ARTICLE

Establishment of isogenic induced pluripotent stem cells with or without pathogenic mutation for understanding the pathogenesis of myeloproliferative neoplasms

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Identification and functional characterization of disease-associated genetic traits are crucial for understanding the pathogenesis of hematologic malignancies. Various *in vitro* and *in vivo* models, including cell lines, primary cells, and animal models, have been established to examine these genetic alterations. However, their nonphysiologic conditions, diverse genetic backgrounds, and species-specific differences often limit data interpretation. To evaluate somatic mutations in myeloproliferative neoplasms (MPNs), we used CRISPR/Cas9 combined with the piggyBac transposon system to establish isogenic induced pluripotent stem (iPS) cell lines with or without *JAK2*V617F mutation, a driver mutation of MPNs. We induced hematopoietic stem/progenitor cells (HSPCs) from these iPS cells and observed phenotypic differences during hematopoiesis using fluorescence-activated cell sorting analysis. HSPCs with pathogenic mutations exhibited cell-autonomous erythropoiesis and megakaryopoiesis, which are hallmarks in the bone marrow of patients with MPNs. Furthermore, we used these HSPCs as a model to validate therapeutic compounds and showed that interferon alpha selectively inhibited erythropoiesis and megakaryopoiesis in mutant HSPCs. These results demonstrate that genome editing is feasible for establishing isogenic iPS cells, studying genetic elements to understand the pathogenesis of MPNs, and evaluating therapeutic compounds against MPNs. © 2022 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.

HIGHLIGHTS

- Isogenic iPS cells with or without *JAK2*V617F mutation were established.
- *JAK2*V617F mutation induced cell-autonomous erythropoiesis and megakaryopoiesis.
- IFN-α inhibits erythropoiesis and megakaryopoiesis in cells with *JAK2*V617F mutation.

BCR-ABL-negative myeloproliferative neoplasms (MPNs), including polycythemia vera, essential thrombocythemia, and primary myelofibrosis, are characterized by the expansion of at least one myeloid lineage cells caused by acquired somatic mutations [1]. A point mutation of valine to phenylalanine at position 617 (V617F) on Janus kinase 2 (JAK2) is the most common mutation found in MPNs [2−5]. JAK2 normally binds to the intracellular domain of cytokine receptors and transduces signals into cells in response to cytokines. However, when the V617F mutant is present, JAK2 becomes constitutively active, promoting oncogenic hematopoietic cell expansion [1]. Although *in vivo* models such as bone marrow transplantation and transgenic mouse models [6−9] recapitulate the phenotypes observed in patients, they show nonphysiologic levels of mutant gene expression following viral vector transduction, inconsistent *JAK2*V617F copy numbers, and irregularity in promoter activation. The inherent differences between human and mouse *JAK2*V617F protein have also been discussed previously [10].

Primary and patient-derived induced pluripotent stem (iPS) cells have been used to examine the function of mutant JAK2 under physiologic conditions in human cells [4,11−14]. iPS cells are suitable for research because of their close to unlimited resources and fewer ethical issues. Although iPS cells are convenient to acquire, iPS cells from different patients may be influenced by genetic trait variants and/or concomitant mutations, some of which appear to impact

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hematopoiesis [1,15]. To overcome these issues, we introduced the *JAK2*V617F mutation into iPS cells derived from a healthy individual using genome editing to generate isogenic iPS cell lines harboring the *JAK2*V617F mutation. These cell lines will be useful for elucidating other genetic traits found in patients with MPNs, as well as for evaluating therapeutic agents in these diseases.

METHODS

Plasmids

Guide RNA sequences (Supplementary Table E1), designed using the online tool CRISPRdirect [16], were cloned into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (#42230, Addgene). We constructed a donor vector containing a drug selection cassette and piggyBac transposon flanked by homology arms bearing an intended substitution of V617F and a silent mutation in the PAM sequence. Briefly, the intended region containing *JAK2*V617F (chr9:5072285−5075085) was amplified by polymerase chain reaction (PCR) from the genomic DNA of a patient with MPNs, and the left and right homology arms were generated using PCR. The primers used in PCR are shown in Supplementary Table E1. A silent mutation from G to A was introduced into the PAM sequence (TGG) of the *JAK2* sequence to prevent recleavage of the donor sequence by Cas9 after successful insertion. Using overlap extension PCR, both homology arms were conjugated and inserted into the BamHI and PstI sites in the pSP73 vector (P2221, Promega). The gateway destination cassette NeoPheS fragment sandwiched with inverted terminal repeat sequences for piggyBac transposase in pPB-R1R2_NeoPheS [17] (a kind gift from the Sanger Institute) was inserted between the NsiI and BsiWI sites of the intermediate plasmid, and the puromycin resistance cassette in the pENTR-PGKpuroΔtk [17] (a kind gift from the Sanger Institute) vector was introduced using the gateway system [18]. To excise the transposon element, the pCMV-HAhyPBase [19] (a kind gift from the Sanger Institute) vector was used.

