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Original Article

Primary evaluation of induced pluripotent stem cells using flow cytometry

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Induced pluripotent stem (iPS) cells are an attractive cell source in regenerative medicine; however, some problems must be overcome to improve clinical applications. iPS cells generated using the genomic integration method increase the risk of tumor generation because of transgene reactivation and the disruption of endogenous genes. The somatic cell sources of iPS cells also affect teratoma formation. Therefore, it is important to select a suitable cell source from among adult somatic cells, to generate iPS cells using a transgene insertion-free method, and to screen for good iPS clones that do not contain any differentiation-resistant cells after differentiation induction. Recently, we reported a method for obtaining high-quality iPS cells using purified mesenchymal stem cells (MSCs). In this report, we produced genomic integration-free iPS cells from adult tissues, purified MSCs, and tail tip fibroblasts using the Sendai virus. Then, we evaluated the residual undifferentiated cells in secondary neurospheres generated from retroviral induction iPS cell lines and non-integration iPS cell lines derived from adult MSCs. As a result, we could generate integration-free iPS cells only from MSCs. Nevertheless, some iPS cell lines generated by the non-integration method contained undifferentiated cells. Interestingly, the integration-free iPS cells that could not differentiate correctly showed a higher side scatter (SSC) intensity than the other ES/iPS cells. Some somatic cell-derived iPS cells had a higher SSC intensity, and these cells also could not differentiate normally. Our findings suggested that an SSC intensity analysis may be efficient method for evaluating individual iPS cells before their use in therapies.

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Introduction

iPS cells were originally generated from somatic cells by the ectopic overexpression of four transcriptional factors, Oct3/4, Sox2, Klf4 and c-Myc[1], using a retroviral vector. For regenerative medicine, this approach can be used to generate clinically useful cells for autologous transplantation therapies. However, several problems must be overcome before practical application as a cell source becomes feasible.

First, iPS cells that are generated using a genomic integration method have a risk of insertional mutation that interferes with the normal function of iPS cell derivatives. Furthermore, residual transgene expression may influence the differentiation capacity into specific lineages. Reactivation of c-Myc results in tumorigenesis in the chimeric mice[2]. To overcome these problems, several approaches have been established using an adenovirus vector[3], plasmid[4], PiggyBac transposon[5, 6], Cre-loxP based viral vector[7], episomal vectors[8], recombinant proteins[9], and synthetic modified mRNA[10] for genomic integration-free iPS cell induction. However, these methods have so far exhibited low iPS cell derivation efficiencies, compared with retroviral transduction.

Second, although iPS cells have also been derived from various tissues, iPS cells generated using somatic cells from adult donors often exhibit either a propensity to differentiate into a specific cell lineage or a strong differentiation resistance[11]. However, recent studies have shown that mouse neural stem cells (NSCs) can be reprogrammed at a higher efficiency using the four defined transcription factors[12] than tail-tip fibroblasts (TTFs)[1, 2]. We have also reported that purified mesenchymal stem cells (MSCs) are an effective cell source for iPS cell induction[13]. Based on the expressions of PDGFRα and Sca-1 (PαS), we were able to obtain highly enriched MSCs[14, 15] that were 120,000-fold more enriched for clonogenic cells than traditional unfractionated bone marrow[16-18]. We showed that homogeneous, high-quality iPS cells could be generated more efficiently from PαS cells, as compared to that from TTFs and mouse embryonic fibroblasts (MEFs). These findings suggest that immature tissue stem cells (PαS cells) may be more efficiently reprogrammed than somatic cells.

In this study, we produced genomic integration-free iPS cells from adult mouse PαS cells using SeV[19-21]. Integration-free iPS cells were generated from PαS, but not from TTF. Some of the integration-free iPS clones exhibited a neural differentiation potential and did not contain undifferentiated cells. However, some of the iPS clones were not necessarily secure. We accidentally found that iPS cells with a high side scatter (SSC) intensity exhibited a strong differentiation resistance. Selection of iPS cells with a high differentiation potency is essential before application of cell therapies based on iPS cells. As a signature for iPS cells, many evaluation methods can be used, including a methylation analysis of CpG dinucleotides in the Oct4 and Nanog promoter regions[22], an *in vivo* teratoma formation assay[1], and the ability to contribute to the germline[2]. Until now, although these evaluation systems exhibit degrees of reprogramming and pluripotency, effective measures for excluding differentiation-resistant cells have not existed. Our findings suggest that the measurement of SSC intensity using flow cytometry could be a simple and easy method of excluding low-quality iPS cells during the initial stage of selection.

