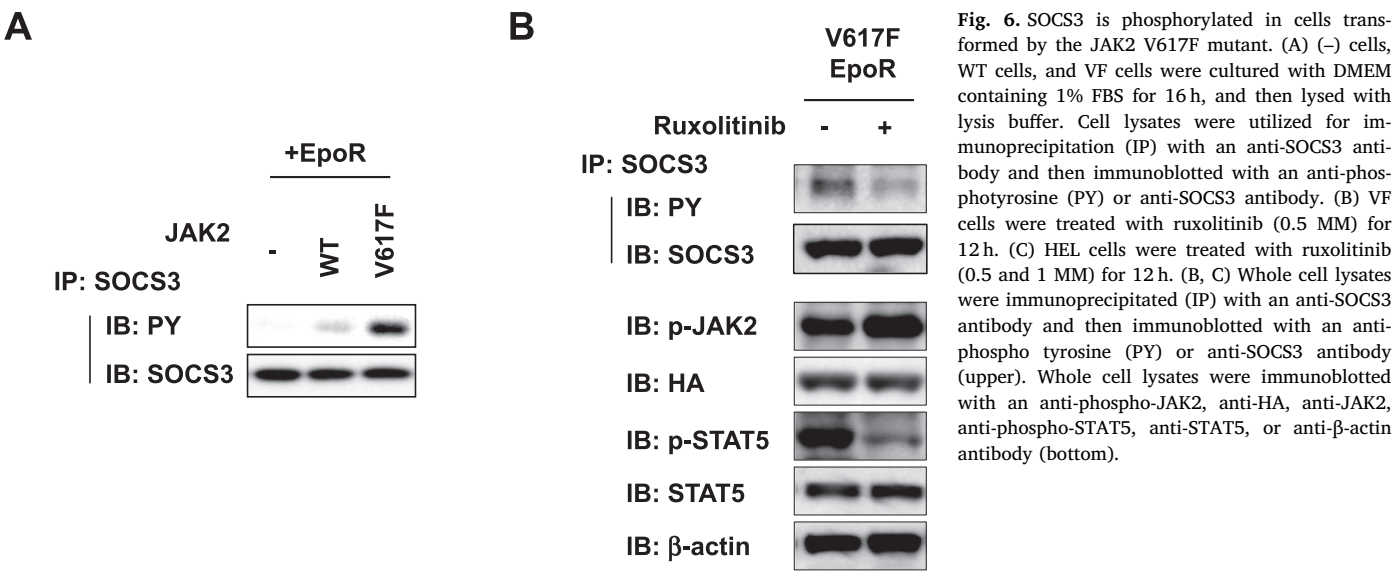

Fig. 6. SOCS3 is phosphorylated in cells transformed by the JAK2 V617F mutant. (A) (–) cells, WT cells, and VF cells were cultured with DMEM containing 1% FBS for 16 h, and then lysed with lysis buffer. Cell lysates were utilized for immunoprecipitation (IP) with an anti-SOCS3 antibody and then immunoblotted with an anti-phosphotyrosine (PY) or anti-SOCS3 antibody. (B) VF cells were treated with ruxolitinib (0.5 MM) for 12 h. (C) HEL cells were treated with ruxolitinib (0.5 and 1 MM) for 12 h. (B, C) Whole cell lysates were immunoprecipitated (IP) with an anti-SOCS3 antibody and then immunoblotted with an anti-phospho tyrosine (PY) or anti-SOCS3 antibody (upper). Whole cell lysates were immunoblotted with an anti-phospho-JAK2, anti-HA, anti-JAK2, anti-phospho-STAT5, anti-STAT5, or anti-β-actin antibody (bottom).