*JAK2*V617F Mutated iPS Cell Generation

iPS-*JAK2*$^{WT/WT}$ cells previously established from healthy individuals were used in this study [20]. iPS cells were maintained in mouse embryonic fibroblasts as previously described [20]. The above-mentioned CRISPR/Cas9 and donor vectors were electroporated into iPS-*JAK2*$^{WT/WT}$ cells using the Amaxa electroporation system (Lonza), and cells were selected using puromycin (Nacalai Tesque) (Figure 1A,B). Individual iPS clones were isolated and subjected to genotyping PCR using the primers listed in Supplementary Table E1. iPS cells were treated with transposase to eliminate the piggyBac cassette, and clones with lost piggyBac cassettes were selected using media containing 500 nM fialuridine (Sigma). iPS cells were screened for clones without reintegration of the transposon by PCR using the primer listed in Supplementary Table E1, and successful knock-in of the *JAK2*V617F mutation was confirmed using Sanger sequencing. This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Juntendo University School of Medicine (IRB#M12-0895). All clinical samples and records from individuals were used after obtaining written informed consent from the donors.

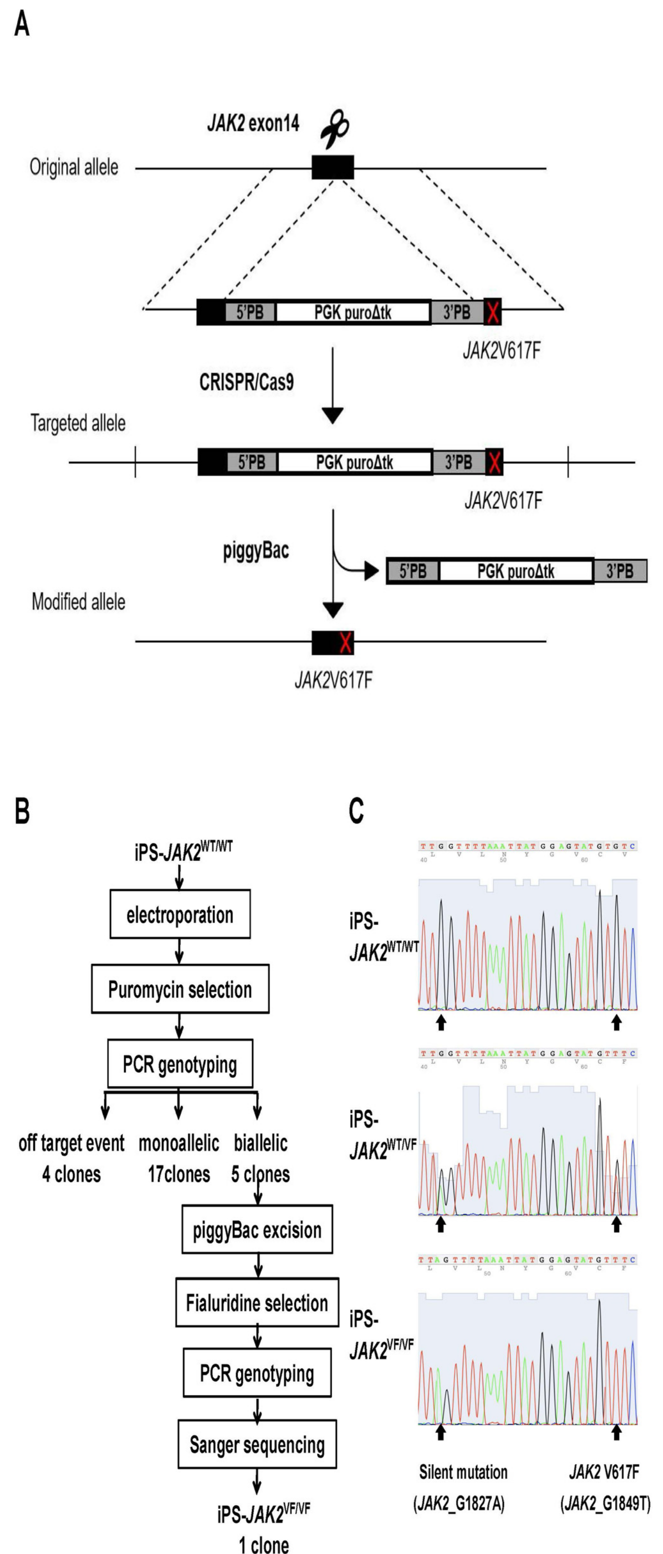

Figure 1 Establishment of iPS cells harboring *JAK2*V617F by genome editing. **(A)** Schematic overview depicting the *JAK2*V617F knock-in strategy using CRISPR/Cas9 genome editing followed by the piggyBac transposon system.

Validation of Pluripotent Marker Expression

Immunofluorescence staining of pluripotent markers and visualization of alkaline phosphatase activity were performed as previously described [20]. Images were captured using BZ-8000 and BZ-X710 fluorescence microscopes (Keyence). *NANOG, SOX2*, and *OCT3/4* expression was detected using reverse transcription PCR as described previously [20] with the primers shown in Supplementary Table E2.

Teratoma Formation Assay

Undifferentiated iPS cells (2×10^6) were injected into the flank region of 7-week-old NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) immunocompromised mice (Jackson laboratory Japan) to induce teratoma formation. After 6−8 weeks, the tumors were excised from anesthetized mice, fixed with 10% formalin, and embedded in paraffin for histologic analysis. Animal experiments were conducted under supervision of the Animal Ethics Committee of Juntendo University School of Medicine (#2021001).