Materials and Method

1)Preparation of bone-marrow cell suspension

Mouse femurs and tibias were dissected out and crushed with a pestle. The crushed bones were washed in HBSS$^+$ (Gibco) supplemented with 2% FBS, 10 mM HEPES, and 1% penicillin/streptomycin (P/S) to remove the hematopoietic cells. The bone fragments were incubated for 1 hour at 37℃ in 0.2% collagenase (Wako) in DMEM (Gibco) containing 10 mM HEPES and 1% P/S. The suspension was filtered through a cell strainer (Falcon) and collected by centrifugation at 280 × *g* for 7 minutes at 4℃. The pellet was suspended for 5-10 seconds in 1 mL of water to burst the red blood cells, after which 1 mL of 2 × PBS containing 4% FBS was added. The cells were suspended in HBSS$^+$ and poured through a cell strainer. All the experimental procedures were approved by the Ethics Committee of Keio University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health).

2)Flow-cytometry analysis and cell sorting

The following fluorescently conjugated antibodies (PE, APC, or FITC) were used for analysis and cell sorting: PE-conjugated CD45 (30-F11) and Ter119 (Ter-119), APC-conjugated PDGFRα (APA5), and FITC-conjugated Sca-1 (Ly6A/E). Flow-cytometry analysis and sorting were performed using a triple-laser MoFlo (Dako) flow cytometer.

Propidium iodide (PI) fluorescence was measured, and a live cell gate was defined that excluded cells that were positive for PI. Additional gates were defined as positive for PDGFRα and Sca-1 and negative for CD45 and Ter119, based on the isotype control fluorescence intensity. iPS-SNSs containing the Nanog-GFP element were dissociated and processed for flow cytometric analysis using a FACS Calibur (Becton-Dickinson). The number of GFP$^+$ cells was presented as the percentage of the total number of cells, excluding dead cells stained by PI.

3)Cell cultures

Mouse MSCs were cultured in maintenance medium (i.e., DMEM supplemented with 20% FBS, 1% P/S, and 10 mM HEPES). As previously described[2, 23], the ES cells and the iPS cells were cultured in ES medium (i.e., DMEM containing 15% FBS, 1 × NEAA, 1 mM sodium pyruvate, 5.5 mM 2-ME, 50 units/50 μg/mL P/S, and 50 μg/mL streptomycin) on feeder layers of mitomycin C-treated STO cells.

4)Generation of induced pluripotent stem cells using SeV

The iPS induction was performed as described previously except for the use of SeV vectors instead of a retrovirus. Briefly, PαS cells and TTFs were isolated from 8-week-old Nanog-reporter mice and then cultured in alpha-MEM containing 10% FCS for two weeks. Thereafter, the cells were transferred at a density of 0.82 × 10^5 cells/well to a 12-well plate and incubated for an additional 24 hours. Then, a solution containing SeV vectors that individually carried each of OCT3/4, SOX2, KLF4, and c-MYC (DNAVEC) was added to the wells at an MOI of 3-10. After 24 hours of infection, the medium was changed to fresh 10% FBS alpha-MEM medium. At 48 hours after infection, the cells were collected and transferred to a 6-well plate that contained mitomycin C-inactivated puromycin-resistant feeder cells at 0.6 × 10^5 to 1.2 × 10^6 cells/well. On the next day, the medium was changed to ES medium supplemented with LIF. Three weeks after infection, the cells were selected using puromycin (Sigma) at a final concentration of 1.5 μg/mL. One week after puromycin selection, the Nanog GFP-positive colonies were selected and expanded into 24-well plates for cryopreservation and further expansion. The ES culture medium was changed every 2-3 days.

5)RT-PCR

Total RNA was purified using Trizol (Invitrogen) and treated with DNAse (Qiagen) to remove genomic DNA contamination. The total RNA was used for a reverse transcription reaction using a cDNA synthesis kit (Stratagene). PCR was performed using ExTaq (Takara).