3.8. The SH2 domain and phosphorylation of the SOCS domain at Y221 are critical for the inhibitory function of SOCS3 on JAK2 V617F mutant-induced tumorigenesis

Based on the experimental results shown in Figs. 4 and 8, SOCS3 requires its SH2 domain and phosphorylated Tyr-221 in the SOCS box to exert inhibitory effects on JAK2 V617F mutant-induced cellular transformation. To clarify the importance of these domains and the modification of SOCS3 in the inhibition of JAK2 V617F-indcued tumorigenesis, nude mice were inoculated s.c. with VF (–) cells, SOCS3 cells, R71E cells, Y204F cells, Y221F cells, and Y204/221F cells. The sizes and weights of tumors, the liver, spleen, and lymph nodes were significantly smaller in mice inoculated with SOCS3 cells and Y204F cells than in mice inoculated with VF (–) cells. On the other hand, no significant differences were observed in tumor formation or the sizes of the liver, spleen, and lymph nodes between mice inoculated with VF (–) cells and mice inoculated with R71E cells, Y221F cells, and Y204/221F cells (Fig. 9A–C). Furthermore, the life span of mice inoculated with SOCS3 cells and Y204F cells was markedly longer than that of nude mice inoculated with VF (–) cells. In contrast, no significant difference was noted in the life span of mice inoculated with VF (–) cells, Y221F cells, and Y204F/221F cells (Fig. 9D). These results clearly indicate that the SH2 domain and phosphorylation at Tyr-221 in the SOCS box are critical for SOCS3 to inhibit JAK2 V617F mutant-induced tumorigenesis.

In the present study, our experimental results demonstrated that SOCS3 functions as a negative regulator in the JAK2 V617F mutant-induced signals in oncogenic processes. SOCS3 interacts with the JAK2 V617F mutant and negatively regulates the expression level of the JAK2 V617F mutant, leading to the inhibition of its caused tumorigenesis (Fig. 10).

4. Discussion

The expression of SOCS3 was previously shown to be induced by the enforced expression of the JAK2 V617F mutant in HEK293 cells [18]. However, the expression of SOCS3 was detected in Ba/F3 cells and its expression level was not altered regardless of the enforced expression of wild-type JAK2 or the JAK2 V617F mutant (Fig. 1). This difference may be attributed to the different cell types analyzed in each investigation. A previous study showed that the expression of SOCS2 mRNA was completely inhibited, whereas that of SOCS3 mRNA was slightly reduced in