In Vitro Differentiation

iPS cells were differentiated into hematopoietic stem/progenitor cells (HSPCs), which were then differentiated into erythrocytes and megakaryocytes as previously described [20]. Briefly, clumps of iPS cells formed on mouse embryonic fibroblasts were transferred onto mitomycin-treated C3H10T1/2 feeder cells (RIKEN Cell Bank) in Iscove's modified Dulbecco's medium (IMDM) (Thermo Fisher) supplemented with 15% fetal bovine serum (FBS), penicillin-streptomycin-glutamine (Thermo Fisher), insulin-transferrin-selenium (Thermo Fisher), 0.45 mM 1-thioglycerol (Sigma), 50 μg/mL ascorbic acid (Sigma), and 20 ng/mL recombinant human vascular endothelial growth factor (VEGF) (BioLegend). On day 14, bulk HSPCs were collected by thoroughly disrupting the iPS-sacs via pipetting, and the samples were passed through a 40 μm strainer. To further induce hematopoietic cell differentiation, CD34+ HSPCs were purified with the CD34+ MicroBead Kit UltraPure (Miltenyi Biotec) and then cultured in IMDM supplemented with 15% FBS in the presence of 50 ng/mL stem cell factor (PeproTech) and various concentrations of erythropoietin (EPO, Kyowa Hakko Kirin) with the presence of C3H10T1/2 cells for erythroid cell differentiation. For megakaryocytic cell differentiation, purified CD34+ HSPCs were cultured in IMDM supplemented with 15% FBS in the presence of various concentrations of human thrombopoietin (TPO, Kyowa Hakko Kirin) and 0.1% bovine serum albumin. HSPCs, erythroid cells, and megakaryocytic cells were defined by expression of the markers CD34, CD235ab, and CD42b, which was detected using fluorescence-activated cell sorting (FACS) analysis with a FACSCalibur (BD Biosciences) and FlowJo software (TreeStar). Antibodies used are listed in Supplementary Table E3.

(B) Schematic presentation of screening steps for establishing *JAK2*V617F knock-in iPS cells. **(C)** Sanger sequencing results of genomic DNA purified from the original (iPS-*JAK2*$^{WT/WT}$) and genome-edited (iPS-*JAK2*$^{WT/VF}$ and iPS-*JAK2*$^{VF/VF}$) iPS cells. The silent mutation was introduced to block recleavage event by Cas9 on the donor sequence after successful insertion (details described in the Materials and Methods section).

Immunoblot Analysis

Immunoblot analysis was performed as described previously [21]. Briefly, purified CD34+ HSPCs were lysed in RIPA buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 1% sodium deoxycholate) supplemented with phosphatase and protease inhibitor cocktails. Equal amounts of cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes, which were then blocked with TBST buffer (24 mM Tris [pH 7.4], 147 mM NaCl, 2.7 mM KCl, and 0.1% Tween-20) containing 5% skimmed milk. The membranes were reacted with the following primary antibodies purchased from Cell Signaling Technology: anti-JAK2 (#3230), anti-pJAK2 (#3771), anti-STAT5 (#94205), anti-pSTAT5 (#9359), and anti-β-actin (#4967). Horseradish peroxidase−conjugated goat anti-rabbit IgG (#111-035-003, Jackson Immuno Research) was used as a secondary antibody. The chemiluminescence reaction was performed using ECL Western Blotting Femto (Thermo Fisher), and images were captured using Fusion Fx System (Vilber Lourmat).

Validation of Therapeutic Compounds on Erythroid and Megakaryocytic Differentiation

Bulk HSPCs were treated with the indicated amounts of ruxolitinib (INCB018424, Selleck Chemicals), interferon (IFN)-α (Sumiferon, Sumitomo Pharmaceuticals), or vehicle (dimethyl sulfoxide for ruxolitinib, phosphate-buffered saline for IFN-α) in the presence of 50 ng/mL stem cell factor and 1 U/mL EPO or 5 ng/mL TPO and analyzed at 1 week after treatment. The number of erythroid and megakaryocytic cells, defined by expression of CD235ab+ and CD42b+, respectively, was determined using flow cytometry with counting beads (CountBright Absolute Counting Beads, Invitrogen). EC_{50} values were determined by Prism 9 (GraphPad software).

Statistical Analysis

Statistical analyses were performed using Prism 7 (GraphPad software). For parametric data with equal variance, Student t test was used; for those with unequal variance, Welch's correction was used. For nonparametric data, we applied the Mann-Whitney U test. Statistical significance was set at $p < 0.05$.

RESULTS

Establishment of *JAK2*V617F Knock-in iPS Cells

To introduce the *JAK2*V617F mutation into iPS cells derived from a healthy individual (hereinafter named as iPS-*JAK2*$^{WT/WT}$ cells), we employed the CRISPR/Cas9 system combined with piggyBac technology (Figure 1A) [18]. After transfecting the targeting construct with the CRISPR/Cas9 vector and selection with puromycin (Figure 1B), 36 drug-resistant clones were obtained. Genotyping PCR identified five biallelic, 17 monoallelically targeted, and four off-target clones (10 left undefined) (Figure 1B). Sanger sequencing analysis of the CRISPR targeting sequences for six randomly chosen monoallelic targeted clones revealed that only two clones maintained intact sequences in the remaining allele. To excise the integrated marker construct, randomly chosen biallelic and monoallelic clones were transiently transfected with the hyPBase vector, and the cells were cultured in the presence of fialuridine for negative selection of clones that had lost the thymidine kinase marker. Sanger sequencing

confirmed that two of the three clones for each allelic type excised the marker construct; these clones were named as iPS-*JAK2*$^{WT/VF}$ and iPS-*JAK2*$^{VF/VF}$ for the monoallelic and biallelic *JAK2*V617F mutant iPS cells, respectively. Even after negative selection, reintegration of the excised DNA into the genome was strongly suspected based on PCR specific for the cassette element; thus, an additional round of excision and negative selection was performed for one clone from each allelic type. Finally, two clones of iPS-*JAK2*$^{WT/VF}$ and one clone of iPS-*JAK2*$^{VF/VF}$ with no random reintegration in the genome were isolated. Introduction of a silent mutation and the *JAK2*V617F mutation was confirmed using Sanger sequencing (Figure 1C). Although the iPS-*JAK2*$^{WT/VF}$ and iPS-*JAK2*$^{VF/VF}$ clones were stable for more than 6 months, both iPS-*JAK2*$^{WT/VF}$ clones lost their pluripotency; thus, only iPS-*JAK2*$^{VF/VF}$ was used in subsequent analyses.