6)Neural induction

The neural induction of iPS cells was performed as previously described[11, 23]. EBs were prepared in the presence of 1 × 10^{-8}M retinoic acid. To culture the primary neurospheres (PNSs), day 6 EBs were collected and dissociated, then cultured in a suspension at 5 × 10^4 cells/mL in Media hormone mix (MHM) supplemented with B27 and 20 ng/mL of FGF-2 (Wako) for 7 days. Day 4 PNSs were transferred from the cell culture flask (Nunc) to the Ultra-Low Attachment Dish (Corning). To culture the SNSs, the PNSs were collected and dissociated with TrypLE Select (Invitrogen) and cultured in the same culture medium. Day 4 SNSs were transferred from the cell culture flask (Nunc) to an Ultra-Low Attachment Dish (Corning). For further differentiation, the neurospheres were plated onto poly-L-ornithine/fibronectin-coated coverslips and cultured without FGF-2 for 7 days.

7)Immunocytochemical analysis

Immunocytochemical analyses of the cultured cells were performed as described previously[23]. Differentiated neurospheres were stained with the following primary antibodies: anti-β-III tubulin (mouse IgG_{2b}, 1:1000; Sigma), anti-GFAP (rabbit IgG, 1:4000; DAKO), anti-CNPase (mouse IgG1, 1:1000; Sigma).

Results

1)Generation of iPS cells using Sendai virus from Nanog-GFP-IRES-Puro^r mice

To compare the induction efficiency of integration-free iPS cells from mouse adult tissues, PαS and tail-tip fibroblasts (TTF) were isolated from Nanog-GFP-IRES-Puro^r[2] and Yamanaka's 4 factors (Oct3/4, Sox2, c-Myc, and Klf4) were individually introduced using the Sendai virus (SeV). The multiplicity of infection (MOI) was varied, ranging from 3 to 10. Three weeks after SeV infection, we obtained 10 Nanog-GFP$^+$ colonies using an MOI of 3, 22 using an MOI of 5, and 1 using an MOI of 10 from PαS cells. By contrast, no GFP$^+$ colonies emerged from the TTF cells (Fig.

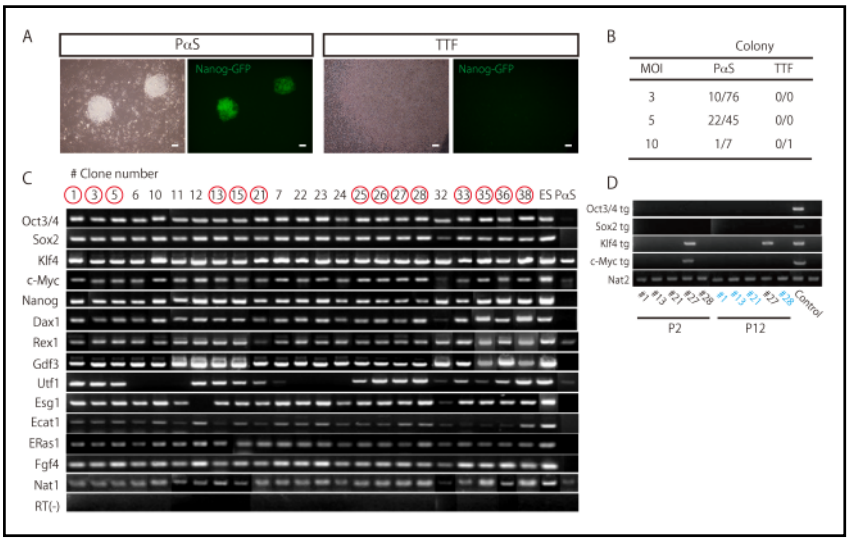
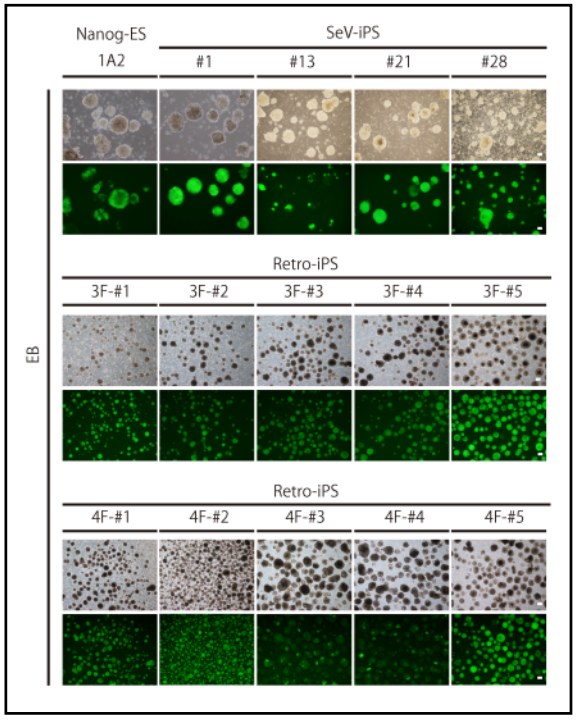