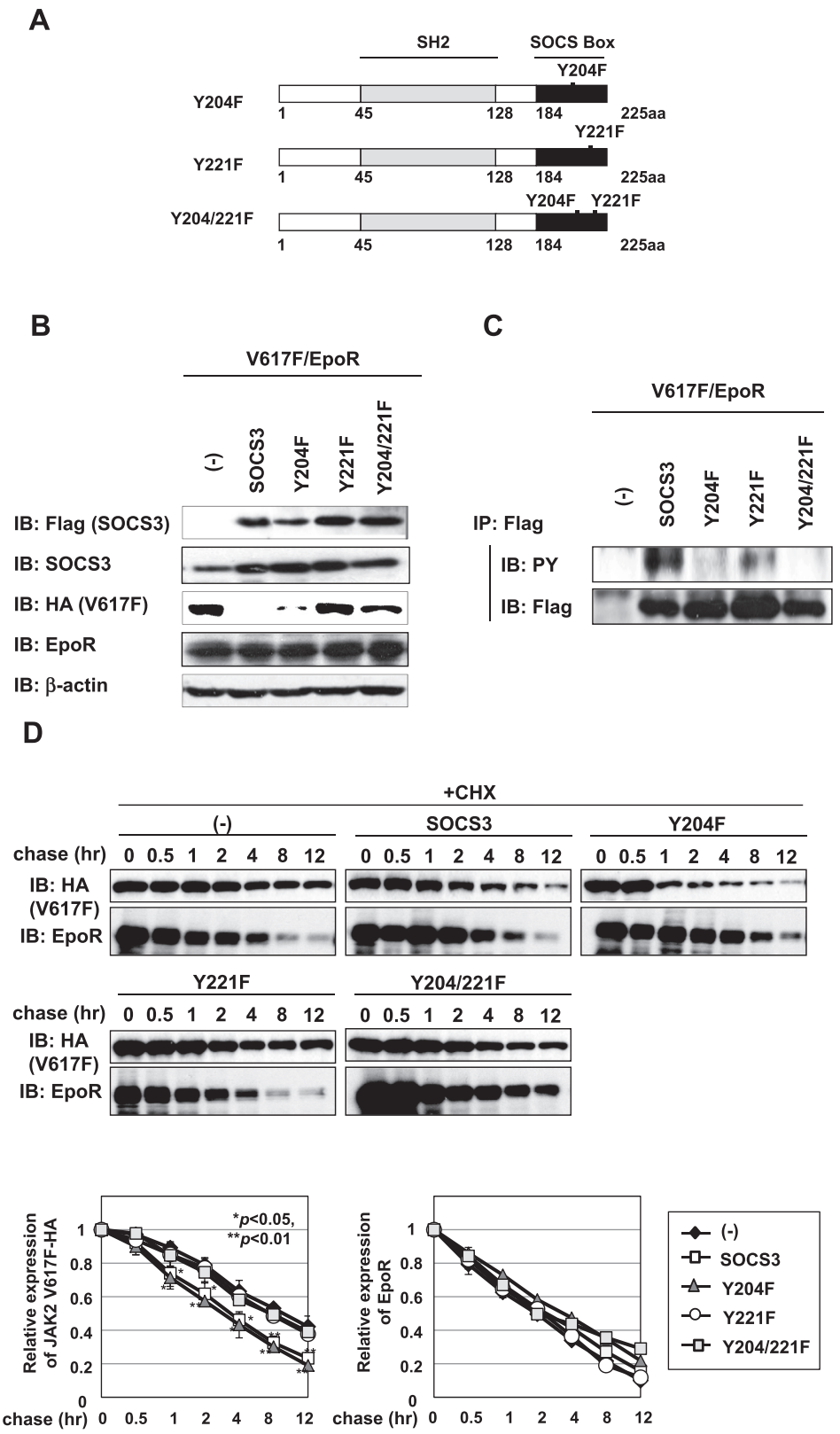

Fig. 7. SOCS3 is phosphorylated at Y204 and Y221 in cells transformed by the JAK2 V617F mutant. (A) Scheme of SOCS3 mutants, such as the Y204F mutant, Y221F mutant, and Y204/221F mutant. Tyrosine 204 and tyrosine 221 in the SOCS box of SOCS3 are phosphorylated by JAK2. These tyrosine residues were substituted into phenylalanine. (B and C) VF cells were infected with an empty virus (–) and a retrovirus encoding Flag-tagged wild-type SOCS3 and its mutants, such as Y204F, Y221F, and Y204/221F, and named VF (–) cells, SOCS3 cells, Y204F cells, Y221F cells, and Y204/221F cells, respectively. (B) Whole cell lysates were immunoblotted with an anti-Flag, anti-SOCS3, anti-HA, anti-EpoR, or anti-β-actin antibody. (C) Whole cell lysates were immunoprecipitated (IP) with an anti-Flag antibody and then immunoblotted with an anti-phosphotyrosine (PY) or anti-Flag antibody. (D) VF (–) cells, SOCS3 cells, Y204F cells, Y221F cells, and Y204/221F cells were cultured in the medium including cycloheximide (100 mg/ml) for the indicated periods. Whole cell lysates were immunoblotted with an anti-HA or anti-EpoR antibody. The relative amount of JAK2 V617F mutant c-HA and EpoR was graphed. Data are shown as the mean ± SD of three independent experiments. * and ** indicate significant differences of $p < 0.05$ and $p < 0.01$, respectively.

C2C12 cells that are deficient in STAT5 signaling [29], supporting the expression of SOCS3 not mainly being regulated by STAT5, in contrast to c-Myc and Pim1.

Contradictory findings have been reported for the effects of SOCS3 on JAK2 V617F mutant-provoked oncogenic signaling. Haan et al. showed the negative regulation of JAK2 V617F mutant by SOCS3 [18]. In another study, SOCS3 was found to induce the ubiquitination of JAK2 by recruiting components of an E3 ubiquitin ligase complex, such as Cullin5 [30], suggesting that the inhibition of JAK2 V617F by SOCS3 is mediated by the ubiquitination of the JAK2 V617F protein. The phosphorylation of SOCS3 was also shown to be required to prevent IL-2-induced STAT5 activation and sustain ERK activation by binding to