Pluripotency of Established iPS Cells

Expression of the endogenous pluripotency markers *NANOG, SOX2*, and *OCT3/4* was detected using reverse transcription PCR in both iPS-*JAK2*$^{WT/WT}$ and iPS-*JAK2*$^{VF/VF}$ clones (Figure 2A). Immunofluorescence staining revealed expression of the pluripotency markers NANOG, SSEA-4, OCT3/4, and TRA-1-81 (Figure 2B). The established clones were positive for alkaline phosphatase staining (Figure 2C). A teratoma formation assay demonstrated the trilineage differentiation capacity of the established iPS cells (Figure 2D). These results show that the iPS cells were pluripotent following genome editing and drug selection.

Induction of HSPCs

To induce HSPCs differentiation, iPS cells were cultured on C3H10T1/2 feeder cells for 2 weeks in the IMDM containing 15% FBS, 20 ng/mL VEGF, and other components (details can be found in the *Materials and Methods* section). On day 14, the iPS-sacs were disrupted and analyzed using FACS to count the CD34+ HSPCs (Figure 3A). Both iPS-*JAK2*$^{WT/WT}$ and iPS-*JAK2*$^{VF/VF}$ cells induced HSPCs at similar frequencies (38.83% ± 5.57% and 41.13% ± 5.15%, respectively), with no significant difference between cell types regardless of the presence of the *JAK2*V617F mutation (Figure 3B,C). Immunoblot analysis of cell lysates prepared from CD34+ cells exhibited increased levels of phosphorylation on JAK2 and STAT5 in HSPCs with *JAK2*V617F mutation compared with those without the mutation (Figure 3D), demonstrating the activation of JAK2 and downstream molecules via the introduction of *JAK2*V617F mutation.

iPS-*JAK2*$^{VF/VF}$ HSPCs Showed Predisposition to Both Erythroid and Megakaryocytic Lineages

To determine the differentiation capacity of HSPCs harboring the *JAK2*V617F mutation, the cells were differentiated into erythroid and megakaryocytic lineages (Figure 3A). HSPCs harboring the *JAK2*V617F mutation exhibited cell-autonomous erythropoiesis, whereas *JAK2* wild-type HSPCs induced a minimal number of erythrocytes defined by CD235ab positivity in the absence of EPO (5.62% ± 0.94% and 0.82% ± 0.30%, respectively, $p < 0.01$) and in the presence of 0.01 U/mL EPO (Figure 3E,F). These results show that the EPO hypersensitivity observed in primary HSPCs in patients with polycythemia vera was recapitulated [14]. In the presence of 1 U/mL EPO, no significant difference in erythroid differentiation was observed between HSPCs with and without the *JAK2*V617F mutation (34.22% ± 4.97% and 30.48% ± 5.82%, respectively) (Figure 3E,F).

In megakaryopoiesis, HSPCs harboring the *JAK2*V617F mutation showed more robust factor-independent differentiation than erythropoiesis, with approximately 12% of CD42b+ cells induced in the absence of TPO; this value was significantly higher than that of wild-type HSPCs (12.27% ± 2.59% and 2.08% ± 0.61%, respectively, $p < 0.05$) (Figure 3E,F). HSPCs harboring the *JAK2*V617F mutation induced from iPS cells resembled cell-autonomous megakaryopoiesis observed in HSPCs isolated from patients with MPNs [22,23]. In the presence of 50 ng/mL TPO, the rate of megakaryocyte induction did not significantly differ between HSPCs with and without the *JAK2*V617F mutation (23.48 ± 3.30 and 16.40 ± 3.30, respectively) (Figure 3E,F). These results suggest that introduction of the *JAK2*V617F mutation was sufficient to induce the formation of normal human HSPCs from iPS cells to confer cell-autonomous erythropoiesis and megakaryopoiesis. We also demonstrated the feasibility of validating somatic mutations associated with MPNs in the same genetic background using the iPS model system.

Inhibitory Effect of Ruxolitinib and IFN-α on Erythropoiesis and Megakaryopoiesis

To demonstrate the potential of using established iPS cells to validate therapeutic compounds for MPNs, HSPCs were differentiated into erythroid and megakaryocytic lineages in the presence of the JAK1/2 inhibitor ruxolitinib or IFN-α (Figure 4A). The results showed that, as described previously [24], ruxolitinib exhibited potent inhibition of erythropoiesis for both HSPCs with and without *JAK2*V617F (EC_{50} = 0.20 and 0.14 μM, respectively) (Figure 4B). Ruxolitinib also inhibited megakaryopoiesis for both HSPCs with and without *JAK2*V617F (EC_{50} = 0.39 and 1.01 μM, respectively) (Figure 4C). *JAK2* wild-type HSPCs exhibited weak resistance to ruxolitinib in megakaryopoiesis, particularly at a concentration of 0.37 μM (Figure 4C). Additionally, inhibition of erythropoiesis and megakaryopoiesis by ruxolitinib did not significantly differ between HSPCs with and without the *JAK2*V617F mutation (Figure 4B,C). Thus, the *JAK2*V617F mutant was not selectively inhibited by ruxolitinib in erythropoiesis and megakaryopoiesis.