Fig.1 Generation of iPS cells from PαS of Nanog-GFP-Ires-Puror transgenic mice using Sendai virus

(A) Phase and fluorescence image of a Sendai virus-iPS cell (SeV-iPS cell) GFP⁺ colony. Scale bar, 100 μm. (B) Effect of increasing the MOI of SeV. The number of GFP-positive colonies/total colonies obtained from the 4F-SeV-transfection of PαS cells and TTFs is shown. (C) RT-PCR of SeV-iPS cells for ES-cell marker genes. The red circle clones expressed the ES cell marker genes uniformly (14/23 colony). (D) Transgene expression determined using RT-PCR with specific primers in five selected clones from the iPS cells circled in red. The transgenes were silenced in the blue-colored clones.



Supplementary Fig.1 Embryoid body formation of ES and iPS cell clones

Embryoid body (EB) formation of ES (1A2), SeV-iPS (-#1, #13, #21, and -#28), 3F-Retro-iPS (-#1 to -#5), and 4F-Retro-iPS (-#1 to -#5).

1A and B). Therefore, the PαS cells were reprogramed through gene induction with integration-free SeV at a high efficiency, as previously reported for retroviral gene transduction[13].

To determine whether these colonies fulfilled the more stringent criteria for iPS, we randomly selected GFP⁺ colonies and established 23 clones. Fourteen of these clones expressed all the ES cell marker genes that were tested (Fig.1C). The SeV RNA genome was usually diluted and disappeared during cell growth[19]. To investigate the remaining SeV RNA genome, we randomly selected five clones from among 14 iPS clones expressing the ES cell marker genes and performed RT-PCR analysis using specific primers. Clone #27 showed exogenous Klf4 and a c-Myc RNA genome after the second passage, and Klf4 persisted even after P12, although the c-Myc expression disappeared. Four of the five clones did not contain the SeV RNA genome after passage 2 or 12 (Fig.1D). These results suggested that iPS clones containing the SeV genome should be excluded during early passage to obtain integration-free iPS cells.

2)Correlation of neural differentiation potential and SSC intensity of SeV-iPS cells

In our previous paper, the frequency of *in vivo* teratoma formation was strongly correlated with the residual ratio of Nanog-GFP⁺ cells after *in vitro* differentiation[11]. Therefore, the iPS clones derived from MSCs using either retrovirus or SeV gene induction were evaluated using a neurosphere assay, as previously described[23]. Four integration-free SeV-iPS cell clones (SeV-iPS-#1, -#13, -#21, -#28), ten retrovirally induced iPS clones characterized by the presence (4F-Retro-iPS-#1 to #5) or absence (3F-Retro-iPS-#1 to #5) of c-Myc, and an ES cell clone carrying the Nanog-GFP reporter (Nanog-ES 1A2) were tested. All the iPS cell clones, as well as the ES cells, formed embryoid bodies