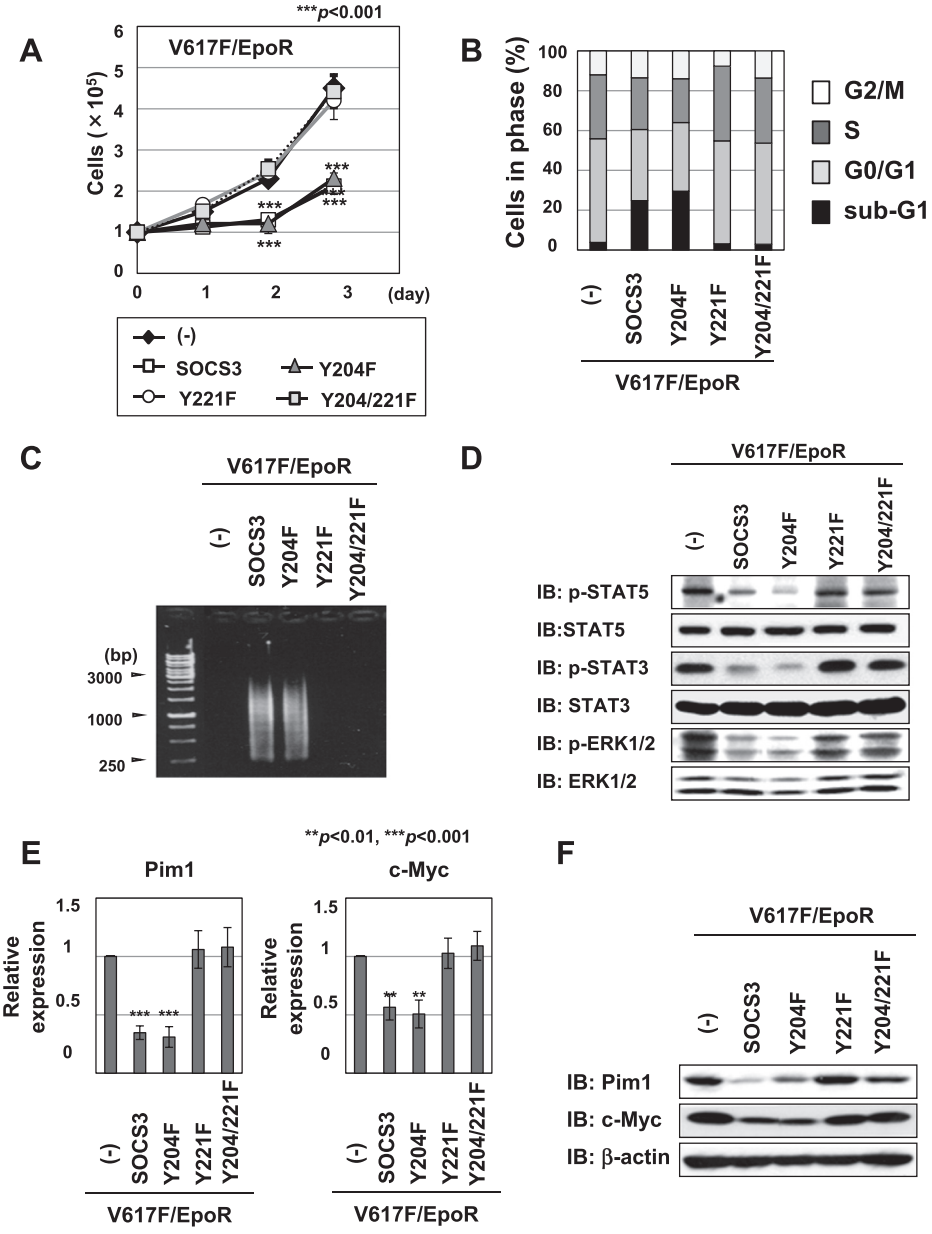

Fig. 8. The phosphorylation of SOCS3 at Y221 is critical for its inhibitory effects on the proliferation of cells transformed by the JAK2 V617F mutant. (A) VF (–) cells, SOCS3 cells, Y204F cells, Y221F cells, and Y204/221F cells were incubated with DMEM containing 1% FBS for 3 days. Viable cells were counted by the trypan blue exclusion method. Results represent the mean ± SD of three independent experiments. *** indicates $p < 0.001$. (B-F) Cells were incubated with DMEM containing 1% FBS for 24 h. (B) Cells were fixed, treated with propidium iodide, and the cell cycle was analyzed by a flow cytometric analysis. (C) DNA was isolated from cells and subjected to agarose gel electrophoresis. (D) Whole cell lysates were immunoblotted with an anti-phospho-STAT5, anti-STAT5, anti-phospho-STAT3, anti-STAT3, anti-phospho-ERK1/2, or anti-ERK1/2 antibody. (E) Total RNA was prepared and the expression of Pim1 mRNA and c-Myc mRNA was assessed by a quantitative RT-PCR analysis. GAPDH mRNA was used as an internal control. Data are shown as the mean ± SD of three independent experiments. ** and *** indicate significant differences of $p < 0.01$ and $p < 0.001$. (F) Whole cell lysates were immunoblotted with an anti-Pim1, anti-c-Myc, or anti-β-actin antibody.

the Ras inhibitor p120 RasGAP [29]. The present results revealed similar inhibitory effects for SOCS3 on JAK2 V617F-caused oncogenic signals. Furthermore, we demonstrated that the phosphorylation of SOCS3 at Tyr-221 was induced by the JAK2 V617F mutant and SOCS3 Y221F mutant failed to exert inhibitory effect on the protein expression of JAK2 V617F mutant (Fig. 7). These results suggest the possibility that the phosphorylation at Tyr-221 in SOCS3 was required for its inhibitory activity against the JAK2 V617F mutant. In addition, these results provide the other possibilities that the SOCS3 Y221F mutant may be non-functional for structural change caused by introduction