Unlike ruxolitinib, IFN-α showed a more robust effect on the differentiation of HSPCs harboring *JAK2*V617F mutation than of those harboring wild-type *JAK2*. Erythropoiesis of HSPCs with the *JAK2*V617F mutation was significantly inhibited by 1 and 3 U/μL IFN-α compared with that of cells without the mutation (Figure 4D). In megakaryopoiesis, 0.33, 0.5, and 1.0 U/μL IFN-α promoted megakaryocytic differentiation of HSPCs with wild-type *JAK2*, whereas differentiation was suppressed in a dose-dependent manner in HSPCs with the *JAK2*V617F mutation (Figure 4E). These data indicate that IFN-α can inhibit erythropoiesis and megakaryopoiesis in HSPCs harboring the *JAK2*V617F mutation.

DISCUSSION

We established isogenic iPS-*JAK2*$^{VF/VF}$ cells from iPS-*JAK2*$^{WT/WT}$ cells using CRISPR/Cas9 combined with the piggyBac system [18]. The induction rate of HSPCs was equivalent between iPS cells. For terminal differentiation, however, HSPCs harboring the *JAK2*V617F mutation exhibited more pronounced cell-autonomous differentiation

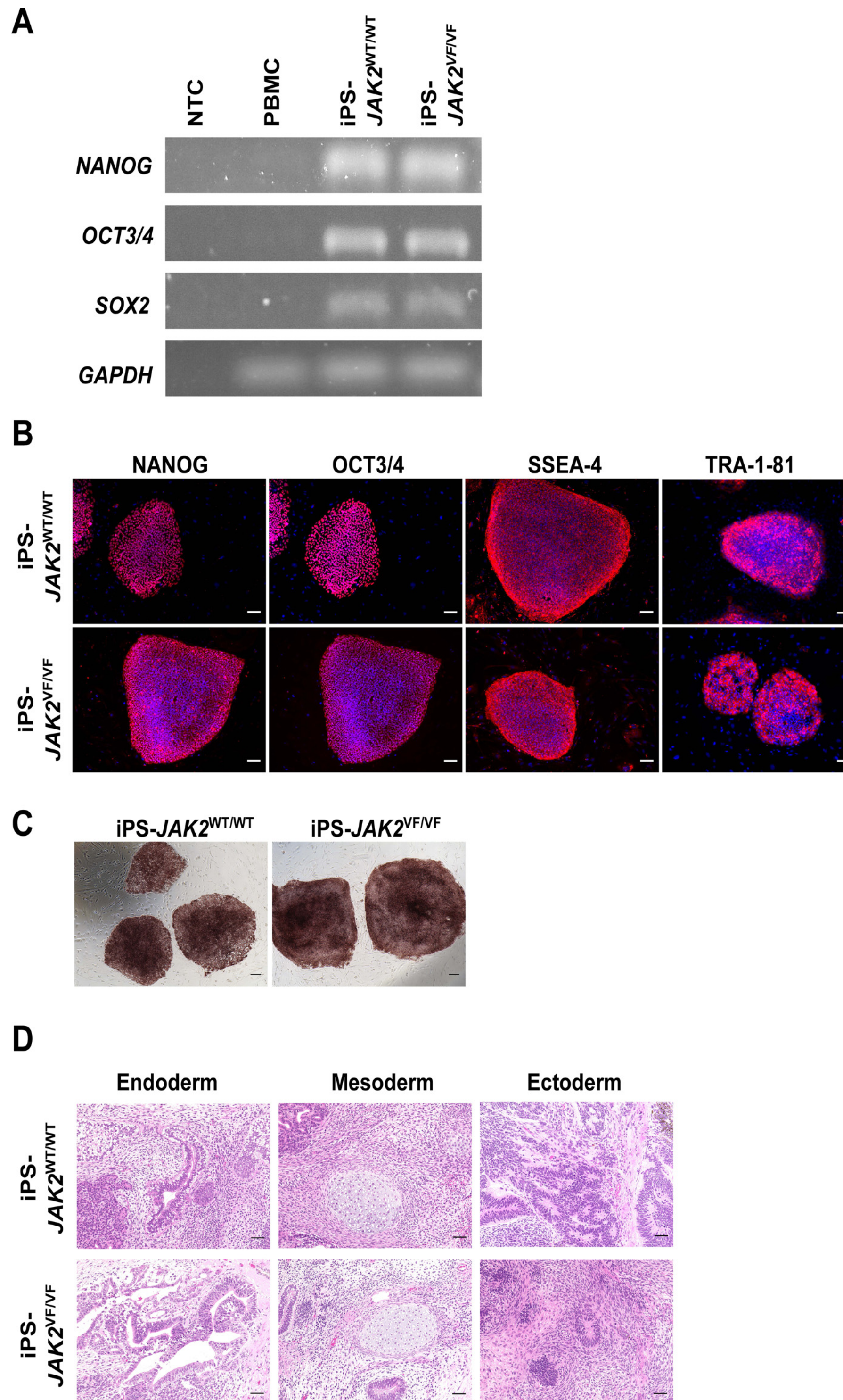

Figure 2 Validation of pluripotency of genome-edited iPS cells. **(A)** Gel electrophoresis images of PCR products from reverse-transcribed mRNA of indicated sources. Human peripheral blood cells (PBMCs) were used as a negative control of pluripotent markers. *GAPDH* was used as a positive control in reverse transcription. **(B)** Fluorescence microscopic images of iPS cells stained with antibodies recognizing indicated pluripotent markers merged with 4′,6-diamidino-2-phenylindole. Artificial red was used in images stained with OCT3/4 and TRA-1-81. Scale bars represent 100 μm. **(C)** Microscopic images of alkaline phosphatase activity in iPS cells. Scale bars represent 200 μm. **(D)** Hematoxylin and eosin-stained specimens of teratoma formed from indicated iPS cells in immunodeficient mice. Scale bars represent 50 μm. *NTC*=Non-template control.