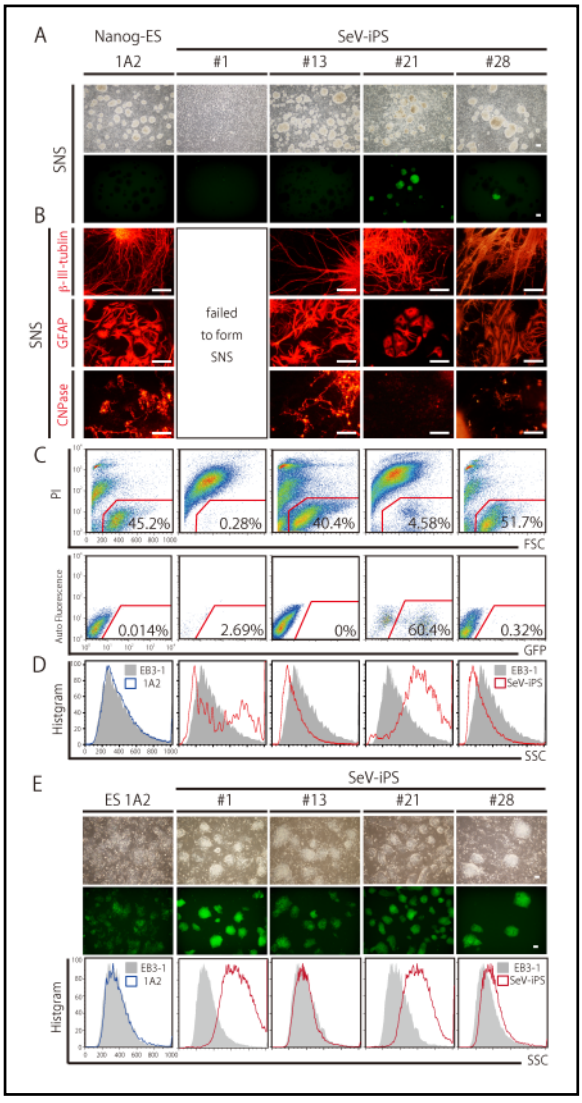
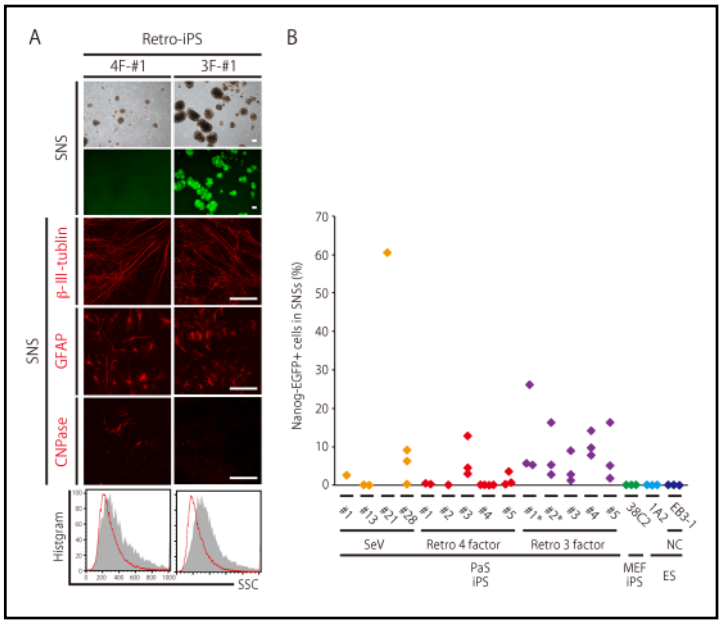


Fig.2 SNS formation from mouse iPS cells and SSC analysis

(A) SNS derived from ES cells (1A2) and SeV-iPS cells (SeV-iPS-#1, -#13, -#21, and -#28). Scale bar, 100 μm (B) Immunocytochemical analysis of cells differentiated from SNS for β-III-tubulin (neurons), GFAP (astrocytes), and CNPase (oligodendrocytes). Scale bar, 100 μm. (C) Flow cytometric analysis of the content of Nanog-EGFP+ cells in SNS. (D) Side scatter (SSC) intensity of SNS. (E) Flow cytometric analysis of the SSC intensity, phase, and fluorescence images of ES and SeV-iPS cells. Scale bar, 100 μm.



Supplementary Fig.2 SNS formation from iPS cells and SSC analysis

(A) SNS derived from 3F-Retro-iPS-#1 and 4F-Retro-iPS-#1. Scale bar, 100 μm. Immunocytochemical analysis of cells differentiated from SNS for β-III-tubulin (neurons), GFAP (astrocytes), and CNPase (oligodendrocytes). Scale bar, 100 μm. The side scatter (SSC) intensity of the SNS is shown. (B) Comparison of the Nanog-GFP+ cells in SNS derived from ES cells (1A2 and EB3-1 as negative control), MEF38C2, SeV-iPS cells, 4F-Retro-iPS cells, and 3F-Retro-iPS cells. * 3F-Retro-iPS-#1 and #2 have a germline transmission capacity.

(Supplemental Fig.1). The embryoid bodies were then dissociated to form primary neurospheres prior to the formation of secondary ones. Although all the iPS cell lines formed primary neurospheres, SeV-iPS-#1 and -#21 contained many scattered cells (Fig.2A).