of the Y221F substitution, and that Y221 in SOCS3 may be required for interaction with binding partner proteins involved in its inhibitory effect against JAK2 V617F mutant. We also observed opposite effects by the knockdown of SOCS3 on ERK, which is also activated by the JAK2 V617F mutant. Hookham and colleagues reported that SOCS3 significantly accelerated the activity of the JAK2 V617F mutant in the presence of EpoR in 293 T cells [20]. Although we currently cannot provide a clear explanation for the different SOCS3-induced effective directions in the JAK2V617F mutant-induced signaling pathway, the expression levels of each signaling molecule in each cell type or the presence or absence of additional unknown signaling factors may be contributing factors.

These findings strongly suggest that SOCS3 functions as a tumor suppressor against JAK2 V617F-induced cellular transformation. A previous study reported that tumor suppressor genes were mutated or deleted in several types of tumors [31]. In other cases, tumor suppressor genes were methylated to abolish gene expression [32]. The DNA methylation of the promoters of CIS/SOCS family genes was detected in MPN patients, suggesting that their epigenetic down-regulation contributes to the pathogenesis of MPNs. For example, the hypermethylation of the SOCS2 promoter was detected in MPN patients [33]. Furthermore, in the case of SOCS3, the hypermethylation of its promoter was identified in 32% of patients with PMF, but not in patients with ET and PV, suggesting that SOCS2 and SOCS3 function as tumor suppressors against PMF [34]. In addition, the methylation of the SOCS3 promoter was also detected in hepatocellular carcinoma (HCC) cell lines. The restoration of SOCS3 in cells lacking SOCS3 expression suppressed the phosphorylation of STAT3 and cell proliferation. SOCS3 physically interacted with phosphorylated FAK and Elongin B in HCC cells, resulting in a decrease in FAK protein levels [35]. These findings