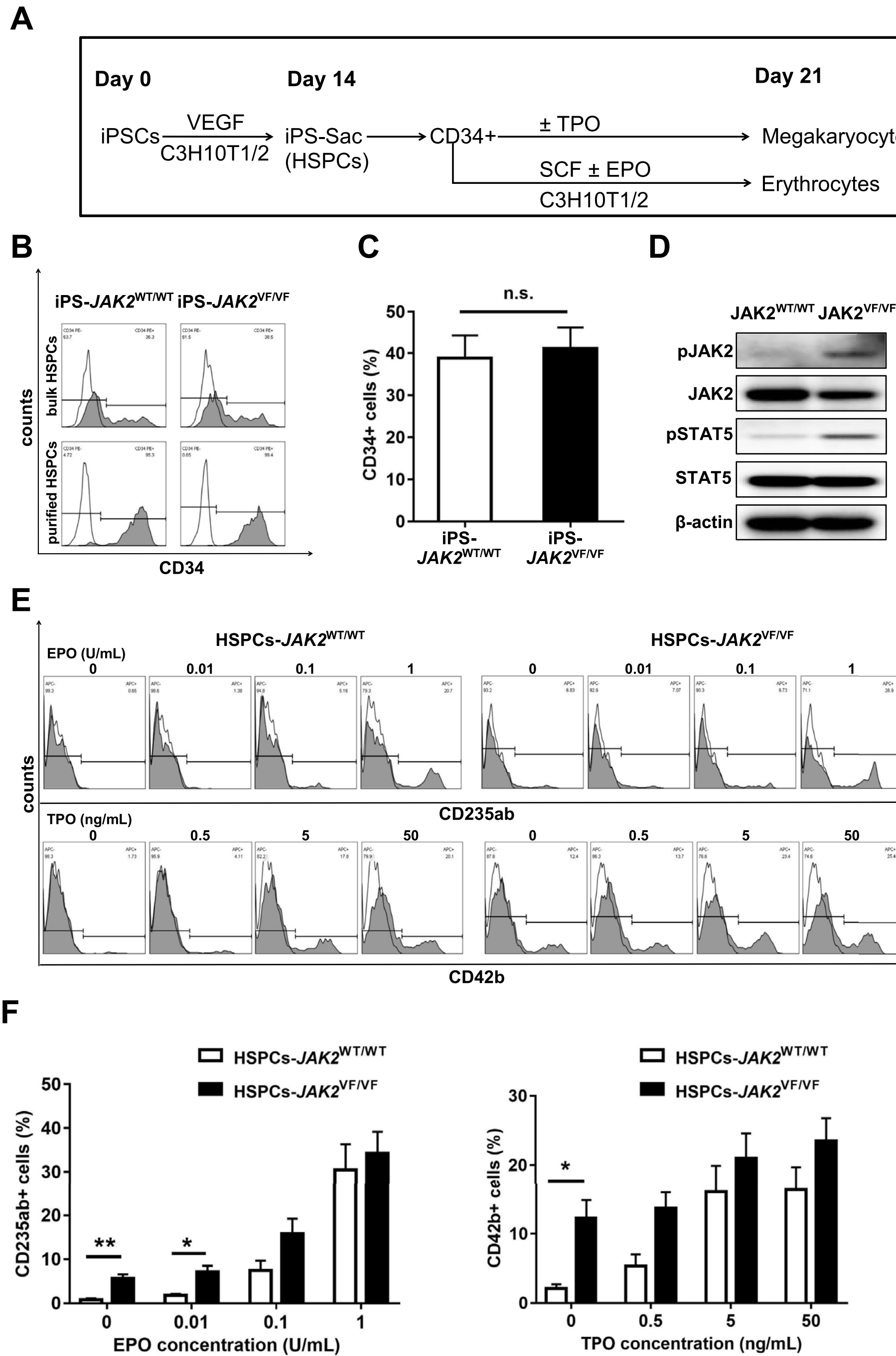

Figure 3 Increased factor-independent erythropoiesis and megakaryopoiesis in HSPCs induced from genome-edited iPS cells with *JAK2*V617F. **(A)** Schematic representation of HSPCs, erythroid, and megakaryocytic cell induction. HSPCs were induced from iPS-sac in IMDM supplemented with 15% FBS, 20 ng/mL vascular endothelial growth factor, and other components (further details in the *Materials and Methods* section) on C3H10T1/2 feeder cells. Purified CD34+ cells were induced for erythorid and megakaryocytic differentiation in the IMDM containing 15% FBS with the indicated components and conditions. (**B**) Representative FACS profiles of bulk HSPCs and purified CD34+ HSPCs induced from iPS-$JAK2^{WT/WT}$ and iPS-$JAK2^{VF/VF}$ cells. (**C**) Comparison of the rate of CD34+ HSPC induction between iPS-$JAK2^{WT/WT}$ and iPS-$JAK2^{VF/VF}$ cells. Data are shown as the mean ± SEM of nine independent experiments. (**D**) Immnunoblot analysis of cell lysates prepared from HSPCs with ($JAK2^{VF/VF}$) or without ($JAK2^{WT/WT}$) *JAK2*V617F mutation. β-actin served as loading control. (**E**) Representative FACS profiles of erythroid, defined by CD235ab+ (upper panel), and megakaryocytic, defined by CD42b+ (lower panel), cells induced from HSPCs with ($JAK2^{VF/VF}$) or without ($JAK2^{WT/WT}$) *JAK2*V617F mutation. (**F**) Frequencies of erythroid (left) and megakaryocytic (right) cell differentiation. Data are shown as the mean ± SEM of five independent experiments (*$p < 0.05$; **$p < 0.01$).