Each secondary neurosphere (SNS) derived from the four SeV-iPS clones, 4F-Retro-iPS-#1, and 3F-Retro-iPS-#1 was cultured under adherent conditions, and the ability to differentiate into tri-lineage neural cells (neurons, astrocytes, and oligodendrocytes) was examined (Fig.2B, Supplemental Fig.2A). Furthermore, we used these SNSs containing a Nanog-GFP reporter to detect undifferentiated cells using flow cytometry (Fig.2C, Supplemental Fig.2B).

The SNSs showed a significant variation in each clone. SeV-iPS-#1 failed to form an SNS, while #21 exhibited a much lower SNS forming ability and most of the spheres contained Nanog-GFP+ undifferentiated cells. Interestingly, SNS derived from 4F-Retro-iPS clones, except for 4F-Retro-iPS-#3 and -#5, contained few Nanog-EGFP+ cells regardless of the use of c-Myc retrovirus transduction. In contrast, SNS from 3F-Retro-iPS-#1 and -#2 also contained many Nanog-GFP+ undifferentiated cells despite its germline transmission capacity *in vivo*. The iPS clones that formed Nanog-GFP- SNS (SeV-iPS-#13, SeV-#28, and 4F-Retro-iPS-#1) differentiated into tri-lineage neuronal cells. In contrast, SeV-iPS-#21 and 3F-Retro-iPS-#1 that formed Nanog-GFP+ SNS exhibited absent or abnormal oligoden-

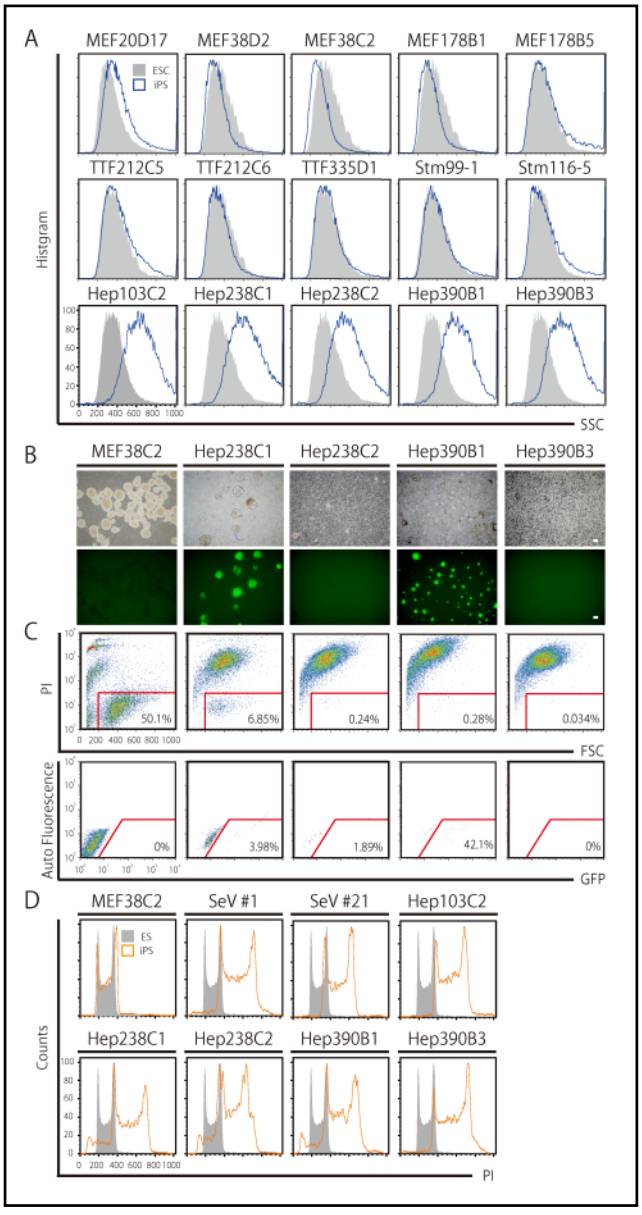
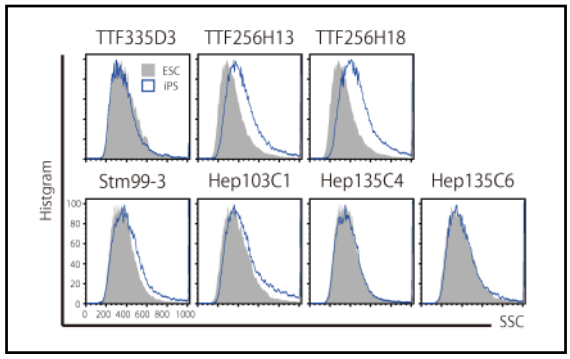