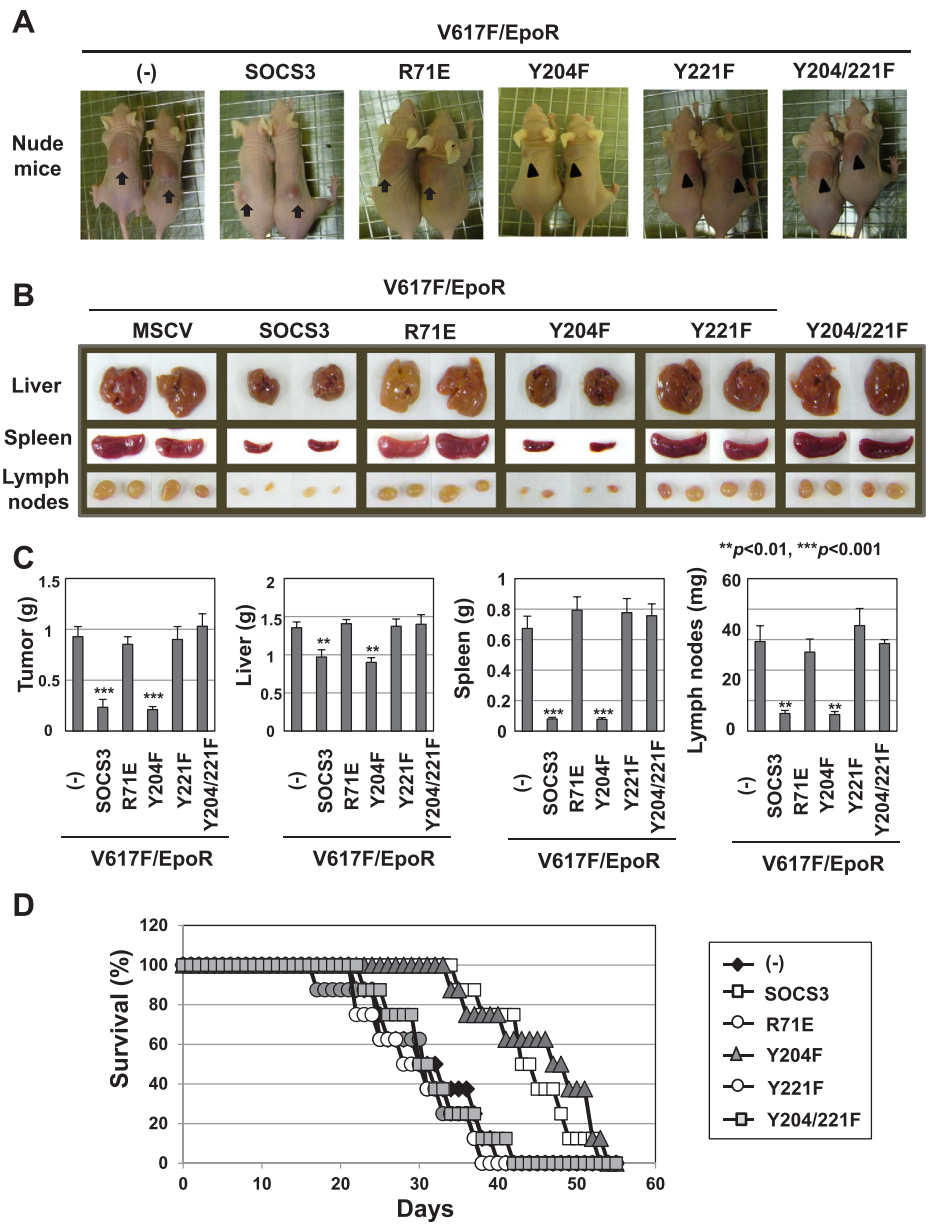

Fig. 9. The phosphorylation of SOCS3 at Y221 is critical for its inhibitory effects on the tumorigenesis provoked by the JAK2 V617F mutant. VF (–) cells, SOCS3 cells, R71E cells, Y204F cells, Y221F cells, and Y204/221F cells (1×10^7 cells) were subcutaneously transplanted into nude mice. (n = 8) (A) Nude mice inoculated with transduced cells were photographed 18 days post-inoculation. Arrowheads indicate tumors in nude mice. (B) Eighteen days post-inoculation, mice were sacrificed and morphological changes in the liver, spleen, and lymph nodes were photographed. (C) In each experimental condition, mice were sacrificed, and the tumor, liver, spleen, and lymph nodes were isolated, and then weighed and plotted. ** and *** indicate significant differences of $p < 0.01$ and $p < 0.001$, respectively. (D) The survival of eight nude mice inoculated with transduced cells was monitored daily for 55 days post-inoculation.