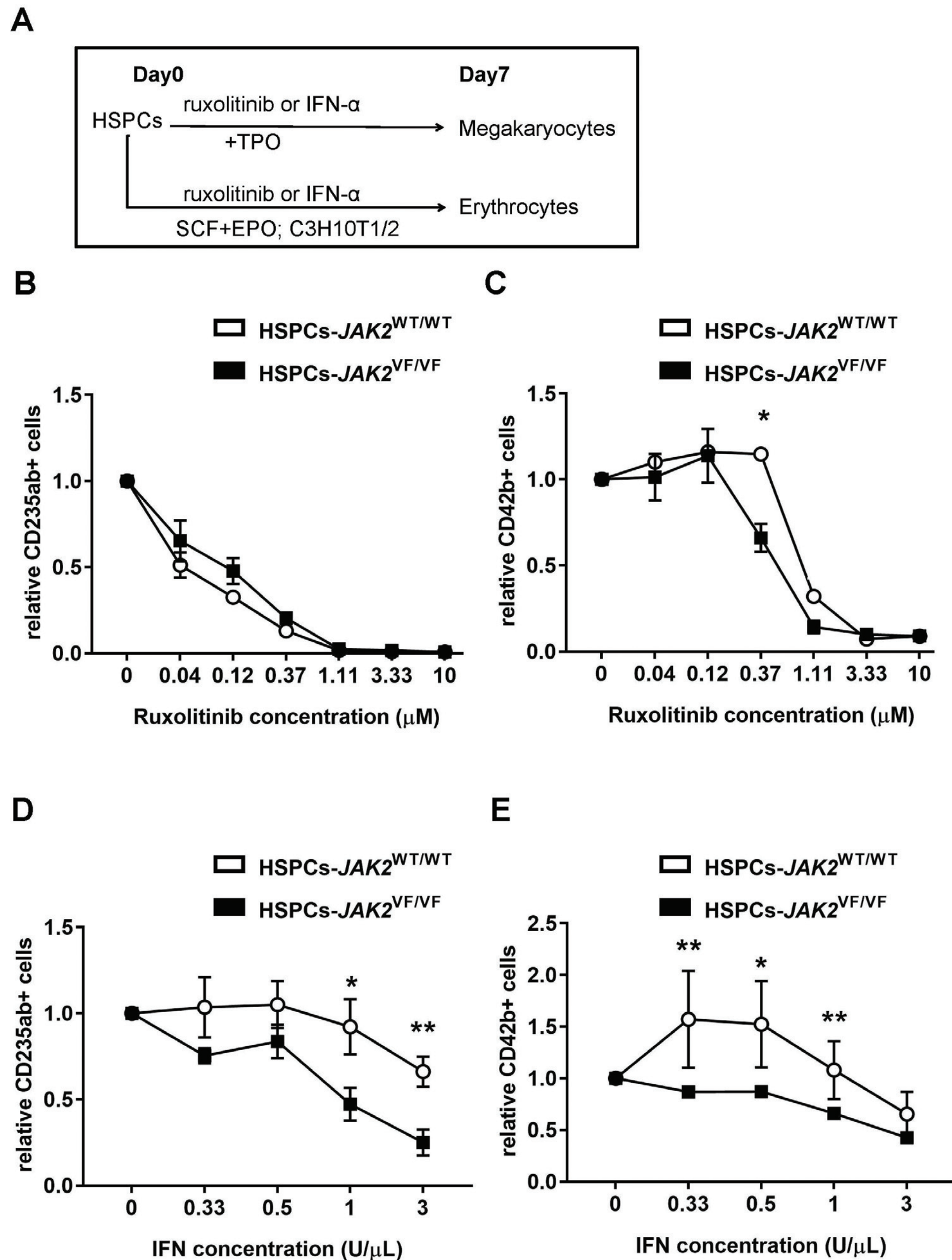

Figure 4 Use of isogenic iPS cells with or without *JAK2*V617F mutation to validate the effects of therapeutic compounds. **(A)** Schematic representation of the treatment of hematopoietic stem/progenitor cells (HSPCs) harboring *JAK2*V617F mutation ($JAK2^{VF/VF}$) or wild-type *JAK2* ($JAK2^{WT/WT}$) with ruxolitinib or IFN-α. (**B,C)** Relative number of erythroid (CD235ab+) (**B**) and megakaryocytic (CD42b+) (**C**) cells following treatment with the indicated concentrations of ruxolitinib relative to vehicle-treated cells. Data are shown as the mean ± SEM, $n = 5$ (**B**) vs. 4 (**C**). (**D,E)** Relative number of erythroid (CD235ab+) (**D**) and megakaryocytic (CD42b+) (**E**) cells treated with the indicated concentrations of IFN-α relative to vehicle-treated cells. Data are shown as the mean ± SEM of five independent experiments.

compared with cells with wild-type *JAK2*. Furthermore, therapeutic compounds showed differential effects against hematopoiesis between *JAK2* wild-type and mutant HSPCs. Taken together, we demonstrated the feasibility of using genome editing to establish isogenic iPS cell lines with or without the *JAK2*V617F mutation and that these cells can be used to validate the effects of therapeutic compounds against MPNs.