Fig.3 SSC and DNA content analysis using flow cytometry

(A) SSC intensity of MEF-iPS (20D17, 38D2, 38C2, 178B1, and 178B5), TTF-iPS (212C5, TTF212C6, and TTF335D1), Stm-iPS (Stm99-1 and Stm116-5), and Hep-iPS cells (Hep103C2, Hep390B1, Hep390B3, Hep238C1, and Hep238C2). (B) SNS formation from a low-SSC-intensity iPS clone (MEF38C2) and high-SSC-intensity iPS clones (Hep238C1, Hep238C2, Hep390B1, and Hep390B3). (C) FACS analysis of the content of Nanog-EGFP$^+$ cells and the SSC intensity in SNS. (D) FACS analysis of the DNA content using propidium iodide (PI) staining in a low-SSC-intensity iPS clone (MEF38C2) and high-SSC-intensity iPS clones (SeV-iPS-#1, SeV-iPS#21, Hep103C2, Hep238C1, Hep238C2, Hep390B1, and Hep390B3).

drocyte differentiation.

In flow cytometric analyses, analyzed cells were mostly alive in the SNSs that contained none or less than 1% of



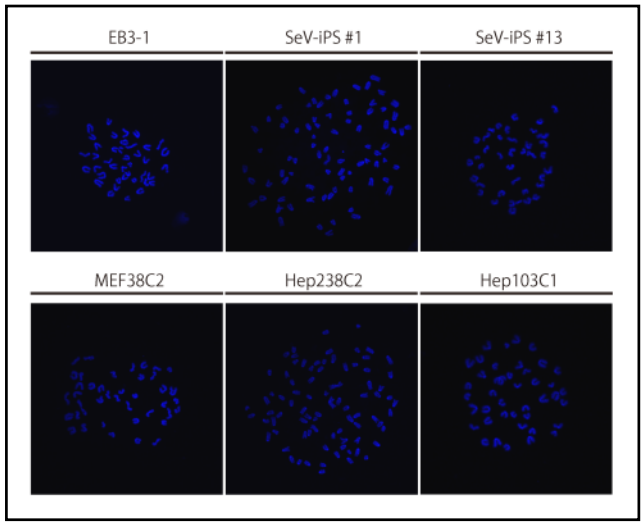
Supplementary Fig.3 SSC analysis using flow cytometry

The SSC intensity of TTF-iPS (335D3, 256H13, and 256H18), Stm-iPS (99-3), and Hep-iPS (103C1, 135C4, and 135C6) is shown.

Nanog-GFP$^+$ cells (derived from SeV-iPS-#13 and -#28). In contrast, SNSs that contained significantly higher amounts of Nanog-GFP$^+$ undifferentiated cells (derived from SeV-iPS-#1 and -#21) also contained larger number of PI$^+$ dead cells (Fig.2C). Interestingly, these two clones showed a high side scatter (SSC) intensity compared with the EB-3 ES cells, SeV-iPS-#13 and -#28 (Fig.2D). We also examined the SSC intensity of these undifferentiated SeV-iPS cells. Although all the SeV-iPS cell clones were morphologically indistinguishable from the ES cells, SeV-iPS-#1 and -#21 had a higher SSC intensity (Fig.2E). These results suggest that iPS-clones with a high SSC intensity did not have the ability to differentiate or included differentiation-resistant cells.

3)High-SSC-intensity iPS clones failed to differentiate into neurospheres and showed double the DNA content

To test our hypothesis, we investigated 22 iPS cells for which the cellular characteristics had already been individually defined. A flow cytometric analysis revealed that five iPS clones (Hep103C2, Hep238C1, Hep238C2, Hep390B1, and Hep390B3) showed a higher SSC intensity than the other ES/iPS clones (Fig.3A, Supplemental Fig.3). A neurosphere assay was performed to test the differentiation potential of these high SSC iPS cells, except for Hep103C2 carrying the CAG-EGFP reporter. Although Hep238C1 and Hep390B1 formed SNS, the sizes and shapes were heterogeneous and the vast majority of the cells were dead. Hep238C2 and Hep390B3 did not have the ability to form SNS. By contrast, low SSC iPS MEF38C2



Supplementary Fig.4 Metaphase spreads stained with Hoechst
Representative metaphase spreads of the iPS clones with high SSC intensity (SeV-iPS #1 and Hep238C2) and iPS clones with low SSC intensity (MEF38C2, SeV-iPS #13, and Hep103C1).

formed a normal SNS, and no Nanog-GFP$^+$ cells were detected (Fig.3B).