demonstrated that the loss of SOCS3 by DNA methylation confers cells advantage in growth of various tumor cells by enhancing not only the JAK/STAT pathway, but also FAK signaling and other tyrosine kinases. The ectopic overexpression of SOCS2 in 32D cells expressing BCR-ABL slowed growth and inhibited clonogenicity, suggesting that SOCS2 is a component of a negative feedback mechanism in BCR-ABL-induced chronic myeloid leukemia (CML) [36]. Additionally, the constitutive expression of SOCS1 was shown to occur at an early stage in the pathogenesis of CML, suggesting that SOCS1 influences the clinical behavior of the disease [37]. On the other hand, Qiu et al. reported that the BCR-ABL-dependent tyrosine phosphorylation of SOCS1 at Tyr-155 and Tyr-204 and SOCS-3 at Tyr-221 diminished their inhibitory effects on the activation of JAK and STAT5, thereby enhancing JAK/STAT5 signaling [38]. It was also reported that the co-expression of SOCS1 with BCR-ABL led to the development of a MPN phenotype with a longer disease latency than BCR-ABL alone in a murine bone marrow transplantation model [39]. Based on the present results and previous findings, CIS/SOCS family members appear to possess diverse functional directions depending on the types of diseases and their related signaling pathways. Furthermore, to clarify the mechanisms by which CIS/SOCS family members function as tumor suppressors, the target proteins of each CIS/SOCS family member need to be identified in various tumors.

5. Conclusions

We demonstrated that SOCS3 completes a negative feedback loop in JAK2 V617F mutant-induced tumorigenesis. The activated JAK2 V617F mutant interacts with SOCS3 and induces the phosphorylation of SOCS3, which may be required to facilitate the degradation of the JAK2 V617F mutant. The present results provide novel perspectives for developing therapeutic strategies against MPNs by utilizing CIS/SOCS family proteins.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

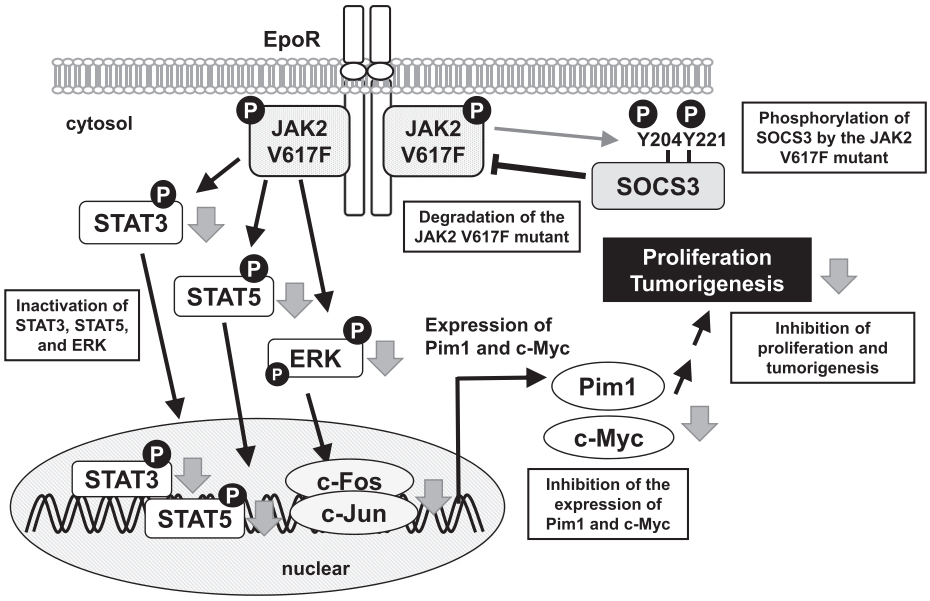

Fig. 10. Hypothetical model for SOCS3 to tumor suppressive function against the transformation provoked by the JAK2 V617F mutant. The JAK2 V617F mutant phosphorylates SOCS3 at Y204 and Y221 in the SOCS box. Phosphorylated SOCS3 reduces the expression protein level of the JAK2 V617F mutant and the activation of STAT3, STAT5, and ERK. As a result, SOCS3 inhibits the proliferation and tumorigenesis of cells transformed by the JAK2 V617F mutant and functions as a critical negative regulator against the JAK2 V617F-induced oncogenic signaling pathway.

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