HSPCs can be formed from iPS cells using various approaches, such as embryoid body formation, feeder cell coculture, extracellular matrix-coated culture, or teratoma formation [25,26]. We induced HSPCs from iPS cells in the presence of C3H10T1/2 feeder cells; comparison of the induction rate of HSPCs between iPS-$JAK2^{VF/VF}$ and iPS-$JAK2^{WT/WT}$ cells showed that the *JAK2*V617F mutation did not strongly impact reprogramming from iPS cells to HSPCs under

our assay conditions. This result is consistent with that of a previous study, which compared patient-derived iPS cells harboring the *JAK2*V617F mutation and control iPS cells without the mutation and demonstrated no major selective advantage between mutant and wild-type HSPCs despite the use of different protocols [24].

*JAK2*V617F knocked-in HSPCs recapitulated the cellular phenotypes observed in HSPCs from MPNs patients harboring the *JAK2*V617F mutation [27−29]. This result is consistent with those of previous studies of HSPCs induced from patient-derived iPS cells, in which increased erythropoiesis and megakaryopoiesis were observed [11,13]. However, a study using iPS cells derived from a patient with secondary myelofibrosis showed no increase in megakaryopoiesis in *JAK2*V617F homozygous clones [12]. This occurrence is likely owing to the acquisition of additional mutation(s) that promote the development of myelofibrosis without excess platelet production [30]. Compared with that reported in a previous research [24], the frequency of erythroid differentiation was less efficient in the present study presumably due to the use of different protocols for the induction of HSPCs and differentiation of erythrocytes. Besides, CD34+ HSPCs in the present assay contain approximately 20% of CD43-negative nonhematopoietic cells, which may underscore the hematopoietic cell differentiation. Further study including an assessment for the myeloproliferative phenotypes is required for the validation of genome-edited iPS clones.

Consistent with previous studies showing the feasibility of genome editing at the *JAK2* locus [31,32], the present study demonstrated the prospect of introducing the *JAK2*V617F mutation into iPS cells to establish isogenic iPS cells with or without a pathogenic mutation. Although we performed functional analysis, including hematopoietic differentiation, confirmation of JAK2 pathway activation, and validation of therapeutic compounds, our study was limited to a pair of parental and genome-edited iPS clones because only one clone was obtained after the genome editing and subsequent elimination of transposon element. Because isogenic iPS clones show diverse characteristics in a variety of assays, including in *in vitro* hematopoietic differentiation, further study with a large number of iPS clones are required for the generalization of the results of this study. Additionally, because gene editing and subsequent elimination of marker constructs while maintaining the iPS status are labor- and time-consuming, iPS clones that we handled during the screening process were limited, resulting in a loss of heterozygous clones due to the small number of stocks. Therefore, technical advancements are needed to overcome these issues to generate multiple genome-edited iPS clones for use in studies.

Our results were similar to those of previous studies using HSPCs induced from iPS cells derived from patients with MPNs [13], where erythroid progenitor cells induced from iPS cells harboring *JAK2*V617F mutation were equally sensitive to ruxolitinib in erythropoiesis compared with those without the mutation. This is also consistent with the ruxolitinib response that occurs in both lineages without the selective effects observed in clinical settings [33,34]. In contrast to ruxolitinib, IFN-α preferentially suppressed erythropoiesis and megakaryopoiesis in HSPCs harboring the *JAK2*V617F mutation. A recent study has shown that IFN-α selectively reduces the long-term hematopoietic stem cells harboring *JAK2*V617F mutation by promoting the generation of megakaryo-biased hematopoietic stem cells [35], which provides a possible mechanism for the observation that HSPCs harboring the *JAK2*V617F mutation regressed in a group of MPNs patients treated with IFN-α [36]. The effect of IFN-α on HSPCs remained elusive in our assay, and our study was limited to a genome-edited iPS clone. Thus, further studies with a larger number of iPS clones are required to use the genome-edited iPS clones for the validation of therapeutic compounds.

In conclusion, we established isogenic iPS cells with or without pathogenic mutations through genome editing and demonstrated the potential of using established disease models to study disease pathogenesis and therapeutic strategies.

Conflict of Interest Disclosure

M.Araki. and M.I. served as employees of Meiji Seika Pharma Co., and N.K. has received a salary from PharmaEssentia Japan where he is a board member; all other authors declare no conflicts of interest.

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Author Contributions

Contribution: C.L. designed and performed experiments, analyzed the data, and wrote the article; M.I. and Y.E. designed the research study, analyzed the data, and revised the article; S.Mano, H.T., M.N., and S.Morishita performed experiments, analyzed the data, and wrote the article; A.K., M.Ando, and S.T. contributed to data interpretation and revised the article; M.Araki and N.K. designed the research study, performed data interpretation, and wrote the article. All authors approved the final manuscript version for the submission.

SUPPLEMENTARY MATERIALS

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