The SSC intensity depends on intracellular granules. Furthermore, the SSC intensity and the DNA content are known to be correlated with each other[24]. Thus, we compared the DNA contents of low and high SSC intensity iPS clones. The iPS clones with a high SSC intensity, SeV-iPS-#1, -#21, Hep103C2, Hep238C1, Hep238C2, Hep390B1, and Hep390B3, exhibited double the DNA content. In contrast, the DNA content of MEF38C2, which had a low SSC intensity, was similar to that of ES cells (Fig.3C). Furthermore, we compared the karyotypes of the iPS clones with high SSC intensity (SeV-iPS-#1 and Hep238C2) and those with low SSC intensity (MEF38C2, SeV-iPS-#13, and Hep103C1). SeV-iPS-#1 and Hep238C2 were tetraploid in metaphase (Supplemental Fig.4).

Discussion

Here, we demonstrated that highly enriched mesenchymal stem cells (PαS cells) produce iPS clones using SeV at a higher frequency than TTF. One of the reasons is that PαS cells express c-Myc and Klf4 mRNA at similar levels to ES cells, whereas their expression levels in TTF[13]. However, all the SeV-iPS cell clones did not necessarily have a high differentiation capacity, compared with Retro-iPS cells. Nevertheless, SeV is regarded as a safe and effective way of generating iPS cells, since the SeV vector expresses transgenes stably in cytoplasm without the risk of modifying the host genome[25], is efficient at generating iPS cells[19], and can erase the viral genome from the target cell[21]. Therefore, a method of removing iPS cell clones that show resistance to differentiation is needed.

Meanwhile, Retro-3F-iPS (without c-Myc) cells are known to reduce the incidence of tumorigenicity in chimeras and progeny mice, compared with Retro-4F-iPS cells[26]. As previously reported, 3F-iPS (without c-Myc) cells capture the full pluripotency through tetraploid complementation[27]. In our previous description[13], 3F-Retro-iPS showed a higher chimerism efficiency and a higher frequency of germ-line transmission than 4F-Retro-iPS cell clones. In contrast, we found a tendency for undifferentiated 3F-Retro-iPS to persist in SNS. Therefore, we found no significant correlation between the neural differentiation potential and the germline transmission competency in PαS-iPS. iPS cells, on the other hand, retain an epigenetic memory of their tissue of origin[28]. If our MSCs-derived iPS cells were differentiated into a mesenchymal cell lineage, rather than ectoderm, the appearance of differentiation-resistant cells might have been suppressed.

Our results also indicated that iPS cells with a high SSC intensity contain a large number of differentiation-resistant cells that undergo cell death during the differentiation stage. This was an interesting observation, since it is known that an elevated SSC is characteristic of accumulating granules[29], an increment in the DNA content[24], senescent cells[30], and inferior survival[31]. These findings were also consistent with our observation that high-SSC intensity SNS derived from SeV-iPS and Hep-iPS cell clones caused cell death. Moreover, these iPS cells had approximately double the DNA content. Because of the SeV vector defect fusion protein, cell-to-cell fusion does not occur[25]. Some iPS cells from liver cells using an adenovirus vector have been shown to be tetraploid[3]. Additionally, liver cell tetraploidy has been described as a morphological feature appearing during postnatal growth[32, 33]. Liver cell tetraploidy is thought to lead to senescence[34] and the loss of cell pluripotency and proliferation[35]. Therefore, our results suggest that Hep-iPS cells with a high SSC intensity and double the DNA content include many differentiation-resistant cells or undergo cell death during the differentiation stage.

In conclusion, we have noted that low-quality iPS cells appear despite the use of a reprograming method without genomic modification and regardless of the tissue of ori-

gin. Furthermore, we have shown that flow cytometry is a convenient means of assessing the quality of iPS cells as a primary screening method. Before cell therapies based on iPS cells can advance to clinical applications, our simple technique may be a very effective means of excluding extremely low-quality iPS as a primary screening method.

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Conflict of interest

The authors declare no competing financial interests.

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